DIETARY SUPPLEMENTS, BOTANICALS AND HERBS AT THE INTERFACE OF FOOD AND MEDICINE

EDITED BY: Alessandra Durazzo, Massimo Lucarini and Michael Heinrich
PUBLISHED IN: Frontiers in Pharmacology, Frontiers in Plant Science and Frontiers in Nutrition

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-654-3
DOI 10.3389/978-2-88976-654-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

DIETARY SUPPLEMENTS, BOTANICALS AND HERBS AT THE INTERFACE OF FOOD AND MEDICINE

Topic Editors:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy
Massimo Lucarini, Council for Agricultural Research and Economics, Italy
Michael Heinrich, University College London, United Kingdom

Citation: Durazzo, A., Lucarini, M., Heinrich, M., eds. (2022). Dietary Supplements, Botanicals and Herbs at The Interface of Food and Medicine.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-654-3

Table of Contents

frontiers | Frontiers in Pharmacology

EDITORIAL
published: 04 July 2022
doi: 10.3389/fphar.2022.899499

Editorial: Dietary Supplements, Botanicals and Herbs at the Interface of Food and Medicine

Alessandra Durazzo[1]*, *Massimo Lucarini*[1]* and *Michael Heinrich*[2]*

[1]Research Centre for Food and Nutrition, Council for Agricultural Research and Economics, Rome, Italy, [2]School of Pharmacy, Faculty of Life Sciences, University College London, London, United Kingdom

Keywords: dietary supplements, nutraceuticals, dietary supplements labeling, chemometrics, dietary supplement databases, metrology, control quality, food

Editorial on the Research Topic

Dietary Supplements, Botanicals and Herbs at The Interface of Food and Medicine

OPEN ACCESS

Edited by:
Javier Echeverria,
University of Santiago, Chile

Reviewed by:
Anna Rita Bilia,
University of Florence, Italy
Rolf Teschke,
Hospital Hanau, Germany

***Correspondence:**
Alessandra Durazzo
alessandra.durazzo@crea.gov.it
Massimo Lucarini
massimo.lucarini@crea.gov.it
Michael Heinrich
m.heinrich@ucl.ac.uk

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *18 March 2022*
Accepted: *14 June 2022*
Published: *04 July 2022*

Citation:
Durazzo A, Lucarini M and Heinrich M (2022) Editorial: Dietary Supplements, Botanicals and Herbs at the Interface of Food and Medicine. Front. Pharmacol. 13:899499. doi: 10.3389/fphar.2022.899499

INTRODUCTION

The Research Topic "Dietary Supplements, Botanicals and Herbs at The Interface of Food and Medicine" provide a detailed and up to date picture on a key interdisciplinary field.

The focus of this Research Topic has been the relationship between dietary supplements (according to FDA definition)/food supplements (according to EFSA definition), botanicals, herbal medicines, herbal medicinal products and, foods with potential beneficial properties from a global and interdisciplinary perspective with the aim of understanding their potential health benefits. In order to simplify the discussion in the context of the complex terminology, herbal remedies, herbal drugs, herbal medicinal products, herbal medicines, botanical drugs are all labelled as botanicals, a term commonly linked to the framework in the USA, but now also used, for example, in Europe (EFSA Scientific Committee, 2009; European Food safety Authority, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), 2016; [1]).

The use of plants in new formulations of dietary supplements was explored.

The beneficial properties of medicinal plants (often having a restricted regional uses) trigger interest in the possibility of developing novel nutraceutical botanical supplements formulations, which can help to support health conditions reducing the need for pharmacological interventions, in particular for individuals who do not qualify for conventional drug-based treatment.

The Areas Explored

i) Interdisciplinary approaches using emerging and innovative techniques with chemometrics on dietary supplements, the production of standardized formulations, authentication of individual plants, monitoring of the quality of herbs and plants used for botanical supplements, detection of adulteration or contamination of herbs;
ii) studies on the description and use of bioactive compounds i.e., antioxidants in medicinal plants as well as on physiological mechanism and bioaccessibility of compounds;

[1]https://ods.od.nih.gov/factsheets/list-Botanicals/.

iii) the application of nanotechnologies for developing novel plant-based products with improved bioavailability, solubility, and efficacy;
iv) studies of medicinal plants using "large data" e.g., extracted from healthcare or other databases e.g., dietary supplement ones, including the application of classification systems, harmonization, and coding procedures;
v) investigations on the functional/nutraceutical characteristics of foods in order to integrate intrinsic nutritional properties;
vi) the use of food waste as a sustainable alternative source of biologically active compounds for botanicals.

The papers published cover an exciting range of themes within this fast evolving area of research.

The strict linkage of territory, medicinal plants and health was underlined in this Research Topic. For instance, Mudau et al. presented a systematic review of the potential of South African neglected and underutilized crops as food and herbal medicinal crops. Vujicic and Cohall reported the knowledge, attitudes and practices on the use of botanical medicines in a rural Caribbean territory. Ojha et al. described the traditional dietary knowledge of a marginal hill community in the central Himalaya, with particular focus on the implications for food, nutrition, and medicinal security. Cordero et al. reported the ethnobotanical documentation of medicinal plants used by the indigenous Panay Bukidnon in Lambunao, Iloilo, Philippines. Okagu et al. provided a stimulating review of *Zanthoxylum* species and their traditional uses, phytochemistry and pharmacology in relation to cancer, infectious diseases and sickle cell anemia. Wu et al. reviewed the ethnopharmacological understanding of *Houttuynia cordata* Thunb. Kasali et al. presented a critical review of ethnopharmacology and bioactivity data of antidiabetic medicinal plants used in Democratic Republic of Congo. Belhouala and Benarba presented a multiregional ethnobotanical study of medicinal plants used by traditional healers in Algeria. Brendler1 and Abdel-Tawab reviewed the features of Buchu (*Agathosma betulina* (P.J.Bergius) Pillans and *Agathosma crenulata* (L.) Pillans).

Research on bioactive metabolites and beneficial properties and effects of medicinal plants is another core focus. Rahim et al. studied the phytochemical analysis, antioxidant and bone anabolic effects of *Blainvillea acmella* (L.) Philipson. Otari et al. reported the phytochemistry of two unexplored endemic medicinal plants of India, *Barleria terminalis* Nees and *Calacanthus grandiflorus* (Dalzell) Radlk. Divyashri et al. showed experimental evidence of neuroprotective potential of non-digestible oligosaccharides. Sadgrove et al. described the pharmacology of natural volatiles and essential oils in food, therapy, and disease prophylaxis.

An overview of meta-analyses of clinical trials of medicinal plants used for glycaemic control in type 2 diabetes is given by Willcox et al. Razali et al. studied *in vitro* anticancer activity of *Neolamarckia cadamba* (Roxb.) Bosser leaf extract and its metabolite profile. Kudera et al. reported on selective *in vitro* antibacterial and antiproliferative effects of ethanolic extracts from Cambodian and Philippine plants used in local medical system for diarrhea treatment.

Zheng et al. reported how the artesunate combined with Metformin ameliorate on diabetes-induced xerostomia by mitigating superior salivatory nucleus and salivary glands injury in type 2 diabetic rats *via* the PI3K/AKT pathway. Mohanty et al. described the ameliorative effects of dietary ellagic acid against severe malaria pathogenesis by reducing cytokine storms and oxidative stress. Rahmi et al. reported how the extracts of *Andrographis paniculata* (Burm.f.) Nees leaves exert anti-gout effects by lowering uric acid levels and reducing monosodium urate cystal-induced inflammation.

Moving towards the field of innovative procedures for medicinal plants and herbs research in quality control area, the international perspective by Durazzo et al. addresses analytical challenges and metrological approaches to ensuring dietary supplement quality. An example is given by Xu et al. that described the authentication of three source spices of *Arnebiae Radix -Arnebia decumbens* (Vent.) Coss. et Kralik, *Arnebia euchroma* (Royle ex Benth.) I.M.Johnst. and *Arnebia guttata* Bunge-using DNA barcoding and HPLC. Jiang et al. showed a value chain perspective for the quality monitoring of *Cistanche deserticola* Ma.

Schreiner et al. investigated 68 powdered plant extracts (botanicals) which are added to food products in food industry throughout 8-dimensional hyphenation of normal-phase high-performance thin-layer chromatography with multi-imaging by ultraviolet, visible and fluorescence light detection as well as effect-directed assay and heart-cut of the bioactive zone to orthogonal reversed-phase high-performance liquid chromato-graphy–photodiode array detection–heated electrospray ionization mass spectrometry: the array of 1,292 profiles (68 samples × 19 detections) showed the versatile bioactivity potential of natural food.

One particular focus is the interface between food and medicine. *Prunus mira* Koehne in Sichuan (PR China) is the focus of a review covering its history, distribution, modern application and ethnobotanical investigation (Zhang et al.; Martínez-Francés et al. analysed the medicinal plants in traditional herbal wines and liquors in the east of Spain and the Balearic Islands. Asdaq et al. reported the potential benefits of using garlic oil and its active constituent, dially disulphide, in combination with carvedilol in ameliorating isoprenaline-induced cardiac damage in rats. Otunola et al. reviewed the properties of culinary spices in food and medicine, with focus on *Syzygium aromaticum* (L.) Merr. and L. M. Perry [Myrtaceae].

Iñiguez-Luna et al. studied the natural bioactive compounds of *Sechium* spp. P. Br (chayotes)for therapeutic and nutraceutical supplements. Li et al. reviewed the promising traditional uses, pharmacological effects, aspects, and potential applications *Citrullus colocynthis* (L.) Schrad (Bitter Apple Fruit). Attah et al. studied effect of extracts of moringa oleifera seed on some reproductive parameters, hepatic and renal histology.

Chao et al. presented an ethnobotanical survey on bitter tea in Taiwan.

Krepkova et al. described valuable hepatoprotective plants i.e. milk thistle, artichoke, and chicory and the use of their waste products as rich sources of active components. Alyahya et al. reported the quantification of chlorogenic acid and vanillin from

coffee peel extract and its effect on α-Amylase activity, immunoregulation, mitochondrial oxidative stress, and tumor suppressor gene expression levels in H_2O_2-induced human mesenchymal stem cells.

Bilal et al. reviewed the nutritional applications, beneficial health aspects of olive oil and its prospective application in poultry production. Oshiomame Unuofin et al. reviewed nutritional and pharmacological applications of ginger from farmyard to town.

Lastly, the development carrot nutraceutical products as an alternative supplement for the prevention of nutritional diseases Riyaz et al.

The research topic has highlighted the diversity of approaches to understand plants which may be a food or a medicine, depending on the context, regulatory status and the interpretation of the evidence. The articles provide fascinating spectrum of perspectives on this important them, relevant for human health and one of the long-standing challenges in ethnopharmacology. They provide not only new insights into specific topics, but also advance our Frontier in research at this interface, which does require considerable more research related to the health benefits and their limitations of such products.

AUTHOR CONTRIBUTIONS

AD, ML, and MH have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

We would like to acknowledge the authors and the reviewers of the publications in this Research Topic for their invaluable contributions and effort. We are also grateful to the editorial board members and support staff of the journal for their kind support during the preparation of this Research Topic.

REFERENCES

EFSA Scientific Committee (2009). Guidance on Safety Assessment of Botanicals and Botanical Preparations Intended for Use as Ingredients in Food Supplements, on Request of EFSA. *EFSA J.* 7, 1249–1257. doi:10.2903/j.efsa.2009.1249

European Food safety Authority (2012). Compendium of Botanicals Reported to Contain Naturally Occurring Substances of Possible Concern for Human Health when Used in Food and Food Supplements. *EFSA J.* 10, 2663–2668. doi:10.2903/j.efsa.2012.2663

U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) (2016). Botanical Drug Development Guidance for Industry. December 2016. Pharmaceutical Quality/CMC Revision 1. Available online: https://www.fda.gov/files/drugs/published/Botanical-Drug-Development–Guidance-for-Industry.pdf.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 01 July 2021
doi: 10.3389/fphar.2021.677014

Authentication of Three Source Spices of *Arnebiae Radix* Using DNA Barcoding and HPLC

Haiyan Xu[1,2], *Ping Li*[1], *Guangxi Ren*[1], *Yanjiao Wang*[3], *Dan Jiang*[1]* *and Chunsheng Liu*[1]*

[1]School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China, [2]College of Traditional Chinese Medicine, Xinjiang Medical University, Xinjiang, China, [3]Department of Basic Medical Sciences, Xinjiang Medical University, Xinjiang, China

Arnebia decumbens (Vent.) Coss. et Kralik, *A. euchroma* (Royle) Johnst and *A. guttata* Bunge, three commonly used traditional Chinese medicinal plants have been widely used for the clinical treatment of inflammatory diseases caused by fungal, bacterial, oxidation, and other related pathogens. However, precise identification at the similar species level is usually challenging due to the influence of the source of medicinal materials, traditional ethnic medicine and medicinal habits. Here we developed a comprehensive and efficient identification system for three source spices of *Arnebiae Radix* based on DNA barcoding and HPLC fingerprinting. A total of 599 samples from thirty-five wild populations were collected and identified by using DNA barcodes of ITS2 regions, and the chemotypes of seven naphthoquinoneswere revealed by HPLC quantitative analysis including principal component analysis and hierarchical clustering analysis. Our results showed that the ITS2 sequences can distinguish three source spices of *Arnebiae Radix* from adulterants. However, it was difficult to identify them by HPLC-specific chromatograms combined with chemometric analysis. These results indicated that DNA barcoding was a more powerful method than HPLC fingerprinting for the identification of related species that were genetically similar. DNA barcoding analysis could be a promising and reliable tool to accurately confirm the identities of medicinal materials, especially for those whose sources are multiple and difficult to be identified by conventional chromatography.

Keywords: *Arnebiae Radix*, DNA barcoding, ITS2, HPLC, identification

OPEN ACCESS

Edited by:
Massimo Lucarini, Council for Agricultural Research and Economics, Italy

Reviewed by:
Yuelin Song, Beijing University of Chinese Medicine, China
Ina Yosifova Aneva, Bulgarian Academy of Sciences, Bulgaria

***Correspondence:**
Dan Jiang
jiangdan1027@163.com
Chunsheng Liu
max_liucs@263.net

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *07 March 2021*
Accepted: *19 May 2021*
Published: *01 July 2021*

Citation:
Xu H, Li P, Ren G, Wang Y, Jiang D and Liu C (2021) Authentication of Three Source Spices of Arnebiae Radix *Using DNA Barcoding and HPLC. Front. Pharmacol. 12:677014. doi: 10.3389/fphar.2021.677014*

INTRODUCTION

Arnebiae Radix (Zicao in Chinese), a kind of traditional Chinese medicine, is the dried root bark of *A. euchroma* (Royle) Johnst. and *A. guttata* Bunge in the Chinese Pharmacopoeia (2020 version). Shikonin and its derivatives, red naphthoquinones, are widely found in the epidermis of the roots of *Arnebiae Radix* (Zhan et al., 2015) and have been widely demonstrated to possess various biological activities, such as anti-inflammatory (Fu et al., 2016; Sun et al., 2017; Guo et al., 2019), antibacterial (Zhao et al., 2017; Huang et al., 2020), and antiangiogenic (Liu C. et al., 2020) activities. Recently, it was reported that shikonin and its derivatives could induce apoptosis of many types of cancer cells and exhibit anticancer activities and antitumorigenic properties (Liao et al., 2020). *Amebia Radix* has been widely used in the medicine, printing and dyeing industry, cosmetics and food industries (Xu et al., 2014; Ma et al., 2021).

TABLE 1 | Sample information of *Arnebiae Radix* and its adulterants in this study.

Taxon	Sample	Locality	Longitude(E)	Latitude(N)	Altitude(m)	Sample Size
A. decumbens (vent.) coss. et kralik	Ad(FK)	Fukang city	88°17′34″	44°24′39″	487	18
	Ad(TKS)	Tekesi county	81°54′41.78″	43°12′10.64″	1375	16
	Ad(MQ)	Miquan county	87°26′47″	44°36′42″	707	15
	Ad(KLMY)	Kelamayi city	84°57′40″	45°11′48″	436	18
	Ad(WS)	Wusu county	84°57′40″	45°11′48″	379	15
	Ad(SHZ)	Shihezi city	86°14′28″	45°1′42″	472	15
	Ad(BEJ)	Buerjin county	86°92′34.51″	47°07′33.08″	497	11
	Ad(SHW)	Shawan county	85°55′17″	44°55′52″	564	12
	Ae(WLMQ)	Wulumuqi county	87°07′28.67″	43°17′19″	2,507	14
	Ae(ML)	Mulei county	90°31′09.79″	43°33′21.42″	2,568	5
	Ae(BCH)	Baicheng county	81°84′55.37″	41°82′11.06″	2,606	20
	Ae(NLT)	Nalati town	83°56′10.920″	43°10′11.24″	2,500	16
	Ae(TSHKEG)	Tashikuergan county	75o04′52.2″	37o49′58.7″	4,234	18
A. euchroma (royle) Johnst	Ae(ATSH)	Atushi county	76°12′33.08″	39°27′43.50″	2,300	20
	Ae(WQ)	Wenquan county	80°32′18″	45°2′18″	2,299	19
	Ae(HJ)	Hejing county	84o07′13.3″	42o42′00.5″	2,456	19
	Ae(HCH)	Huocheng county	81°09′53.83″	44°27′32.39″	2,502	21
	Ae(JH)	Jinghe county	83o15′44.7″	44o23′42.6″	2,144	19
	Ae(GL)	Gongliu county	82°23′19.10″	43°35′42.30″	2,530	19
	Ae(AKS)	Akesu county	80°29′31.22″	41°15′33.28″	2030	19
	Ae(XY)	Xinyuan county	83°27′66.19″	43°41′55.40″	2,201	18
	Ag(HM)	Hami county	93°50′53.05″	43°07′42.63″	861	18
	Ag(FY)	Fuyun county	89°1′2″	45°2′46″	1065	22
	Ag(BLK)	Balikun county	91°39′48.60″	43°47′25,6″	1632	17
	Ag(NLK)	Nileke county	82°10′3″	43°36′41″	765.4	16
	Ag(TSHKEG)	Tashikuergan county	75o28′58.3″	37o13′44.7″	3,780	14
	Ag(QT)	Qitai county	091°22′52.9″	44°58′0.5″	1167	19
	Ag(HJ)	Hejing county	86°0′23.56″	43°01′1.89″	2,253	17
A. guttata bunge	Ag(YW)	Yiwu county	94°48′51.30″	43°19′25.3″	1414	19
	Ag(SHSH)	Shanshan county	89°56′37.05″	43°06′2,0″	653	15
	Ag(TL)	Tuoli county	82°34′40″	45°35′0″	1532.3	17
	Ag(ML)	Mulei county	091°23′22.5″	45°03′20″	1312	23
	Ag(QH)	Qinghe county	90°22′25″	45°33′7″	1193	20
	Ag(WQ)	Wenquan county	81°8′18″	44°46′32″	1801.7	16
	Ag(XY)	Xinyuan county	82°29′45″	43°24′17″	894.1	19

Furthermore, there are other plants of the genus *Arnebia* (Boraginaceae) that are also named Zicao in Xinjiang of China, such as *A. decumbens* (Vent.) Coss. et Kralik and *A. tschimganica* (Fedtsch.) G. L. Chu (Jia-Xin et al., 2018). The market for *Arnebiae Radix* is complicated due to the influence of the source of medicinal materials, traditional ethnic medicine and medicinal habits. Thus, it is difficult to identify the authenticity of medicinal *Arnebiae Radix*, and the identification problem needs to be solved urgently.

Currently, DNA barcoding has emerged as an effective tool for the identification of traditional Chinese medicine due to its species specificity. DNA barcoding has been performed to recognize animals, plants, and fungi (Chen et al., 2014; Gunnels et al., 2020; Behrens-Chapuis et al., 2021; Selva Pandiyan et al., 2020). As a valuable tool for biological identification, DNA barcoding can identify species efficiently and conveniently (Yu et al., 2021). Chen et al. found that the internal transcribed spacer 2 (ITS2) region can potentially be used as a standard DNA barcode to identify medicinal plants and their closely related species (Chen et al., 2010). ITS2 can serve as a novel universal barcoding for the identification of a broader range of plant taxa (Liu et al., 2012; Zhang et al., 2018; Khan et al., 2019). Moreover, high-performance liquid chromatography (HPLC) specific chromatograms, which can effectively determine the content of compounds, are widely used for authenticity confirmation and quality control of traditional Chinese medicines (Hu et al., 2020; Liu B. et al., 2020). Some researchers have indicated that the chemical components of different *Arnebiae Radix* vary, some of which may have good bioactivities (Feng et al., 2020; Liao et al., 2020; Mei et al., 2020).

Thus, authenticity assurance is crucial for their quality control. In this study, we intended to use the DNA barcoding technique and HPLC-specific chromatograms to identify three source spices of *Arnebiae Radix*. The results will facilitate exploring the genetic basis of chemical variations and developing strategies for the utilization and conservation of *Arnebiae Radix*.

MATERIALS AND METHODS

Plant Materials

A total of 599 samples from thirty-five wild populations were collected and analyzed in this study (**Table 1**), including *A. decumbens* (Vent.) Coss. et Kralik (Ad:120 individuals from eight wild populations), *A. euchroma* (Royle) Johnst (Ae: 227 individuals from 13 wild populations), and *A. guttata* Bunge (Ag:

TABLE 2 | Variable sites of the ITS2 region of the three *Arnebiae Radix* species.

Site	Ad	Ae	Ag
1	–/A	–/A	–/A/G
2	–/A	–/G	–/A/G
13	C	C	C/T
26	C/T	C	C
40	C	C/T	C/T
41	G	A	A/G
42	C	C/T	C
44	T	T	A/T
45	C	C/T	A/C
46	G	C/G	G/T
52	C	C	C/T
60	C	A	A/C
63	A	A	A/T
64	G	A	A/G
66	A	A	A/G
67	C	A/C	A
68	T	T	C/T
69	T	G	G/T
70	G	T	A/T
71	C	T	T
77	T	G/T	C/T
98	G	G	A/G
101	C	T	T
102	C/T	T	T
103	G	G	G/T
104	G	A	A/T
118	A/G	G	A/G
122	A/T	T	T
124	C/T	C	C/T
130	C/T	C	C/T
137	G/T	T	G/T
149	A/G/T	A	A/T
180	A/C	A	A/C
184	C/T	C/T	C
194	A/G	A/G	A
200	A/G	G	A/G
201	C/T	T	T
202	G/T	G	G/T
203	C/G/T	T	C/T
207	C/T	C	C/T
208	C/G	G	G
210	A/G	A	A/G
218	A/T	A	A
219	G/T	G/T	G/T
223	C/T	C	C/T
226	G/T	G	G/T
227	C/T	C	C/T
231	C/T	C	C/T
232	A/C	C	A/C
233	A/G	A	A
236	C/T	T	C/T
238	G/T	G	G/T
239	C/T	T	T
240	G/T	T	G/T
241	C/G/T	C	C/T
252	C/T	C	C/T
254	G/T	G	G/T
261	C/G	C/G	G
263	C/T	C	C/T
264	C/T	C	C/T
267	C/G	C/G	G
286	C/T	T	C/T
348	A/G	A/G	G
359	C/T	C	C

(Continued in next column)

TABLE 2 | (*Continued*) Variable sites of the ITS2 region of the three *Arnebiae Radix* species.

Site	Ad	Ae	Ag
371	A/T	A	A/T
380	–/G/T	G/T	–/T
389	–/A/G	G	–/G
390	–/A/G	G	–/A/G
391	–/C/T	C	–/C/T
396	–/C/T	C/T	–/C/T
399	–/G/T	–/T	–/G/T

Ad, A. decumbens *(Vent.) Coss. et Kralik; Ae,* A. euchroma *(Royle) Johnst; Ag,* A. guttata *Bunge; -missing variant site.*

252 individuals from 13 wild populations). This study contained most of the *Arnebiae radix* species in Taiwan and China but did not include *C. quinquesecta*, because the species is a critically endangered medicinal plant and was not found in the field. Sampling from plantation populations or within short distances was avoided (>50 km). All samples were dried and stored immediately in silica gel after collection. Voucher specimens were deposited at Xinjiang Medical University. The geographic localities of each sampled population were determined using a Garmin GPS unit (**Table 1**).

Chemical Apparatus

Chemical standards including (β,β-dimethylacryl)shikonin (15102821), alkannin (15102721), deoxyalkannin (15062422) and acetylshikonin (15120431) were purchased from Tauto Biotech (Shanghai, China). β-Acetoxyisovalerylalkannin (P05M7F14235) was purchased from Yuanye Biotech (Shanghai, China). Isobutylshikonin (wkq16101302) was purchased from Weikeqi Biotech (Sichuan, China). (2-Methyl-n-nbutyl) shikonin (AV51-LDQR) was purchased from Tokyo Chemical Industry (Tokyo, Japan). The purity of the standards was above 98%. The petroleum ether (60–90°C) was analytically pure. All of the chemicals and reagents used in this study were of HPLC analytical grade.

DNA Extraction, PCR Amplification and DNA Sequencing

The material specimens were dried by natural methods, and 20 mg of dried plant material was used for DNA extraction. Genomic DNA was extracted with a DNA Secure Plant Kit from Tiangen Biotech (Beijing, China). The relative purity and concentration of extracted DNA were estimated by ethidium bromide staining on agarose gels and compared with known DNA concentration markers.

The extracted genomic DNA was amplified by polymerase chain reaction (PCR), using the ITS2 (ITS2F, 5′-ATGCGATAC TTGGTGTGAAT-3′ and ITS2R, 5′-GACGCTTCTCCAGAC TACAAT-3′). PCR amplifications were carried out in a volume of 20 μL using 1 μL of template DNA (50–100 ng), 2 μL of 10 × reaction buffer, 1.6 μL of dNTP mix (2.5 mM),

1.25 μL of 10 μM of each primer, 0.2 μL of Ex-Taq DNA polymerase (Takara Shuzo Co., Ltd., Otsu, Japan), and 12.7 μL of sterile distilled water. Reactions were run on a Veriti thermocycler (Applied Biosystems, United States). The PCR conditions for amplification consisted of one cycle of denaturation at 95°C for 5 min, 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 1 min 30 s of extension at 72°C, followed by an 8 min extension step at 72°C. PCR products were electrophoresed on 1.5% (w/v) agarose gels and purified through precipitation with 95% ethanol and 3 M sodium acetate (pH 5.2). All the purified PCR products were sequenced directly in both directions on an ABI 3730XL automated sequencer (Applied Biosystem, Foster City, CA, United States).

DNA Barcoding Analysis

Cutting and splicing of all ITS2 sequences, removal of the primer region and low-quality regions, manual correction, and stitching were performed by ContigExpress software. Then, the modified ITS2 sequences were submitted to DNAMAN software to compare the similarities of the samples. Finally, the modified ITS2 sequences were aligned, and the inter/intraspecific genetic distances were measured using MEGAX 10.2.4 software. A phylogenetic tree using GenBank sequences as outgroups was constructed based on standard parameters with bootstrap testing of 1,000 replicates.

HPLC Conditions

The medicinal powder precisely was weighed to approximately 1 g and placed in an Erlenmeyer flask; 50 ml of petroleum ether (60–90°C) was added. Then, the sample was accurately weighed and extracted with 30 min ultrasonication steps. After cooling and adding petroleum ether (60–90°C) to compensate for the decrease in weight, the sample was filtered. The resulting filtrate was measured (10 ml of *A. euchroma*, 30 ml of *A. guttata* Bunge, 30 ml of *A. tschimganica*, and 30 ml of *A. decumbens* (Vent.) Coss. et Kralik), evaporated to dryness, dissolved the residue in acetonitrile, transferred to a 10 ml volumetric flask, and dissolved in acetonitrile. The sample was then transferred to a 10 ml volumetric flask, diluted with acetonitrile to scale, shaken to mix well and prepared for analysis. HPLC chromatographic conditions were conducted as described by Ding et al. (Ding et al., 2019).

Chemometric Analysis

The reference chromatogram was generated using a Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2012). Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed by the professional software SIMCA 14.1 to demonstrate the variability of the relative peak areas of bioactive compounds. Outliers were identified and removed by DmodX.

RESULTS

Genetic Divergence Determination

The length of the aligned ITS2 sequences was 399 bp, and the number of variable sites was 71 (**Table 2**). We used four parameters to characterize divergence. The intraspecific distance of ITS2 was 0.0025–0.006, and the interspecific distance was 0.0745–0.0915. Additionally, after grouping samples according to locality, the genetic distance within a group was 0–0.0494, and the genetic distance between groups was 0–0.0941. The analysis of the distribution of genetic distance (**Figure 1**) showed obvious barcoding gaps between samples, indicating that the ITS2 sequence has a strong ability to identify *Arnebia* genus samples at the species level.

Identification of *Arnebiae Radix* by DNA Barcoding

To identify the species of the 599 *Arnebiae Radix* samples more accurately and visually, we constructed a neighbor-joining tree based on the ITS2 sequences obtained from the samples and four ITS2 sequences of Boraginaceae downloaded from NCBI

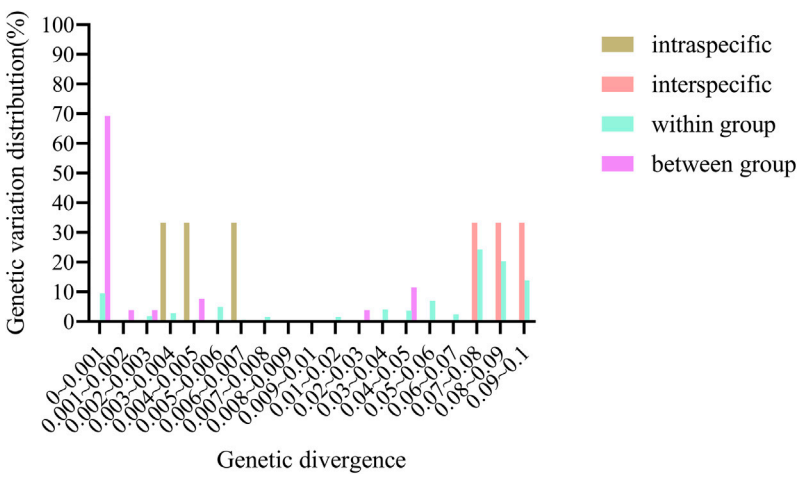


FIGURE 1 | Genetic divergence of the ITS2 region of the three *Arnebiae Radix* species.

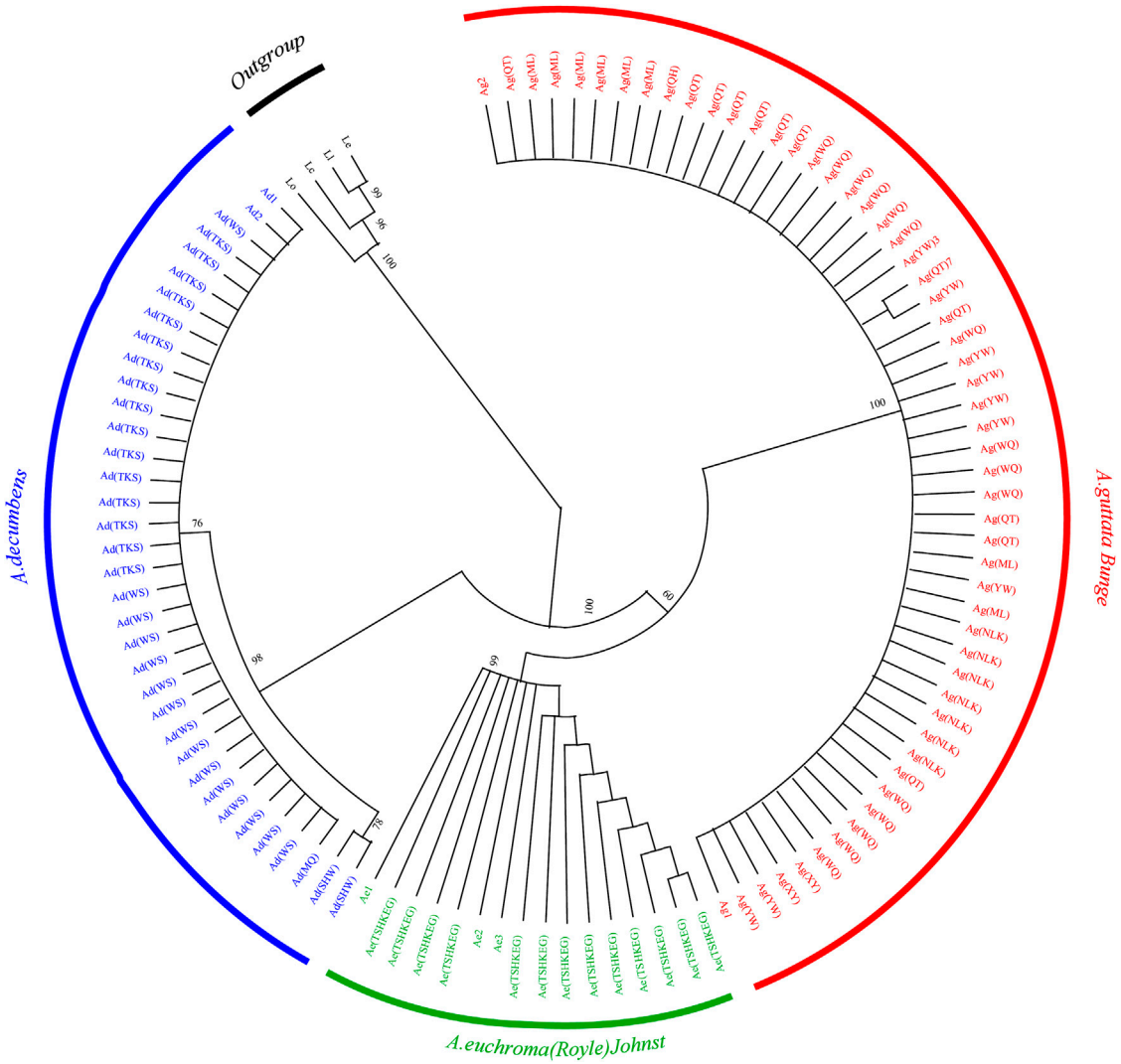


FIGURE 2 | Results of the neighbor-joining tree based on ITS2 sequences of the 599 *Arnebiae Radix* samples. Supporting values (>50%) are indicated above or below the relevant branches. Some subtrees are compressed to show the samples completely in a picture. Ad, *A. decumbens* (Vent.) Coss. et Kralik; Ae, *A. euchroma* (Royle) Johnst; Ag, *A. guttata* Bunge. Ad1:17 samples of Ad(FK),18 samples of Ad(KLMY),nine samples of Ad(SHW) and 15 samples of Ad(SHZ); Ad2:11 samples of Ad(BEJ),14 samples of Ad(MQ),one samples of Ad(SHW), and one sample of Ad(FK);Ae1:19 samples of Ae(WQ), 14 samples of Ae(WLMQ), 19 samples of Ae(HJ), 16 samples of Ae(NLT),15 samples of Ae(XY), 20 samples of Ae(JH), 21 samples of Ae(HCH), 19 samples of Ae(GK) and 20 samples of Ae(ATSH);Ae2: six samples of Ae(TSHKEG);Ae3: five samples of Ae(ML) ,20 samples of Ae(BCH) and 19 samples of Ae(AKS);Ag1: nine samples of Ag(NLK), and 17 samples of Ag(XY);Ag2: four samples of Ag(TL), 14 samples of Ag(HJ), three samples of Ag(SHSH), and one samples of Ag(TSHKEG).

(Supplementary Table S1). The ITS2 sequences were divided into mutually exclusive monophyletic clades. *A. guttata* Bunge clustered into one subgroup, *A. euchroma* (Royle) Johnst could be clustered into one group, and *A. decumbens* (Vent.) Coss. et Kralik could be clustered into one group (**Figure 2**). The pairwise distance analysis supports this interpretation, revealing that ITS2, as a barcode, is able to distinguish between the three species of *Arnebiae Radix*.

HPLC Fingerprint of *Arnebiae Radix* Samples

The results of the determination of seven naphthoquinones in the roots of three species of *Arnebiae Radix* from different habitats in Xinjiang are shown in **Table 3**. Naphthoquinones were found in the roots of all three *Arnebiae Radix* species, and there were differences among different species. The total amount of naphthoquinones in Ae was the highest (43.3505 mg/g), followed by that in Ag (11.4042 mg/g), and the lowest amount (6.0462 mg/g) was found for Ad. It was assumed that Ad was an annual herb, the rest were perennial herbs, and the accumulation of each component in plants was different. Ag and Ae both contain seven components, while β-acetoxyisovaleryl acarnin β and β'-dimethylacrylamine were not detected in Ad. According to the Chinese Pharmacopoeia (2020 edition), the content of β,β'-dimethylacrylamine in *Arnebiae Radix* should not be less than 0.30%. This study found that this component in Ae and

TABLE 3 | Content of seven bioactive components of the 35 *Arnebiae Radix* samples. (mg/g).

	L-shikonin	Acetylshikonin	β-Acetoxyisovaleryl acarnin	Deoxyshikonin	Isobutyryl shikonin	β. β′-dimethylacrylamine	2-methylbutyl Shikonin	Total
Ag(HM)	–	0.69	1.0	–	–	0.84	1.5	4.0
Ag(FY)	–	0.46	0.74	–	–	0.67	1.1	3.0
Ag(BLK)	–	1.1	0.87	0.26	–	0.65	1.2	4.1
Ag(NLK)	0.10	1.6	3.0	0.19	0.38	0.73	2.4	8.4
Ag(TSHKEG)	0.57	8.1	9.8	0.58	2.0	1.3	11	34
Ag(QT)	0.12	2.2	1.3	0.29	–	1.6	1.9	7.3
Ag(HJ)	0.11	1.6	3.2	0.21	0.55	0.95	4.0	11
Ag(YW)	–	0.96	1.0	0.23	–	0.82	1.4	4.5
Ag(SHSH)	0.94	2.9	14	0.21	1.8	1.33	15	36
Ag(TL)	–	1.3	1.2	–	–	0.44	1.0	4.0
Ag(ML)	0.15	3.3	0.81	0.23	–	0.55	1.1	6.1
Ag(QH)	0.14	1.5	1.6	0.26	–	1.31	2.1	6.9
Ag(WQ)	–	0.61	1.2	–	–	0.42	0.72	3.0
Ag(XY)	0.13	4.0	3.5	0.49	0.62	1.33	5.7	16
Average	0.28	2.2	3.1	0.29	1.1	0.92	3.6	11
Ae(WLMQ)	0.23	4.4	1.9	–	1.7	4.6	7.9	21
Ae(ML)	0.21	2.8	1.9	–	1.4	3.0	7.1	16
Ae(BCH)	0.25	2.7	1.3	0.23	1.2	3.0	6.6	15
Ae(NLT)	0.68	22	3.6	0.67	6.0	11	32	76
Ae(TSHKEG)	0.19	1.4	8.4	–	3.2	0.60	4.1	18
Ae(ATSH)	0.67	9.1	2.7	0.30	2.9	5.5	14	35
Ae(WQ)	1.07	9.4	3.7	0.37	8.6	8.0	17	48
Ae(HJ)	0.54	18.9	3.1	0.63	4.4	12	24	63
Ae(HCH)	0.68	12.6	5.4	0.36	7.1	4.1	17	47
Ae(JH)	0.75	12.2	4.7	0.69	6.8	5.1	15	46
Ae(GL)	0.80	20.0	4.2	0.64	4.2	14	26	70
Ae(AKS)	0.36	9.0	1.7	–	2.3	4.2	11	29
Ae(XY)	0.84	25.2	3.8	0.90	5.6	13	29	78
Average	0.56	11.5	3.6	0.53	4.2	6.8	16	43
Ad(FK)	–	4.4	–	0.25	0.43	–	3.3	8.4
Ad(TKS)	–	4.1	–	0.93	0.22	–	1.2	6.4
Ad(MQ)	–	1.7	–	0.29	0.14	–	0.81	3.0
Ad(KLMY)	0.13	8.2	–	0.66	0.32	–	2.4	12
Ad(WS)	–	2.5	–	0.21	0.25	–	1.8	4.7
Ad(SHZ)	–	3.1	–	0.42	0.25	–	1.3	5.0
Ad(BEJ)	–	2.4	–	0.20	0.32	–	2.4	5.3
Ad(SHW)	–	1.5	–	0.20	0.22	–	0.92	2.8
Average	0.13	3.5	–	0.39	0.27	–	1.8	6.0

Ad, A. decumbens*; Ae,* A. euchroma *(Royle) Johnst; Ag,* A. guttata *Bunge.*

Ag met the pharmacopoeia requirements. This study will provide a reference basis for exploring new drug sources.

To establish the chromatographic fingerprint, 35 *Arnebiae Radix* samples from different species were analyzed under the optimized chromatographic analysis conditions. All chromatograms were matched through multipoint correction and free matching, and the bottom sample was the reference sample (Supplementay Figures S1, S2). The representative HPLC fingerprints were so similar that it was difficult to separate the three spaces visually (**Figure 3**).

Principal Component Analysis of HPLC Fingerprint of *Arnebiae Radix* Samples

PCA, a multivariate method, is widely used in data analysis to summarize variation, and is implemented as a data-reduction technique to generate a visual scatter plot for the qualitative evaluation of similarities and differences within multivariate data. To differentiate all the *Arnebiae Radix* samples clearly, we carried out PCA according using the data for the seven common characteristic peaks. The score plot was structured based on the first three principal components, which accounted for more than 94.3% of the total variability. We discarded the other principal components because they had little effect on the model. The results showed that all samples were divided into six groups according to their different sources (**Figure 4**). Group 1 contained two samples belonging to Ag, Group 2 contained four samples belonging to Ae, and Group 3 contained four samples belonging to Ae. Group 4 included five samples belonging to Ae, Group 5 included two samples belonging to Ad, and Group 6 included 12 samples belonging to Ag and six samples belonging to Ad. The results were consistent with the HPLC fingerprint analysis. The results of the HPLC-specific

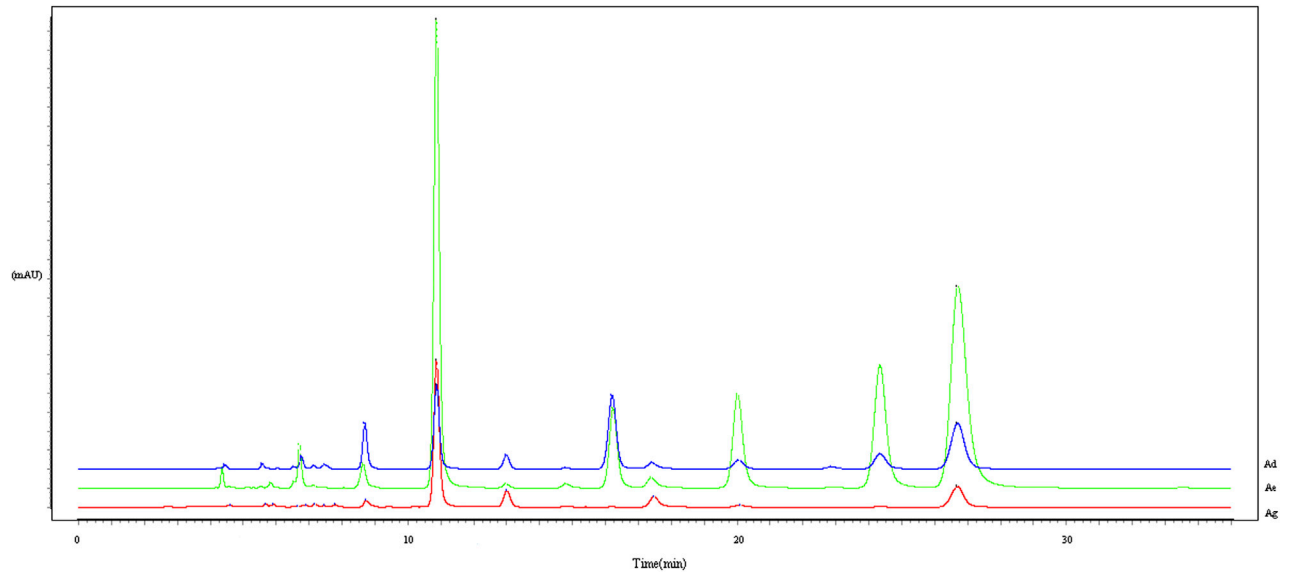


FIGURE 3 | Representative HPLC fingerprints of three *Arnebiae radix* species. Ad, *A. decumbens*; Ae, *A. euchroma* (Royle) Johnst; and Ag, *A. guttata* Bunge.

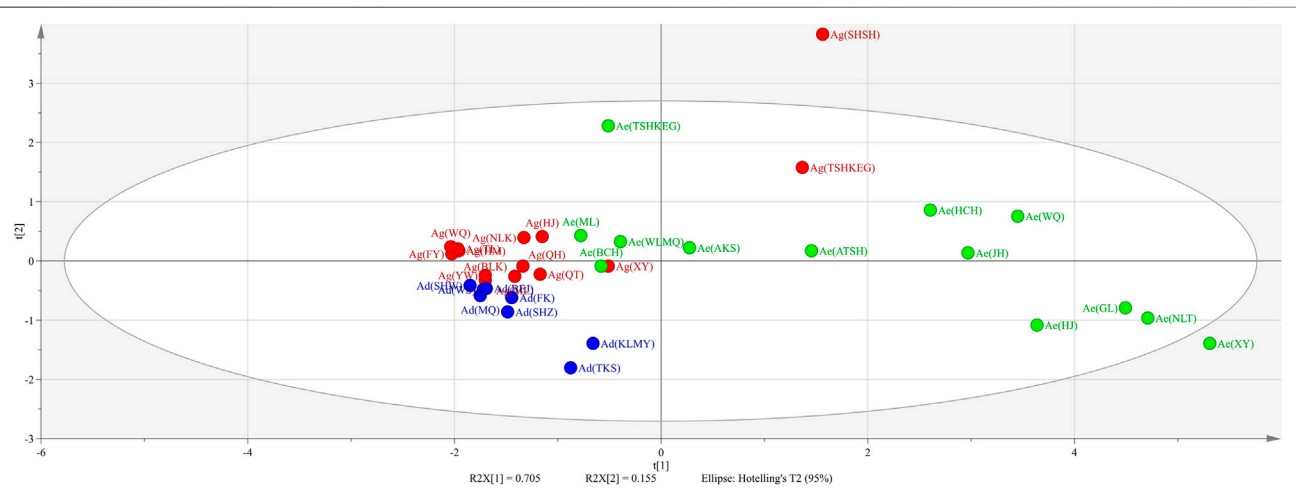


FIGURE 4 | Score plot of PCA of the 35 Arnebiae Radix samples. Ad, *A. decumbens*; Ae, *A. euchroma* (Royle) Johnst; Ag, *A. guttata* Bunge.

chromatograms combined with PCA were not as accurate as those of DNA barcoding.

Hierachical Cluster Analysis of HPLC Fingerprint of *Arnebiae Radix* Samples

HCA is one of the most commonly used unsupervised pattern recognition methods, and is a useful multivariate statistical technique. It can create a cluster tree to assign a data set into groups according to similarity. To show the degree of similarity and difference among the 35 *Arnebiae Radix* samples more clearly, HCA was performed based on the results of PCA of all common characteristic peaks. As shown in **Figure 5**, all samples were divided into two main clusters according to their similarities and differences. Cluster one included three groups: Group 1 contained two samples belonging to Ag, Group 2 contained four samples belonging to Ae, and Group 3 contained four samples belonging to Ae. Cluster two was divided into three groups: Group 4 included five samples belonging to Ae, Group 5 included two samples belonging to Ad, and Group 6 included 12 samples belonging to Ag and six samples belonging to Ad. Group 4 merged with Group 5 to form a larger branch. All samples in the branch were gathered from

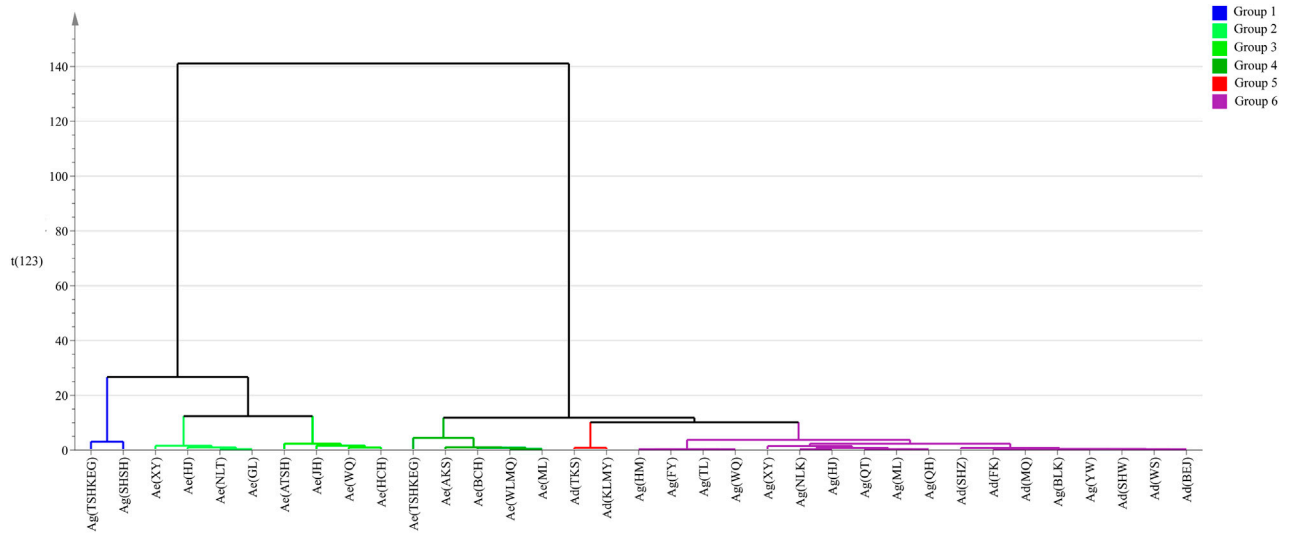


FIGURE 5 | The results of HCA of the 35 *Arnebiae Radix* samples. Calculated with Ward and stored by size. Ad, *A. decumbens*; Ae, *A. euchroma* (Royle) Johnst; and Ag, *A. guttata* Bunge.

Ad, Ae, and Ag. The results of the HPLC-specific chromatograms combined with HCA were not as accurate as those of DNA barcoding.

DISCUSSION

Arnebiae Radix, a commonly used herbal medicine in China, is also widely used in the food and cosmetics industries. As the ecological environment has been constantly destroyed, the wild resources of *Arnebiae Radix* have been sharply reduced, and cultivation is very difficult. There is a serious shortage of *Arnebiae Radix* supplies, leading to a complex and confusing market. The traditional classification method is based on the roots, leaves, flowers, fruits, and other organs of plants. Due to the lack of accurate identification characteristics, the processed commodities only retain the root, which creates great difficulties in the identification of *Arnebiae Radix*. However, the accuracy of the original medicinal materials is required to ensure the effectiveness and safety of clinical medication. DNA barcoding technology techniques are not influenced by organs, growth conditions, tissue differences or the external environment, among other factors (Bhargava and Sharma 2013; Mohammed Abubakar et al., 2017). In this study, the DNA barcoding technique showed the ability to scientifically and accurately identify the species. In the preliminary experimental stage, another three sequences, *matK*, *rbcL*, and ITS, were also considered, but it was found that there were many nested peaks within ITS, and the variable sites measured by *matK* and *rbcL* were not obvious. The established PCR-RFLP method based on the ITS2 sequence can identify *A. euchroma* (Royle) Johnst and *A. guttata* Bunge, as well as other plants also named Zicao in China (Qian et al., 2019). Thus, we examined the ITS2 sequence similarity, genetic distance and phylogenetic tree by using DNA barcoding technology based on its ability to differentiate *Arnebiae Radix* species. Our results suggested that ITS2 can discriminate *A. euchroma* (Royle) Johnst, *A. guttata* Bunge and *A. decumbens* (Vent.) Coss. et Kralik (**Figure 2**). As one of the most important markers in molecular phylogenetic research, the ITS2 sequence has obvious sequence variation at the species level or subspecies level, and it is an important candidate barcode for identification at the species level or subspecies level (Sickel et al., 2015; Cheng et al., 2016).

HPLC-specific chromatograms were established, and the contents of six hydroxyl naphthoquinones can be used to distinguish the different origins of *Arnebiae Radix* herbs (Ke et al., 2016). Therefore, we utilized HPLC methods to differentiate the three *Arnebiae Radix* species. Our results indicated that this method allows for the simultaneous discrimination of the seven main naphthoquinones in these samples. The lichen *A. euchroma* (Royle) Johnst shows high intraspecific chemical variations in Xinjiang, while *A. guttata* Bunge and *A. decumbens (Vent.) Coss. et Kralik*show relatively less variation (**Table 3**). This result could partly be explained by the limited distribution of Ag and Ad, resulting in less variation. Ag and Ad have restricted geographic distributions in western and northern Xinjiang, whereas Ae has a rather wide distribution around Xinjiang. However, the samples could not be distinguished based on HPLC fingerprint (**Figure 3**), PCA (**Figure 4**) and HCA methods (**Figure 5**). Ecological factors, especially altitude, may be responsible for this result. Additionally, other environmental factors, such as light, temperature, air, soil, and moisture, also affect the content of the chemical components of plants. To further develop and utilize the plant resources of *Arnebiae Radix*, it is necessary to study more about the how genetic and

environmental factors influence the metabolites of *Arnebiae Radix*.

In summary, this study has established a system for identifying the three *Arnebiae Radix* species based on DNA barcoding and chemical analysis methods. The results revealed that although the HPLC method cannot differentiate these samples, DNA barcoding can transcend the limitations of HPLC methods to ensure effective and universal proof of medicinal plants from different species. Similar results showed that DNA barcoding was a promising and reliable tool for the identification of three kinds of *Plumeria* flowers compared to HPLC-specific chromatograms, which are generally used (Zhao et al., 2018). Thus, DNA barcoding is more powerful than HPLC fingerprinting for species traceability in related species that are genetically similar. Our findings may be useful for the determination of naphthoquinones of *Arnebiae Radix* and provide a reference for the identification of traditional Chinese medicine based on DNA barcoding. Due to the narrow distribution of *A. tschimganica* (Fedtsch.) G. L. Chu, no sample was used in this experiment. It is necessary to expand the sample size and investigate the corresponding response of different growth periods and growing environments to provide a reference for the quality control and expansion of new drug sources of *Arnebiae Radix*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

CL, DJ and HX designed researches, analyzed data and prepared the manuscript. HX and YW performed the experiment. PL and GR participated in the data analysis and prepared the manuscript. CL and DJ revised the manuscript. All authors read and approved the final article.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 82060696) and Xinjiang Tianshan talent projects. We gratefully thank Wenhuan Ding, Dongdong Wang, Xuejia Zhang and Li Ma for the collection of wild Arnebiae Radix samples.

REFERENCES

Behrens-Chapuis, S., Herder, F., and Geiger, M. F. (2021). Adding DNA Barcoding to Stream Monitoring Protocols - What's the Additional Value and Congruence between Morphological and Molecular Identification Approaches? *PLoS One* 16 (1), e0244598. doi:10.1371/journal.pone.0244598

Bhargava, M., and Sharma, A. (2013). DNA Barcoding in Plants: Evolution and Applications of In Silico Approaches and Resources. *Mol. Phylogenet. Evol.* 67 (3), 631–641. doi:10.1016/j.ympev.2013.03.002

Chen, S., Pang, X., Song, J., Shi, L., Yao, H., Han, J., et al. (2014). A Renaissance in Herbal Medicine Identification: from Morphology to DNA. *Biotechnol. Adv.* 32 (7), 1237–1244. doi:10.1016/j.biotechadv.2014.07.004

Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., et al. (2010). Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS One* 5 (1), e8613. doi:10.1371/journal.pone.0008613

Cheng, T., Xu, C., Lei, L., Li, C., Zhang, Y., and Zhou, S. (2016). Barcoding the Kingdom Plantae: New PCR Primers forITSregions of Plants with Improved Universality and Specificity. *Mol. Ecol. Resour.* 16 (1), 138–149. doi:10.1111/1755-0998.12438

Ding, W. H, Yan, X. H, Ge, L., and Xu, H. Y. (2019). Determination of Naphthoquinones in Roots of Four Species of Arnebia in Xinjiang by HPLC. *Chin. Traditional Patent Med.* 41 (04), 936–939. doi:10.3969/j.Issn.1001-1528.2019.04.047

Feng, J., Yu, P., Zhou, Q., Tian, Z., Sun, M., Li, X., et al. (2020). An Integrated Data Filtering and Identification Strategy for Rapid Profiling of Chemical Constituents, with Arnebiae Radix as an Example. *J. Chromatogr. A* 1629, 461496. doi:10.1016/j.chroma.2020.461496

Fu, D., Shang, X., Ni, Z., and Shi, G. (2016). Shikonin Inhibits Inflammation and Chondrocyte Apoptosis by Regulation of the PI3K/Akt Signaling Pathway in a Rat Model of Osteoarthritis. *Exp. Ther. Med.* 12 (4), 2735–2740. doi:10.3892/etm.2016.3642

Gunnels, T., Creswell, M., McFerrin, J., and Whittall, J. B. (2020). The ITS Region Provides a Reliable DNA Barcode for Identifying Reishi/lingzhi (Ganoderma) from Herbal Supplements. *PLoS One* 15 (11), e0236774. doi:10.1371/journal.pone.0236774

Guo, H., Sun, J., Li, D., Hu, Y., Yu, X., Hua, H., et al. (2019). Shikonin Attenuates Acetaminophen-Induced Acute Liver Injury via Inhibition of Oxidative Stress and Inflammation. *Biomed. Pharmacother.* 112, 108704. doi:10.1016/j.biopha.2019.108704

Hu, L., Zhou, C., Huang, Y. C., Wang, Y., Wei, G., Liang, Z., et al. (2020). HPLC Coupled with Electrospray Ionization Multistage MS/MS and TLC Analysis of flavones-C-glycosides and Bibenzyl of Dendrobium Hercoglossum. *J. Sep. Sci.* 43 (20), 3885–3901. doi:10.1002/jssc.202000647

Huang, X. Y., Fu, H. L., Tang, H. Q., Yin, Z. Q., Zhang, W., Shu, G., et al. (2020). Optimization Extraction of Shikonin Using Ultrasound-Assisted Response Surface Methodology and Antibacterial Studies. *Evid. Based Complement. Alternat Med.* 2020, 1208617. doi:10.1155/2020/1208617

Jia-Xin, T., F., G., Zhi-Lai, Z., et al. (2018). Herbalogical Textual of Arnebiae Radix and Analysis of Medicinal Resources. *Mod. Chin. Med.* 20 (9), 1064–1067. doi:10.13313/j.issn.1673-4890.20180428002

Ke, Z., Rui, S., Aijun, T., Hong, W., Jie, L., Linong, G., et al. (2016). HPLC Specific Chromatogram of Arnebia Euchroma and Determination of Six Naphthoquinones in Boraginaceous Herbs. *Chin. J. Pharm. Anal.* 36 (9), 1526–1535. doi:10.16155/j.0254-1793.2016.09.03

Khan, S. A., Baeshen, M. N., Ramadan, H. A., and Baeshen, N. A. (2019). ITS2: An Ideal DNA Barcode for the Arid Medicinal Plant Rhazya Stricta. *Pharm. Med.* 33 (1), 53–61. doi:10.1007/s40290-019-00266-3

Liao, M., Yan, P., Liu, X., Du, Z., Jia, S., Aybek, R., et al. (2020). Spectrum-effect Relationship for Anti-tumor Activity of Shikonins and Shikonofurans in Medicinal Zicao by UHPLC-MS/MS and Chemometric Approaches. *J. Chromatogr. B* 1136, 121924. doi:10.1016/j.jchromb.2019.121924

Liu, B., Hu, T., and Yan, W. (2020). Authentication of the Bilberry Extracts by an HPLC Fingerprint Method Combining Reference Standard Extracts. *Molecules* 25 (11), 2514. doi:10.3390/molecules25112514

Liu, C., He, L., Wang, J., Wang, Q., Sun, C., Li, Y., et al. (2020). Anti-angiogenic Effect of Shikonin in Rheumatoid Arthritis by Downregulating PI3K/AKT and MAPKs Signaling Pathways. *J. Ethnopharmacology* 260, 113039. doi:10.1016/j.jep.2020.113039

Liu, Z., Zeng, X., Yang, D., Ren, G., Chu, G., Yuan, Z., et al. (2012). Identification of Medicinal Vines by ITS2 Using Complementary Discrimination Methods. *J. Ethnopharmacology* 141 (1), 242–249. doi:10.1016/j.jep.2012.01.057

Ma, S. J., Geng, Y., Ma, L., et al. (2021). Advances in Studies on Medicinal Radix Arnebia. *Mod. Chin. Med.* 23 (01), 177–184. doi:10.13313/j.issn.1673-4890.20190719004

Mei, L., Ling-Feng, W., and Hong-Liang, J. (2020). Reviews on Natural Quinones and Their Bioactivities of Medicinal Zicao. *Nat. Product. Res. Dev.* 32 (04), 694–707. doi:10.16333/j.1001-6880.2020.4.021

Mohammed Abubakar, B., Mohd Salleh, F., Shamsir Omar, M. S., and Wagiran, A. (2017). Review: DNA Barcoding and Chromatography Fingerprints for the Authentication of Botanicals in Herbal Medicinal Products. *Evid. Based Complement. Alternat Med.* 2017, 1352948. doi:10.1155/2017/1352948

Qian, L., LIu, J., Guo, L., Zheng, J. I., and Ma, S. (2019). Molecular Identification of Arnebiae Radix by PCR-RFLP Based on the ITS2 Sequence. *Chin. J. Pharm. Anal.* 36 (9), 1611–1617. doi:10.16155/j.0254-1793.2016.09.14

Selva Pandiyan, A., Siva Ganesa Karthikeyan, R., Rameshkumar, G., Sen, S., and Lalitha, P. (2020). Identification of Bacterial and Fungal Pathogens by rDNA Gene Barcoding in Vitreous Fluids of Endophthalmitis Patients. *Semin. Ophthalmol.* 35, 358–364. doi:10.1080/08820538.2020.1864416

Sickel, W., Ankenbrand, M. J., Grimmer, G., Holzschuh, A., Härtel, S., Lanzen, J., et al. (2015). Increased Efficiency in Identifying Mixed Pollen Samples by Meta-Barcoding with a Dual-Indexing Approach. *BMC Ecol.* 15, 26194794. doi:10.1186/s12898-015-0051-y

Sun, W. X., Liu, Y., Zhou, W., Li, H. W., Yang, J., and Chen, Z. B. (2017). Shikonin Inhibits TNF-Alpha Production through Suppressing PKC-NF-kappaB-dependent Decrease of IL-10 in Rheumatoid Arthritis-like Cell Model. *J. Nat. Med.* 71 (2), 349–356. doi:10.1007/s11418-016-1064-3

Xu, C. M., Li, D., Wang, Y. Y., et al. (2014). Research Development of Food Pigment Biosynthesis. *J. Chin. Inst. Food Sci. Technol.* 14 (02), 225–233. doi:10.16429/j.1009-7848.2014.02.046

Yu, J., Wu, X., Liu, C., Newmaster, S., Ragupathy, S., and Kress, W. J. (2021). Progress in the Use of DNA Barcodes in the Identification and Classification of Medicinal Plants. *Ecotoxicol. Environ. Saf.* 208, 111691. doi:10.1016/j.ecoenv.2020.111691

Zhan, Z. L., Hu, J., Liu, T., Kang, L. P., Nan, T. G., and Guo, L. P. (2015). Advances in Studies on Chemical Compositions and Pharmacological Activities of Arnebiae Radix. *Zhongguo Zhong Yao Za Zhi* 40 (21), 4127–4135.

Zhang, J., Hu, X., Wang, P., Huang, B., Sun, W., Xiong, C., et al. (2018). Investigation on Species Authenticity for Herbal Products of Celastrus Orbiculatus and Tripterygum Wilfordii from Markets Using ITS2 Barcoding. *Molecules* 23 (4). doi:10.3390/molecules23040967

Zhao, L., Yu, X., Shen, J., and Xu, X. (2018). Identification of Three Kinds of Plumeria Flowers by DNA Barcoding and HPLC Specific Chromatogram. *J. Pharm. Anal.* 8 (3), 176–180. doi:10.1016/j.jpha.2018.02.002

Zhao, X., Zhou, Y., Wang, L., Li, M., Shi, D., Li, D., et al. (2017). Shikonin Alleviates the Biotoxicity Produced by Pneumococcal Pneumolysin. *Life Sci.* 177, 1–7. doi:10.1016/j.lfs.2017.04.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer (YS) declared a shared affiliation with several of the authors, DJ, PL, GR, CL, to the handling editor at time of review

frontiers in Pharmacology

REVIEW
published: 23 August 2021
doi: 10.3389/fphar.2021.712531

Neuroprotective Potential of Non-Digestible Oligosaccharides: An Overview of Experimental Evidence

Gangaraju Divyashri[1], Bindu Sadanandan[1], Kotamballi N Chidambara Murthy[2], Kalidas Shetty[3] and Kumari Mamta[1]*

[1]Department of Biotechnology, M S Ramaiah Institute of Technology, Bengaluru, India, [2]Central Research Laboratory and Division of Research and Patents, Ramaiah Medical College and Hospital, Bengaluru, India, [3]Department of Plant Science, North Dakota State University, Fargo, ND, United States

Non-digestible oligosaccharides (NDOs) from dietary sources have the potential as prebiotics for neuroprotection. Globally, diverse populations suffering from one or the other forms of neurodegenerative disorders are on the rise, and NDOs have the potential as supportive complementary therapeutic options against these oxidative-linked disorders. Elevated levels of free radicals cause oxidative damage to biological molecules like proteins, lipids, and nucleic acids associated with various neurological disorders. Therefore, investigating the therapeutic or prophylactic potential of prebiotic bioactive molecules such as NDOs as supplements for brain and cognitive health has merits. Few prebiotic NDOs have shown promise as persuasive therapeutic solutions to counter oxidative stress by neutralizing free radicals directly or indirectly. Furthermore, they are also known to modulate through brain-derived neurotrophic factors through direct and indirect mechanisms conferring neuroprotective and neuromodulating benefits. Specifically, NDOs such as fructo-oligosaccharides, xylo-oligosaccharides, isomalto-oligosaccharides, manno-oligosaccharides, pectic-oligosaccharides, and similar oligosaccharides positively influence the overall health *via* various mechanisms. Increasing evidence has suggested that the beneficial role of such prebiotic NDOs is not only directed towards the colon but also distal organs including the brain. Despite the wide applications of these classes of NDOs as health supplements, there is limited understanding of the possible role of these NDOs as neuroprotective therapeutics. This review provides important insights into prebiotic NDOs, their source, and production with special emphasis on existing direct and indirect evidence of their therapeutic potential in neuroprotection.

Keywords: free radical, oxidative stress, prebiotics, non-digestible oligosaccharides, neuromodulation, neuroprotection

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Murali Muralidhara, Central Food Technological Research Institute (CSIR), India
Loh Teng Hern Tan, Monash University Malaysia, Malaysia
Vijay Omprakash Kothari, Nirma University, India

****Correspondence:***
Bindu Sadanandan
bindu@msrit.edu

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *20 May 2021*
Accepted: *26 July 2021*
Published: *23 August 2021*

Citation:
Divyashri G, Sadanandan B, Chidambara Murthy KN, Shetty K and Mamta K (2021) Neuroprotective Potential of Non-Digestible Oligosaccharides: An Overview of Experimental Evidence. Front. Pharmacol. 12:712531. doi: 10.3389/fphar.2021.712531

INTRODUCTION TO PREBIOTICS AND ITS TYPES

A balanced diet with good nutrition and physical exercise confers beneficial effects on human health and wellness. On the other hand, unhealthy lifestyle and environmental stresses can lead to several associated challenges like the onset of various diseases including mental disorders. Currently, mental disorders affect around 450 million people worldwide, causing neuro-cognitive breakdowns that are the leading causes of poor health (WHO, 2019). Enhanced levels of reactive oxygen species (ROS)

and insufficient antioxidant defense mechanisms to counter them have been associated with the pathogenesis of various mental disorders including anxiety, depression, schizophrenia, Parkinson's disease (PD), Alzheimer's disease (AD), and many others (Manoharan et al., 2016). Prebiotics are a group of non-digestible oligosaccharides (NDOs) that are biotransformed by beneficial colonic microorganisms (probiotics) with potential health benefits to the host. Probiotics are live microorganisms which, when administered in adequate amounts, confer health benefits on the host. These gut-residing probiotics support the host health by offering resistance to pathogens, regulating the immune system, modifying insulin resistance and metabolic profile (Kelly et al., 2015), and also influencing behavioral and neurological functions (Hansan and Yang 2019). Prebiotics are widely known to modulate probiotics in humans and animals, with an overall beneficial impact on health. Although the focus of the use of prebiotics was initially towards treating digestive ailments over the last decade, the efficacy of prebiotics to suppress ROS and to positively influence mental health *via* modulation of gut-residing probiotics and through impact on the gutbrain axis is also documented (Ansari et al., 2020). Nutritional modulation of gut probiotics by prebiotics results in the formation of key beneficial metabolites such as short-chain fatty acids-SCFA, and inflammatory and immune markers which potentially offer "distal" health benefits to the brain (Cerdo et al., 2019). However, the challenge with understanding the possible role of these prebiotic functional components in brain development and function is the lack of clarity on the metabolic function and benefits of the interaction of the intestinal microbiota with the central nervous system (CNS). Considering the therapeutic benefits and applications of prebiotics, it is important to understand their neuroprotective function to integrate them into health solutions and overall wellness.

Definitions of prebiotics and global demand: Prebiotic oligosaccharides are microbiota-modulating compounds as they can serve as a carbon source that supports the growth of probiotics, thereby conferring specific or selective change in the gut to support host health *via* improvements in metabolic functions (Carlson et al., 2018). There have been several studies to develop prebiotics towards improving human health. With the increased demand for healthier foods, the interest in prebiotics has grown. The idiom "prebiotics" has caused some discrepancy and confusion among consumers worldwide. The term "prebiotics" was coined by Gibson and Roberfroid in 1995 as "non-digestible food ingredients that can be useful, affecting the host by selectively stimulating the growth and/or activity of one or more limited number of bacteria in the colon, thus improving host health (Gibson and Roberfroid 1995). In line with this definition, only a few compounds of the carbohydrate group mainly oligosaccharides, *viz.*, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), manno-oligosaccharides (MO), xylo-oligosaccharides (XOS) are classified as potential prebiotics (Davani-Davari et al., 2019). Since then many scientific definitions of prebiotics have evolved. The present scientific definition of prebiotics was presented in 2017 at the International Scientific Association for Prebiotics and Probiotics (ISAPP) by a panel of experts from the domains of microbiology, nutrition, and clinical research (Gibson et al., 2017). The panel-defined prebiotics as a substrate that is selectively utilized by host microorganisms conferring a health benefit (Scott et al., 2020). Thus, according to the above scientific consensus, for any compound to be called a prebiotic, it should act as a substrate for health-promoting gut microorganisms and must possess a physiological effect benefiting the host. In addition to oligosaccharide-based prebiotics, dietary fibers such as resistant starch, inulin, pectin, and beta-glucans also fit the definition of prebiotics. This updated definition paves the way for deepening our perception and understanding of prebiotics.

Regulatory agencies across the globe have their own definitions for prebiotics. Almost all the regulatory and scientific definitions consider prebiotics as dietary fibers (**Table 1**). However, there is more than one prebiotic that will not fall under the dietary fiber category (Carlson et al., 2018). The U.S. Food and Drug Administration (USFDA) and Food and Agriculture Organization/World Health Organization (FAO/WHO) clearly distinguish between prebiotic dietary fiber and other qualifying prebiotic compounds. The US-FDA defines the prebiotic dietary fiber as isolated or synthetic non-digestible soluble or insoluble carbohydrates with monomeric units ≥ 3, possessing physiologic effects that are beneficial to human health. The FAO/WHO states prebiotic dietary fibers as carbohydrate polymers comprising monomeric units (10 or more) that resist hydrolysis in the human small intestine by the endogenous enzymes. On the other hand, the US-FDA allows a biologically based group of foods conferring health benefits on the host to qualify as prebiotic compounds. On similar lines, the FAO/WHO defines other qualifying compounds as prebiotics when any non-viable food component results in conferring health benefits associated with microbiota modulation. Conversely, other regulatory agencies across the globe, *viz.*, the Food for Specified Health Use (FOSHU), European Food Safety Authority (EFSA), Health Canada, and Food Safety and Standards Association of India (FSSAI), have a general definition for prebiotics (**Table 1**). These definitions make a point that not all prebiotics are carbohydrates and all dietary fibers are prebiotics (Davani-Davari et al., 2019). The criteria used for categorizing a compound as prebiotics are that it should be resistant to the stomach's acidic pH, resistant to hydrolysis by mammalian enzymes, can be fermented selectively by the intestinal microbiota, promote the activity, and/or growth of the microbes present in the intestine. A prebiotic fiber should possess a degree of polymerization equal to or higher than 3 (Davani-Davari et al., 2019).

It has been difficult to assess and measure the global consumption of prebiotics since they are found in varied food groups from natural sources, *viz.*, vegetables, fruits, milk, and honey, to wide ranges of supplements (de Souza Aquino et al., 2017; Carlson et al., 2018). The prebiotics from natural sources include resistant starch, GOS, FOS, XOS, POS, and other oligosaccharides (de Souza Aquino et al., 2017). Without the inclusive list of food ingredients on the food package, epidemiologic tracking of prebiotic consumption pattern is difficult to ascertain (Carlson et al., 2018). However, growing

TABLE 1 | Regulatory definitions of prebiotic dietary fiber and other qualifying compounds.

Regulatory agency	Definition of Prebiotic Dietary Fiber	Definition of other qualifying compounds as Prebiotics
United States-Food and Drug Administration (US-FDA)	Synthetic or isolated non-digestible (soluble or insoluble) carbohydrates made up of monomeric units ≥ 3, and possessing physiologic effects that are beneficial to human health	Biologically-based group of foods conferring health benefits on the host
Food and Agriculture Organization/World Health Organization (FAO/WHO)	Carbohydrate polymers comprising of monomeric units (10 or more) that resist hydrolysis by the endogenous enzymes that naturally occur in the human small intestine	Non-viable food components that confer health benefits on the host by modulating beneficial gut microbiota
Food for Specified Health Use (FOSHU), Japan	Principal food ingredient that is officially approved to claim its physiological effects on human body (Brown well et al., 2012)	
European Food Safety Authority (EFSA)	Non-viable food ingredient that confers health benefits to host by modulating the gut microbiota (Definition adapted from the FAO/WHO)	
Health Canada	Term "prebiotic" is allowed only for products that are required for an approved health claim	
Food Safety and Standards Association of India (FSSAI), India	Non-viable food ingredient that provides health benefits to host by modulating the gut microbiota (Definition adapted from the FAO/WHO)	

awareness of gut–brain connection and the consequential focus on maintaining gut health have enhanced the demand and the need for bioactive prebiotics (Hwang and Lee, 2019). Worldwide, there is a substantial growth in prebiotics demand, and this is anticipated to grow, beyond $7.5 billion by 2023 (Carlson et al., 2018). Currently, there are no authorized dietary recommendations for the adequate intake of prebiotics in healthy individuals. To maintain gut health, most prebiotics require an average oral dose of 3–5 g/day. However, daily dose recommendations depend on the nature of the food containing a prebiotic compound whether it is naturally present or specifically added (de Souza Aquino et al., 2017). Scientific evidence indicates that the consumption of inulin (5–8 g/day), FOS (3 g/day), and GOS (4 g/day) led to a significant increase in fecal probiotic bifidobacteria (de Souza Aquino et al., 2017).

Occurrence: Prebiotics exist naturally in several foods and can also be synthesized commercially using various enzymes or substrates (Lockyer and Stanner, 2019). Presently, a number of prebiotics are being investigated, and majority of them belong to a carbohydrate group and are generally oligosaccharide carbohydrates (Davani-Davari et al., 2019). These oligosaccharide carbohydrates include FOS, GOS, IMO, MO, POS, XOS, and chitin oligosaccharides (COS). FOS and GOS are most in demand on the global market currently. Published literature mostly discusses non-digestible oligosaccharide carbohydrates (NDOs) and polysaccharide prebiotics, *viz.*, inulin and resistant starch, which is well established to possess prebiotic activity. There are reports on the effects of NDOs and polysaccharide prebiotics on gut microbiota modulation (Dahiya et al., 2017; Gibson et al., 2017). Inulin is shown to counter the detrimental effects of high-fat diets on the mucus layer penetrability and metabolic functions (Schroeder et al., 2018). The mixture of FOS and GOS was able to modulate bifidobacteria by suppressing *Clostridium* levels in the gut whereas GOS alone enhanced *Lactobacillus* levels (Vandenplas, Zakharova and Dmitrieva, 2015). In addition, there is also some evidence for disaccharides such as lactulose and lactitol as potent prebiotics. Lactulose, an isomer of lactose, was shown to modulate gut microbiota by stimulating the growth of beneficial microorganisms. Gibson et al. (2010) synthesized lactulose-derived GOS, and lactulose was demonstrated to improve the quality of human life suffering from hepatic encephalopathy (Shukla et al., 2011). Investigations on evaluating the neuroprotective therapeutic mechanism of lactulose are also highlighted in the sections below (Lee et al., 2021). This review discusses prebiotic NDOs production in the introduction section, and their probable mechanisms/routes through which they offer mental health protection are highlighted in the subsequent section. Evidences in rodent models and humans are explained by the observed therapeutic effects of prebiotics. The probable mechanism through which neuroprotection effect is observed is highlighted in a separate section. The last section identifies the obvious gaps in current knowledge and avenues for future investigations.

Prebiotic Oligosaccharide Carbohydrates

Prebiotic oligosaccharides are carbohydrates that are chemically stable at a wide range of temperatures and pH and are classified as non-digestible oligosaccharides (NDOs) (Singh et al., 2017). Health attributes of prebiotic NDOs have been extensively reviewed and are accepted worldwide (**Figure 1**). However, these effects have been primarily observed towards the colon, but evidence indicates that the NDO prebiotics have the ability to modulate beyond the GIT. Beneficial gut microorganisms, *viz.*, *Lactobacillus* sp. and *Bifidobacterium* sp. selectively ferment NDOs, thereby producing a wide range of metabolites in the gut, *for example*, short-chain fatty acids (SCFAs), including butyric acid, acetic acid, and propionic acid. (Hutkins et al., 2016; Davani-Davari et al., 2019). These SCFAs (straight or branched volatile fatty acids) as metabolic products are reported to have beneficial effects on the human body (Singh et al., 2017). Acetic acid (C2) is a key metabolite in the ability of bifidobacteria to inhibit gut-related pathogens (Rios-Covian et al., 2016). Treatment for subcutaneous adipose tissue with propionic acid (C3) results in the downregulation of macrophage markers (CD163 and MMP-9) and inflammatory parameters, *viz.*, TNF-α and IP-10 (Stinson et al., 2017; Rezaee, 2019). Butyric acid (C4) is reported to alter bacterial adhesion to the gut wall by increasing mucus production (Jung et al., 2015). Therefore, SCFA appears to be very important in maintaining gut barrier function and acts as a mediator in the link between

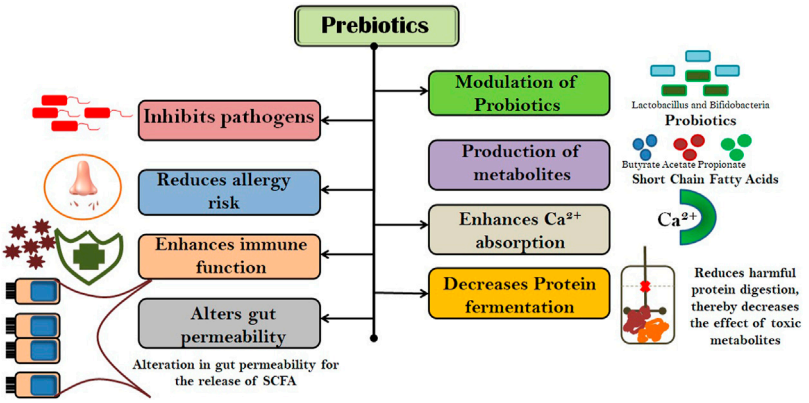


FIGURE 1 | Health attributes of prebiotic NDOs.

nutrition, gut microbiota, and human physiology. A study by Clarke et al. (2010) showed that prebiotic fermentation also produces peptidoglycan that enhances the innate immune system *via* downregulating bone-marrow-derived neutrophils against *Staphylococcus aureus* and *Streptococcus pneumoniae*. Thus, the effect of prebiotics on human health is mediated through their fermentation products in the gut.

Fructo-oligosaccharides (FOS): FOS occur naturally in various plant sources including onion, asparagus, *Jerusalem artichokes*, and wheat. However, FOS concentration in these sources is not sufficient to offer prebiotic activity, and therefore, there is a need for its synthesis to provide a higher dose. FOS are commercially produced using 2 processing methods: 1) batch production of FOS using sucrose as a substrate and employing fructosyltransferase (FTase) and 2) continuous production using sucrose as a substrate and immobilized FTase (Ashwini et al., 2019). Several microbes produce significant titers of FTase. These include species in the genera of *Aspergillus*, *Aureobasidium*, *Penicillium*, and *Fusarium* (Davani-Davari et al., 2019). FOS produced from FTase are a mixture of oligosaccharides with a degree of polymerization (DP) 3–5. Structurally, FOS consist of fructose units linked to the terminal glucose *via* β-(1, 2) linkages. Galacto-oligosaccharides (GOS) are commercially produced from lactose by β-galactosidase. However, previous attempts in commercial production of GOS using galactosyl-transferase were not very successful and economical because galactosyl-transferase is more stereoselective than β-galactosidase, and it requires nucleotide sugars as the donor (Davani-Davari et al., 2019). A wide range of microorganisms (bacteria, fungi, and yeast) produce β-galactosidase, and this affects GOS production in terms of the degree of polymerization (DP), amount of GOS produced, and glycosidic linkages (Osman et al., 2012). Structurally, GOS consist of oligosaccharides mixture comprising of galactose units linked to a terminal glucose moiety with DP ranging from 3 to 8 linked *via*., β (1–6), β (1–3), and α (1–4) linkages (Torres et al., 2010; Vera et al., 2016). Xylo-oligosaccharides (XOS): XOS occur naturally in honey, bamboo shoots, milk, fruits, and vegetables (Acharya and Prapulla 2010). XOS consist of xylose molecules connected by β (1–4) linkages with DP ranging from 2 to 10. Xylan, being the main hemicellulosic component in the lignocellulosic materials (LCMs), represents a potential source for XOS production (Jain et al., 2015). XOS can be produced by the hydrolysis of LCMs *via* chemical, enzymatic, or combination of chemical and enzymatic (chemo-enzymatic) methods at an industrial scale (Jain et al., 2015). The enzymatic process for XOS production from xylan has proven to be favorable for the manufacture of pharmaceutically important and food-grade XOS using food-grade xylanolytic enzymes (endo-xylanase, exo-xylanase, β-xylosidase, and debranching enzymes) (Acharya and Prapulla, 2011; Mamo et al., 2013). Attempts have also been made to use immobilized xylanase to produce XOS with lower DP (2 and 3) for its potential biotechnological applications (Sukri and Sakinah, 2018). Isomalto-oligosaccharides (IMO): IMO are made up of glucose units linked through α (1–6) glycosidic bonds using glucose units. Natural sources of IMO include honey, miso, soy sauce, and sake. Commercially, it is produced through a two-stage process using starch as a substrate. Starch is first hydrolyzed to liquefied starch by using a mixture of α-amylase and pullulanase. Then, α-amylase hydrolyses liquefied starch to maltose. In the second stage using the transglucosidase activity of α-glucosidase, IMO are produced from maltose (Callaway and Ricke, 2011). Other emerging prebiotic oligosaccharides: In addition to the above-mentioned well-researched NDOs, there are other oligosaccharides too which are emerging and have good potential. These include pectic-oligosaccharides (POS), manno-oligosaccharides (MO), and chitin-oligosaccharides (COS). POS are synthesized from pectin using pectinase. Many investigations have been directed at the use of pectin-rich agro-industrial by-products (citrus peel, orange peel, apple pomace) as a source of POS (Babbar et al., 2016). MO are produced by enzymatic hydrolysis of the commercial substrate using β-mannanase (Singh et al., 2018). Attempts have also been made to produce MO from mannan-rich agricultural by-products (Jian et al., 2013; Jana and Kango, 2020). COS can be isolated from natural sources or can be produced by chitin hydrolysis using chitinase (Ahmed et al., 2012; Qin and Zhao, 2019). Recently, the neuroprotective properties of feruloylated oligosaccharides (FOs) from maize bran have been evaluated (Li et al., 2020).

Ideal Physicochemical and Biological Environment for Optimum Benefits of Prebiotics

Prebiotics promote the activation of microflora in the intestine and enhance the absorption of essential bioactive molecules through fermentation or other enzymatic activity. Therefore, the micro-environment in the gut plays a vital role in the optimum benefits of these compounds. The majority of the prebiotics are active at a pH range of 7.0–8.0. Other factors including temperature, oxygen status, digestive enzymes, and the presence of H_2O_2 are also known to influence the activity of both prebiotics and probiotics. Since fermentation is the major process in the enhancement of the growth of probiotics by prebiotics, the presence of antimicrobial peptides, immunoglobulin A, microRNA, and fecal microbiota transplantation are some of the non-specific host factors known to influence the outcome (Hasan and Yang 2019) and the host-associated factors are age, disease status, genetic makeup, diet, and lifestyle. The presence of metal ions is also known to influence the activity of prebiotics. Metal ions like Mg^{2+}, $Fe^{2+,}$ Mn^{2+}, and Zn^{2+}mediate enzyme activation and protein translation. Fe^{2+} and Mn^{2+} are said to be associated extensively with the ribosome and can replace the activity of Mg^{2+} (Bray et al., 2018). Therefore, it is essential to consider host, external, and other factors (diet, lifestyle use of drugs and antibiotics) while considering the health benefits or influence of prebiotics on metabolic changes in probiotics.

METHODOLOGY

Biomedical literature databases (PubMed and Google Scholar) were searched using neuroprotection, oligosaccharides, prebiotics, non-digestible oligosaccharides, brain health, oxidative stress, or neurodegeneration as keywords within the article title. Peer-reviewed articles dealing with neuroprotective prebiotic oligosaccharides were critically read and included in the review. Research and review articles about preclinical or clinical trials on the therapeutic potential of prebiotics in brain health were analyzed for inclusion in this review. ClinicalTrails.gov website was referred to analyze the articles on human trials. At the preclinical and clinical level, analysis focused on prebiotics type and dose, subjects, mode of administration, treatment duration, and measurable biochemical outcomes.

THERAPEUTIC SPECTRUM OF PREBIOTIC OLIGOSACCHARIDES AND THEIR NEUROPROTECTIVE EFFECT: VALIDATED EXPERIMENTAL EVIDENCE ON PROBABLE MECHANISMS

Evidence in Animal Models

A substantial body of evidence suggests that SCFA produced *via* the fermentation of prebiotic NDOs by colonic probiotics plays a key role in maintaining brain homeostasis (van de Wouw et al., 2018; Silva et al., 2020). Results have indicated that approximately 500–600 nmol of SCFAs/day was produced in the molar proportion of 60:20:20 of acetate, propionate, and butyrate, respectively (Dalile et al., 2019). After their production in the colon, SCFAs subsequently get rapidly absorbed by colonocytes and the unabsorbed SCFAs get transported into the portal circulation. Furthermore, a small amount of the colon-derived SCFAs reaches the peripheral tissues and systematic circulation (Boets et al., 2015). The complete mechanism of how SCFAs influence brain function is not understood well.

Several animal studies have determined that SCFAs exert prevalent effects on key neurological and behavioral processes and are implicated in the crucial phases of neurodevelopmental and neurodegenerative disorders (Dalile et al., 2019; Fung et al., 2017) (**Table 2**). SCFAs influence brain functions by binding as endogenous ligands to G protein-coupled free fatty acid receptors (FFARs) and/or by inhibiting histone deacetylase in the brain (Vijay and Morris, 2014; Dalile et al., 2019). Multiple evidence has revealed that the functional SCFA receptors are localized in the gastrointestinal mucosa and the CNS (Parada Venegas et al., 2019). SCFAs are known to regulate systemic function through the inhibition of histone deacetylase activity (Lin et al., 2015). Histone deacetylase (HDAC) enzymes catalyze the exclusion of acetyl groups resulting in the interaction of the positively charged histones with the negatively charged DNA, thereby leading to a transcriptionally repressive, more compacted chromatin conformation (Licciardi et al., 2011). Intracellular butyrate and other SCFAs are reported to inhibit HDAC activity (Dalile et al., 2019), and the intracellular HDAC inhibition signaling found in the gut and remote organs is on binding to cell surface receptors (Stilling et al., 2016). However, the mechanism of this signaling is not clearly understood.

Psychiatric disorders including depression and anxiety are closely associated with histone acetylation (Stilling et al., 2016). In animal models, SCFA-induced histone hyperacetylation such as a reduction in depressive behavior has been observed (Schroeder et al., 2007; Wei et al., 2015). Schroeder et al. (2007) demonstrated the ability of sodium butyrate (single dose: 1.2 g/kg BW) as an HDAC inhibitor in combination with a selective serotonin reuptake inhibitor, fluoxetine (10 mg/kg BW, antidepressant drug). Acute and chronic administration of sodium butyrate alone or in combination with fluoxetine in mice (C57BL/6J) for a period of 28 days induced short-lasting histone hyperacetylation in the hippocampus and frontal cortex, thereby exerting anti-depressant like effects. On similar lines, histone hyperacetylation following intraperitoneal injection of sodium butyrate (1.2 g/kg BW daily for 4 weeks) in CK-p25 Tg mice improved learning and memory in wild-type mice and in mice with brain atrophy (Fischer et al., 2007). Conversely, excessive levels of SCFA might have had adverse effects on brain health and behavior. Elevations in propionic acid are shown to induce autism-like symptoms in animal models through the formation of propionyl coenzyme A (CoA) and sequestration of carnitine (MacFabe, 2012; MacFabe, 2015). In another study, prenatal and early life administration of propionic

TABLE 2 | Selected *in vivo* studies at a preclinical level using prebiotics and/or their metabolites (SCFA) and probiotics to modulate brain physiology and function.

Prebiotics/Probiotics/SCFA	Feeding duration	Model organism	Observation	References
Sodium butyrate (1.2 g/kg BW) in combination with fluoxetine (10 mg/kg BW)	4 weeks	C57BL/6J mice	Histone hyperacetylation in the hippocampus and frontal cortex for short-term, thereby exerted anti-depressant like effects	Schroeder et al. (2007)
Sodium butyrate (1.2 g/kg BW)	4 weeks	CK-p25 Tg mice	Histone hyperacetylation improved learning and memory	Fischer et al. (2007)
Propionic acid (500 mg/kg BW, twice a day)	3 weeks	Pregnant Long-Evans rats	Enhancement in repetitive behavior associated with ASD	Foley et al. (2014)
Sodium butyrate (1.2 g/kg BW)	5 weeks	Female ICR (CD1) mice	Increased histone H4 acetylation in hippocampus facilitated amelioration of memory impairment	Takuma et al. (2014)
GOS (4 g/kg BW)	Postnatal days (3–21)	Neonatal rats	Alterations in BDNF levels, synaptic proteins (synaptophysin, MAP2, and GAP43), and NMDAR subunits (GluN1, GluN2A, GluN2B)	Williams et al. (2016)
E. faecium (4×10^8 CFU) Inulin (860 mg/kg BW) *E. faecium* + inulin (4×10^8 CFU + 860 mg/kg BW)	5 weeks	Sprague-Dawley male rats	Lower levels of pro-inflammatory cytokines and higher levels of BDNF in the hippocampus region of synbiotic and prebiotic treated animals Significant increase in butyrate concentration after synbiotic and prebiotic supplementation	Araiza et al., 2018
GOS (15 g/L)	3 weeks	SPF Sprague-Dawley male adult rats	Downregulated activation of microglial cells, thereby reducing surgery-induced cognitive impairments	Yang et al. (2018)
Polydextrose-GOS (7 and 15 g/kg BW for mice and rats, respectively)	Postnatal days (21–50)	Weanling male C57BL/6J mice SD rats LE rats	Improvement of memory and reducing anxiety-related behaviors in normally developing rodents	Waworuntu et al. (2014)
Polydextrose-GOS (2 g/L each)	Postnatal days (2–33)	Translational piglet model	Higher recognition memory	Fleming et al. (2019)
FOS GOS FOS + GOS (0.3–0.4 g/mouse/day)	10 weeks	Male C57BL/6J mice	Improvement in brain chemistry and social behavior related to anxiety and depression Enhanced levels of SCFA in the caecum reduction in stress-induced corticosterone levels in plasma Anti-anxiety levels in open field and elevated plus-maze	Burokas et al. (2017)
XOS (10%) *L. paracasei* HIIO1 (1×10^8 CFU) Synbiotics (XOS + *L. paracasei* HIIO1; 1:1 ratio)	12 weeks	Male Wister rats	Enhancement in brain mitochondrial function and synaptic plasticity, thereby restoring cognitive function	Chunchai et al. (2018)
Lactoferrin (0.3 g/100 g milk powder) Milk fat globule membrane (0.25 g/100 g milk powder) Blend of polydextrose (1.3 g/100 g milk powder)/GOS (3.5 g/100 g milk powder)	Postnatal days (2–31)	Piglets	Positively influenced brain development as evidenced with neuroimaging outcomes	Mudd et al. (2016)
FOS + XOS (3 g/kg/day)	Gestation days (0–19)	Pregnant Wistar rats	Enhanced exploratory behavior in the open field test Reduction in acrylamide-induced oxidative stress markers level	Krishna et al. (2015)
Inulin (2 g/kg/day)	Gestation days (0–19)	Pregnant Wistar rats	Reduction in acrylamide induced increase in oxidative markers in the fetal and the brain tissues	Krishna and Muralidhara (2015)
Inulin (2 g/kg BW, twice a day)	Gestation days (6–19)	Pregnant Wistar rats	Inulin supplementation diminished gestational rotenone induced increase in oxidative markers in the regions of the maternal brain and affected the whole fetal brain	Krishna and Muralidhara (2018)
FOs (200 ul)	4 weeks	Young adult female C57/BL mice	Ameliorated behavioral recovery following spinal cord injury *via* modulating the expression of inflammatory mediators	Li et al. (2020)
COS (0.2 mM)	1 day	*C. elegans*	Enhancement in the antioxidant potential with an increase in dopamine levels, thereby attenuating monocrotophos induced oxidative stress	Nidheesh et al. (2016)

acid (500 mg/kg BW, twice a day for 3 weeks) to pregnant Long-Evans rats on gestation days G12–16 resulted in enhanced repetitive behavior in the open-field test contributing to ASD (Foley et al., 2014). Prenatal exposure to valproic acid results in autism spectrum disorder (ASD) with a spatial learning disability and anxiety-like behavior. Interestingly, chronic treatment (5 weeks) to female ICR (CD1) mice with sodium butyrate (1.2 g/kg BW/day, i.p.; starting at 4 weeks of age) reversed valproic acid-induced ASD-like behavior in the offspring. Increased histone H4 acetylation with sodium butyrate administration in the mice hippocampus facilitated the amelioration of the memory impairment in prenatally valproic acid-exposed mice (Takuma et al., 2014). Therefore, these research studies suggest that extreme care should be taken to evaluate the potential use of SCFAs to treat ASD (Stilling et al., 2016).

Aging is associated with degenerative loss of neurons in the CNS, ultimately leading to memory loss and impaired learning (Romo-Araiza et al., 2018). Furthermore, it is also associated with the reduced expression of brain-derived neurotrophic factors (BDNFs) in the hippocampal region that is closely associated with the regulation of synaptic transmission and plasticity (Ryan and Nolan, 2016). Thus, aging is associated with reducing levels of BDNF. The maintenance of sufficient BDNF concentrations is suggested to delay the onset of cognitive impairments (Pineda-Rodriguez et al., 2017). Several studies have highlighted the role of prebiotics and their metabolites (SCFA) in psychophysiological modulation *via* BDNF (Sarkar et al., 2016; Park et al., 2017; Haghighat et al., 2019; Heyck and Ibarra, 2019). Williams et al. (2016) studied the effect of GOS supplementation on brain development and maturation in neonatal rats from the postnatal days (3–21). GOS supplementation brought alterations in the levels of BDNF, synaptic proteins (synaptophysin, MAP2, and GAP43) and NMDAR subunits (GluN1, GluN2A, GluN2B) on postnatal days (22 and 56). An increase in the levels of BDNF, NMDAR subunit, GluN2A, and synaptic protein, *viz*., synaptophysin (but not MAP2) imply that neonatal GOS supplementation alters neurotransmission instead of synaptic architecture. Thus, the study confirms that prebiotic oligosaccharides are capable of manipulating gut microbiota in early life and its positive effects on the brain persist at least up to young adulthood. On similar lines, Romo-Araiza et al. (2018) investigated the modification of intestinal microbiota through supplementation of prebiotics/probiotics/synbiotics and thereby studied their effect on brain health. Middle-aged Sprague-Dawley male rats were randomly assigned into 4 groups and each group (one group served as a control) was supplemented (5 weeks through oral gavaging) with either probiotic *Enterococcus faecium* (4×10^8 CFU) or prebiotic, inulin (860 mg/kg BW) or synbiotic, comprising of *E. faecium* and inulin (4×10^8 CFU + 860 mg/kg BW). The impact of probiotic, prebiotic, or synbiotic supplementation on spatial and associative memory in middle-aged rats was assessed at the end of the study. Their findings revealed that synbiotic and prebiotic supplemented groups performed significantly better in the spatial memory test. This improvement is correlated with lower levels of pro-inflammatory cytokines and higher levels of BDNF in the hippocampus region of synbiotic and prebiotic-treated animals. A significant increase in butyrate concentration after synbiotic and prebiotic supplementation is potentially the reason for the enhanced levels of BDNF and progression of spatial memory. Delta butyrate concentration for each group was found as follows: control (0.45 ± 0.008), probiotic (−0.008 ± 0.009), prebiotic (0.87 ± 0.008), and synbiotic (1.17 ± 0.01). These values clearly indicated that the inulin supplementation resulted in the improvement of butyrate concentrations. Furthermore, a decrease in pro-inflammatory cytokine concentrations and an increase in BDNF levels in the hippocampus region offered a positive outcome. Thus, these research findings clearly indicate that supplementation of prebiotics alone or in combination with probiotics positively impacts brain health *via* modulation of BDNF.

Microglia are reported to play a crucial role in brain development and therefore represent the principal immune cells in the human brain (Chen and Trapp, 2016). These cells populate the CNS *in utero* and assist in brain development. Once neuronal development is complete, they serve as resident innate immune cells of the CNS and get activated only when the CNS is challenged with injury, infection, and/or disease. During the inflammatory state, microglial activation is not only associated with neuroprotective effects (*viz*., phagocytosis of dead neurons and clearance of debris) but also has neurotoxic consequences (Polazzi and Monti, 2010). Thus, microglia are the first cells to induce neuroinflammatory response and thereby play a crucial role in the initiation of various mental disorders (Yang et al., 2018). It is found that postoperative cognitive dysfunction, Alzheimer's and Parkinson's diseases are associated with enhanced concentrations of pro-inflammatory cytokines along with microglial activation in the brain (Chunchai et al., 2018; Yang et al., 2018; Liu et al., 2019). Recent studies have identified a communication link between prebiotic NDOs and microglia. Yang et al. (2018) evaluated whether supplementation of GOS would attenuate postoperative cognitive dysfunction and surgery-induced neuroinflammation. To assess the effect of GOS supplementation, abdominal surgery under isoflurane anesthesia was performed on SPF Sprague-Dawley male adult rats. Over the course of 3 weeks of the study, the GOS-treated group received GOS at a dose of 15 g/L in water. At the end of the study, supplementation of GOS significantly attenuated surgery-induced cognitive impairments and downregulated activation of microglial cells in comparison to the control group. A detailed analysis of the gut revealed that GOS supplementation significantly altered the diversity of the gut microbiome and enhanced the proliferation of anti-inflammatory microbes, *viz*., bifidobacteria.

Dietary oligosaccharides have shown potential to improve memory, cognition ability, and social behavior (Waworuntu et al., 2014; Collins and Reid, 2016). A mixture of polydextrose-GOS fed to mice (15 g/kg BW) and rats (7 g/kg BW) from postnatal days 21–50 showed increased positive social interactions and higher object recognition index in comparison to the control group. Thus, these results indicate the ability of the tested prebiotics in the improvement of memory and reduced anxiety-related behavior in normally developing rodents (Waworuntu et al., 2014). The same mixture (2 g/L each) was further evaluated by Fleming et al. (2019) in young pigs administered from postnatal days 2–33. The study found that early life consumption of this mixture supported higher recognition memory. In line with this, the beneficial role of FOS and GOS in stress-related behavior has been evaluated (Burokas et al., 2017). Male C57BL/6J mice that received either FOS, GOS, or both at a dose of 0.3–0.4 g/mouse/day for 10 weeks showed improvement in brain chemistry and social behavior related to anxiety and depression. Moreover, combination treatment was found to be more effective in altering the microbial community, and this led to the increase in SCFA levels in the caecum. Furthermore, marked reduction in stress-induced corticosterone levels in plasma and anti-anxiety

levels in an open field and elevated plus-maze were recorded. All these evidences confirm the potential of combination treatment, that is, polydextrose-GOS and FOS-GOS in the successful reduction of anxiety-related symptoms.

High-fat diets can cause cognitive decline and microglial hyperactivity in addition to obesity-induced insulin resistance. In line with this, Chunchai et al. (2018) explored the effect of prebiotics (XOS; 10%), probiotics (*L. paracasei* HIIO1; 1×10^8 CFU), and synbiotics (XOS + *L. paracasei* HIIO1; 1:1 ratio) on microglia in obese-insulin-resistant male Wistar rats. Male Wister rats used in the study for 12 weeks were fed with either a high-fat or normal diet. At the beginning of the 13th week, the rats were randomly assigned into 4 subgroups in each of the dietary treatments (vehicle, prebiotic, probiotic, and synbiotic groups) followed by intervention with the assigned dietary supplement. After 12 weeks of treatment, the cognitive function and microglial activation status of each rat group were assessed. Prebiotic, probiotic, and symbiotic-fed groups showed a significant decrease in high-fat diet-induced cognitive impairments and microglia activation. Prebiotic, probiotic, and synbiotic treatments could significantly reduce microglial activation. Overall, the animals that received the supplements had better cognitive functions in comparison to the control group clearly suggesting that prebiotic, probiotic, and synbiotic treatments could effectively attenuate cognitive impairments and inhibit microglial activation thereby conferring neuroprotection.

Like all other tissues in the human body, brain tissues require oxygen to meet their energy needs. The human brain constitutes 2% of total body mass and needs 20% of total body oxygen demand to support neuronal activity (Devi and Satpati, 2017). The oxygen consumption rate of the normal human brain is 3.5 ml/min/100 g brain tissue (Rink and Khanna, 2011). The brain is an active site for the production of free radicals from reactive oxygen species (ROS) as a result of inefficiencies in oxidative phosphorylation in the mitochondria (Black et al., 2015). An excessive level of free radicals can impair brain function. Therefore, a balance between free radicals and antioxidants is essential to support brain function from any degeneration. Several defensive mechanisms exist to counteract and protect brain cells against oxidative stress-mediated neuronal degeneration such as upregulation of endogenous antioxidants and removal of damaged proteins and organelles by autophagy (Divyashri et al., 2015). However, higher rates of oxidative stress can initiate the oxidation of proteins and lipids, thereby changing their structure and functions that can subsequently result in cell death (Dumitrescu et al., 2018; Lobo et al., 2010). Therefore, any excess of free radical production due to oxidative stress could be associated with damage to a wide range of molecular species. Studies on prebiotic NDOs alone or in combination with probiotics have the potential to modulate and counter oxidative stress in the brain. This is gaining significant attention and relevance as a strategy to treat/prevent neurological pathologies, including Alzheimer's and Parkinson's diseases.

Early life intake of key nutritional components (prebiotics) enriches neurodevelopmental activities (Kao et al., 2016). The beneficial effects of lactoferrin (0.3 g/100 g milk powder), milk fat globule membrane (0.25 g/100 g milk powder), and a blend of polydextrose (1.3 g/100 g milk powder)/GOS (3.5 g/100 g milk powder) as potent prebiotics on the early developing brain are elucidated using piglets (dosage from 2–31 days). It is notable that dietary supplementation was well tolerated and positively influenced brain development (Mudd et al., 2016). Krishna et al. (2015) evaluated the physiological benefits of prebiotic oligosaccharides during pregnancy. Attempts were made to test the effectiveness of a prebiotic combination (FOS + XOS) to attenuate acrylamide-induced oxidative impairments, mitochondrial dysfunction, and neurotoxicity in maternal and fetal rat brains. A dose of 3 g/kg/day of oligosaccharides (XOS and FOS) was supplemented to pregnant dams (Wistar rats) during the gestation period of 0–19 days. Simultaneously, the rats were exposed to acrylamide (200 ppm in drinking water). Prebiotics fed acrylamide dams displayed better exploratory behavior in the open field test. Furthermore, prenatal assessment proved that prebiotic supplementation could effectively restore acrylamide-induced decrements of placental/fetal weights. In addition, prebiotics supplementation could significantly lower the markers of oxidative stress (ROS, reduced glutathione, and protein carbonyls) and restore activities of antioxidant enzymes (acetylcholinesterase and glutathione peroxidase), in the maternal and fetal brains with a concomitant increase in dopamine and γ-aminobutyric acid levels. This study suggested that prenatal prebiotic oligosaccharide supplements can effectively safeguard the developing brain against acrylamide-induced oxidative stress-mediated neurotoxicity.

Scientific evidence suggests that the consumption of prebiotic oligosaccharides influences the human brain positively. The influence of inulin supplementation during gestation in acrylamide-induced oxidative impairments and neurotoxicity in maternal and fetal rat brains was examined by Krishna and Muralidhara (2015). Pregnant rats were co-fed with inulin (2 g/kg/day; gestation days 0–19) and acrylamide at a dose of 0.2 g/L (gestation days 6–19). Their results revealed that inulin supplementation could significantly increase placental weight among acrylamide-exposed rats. A detailed analysis of oxidative stress markers (ROS, hydroperoxide, lipid peroxidation, reduced glutathione, and protein carbonyls) revealed that inulin supplementation could effectively lower acrylamide-induced increase in oxidative markers in the fetal and the brain tissues. This study reveals the neuroprotective role of prebiotic NDOs towards developmental neurotoxicants such as acrylamide during pregnancy.

The impact of increased pesticide exposure on developmental neurotoxicity due to entry into the immature brain is of concern. Investigations have identified the influence of maternal gut microbiota on utero fetal development by modulating the host gut microbial composition with prebiotic NDOs. Krishna and Muralidhara (2018) examined the beneficial role of inulin in a developmental model of rotenone neurotoxicity. Pregnant rats gavaged orally with inulin (2×/day, 2 g/kg BW; during gestation days 0–21), also received rotenone (50 mg/kg BW, gestation days 6–19) to potentially counter the developmental effects of general fetotoxicity, cholinergic activities, and oxidative stress in maternal

and whole fetal brain. It was found that inulin supplementation resulted in a significant increase in maternal caecal bacterial numbers, with a concomitant increase in exploratory behavior among rotenone-treated rats. Furthermore, inulin supplementation diminished gestational rotenone-induced increase in oxidative markers (ROS protein carbonyls and lipid peroxidation) in the regions of the maternal brain (cerebellum, striatum, and cortex), and the fetal brain. This study indicates the prebiotics potential in lowering oxidative stress-mediated neurodegenerative disorders.

Deterioration in neuronal survival and irreversible motor and sensory dysfunction is reported to occur following spinal cord injury. The neuroprotective effect of FOs (200 ul, 4 weeks) was evaluated using the spinal cord injury model of young adult female C57/BL mice. FOs modulated the expression of inflammatory mediators (downregulated TNF-α, IL-2, IL-6, IL-18 levels, and upregulated IL-10 and BDNF) and ameliorated behavioral recovery following spinal cord injury. Furthermore, the study demonstrated that FOs exhibited anti-inflammatory and neuroprotective effects *via* the mitogen-activated protein kinase signaling pathway with enhanced expression of BDNF levels (Li et al., 2020).

Many studies have attempted to use models outside of the rodent system to evaluate the neuroprotective potential. *Drosophila melanogaster* is a small insect, encompasses noteworthy cellular, molecular, and biological signaling complexity with relevance to humans (Westfall et al., 2018). Thus, *Drosophila* has become one of the prominent model systems to evaluate neuroprotection. However, investigations on the effect of NDOs on neurological disorders using *Drosophila* are limited. *Caenorhabditis elegans* also represents a model system for studying neuroprotective effects as it has relevant homology with mammalian systems. However, investigations on the NDOs effect on neurological disorders using *C. elegans* are comparatively few. A study by Nidheesh et al. (2016) tested the efficiency of chito-oligomers (COS) to ameliorate monocrotophos-induced oxidative stress in *C. elegans*. COS exhibited a significant neuroprotective effect by enhancing the antioxidant potential of the brain and thereby attenuating oxidative stress. But this systematic review provides an interesting perspective on the current evidence base for the effects of prebiotics on symptoms of oxidative stress reduction and improving brain health using rodent models.

Evidence in Humans

Prebiotic administration at the clinical level to investigate their central effects as neuroprotective agents is currently lacking (Kao et al., 2016; Johnstone et al., 2021). However, we have summarized the limited progress made in the clinical studies with NDOs as medicinal therapeutics (**Table 3**). A study in 45 healthy male volunteers found that the consumption of FOS (5.5 g/day) and GOS (5.5 g/day) for 2 weeks helped to proliferate host gut microbiota with a reduction in salivary cortisol levels. In comparison to FOS, GOS were more successful in regulating the hypothalamic–pituitary–adrenal (HPA) axis to restore emotional perturbations in healthy volunteers (Schmidt et al., 2015). This study demonstrates the ability of prebiotic oligosaccharides in the reduction of anxiety-related psychological mechanisms. A randomized double-blind study to assess the effect of short-chain FOS (5 g/day; 4 weeks) on clinical outcomes of anxiety/depression was performed on 79 irritable bowel syndrome (IBS) volunteers. FOS significantly reduced anxiety by modulating gut microbiota (Azpiroz et al., 2017). The influence of GOS (7.5 g/day for 4 weeks) on gut microbiota to improve mental ailments, *viz.*, anxiety and moodiness was evaluated in late adolescence and early adulthood using healthy female volunteers (64 no.; aged between 18–25 years) (Johnstone et al., 2021). The authors reported that through a dot-probe task, GOS reduced negative emotional bias and increased positive bias associated with high anxious participants. This finding was further supported by the increased beneficial bacterial abundance in the GOS supplemented group.

It is proven at the preclinical level that the early life intake of various prebiotic oligosaccharides supported neurodevelopmental activities (Krishna et al., 2015; Fleming et al., 2019; Upadhyay et al., 2020). However, at the clinical level, these studies were examined with a smaller experimental window. Preterm infants (77 no.) with a gestational age of <32 underwent a randomized double-blind trial and received a mixture of short-chain GOS/long-chain FOS/pectic-derived acidic oligosaccharides (1.5 g/kg BW/day) through breast/formula milk from day 3 to 30 (van den Berg et al., 2016). Neurodevelopmental outcome at the corrected age of 2 years revealed that the supplementation of the above mixture resulted in no improvement in comparison to the placebo group. This is attributed to the higher serum cytokine levels with lower bifidobacterial counts indicating the importance of gut microbiota in the immune response during the brain development process. A similar observation was made by LeCouffe et al. (2014) wherein enteral supplementation of a prebiotic mixture (for the short term) displayed no beneficial effect on neurodevelopmental outcome in preterm infants in the first year of life. Although these studies are acknowledged for conducting prebiotic research involving preterm infants, the results seem not conclusive even though the beneficial effects of early life intake of prebiotic oligosaccharides in rodent models are well-established. Therefore, further studies are required to evaluate the early life intake of prebiotic oligosaccharides on neurodevelopment to draw accurate conclusions. The effect of a prebiotic intervention on human mood, learning, affective, and cognitive processes was previously reported. Smith et al. (2015) involved 47 subjects (1 week) to evaluate the effect of oligofructose-enriched inulin (5 g/day) on human well-being and cognitive performance. Selective improvement following inulin ingestion was observed with recall and recognition memory. However, no effect on mood and sustained behavior was observed.

Hemodialysis procedure is reported to have an adverse effect on the patient's mental health (Teles et al., 2014). Researchers in this study were also making efforts to combine prebiotics with specific probiotics for the formulation of a symbiotic mixture to maximize their health benefits. To investigate the beneficial effect of prebiotics/probiotics/synbiotics in hemodialysis patients suffering from anxiety and depression, Haghighat et al. (2019)

TABLE 3 | Selected studies at a clinical level using prebiotics and/or their metabolites (SCFA) and probiotics to modulate brain physiology and function.

Prebiotics and/or Probiotics	Duration	Subjects	Outcome	References
FOS (5.5 g/day) GOS (5.5 g/day)	2 weeks	45 healthy male and female volunteers	Reduction in salivary cortisol levels Regulation of HPA axis to restore emotional perturbations	Schmidt et al. (2015)
FOS (5 g/day)	4 weeks	79 irritable bowel syndrome (IBS) volunteers	Reduction in anxiety by modulating gut microbiota	Azpiroz et al. (2017)
GOS (7.5 g/day)	4 weeks	64 healthy female volunteers	Increased beneficial bacterial abundances Reduction in negative emotional bias and increase in positive bias associated with high anxious participants	Johnstone et al. (2021)
Mixture of GOS/FOS/pectic-derived acidic oligosaccharides (1.5 g/kg BW/day)	Gestation days (3–30)	77 preterm infants with a gestational age of less than 32 weeks	No improvement in the neurodevelopmental outcome was observed at the corrected age of 2 years	van den Berg et al. (2016)
Mixture of GOS/FOS and pectic-derived acidic oligosaccharides (80% + 20%)	Gestation days (3–30)	93 preterm infants with a gestational age of less than 32 weeks	No beneficial effect on neurodevelopmental outcome in preterm infants in the first year of life	LeCouffe et al. (2014)
Oligofructose-enriched inulin (5 g/day)	1 week	47 subjects	Selective improvement was observed with recall and recognition memory No effect on mood and sustained behavior was reported	Smith et al. (2015)
Probiotic mixture (5 g) of 2.7×10^7 CFU/g each of *L. acidophilus* T16, *B. bifidum* BIA-6, *B. lactis* BIA-7, and *B. longum* BIA-8 Prebiotic mixture: 5 g each of FOS, GOS, and inulin Synbiotic mixture: 15 g prebiotics and 5 g probiotics	12 weeks	75 hemodialysis patients	Reduction in depression associated symptoms with a concomitant increase in serum BDNF levels in the synbiotic treated group	Haghighat et al. (2019)

enrolled 75 patients for the study. The patients (n = 75) were randomly assigned to synbiotic group (n = 25; prebiotics, 15 g and probiotic mixture, 5 g), probiotic group (n = 25; probiotic mixture, 5 g and maltodextrin, 15 g), and a placebo group (n = 25; maltodextrin, 20 g). Probiotic mixture (5 g) comprised of 2.7×10^7 CFU/g each of *Lactobacillus acidophilus* T16, *Bifidobacterium bifidum* BIA-6, *Bifidobacterium lactis* BIA-7, and *Bifidobacterium longum* BIA-8. The prebiotic group received 5 g each of FOS, GOS, and inulin. Synbiotic supplementation for 12 weeks resulted in a greater decrease in depression symptoms measured as Hospital Anxiety and Depression Scale (HADS) (HADS-DEP ≥ 8) with a concomitant increase in serum BDNF in comparison to the probiotics and placebo groups.

Mechanism-Based Studies

SCFAs provide benefits to peripheral tissues, and therefore, the colon is well supported, and it is also suggested that potentially they exert crucial physiological effects on distal organs, including the brain (Silva et al., 2020). SCFAs can cross the blood–brain barrier *via* monocarboxylate transporters located on endothelial cells, and hence, it can alter the neurotransmitter (γ-aminobutyric acid (GABA), and serotonin (5-HT)) and hormone concentrations (glucagon-like peptide 1 (GLP1) and peptide YY (PYY)) by promoting their secretion (Silva et al., 2020). In addition, they are also reported to prevent neurodegeneration and promote neuronal regeneration (Sampson and Mazmanian, 2015) and thereby have a protective effect on the brain *via* direct and indirect means (**Figure 2**). Direct pathways influencing brain function: SCFAs can cross the blood–brain barrier to reach the brain, and the average concentrations of butyrate and propionate in the human brain tissue were found to be 17.0 and 18.8 pmol/mg of brain tissue, respectively (Silva et al., 2020). Further evidence suggests that SCFAs can exhibit neuroactive properties in the CNS (Tran and Mohajeri, 2021). However, the mechanism by which these SCFAs offer neuroprotection is still not clear. Indirect pathways influencing brain function: Gutbrain axis is composed of the CNS, enteric nervous system (ENS), afferent, and efferent neurons that are associated with the signal transduction between the gut and the brain (Chen et al., 2017; Liu et al., 2018). Numerous scientific evidences indicate the widespread communication between the gut and the brain *via* the gutbrain axis (Chen et al., 2013; Liu et al., 2015). The bidirectional communication between the gut and the brain occurs through the vagus nerve, neuroimmune, humoral, and neuroendocrine pathways (Sandhu et al., 2017). By interacting with FFAR receptors on enteroendocrine cells, SCFA promotes indirect signaling to the brain *via* the systemic circulation or vagal pathways by inducing the secretion of neurotransmitters (GABA and 5-HT) and gut hormones (GLP1 and PYY) (Silva et al., 2020). SCFAs, particularly butyrate is known to influence brain activity indirectly by acting through the gutbrain axis (Stilling et al., 2016). Butyrate, with its ability to cross the blood–brain barrier, is reported to activate the vagus nerve, thereby indirectly influencing the brain by modulating host appetite and eating behavior (van de Wouw et al., 2017). Furthermore, butyrate also modulates the activity of cholinergic enteric neurons through epigenetic mechanisms (Soret et al., 2010). Butyrate, upon binding to its specific receptors in the intestine, is recognized to modulate signals to the brain *via* the gutbrain neural circuit through cAMP signaling pathways (Keenan et al., 2015).

Surgical trauma is reported to attenuate behavioral deficits, thereby enhancing neuroinflammatory responses with reduced SCFA and BDNF levels. The pretreatment effect (4 weeks) of exogenous SCFA to restore physiological and behavioral deficits

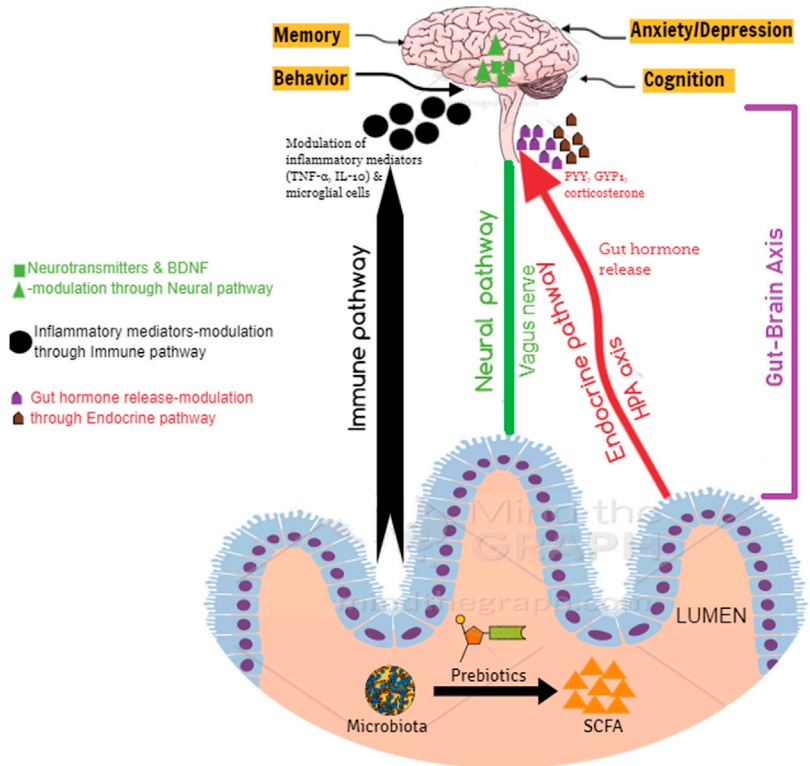


FIGURE 2 | Protective mechanism of prebiotic NDOs on the brain.

was studied using 8- to 10-week-old adult male C57BL/6J mice (Xu et al., 2021). Mice were randomly assigned into 5 groups. First group served as control; second group of mice received SCFA (67. 5 mM sodium acetate, 25 mM sodium propionate, 40 mM sodium butyrate); third group served as surgery control; fourth group underwent surgery and received SCFA (dose similar to the second group); and fifth group received fecal microbiota transplantation. The impact of SCFA supplementation prior to surgery on spatial learning and memory was assessed at the end of the study. Their findings revealed that pretreatment with SCFA prior to surgery partially improved the locomotor activity and anxiety-like behaviors. SCFA feeding has also been shown to result in the reduction of surgical trauma-induced upregulation of IL-1β and IL-6 in the hippocampus region. Furthermore, upregulated levels of hippocampal Iba-1 levels were downregulated, thereby suggesting that SCFA treatment could effectively reverse microglial overactivation. This data clearly demonstrates the ability of exogenously administrated SCFA or gut-derived SCFA in the restoration of microglial over-activation and modulation of neuroinflammatory responses to rescue surgical trauma.

The neuroprotective effect of exogenously administered sodium butyrate was explored by Sun et al. (2016) using chronic unpredictable mild stress (CUMS)-induced male C57BL/B6 mice model. Sodium butyrate treatment (200 mg/kg BW, 2 weeks) reversed CUMS induced depressive status by enhancing BDNF expression with a concomitant increase in 5-HT concentration in the hippocampus region of mice brain. These data clearly suggest the ability of SCFA, more specifically butyrate in amelioration of depressive behaviors by enhancing 5-HT and BDNF levels.

Intragastric injections of sodium butyrate for 2 weeks on chronic unpredictable mild stress (CUMS)-induced depression-like behaviors in male C57BL/B6 mice was studied (Sun et al., 2016). It was observed that butyrate could ameliorate CUMS-induced alterations in BDNF expressions, thereby offering anti-depressive effects. Another validated mechanism by which SCFA offers neuroprotection is by modulation of the gut hormone release from enteroendocrine cells (Kao et al., 2016). SCFA produced through the ingestion of prebiotic oligosaccharides modulates the secretion of peptide tyrosine (PYY) and glucagon-like peptide-1 (GLP-1) from enteric L-cells. The effect of intra-colonic administration of propionic acid (180 mmol/L) on the release of GLP-1 and PYY was investigated using rodent models (male C57BL6 Wistar rats). Analysis of plasma gut hormone profile using radio-immunoassay revealed that the propionic acid administration stimulated the release of GLP-1 and PYY, thereby playing a significant role in the central effects (Psichas et al., 2015). It is a well-established fact in animal models that exogenous PYY influences behavioral and cognitive functions (Stadlbauer et al., 2015). Furthermore, PYY is also known to modulate vagal nerve activity through local BNDF signaling pathways (Kao et al., 2015). In addition to the above studies on PYY secretion and modulation by SCFA, prebiotic oligosaccharide, *viz.*, GOS (6%; 3 weeks) are also known to induce the expression of circulating PYY in male Wister rats (Overduin et al., 2013). GOS fed

TABLE 4 | Mechanism-based evidences of prebiotics and/or their metabolites (SCFA) to modulate brain physiology and function.

Pathway	Prebiotics and/or their metabolites (SCFA)	Model	Mechanism	Reference
Immune	SCFA mixture (67. 5 mM sodium acetate, 25 mM sodium propionate, 40 mM sodium butyrate)	Surgical trauma-induced adult male C57BL/6J mice model	Partial improvement in the locomotor activity and anxiety-like behaviors by The upregulation of IL-1β and IL-6 in the hippocampus region with the restoration of surgery-induced microglial over-activation.	Xu et al. (2021)
Neural	Sodium butyrate (200 mg/kg BW)	Chronic unpredictable mild stress (CUMS)-induced male C57BL/B6 mice model	Amelioration of CUMS induced alterations in BDNF expressions, thereby offering anti-depressive effects.	Sun et al. (2016)
Endocrine	Propionic acid (180 mmol/L)	Male C57BL6 Wistar rats	Modulates the secretion of gut hormones, PYY, and GLP-1 from enteric L-cells	Psichas et al. (2015)
Endocrine	GOS (6%)	Male Wistar rats	Elevations in the levels of PYY and GLP-P in colonic mucosal, thereby influencing brain health	Overduin et al., 2013
Neural	Sodium butyrate (100 mg/kg BW/day)	BTBR mouse model of autism	Promotes transcription of inhibitory pathway transcripts *via* an increase in inhibitory neurotransmitter genes (Drd2 and Gabrg1) with a decrease in neuronal activation and excitatory neurotransmitter marker genes (cFos Grin2b, and Adra1)	Kratsman et al., 2016
Immune	COS (200, 400 or 800 mg/kg BW)	Amyloid-β-$_{1-42}$-induced Alzheimer's disease (AD) rats	Reduction in the levels of IL-1β and TNF-α to influence cognitive functions	Jia et al. (2016)
Immune	FOS (2.5 and 5%)	D-Galactose AD rat model	Improvement in spatial learning and memory by reducing Aβ density in the cortex and hippocampus with improvement in the plasma ascorbic acid level in a dose-dependent manner	Yen et al. (2017)
Neural	FOS extract (50 and 100 mg/kg BW/day)	D-Galactose AD rat model	Enhancement in the levels of neurotransmitters (norepinephrine, dopamine, 5-hydroxytryptamine, and 5-hydroxyindole acetic acid) with down-regulate the expression of AD-related intracellular markers (Tau and Aβ_{1-42})	Chen et al. (2017)
Immune	Sodium butyrate (300 mg/kg BW)	Hypoxic–ischemic-injured immature rat model	Stimulation of oligodendrocyte precursor cell proliferation in the hippocampal dentate gyrus with a reduction in the microglial cell number in the rat ipsilateral hemisphere and enhancement in the BDNF levels	Ziemka-Nalecz et al. (2017)
Neural	Sodium butyrate (300 mg/kg BW)	Neonatal rat model of hypoxia–ischemia	Increased BDNF levels with enhanced activation of the TrkB receptor (BDNF receptor) and the phosphorylation of the transcription factor-CREB-in the ipsilateral hemisphere suggests the involvement of BDNF-TrkB signaling pathways	Jaworska et al. (2019)
Immune	COS (0.1 ml/10 g BW)	Neonatal rat model of hypoxia–ischemia	Inhibition of astrocytes and microglia activation by reducing the expression of inflammatory markers, *viz.*, TNF-α and IL-1β, with an increase in the expression of IL-10 protein	Wu et al. (2017)

rats expressed elevated levels of PYY and GLP-P in colonic mucosal, thereby influencing brain health. These studies clearly explain the possible mechanisms by which NDOs and their products (SCFA) modulate brain chemistry.

In rodent models, HDAC inhibition with altered GABAergic signaling is closely connected to autism spectrum disorders (ASD) (Stilling et al., 2016). The effect of butyrate (i.p. 100 mg/kg BW/day, 10 days) on ASD-associated social behavior was evaluated by Kratsman et al. (2016) using the BTBR mouse model of autism. Administered dose of butyrate affected genes involved in neuronal excitation and inhibition. An increase in inhibitory neurotransmitter genes (Drd2 and Gabrg1) with the decrease in neuronal activation and excitatory neurotransmitter marker genes (cFos Grin2b, and Adra1) supports the fact that the butyrate promotes the transcription of inhibitory pathway transcripts. Furthermore, the tested dose of butyrate failed to induce any significant difference in histone acetylation in the prefrontal cortex; however, there was an increase in ASD-associated social behavior through the modulation of the excitatory/inhibitory balance.

All these evidences prove that prebiotics and their metabolites communicate to the brain *via* neural (vagus nerve, BDNF, neurotransmitters), endocrine (HPA axis and associated hormones), and immune (immune cells and markers, *viz.*, TNF-α and IP-10) pathways (**Table 4**).

Specific Evidence in Neurodegenerative Models

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by cognitive and memory impairments (Kumar and

Singh 2015). It also results in the formation of neurofibrillary tangles from abnormally phosphorylated tau and abnormal accumulation of amyloid plaques (Drummond and Wisniewski 2017). Animal models play a crucial role in defining disease-associated mechanisms and have been of prime importance in evaluating the effectiveness of novel therapeutic agents (Drummond and Wisniewski 2017). In a rodent model of AD, COS (chitosan oligosaccharides) (200, 400, or 800 mg/kg BW for 2 weeks) were found effective in reducing cognitive deficits in amyloid-β-$_{1-42}$-induced rats. Inhibition of oxidative stress and suppression of inflammatory response *via* the reduction in the levels of IL-1β and TNF-α are reported to influence cognitive functions (Jia et al., 2016). Similar observations were reported for D-galactose AD rat models wherein supplementation of FOS (2.5 and 5% w/w; for 49 days) improved spatial learning and memory by reducing Aβ density in the cortex and hippocampus with improvement in the plasma ascorbic acid level in a dose-dependent manner (Yen et al., 2017). A study by Chen et al. (2017) indicated that FOS from *Gynochthodes officinalis (F.C.How) Razafim. & B.Bremer* exerted memory improvements in adult male Sprague-Dawley rats model of AD. They investigated the role of FOS extract in alleviating symptoms of AD by targeting the microbiota effects of the gutbrain axis. Rats were randomly assigned into 4 groups. The first group served as control; the second group of rats received D-galactose (100 mg/kg BW/day); the third group received low dose of FOS extract (50 mg/kg BW/day) + D-galactose (100 mg/kg BW/day); the fourth group received high dose of FOS extract (100 mg/kg BW/day) + D-galactose (100 mg/kg BW/day). FOS extract administration showed a marked effect on the AD-associated cognitive behavior by improving oxidative stress, enhancing neurotransmitter synthesis (norepinephrine, dopamine, 5-hydroxytryptamine, and 5-hydroxyindole acetic acid) in rats. Furthermore, FOS administration could significantly down-regulate the expression of AD-related intracellular markers (tau and Aβ_{1-42}). This effect is attributed to the fact that FOS extract administration could modulate the interaction between gut ecology and brain physiology *via* the gutbrain axis. The disaccharide, lactulose is reported to offer beneficial effects towards AD. Pretreatment of lactulose offered neuroprotection in the mice model by increasing the levels of the autophagic pathways and decreasing neuroinflammation, thereby attenuating short-term memory and the learning retrieval associated with AD (Lee et al., 2021).

A significant number of research findings have demonstrated the link between gut microbiota alterations on the onset of ASD. The beneficial effect of probiotic administration in ASD children is evaluated by many researchers as they are known to restore gut microbiota and downregulate ASD symptoms. Probiotic strains, *viz.*, *Lactobacillus acidophilus* (Liu et al., 2017), *Lactobacillus plantarum* (Shaaban et al., 2018), *Lactobacillus rhamnosus* (Kałużna-Czaplińska and Błaszczyk, 2012), and *Bifidobacterium longum* (Niu et al., 2019) have shown therapeutic potential towards ASD. In line with this, there have been few studies that have examined the use of prebiotics in ASD children (Grimaldi et al., 2018; Sanctuary et al., 2019). Tolerability and efficacy of a probiotic strain (*Bifidobacterium infantis*; 20 billion CFU/d) in combination with prebiotic oligosaccharides (bovine colostrum product) to improve immune functions in ASD children and gastrointestinal co-morbidities were evaluated (Sanctuary et al., 2019). Children (9 no.) in the age group 2–11 with a history of frequent gastrointestinal discomfort (constipation, diarrhea, and IBS) were evaluated in the study (12 weeks). The study period comprised of pro-prebiotic supplementation for 5 weeks, followed by a wash-out period (2 weeks) and prebiotic supplementation (5 weeks). Pro-prebiotic supplementation was found to be well tolerated as assessed using the validated questionaries on pediatric gastrointestinal symptoms. Reduction in gastrointestinal symptoms is attributed to the reduction in IL-13 and TNF-α levels after supplementation. Community-level analyses were performed to study how supplementation affects gut microbiome enterotypes. It was found that few participants shifted from *Prevotella* enterotype levels to high *Bifidobacterium* enterotype levels. However, a detailed analysis of the enterotype data revealed that the treatment showed an inconsistent effect on enterotype levels. Grimaldi et al. (2018) studied the effect of exclusion diets (gluten and casein-free diets) and the impact of GOS (6 weeks) on gut microbiota and metabolism in ASD children (30 no.; age group: 5–10). Combining GOS supplementation with an exclusion diet resulted in a significant reduction in gastrointestinal discomfort and anti-sociability scores. Prior to this, the same group of researchers had validated the ability of GOS to alter gut community positively (increased bifidobacterial populations with enhanced levels of SCFA) in autistic children (Grimaldi et al., 2017).

Neonatal hypoxic–ischemic brain injury is a leading cause of neurodevelopmental disabilities in infants. Few research studies have demonstrated the fact that HDAC inhibitors play a beneficial role in adult ischemia models (Fleiss et al., 2012; Ziemka-Nalecz et al., 2017). Ziemka-Nalecz et al. (2017) evaluated the neuroprotective potential of sodium butyrate (as an HDAC inhibitor) in the dentate gyrus of hypoxic–ischemic-injured immature rats. Administration of 300 mg/kg BW sodium butyrate (immediately starting after hypoxic exposure; for 5 consecutive days) resulted in the stimulation of oligodendrocyte precursor cell proliferation in the hippocampal dentate gyrus with a reduction in the microglial cell numbers in the rat ipsilateral hemisphere and enhancement in the BDNF levels in the ipsilateral hemisphere after hypoxic–ischemic brain injury. All these observed parameters demonstrated the neuroprotective effect of sodium butyrate treatment in neonatal rats subjected to hypoxiaischemia. The underlying mechanism for sodium-butyrate-induced HDAC inhibition was explored using the neonatal rat model of hypoxia–ischemia (Jaworska et al., 2019). Wistar rats (7 days old) received sodium butyrate at a dose of 300 mg/kg BW for consecutive 5 days, immediately after hypoxic exposure. The neuroprotective effect of sodium butyrate in hypoxiaischemia is attributed to the neurogenic effect associated with increased BDNF levels with enhanced activation of the TrkB receptor (BDNF receptor) and the phosphorylation of the transcription factor CREB in the ipsilateral hemisphere. This study suggests

that BDNF-TrkB signaling plays a vital role in sodium butyrate-induced neurogenesis after hypoxiaischemia. The protective effect of prebiotic NDO, COS (0.1 ml/10 g BW, injected for 2 consecutive days every 12 h after hypoxic exposure) in neonatal hypoxicischemic brain damage was evaluated using Sprague-Dawley rats (7 days) (Wu et al., 2017). Posttreatment with COS resulted in the upregulation of antioxidant enzymes (GSH-PX, SOD, and T-AOC) and downregulation of lactic acid, MPO, and MDA levels in ischemic hemispheres. Furthermore, COS treatment also inhibited astrocytes and microglia activation by reducing the expression of inflammatory markers, *viz.*, TNF-α and IL-1β, with an increase in the expression of IL-10 protein. In conclusion, this study proves the ability of COS as a potential neuroprotective compound against neonatal hypoxicischemic brain damage.

CURRENT LIMITATIONS AND FUTURE PERSPECTIVE

Prebiotic NDOs are a broad category of beneficial compounds supporting good overall nutrition that provide health benefits by stimulating the growth of beneficial microorganisms (probiotics). They support the carbon-based energy needs of intestinally residing probiotics which results in products of metabolism such as SCFAs that get released into the bloodstream. These SCFAs are reported to have a beneficial effect on the GI tract and other distal organs, *viz.*, the brain. Minute quantities of prebiotics that naturally occur in food may not be effective in conferring health benefits. Therefore, strategies are being advanced to produce prebiotics on an industrial scale and incorporate them into designed food and supplement products for improved health benefits. SCFAs produced through the process of bacterial fermentation of prebiotics in the GI tract are hypothesized to confer neuroprotection and sustain mental health by modulating the physiology of the gutbrain axis through various neuro-immunological pathways. Studies have suggested that the gut–brain axis which links and aligns the CNS and ENS, corresponds to a key bidirectional pathway in conferring neuroprotection. The ability of prebiotics to regulate CNS processes through direct and indirect mechanisms by normalizing the gut microbiota, and they offer beneficial effect against various disorders including mental health which ultimately shape cognitive behavior and function. Research advances to date using animal models, though neural (vagus nerve, BDNF, neurotransmitters), endocrine (HPA axis and associated hormones), and immune (immune cells and markers, *viz.*, TNF-α and IP-10) pathways, have been identified to be associated with prebiotic NDOs gutbrain communication. There is an essential need for other mechanistic pathways to be explored. SCFAs influence brain function by inhibiting HDAC activity and enhancing BDNF levels. The available literature has indicated butyrate to be an HDAC inhibitor, and other SCFA, *viz.*, propionic acid when administered at a larger dose has shown autistic (ASD)-like symptoms. Thus, in-depth research studies are required to evaluate the potential use of SCFAs to treat ASD. A thorough understanding of the functional effects of prebiotics and their metabolites (SCFAs) in gutbrain interactions would support the design and development of novel prebiotic therapeutic targets for treating various neurological ailments. A limited number of publications support the fact that peptidoglycan, another prebiotic fermentation product, offers health benefits by influencing the innate immune system against host pathogens (Clarke et al., 2010). However, in-depth studies are required to evaluate the beneficial role of this metabolite. Even though there is a long history of safe consumption of prebiotics, more research is needed on the safety and toxicity considerations of various prebiotic NDOs, especially as novel prebiotics emerge in the market. FOS (> 20 g/day) are reported to enhance the fecal output with flatulence, bloating, abdominal pain, cramps, and diarrhea as side effects. However, these side effects are unlikely to occur when FOS administered is below 20 g/day (Serra et al., 2019). Studies exploring 90 days oral toxicity using animal model found that GOS up to 5,000 mg/kg BW/d resulted in a decrease in food consumption (7–13%) with no significant adverse toxicological effects attributed to clinical pathologies (blood biochemistries, hematology, coagulation, and urinalysis) (Anthony et al., 2006). A single dose of XOS (5,000 mg/kg BW) was found to be well-tolerated and non-toxic in the acute oral toxicity studies (Boonchuay et al., 2021). In line with this, tolerability and toxicity studies for other prebiotics are still underway. Despite promising preclinical findings, prebiotics have demonstrated limited efficacy in the management of behavioral symptoms at the clinical level. Preliminary evidence available at the clinical level supports the fact that prebiotic NDOs are capable of improving brain function and behavior. However, the studies are mainly of short duration (4–12 weeks) and are limited to healthy, young, and middle-aged adults. More research is needed to identify safe and effective dose, delivery method, and duration of application particularly among diseased adults and the geriatric population. Even though early life intake of various prebiotic NDOs is validated to support neurodevelopmental activities at the preclinical level, however, at the clinical level, the results seem to be detrimental. Thus, more reproducible and rigorous research is needed to evaluate early life intake of prebiotic NDOs on neurodevelopmental outcomes to draw an accurate conclusion. Finally, the available preclinical and clinical studies advance the potential application of prebiotics and/or probiotics and their combination as therapeutics in the treatment of brain disorders. Easier production, formulation procedures, and storage advantages in comparison to probiotics make prebiotics promising candidates for promoting better health. Further research on effective formulation with clinical studies is essential to advance the important potential of prebiotics to improve brain health and wellness and advance them as essential therapeutic candidates to incorporate in dietary and nutraceutical formulations.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Aachary, A. A., and Prapulla, S. G. (2011). Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications. *Compr. Rev. Food Sci. Food Saf.* 10 (1), 2–16. doi:10.1111/j.1541-4337.2010.00135.x

Ahmed, A. B. A., Taha, R. M., Mohajer, S., Elaagib, M. E., and Kim, S. K. (2012). Preparation, Properties and Biological Applications of Water Soluble Chitin Oligosaccharides from marine Organisms. *Russ. J. Mar. Biol.* 38 (4), 351–358. doi:10.1134/s1063074012040025

Ansari, F., Pourjafar, H., Tabrizi, A., and Homayouni, A. (2020). The Effects of Probiotics and Prebiotics on Mental Disorders: a Review on Depression, Anxiety, Alzheimer, and Autism Spectrum Disorders. *Curr. Pharm. Biotechnol.* 21 (7), 555–565. doi:10.2174/1389201021666200107113812

Anthony, J. C., Merriman, T. N., and Heimbach, J. T. (2006). 90-Day Oral (Gavage) Study in Rats with Galactooligosaccharides Syrup. *Food Chem. Toxicol.* 44 (6), 819–826. doi:10.1016/j.fct.2005.10.012

Asha Devi, S., and Satpati, A. (2017). Oxidative Stress and the Brain: an Insight into Cognitive Aging. In Topics in Biomedical Gerontology. Singapore: Springer, 123–140. doi:10.1007/978-981-10-2155-8_8

Ashwini, A., Ramya, H. N., Ramkumar, C., Reddy, K. R., Kulkarni, R. V., Abinaya, V., et al. (2019). Reactive Mechanism and the Applications of Bioactive Prebiotics for Human Health: Review. *J. Microbiol. Methods* 159, 128–137. doi:10.1016/j.mimet.2019.02.019

Azpiroz, F., Dubray, C., Bernalier-Donadille, A., Cardot, J. M., Accarino, A., Serra, J., et al. (2017). Effects of scFOS on the Composition of Fecal Microbiota and Anxiety in Patients with Irritable Bowel Syndrome: a Randomized, Double Blind, Placebo Controlled Study. *Neurogastroenterol Motil.* 29 (2), e12911. doi:10.1111/nmo.12911

Babbar, N., Dejonghe, W., Gatti, M., Sforza, S., and Elst, K. (2016). Pectic Oligosaccharides from Agricultural By-Products: Production, Characterization and Health Benefits. *Crit. Rev. Biotechnol.* 36 (4), 594–606. doi:10.3109/07388551.2014.996732

Black, C. N., Bot, M., Scheffer, P. G., Cuijpers, P., and Penninx, B. W. (2015). Is Depression Associated with Increased Oxidative Stress? A Systematic Review and Meta-Analysis. *Psychoneuroendocrinology* 51, 164–175. doi:10.1016/j.psyneuen.2014.09.025

Boets, E., Deroover, L., Houben, E., Vermeulen, K., Gomand, S. V., Delcour, J. A., et al. (2015). Quantification of *In Vivo* Colonic Short Chain Fatty Acid Production from Inulin. *Nutrients* 7 (11), 8916–8929. doi:10.3390/nu7115440

Boonchuay, P., Wongpoomchai, R., Jaturasitha, S., Mahatheeranont, S., Watanabe, M., and Chaiyaso, T. (2021). Prebiotic Properties, Antioxidant Activity, and Acute Oral Toxicity of Xylooligosaccharides Derived Enzymatically from Corncob. *Food Biosci.* 40, 100895. doi:10.1016/j.fbio.2021.100895

Bray, M. S., Lenz, T. K., Haynes, J. W., Bowman, J. C., Petrov, A. S., Reddi, A. R., Williams, L. D., and Glass, J. B. (2018). Multiple Prebiotic Metals Mediate Translation. *Proc. Natl. Acad. Sci. U S A.* 115 (48), 12164–12169. doi:10.1073/pnas.1803636115

Burokas, A., Arboleya, S., Moloney, R. D., Peterson, V. L., Murphy, K., Clarke, G., et al. (2017). Targeting the Microbiota-Gut-Brain axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice. *Biol. Psychiatry* 82 (7), 472–487. doi:10.1016/j.biopsych.2016.12.031

Carlson, J. L., Erickson, J. M., Lloyd, B. B., and Slavin, J. L. (2018). Health Effects and Sources of Prebiotic Dietary Fiber. *Curr. Dev. Nutr.* 2 (3), nzy005. doi:10.1093/cdn/nzy005

Cerdó, T., García-Santos, J., G. Bermúdez, M., and Campoy, C. (2019). The Role of Probiotics and Prebiotics in the Prevention and Treatment of Obesity. *Nutrients* 11 (3), 635. doi:10.3390/nu11030635

Chen, D., Yang, X., Yang, J., Lai, G., Yong, T., Tang, X., et al. (2017). Prebiotic Effect of Fructooligosaccharides from Morinda Officinalis on Alzheimer's Disease in Rodent Models by Targeting the Microbiota-Gut-Brain Axis. *Front. Aging Neurosci.* 9, 403. doi:10.3389/fnagi.2017.00403

Chen, X., D'Souza, R., and Hong, S. T. (2013). The Role of Gut Microbiota in the Gut-Brain axis: Current Challenges and Perspectives. *Protein Cell* 4 (6), 403–414. doi:10.1007/s13238-013-3017-x

Chen, X., Eslamfam, S., Fang, L., Qiao, S., and Ma, X. (2017). Maintenance of Gastrointestinal Glucose Homeostasis by the Gut-Brain axis. *Curr. Protein Pept. Sci.* 18 (6), 541–547. doi:10.2174/1389203717666160627083604

Chen, Z., and Trapp, B. D. (2016). Microglia and Neuroprotection. *J. Neurochem.* 136, 10–17. doi:10.1111/jnc.13062

Chunchai, T., Thunapong, W., Yasom, S., Wanchai, K., Eaimworawuthikul, S., Metzler, G., et al. (2018). Decreased Microglial Activation through Gut-Brain axis by Prebiotics, Probiotics, or Synbiotics Effectively Restored Cognitive Function in Obese-Insulin Resistant Rats. *J. Neuroinflammation.* 15 (1), 11. doi:10.1186/s12974-018-1055-2

Clarke, T. B., Davis, K. M., Lysenko, E. S., Zhou, A. Y., Yu, Y., and Weiser, J. N. (2010). Recognition of Peptidoglycan from the Microbiota by Nod1 Enhances Systemic Innate Immunity. *Nat. Med.* 16 (2), 228–231. doi:10.1038/nm.2087

Collins, S., and Reid, G. (2016). Distant Site Effects of Ingested Prebiotics. *Nutrients* 8 (9), 523. doi:10.3390/nu8090523

Dahiya, D. K., Renuka, M., Puniya, M., Shandilya, U. K., Dhewa, T., Kumar, N., et al. (2017). Gut Microbiota Modulation and its Relationship with Obesity Using Prebiotic Fibers and Probiotics: a Review. *Front. Microbiol.* 8, 563. doi:10.3389/fmicb.2017.00563

Dalile, B., Van Oudenhove, L., Vervliet, B., and Verbeke, K. (2019). The Role of Short-Chain Fatty Acids in Microbiota-Gut-Brain Communication. *Nat. Rev. Gastroenterol. Hepatol.* 16, 461–478. doi:10.1038/s41575-019-0157-3

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S. J., et al. (2019). Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. *Foods* 8 (3), 92. doi:10.3390/foods8030092

de Souza Aquino, J., Batista, K. S., Menezes, F. N. D. D., Lins, P. P., de Sousa Gomes, J. A., and da Silva, L. A. (2017). Models to Evaluate the Prebiotic Potential of Foods. *Funct. Food Improve Health Through Adequate Food*, 235–256. doi:10.5772/intechopen.69174

Divyashri, G., Krishna, G., Muralidhara, M., and Prapulla, S. G. (2015). Probiotic Attributes, Antioxidant, Anti-inflammatory and Neuromodulatory Effects of *Enterococcus Faecium* CFR 3003: *In Vitro* and *In Vivo* Evidence. *J. Med. Microbiol.* 64 (12), 1527–1540. doi:10.1099/jmm.0.000184

Drummond, E., and Wisniewski, T. (2017). Alzheimer's Disease: Experimental Models and Reality. *Acta Neuropathol.* 133 (2), 155–175. doi:10.1007/s00401-016-1662-x

Dumitrescu, L., Popescu-Olaru, I., Cozma, L., Tulbă, D., Hinescu, M. E., Ceafalan, L. C., et al. (2018). Oxidative Stress and the Microbiota-Gut-Brain axis. *Oxid. Med. Cel. Longev.* 2018, 2406594. doi:10.1155/2018/2406594

Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M., and Tsai, L. H. (2007). Recovery of Learning and Memory Is Associated with Chromatin Remodelling. *Nature* 447 (7141), 178–182. doi:10.1038/nature05772

Fleiss, B., Nilsson, M. K., Blomgren, K., and Mallard, C. (2012). Neuroprotection by the Histone Deacetylase Inhibitor Trichostatin A in a Model of Lipopolysaccharide-Sensitised Neonatal Hypoxic-Ischaemic Brain Injury. *J. Neuroinflammation* 9 (1), 70–15. doi:10.1186/1742-2094-9-70

Fleming, S. A., Monaikul, S., Patsavas, A. J., Waworuntu, R. V., Berg, B. M., and Dilger, R. N. (2019). Dietary Polydextrose and Galactooligosaccharide Increase Exploratory Behavior, Improve Recognition Memory, and Alter Neurochemistry in the Young Pig. *Nutr. Neurosci.* 22 (7), 499–512. doi:10.1080/1028415X.2017.1415280

Foley, K. A., Ossenkopp, K. P., Kavaliers, M., and MacFabe, D. F. (2014). Pre- and Neonatal Exposure to Lipopolysaccharide or the Enteric Metabolite, Propionic Acid, Alters Development and Behavior in Adolescent Rats in a Sexually Dimorphic Manner. *PLoS One* 9 (1), e87072. doi:10.1371/journal.pone.0087072

Fung, T. C., Olson, C. A., and Hsiao, E. Y. (2017). Interactions between the Microbiota, Immune and Nervous Systems in Health and Disease. *Nat. Neurosci.* 20 (2), 145–155. doi:10.1038/nn.4476

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert Consensus Document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) Consensus Statement on the Definition and Scope of Prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 14 (8), 491–502. doi:10.1038/nrgastro.2017.75

Gibson, G. R., and Roberfroid, M. B. (1995). Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. *J. Nutr.* 125 (6), 1401–1412. doi:10.1093/jn/125.6.1401

Gibson, G. R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., et al. (2010). Dietary Prebiotics: Current Status and New Definition. *Food Sci. Tech. Bull. Funct. Foods* 7 (1), 1–19. doi:10.1616/1476-2137.15880

Grimaldi, R., Cela, D., Swann, J. R., Vulevic, J., Gibson, G. R., Tzortzis, G., et al. (2017). *In Vitro* fermentation of B-GOS: Impact on Faecal Bacterial Populations and Metabolic Activity in Autistic and Non-autistic Children. *FEMS Microbiol. Ecol.* 93 (2), 1–13. doi:10.1093/femsec/fiw233

Grimaldi, R., Gibson, G. R., Vulevic, J., Giallourou, N., Castro-Mejía, J. L., Hansen, L. H., et al. (2018). A Prebiotic Intervention Study in Children with Autism Spectrum Disorders (ASDs). *Microbiome* 6 (1), 133–213. doi:10.1186/s40168-018-0523-3

Haghighat, N., Rajabi, S., and Mohammadshahi, M. (2019). Effect of Synbiotic and Probiotic Supplementation on Serum Brain-Derived Neurotrophic Factor Level, Depression and Anxiety Symptoms in Hemodialysis Patients: a Randomized, Double-Blinded, Clinical Trial. *Nutr. Neurosci.* 1, 1–10. doi:10.1080/1028415X.2019.1646975

Hasan, N., and Yang, H. (2019). Factors Affecting the Composition of the Gut Microbiota, and its Modulation. *PeerJ* 7, e7502. doi:10.7717/peerj.7502

Heyck, M., and Ibarra, A. (2019). Microbiota and Memory: A Symbiotic Therapy to Counter Cognitive Decline? *Brain Circ.* 5 (3), 124–129. doi:10.4103/bc.bc_34_19

Hutkins, R. W., Krumbeck, J. A., Bindels, L. B., Cani, P. D., Fahey, G., Jr, Goh, Y. J., et al. (2016). Prebiotics: Why Definitions Matter. *Curr. Opin. Biotechnol.* 37, 1–7. doi:10.1016/j.copbio.2015.09.001

Hwang, H. W., and Lee, D. W. (2019). Prebiotics: An Overview of Current Researches and Industrial Applications. *Food Sci. Ind.* 52 (3), 241–260.

Jain, I., Kumar, V., and Satyanarayana, T. (2015). Xylooligosaccharides: an Economical Prebiotic from Agroresidues and Their Health Benefits. *Indian J. Exp. Biol.* 53, 131–142.

Jana, U. K., and Kango, N. (2020). Characteristics and Bioactive Properties of Mannooligosaccharides Derived from Agro-Waste Mannans. *Int. J. Biol. Macromol.* 149, 931–940. doi:10.1016/j.ijbiomac.2020.01.304

Jaworska, J., Zalewska, T., Sypecka, J., and Ziemka-Nalecz, M. (2019). Effect of the HDAC Inhibitor, Sodium Butyrate, on Neurogenesis in a Rat Model of Neonatal Hypoxia-Ischemia: Potential Mechanism of Action. *Mol. Neurobiol.* 56 (9), 6341–6370. doi:10.1007/s12035-019-1518-1

Jia, S., Lu, Z., Gao, Z., An, J., Wu, X., Li, X., et al. (2016). Chitosan Oligosaccharides Alleviate Cognitive Deficits in an Amyloid-B1-42-Induced Rat Model of Alzheimer's Disease. *Int. J. Biol. Macromol* 83, 416–425. doi:10.1016/j.ijbiomac.2015.11.011

Jian, H. L., Zhu, L. W., Zhang, W. M., Sun, D. F., and Jiang, J. X. (2013). Enzymatic Production and Characterization of Manno-Oligosaccharides from Gleditsia Sinensis Galactomannan Gum. *Int. J. Biol. Macromol.* 55, 282–288. doi:10.1016/j.ijbiomac.2013.01.025

Johnstone, N., Milesi, C., Burn, O., van den Bogert, B., Nauta, A., Hart, K., et al. (2021). Anxiolytic Effects of a Galacto-Oligosaccharides Prebiotic in Healthy Females (18-25 Years) with Corresponding Changes in Gut Bacterial Composition. *Sci. Rep.* 11 (1), 8302–8311. doi:10.1038/s41598-021-87865-w

Jung, T. H., Park, J. H., Jeon, W. M., and Han, K. S. (2015). Butyrate Modulates Bacterial Adherence on LS174T Human Colorectal Cells by Stimulating Mucin Secretion and MAPK Signaling Pathway. *Nutr. Res. Pract.* 9 (4), 343–349. doi:10.4162/nrp.2015.9.4.343

Kao, A. C., Harty, S., and Burnet, P. W. (2016). The Influence of Prebiotics on Neurobiology and Behavior. *Int. Rev. Neurobiol.* 131, 21–48. doi:10.1016/bs.irn.2016.08.007

Kałużna-Czaplińska, J., and Błaszczyk, S. (2012). The Level of Arabinitol in Autistic Children after Probiotic Therapy. *Nutrition* 28 (2), 124–126. doi:10.1016/j.nut.2011.08.002

Keenan, M. J., Zhou, J., Hegsted, M., Pelkman, C., Durham, H. A., Coulon, D. B., et al. (2015). Role of Resistant Starch in Improving Gut Health, Adiposity, and Insulin Resistance. *Adv. Nutr.* 6 (2), 198–205. doi:10.3945/an.114.007419

Kelly, C. J., Zheng, L., Campbell, E. L., Saeedi, B., Scholz, C. C., Bayless, A. J., et al. (2015). Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell Host Microbe* 17 (5), 662–671. doi:10.1016/j.chom.2015.03.005

Krishna, G., Divyashri, G., Prapulla, S. G., and Muralidhara (2015). A Combination Supplement of Fructo- and Xylo-Oligosaccharides Significantly Abrogates Oxidative Impairments and Neurotoxicity in Maternal/Fetal Milieu Following Gestational Exposure to Acrylamide in Rat. *Neurochem. Res.* 40 (9), 1904–1918. doi:10.1007/s11064-015-168710.1007/s11064-015-1687-x

Krishna, G., and Muralidhara (2015). Inulin Supplementation during Gestation Mitigates Acrylamide-Induced Maternal and Fetal Brain Oxidative Dysfunctions and Neurotoxicity in Rats. *Neurotoxicol. Teratol.* 49, 49–58. doi:10.1016/j.ntt.2015.03.003

Krishna, G., and Muralidhara (2018). Oral Supplements of Inulin during Gestation Offsets Rotenone-Induced Oxidative Impairments and Neurotoxicity in Maternal and Prenatal Rat Brain. *Biomed. Pharmacother.* 104, 751–762. doi:10.1016/j.biopha.2018.05.107

Kumar, A., Singh, A., and Ekavali, fnm. (2015). A Review on Alzheimer's Disease Pathophysiology and its Management: an Update. *Pharmacol. Rep.* 67 (2), 195–203. doi:10.1016/j.pharep.2014.09.004

LeCouffe, N. E., Westerbeek, E. A., van Schie, P. E., Schaaf, V. A., Lafeber, H. N., van Elburg, R. M., et al. (2014). Neurodevelopmental Outcome during the First Year of Life in Preterm Infants after Supplementation of a Prebiotic Mixture in the Neonatal Period: a Follow-Up studyPrebiotic Lactulose Ameliorates the Cognitive Deficit in Alzheimer's Disease Mouse Model through Macroautophagy and Chaperone-Mediated Autophagy Pathways. *NeuropediatricsJournal Agric. Food Chem.* 45 (01), 22–29. doi:10.1055/s-0033-1349227

Li, J., Huang, L., Yu, L. T., Tao, G., Wang, Z. Y., Hao, W. Z., et al. (2020). Feruloylated Oligosaccharides Alleviate Central Nervous Inflammation in Mice Following Spinal Cord Contusion. *J. Agric. Food Chem.* 68 (52), 15490–15500. doi:10.1021/acs.jafc.0c05553

Licciardi, P. V., Ververis, K., and Karagiannis, T. C. (2011). Histone Deacetylase Inhibition and Dietary Short-Chain Fatty Acids. *ISRN Allergy* 2011, 869647. doi:10.5402/2011/869647

Lin, M. Y., de Zoete, M. R., van Putten, J. P., and Strijbis, K. (2015). Redirection of Epithelial Immune Responses by Short-Chain Fatty Acids through Inhibition of Histone Deacetylases. *Front. Immunol.* 6, 554. doi:10.3389/fimmu.2015.00554

Liu, C. Y., Wang, X., Liu, C., and Zhang, H. L. (2019). Pharmacological Targeting of Microglial Activation: New Therapeutic Approach. *Front. Cel. Neurosci.* 13, 514. doi:10.3389/fncel.2019.00514

Liu, H., Wang, J., He, T., Becker, S., Zhang, G., Li, D., et al. (2018). Butyrate: a Double-Edged Sword for Health? *Adv. Nutr.* 9 (1), 21–29. doi:10.1093/advances/nmx009

Liu, J., Liu, X., Xiong, X. Q., Yang, T., Cui, T., Hou, N. L., et al. (2017). Effect of Vitamin A Supplementation on Gut Microbiota in Children with Autism Spectrum Disorders - a Pilot Study. *BMC Microbiol.* 17 (1), 204–214. doi:10.1186/s12866-017-1096-1

Liu, X., Cao, S., and Zhang, X. (2015). Modulation of Gut Microbiota-Brain Axis by Probiotics, Prebiotics, and Diet. *J. Agric. Food Chem.* 63 (36), 7885–7895. doi:10.1021/acs.jafc.5b02404

Lockyer, S., and Stanner, S. (2019). Prebiotics - an Added Benefit of Some Fibre Types. *Nutr. Bull.* 44 (1), 74–91. doi:10.1111/nbu.12366

MacFabe, D. F. (2015). Enteric Short-Chain Fatty Acids: Microbial Messengers of Metabolism, Mitochondria, and Mind: Implications in Autism Spectrum Disorders. *Microb. Ecol. Health Dis.* 26 (1), 28177. doi:10.3402/mehd.v26.28177

MacFabe, D. F. (2012). Short-chain Fatty Acid Fermentation Products of the Gut Microbiome: Implications in Autism Spectrum Disorders. *Microb. Ecol. Health Dis.* 23 (1), 19260. doi:10.3402/mehd.v23i0.19260

Mamo, G., Faryar, R., and Karlsson, E. N. (2013). Microbial Glycoside Hydrolases for Biomass Utilization in Biofuels Applications, *Biofuel Technologies*. Berlin, Heidelberg: Springer, 171–188. doi:10.1007/978-3-642-34519-7_7

Manoharan, S., Guillemin, G. J., Abiramasundari, R. S., Essa, M. M., Akbar, M., and Akbar, M. D. (2016). The Role of Reactive Oxygen Species in the Pathogenesis of Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease: A Mini Review. *Oxid. Med. Cel. Longev.* 2016, 8590578. doi:10.1155/2016/8590578

Mudd, A. T., Alexander, L. S., Berding, K., Waworuntu, R. V., Berg, B. M., Donovan, S. M., et al. (2016). Dietary Prebiotics, Milk Fat Globule Membrane, and Lactoferrin Affects Structural Neurodevelopment in the Young Piglet. *Front. Pediatr.* 4, 4. doi:10.3389/fped.2016.00004

Nidheesh, T., Salim, C., Rajini, P. S., and Suresh, P. V. (2016). Antioxidant and Neuroprotective Potential of Chitooligomers in *Caenorhabditis elegans*

Exposed to Monocrotophos. *Carbohydr. Polym.* 135, 138–144. doi:10.1016/j.carbpol.2015.08.055

Niu, M., Li, Q., Zhang, J., Wen, F., Dang, W., Duan, G., et al. (2019). Characterization of Intestinal Microbiota and Probiotics Treatment in Children with Autism Spectrum Disorders in China. *Front. Neurol.* 10, 1084. doi:10.3389/fneur.2019.01084

Osman, A., Tzortzis, G., Rastall, R. A., and Charalampopoulos, D. (2012). BbgIV Is an Important Bifidobacterium β-Galactosidase for the Synthesis of Prebiotic Galactooligosaccharides at High Temperatures. *J. Agric. Food Chem.* 60 (3), 740–748. doi:10.1021/jf204719w

Parada Venegas, D., De la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., et al. (2019). Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and its Relevance for Inflammatory Bowel Diseases. *Front. Immunol.* 10, 277. doi:10.3389/fimmu.2019.00277

Park, J., Lee, J., Yeom, Z., Heo, D., and Lim, Y. H. (2017). Neuroprotective Effect of *Ruminococcus Albus* on Oxidatively Stressed SH-Sy5y Cells and Animals. *Sci. Rep.* 7 (1), 1–13. doi:10.1038/s41598-017-15163-5

Pineda-Rodriguez, B., Toscano-Tejeida, D., García-Vences, E., Rodriguez-Barrera, R., Flores-Romero, A., Castellanos-Canales, D., et al. (2017). Anterior Chamber Associated Immune Deviation Used as a Neuroprotective Strategy in Rats with Spinal Cord Injury. *Plos one* 12 (11), e0188506. doi:10.1371/journal.pone.0188506

Polazzi, E., and Monti, B. (2010). Microglia and Neuroprotection: from *In Vitro* Studies to Therapeutic Applications. *Prog. Neurobiol.* 92 (3), 293–315. doi:10.1016/j.pneurobio.2010.06.009

Psichas, A., Sleeth, M. L., Murphy, K. G., Brooks, L., Bewick, G. A., Hanyaloglu, A. C., et al. (2015). The Short Chain Fatty Acid Propionate Stimulates GLP-1 and PYY Secretion via Free Fatty Acid Receptor 2 in Rodents. *Int. J. Obes. (Lond)* 39 (3), 424–429. doi:10.1038/ijo.2014.153

Qin, Z., and Zhao, L. (2019). The History of Chito/Chitin Oligosaccharides and its Monomer, *Oligosaccharides of Chitin and Chitosan*. Singapore: Springer, 3–14. doi:10.1007/978-981-13-9402-7_1

Rezaee, F. (2019). Propionic Acid Counteracts the Inflammation of Human Subcutaneous Adipose Tissue: a New Avenue for Drug Development. *DARU J. Pharm. Sci.* 27 (2), 645–652.

Rink, C., and Khanna, S. (2011). Significance of Brain Tissue Oxygenation and the Arachidonic Acid cascade in Stroke. *Antioxid. Redox Signal.* 14 (10), 1889–1903. doi:10.1089/ars.2010.3474

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavilán, C. G., and Salazar, N. (2016). Intestinal Short Chain Fatty Acids and Their Link with Diet and Human Health. *Front. Microbiol.* 7, 185. doi:10.3389/fmicb.2016.00185

Romo-Araiza, A., Gutiérrez-Salmeán, G., Galván, E. J., Hernández-Frausto, M., Herrera-López, G., Romo-Parra, H., et al. (2018). Probiotics and Prebiotics as a Therapeutic Strategy to Improve Memory in a Model of Middle-Aged Rats. *Front. Aging Neurosci.* 10, 416. doi:10.3389/fnagi.2018.00416

Ryan, S. M., and Nolan, Y. M. (2016). Neuroinflammation Negatively Affects Adult Hippocampal Neurogenesis and Cognition: Can Exercise Compensate? *Neurosci. Biobehav. Rev.* 61, 121–131. doi:10.1016/j.neubiorev.2015.12.004

Sampson, T. R., and Mazmanian, S. K. (2015). Control of Brain Development, Function, and Behavior by the Microbiome. *Cell Host Microbe* 17 (5), 565–576. doi:10.1016/j.chom.2015.04.011

Sanctuary, M. R., Kain, J. N., Chen, S. Y., Kalanetra, K., Lemay, D. G., Rose, D. R., et al. (2019). Pilot Study of Probiotic/colostrum Supplementation on Gut Function in Children with Autism and Gastrointestinal Symptoms. *PloS one* 14 (1), e0210064. doi:10.1371/journal.pone.0210064

Sandhu, K. V., Sherwin, E., Schellekens, H., Stanton, C., Dinan, T. G., and Cryan, J. F. (2017). Feeding the Microbiota-Gut-Brain axis: Diet, Microbiome, and Neuropsychiatry. *Transl. Res.* 179, 223–244. doi:10.1016/j.trsl.2016.10.002

Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F., and Burnet, P. W. J. (2016). Psychobiotics and the Manipulation of Bacteria-Gut-Brain Signals. *Trends Neurosci.* 39 (11), 763–781. doi:10.1016/j.tins.2016.09.002

Schmidt, K., Cowen, P. J., Harmer, C. J., Tzortzis, G., Errington, S., and Burnet, P. W. (2015). Prebiotic Intake Reduces the Waking Cortisol Response and Alters Emotional Bias in Healthy Volunteers. *Psychopharmacology (Berl)* 232 (10), 1793–1801. doi:10.1007/s00213-014-3810-0

Schroeder, B. O., Birchenough, G. M. H., Ståhlman, M., Arike, L., Johansson, M. E. V., Hansson, G. C., et al. (2018). Bifidobacteria or Fiber Protects against Diet-Induced Microbiota-Mediated Colonic Mucus Deterioration. *Cell Host Microbe* 23 (1), 27–e7. doi:10.1016/j.chom.2017.11.004

Schroeder, F. A., Lin, C. L., Crusio, W. E., and Akbarian, S. (2007). Antidepressant-like Effects of the Histone Deacetylase Inhibitor, Sodium Butyrate, in the Mouse. *Biol. Psychiatry* 62 (1), 55–64. doi:10.1016/j.biopsych.2006.06.036

Scott, K. P., Grimaldi, R., Cunningham, M., Sarbini, S. R., Wijeyesekera, A., Tang, M. L. K., et al. (2020). Developments in Understanding and Applying Prebiotics in Research and Practice-An ISAPP Conference Paper. *J. Appl. Microbiol.* 128 (4), 934–949. doi:10.1111/jam.14424

Serra, M. C., Nocera, J. R., Kelleher, J. L., and Addison, O. (2019). Prebiotic Intake in Older Adults: Effects on Brain Function and Behavior. *Curr. Nutr. Rep.* 8 (2), 66–73. doi:10.1007/s13668-019-0265-2

Shaaban, S. Y., El Gendy, Y. G., Mehanna, N. S., El-Senousy, W. M., El-Feki, H. S. A., Saad, K., et al. (2018). The Role of Probiotics in Children with Autism Spectrum Disorder: A Prospective, Open-Label Study. *Nutr. Neurosci.* 21 (9), 676–681. doi:10.1080/1028415X.2017.1347746

Shukla, S., Shukla, A., Mehboob, S., and Guha, S. (2011). Meta-analysis: the Effects of Gut flora Modulation Using Prebiotics, Probiotics and Synbiotics on Minimal Hepatic Encephalopathy. *Aliment. Pharmacol. Ther.* 33 (6), 662–671. doi:10.1111/j.1365-2036.2010.04574.x

Silva, Y. P., Bernardi, A., and Frozza, R. L. (2020). The Role of Short-Chain Fatty Acids from Gut Microbiota in Gut-Brain Communication. *Front. Endocrinol. (Lausanne)* 11, 25. doi:10.3389/fendo.2020.00025

Singh, S., Ghosh, A., and Goyal, A. (2018). Manno-oligosaccharides as Prebiotic-Valued Products from Agro-Waste. In Biosynthetic Technology and Environmental Challenges. Singapore: Springer, 205–221. doi:10.1007/978-981-10-7434-9_12

Singh, S. P., Jadaun, J. S., Narnoliya, L. K., and Pandey, A. (2017). Prebiotic Oligosaccharides: Special Focus on Fructooligosaccharides, its Biosynthesis and Bioactivity. *Appl. Biochem. Biotechnol.* 183 (2), 613–635. doi:10.1007/s12010-017-2605-2

Smith, A. P., Sutherland, D., and Hewlett, P. (2015). An Investigation of the Acute Effects of Oligofructose-Enriched Inulin on Subjective Wellbeing, Mood and Cognitive Performance. *Nutrients* 7 (11), 8887–8896. doi:10.3390/nu7115441

Soret, R., Chevalier, J., De Coppet, P., Poupeau, G., Derkinderen, P., Segain, J. P., et al. (2010). Short-chain Fatty Acids Regulate the Enteric Neurons and Control Gastrointestinal Motility in Rats. *Gastroenterology* 138 (5), 1772–1782. doi:10.1053/j.gastro.2010.01.053

Stadlbauer, U., Woods, S. C., Langhans, W., and Meyer, U. (2015). PYY3-36: Beyond Food Intake. *Front. Neuroendocrinol* 38, 1–11. doi:10.1016/j.yfrne.2014.12.003

Stilling, R. M., van de Wouw, M., Clarke, G., Stanton, C., Dinan, T. G., and Cryan, J. F. (2016). The Neuropharmacology of Butyrate: the Bread and Butter of the Microbiota-Gut-Brain axis? *Neurochem. Int.* 99, 110–132. doi:10.1016/j.neuint.2016.06.011

Stinson, L. F., Payne, M. S., and Keelan, J. A. (2017). Planting the Seed: Origins, Composition, and Postnatal Health Significance of the Fetal Gastrointestinal Microbiota. *Crit. Rev. Microbiol.* 43 (3), 352–369. doi:10.1080/1040841X.2016.1211088

Sukri, S. S. M., and Mimi Sakinah, A. M. (2018). Production of High Commercial Value Xylooligosaccharides from Meranti wood Sawdust Using Immobilised Xylanase. *Appl. Biochem. Biotechnol.* 184 (1), 278–290. doi:10.1007/s12010-017-2542-0

Sun, J., Wang, F., Hong, G., Pang, M., Xu, H., Li, H., et al. (2016). Antidepressant-like Effects of Sodium Butyrate and its Possible Mechanisms of Action in Mice Exposed to Chronic Unpredictable Mild Stress. *Neurosci. Lett.* 618, 159–166. doi:10.1016/j.neulet.2016.03.003

Takuma, K., Hara, Y., Kataoka, S., Kawanai, T., Maeda, Y., Watanabe, R., et al. (2014). Chronic Treatment with Valproic Acid or Sodium Butyrate Attenuates Novel Object Recognition Deficits and Hippocampal Dendritic Spine Loss in a Mouse Model of Autism. *Pharmacol. Biochem. Behav.* 126, 43–49. doi:10.1016/j.pbb.2014.08.013

Teles, F., Azevedo, V. F., Miranda, C. T., Miranda, M. P., Teixeira, Mdo. C., and Elias, R. M. (2014). Depression in Hemodialysis Patients: the Role of Dialysis Shift. *Clinics (Sao Paulo)* 69 (3), 198–202. doi:10.6061/clinics/2014(03)10

Torres, D. P. M., Gonçalves, M. D. P. F., Teixeira, J. A., and Rodrigues, L. R. (2010). Galacto-Oligosaccharides: Production, Properties, Applications, and

Significance as Prebiotics. *Compr. Rev. Food Sci. Food Saf.* 9 (5), 438–454. doi:10.1111/j.1541-4337.2010.00119.x

Tran, S. M.-S., and Mohajeri, M. H. (2021). The Role of Gut Bacterial Metabolites in Brain Development, Aging and Disease. *Nutrients* 13 (3), 732. doi:10.3390/nu13030732

Callaway, T. R., and Ricke, S. C. (2011). in *Direct-fed Microbials and Prebiotics for Animals: Science and Mechanisms of Action* (Springer Science & Business Media).

Upadhyay, R. P., Taneja, S., Chowdhury, R., Strand, T. A., and Bhandari, N. (2020). Effect of Prebiotic and Probiotic Supplementation on Neurodevelopment in Preterm Very Low Birth Weight Infants: Findings from a Meta-Analysis. *Pediatr. Res.* 87 (5), 811–822. doi:10.1038/s41390-018-0211-9

van de Wouw, M., Boehme, M., Lyte, J. M., Wiley, N., Strain, C., O'Sullivan, O., et al. (2018). Short-chain Fatty Acids: Microbial Metabolites that Alleviate Stress-Induced Brain-Gut axis Alterations. *J. Physiol.* 596 (20), 4923–4944. doi:10.1113/JP276431

van de Wouw, M., Schellekens, H., Dinan, T. G., and Cryan, J. F. (2017). Microbiota-gut-brain axis: Modulator of Host Metabolism and Appetite. *J. Nutr.* 147 (5), 727–745. doi:10.3945/jn.116.240481

van den Berg, J. P., Westerbeek, E. A., Bröring-Starre, T., Garssen, J., and van Elburg, R. M. (2016). Neurodevelopment of Preterm Infants at 24 Months after Neonatal Supplementation of a Prebiotic Mix: A Randomized Trial. *J. Pediatr. Gastroenterol. Nutr.* 63 (2), 270–276. doi:10.1097/MPG.0000000000001148

Vandenplas, Y., Zakharova, I., and Dmitrieva, Y. (2015). Oligosaccharides in Infant Formula: More Evidence to Validate the Role of Prebiotics. *Br. J. Nutr.* 113 (9), 1339–1344. doi:10.1017/S0007114515000823

Vera, C., Córdova, A., Aburto, C., Guerrero, C., Suárez, S., and Illanes, A. (2016). Synthesis and Purification of Galacto-Oligosaccharides: State of the Art. *World J. Microbiol. Biotechnol.* 32 (12), 197. doi:10.1007/s11274-016-2159-4

Vijay, N., and Morris, M. E. (2014). Role of Monocarboxylate Transporters in Drug Delivery to the Brain. *Curr. Pharm. Des.* 20 (10), 1487–1498. doi:10.2174/13816128113199990462

Waworuntu, R., Hain, H., Chang, Q., Thiede, L., Hanania, T., and Berg, B. (2014). Dietary Prebiotics Improve Memory and Social Interactions while Reducing Anxiety when provided Early in Life to Normally Developing Rodents (637.5). *FASEB J.* 28, 637–645. doi:10.1096/fasebj.28.1_supplement.637.5

Wei, Y., Melas, P. A., Wegener, G., Mathé, A. A., and Lavebratt, C. (2015). Antidepressant-like Effect of Sodium Butyrate Is Associated with an Increase in TET1 and in 5-hydroxymethylation Levels in the Bdnf Gene. *Int. J. Neuropsychopharmacol.* 18 (2). doi:10.1093/ijnp/pyu032

Westfall, S., Lomis, N., and Prakash, S. (2018). A Novel Polyphenolic Prebiotic and Probiotic Formulation Have Synergistic Effects on the Gut Microbiota Influencing *Drosophila melanogaster* Physiology. *Artif. Cell Nanomed Biotechnol* 46 (Suppl. 2), 441–455. doi:10.1080/21691401.2018.1458731

Williams, S., Chen, L., Savignac, H. M., Tzortzis, G., Anthony, D. C., and Burnet, P. W. (2016). Neonatal Prebiotic (BGOS) Supplementation Increases the Levels of Synaptophysin, GluN2A-Subunits and BDNF Proteins in the Adult Rat hippocampus. *Synapse* 70 (3), 121–124. doi:10.1002/syn.218810.1002/syn.21880

World Health Organization (2019). *The WHO Special Initiative for Mental Health (2019-2023): Universal Health Coverage for Mental Health*. No. WHO/MSD/19.1. Geneva: World Health Organization

Wu, W., Wei, W., Lu, M., Zhu, X., Liu, N., Niu, Y., et al. (2017). Neuroprotective Effect of Chitosan Oligosaccharide on Hypoxic-Ischemic Brain Damage in Neonatal Rats. *Neurochem. Res.* 42 (11), 3186–3198. doi:10.1007/s11064-017-2356-z

Xu, X., Wang, K., Cao, X., Li, Z., Zhou, Y., Ren, J., et al. (2021). Gut Microbial Metabolite Short-Chain Fatt Acids Partially Reverse Surgery and Anesthesia-Induced Behavior Deficits in C57BL/6J Mice. *Front. Neurosci.* 15, 593. doi:10.3389/fnins.2021.664641

Yang, X. D., Wang, L. K., Wu, H. Y., and Jiao, L. (2018). Effects of Prebiotic Galacto-Oligosaccharide on Postoperative Cognitive Dysfunction and Neuroinflammation through Targeting of the Gut-Brain axis. *BMC Anesthesiol* 18 (1), 177. doi:10.1186/s12871-018-0642-1

Yen, C. H., Wang, C. H., Wu, W. T., and Chen, H. L. (2017). Fructo-oligosaccharide Improved Brain β-amyloid, β-secretase, Cognitive Function, and Plasma Antioxidant Levels in D-Galactose-Treated Balb/cJ Mice. *Nutr. Neurosci.* 20 (4), 228–237. doi:10.1080/1028415X.2015.1110952

Ziemka-Nalecz, M., Jaworska, J., Sypecka, J., Polowy, R., Filipkowski, R. K., and Zalewska, T. (2017). Sodium Butyrate, a Histone Deacetylase Inhibitor, Exhibits Neuroprotective/neurogenic Effects in a Rat Model of Neonatal Hypoxia-Ischemia. *Mol. Neurobiol.* 54 (7), 5300–5318. doi:10.1007/s12035-016-0049-2

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 25 August 2021
doi: 10.3389/fphar.2021.723040

Olive Oil: Nutritional Applications, Beneficial Health Aspects and its Prospective Application in Poultry Production

Rana M. Bilal[1,2], Chunjie Liu[1], Haohan Zhao[1], Yanzhou Wang[1,3], Mayada R. Farag[4], Mahmoud Alagawany[5], Faiz-ul Hassan[6], Shaaban S. Elnesr[7], Hamada A. M. Elwan[8], Huajiao Qiu[1] and Qian Lin[1,3]**

[1]Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha, China, [2]University College of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan, [3]Hunan Deren Husbandry Technology Co., Ltd., Changde, China, [4]Forensic Medicine and Toxicology Department, Veterinary Medicine Faculty, Zagazig University, Zagazig, Egypt, [5]Poultry Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt, [6]Institute of Animal and Dairy Sciences, Faculty of Animal Husbandry, University of Agriculture, Faisalabad, Pakistan, [7]Poultry Production Department, Faculty of Agriculture, Fayoum University, Fayoum, Egypt, [8]Animal and Poultry Production Department, Faculty of Agriculture, Minia University, El-Minya, Egypt

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Ioannis Zabetakis, University of Limerick, Ireland
Homero Rubbo, Universidad de la República, Uruguay
Cristiano Capurso, University of Foggia, Italy
Rodrigo Valenzuela, University of Chile, Chile

***Correspondence:**
Huajiao Qiu
qiuhuajiao@caas.cn
Qian Lin
kinglli@163.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *09 June 2021*
Accepted: *12 July 2021*
Published: *25 August 2021*

Citation:
Bilal RM, Liu C, Zhao H, Wang Y, Farag MR, Alagawany M, Hassan F, Elnesr SS, Elwan HAM, Qiu H and Lin Q (2021) Olive Oil: Nutritional Applications, Beneficial Health Aspects and its Prospective Application in Poultry Production. Front. Pharmacol. 12:723040. doi: 10.3389/fphar.2021.723040

Plant polyphenols have promoting health features, including anti-mutagenic, anti-inflammatory, anti-thrombotic, anti-atherogenic, and anti-allergic effects. These polyphenols improve the immune system by affecting the white blood cell proliferation, as well as by the synthesis of cytokines and other factors, which contribute to immunological resistance. Olive trees are one of the most famous trees in the world. Whereas, olive olive oil and derivatives represent a large group of feeding resource for farm animals. In recent years, remarkable studies have been carried out to show the possible use of olive oil and derivatives for improvement of both animal performance and product quality. In vivo application of olive oil and its derived products has shown to maintain oxidative balance owing to its polyphenolic content. Consumption of extra virgin olive oil reduces the inflammation, limits the risk of liver damage, and prevents the progression of steatohepatitis through its potent antioxidant activities. Also, the monounsaturated fatty acids content of olive oil (particularly oleic acid), might have positive impacts on lipid peroxidation and hepatic protection. Therefore, this review article aims to highlight the nutritional applications and beneficial health aspects of olive oil and its effect on poultry production.

Keywords: Health, olive oil, poultry, nutrition, feed

INTRODUCTION

Plant-derived supplements are usually used to improve the public health and growth performance of animals (Elwan H. A. M. et al., 2019; Alagawany et al., 2019). The active molecules of plant seed oils can activate the immunity and enhance the secretion of digestive enzymes (Reda et al., 2020; Alagawany et al., 2021. (Nutrients and phytochemicals, especially polyphenols and fatty acids have shown to improve the immune system, rendering the development of dietary approaches for non-pharmacological prevention and management of the diseases (Alagawany et al., 2020a; El-Tarabily

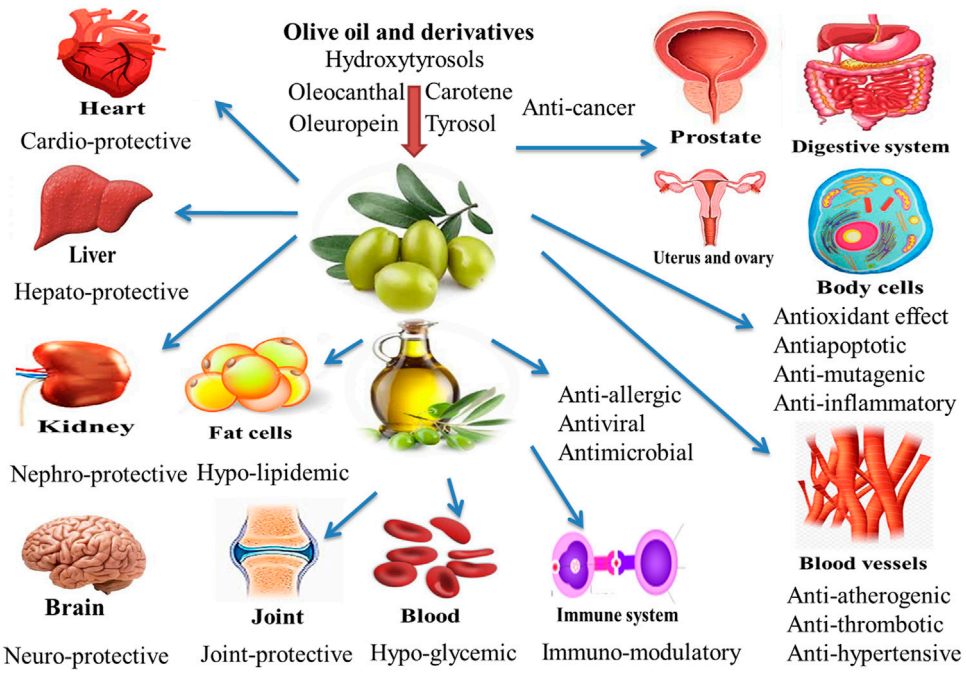


FIGURE 1 | Health benefits and nutritional applications of olive oil.

et al., 2021). One of such non-pharmacological substances is olive (*Olea europaea* L.) fruit and its by-products, including olive oil, which is isolated by the physical methods or by solvent extraction or reorganization processes. The European Union (EU) regulations (EEC Regulation 1513/2001; EU Regulation No. 29/2012 and EU Regulation 1348/2013) classify and define olive oil into different types such as: Virgin olive oil (VOO), Extra Virgin olive oil (EVOO), refined olive oil, olive oil, olive-pomace oils and lampante olive oil. Particularly, EVOO containing glycerol or saponifiable fraction represents about 90–99% of the oil. Fatty acids represent the major portion of the saponifiable compounds, mainly including monounsaturated fatty acids (MUFAs), where oleic acid make up to 80% of the total oil. Polyunsaturated fatty acids (PUFAs) constitute 3–22% of the olive oil, where saturated fatty acids (SFAs) and linoleic acid from 8 to 26% of it (Quintero-Flórez et al., 2015). Moreover, olive oil also contains minor phytochemical compounds that have many biological functions and represent 1–2% (La Lastra et al., 2001).

The positive effects of EVOO are attributed to its higher MUFA contents, especially oleic acid, which has shown several favorable properties (Bermudez et al., 2011). MUFAs have the ability to modulate the immune response and can be useful in treating certain autoimmune diseases and in general regulation of immunity (Miles and Calder, 2015). Polyphenols of olive oil may be associated with some properties, including hypoglycemics, anti-atherogenic, antitumor, anti-inflammatory, immunomodulatory, and antiviral properties which are partly attributed to the antioxidant effect of these products (Rigacci and Stefani, 2016). Also, hydroxytyrosol (HT) ((3,4-Dihydroxyphenyl)ethanol) is a polyphenol found in extra virgin olive oil (EVOO) and red wine. It has a strong antioxidant effect due to hydrogen donation, and it can enhance radical stability. Humans, as well as cellular and animal models, have been researched for the positive benefits of HT, most notably in connection to EVOO intake. Aside from its antioxidant potential, this polyphenol has been linked to a slew of other benefits. The purpose of this study was to evaluate the major characteristics of HT for human health, with a focus on those linked to the potential prevention and/or treatment of noncommunicable illnesses (Echeverría et al., 2017). Besides, unsaturated fatty acids can perform critical biological activities such as anti-persistent role and positive impacts on endothelial function and regulation of specific parameters for inflammatory diseases (Cárdeno et al., 2014). A variety of wastes and byproducts are produced during the olive oil processing process. The main ones with significant nutritional and technological interests are olive pomace, olive mill waste waters, olive leaves, and olive stone and seed (Nasopoulou and Zabetakis 2013; Nunes et al., 2016; Nasopoulou et al., 2018). In this review, we reviewed existing information of olive oil and derivatives positive health uses and prospective effects on poultry production in this review (**Figure 1**).

METHODOLOGY AND CRITERIA USED

The current review was based on literature and patents already available on various scientific databases highlighting the nutritional applications and beneficial health aspects of olive oil and its effect on poultry production. The databases included under study were PubMed, Medline, PubMed Central, Science Direct and few other scientific databases. The

information obtained through these diverse databases is compiled, critically interpreted and presented in the current study. The following inclusion criteria were used: 1) articles from any year, referring to any country; 2) articles that contained the clear information; and 3) articles in English.

Anti-Inflammatory Activity of Olive Oil

Inflammation plays a driving role in the pathogenesis and prevalence of the joint degenerative diseases. Hence, it is imperative to control inflammation through proper pharmacological measures. The regular consumption of olive oil may alter inflammatory markers and cytokines associated with coronary artery disease (Patrick and Uzick, 2001). These beneficial and health-promoting effects of olive oil are attributed to its polyphenolic contents which have potent anti-mutagenic, anti-inflammatory, anti-thrombotic, anti-atherogenic and anti-allergic effects. Plant polyphenols have shown to decrease the morbidity or/and slow down the development of neurodegenerative and cardiovascular diseases. Approximately, 36 phenolic constituents are found in EVOO, including mainly oleocanthal, carotenes, hydroxytyrosols, oleuropein and tyrosol. These beneficial compounds, when get entry into the brain, exhibit neuroprotective actions by their potent anti-inflammatory, antioxidant and antiapoptotic properties (de Souza et al., 2017; Shi et al., 2017; Farooqui and Farooqui, 2018). A concentration of 284–711 mg/kg oleocanthal is present in EVOO. Oleocanthal plays an exclusive role in perpetual anti-inflammatory characteristics. Oleocanthal has gained attention of scientific community due to its well-known pharmacological properties. It has been found to inhibit the spread of neuro-degenerative and joint-degenerative diseases through various mechanisms. Oleocanthal in conjunction with other phenolics exhibits a neuro-therapeutic potential, which ultimately reduces neurodegenerative diseases in populations that consume EVOO on a regular basis. Hence, a continuous intake of EVOO is advised by researchers to promote human health (Parkinson and Keast, 2014).

Ibuprofen is well recognized to show a positive impact on the markers of neurodegenerative disease (Parkinson and Keast, 2014). Cyclooxygenase (COX) is a kind of oxidoreductase enzyme involved in the regulation of platelet and kidney. However, the long term use of low doses of ibuprofen and other COX cause severe side effects leading to strong inflammatory effects. *In-vivo* and *in-vitro* reports suggest that the phenolics from EVOO positively influence the inflammation, antioxidant status, and antimicrobial activity (Cicerale et al., 2012). Extra virgin olive oil contains compounds which can induce a localized irritation in the oropharyngeal region because of the perceptual similarities, hence produce an anti-inflammatory action similar to Ibuprofen (Beauchamp et al., 2005). The use of naturally occurring non-steroidal anti-inflammatory drugs (NSAIDs), like oleocanthal, may alleviate inflammation and contribute towards a substantial reduction in the development of chronic inflammatory diseases (Parkinson and Keast 2014). Similarly, oleocanthal has been considered as natural NSAIDs for an effective cure for the degenerative joint disease (Scher et al., 2007). Indeed, oleocanthal displays an anti-inflammatory action in the body equivalent to ibuprofen, as it works on exactly the same pathways as a non-steroidal anti-inflammatory drug. Hence, EVOO is a potential NSAID replacer of ibuprofen (Beauchamp et al., 2005).

Frying results in the production of free radicals in conventional cooking oils, which cause inflammation in the body after consumption. Generally, the Mediterranean people use 25–30 ml of vegetable oil in cooked foods and salad dressings (Corona et al., 2009). An excessive use of conventional oil in food products can cause substantial increase in body weight (BW), and a plethora of obesity related diseases. However, olive oil has been found beneficial in counteracting the obesity-related diseases (Scoditti et al., 2019). The higher price of EVOO than conventional cooking oils limits its frequent use. However, its therapeutic benefits make it a cost-effective alternative in the long run. EVOO is much resistance to high temperatures and contains essential antioxidant compounds which scavenge free radicals, hence perform potent anti-inflammatory actions (Dick, 2018). *In vivo* study by Molnar et al. (2021) stated that olive oil at a dose of 0.3 g/animal/day had the cell membrane protective and anti-inflammatory effects. Due to its effective anti-inflammatory action, regular intake of olive oil has shown provide relief rheumatoid arthritis, which is an autoimmune disease characterized by inflammation and pain of joints (Wahle, 2004). Moreover, the combined intake of fish and olive oils effectively controlled rheumatoid arthritis as compared to the intake of dietary fish oil alone (Berbert et al., 2005).

Chemopreveintive Effects of Olive Oil

Olive oil has been reported to provide protection against leukemia in children and many types of cancer, such as the colon cancer and esophageal squamous cell cancer (Mosby et al., 2012). The dietary olive oil has shown to reduce the number of cancerous lesions and number of tumors (Grosso et al., 2013). It has been observed that the fatty acids present in olive oil can reduce the production of prostaglandins which in turn, can potentially inhibit the tumor development and production (de Souza et al., 2017). MUFAs of olive oil have the ability of positively altering the fatty acid profile in the animal body (Stark and Madar, 2002; Nakbi et al., 2010). Chemo-preventive property of olive oil has been associated with its phenolic contents, including hydroxytyrosol (3, 4-dihydroxyphenylethanol), (p-hydroxyphenylethanol) tyrosol and phenolic alcohols, as well as their secondary derivatives p-HPEA-EDA (oleocanthal), oleuropein, p-HPEA-EA (ligstroside aglycon), 3,4-DHPEA-EA (oleuropein aglycon) and 3,4-DHPEA-EDA (Bendini et al., 2007). Moreover, polyphenols have shown to slow down the development and progression of cancer. These polyphenols can modify the immune system through proliferation of white blood cells as well as by the synthesis of cytokines and other factors, which contribute to the immunological resistance (Ding et al., 2018). Various *in vivo* and *in vitro* reports have suggested that the olive oil might reverse or inhibit cancer progression owing to its phenolic and polyphenolic contents which scavenge free radicals or other reactive oxygen species (Wahle et al., 2010). Dietary EVOO has shown to decrease the incidence of different types of

cancer such as breast, prostate and digestive system cancers (Psaltopoulou et al., 2011).

Oleocanthal, a kind of natural phenolic compounds present in EVOO, is a robust anti-inflammatory agent (Beauchamp et al., 2005). It has become a center of interest in cancer prevention programs, because it can be used as a potent natural *cyclooxygenase* (*COX*) inhibitor. It has the capability of breaking the inflammatory cascade by reducing the secretion of *COX* inflammatory enzymes (Zarghi et al., 2011).

Hydroxytyrosol (3,4-dihydroxyphenylethanol, HTyr) is another powerful polyphenol obtained from olives and its derived products that has been reported to exert anticancer activities (Vilaplana-Pérez et al., 2014). In mice, no mortality or morbidity was found with an aqueous extract of olive-pulp containing pure HTyr (about 1,400 mg/kg mg/day for 90 days) (Christian et al., 2004). The *HTyr* exerted anti-proliferative impacts on human *colon carcinoma cell* lines. Moreover, it significantly promoted the regulation of glomerular filtration rate in human with colorectal adenocarcinoma (Terzuoli et al., 2016). Furthermore, *HTyr also* acts as an effective cytotoxic factor against cancer cell lines in the breast. It arrests the cell cycle in the G0/G1 stage by lowering the concentration of cyclin D1 (Han et al., 2009).

Oleuropein inhibited the cell growth and induced apoptosis in different cancer cell lines (Emma et al., 2021). Oleuropein (125 mg/kg of diet), another potent polyphenol, has anticancer property. It has shown anticancer activties in human cancer cell lines (Sepporta et al., 2014). Oleuropein possesses potent anti-breast cancer properties, as demonstrated against the *mammary tumor MCF-7 cell line* (Hassan et al., 2013; Sepporta et al., 2014). The anti-breast cancer property of oleuropein is attributed to cytochrome P450 enzyme, which is a potent aromatase inhibitor and is a significant pharmacological target in the breast cancer therapy (Neves et al., 2007). Furthermore, it has shown to enhance (>1000-fold) the sensitivity of trastuzumab-conditioned SKBR3/Tzb100 breast cancer cells (Rigacci and Stefani, 2016).

Antimicrobial Activity of Olive Oil

An *antimicrobial* agent is a substance that kills or inhibits the spread of microorganisms. Polyphenols are bioactive molecules that have been well documented for their antimicrobial and antioxidant activities (Zbakh and Abbassi, 2012). *In vitro* studies have shown that olive oil contains substantial phenolic contents, with strong antimicrobial and antioxidant activities capable to reduce the growth and propagation of several bacteria. The HTyr content of olive oil has the capability of reducing the growth of a variety of harmful microorganisms. The beneficial properties of olive include some antiatherogenic, hypocholesterolemic, antitumor, antihypertensive, cardioprotective, anti-inflammatory, antiviral, antimicrobial, antioxidant and hypoglycemic properties (Cayan and Erener, 2015). A remarkable antimicrobial response with the intake of olive extract has been attributed to its polyphenolic contents (Ritchason, 2000). Oral doses of pulverized olive leaves have been used to treat the malarial infections (Benavente-Garcõa, 2000). Polyphenols have shown to negatively affect the growth and propagation of *Bacillus cereus* and *Klebsiella pneumoniae, Salmonella typhi* and *Escherichia coli* (Appendini and Hotchkiss, 2002). Moreover, the growth and propagation of both Gram negative Pseudomonas syringae and Gram positive Corynebacterium michiganense have also been successfully inhibited by the treatment with co-products derived from the processing of olive oil (Capasso et al., 1995). *In vivo* application of olive oil and its derived products indicated a positive oxidative balance due to the presence of polyphenols (Soni et al., 2006). *Dietary olive oil showed a possible antimicrobial activity against intestinal and respiratory infections (*Sudjan et al., 2009). Campylobacter induces food related human campylobacteriosis (European Food Safety Authority, 2015).

Hepatoprotective Activity of Olive Oil

The liver is a multifunctional organ which regulates the internal chemical environment of the body (Thirumalai et al., 2011). The liver controls the absorption and metabolism of medications and other xenobiotics in the body by purifying and removing them, hence, protects the body against external contaminants (Saleem et al., 2010). Ortiz et al. (2020) indicate that the hydroxytyrosol prevents the development of liver steatosis and the associated mitochondrial dysfunction induced by high-fat diet. The liver is often affected by free radicals, which induce the onset of liver cancer, cirrhosis, hepatitis and other diseases (Ilavenil et al., 2015), which lead to morbidity and mortality. These issues highlight the need to explore the potential of plant-derived products as hepatoprotective and therapeutic agents (Saeed et al., 2021). Historically, olive oil has been a primary ingredient in the Mediterranean diet. The Mediterranean diet has been suggested to prevent the metabolic syndrome associated with the liver (Martínez-González and Sánchez-Villegas, 2004). Antioxidant properties of olive oil play a remarkable role in reducing the malignant neoplasms (Rodriguez-Rodriguez et al., 2006). Moreover, anti-inflammatory and antioxidant properties of olive oil are useful in protecting the humans from various ailments (Fang et al., 2008). Another favorable influence of the Mediterranean diet is its rich energy contents mainly derived from MUFAs portion of olive oil. The palmitate and oleate are main fatty acid esters found in the normal liver. The proportion of linoleate and linolenic acids was reduced in patients that suffer from alcoholic fatty liver. Moreover, concentration of oleate is higher in the normal liver as compared to fatty liver and after liver biopsies (Aghdassi et al., 2007). Moreover, dietary MUFAs (from olive oil) have shown to control the hepatic steatosis mainly through activation of PPARα and PPARγ by reducing insulin resistance while enhancing lipid oxidation (Soriguer et al., 2006). EVOO contains oleic acid and polyphenols, which have been reported to exert protective impacts on the liver in several experimental models, particularly in animal studies and cell cultures (Pirozzi et al., 2016) These components of olive oil have also shown to prevent various hepatic disorders, like hepatic fibrinogenesis, hepatocyte ballooning, and liver steatosis, hence aid in prevention of hepatic tissue damage induced by CCl4 (0.6 ml/kg, intraperitoneally (i.p.)) twice a week for 6 weeks (Han et al., 2016). The MUFAs in EVOO play a pivotal role in the treatment and prevention of liver

steatosis, induced by CCl4 (0.6 ml/kg, intraperitoneally (i.p.)) twice a week for 6 weeks, both alone or in combination with other components such as n-3 PUFAs particularly þdocosahexaenoic acid (DHA, C22:6 n-3) and eicosapentaenoic acid (EPA, C20: n-3) (Valenzuela et al., 2016). The consumption of EVOO reduces the inflammation, inhibits the risk of liver damage, and prevents the progression of steatohepatitis through the antioxidant action of its polyphenolic contents (Rincón-Cervera et al., 2016). Moreover, it has also shown to remarkably control the CCl_4-induced liver cirrhosis in a rat model. Moreover, Wang et al. (2014) demonstrated that EVOO consumption reduced the derangements in hepatic tissue, as well as decreased the formation of fibrous tissue in rats intoxicated with (0.1 ml/100 g body weight, 1:1 mixed with soybean oil) CCl_4. The mechanisms responsible for decreasing hepatic fibrosis by using EVOO include reduction in lipid peroxidation and expression of α-smooth muscle actin (α-SMA), a protein that participates in the structure of cell (Wang et al., 2014). Another anticancer effect of polyphenolic contents of EVOO was also reported by Vilaplana-Pérez et al. (2014). Moreover, the risk of hepatocellular carcinoma was limited through inhibition of the enzyme xanthine oxidase by HTyr accompanied by a decline in superoxide anion production, which protected against DNA damage (Zhao et al., 2014). Another study reported that about 10–80 μM of HTyr precursor oleuropein found in EVOO when added to human hepatoma cell lines exhibited a dose-dependent increase in the cellular apoptosis, as well as inhibition of colony formation and cell growth, which resulted in the PI3K/AKT pathway inactivation (Yan et al., 2015). Sánchez-Calvo et al. (2021) reported a clearly imply a link between the production of NO_2-OA from EVOO and the observed improvement in mitochondrial function in NAFLD. NO_2-FA formation may be responsible for the health advantages linked with EVOO intake.

Ischemic/Reperfusion (I/R) is a condition in which an organ is temporarily or permanently deprived of blood flow for a specific duration of time. In the ischemic condition of the liver, there is less supply of oxygen to hepatic tissues. Moreover, there is a transformation of hepatocellular metabolism to anaerobic pathways that persuades a pro-inflammatory condition, which makes the tissue susceptible to reperfusion (Jaeschke, 2003). The constituents of EVOO have been reported to reduce the hepatic I/R injury (Pan et al., 2013). Moreover, combined treatment of olive oil with camel milk in mice exhibited hepatoprotective action against single dose (500 mg/kg) of acetaminophen-induced hepatotoxicity owing to the pronounced antioxidant action (Ibrahim et al., 2017).

Regarding, liver steatosis progresses into non-alcoholic steatohepatitis (NASH) under persistent oxidative stress conditions, (Videla et al., 2004; Rolo et al., 2012) a last-stage disease of the liver, the AR EVOO supplementation provides an appropriate therapeutically strategy to prevent or resolve the development of liver steatosis. An iron-rich diet causes oxidative stress in the liver, with increased lipid peroxidation and protein oxidation, responses associated with mitochondrial dysfunction and membrane unsaturation, the latter effect triggering a drastic increase in the SREBP1c/PPAR- ratio with the development of hepatic steatosis, thus representing a type of nonalcoholic fatty liver disease (NAFDL). In this situation, AR-EVOO supplementation corrected IRD-induced alterations, resulting in an efficient anti-steatotic natural product, the protective benefits of which may be attributed to the molecular pathways established by its primary components, namely, HT, OA, tocopherols, and PUFAs (Barrera et al., 2018).

The oxidative stress is defined as an imbalance of metabolic and radical constituents called reactive (chlorine, oxygen or nitrogen) species at the cellular level (Elwan H. et al., 2019; Elnesr et al., 2019). Olive oil has been shown its excellent potential to effectively mitigate liver oxidative stress through regulation of various pathways (Kyle et al., 1987). The rich MUFAs content of olive oil (35% ethanol solution of 3 g/kg body weight) positively influenced the lipid profiles and peroxidation of hepatic mitochondria in rabbits (Kasdallah-Grissa et al., 2008). No doubt that EVOO has remarkable health boosting properties, which are mainly associated with its abundant MUFAs and polyphenolic contents. Studies have shown that olive oil enhances circulating lipoproteins which are less sensitive to peroxidation hence decreases risk of onset of diseases in the human body (Kasdallah-Grissa et al., 2008; Necib et al., 2013). Similarly, Berrougui et al. (2015) reported that cholesterol efflux, a way of transferring intracellular cholesterol and improvement in the high-density lipoprotein-cholesterol is observed in individuals consuming the olive oil. Ortiz et al. (2020) indicated that the combination of docosahexaenoic acid and hydroxytyrosol prevents the development of liver steatosis and the associated mitochondrial dysfunction caused by a high-fat diet, highlighting the importance of this protocol as a therapeutic strategy for preventing disease progression into more irreversible forms characterized by the absence of adequate pharmacological treatment.

Dietary supplementation with virgin olive oil (VOO) at either low or high dose in rats caused a significant decrease in the serum triglycerides, total cholesterols, low density lipoprotein (LDL), and glucose, but increased high density lipoprotein (HDL) levels. This positive effect was attributed to its higher content of MUFAs (such as oleic acid), which exerted beneficial effects on the cardiovascular system of the male albino rats (Farahat et al., 2019). The MUFAs content of the olive oil exhibited an important role in modulating atherosclerosis, which ultimately affected the lipid profile and peroxidation in the hepatic mitochondria of rabbit (Massimo et al., 2009; Qian et al., 2016).

Benefits of Olive Oil on Kidney

Natural compounds and medicinal herbs are playing a vital role in the control and prevention of many communicable and viral diseases (Dhama et al., 2018; Tiwari et al., 2018). It is well known that the VOO effectively inhibits the progression of nephrotoxicity induced by various chemical agents as dietary VOO and olive leaf extract have shown to control the nephrotoxicity in animal models (Abdel-Gayoum et al., 2015; Khalatbary et al., 2017). Cyclosporine may cause side effects like nephrotoxicity and the scientific community is searching for its replacement with some natural agents. It was found that the combined use of olive oil (1.25 ml/kg/d virgin olive oil) and naringenin, (100 mg/kg/d naringenin), respectively, reduced the

cyclosporine-induced (25 mg/kg/d cyclosporine) nephrotoxicity by improving renal functionality and reducing the concentration of serum urea and creatinine in rats during a 45 days treatment period (Elshama et al., 2016). In another study, EVOO (2 ml/kg/day) reduced the signs of nephrotoxicity in rats exposed to ethopon (150 mg per kg daily) and improved the antioxidant and health status (Mokhtari et al., 2020). Moreover, administration of EVOO markedly reduced the tumor necrosis factor α (TNFα), interleukine-1, interleukine-6, uric acid, creatinine and urea levels in the serum and reduced the lethal effects caused by mercuric chloride ($HgCl_2$) in the kidney (Necib et al., 2013).

According to Nekooeian et al. (2011), the control of nephrotoxicity and other diseases by olive oil feeding may be attributed to its phenolic compounds which behaved as potent lipid peroxidation inhibitors in addition to its ability to work as chelators which prevented toxicity by detoxifying metal ions. Various reports suggested that dietary VOO or its derived products have the therapeutic ability to address kidney related diseases in animals (Abdel-Gayoum et al., 2015; Khalatbary et al., 2017).

Human and experimental animals are contagiously suffering from renal toxicity due to the excessive use of a large number of pesticides and drugs (Ullah et al., 2018). Amikacin (a semisynthetic aminoglycoside antibiotic) is used to treat Gram-negative infections; however, it is known to cause nephrotoxicity (Abdel-Gayoum et al., 2015). In a study on rats, amikacin significantly increased the serum values of urea and creatinine, while co-administration of OLE and VOO significantly decrease their values, providing protection against amikacin-induced nephrotoxicity (Abdel-Gayoum et al., 2015).

Anti-Neurodegenerative Effects

Neurological diseases like stroke, Parkinson's and Alzheimer's diseases, are serious concerns for human health. These ailments share frequent pathological appearances, such as the induction of inflammation, abnormal protein aggregation, apoptosis, oxidative stress, excitotoxicity and perturbed Ca^{2+} homeostasis. There is a large body of evidence supporting the favorable impacts of the Mediterranean diet in the prevention of neurodegeneration in humans (Angeloni et al., 2017). Mediterranean diets impart beneficial effects on human health owing to its rich polyphenolic contents of EVOO (Angelon et al., 2017). Notably, olive oil has been reported to have a positive influence on Parkinson's Disease as the polyphenols present in olive oil can modify a different cellular mechanism involved in the onset and development of the disease (Sarrafchi et al., 2016; Maher, 2017).

The most critical and active component of olive oil is oleuropein, which has shown to reduce the cell damage, apoptosis and oxidative stress in PC12 cells induced by 6-OHDA in an *in vitro* model of Parkinson's disease. Moreover, olive extract (400 and 600 μg/ml) or oleuropein (20 and 25 μg/ml) exhibited neuroprotective effects on PC12 cells subjected to 150 μM 6-OHDA. (Pasban-liabadi et al., 2013). Similarly, HTyr is another active component of olive oil which has shown to reduce the 5-S-cysteinyl-dopamine levels induced by monoamine oxidase inhibitors, which supported the clinical therapy of the Parkinson's disease (Vauzour et al., 2010). Since most of the investigations have been conducted in cell cultures, *in vivo* experiments are required to prove the protective impacts of phenolic contents of olive oil that have been detected under *in vitro* studies. Furthermore, it would be essential to detect the protective impact of this oil in Parkinson's disease.

Multiple Sclerosis (MS) is a complicated neurodegenerative ailment of the central nervous system that causes inflammation, axonal and oligodendrocyte injury, blood-brain barrier breakdown, demyelination and gliosis (Browne et al., 2014). Globally, the prevalence of MS has increased substantially from 2.1 million in 2008 to 2.3 million in 2013 (Browne et al., 2014) and it represents the first reason for disability in young individuals after traumatic brain injury (Sospedra, 2005).

Olive oil is suggested to be a powerful tool to cure and treat MS disorders in patients (Riccio et al., 2010). Similarly, Amyotrophic Lateral Sclerosis (ALS) is a progressive complex of neurological diseases refers to spinal cord muscle weakness and adversely affects the brain, causing paralysis and death due to constriction of respiratory muscles (Shneider, 2001). In this perspective, olive oil intake has been reported to delay the onset of ALS, while improving the motor performance and increase muscle fiber area. Moreover, supplementation of (20%, w/w) extra virgin olive oil folive oil upgraded the muscle status as confirmed by the augmented expression of myogenic regulatory factors (MRFs) (MyoG and MyoD) and reduced endoplasmic reticulum stress (Oliván et al., 2014).

De Paola et al. (2016) suggested that the phenolic extract derived from the commercial olive oil may modulate Toll-like receptor 4 signalling pathway involved in the pathogenic mechanisms of ALS. Furthermore, it is well endorsed that phenols of olive oil were able to provide neuroprotective impacts associated with modulation of inflammation. The positive impacts of olive oil or its phenolic components on the neurological problems through addressing different cellular pathways have been widely studied. Olive oil and its essential compounds such as oleuropein, ployphenol, HTyr, tyrosol and oleocanthal have been frequently investigated regarding their effects on the spinal cord and acute brain injuries. Additionally, tyrosol, oleuropein, and HTyr have been shown to decrease apoptosis, infarct volume and mitigate the outcome of these damages.

Finally, olive oil has various potent effects including antimicrobial, antioxidant, immunomodulatory, anticancer, anti-inflammatory, hepatoprotective, anti-neurodegenerative, neuroprotective, and other beneficial health effects. We summarized the biological effects and health benefits of olive oil in **Table 1**.

Application of Olive Oil and Derivatives in Poultry Nutrition

Health benefits and potential uses of olive oil and derivatives in poultry nutrition are illustrated in **Figure 2**. The phenolic components and carotenoids found in olive oil are naturally lipophilic and hydrophilic (Mahmoud et al., 2013). The phenolic components of olive oil such as oleuropein or HTyr

TABLE 1 | Biological effects and health benefits of olive oil.

Activities	Results/Mechanisms	References
Antioxidant position, and antimicrobial activity	Phenolics of EVOO positively influence the inflammation, antioxidant position, and antimicrobial activity EVOO is much resistant to high temperatures and contains essential antioxidant compounds which scavenge free radicals, hence perform anti-inflammatory roles	Cicerale et al. (2012), Dick (2018)
Antiobesity and Anti-hyperglycemic effect	The excessive use of conventional oil in food products increased body weight, and a plethora of obesity related diseases. Olive oil has been found beneficial in counteracting the obesity-related diseases Moreover, the dietary 5% olive oil increased serum HDL concentration, but decreased triglyceride level	Abo-Omar (2000); that; Scoditti et al. (2019)
Immunomodulatory	Polyphenols of olive oil modify the immune system by increasing white blood cell proliferation, as well as by synthesis of cytokines and other factors, which contribute to immunological resistance	Psaltopoulou et al. (2011)
Anti-neurodegenerative effects	Phenolic extract derived from the commercial olive oil may modulate Toll-like receptor 4 signal pathway involved in the pathogenic mechanisms of Amyotrophic Lateral Sclerosis	De Paola et al. (2016)
Antimicrobial effect	Olive oil has a high quantity of phenols, which have strong antimicrobial and antioxidant compounds capable to reduce the growth and propagation of several bacteria. A remarkable antimicrobial response with the intake of olive extract has been attributed to its polyphenolic contents	Ritchason (2000), Cayan and Erener (2015)
Antioxidant and renoprotective	EVOO (2 ml/kg/day) reduced the signs of nephrotoxicity in rats exposed to ethopone (150 mg per kg daily) and improved antioxidant and health status. Various reports suggested that dietary virgin olive oil or the products derived from it have the capability of treating kidney related diseases in animals	Abdel-Gayoum et al. (2015), Khalatbary et al., (2017), Makhtari et al. (2019)
Antioxidant and Neuroprotection	Oleic acid in the olive oil played an important role in modulating atherosclerosis, which ultimately affected the lipid profile and peroxidation in the hepatic mitochondria of rabbit	Qian et al. (2016)
Growth enhancer and immunomodulatory effect	Higher antibody titers against Newcastle disease and improvement in growth and development were observed in the broilers given a diet enriched with olive oil relative to the control group EVOO significantly improved the BW gain, and feed efficiency, but reduced lipid peroxidation level of the chicks mainly through strengthening the antioxidant defense system	El-Bahra and Ahmed (2012), Tufarelli et al. (2016)
Anti-cancer and antitumor	The dietary olive oil has been associated with a reduced number of cancerous lesions. Also, the fatty acids present in olive oil reduce the prostaglandins production obtained from the arachidonic acid that, in turn, plays an integral part in the tumor development and production	Nakbi et al. (2010), Grosso et al. (2013)
Anti-cancer effect	Diets enriched with EVOO have shown to limit the prevalence of several cancer types such as breast, prostate and digestive system cancer	Psaltopoulou et al. (2011)
Hepato-protective effect	EVOO contains oleic acid and polyphenols, which have been reported to exert protective impacts on the liver in several experimental models, particularly in animal studies and cellular cultures. These components of olive oil have been reported to prevent various hepatic disorders, like hepatic fibrinogenesis, hepatocyte ballooning, and liver steatosis, hence aid in prevention of hepatic tissue damage	Han et al. (2016), Pirozzi et al. (2016)

induce antioxidant action in the gastrointestinal tract and its metabolites can effectively exhibit antioxidant properties (Omar, 2010). The protective impacts of oleuropein against H_2O_2-induced apoptosis was confirmed in human liver cells where the significant increase in expression of superoxide dismutase (SOD1), catalase, and glutathione peroxidase 1 were observed (Shi et al., 2017). In broilers, the supplementation of olive oil improved the growth rate (BW and body weight gain) and antibody titers against Newcastle disease virus as compared with the control group (El-Bahra and Ahmed, 2012).

Plant derived materials contain plenty of polyphenolic content, which positively affect the growth performance of the poultry (Abd El-Hack et al., 2020a,b; Abo Ghanima et al., 2020; Alagawany et al., 2020b; Ebrahim et al., 2020). No adverse effects on the carcass characteristics, inner body organs, growth performance and blood profile were observed in response to the feeding of olive cake (OC) in broilers (Zangeneh and Torki, 2011). Similarly, El Hachemi et al. (2007) reported that OC might successfully be included up to 15% without harmful effect on the feed intake and feed efficiency. On the other hand, significantly higher feed efficiency was obtained by the inclusion of 10% OC in broilers diet (Al-Shanti, 2003). Similarly, Al-Harthi (2017) reported a positive impact of including OC in poultry diet from 1 to 28 days trial period. Further, discussing the potential effects of OC, it was observed that its inclusion maintains a remarkably higher survival rate owing to its valuable nutrients such as essential fatty acids, essential amino acids, polyphenols and important elements which may be factors for this appreciable change.

In commercial poultry production systems, stress (induced by many factors such as, environmental, pathogenic and nutritional factors) negatively affects the growth and health of the birds. In

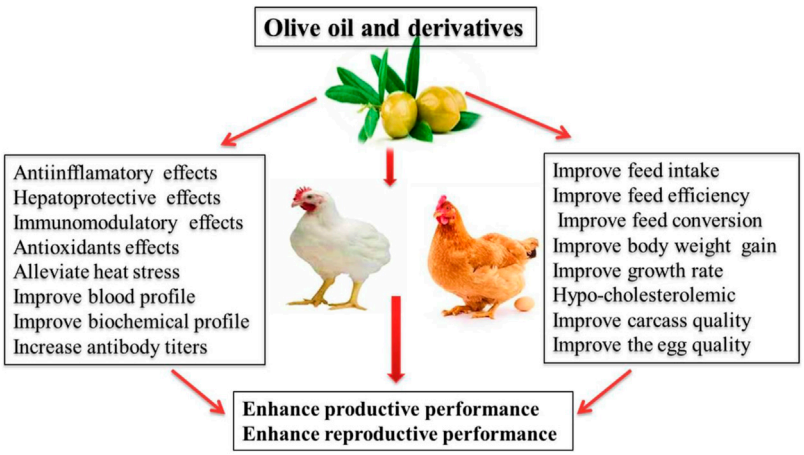


FIGURE 2 | Application of olive oil and derivatives in poultry nutrition.

contrast, *oxidative stress* ruins animal productivity by damaging the body proteins, lipids, DNA, and cellular structures (Lykkesfeldt and Svendsen, 2007).

The products derived from olive contain various vital nutrients like organic compounds, sugars, oils, fiber contents and polyphenols which may be recycled. The extract of olive leaves can reduce the dependency of poultry on grains and may reduce the competition for grains between human and animals (Tabera et al., 2004). Olive meal (OM) is yielded as a by-product during olive oil processing. The OM contains hefty amounts of lipids, including 73% oleic acid, 13% palmitic acid and 7% linoleic acid, which make it as an economical feed ingredient for animal (Ranalli et al., 2002). Preliminary findings revealed feeding of OM up to 9% exhibited no harmful effect on the growth performance while significantly improved the fiber intake in broilers (Zangeneh and Torki, 2011). Since OM is a waste of olive processing, its utilization as a feed ingredient in commercial poultry is an environment friendly technology particularly under resource limited set ups (Sateri et al., 2017).

Inclusion of 2% olive oil in feed facilitated the birds to gain higher BW with a better feed conversion ratio (FCR). Moreover, it significantly lowered the blood cholesterol, triglycerides and LDL, but elevated the level of HDL in chicken (Hadi and Al-Khalisy, 2018). Moreover, the addition of 400 ppm oleuropein promoted feed conversion efficiency of Japanese quails (Bahşi et al., 2016). Furthermore, feeding of olive oil resulted in an increased anti-inflammatory activity in chicken (Korver et al., 1998; Berbert et al., 2005). Similarly, broilers supplemented with olive extract led to an increased BW, as well as improvement of FCR (Erener et al., 2009). Similar incremental effects of feeding olive oil have been observed in the treated group when compared to control diet (Mujahid et al., 2009). In another study, the dietary (86 g/kg) supplementation of olive pulp did not show any adverse effects on production parameters of laying hens (Zarei et al., 2011). Moreover, Abo-Omar (2000) reported that the dietary olive oil (5%) increased serum HDL concentration, but decreased triglyceride level.

In another study, Ahmed et al. (2013) investigated the performance, immunity and biochemical profile of broilers by supplementing two sources of oil, i.e. olive and canola with four diets *viz*. control (no test diet), diet II (2% canola oil), diet III (2% olive oil), diet IV (1% olive oil + 1% canola oil). The significantly higher antibody response was observed with diet IV at 3, 7 and 10 days. Olive oil also alleviated the lethal effects of heat stress during hot climatic conditions by promoting the immunity of the birds. Furthermore, olive oil has shown to down-regulate the oxidative damages from heat stress and modified respiratory chain in the mitochondria of skeletal muscle. Moreover, liver enzyme activity was significantly higher in the birds fed with diet containing olive and canola oils. Additionally, the combination of olive and canola oils enhanced the weight gain and feed efficiency of the birds. In another study, the administration of olive oil in the rats intoxicated with 2-4 dichlorophenoxy acetic acid impacted the concentration of ALT and AST (Nakbi et al., 2010; Ahmad et al., 2013).

The dietary inclusion of processed olive pulp improved the FCR in broilers (Sayehban et al., 2016), mainly due to the presence of substances like flavanols, flavonoids, oleuropeosides and simple phenolic components (Herrero-Encinas et al., 2020). Zangeneh and Torki (2011) observed that olive leaf powder improved the color of egg yolk in layers. Furthermore, the phenolic components in olive leaf exhibited hypo-cholesterolemic activities by reducing the levels of hepatic and serum triglyceride, while modulating cholesterol metabolism (Sarica and Toptas, 2014).

It is well known that the oleuropein and HTyr derived from olive leaves inhibit LDL oxidation and decrease the secretion of an enzyme (3-hydroxy-3-methyglutaryl coenzyme A), responsible for the synthesis of cholesterol. This ability of oleuropein and HTyr may be attributed to the decreased yolk cholesterol concentration (Patrick and Uzick, 2001). The expressions of antioxidative enzymes while decreasing lipid peroxidation such as thiobarbituric acid-reactive substances content. Feeding of EVOO (2.5%) in male Hubbard broiler significantly improved the BW gain, and feed efficiency, but reduced lipid peroxidation levels

mainly through strengthening the antioxidant defense system (Tufarelli et al., 2016).

CONCLUSION

Studies reviewed in this article convincingly revealed that the use of olive oil and its bioactive molecules have shown a wide range of promising activities in various inflammatory and disease conditions. Olive oil may be a consistent approach to prevent and manage nutritional and health disorders. Besides, it may be used as an anti-inflammatory, anticancer, antimicrobials, hepatoprotective, renoprotective, and anti-neurodegenerative agent. However, further investigations are required to further explore the biological activities of olive oil and its derived compounds in poultry to improve bird health and produce enriched products.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

The authors thank and acknowledge the support of their respective universities and institutes. This article was supported by the Central Public-interest Scientific Institution Basal Research Fund (Grant: 1610242021005/1610242021006), The Agricultural Science and Technology Innovation Program (ASTIP-IBFC) and Hunan Key Scientific and Technological Project (Grant: 2020NK2061).

REFERENCES

Abd El-Hack, M. E., Alagawany, M., Shaheen, H., Samak, D., Othman, S. I., Allam, A. A., et al. (2020a). Ginger and its Derivatives as Promising Alternatives to Antibiotics in Poultry Feed. *Animals*, 10(3), 452.þdoi:10.3390/ani10030452

Abd El-Hack, M. E., Elnesr, S. S., Alagawany, M., Gado, A., Noreldin, A. E., and Gabr, A. A. (2020b). Impact of green tea (*Camellia Sinensis*) and Epigallocatechin Gallate on Poultry. *World's Poult. Sci. J.* 76 (1), 49–63. doi:10.1080/00439339.2020.1729672þ

Abdel-Gayoum, A. A., Al-Hassan, A. A., Ginawi, I. A., and Alshankyty, I. M. (2015). The Ameliorative Effects of virgin Olive Oil and Olive Leaf Extract on Amikacin-Induced Nephrotoxicity in the Rat. *Toxicol. Rep.* 2, 1327–1333. doi:10.1016/j.toxrep.2015.09.007

Abo Ghanima, M. M., Alagawany, M., Abd El-Hack, M. E., Taha, A., Elnesr, S. S., Ajarem, J., et al. (2020). Consequences of Various Housing Systems and Dietary Supplementation of Thymol, Carvacrol, and Euganol on Performance, Egg Quality, Blood Chemistry, and Antioxidant Parameters. *Poult. Sci.* 99 (9), 4384–4397. doi:10.1016/j.psj.2020.05.028þ

Abo-Omar, J. (2000). Effect of Different Levels of Olive Pulp Onthe Digestibility of Broiler Chicks. *Bethlehem Univ. J.* 12, 34–40.

Aghdassi, E., Wendland, B. E., Stapleton, M., Raman, M., and Allard, J. P. (2007). Adequacy of Nutritional Intake in a Canadian Population of Patients with Crohn's Disease. *J. Am. Diet. Assoc.* 107 (9), 1575–1580. doi:10.1016/j.jada.2007.06.011

Ahmed, A. S., El-Bahr, S. M., and Al-Azraqi, A. A. (2013). Effect of Canola and Olive Oils on Productive, Immunological and Some Biochemical Parameters of Broiler Chickens Fed Iso Caloric and High Caloric Diets. *Int. J. Poult. Sci.* 12 (12), 726–734. doi:10.3923/ijps.2013.726.734

Al-Harthi, M. (2017). The Effect of Olive Cake, with or without Enzymes Supplementation, on Growth Performance, Carcass Characteristics, Lymphoid Organs and Lipid Metabolism of Broiler Chickens. *Rev. Bras. Cienc. Avic.* 19, 83–90. doi:10.1590/1806-9061-2016-0311

Al-Shanti, H. A. (2003). Effect of Using Olive Cake or Extrudedfull-Fat Soybean in Broiler Chicks' Diets. *Egypt. Poult. Sci. J.* 23, 1–13.

Alagawany, M., El-Saadony, M. T., Elnesr, S. S., Farahat, M., Attia, G., Madkour, M., et al. (2021). Use of Lemongrass Essential Oil as a Feed Additive in Quail's Nutrition: its Effect on Growth, Carcass, Blood Biochemistry, Antioxidant and Immunological Indices, Digestive Enzymes and Intestinal Microbiota. *Poult. Sci.*, þ101172.

Alagawany, M., Elnesr, S. S., Farag, M. R., El-Sabrout, K., Alqaisi, O., Dawood, M. A. O., et al. (2020a). Nutritional Significance and Health Benefits of omega-3, -6 and -9 Fatty Acids in Animals. *Anim. Biotechnol.* 1–13. doi:10.1080/10495398.2020.1869562

Alagawany, M., Elnesr, S. S., and Farag, M. R. (2019). Use of Liquorice (Glycyrrhiza Glabra) in Poultry Nutrition: Global Impacts on Performance, Carcass and Meat Quality. *World's Poult. Sci. J.* 75 (2), 293–304. doi:10.1017/s0043933919000059

Alagawany, M., Farag, M. R., Sahfi, M. E., Elnesr, S. S., Alqaisi, O., El-Kassas, S., et al. (2020b). Phytochemical Characteristics of Paulownia Trees Wastes and its Use as Unconventional Feedstuff in Animal Feed. *Anim. Biotechnol.*, 1–8. doi:10.1080/10495398.2020.1806074

Angeloni, C., Malaguti, M., Barbalace, M., and Hrelia, S. (2017). Bioactivity of Olive Oil Phenols in Neuroprotection. *Ijms* 18 (11), 2230. doi:10.3390/ijms18112230

Appendini, P., and Hotchkiss, J. H. (2002). Review of Antimicrobial Food Packaging. *Innovative Food Sci. Emerging Tech.* 3 (2), 113–126. doi:10.1016/s1466-8564(02)00012-7

Bahşi, M., Ciftci, M., Şimşek, Ü. G., Azman, M. A., Özdemir, G., Yilmaz, Ö., et al. (2016). Effects of Olive Leaf Extract (Oleuropein) on Performance, Fatty Acid Levels of Breast Muscle and Some Blood Parameters in Japanese Quail (*Coturnix coturnix* Japonica) Reared in Different Stocking Densities. *Ankara Üniversitesi Veteriner Fakültesi Dergisi* 63 (1), 61–68.þ

Barrera, C., Valenzuela, R., Rincón, M. Á., Espinosa, A., Echeverria, F., Romero, N., et al. (2018). Molecular Mechanisms Related to the Hepatoprotective Effects of Antioxidant-Rich Extra virgin Olive Oil Supplementation in Rats Subjected to Short-Term Iron Administration. *Free Radic. Biol. Med.* 126, 313–321. doi:10.1016/j.freeradbiomed.2018.08.030

Beauchamp, G. K., Keast, R. S. J., Morel, D., Lin, J., Pika, J., Han, Q., et al. (2005). Ibuprofen-like Activity in Extra-virgin Olive Oil. *Nature* 437 (7055), 45–46. doi:10.1038/437045a

Benavente-Garcõa, O., Castillo, J., Lorente, J., Ortuno, A., and Del Rio, J. A. (2000). Antioxidant Activity of Phenolics Extracted from Olea Europaea L. Leaves. *Food Chem.* 68, 457–462.

Bendini, A., Cerretani, L., Carrasco-Pancorbo, A., Gómez-Caravaca, A., Segura-Carretero, A., Fernández-Gutiérrez, A., et al. (2007). Phenolic Molecules in virgin Olive Oils: a Survey of Their Sensory Properties, Health Effects, Antioxidant Activity and Analytical Methods. An Overview of the Last Decade Alessandra. *Molecules* 12 (8), 1679–1719. doi:10.3390/12081679

Berbert, A. A., Kondo, C. R. M., Almendra, C. L., Matsuo, T., and Dichi, I. (2005). Supplementation of Fish Oil and Olive Oil in Patients with Rheumatoid Arthritis. *Nutrition* 21, 131–136. doi:10.1016/j.nut.2004.03.023

Bermudez, B., Lopez, S., Ortega, A., M. Pacheco, L. Y., Pacheco, Y. M., J.G. Muriana, F., et al. (2011). Oleic Acid in Olive Oil: from a Metabolic Framework toward a Clinical Perspective. *Curr. Pharm. Des.* 17, 831–843. doi:10.2174/138161211795428957

Berrougui, H., Ikhlef, S., and Khalil, A. (2015). Extra virgin Olive Oil Polyphenols Promote Cholesterol Efflux and Improve HDL Functionality. *Evidence-Based Complement. Altern. Med.* 2015.

Browne, P., Chandraratna, D., Angood, C., Tremlett, H., Baker, C., TaylorThompson, B. V. A. J., et al. (2014). Atlas of Multiple Sclerosis 2013: a Growing Global Problem with Widespread Inequity. *Neurology* 83 (11), 1022–1024. doi:10.1212/wnl.0000000000000768

Capasso, R., Evidente, A., Schivo, L., Orru, G., Marcialis, M. A., and Cristinzio, G. (1995). Antibacterial Polyphenols from Olive Oil Mill Waste Waters. *J. Appl. Bacteriol.* 79 (4), 393–398. doi:10.1111/j.1365-2672.1995.tb03153.x

Cárdeno, A., Magnusson, M. K., Strid, H., Alarcón de La Lastra, C., Sánchez-Hidalgo, M., Öhman, L., et al. (2014). The Unsaponifiable Fraction of Extra

virgin Olive Oil Promotes Apoptosis and Attenuates Activation and Homing Properties of T Cells from Patients with Inflammatory Bowel Disease. *Food Chem.* 161, 353–360. doi:10.1016/j.foodchem.2014.04.016

Cayan, H., and Erener, G. (2015). Effect of Olive Leaf (*Olea Europaea*) Powder on Laying Hens Performance, Egg Quality and Egg Yolk Cholesterol Levels. *Asian Australas. J. Anim. Sci.* 28 (4), 538–543. doi:10.5713/ajas.14.0369

Christian, M. S., Sharper, V. A., Hoberman, A. M., Seng, J. E., Fu, L., Covell, D., et al. (2004). The Toxicity Profile of Hydrolyzed Aqueous Olive Pulp Extract. *Drug Chem. Toxicol.* 27 (4), 309–330. doi:10.1081/dct-200039714þ

Cicerale, S., Lucas, L., and Keast, R. (2012). Antimicrobial, Antioxidant and Anti-inflammatory Phenolic Activities in Extra virgin Olive Oil. *Curr. Opin. Biotechnol.* 23 (2), 129–135. doi:10.1016/j.copbio.2011.09.006

Corona, G., Deiana, M., Incani, A., Vauzour, D., Dessì, M. A., and Spencer, J. P. E. (2009). Hydroxytyrosol Inhibits the Proliferation of Human colon Adenocarcinoma Cells through Inhibition of ERK1/2 and Cyclin D1. *Mol. Nutr. Food Res.* 53 (7), 897–903. doi:10.1002/mnfr.200800269þ

De Paola, M., Sestito, S. E., Mariani, A., Memo, C., Fanelli, R., Freschi, M., et al. (2016). Synthetic and Natural Small Molecule TLR4 Antagonists Inhibit Motoneuron Death in Cultures from ALS Mouse Model. *Pharmacol. Res.* 103, 180–187. doi:10.1016/j.phrs.2015.11.020

Dhama, K., Karthik, K., Khandia, R., Munjal, A., Tiwari, R., Rana, R., et al. (2018). Medicinal and Therapeutic Potential of Herbs and Plant Metabolites/Extracts Countering Viral Pathogens - Current Knowledge and Future Prospects. *Cdm* 19 (3), 236–263. doi:10.2174/1389200219666180129145252

Dick, L. (2018). The Prevention of Cardiovascular Disease through the Mediterranean Diet. *J. Nutr. Edu. Behav.* 50 (7), 53. doi:10.1016/j.jneb.2018.01.016

Ding, S., Jiang, H., and Fang, J. (2018). Regulation of Immune Function by Polyphenols. *J. Immunol. Res.* 2018, 1–8. doi:10.1155/2018/1264074

Ebrahim, A. A., Elnesr, S. S., Abdel-Mageed, M. A. A., and Aly, M. M. M. (2020). Nutritional Significance of Aloe Vera (*Aloe Barbadensis* Miller) and its Beneficial Impact on Poultry. *World's Poult. Sci. J.* 76 (4), 803–814. doi:10.1080/00439339.2020.1830010

Echeverría, F., Ortiz, M., Valenzuela, R., and Videla, L. (2017). Hydroxytyrosol and Cytoprotection: A Projection for Clinical Interventions. *Ijms* 18 (5), 930. doi:10.3390/ijms18050930

El Hachemi, A., El, M. K. E., Benzineb, K., Saidi, D., and Kheroua, O. (2007). Supplementation of Olive Mill Wastes in Broiler Chicken Feeding. *Afr. J. Biotechnol.* 6, 1848–1853. doi:10.5897/ajb2007.000-2274

El-Bahra, S. M., and Ahmed, A. S. (2012). Effect of Vegetable Oil on Growth, Lipid Profile and Immunological Response in Broiler Chicken Fed Iso-Energetic Diet. *Revista Cientifica UDO Agricola J.* 12, 202–207.

El-Tarabily, K. A., El-Saadony, M. T., Alagawany, M., Arif, M., Batiha, G. E., Khafaga, A. F., et al. (2021). Using Essential Oils to Overcome Bacterial Biofilm Formation and Their Antimicrobial Resistance. *Saudi J. Biol. Sci.* doi:10.1016/j.sjbs.2021.05.033

Elnesr, S. S., Elwan, H. A. M., Xu, Q. Q., Xie, C., Dong, X. Y., and Zou, X. T. (2019). Effects of in Ovo Injection of Sulfur-Containing Amino Acids on Heat Shock Protein 70, Corticosterone Hormone, Antioxidant Indices, and Lipid Profile of Newly Hatched Broiler Chicks Exposed to Heat Stress during Incubation. *Poult. Sci.* 98 (5), 2290–2298. doi:10.3382/ps/pey609

Elshama, S. S., Osman, H. E. H., and El-Kenawy, A. E. M. (2016). Renoprotective Effects of Naringenin and Olive Oil against Cyclosporine-Induced Nephrotoxicity in Rats. *Iranian J. Toxicol.* 10 (5), 27–37.

Elwan, H. A. M., Elnesr, S. S., Mohany, M., and Al-Rejaie, S. S. (2019a). The Effects of Dietary Tomato Powder(Solanum lycopersicum L.)supplementation on the Haematological, Immunological, Serum Biochemical and Antioxidant Parameters of Growing Rabbits. *J. Anim. Physiol. Anim. Nutr.* 103 (2), 534–546. doi:10.1111/jpn.13054þ

Elwan, H., Elnesr, S., Xu, Q., Xie, C., Dong, X., and Zou, X. 2019b. Effects of in Ovo Methionine-Cysteine Injection on Embryonic Development, Antioxidant Status, IGF-I and TLR4 Gene Expression, and Jejunum Histomorphometry in Newly Hatched Broiler Chicks Exposed to Heat Stress during Incubation. *Animals*, 9 (1), 25. þdoi:10.3390/ani9010025

Emma, M. R., Augello, G., Di Stefano, V., Azzolina, A., Giannitrapani, L., Montalto, G., et al. 2021. Potential Uses of Olive Oil Secoiridoids for the Prevention and Treatment of Cancer: A Narrative Review of Preclinical Studies. *Ijms* 22(3), 1234.þ doi:10.3390/ijms22031234

Erener, G., Ocak, N., Ozturk, E., Cankaya, S., and Ozkanca, R. (2009). The Scientific and Technological Research Council of Turkey, Agriculture, Forestry and Veterinary Research Group, AFVRG-Project No: 1070820 Report of Final Results, in *The Effects of Olive Leaf Extract on Performance, Some Blood Parameters and Cecal Microflora of Broilers*.

European Food Safety Authority (2015). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks in 2013. *EFSA J.* 13, 3991–4156.

Fang, H.-L., Lai, J.-T., and Lin, W.-C. (2008). Inhibitory Effect of Olive Oil on Fibrosis Induced by Carbon Tetrachloride in Rat Liver. *Clin. Nutr.* 27 (6), 900–907. doi:10.1016/j.clnu.2008.08.004

Farahat, A. A., Sawiress, F. A., S., and Aghwider, A. A. (2019). Effect of virgin Olive Oil Supplementation on Some Hematologic and Thyroid Hormones, Levels in Rats. *J. Vet. Med. Res.* 26 (1), 41–47.

Farooqui, A. A., and Farooqui, T. (2018). Effects of Mediterranean Diet Components on Neurodegenerative Diseases. *Role of the Mediterranean Diet in the Brain and Neurodegenerative Diseases*. Academic Press, 1–16. doi:10.1016/b978-0-12-811959-4.00001-8

Grosso, G., Buscemi, S., Galvano, F., Mistretta, A., Marventano, S., Vela, V., et al. (2013). Mediterranean Diet and Cancer: Epidemiological Evidence and Mechanism of Selected Aspects. *BMC Surg.* 13 (S2), S14. doi:10.1186/1471-2482-13-s2-s14

Hadi, D. S., and Al-khalisy, A. F. (2018). The Effect of Different Levels of Olive Oil in Ration Supplementation on Some Biochemical and Productive Traits in Broilers. *Int. J. Sci. Nat.* 9, 137–142.

Han, C. Y., Koo, J. H., Kim, S. H., Gardenghi, S., Rivella, S., Strnad, P., and Kim, S. G. (2016). Hepcidin Inhibits Smad3 Phosphorylation in Hepatic Stellate Cells by Impeding Ferroportin-Mediated Regulation of Akt. *Nat. Commun.* 7 (1), 1–14. doi:10.1038/ncomms13817þ

Han, J., Talorete, T. P. N., Yamada, P., and Isoda, H. (2009). Anti-proliferative and Apoptotic Effects of Oleuropein and Hydroxytyrosol on Human Breast Cancer MCF-7 Cells. *Cytotechnology* 59, 45–53. doi:10.1007/s10616-009-9191-2

Hassan, Z. K., Elamin, M. H., Omer, S. A., Daghestani, M. H., Al-Olayan, E. S., Elobeid, M. A., et al. (2013). Oleuropein Induces Apoptosis via the P53 Pathway in Breast Cancer Cells. *Asian Pac. J. Cancer Prev.* 14, 6739–6742. doi:10.7314/apjcp.2013.14.11.6739

Herrero-Encinas, J., Blanch, M., Pastor, J. J., Mereu, A., Ipharraguerre, I. R., and Menoyo, D. (2020). Effects of a Bioactive Olive Pomace Extract from Olea Europaea on Growth Performance, Gut Function, and Intestinal Microbiota in Broiler Chickens. *Poult. Sci.* 99 (1), 2–10. doi:10.3382/ps/pez467

Ibrahim, M., Wani, F., and Rahiman, S. (2017). Hepatoprotective Effect of Olive Oil and Camel Milk on Acetaminophen-Induced Liver Toxicity in Mice. *Int. J. Med. Sci. Public Health* 6 (1), 186–194. doi:10.5455/ijmsph.2017.07092016614

Ilavenil, S., Karthik, D., Arasu, M. V., Vijayakumar, M., Srigopalram, S., Arokiyaraj, S., et al. (2015). Hepatoprotective Mechanism of Lycorine against Carbon Tetrachloride Induced Toxicity in Swiss Albino Mice - A Proteomic Approach. *Asian Pac. J. Reprod.* 4 (2), 123–128. doi:10.1016/s2305-0500(15)30008-7

Jaeschke, H. (2003). Molecular Mechanisms of Hepatic Ischemia-Reperfusion Injury and Preconditioning. *Am. J. Physiology-Gastrointestinal Liver Physiol.* 284 (1), G15–G26. doi:10.1152/ajpgi.00342.2002

Kasdallah-Grissa, A., Nakbi, A., Koubâa, N., El-Fazaâ, S., Gharbi, N., Kamoun, A., et al. (2008). Dietary virgin Olive Oil Protects against Lipid Peroxidation and Improves Antioxidant Status in the Liver of Rats Chronically Exposed to Ethanol. *Nutr. Res.* 28, 472–479. doi:10.1016/j.nutres.2008.03.014

Khalatbary, A. R., Ghabaee, D. N. Z., Ahmadvand, H., Amiri, F. T., and Lehi, S. T. (2017). Deltamethrin-induced Hepatotoxicity and virgin Olive Oil Consumption: an Experimental Study. *Iran J. Med. Sci.* 42 (6), 586–592.

Korver, D. R., Roura, E., and Klasing, K. C. (1998). Effect of Dietary Energy Level and Oil Source on Broiler Performance and Response to an Inflammatory challenge. *Poult. Sci.* 77 (8), 1217–1227. doi:10.1093/ps/77.8.1217

Kyle, M. E., Miccadei, S., Nakae, D., and Farber, J. L. (1987). Superoxide Dismutase and Catalase Protect Cultured Hepatocytes from the Cytotoxicity of Acetaminophen. *Biochem. Biophysical Res. Commun.* 149, 889–896. doi:10.1016/0006-291X(87)90491-8

La Lastra, C., Barranco, M., Motilva, V., and Herrerías, J. (2001). Mediterrranean Diet and Health Biological Importance of Olive Oil. *Cpd* 7, 933–950. doi:10.2174/1381612013397654

Lykkesfeldt, J., and Svendsen, O. (2007). Oxidants and Antioxidants in Disease: Oxidative Stress in Farm Animals. *Vet. J.* 173, 502–511. doi:10.1016/j.tvjl.2006.06.005

Maher, P. (2017). Protective Effects of Fisetin and Other berry Flavonoids in Parkinson's Disease. *Food Funct.* 8, 3033–3042. doi:10.1039/c7fo00809k

Mahmoud, R., Badawi, Doaa., and El-Sayed, M. (2013). Effect of Supplementation of Broiler Diets with Guava Leaves And/or Olive Oil on Growth, Meat Composition, Blood Metabolites and Immune Response. *Benha Vet. Med. J.* 25 (2), 23–32.

Martínez-González, M. Á., and Sánchez-Villegas, A. (2004). The Emerging Role of Mediterranean Diets in Cardiovascular Epidemiology: Monounsaturated Fats, Olive Oil, Red Wine or the Whole Pattern? *Eur. J. Epidemiol.* 19 (1), 9–13.

Massimo, C., Lucio, T., Jesus, M. A., Giovanni, L., and Caramia, G. M. (2009). Extra virgin Olive Oil and Oleic Acid. *Nutrición Clinica y Dietetica Hospitalaria* 29 (3), 12–24.

Miles, E. A., and Calder, P. C. (2015). Fatty Acids, Lipid Emulsions and the Immune and Inflammatory Systems. *World Rev. Nutr. Diet.* 112, 17–30. doi:10.1159/000365426

Mokhtari, T., Hussein Osman, H.-E., El-Meghawry El-Kenawy, A., and Dashti, N. (2020). Ameliorative Effect of virgin Olive Oil against Nephrotoxicity Following Sub-chronic Administration of Ethephon in Male Rats. *J. Traditional Complement. Med.* 10, 487–495. doi:10.1016/j.jtcme.2019.08.005

Molnar, R., Szabo, L., Tomesz, A., Deutsch, A., Darago, R., Ghodratollah, N., et al. (2021). *In Vivo* effects of Olive Oil and Trans-fatty Acids on miR-134, miR-132, miR-124-1, miR-9-3 and mTORC1 Gene Expression in a DMBA-Treated Mouse Model. *Plos one* 16 (2), e0246022. þdoi:10.1371/journal.pone.0246022

Mosby, T. T., Cosgrove, M., Sarkardei, S., PlattKaina, K. L. B., and Kaina, B. (2012). Nutrition in Adult and Childhood Cancer: Role of Carcinogens and Anti-carcinogens. *Anticancer Res.* 32 (10), 4171–4192.

Mujahid, A., Akiba, Y., and Toyomizu, M. (2009). Olive Oil-Supplemented Diet Alleviates Acute Heat Stress-Induced Mitochondrial ROS Production in Chicken Skeletal Muscle. *Am. J. Physiology-Regulatory, Integr. Comp. Physiol.* 297 (3), R690–R698. doi:10.1152/ajpregu.90974.2008

Nakbi, A., Issaoui, M., Dabbou, S., Koubaa, N., Echbili, A., Hammami, M., et al. (2010). Evaluation of Antioxidant Activities of Phenolic Compounds from Two Extra virgin Olive Oils. *J. Food Compost. Anal.* 23, 711–715. doi:10.1016/j.jfca.2010.05.003

Nasopoulou, C., Lytoudi, K., and Zabetakis, I. (2018). Evaluation of Olive Pomace in the Production of Novel Broilers with Enhanced *In Vitro* Antithrombotic Properties. *Eur. J. Lipid Sci. Technol.* 120 (4), 1700290. doi:10.1002/ejlt.201700290

Nasopoulou, C., and Zabetakis, I. (2013). Agricultural and Aquacultural Potential of Olive Pomace A Review. *Jas* 5 (7). doi:10.5539/jas.v5n7p116

Necib, Y., Bahi, A., Zerizer, S., Abdennour, C., and Boulakoud, M. S. (2013). Effect of virgin Olive Oil (Olea Europea. L) on Kidney Function Impairment and Oxidative Stress Induced by Mercuric Chloride in Rats. *Am. J. Biochem. Biotechnol.* 9 (4), 415–422. doi:10.3844/ajbbsp.2013.415.422

Nekooeian, A., Dehghani, G., Mostafavi, H., and Khalili, A. (2011). The Effect of Hydroalcoholic Extract of Olive Leaves on Blood Pressure in Rat Model of Twokidney, One-Clip Goldblatt Hypertension. *Int. J. Cardiovasc. Res.* 5 (1), 1–6.

Neves, M. A. C., Dinis, T. C. P., Colombo, G., Sá e Melo, M. L., and Melo, M. L. (2007). Combining Computational and Biochemical Studies for a Rationale on the Anti-aromatase Activity of Natural Polyphenols. *ChemMedChem* 2, 1750–1762. doi:10.1002/cmdc.200700149

Nunes, M. A., Pimentel, F. B., Costa, A. S. G., Alves, R. C., and Oliveira, M. B. P. P. (2016). Olive By-Products for Functional and Food Applications: Challenging Opportunities to Face Environmental Constraints. *Innovative Food Sci. Emerging Tech.* 35, 139–148. doi:10.1016/j.ifset.2016.04.016

Oliván, S., Martínez-Beamonte, R., Calvo, A. C., Surra, J. C., Manzano, R., Arnal, C., et al. (2014). Extra virgin Olive Oil Intake Delays the Development of Amyotrophic Lateral Sclerosis Associated with Reduced Reticulum Stress and Autophagy in Muscle of SOD1G93A Mice. *J. Nutr. Biochem.* 25, 885–892. doi:10.1016/j.jnutbio.2014.04.005

Omar, S. H. (2010). Oleuropein in Olive and its Pharmacological Effects. *Sci. Pharm.* 78, 133–154. doi:10.3797/scipharm.0912-18

Ortiz, M., Soto-Alarcón, S. A., Orellana, P., Espinosa, A., Campos, C., López-Arana, S., et al. (2020). Suppression of High-Fat Diet-Induced Obesity-Associated Liver Mitochondrial Dysfunction by Docosahexaenoic Acid and Hydroxytyrosol Co-administration. *Dig. Liver Dis.* 52 (8), 895–904. doi:10.1016/j.dld.2020.04.019þ

Pan, S., Liu, L., Pan, H., Ma, Y., Wang, D., Kang, K., et al. (2013). Protective Effects of Hydroxytyrosol on Liver Ischemia/reperfusion Injury in Mice. *Mol. Nutr. Food Res.* 57 (7), 1218–1227. doi:10.1002/mnfr.201300010

Parkinson, L., and Keast, R. (2014). Oleocanthal, a Phenolic Derived from virgin Olive Oil: a Review of the Beneficial Effects on Inflammatory Disease. *Ijms* 15 (7), 12323–12334. doi:10.3390/ijms150712323

Pasban-Aliabadi, H., Esmaeili-Mahani, S., Sheibani, V., Abbasnejad, M., Mehdizadeh, A., and Yaghoobi, M. M. (2013). Inhibition of 6-Hydroxydopamine-Induced PC12 Cell Apoptosis by Olive (Olea Europaea L.) Leaf Extract Is Performed by its Main Component Oleuropein. *Rejuvenation Res.* 16, 134–142. doi:10.1089/rej.2012.1384

Patrick, L., and Uzick, M. (2001). Cardiovascular Disease: C-Reactive Protein and the Inflammatory Disease Paradigm: HMG-CoA Reductase Inhibitors, Alpha-Tocopherol, Red Yeast rice, and Olive Oil Polyphenols. A Review of the Literature. *Altern. Med. Rev.* 6 (3), 248–271.

Pirozzi, C., Lama, A., Simeoli, R., Paciello, O., Pagano, T. B., Mollica, M. P., et al. (2016). Hydroxytyrosol Prevents Metabolic Impairment Reducing Hepatic Inflammation and Restoring Duodenal Integrity in a Rat Model of NAFLD. *J. Nutr. Biochem.* 30, 108–115. doi:10.1016/j.jnutbio.2015.12.004

Psaltopoulou, T., Kosti, R. I., Haidopoulos, D., Dimopoulos, M., and Panagiotakos, D. B. (2011). Olive Oil Intake Is Inversely Related to Cancer Prevalence: a Systematic Review and a Meta-Analysis of 13800 Patients and 23340 Controls in 19 Observational Studies. *Lipids Health Dis.* 10, 127. doi:10.1186/1476-511x-10-127

Qian, F., Korat, A. A., MalikHu, V. F., and Hu, F. B. (2016). Metabolic Effects of Monounsaturated Fatty Acid-Enriched Diets Compared with Carbohydrate or Polyunsaturated Fatty Acid-Enriched Diets in Patients with Type 2 Diabetes: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Dia Care* 39 (8), 1448–1457. doi:10.2337/dc16-0513

Quintero-Flórez, A., Sinausia Nieva, L., Sánchez-Ortíz, A., Beltrán, G., and Perona, J. S. (2015). The Fatty Acid Composition of Virgin Olive Oil from Different Cultivars Is Determinant for Foam Cell Formation by Macrophages. *J. Agric. Food Chem.* 63 (30), 6731–6738. doi:10.1021/acs.jafc.5b01626

Ranalli, A., Pollastri, L., Contento, S., Di Loreto, G., Iannucci, E., LuceraRussi, L. F., et al. (2002). Acylglycerol and Fatty Acid Components of Pulp, Seed, and Whole Olive Fruit Oils. Their Use to Characterize Fruit Variety by Chemometrics. *J. Agric. Food Chem.* 50, 3775–3779. doi:10.1021/jf011506j

Reda, F. M., Alagawany, M., Mahmoud, H. K., Mahgoub, S. A., and Elnesr, S. S. (2020). Use of Red Pepper Oil in Quail Diets and its Effect on Performance, Carcass Measurements, Intestinal Microbiota, Antioxidant Indices, Immunity and Blood Constituents. *Animal* 14 (5), 1025–1033. doi:10.1017/S1751731119002891.þ

Riccio, P., Rossano, R., and Liuzzi, G. M. (2010). May Diet and Dietary Supplements Improve the Wellness of Multiple Sclerosis Patients? A Molecular Approach. *Autoimmune Dis.* 2010, 1–12. doi:10.4061/2010/249842

Rigacci, S., and Stefani, M. (2016). Nutraceutical Properties of Olive Oil Polyphenols. An Itinerary from Cultured Cells through Animal Models to Humans. *Ijms* 17, 843. doi:10.3390/ijms17060843

Rincón-Cervera, M. A., Rodrigo, V., María Catalina, H-R., Macarena, M., Alejandra, E., Susana, M., et al. (2016). Supplementation with Antioxidant-Rich Extra virgin Olive Oil Prevents Hepatic Oxidative Stress and Reduction of Desaturation Capacity in Mice Fed a High-Fat Diet: Effects on Fatty Acid Composition in Liver and Extrahepatic Tissues. *Nutrition* 32, 1254–1267.

Ritchason, J. (2000). *Olive Leaf Extract-Potent Antimicrobial, Antiviral and Antifungal Agent*. Australia: Woodland Publishing.

Rodriguez-Rodriguez, R., Perona, J. S., Herrera, M. D., and Ruiz-Gutierrez, V. (2006). Triterpenic Compounds from "Orujo" Olive Oil Elicit Vasorelaxation in Aorta from Spontaneously Hypertensive Rats. *J. Agric. Food Chem.* 54 (6), 2096–2102. doi:10.1021/jf0528512

Rolo, A. P., Teodoro, J. S., and Palmeira, C. M. (2012). Role of Oxidative Stress in the Pathogenesis of Nonalcoholic Steatohepatitis. *Free Radic. Biol. Med.* 52, 59–69. doi:10.1016/j.freeradbiomed.2011.10.003

Rowland, L. P., and Shneider, N. A. (2001). Amyotrophic Lateral Sclerosis. *N. Engl. J. Med.* 344, 1688–1700. doi:10.1056/nejm200105313442207

Saeed, M., Arain, M. A., Ali Fazlani, S., Marghazani, I. B., Umar, M., Soomro, J., et al. (2021). A Comprehensive Review on the Health Benefits and Nutritional Significance of Fucoidan Polysaccharide Derived from Brown Seaweeds in Human, Animals and Aquatic Organisms. *Aquacult. Nutr.* 27, 633-654.þ doi:10.1111/anu.13233

Saleem, T. M., Chetty, C. M., Ramkanth, S. V. S. T., Rajan, V. S. T., Kumar, K. M., and Gauthaman, K. (2010). Hepatoprotective Herbs–A Review. *Int. J. Res. Pharm. Sci.* 1 (1), 1–5.

Sánchez-Calvo, B., Cassina, A., Mastrogiovanni, M., Santos, M., Trias, E., Kelley, E. E., et al. (2021). Olive Oil-Derived nitro-fatty Acids: protection of Mitochondrial Function in Non-alcoholic Fatty Liver Disease. *J. Nutr. Biochem.* 94, 108646. doi:10.1016/j.jnutbio.2021.108646

Sarica, S., and Toptas, S. (2014). Effects of Dietary Oleuropein Supplementation on Growth Performance, Serum Lipid Concentrations and Lipid Oxidation of Japanese Quails. *J. Anim. Physiol. Anim. Nutr.* 98 (6), 1176–1186. doi:10.1111/jpn.12192þ

Sarrafchi, A., Bahmani, M., Shirzad, H., and Rafieian-Kopaei, M. (2016). Oxidative Stress and Parkinson's Disease: New Hopes in Treatment with Herbal Antioxidants. *Curr. Pharm. Des.* 22, 238–246. doi:10.2174/1381612822666151112151653

Sateri, S., Seidavi, A., Bouyeh, M., Neumann, P., Kutzler, M., Laudadio, V., et al. (2017). Effect of Olive Meal and Supplemental Enzymes on Performance Traits, Blood Biochemistry, Humoral Immunity Response and Caecal Microbiota of Broilers. *SA J. Sci.* 47 (6), 804–812. doi:10.4314/sajas.v47i6.8

Sayehban, P., Seidavi, A., Dadashbeiki, M., Ghorbani, A., AraújoAlbino, W. L. F. T., and Albino, L. (2016). Effects of Different Levels of Two Types of Olive Pulp with or without Exogenous Enzyme Supplementation on Broiler Performance and Economic Parameters. *Rev. Bras. Cienc. Avic.* 18 (3), 489–500. doi:10.1590/1806-9061-2015-0060

Scher, J. U., Pillinger, M. H., and Abramson, S. B. (2007). Nitric Oxide Synthases and Osteoarthritis. *Curr. Rheumatol. Rep.* 9 (1), 9–15. doi:10.1007/s11926-007-0016-z

Scoditti, E., Carpi, S., Massaro, M., Pellegrino, M., Polini, B., Carluccio, M. A., et al. (2019). Hydroxytyrosol Modulates Adipocyte Gene and miRNA Expression under Inflammatory Condition. *Nutrients* 11 (10), 2493. doi:10.3390/nu11102493

Sepporta, M. V., Fuccelli, R., Rosignoli, P., Ricci, G., Servili, M., Morozzi, G., et al. (2014). Oleuropein Inhibits Tumour Growth and Metastases Dissemination in Ovariectomised Nude Mice with MCF-7 Human Breast Tumour Xenografts. *J. Funct. Foods* 8, 269–273. doi:10.1016/j.jff.2014.03.027

Shi, C., Chen, X., Liu, Z., Meng, R., Zhao, X., Liu, Z., et al. (2017). Oleuropein Protects L-02 Cells against H2O2-Induced Oxidative Stress by Increasing SOD1, GPx1 and CAT Expression. *Biomed. Pharmacother.* 85, 740–748. doi:10.1016/j.biopha.2016.11.092

Soni, M. G., Burdock, G. A., Christian, M. S., BitlerCrea, C. M. R., and Crea, R. (2006). Safety Assessment of Aqueous Olive Pulp Extract as an Antioxidant or Antimicrobial Agent in Foods. *Food Chem. Toxicol.* 44 (7), 903–915. doi:10.1016/j.fct.2006.01.008

Soriguer, F., Morcillo, S., Cardona, F., Rojo-Martínez, G., de la Cruz Almaráz, M., de la Soledad Ruiz de Adana, M., et al. (2006). Pro12Ala Polymorphism of the PPARG2 Gene Is Associated with Type 2 Diabetes Mellitus and Peripheral Insulin Sensitivity in a Population with a High Intake of Oleic Acid. *J. Nutr.* 136 (9), 2325–2330. doi:10.1093/jn/136.9.2325

Sospedra, M., and Martin, R. (2005). Immunology of Multiple Sclerosis. *Annu. Rev. Immunol.* 23, 683–747. doi:10.1146/annurev.immunol.23.021704.115707

de Souza, P., Marcadenti, A., and Portal, V. (2017). Effects of Olive Oil Phenolic Compounds on Inflammation in the Prevention and Treatment of Coronary Artery Disease. *Nutrients* 9 (10), 1087. doi:10.3390/nu9101087

Stark, A. H., and Madar, Z. (2002). Olive Oil as a Functional Food: Epidemiology and Nutritional Approaches. *Nutr. Rev.* 60, 170–176. doi:10.1301/002966402320243250

Sudjan, A. N., D'Orazio, C., Ryan, V., Rasool, N., Ng, J., Islam, N., et al. (2009). Antimicrobial Activity of Commercial *Olea Europaea* (Olive) Leaf Extract. *Int. J. Antimicrob. Agents* 33 (5), 461–463.

Tabera, J., Guinda, Á., Ruiz-Rodríguez, A., Señoráns, F. J., Ibáñez, E., AlbiReglero, T. G., et al. (2004). Countercurrent Supercritical Fluid Extraction and Fractionation of High-Added-Value Compounds from a Hexane Extract of Olive Leaves. *J. Agric. Food Chem.* 52, 4774–4779. doi:10.1021/jf049881+

Terzuoli, E., Giachetti, A., Ziche, M., and Donnini, S. (2016). Hydroxytyrosol, a Product from Olive Oil, Reduces colon Cancer Growth by Enhancing Epidermal Growth Factor Receptor Degradation. *Mol. Nutr. Food Res.* 60 (3), 519–529. doi:10.1002/mnfr.201500498

Thirumalai, T., David, E., Therasa, S. V., and Elumalai, E. (2011). Restorative Effect of Eclipta alba in CCl4 Induced Hepatotoxicity in Male Albino Rats. *Asian Pac. J. Trop. Dis.* 1 (4), 304–307. doi:10.1016/s2222-1808(11)60072-8

Tiwari, R., Latheef, S. K., Ahmed, I., Iqbal, H. M. N., Bule, M. H., Dhama, K., et al. (2018). Herbal Immunomodulators - A Remedial Panacea for Designing and Developing Effective Drugs and Medicines: Current Scenario and Future Prospects. *Cdm* 19 (3), 264–301. doi:10.2174/1389200219666180129125436

Tufarelli, V., Laudadio, V., and Casalino, E. (2016). An Extra-virgin Olive Oil Rich in Polyphenolic Compounds Has Antioxidant Effects in Meat-type Broiler Chickens. *Environ. Sci. Pollut. Res.* 23 (7), 6197–6204. doi:10.1007/s11356-015-5852-1

Ullah, S., Zuberi, A., Alagawany, M., Farag, M. R., Dadar, M., Karthik, K., et al. (2018). Cypermethrin Induced Toxicities in Fish and Adverse Health Outcomes: Its Prevention and Control Measure Adaptationsh and Adverse Health Outcomes: Its Prevention and Control Measure Adaptation. *J. Environ. Manage.* 206, 863–871. doi:10.1016/j.jenvman.2017.11.076

Valenzuela, R., Espinosa, A., Llanos, P., Hernandez-Rodas, M. C., Barrera, C., Vergara, D., et al. (2016). Anti-steatotic Effects of an N-3 LCPUFA and Extra virgin Olive Oil Mixture in the Liver of Mice Subjected to High-Fat Diet. *Food Funct.* 7 (1), 140–150. doi:10.1039/c5fo01086a

Vauzour, D., Corona, G., and Spencer, J. P. E. (2010). Caffeic Acid, Tyrosol and P-Coumaric Acid Are Potent Inhibitors of 5-S-Cysteinyl-Dopamine Induced Neurotoxicity. *Arch. Biochem. Biophys.* 501, 106–111. doi:10.1016/j.abb.2010.03.016

Videla, L. A., Rodrigo, R, Araya, J., and Poniachik, J. (2004). Oxidative Stress and Depletion of Hepatic Long-Chain Polyunsaturated Fatty Acids May Contribute to Nonalcoholic Fatty Liver Disease. *Free Radic. Biol. Med.* 37, 1499–1507. doi:10.1016/j.freeradbiomed.2004.06.033

Vilaplana-Pérez, C., Auñón, D., and García-FloresGil-Izquierdo, L. A. A. (2014). Hydroxytyrosol and Potential Uses in Cardiovascular Diseases, Cancer, and AIDS. *Front. Nutr.* 1, 18.

Wahle, K. W., Brown, I., and RotondoHeys, D. S. D. (2010). *Bio-Farms for Nutraceuticals*. Boston, MA: Springer, 36–51.Plant Phenolics in the Prevention and Treatment of Cancer.

Wahle, K. W. (2004). Olive Oil and Modulation of Cell Signaling in Disease Prevention. *Lipids* 39 (12), 1223–1231.

Wang, H., Sit, W. H., Tipoe, G. L., and Wan, J. M. F. (2014). Differential Protective Effects of Extra virgin Olive Oil and Corn Oil in Liver Injury: a Proteomic Study. *Food Chem. Toxicol.* 74, 131–138.

Yan, C. M., Chai, E. Q., Cai, H. Y., and MiaoMa, G. Y. W. (2015). Oleuropein Induces Apoptosis via Activation of Caspases and Suppression of Phosphatidylinositol 3-kinase/protein Kinase B Pathway in HepG2 Human Hepatoma Cell Line. *Mol. Med. Rep.* 11 (6), 4617–4624.

Zangeneh, S., and Torki, M. (2011). Effects of B-Mannanase Supplementing of Olive Pulp-Included Diet on Performance of Laying Hens, Egg Quality Characteristics, Humoral and Cellular Immune Response and Blood Parameters. *Glob. Vet* 7, 391–398.

Zarei, M., Ehsani, M., and Torki, M. (2011). Productive Performance of Laying Hens Fed Wheat-Based Diets Included Olive Pulp with or without a Commercial Enzyme Product. *Afr. J. Biotechnol.* 10 (20), 4303–4312.

Zarghi, A., and Arfaei, S. (2011). Selective COX-2 Inhibitors: A Review of Their Structure-Activity Relationships. *Iran J. Pharm. Res.* 10 (4), 655–683.

Zbakh, H., and El Abbassi, A. (2012). Potential Use of Olive Mill Wastewater in the Preparation of Functional Beverages: A Review. *J. Funct. Foods* 4, 53–65.

Zhao, B., Ma, Y., Xu, Z., Wang, J., Wang, F., Wang, D., et al. (2014). Hydroxytyrosol, a Natural Molecule from Olive Oil, Suppresses the Growth of Human Hepatocellular Carcinoma Cells via Inactivating AKT and Nuclear Factor-Kappa B Pathways. *Cancer Lett.* 347 (1), 79–87.

Conflict of Interest: YW and QL was employed by the company Hunan Deren Husbandry Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 01 September 2021
doi: 10.3389/fphar.2021.714694

Houttuynia cordata Thunb: An Ethnopharmacological Review

Zhao Wu[1], *Xinyu Deng*[1], *Qichao Hu*[1], *Xiaolin Xiao*[2], *Jing Jiang*[3], *Xiao Ma*[1]* and *Mingquan Wu*[4]*

[1]State Key Laboratory of Southwestern Chinese Medicine Resources, Department of Pharmacy, School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2]Hospital of Chengdu University of Traditional Chinese Medicine, School of Clinical Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [3]School of Physical Education, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [4]Department of Pharmacy, Sichuan Orthopedic Hospital, Chengdu, China

OPEN ACCESS

Edited by:
Michael Heinrich,
UCL School of Pharmacy,
United Kingdom

Reviewed by:
Shelini Surendran,
University of Surrey, United Kingdom
Chia-I Tsai,
Taichung Veterans General Hospital,
Taiwan

***Correspondence:**
Xiao Ma
tobymaxiao@163.com
Mingquan Wu
549057257@qq.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 25 May 2021
Accepted: 05 August 2021
Published: 01 September 2021

Citation:
Wu Z, Deng X, Hu Q, Xiao X, Jiang J, Ma X and Wu M (2021) Houttuynia cordata *Thunb: An Ethnopharmacological Review. Front. Pharmacol. 12:714694. doi: 10.3389/fphar.2021.714694*

Houttuynia cordata Thunb (*H. cordata*; Saururaceae) is widely distributed in Asian regions. It plays an important role in traditional health care and disease treatment, as its aboveground stems and leaves have a long medicinal history in China and are used in the treatment of pneumonia and lung abscess. In clinical treatment, it can usually be combined with other drugs to treat dysentery, cold, fever, and mumps; additionally, *H. cordata* is an edible plant. This review summarizes detailed information on the phytochemistry and pharmacological effects of *H. cordata*. By searching the keywords "*H. cordata* and lung", "*H. cordata* and heart", "*H. cordata* and liver", and "*H. cordata* and inflammation" in PubMed, Web of Science and ScienceDirect, we screened out articles with high correlation in the past ten years, sorted out the research contents, disease models and research methods of the articles, and provided a new perspective on the therapeutic effects of *H. cordata*. A variety of its chemical constituents are characteristic of medicinal plants, the chemical constituents were isolated from *H. cordata*, including volatile oils, alkaloids, flavonoids, and phenolic acids. Flavonoids and volatile oils are the main active components. In pharmacological studies, *H. cordata* showed organ protective activity, such as reducing the release of inflammatory factors to alleviate lung injury. Moreover, *H. cordata* regulates immunity, enhances the immune barriers of the vagina, oral cavity, and intestinal tract, and combined with the antibacterial and antiviral activity of its extract, effectively reduces pathogen infection. Furthermore, experiments *in vivo* and *in vitro* showed significant anti-inflammatory activity, and its chemical derivatives exert potential therapeutic activity against rheumatoid arthritis. Antitumour action is also an important pharmacological activity of *H. cordata*, and studies have shown that *H. cordata* has a notable effect on lung tumour, liver tumour, colon tumour, and breast tumour. This review categorizes the biological activities of *H. cordata* according to modern research papers, and provides insights into disease prevention and treatment of *H. cordata*.

Keywords: Houttuynia cordata thunb, anti-inflammatory, antiviral, immunomodulatory, antibacterial, antitumour

INTRODUCTION

Medicinal plants have a variety of chemical components and biological activities that can effectively prevent and treat common clinical diseases. In India and other Asian regions, people use natural plants to treat diseases, accounting for 70–95% of basic treatments (Drasar and Khripach, 2020). The health function of medicinal plants has also attracted attention, and flavonoids, saponins, polysaccharides and alkaloids isolated from plants show effective antiaging activities, which have great potential in the development of antiaging products (Shen et al., 2017). Investigating the active ingredients of plants that have medicinal use is also an ingenious way to develop drugs. The discovery of artemisinin, which was originally isolated from the plant *Artemisia annua*, is a good example (Fu et al., 2021).

Houttuynia cordata (*H. cordata*), a perennial herb, is a plant of the Saururaceae family that is widely used as a Chinese herbal medicine as well as a food. It prefers to grow in moist soil and warm environments. Its application has been described in China, Korea, Japan, India, and other Asian regions, and particularly in many provinces in China (**Figure 1**). *H. cordata* has been eaten and used as medicine by local people for the past few thousand years. Currently, it is also harvested for daily food and medicine in the Yarlung Zangbo Valley in Assam, India (Kumar et al., 2014).

In general, the whole plant of *H. cordata* can be used as medicine, and it is applied by considering traditional Chinese medical theory, allowing it to be used to treat pneumonia caused by viral infection in combination with Forsythia and Magnolia (Park et al., 2005). Moreover, effective antiviral activity has been reported in many studies, and *H. cordata* has a significant inhibitory effect on virus infection and replication (Muluye et al., 2014). In the severe acute respiratory syndrome (SARS) virus infection outbreak in 2003, *H. cordata* was listed as one of the drugs for the treatment of SARS (Lau et al., 2008).

The disease prevention and potential treatment of *H. cordata* cannot be realized without effective research. Therefore, the objective of this review is to provide a unified governance framework that guides studies on the pharmacological activity of *H. cordata* and its derivatives *in vivo* and *in vitro*. This is achieved by reviewing the therapeutic activity of *H. cordata* in different organs, tissues and cells in the past research. This study is also an attempt to synthesize scattered sources of information obtained regarding the therapeutic activity of *H. cordata* against diseases.

CHEMICAL COMPOSITION

H. cordata contains a variety of chemical components (**Table 1**), and alkaloids were the most abundant ingredients (Ahn et al., 2017); however, volatile oil and flavonoids were the main components that exerted pharmacological activity. Interestingly, the decanoyl acetaldehyde component has a special fishy scent, so it is called Yu-Xing-Cao (traditional Chinese medicinal herb) in China (Ma et al., 2017), and it is

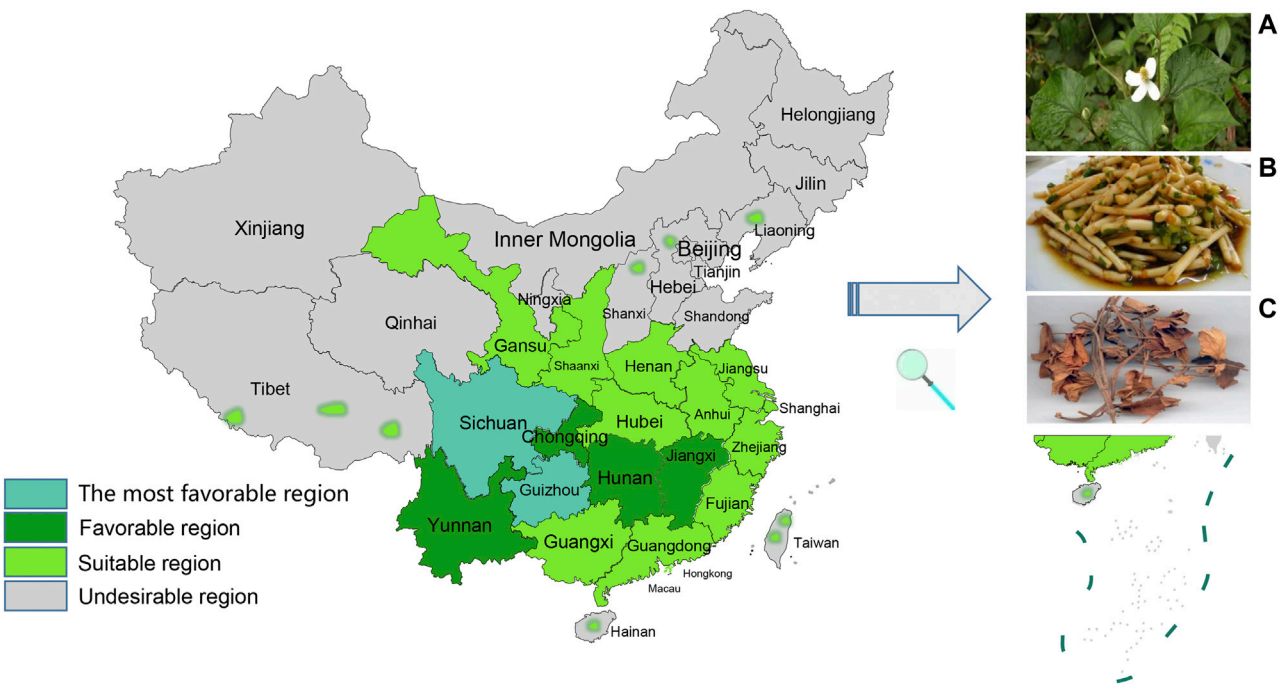


FIGURE 1 | Climatic and ecological adaptability distribution of *H. cordata* in China **(A)** flowers of *H. cordata* **(B)** roots of *H. cordata* as food **(C)** above-ground part of dry stems and leaves of *H. cordata*.

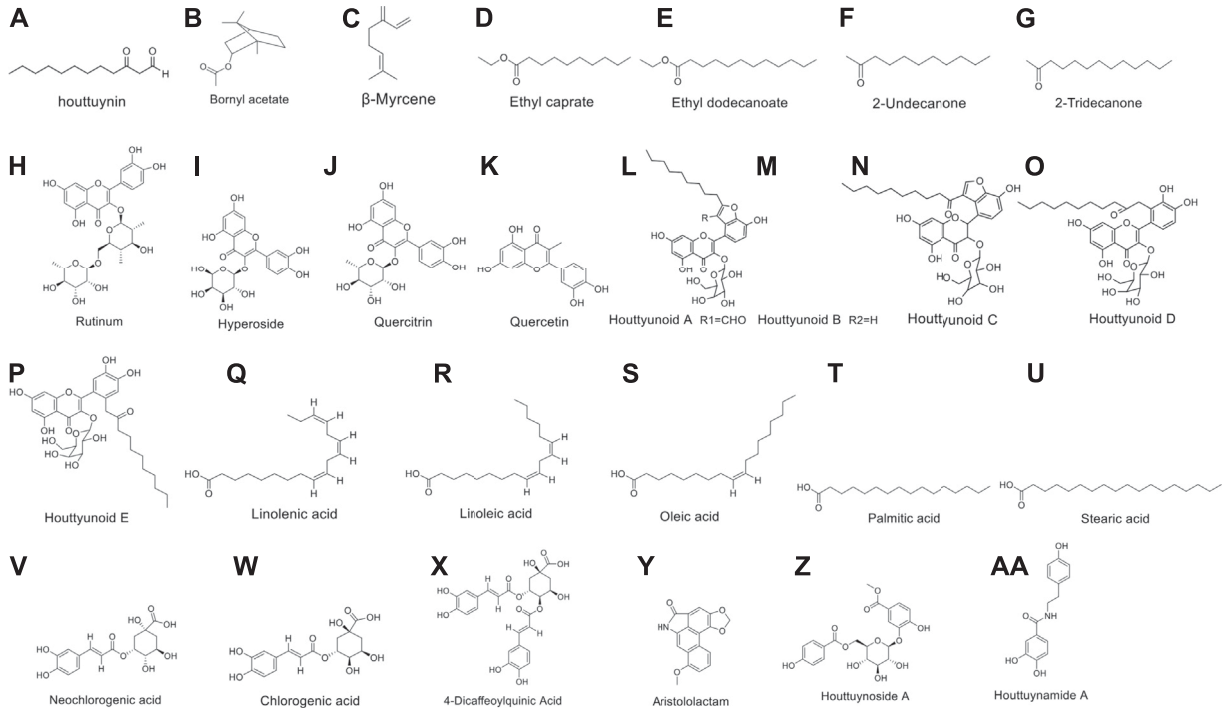


FIGURE 2 | Chemical structures of important constituents of *H. cordata*.

also an antibacterial active ingredient and is easily converted to 2-undecanone (methyl n-nonanone) at high temperature, which can be used to evaluate the quality of *H. cordata* oil to a certain extent (Chen et al., 2014). Moreover, its steam distillation extract contained essential oils including monoterpenes, sesquiterpenes and their oxides, oxidized diterpenes and phenylpropene derivatives (Řebíčková et al., 2020); and nonyl ketones (2.10–40.36%), bornyl acetate (0.4–8.61%) and β-myrcene (2.58–18.47%) are the main components in essential oils (Lu et al., 2006). Interestingly, there were differences in the contents of the aboveground stems and the underground parts, as the contents of 2-undecanone, myrcene, ethyl decanoate, ethyl dodecanoate, 2-tridecanone and decanal in the aboveground parts were higher than those in the underground parts; In particular, 11 ingredients were only isolated in the leaves, while seven ingredients in the roots were not contained in the leaves. Interestingly, there seemed to be variation in different regions. Researchers also reported differences in the antibacterial activity of *H. cordata* from different areas, but these findings still lack sufficient support (Verma et al., 2017).

Moreover, the flavonoids in *H. cordata* include rutin, hyperoside, quercetin, and quercitrin, and most of them are combined with rhamnose in the form of glycosides (Xu et al., 2006; WU et al., 2009; Lu et al., 2011). Chen et al. isolated a new combination of houttuynin and hyperoside (houttuynoids A-E [1–5]), four new flavonoid compounds (Chen et al., 2012; Chen S. D. et al., 2013), and Chou et al. isolated the houttuynoside A and houttuynamide A (Chou et al., 2009). However, phenolic acids are the most isolated components in *H. cordata*, including linolenic acid, linoleic acid, oleic acid, palmitic acid, stearic acid (Bauer et al., 1996), quinic acid derivatives, caffeic acid derivatives, neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid and other ingredients (Nuengchamnong et al., 2009).

Furthermore, alkaloids are the key components of the physical activity of houttuynia-containing herbs, most of which are phenanthrolactam compounds such as aristololactam and piperolactam (Probstle and Bauer, 1992; Ma et al., 2017). The structural formulas of above compounds are shown in **Figure 2**.

PHARMACOLOGICAL ACTIVITY

Lung Protection

Lung diseases have various causes. General symptoms are inflammation of the lungs accompanied by acute lung injury (ALI). The anti-inflammatory activity of *H. cordata* plays an indispensable role in alleviating lung diseases, which might be associated with its flavonoids, sodium houttuyfonate and polysaccharides, in a lipopolysaccharide (LPS)-induced model, proinflammatory cytokine (IL-6) and NO production were obviously reduced by oral administration of quercitrin from *H. cordata* at 100 mg/ml (Lee et al., 2015). Interestingly, in a model of chronic obstructive pulmonary disease (COPD) induced by LPS combined with cigarette smoke for 4 weeks, 24.3 mg/kg sodium houttuyfonate decreased the mRNA levels of TLR4, MyD88, and NF-κB p65 (Wu et al., 2017), however,

considering the differences in the adaptability of humans and rats to cigarette smoke, this research still needs more investigation. Regardless, macromolecular polysaccharides contribute to alleviating lung injury by reducing pulmonary oedema and protein exudation of bronchoalveolar lavage fluid (Lu et al., 2018). Cell transplantation combined with *H. cordata* has also been used to treat lung tissue injury. Injection of 50 mg/g extract via the tail vein downregulated the inflammatory response and reduced the expression of iNOS and ET-1, thereby enhancing the therapeutic effect of endothelial progenitor cells on LPS-induced ALI in rats (Cai et al., 2013). Furthermore, in an acute lung tissue injury model caused by H1N1 virus infection, utilizing 50, 100, and 200 mg/kg *H. cordata* flavonoid glycoside extract compared to 100 mg/kg ribavirin resulted in less weight loss and a lower lung index in 14 days. Antibacterial and anti-inflammatory activity were realized by inhibiting H1N1 neuraminidase activity and the expression of toll-like receptors (TLRs) (Ling et al., 2020), and regulation of polysaccharide on gut mucosal-associated lymphoid tissue (GALT) might be the mechanism that alleviates ALI caused by influenza A virus, as it downregulates Th17 cell differentiation and upregulates Treg cell differentiation to restore Th17/Treg balance from the GALT to the lung, reducing IL-17A and increasing IL-10 to alleviate lung mucosal damage (Shi et al., 2020).

Previous studies have confirmed that pulmonary fibrosis is associated with lung oxidative damage. In bleomycin-induced pulmonary fibrosis in rats, a water extract of *H. cordata* significantly reduced the concentrations of superoxide dismutase, malondialdehyde, and hydroxyproline, showing stronger antioxidant activity than vitamin E. Otherwise, *H. cordata* can relieve the pathological changes of lung tissue caused by bleomycin (Ng et al., 2007), and the increasing level of IFN-γ and inhibition of the TGF-β1/Smad signalling pathway might be a significant mechanism. Meanwhile, 4-terpineol, α-terpineol, L-bornyl acetate and methyl-n-nonyl ketone were significantly decreased in a dose-dependent manner at doses of 3.5–16.5 mg/kg *in vivo*. *In vitro*, the expression of TGF-β1 was inhibited in a dose-dependent manner, and IFN-γ levels were also upregulated in NIH/3T3, thus alleviating the lung fibrosis induced by LPS (Du et al., 2012).

Overall, the above studies revealed that the protective effect of *H. cordata* is related to its anti-inflammatory active ingredients, which mainly include flavonoids, polysaccharides, and sodium houttuyfonate, although the dominant component has not been reported. Mice show consistency in certain symptoms of respiratory diseases with humans, but there are significant differences in the immune system (Shilovskiy et al., 2021), so it is difficult to judge whether the above research results act through the same mechanism in humans.

Digestive System Protection

Reduction of Intestinal Injury

There are mucosal barriers in the intestine to avoid damage, which generally include mechanical barriers, chemical barriers, immune barriers, and biological barriers. In addition, the intestinal flora plays an important role in protecting the intestines (Lu et al., 2019). Recent studies have found that polysaccharides and sodium houttuyfonate in *H. cordata* protect the intestinal flora, a crude polysaccharide extract significantly reduces intestinal goblet cells, and the expression of sIgA and tight junction protein (ZO-1) in the intestine is upregulated to strengthen the intestinal mechanical barriers and immune barrier (Zhu et al., 2018). In a mouse model of intestinal inflammation caused by *Salmonella typhimurium*, Zhang et al. demonstrated the protection of sodium houttuyfonate in restoring the intestinal barrier by regulating the tissue distribution of tight junction proteins, and inflammation was also reduced by inhibiting the NF-κB signalling pathway (Zhang et al., 2020). However, such barrier-enhancing activity lacks effective control. Moreover, the regulation of bacteria is also involved, and polysaccharides composed of Glc, Gal, Ara, and Rha at a 40 mg/kg dosage greatly reduced the relative abundance of the pathogenic bacteria *Vibrio* and *Bacillus* and alleviated the intestinal damage caused by H1N1 infection (CHEN et al., 2019). These findings indicated that *H. cordata* polysaccharides and sodium houttuyfonate exert intestinal protective activity by regulating the intestinal flora and inhibiting the NF-κB signalling pathway to strengthen the intestinal barrier.

Reduction of Liver Damage

Recently, natural products in plants have shown effective activity in the prevention and treatment of liver diseases. Researchers have confirmed that natural products such as terpenoids, alkaloids, glycosides, and coumarins inhibit liver fibrosis (Ma et al., 2020a); moreover, rutin and quercetin show potential therapeutic activity in cholestasis (Ma et al., 2020b). Liver cells are sensitive to oxidative stress, and the ethyl acetate extract of *H. cordata*, which confers liver protection, showed significant antioxidant activity in a CCl_4-induced liver injury mice model. In particular, the 1,000 mg/kg extract remarkably inhibited the increase of glutathione, superoxide dismutase and catalase; furthermore, the levels of serum transaminase and liver malondialdehyde also decreased in mice (Tian et al., 2012), and in ethanol-induced liver damage model, the CYP2E1 activity of mice treated with the mixture of *H. cordata* water and ethanol extract at 300 mg/kg/day for 7 days was significantly reduced, thereby decreasing the level of oxidative factors that CYP2E1 mediated, while the expression of antioxidant enzymes and lipogenic mRNA was increased (You et al., 2018). Overall, in the liver oxidative damage model, *H. cordata* inhibited the increase of glutathione, superoxide dismutase and catalase and regulated the release of oxidative factors mediated by CYP2E1 to relieve oxidative damage. However, the dosages used in different models are not uniform, and whether high-dose *H. cordata* extract has a toxicity needs to be investigate.

Heart Protection

Antioxidant components of *H. cordata* show interventional activity in the process of heart remodelling and functional decline. In diabetic mice, continuous intake of 2% *H. cordata* water extract for 8 weeks downregulated cardiac active oxygen, protein carbonyl, interleukin-6 and inflammatory factors; moreover, intake of 1 and 2% *H. cordata* water extract inhibited the expression of $p47^{phox}$, NF-κB p65 and p-p38 in

the heart caused by diabetes (Hsu et al., 2016). With further research, similar activity of sodium houttuyfonate was found in an isoprenaline-induced myocardial hypertrophy model treated with 90 and 180 mg/kg sodium houttuyfonate for 1 week, and the results showed that the concentration of cyclic adenosine monophosphate, heart weight index, left ventricular weight index, and angiotensin II were simultaneously reduced. In the L-thyroxine-induced cardiac hypertrophy model, the expression of hydroxyproline and the cross-sectional area of cardiomyocytes were downregulated. This myocardial protection might be associated with the suppression of the sympathetic nervous system, renin-angiotensin system and endothelin expression (Gao et al., 2009). Additionally, the activation of the renin-angiotensin-aldosterone system was attenuated by sodium houttuyfonate at dosages of 50 and 100 mg/kg, with the activity of alleviating cardiac inflammation and fibrosis; and it showed inhibition of ventricular remodelling in a model of abdominal artery stenosis-induced ventricular remodelling in rats (Gao et al., 2010). Moreover, the anti-ventricular remodelling mechanism after myocardial infarction of sodium houttuyfonate might be associated with the adenosine monophosphate-activated protein kinase (AMPK) activation and NF-κB pathway inhibition at the same dosage; moreover, the release of myocardial inflammatory factors also declines, thereby relieving the fibrosis process (Zheng et al., 2018). However, in the anti-angiogenic research of *H. cordata*, no effective activity was seen in a zebrafish model (Tu et al., 2016).

Taken together, the *H. cordata* extract reduced the release of inflammatory factors to alleviate myocardial oxidative damage. The sodium houttuyfonate component can affect the sympathetic nervous system and the renin-angiotensin-aldosterone system to reverse myocardial hypertrophy and remodelling, and the molecular mechanism may relate to AMPK activation inhibited.

Kidney Protection

Kidney diseases are usually caused by inflammation, oxidative damage and other factors that seriously affect the body's water and salt metabolism (Gong et al., 2020). In diabetic mice with kidney injury, treatment with 1 and 2% *H. cordata* water extract reduced the level of urea nitrogen and the activity of creatine kinase, and the expression of kidney oxidative factors also decreased. Meanwhile, intake of 2% extract seemed to restrict the expression of membrane-anchored receptor of advanced glycation end products (RAGE), which can induce intracellular reactive oxygen species generation and activate mitogen-activated protein kinase (MAPK) and NF-κB signalling pathways, indicating its renal protective activity (Hsu et al., 2016). Inflammation is also an important mechanism leading to kidney damage. Research by Pan et al. showed that the sodium houttuyfonate significantly reduced the expression of nuclear NF-κB and MCP-1 in a dose-dependent manner at 60–120 mg/kg. In turn, it inhibited cationic bovine serum albumin-induced membranous glomerulonephritis and exhibited renal protective activity (Pan et al., 2010). In summary, the findings suggested that *H. cordata* extract and sodium houttuyfonate reduced kidney oxidative stress damage and inflammation through the glycation polyol pathway, downregulating the expression of NF-κB and MCP-1 and thereby alleviating kidney damage.

Antitumour Activity

Anti-Lung Tumour Activity

Lung cancer usually includes small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). Generally, inducing tumour cell apoptosis and inhibiting tumour cell migration play a vital role in the treatment of lung tumour (Jones et al., 2018). Researchers demonstrated the antitumour activity of *H. cordata* and its active ingredient 2-undecanone in a study of benzo(a)pyrene-induced lung tumour in mice, and activation of the Nrf2-HO-1/NQO-1 pathway might be involved, which in turn inhibits lung cell DNA damage and inflammation. Moreover, there was no obvious systemic toxicity in mice (Lou et al., 2019). In addition, the polysaccharides in *H. cordata* have been found to possess antitumour activity, and the isolated pectin polysaccharide HCA4S1 has been experimentally proven to inhibit tumour cell proliferation by inducing A549 lung tumour/cancer cell cycle arrest and apoptosis. At the same time, the activities of cleaved caspase3 and cyclin B1 in cells after HCA4S1 treatment were significantly increased (Han et al., 2018). Chen et al. further investigated the apoptosis mechanism of lung tumour cells, and the active ingredients of *H. cordata* blocked cell proliferation by acting on the G0/G1 phase of A549 cells. In addition, Fas/CD95 protein levels in A549 cells were upregulated, and caspase-8 and caspase-3 were activated (Chen Y. F. et al., 2013). In another study on the migration of NSCLC, sodium houttuyfonate in *H. cordata* was found to inhibit the migration of tumour cells, and the inhibition of Linc00668 activity might be a vital feature, resulting in the decrease in Slug mRNA levels regulated by miR-147a. This revealed the important role of the Linc00668/miR-147a/slug axis in inhibiting lung tumour cell migration (Jiang et al., 2019). The above research results indicated that *H. cordata* might alleviate DNA damage by activating the Nrf2-HO-1/NQO-1 pathway, blocked cell proliferation by acting on the G0/G1 phase, and regulated the level of lncRNAs to inhibit tumour cell migration. However, the above findings were mainly investigated *in vitro*, and more *in vivo* investigations are still needed.

Anti-Liver Tumour Activity

In a study of human HepG2 cells exposure to high glucose, *H. cordata* extract at a concentration range of 0–80 μg/ml reduced lipid accumulation in HepG2 cells in a dose-dependent manner. This mechanism is related to inhibition of the AMPK signalling pathway, reduction of AMPK-mediated lipid synthesis, and alleviation of the proliferation of liver tumour cells (Kang and Koppula, 2014). Moreover, the apoptosis-inducing activity of *H. cordata* has been investigated, and the levels of factor (HIF)-1A, Forkhead box (FOX)O3, and MEF2A were significantly upregulated in human HepG2 hepatocellular carcinoma cells. At the same time, *H. cordata* enhanced the expression of caspase-3 and caspase-7 through MEF2A, while Bax, Bcl-2 and Bcl-xL protein levels were also disturbed, thus inducing apoptosis of liver tumour cells (Kim et al., 2017). Taken together, lipid accumulation in human HepG2 cells can be reduced after treatment with *H. cordata*, and HIF-1A, FOXO3, and MEF2A factors are significantly activated; however, the chemicals that act

to induce apoptosis in liver tumour and investigation in animal models are still needed.

Anti-Colon Tumour Activity

In research on products for the treatment of colon tumour, Tang et al. found that an ethanol extract of *H. cordata* showed antitumour activity against the colon tumour cell line HT-29. Treatment with a 450 μg/ml extract can significantly induce apoptosis of tumour cells, increase reactive oxygen species and decrease the mitochondrial membrane potential. In particular, cytochrome c, Apaf-1, pro-caspase-9 and AIF are released from mitochondria due to changes in membrane potential by Western blotting and caspase activity assays. This result revealed the mitochondria-dependent mechanism by which *H. cordata* extract induces apoptosis of HT-29 cells (Tang, et al., 2009). Moreover, the investigation of induced cytotoxicity in primary colorectal cancer/tumour cells showed the same results; mitochondrial-dependent apoptosis mechanisms were also involved, and the production of reactive oxygen species increased. After treatment with 250 μg/ml *H. cordata* extract for 24 h, primary colorectal cancer/tumour cells showed chromosome condensation and apoptosis (Lai et al., 2010). Taken together, the molecular mechanism of the cytotoxicity of *H. cordata* extract mainly consists of reducing the mitochondrial membrane potential, thereby increasing the levels of cytochrome c, Apaf-1, caspase-3 and -9 and inducing cancer/tumour cell apoptosis, and the cytotoxicity to colon cells is still lacking.

Anti-Gastric Carcinoma Activity

Gastric carcinoma is the third most fatal tumour and is a prevalent malignancy worldwide, with approximately 1,033,701 new cases reported annually (Bray et al., 2018). The induce apoptosis and inhibit migration activity of herbs at the interface of food and medicine was investigated in gastric carcinoma, and a food composed of six plants, *Coix seed, Lentinula edodes*, *Asparagus officinalis* L., *H. cordata*, *Taraxacum mongolicum* Hand.-Mazz., and *Grifola frondose*, was used to treat gastric carcinoma in nude mice inoculated with SGC-7901 cells; supplementing 43.22, 86.44, and 172.88 g/kg food for 30 days, the serum levels of MMP-2 and MMP-9 decreased, while TNF-α significantly increased. Moreover, treatment notably upregulated the mRNA expression levels of GSK-3β, E-cadherin, Bax, Caspase-3, and Caspase-9 and the Bax/Bcl-2 ratio but substantially downregulated β-catenin, N-cadherin, MMP-2, MMP-9, Snail, and Cyclin D1, especially Ki-67 and N-cadherin, in tumour tissues. The underlying molecular mechanism might be associated with inhibition of the Wnt/β-catenin signalling pathway (Chen X. et al., 2021); this finding indicated the possibility of *H. cordata* forming synergistic interactions with the other five plants to prevent gastric carcinoma as a daily food; however, the specific activity of *H. cordata* among it has not been investigated. Interestingly, researchers compared the anti-gastric carcinoma activity of heated and unheated parts of *H. cordata* separately through DAPI staining and the detection of apoptosis and apoptotic protein levels, and cell viability decreased in SGC-7901, HepG2, NCI-H640, and HO-8910 cells with the increase of the extract concentration at 0, 25, 50, 100, and 150 ml/L. Furthermore, heated ariel stems showed 3–15 times higher effects than stems that were not heated in SGC-7901 cells, and the morphological characteristics of apoptosis, p53 protein, pro-apoptotic protein Bax, Bid, Bak, Apaf-1, activation of PARP, caspase9, and caspase3 were also increased, and the heated sample seemed to have higher activity than the unheated sample (Liu et al., 2020). The above findings suggested the potential of *H. cordata* in preventing gastric carcinoma in the diet.

Other Anti-Tumour Activity

The overexpression of HER2/neu has been shown to be related to breast cell canceration (Dawood et al., 2010; de la Cruz-Merino et al., 2017), which suggests that it may be an anti-breast tumour target. In the research by Zhou et al., houttuyfonate, the active ingredient of *H. cordata*, was modified by adding sulfhydryl groups and showed a significant reduction in tumour volume. At a concentration of 5.52 μg/ml, HER2 phosphorylation and HER2/neu-mediated activation of ERK1/2 and AKT were inhibited (Zhou et al., 2012). In addition, studies have shown that the ethanol extract of *H. cordata* induced breast tumour cell apoptosis. At concentrations of 100–500 μg/ml, MCF-7 and MDA-MB-231 cells stagnated in the G1 phase, and this result might be related to the downregulation of cyclin D1 and CDK4 expression at low concentrations. Moreover, the secretion of MMP-2 and MMP-9 is significantly inhibited, thereby inhibiting the migration and invasion of tumour cells (Subhawa et al., 2020). Overall, *H. cordata* and its derivatives seem to exert anti-breast tumour activity by suppressing tumour volume, inducing apoptosis and inhibiting migration. The regulation of HER2/neu overexpression and cell cycle arrest was also involved.

Leukaemia is a type of disease caused by the malignant cloning of haematopoietic stem cells. In early studies, the ingredients of *H. cordata*, caffeic acid has been found to induce apoptosis in leukaemia cells. At a concentration of 45 μM, caffeic acid treatment for 2 days significantly reduced the activity of U937 leukaemia cells. Additionally, as a typical apoptotic feature, the cleavage of PARP and procaspase-3 was obviously activated, and the apoptotic rate of leukaemia cells treated with 100 mM caffeic acid reached 59.87% (Jang et al., 2011). Furthermore, a study in leukaemic Moult-4 cells revealed the molecular mechanism by which *H. cordata* extract induces apoptosis, the alcohol extract of *H. cordata* decreased the mitochondrial transmembrane potential, the expression of Bcl-xl was downregulated, and the protein levels of Smac/Diablo, Bax and GRP78 increased (Prommaban et al., 2012). In conclusion, *H. cordata* can increase the lysis of PARP and procaspase-3 in leukaemia cells and induce Moult-4 cell apoptosis through the endoplasmic reticulum stress pathway (**Figure 3**).

Anti-Inflammatory Effects and Immunomodulatory Activity

Anti-Inflammatory Effects

The occurrence of inflammation is related to a variety of cells, such as eosinophils, basophils, neutrophils, macrophages, monocytes, and mast cells (Roe, 2021). Mast cells play a vital

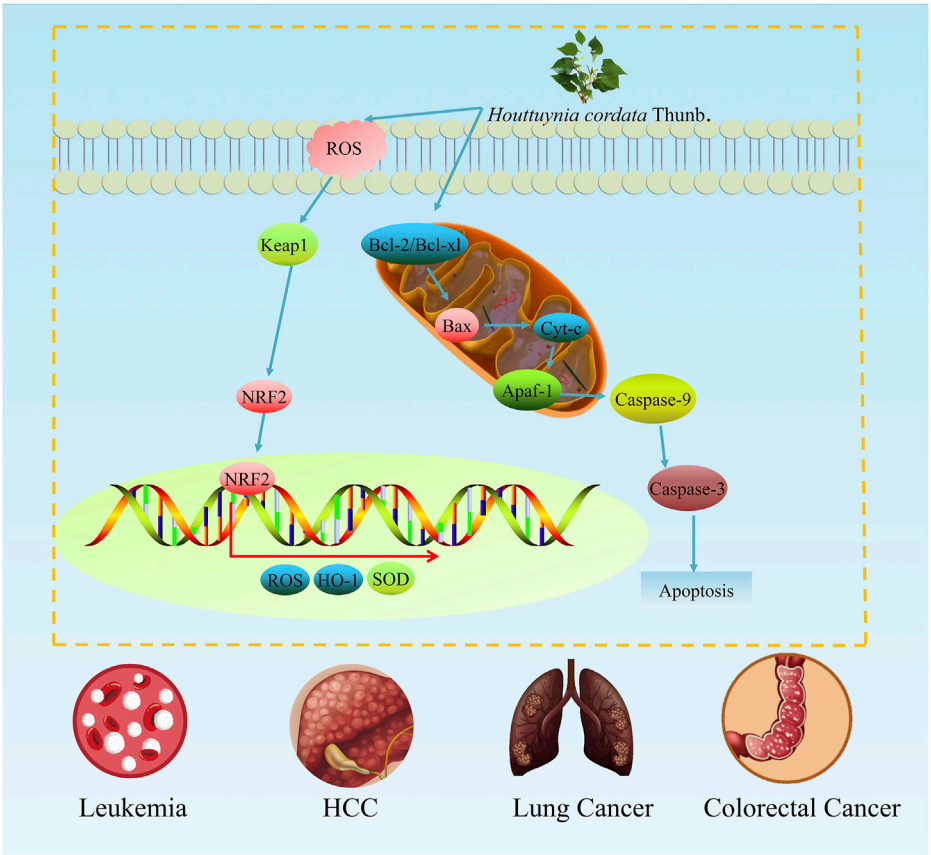


FIGURE 3 | The antitumour effects of *H. cordata*. *H. cordata* might suppress multiple tumours mainly by inhibiting the NRF2 signalling pathway and promoting the process of apoptosis.

role in mediating inflammatory diseases such as asthma and allergies. Interestingly, the ability of an extract of *H. cordata* to inhibit mast cell-mediated inflammatory diseases was investigated by Kim's et al.; the mast cell line HMC-1 was treated with the ethyl acetate *H. cordata* extract at a concentration of 10 μg/ml, and the chemotactic index, secretion, and mRNA levels of inflammatory factors TNF-α and IL-6 were downregulated. In addition, stem cell factor-mediated NF-κB activation was inhibited (Kim et al., 2007; Lee et al., 2013). Moreover, in interstitial bladder inflammation, not only are proinflammatory factors decreased, but the proliferation and activation of mast cells are effectively attenuated, demonstrating the potential value of *H. cordata* in the treatment of interstitial cystitis (Li et al., 2020).

In a RAW264.7 cell experiment induced by LPS, both 2-methyl nonyl ketone and sodium houttuyfonate components isolated from *H. cordata* showed anti-inflammatory activity, and expression of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and Toll-like receptor 4 (TLR4) were decreased while the level of interleukin-10 (IL-10) was upregulated. Moreover, the supercritical extract of *H. cordata* inhibited RAW 264.7 cell inflammation through the TNF-α-NO and cyclooxygenase II-PGE_2 pathways, and the extract at a dose of 200 mg/kg significantly reduced inflammatory cells and albumin exudation after oral administration (Shin et al., 2010). In addition, its fermentation products showed similar activity; the expression of the proinflammatory factors PGE_2, iNOS, IL-1β, TNF-α and IL-6 was downregulated, while the effect on COX-2 activity was weak (Woranam et al., 2020). Furthermore, in LPS-induced peritoneal macrophages, the supercritical extract of *H. cordata* showed similar effects to nonsteroidal anti-inflammatory drugs (NSAIDs) and the COX-2 inhibitor NS398; it not only reduced COX-2 enzyme activity in a dose-dependent manner but also downregulated COX-2 mRNA and protein expression (Li et al., 2011). At the same time, tumour necrosis factor-α (TNF-α) mRNA expression and NO factor levels induced by LPS were significantly inhibited by *H. cordata*. *In vivo*, xylene-induced ear swelling and inflammation in mice were significantly inhibited, and sodium houttuyfonate showed stronger anti-inflammatory activity than 2-methyl nonyl ketone (Chen et al., 2014). Moreover, mouse foot swelling induced by formaldehyde and carrageenan was also effectively relieved by the volatile oil components of *H. cordata* (Li et al., 2013).

Interestingly, inflammation of human keratinocytes was also alleviated by the *H. cordata* ethanol extract, and the secretion of

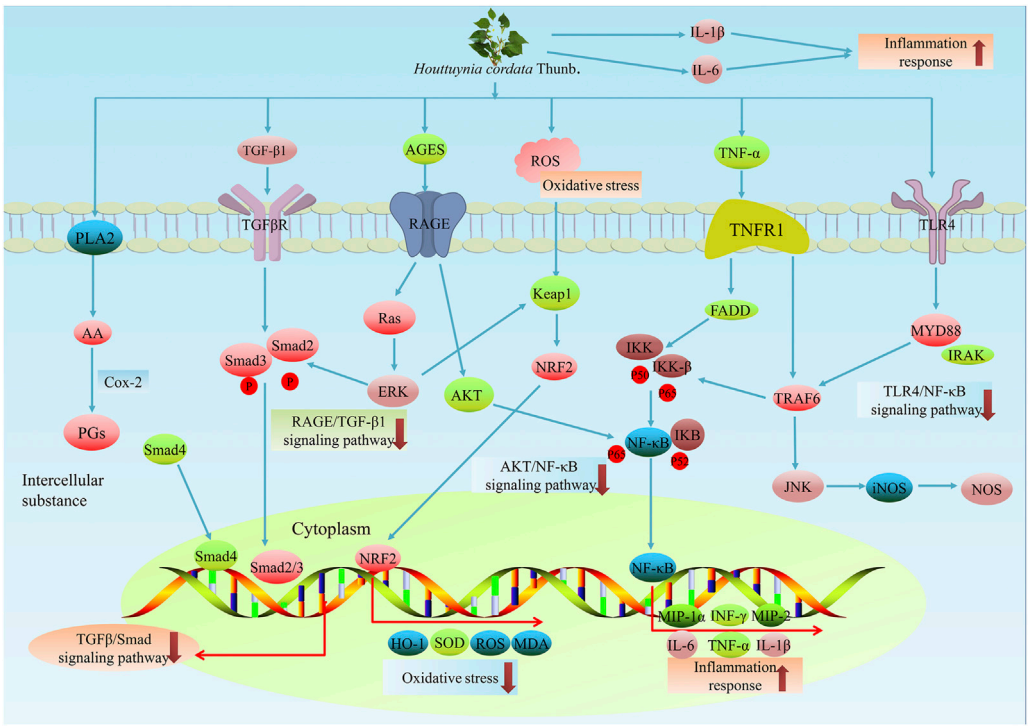


FIGURE 4 | The anti-inflammatory effects of *H. cordata*. *H. cordata* suppresses inflammation mainly via several signalling pathways. H. cordata reduces the production of PGs by acting on PLA2. In addition, *H. cordata* seemed to suppress inflammation by downregulating RAGE/TGFβ1 signals, AKT/NF-κB signals, TLR4/NF-κB signals and TGF-β/Sma d signals.

interleukin-8, CCL20, IP-10, and GRO-α caused by *Porphyromonas gingivalis* was effectively reduced after treatment (**Figure 4**). These research results suggested the application of *H. cordata* in oral infections (Sekita et al., 2017). Sodium houttuyfonate also showed potential therapeutic activity for rheumatoid arthritis, which is the destruction of the joint caused by pathological hyperplasia of the synovium. Through *in vitro* synovial cell experiments, sodium houttuyfonate effectively inhibited the proliferation of synovial cells in a dose-dependent manner within the range of 25 μg/ml~200 μg/ml (Li et al., 2014; Li and Zhao, 2015).

Immunomodulatory Activity

Immune cells, including neutrophils, eosinophils, basophils, and mast cells, play a vital role in ensuring immune function. Immune active substances such as immunoglobulin, interferon, tumour necrosis factor, and interleukin also play important roles (Sattler, 2017). Allergy is a common immune function abnormality that can cause serious diseases such as anaphylactic shock, microcirculation disorders, and central nervous system disorders (Marshall, 2018).

The polyphenols in *H. cordata* have shown antiallergic activity. After basophilic KU812F cells were cultured with extract, the FcεRI level and IgE binding activity decreased significantly. In addition, the mRNA activity of FcεRI-α and γ-chains was also downregulated, and the release of histamine was restricted (Shim et al., 2009). *In vivo*, oral *H. cordata* extract can effectively alleviate passive cutaneous anaphylaxis in mice. FcεRI signalling molecules activated by antigens such as Syk, Lyn, LAT, Gab2, and PLCγ2 and downstream Akt and MAP kinases were also inhibited, but the level of cAMP in mast cells increased, which reveals that *H. cordata* can improve allergic diseases by inhibiting the FcεRI-dependent signal transmission of mast cells (Li et al., 2005; Han et al., 2009). HCP-2, a polysaccharide isolated from *H. cordata*, regulated the expression of T cells in human peripheral blood mononuclear cells at concentrations of 0.1–25 μg/ml, and the levels of the immune molecules interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), and macrophage inhibitory protein-1α and -1β increased significantly; accordingly, the body's immunity was effectively enhanced (Cheng et al., 2014). Moreover, researchers determined the therapeutic effect of *H. cordata* extract on Th2-mediated immune diseases. Ethanol extract not only inhibited the production of TARC in skin fibroblast CCD-986sk cells but also downregulated the level of TARC receptor CC chemokine receptor 4 (CCR4) mRNA in Jurkat T cells, and the migration of T cells induced by TARC was also restricted (Lee et al., 2008). In addition, *H. cordata* extract regulates innate immune mediators. After vaginal epithelial cells were treated with the extract for 18 h, the mRNA levels of human β-defensin 2 and secretory leukocyte protease inhibitor increased remarkably, IL-2 and IL-6 protein secretion increased, and CCL5 secretion decreased (Satthakarn et al., 2015b). Interestingly, the extract has similar effects on oral immune

mediators, and the expression of human β-defensin 2, secretory leukocyte protease inhibitor, IL-8 and CCL20 is regulated by the extract in a dose-dependent manner (Satthakarn et al., 2015a). The above results showed that vaginal and oral immune mediators are upregulated by *H. cordata* extract, suggesting the potential of *H. cordata* to prevent and treat oral diseases in diet.

Antiviral Activity

Anti-Herpes Virus Activity

H. cordata displays obvious activity in inhibiting herpesvirus, its solution extracted with hot water effectively attenuated herpes simplex virus (HSV) infection, which might be associated with the inhibition of the NF-κB pathway; however, the activity of another key pathway, Erk MAPK, was not regulated. Moreover, determination of the anti-infective activity of the powder after lyophilization of the extract revealed the IC_{50} was achieved at a dose of 50 μg/ml, and after the concentration reached 150–450 μg/ml, the inhibitory effect of the extract on HSV-2 exceeded 3 logs (Chen et al., 2011). In addition to inhibiting NF-κB and restricting viral gene expression, the binding and penetration ability of the HSV-1, HSV-2, and acyclovir-resistant HSV-1 viruses at the initial stage of infection is also weakened by the extract, and the replication process of HSV is attenuated. Further research showed that the inhibitory activity of NF-κB involves contributions of the components quercetin and isoquercitrin, and quercetin suppressed the invasion ability of the virus (Hung et al., 2015). Moreover, a monkey kidney cell line (Vero cells) and swine testis cells (ST) were used to investigate the inhibitory activity of *H. cordata* in pseudorabies herpesvirus (PrV). In the Vero cell model, the infection rate was reduced by 70% after treatment with the *H. cordata* extract at a concentration of 2 mg/ml, while infectivity of the virus was completely suppressed at a concentration of 250 mg/ml. In contrast, the same concentration of extract exerted lower infection inhibitory activity on ST cells than Vero cells; nevertheless, single use of high-dose *H. cordata* extract showed apoptosis-inducing activity (Ren et al., 2011).

Furthermore, the new flavonoids houttuynoids A-E and houttuynoids G-J were isolated from the whole *H. cordata* plant, and both groups have been shown to possess anti-herpes virus activity. Through Vero cell experiments, houttuynoid A and houttuynoids G-J inhibited HSV-1 infection. The IC_{50} values of houttuynoids G-J were 38.46, 14.10, 62.00, and 70.76 μM, while houttuynoid A showed a lower value at 33.5 μM in the β-galactosidase activity assay. Moreover, the activity of herpes simplex virus type 2 and varicella-zoster virus was suppressed by houttuynoid A. Plaque reduction experiments and luciferase activity assays proved this effect (Chen et al., 2012; Chen S. D. et al., 2013; Li T. et al., 2017). Moreover, houttuynoid M demonstrated similar activity to houttuynoid A, and plaque formation experiments revealed that the IC_{50} value of houttuynoid M at 17.72 μM could suppress the activity of HSV-1 (Li JJ. et al., 2017).

Anti-Influenza Virus Activity

Human influenza viruses are divided into three types, A, B, and C, and the influenza A virus is the pathogen that causes the body to catch a cold (Luo, 2012). In the determination of neuraminidase activity, *H. cordata* showed effective anti-influenza virus activity and completely inhibited viral neuraminidase at a concentration of 250 mg/ml (HAN et al., 2016). Moreover, *in vivo* and *in vitro* experiments revealed the activity of *H. cordata* flavonoids against influenza virus H1N1, the survival rate and life span of mice infected with H1N1 were significantly improved through the combined action of rutin, hyperoside, isoquercitrin, and quercitrin in the extract, 50–200 μg/ml extract effectively reduced the H1N1 virus titre in the lung tissue, and neuraminidase activity was inhibited both in *in vivo* and *in vitro* experiments (Ling et al., 2020). Moreover, quercetin-3-rhamnoside (Q3R) obtained from *H. cordata* attenuated the replication of influenza A/WS/33 virus, which is associated with the indirect effect of Q3R on virus particles. Through the cytopathic effect, it was observed that Q3R significantly reduced the production of cytopathic changes. Compared with oseltamivir, Q3R showed stronger anti-A/WS/33 virus activity (Choi et al., 2009). The above findings suggested that flavonoids might be the main components acting against different influenza viruses.

Anti-Coronavirus Activity

Coronavirus is a type of virus widely distributed in nature, and it selectively infects humans and other vertebrates. At present, seven kinds of coronaviruses are known to be infectious to humans. Among them, the newly discovered severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is circulating worldwide and has caused millions of deaths (Adhikari et al., 2020). Main protease (Mpro), papain-like protease (PLpro) and ADP ribose phosphatase (ADRP) are the three main replication proteins of SARS-CoV-2, and molecular docking by using Epic, LigPrep and Glide module of Schrödinger suite 2020–3 have shown that the metabolite (ligand) 6-hydroxyondansetron possesses binding affinity towards the receptors Mpro (PDB ID 6LU7) and PLpro (PDB ID 7JRN) with the best Glide scores (G-score) of −7.274 and −5.672, while quercitrin also showed binding affinity towards ADRP (PDB ID 6 W02) with a G-score −6.788. Furthermore, these compounds showed potential inhibition of Mpro and PLpro of SARS-CoV-2 without causing toxicity, although quercitrin showed fewer drug-like properties but demonstrated potential as an inhibitor for ADRP, and the results indicated the potential therapeutic activity of *H. cordata* (Das et al., 2021); however, the reports seems idealized, and more research is needed.

In research against severe acute respiratory syndrome coronavirus (SARS-CoV), enzymes and immune regulation play important roles. Aqueous extracts of *H. cordata* at 0–400 μg/ml effectively promoted the proliferation of mouse spleen lymphocytes in a dose-dependent manner, and increased expression of IL-2 and IL-10 in splenic lymphocytes was observed. The ratio of $CD4^+$ and $CD8^+$ T cells was also upregulated to enhance the body's immunity. Moreover, the activity of 3C-like protease and RNA polymerase is critical for virus replication and was significantly inhibited in a dose-dependent manner at 0–1,000 μg/ml (Lau et al., 2008).

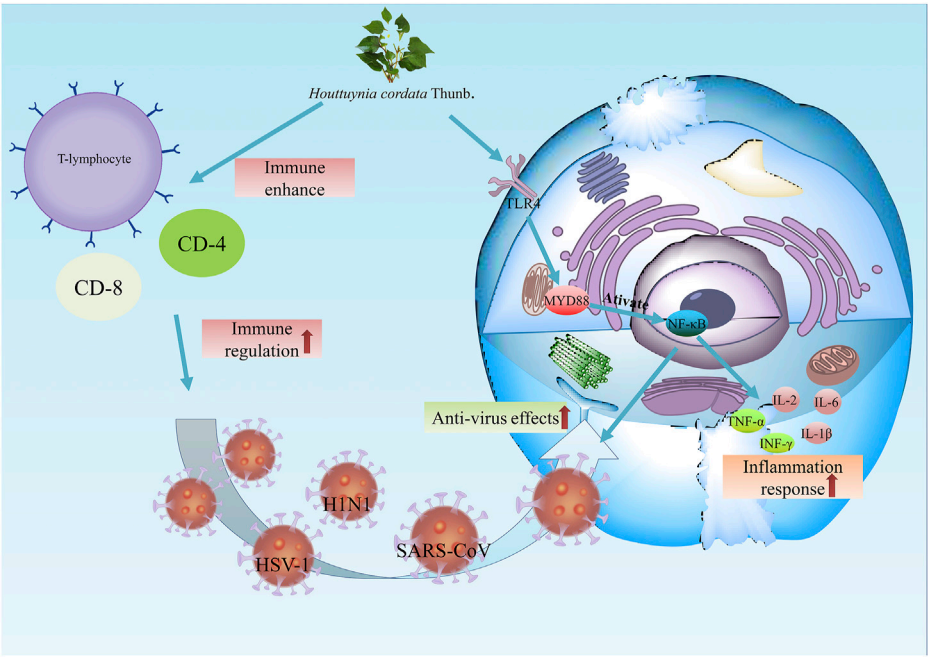


FIGURE 5 | The antiviral effects of *H. cordata*. On the one hand, *H. cordata* enhances immune activity by activating T cells, $CD4^+$ cells and $CD8^+$. On the other hand, *H. cordata* combats viruses such as H1N1, HSV-1 and SARS-CoV by upregulating TLR4/NF-κB signalling.

Researchers used mouse hepatitis virus (MHV) as a coronavirus model to determine the anti-infective activity of the ethyl acetate extract of *H. cordata*, and treatment with the extract solution significantly inhibited the activity of MHV at the stage of virus infection (IC_{50} = 0.98 mg/ml), while a high dose of 2000 mg/kg did not show acute cytotoxicity (Chiow et al., 2016); however, human tolerance has not been effectively investigated.

Moreover, *H. cordata* exerts inhibitory activity against the avian infectious bronchitis virus, similar to coronavirus. Through the detection of plaque reduction and reverse transcription-polymerase chain reaction, 90% of viral infections were inhibited in Vero cells and chicken embryo kidney cells, and more than half of viral invasion was inhibited (Yin et al., 2011) (**Figure 5**).

Antibacterial Activity

Anti-*Staphylococcus aureus* Activity

Staphylococcus aureus, a classical Gram-positive bacterium, usually parasitizes human and animal skin, the nasal cavity, gastrointestinal tract and other parts and is a common food-borne pathogenic microorganism (Lowy, 1998). Sodium houttuyfonate and EDTA-Na_2 synergistically showed inhibitory activity against MRSA. Mice that were not treated with sodium houttuyfonate in combination with EDTA-Na_2 all died 12 days after being infected with MRSA. In contrast, the survival rate of mice in the experimental group treated with sodium houttuyfonate combined with EDTA-Na_2 was 75% after 28 days of MRSA infection, which was much higher than the 50 and 43.75% survival rates of mice treated with sodium houttuyfonate and EDTA-Na_2, respectively (Huang et al., 2015). Moreover, the modified sodium houttuyfonate combined with other antibacterial drugs showed excellent antibacterial effects. In studying the synergistic use of sodium houttuyfonate with oxacillin, cephalosporin, meropenem, and netilmicin, the median fractional inhibitory concentrations monitored by the checkerboard method were all between 0.25 and 0.38. However, time-kill experiments showed that using half the MIC of sodium new houttuyfonate combined with oxacillin and netilmicin, which resulted in MICs that were lower than normal, could significantly decrease the number of viable colonies (Lu et al., 2013). Furthermore, with the long-term use of penicillin and methicillin to produce methicillin-resistant *Staphylococcus* aureus (MRSA), extraction of fresh *H. cordata* leaves with ethanol resulted in higher inhibitory activity than that of aqueous extracts and decoctions of *H. cordata*. By comparing the minimum inhibitory concentration (MIC) of extract on methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA, the concentration range of both was 110 μg/ml~1760 μg/ml, and MSSA and MRSA with MICs below 440 μg/ml accounted for 70%. The inhibition mechanism may be related to preventing the formation of bacterial biofilms (Sekita et al., 2016).

Anti-*Pseudomonas aeruginosa* Activity

Pseudomonas aeruginosa (*PA*) is a common Gram-negative bacterium that can easily cause infection in injured parts of the body and lead to aggravation of disease (Mielko et al., 2019). Sodium houttuyfonate has been shown to have anti-*PA* activity through reverse transcription-quantitative polymerase chain reaction. It was detected that the biosynthesis of alginate, an important substance for *PA* biofilm formation,

was inhibited, which is related to the downregulation of the expression of synthesis-related *algD* and *algR* genes by sodium houttuyfonate. At the same time, after treatment, scanning electron microscopy observations revealed that the morphology of the bacteria changed, and the content of alginate in the bacterial biofilm was also reduced (Wu et al., 2015). Moreover, sodium houttuyfonate and EDTA-Na_2 synergistically enhanced the anti-*PA* activity. After mice were treated with sodium houttuyfonate and EDTA-Na_2 separately for 28 consecutive days, the mortality rate of the mice was 75 and 81.25%, but after 28 days of combined treatment, the case fatality rate was only 43.75% (Huang et al., 2015). Interestingly, Wu et al. proved that sodium houttuyfonate inhibited PA activity through quorum sensing (QS), which is a method of information exchange between bacteria, using transmitted signal molecules to control the population size. *N*-Acyl homoserine lactone (AHL) is a signalling molecule in the *PA* population. In a previous study, sodium houttuyfonate effectively inhibited the synthetic lasl gene of AHL and reduced the level of expression, and the AHL receptor and the transcriptional regulator LasR were also inhibited, thereby downregulating the expression of the virulence factors pyocyanin and LasA; therefore, the *PA* population size can be effectively controlled through AHL mediation (Wu et al., 2014). Moreover, in a treatment evaluation of sodium houttuyfonate, the expression of the *rhll* and *pqsA* genes, which play key roles in the QS system, was significantly reduced and interfered with the production of pyocyanin; additionally, biofilm formation was monitored, and with the exception of *lasA*, the expression of the *LasB*, *LecA*, *phzM, pqsA,* and *pilG* genes was affected, which further inhibited the activation of *PA* virulence factors and biofilm formation (Zhao et al., 2020). These research results provide new insights for anti-*PA* activity.

TOXICITY

As an edible plant, the potential toxicity of *H. cordata* is usually ignored; however, recently, some studies reported that liver cancer is associated with aristolochic acid and aristolactams (Ng et al., 2017), and investigation of the toxicity mechanism of aristolochic acid in microphysiological systems showed that the specific metabolism of aristolochic acid in hepatocytes increased the cytotoxicity of the proximal tubule epithelial cells of the kidney (Chang et al., 2017); which caused people to be concerned about the safety of this plant due to it contains some aristolactam components (Ahn et al., 2017). However, aristolochic acid II is highly toxic *in vivo* due to its mutagenicity but is slightly toxic in *in vitro* cell models and toxicological studies in cell experiments can hardly reflect the true metabolism in the body (Michl et al., 2014). Furthermore, experiments *in vivo* with 95% ethanol extract of *H. cordata* directly demonstrated its potential toxicity, and following oral administration of a single dosage of 2000 mg/kg, no pathological reaction was observed in the rats during 14 days; however, during 28 consecutive days of oral administration of 500–1,000 mg/kg/d, a small number of rats died after 15 days, and histopathological analysis of organ slices showed that vacuum degeneration and inflammatory cell infiltration in liver tissue was present in the 1,000 mg/kg group, compared with the oral administration of ionized water, and a high dose in the kidney caused focal necrosis of renal epithelial cells, although no pathological signs in other organs were observed (Chen H. et al., 2021).

Although the above studies have shown the weak potential toxicity of *H. cordata*, there are no reports about its toxicity in a long-term consumption as vegetable and alone medicinal use in some areas of China and India, and it was listed as one of the plants that can be used as both food and medicine by the National Health Commission of China in 2013, indicating that *H. cordata* is relatively safe for oral administration in humans. Therefore, more sufficient and reliable data is needed to reveal its potential toxicity.

DISCUSSION AND CONCLUSION

H. cordata is a medicinal plant with diverse biological activities. In the studies of organ protective activity, antioxidant stress and inflammation are important properties determining its therapeutic potential (Shingnaisui et al., 2018), inflammation and oxidative stress of the lung, heart, kidney and liver are alleviated. However, aristolochia derivatives in *H. cordata* seem to show nephrotoxicity, which is not consistent with its protective activity, different dosages may be one reason. It is also suggesting that the research on the nephrotoxic components and mechanisms of *H. cordata* is insufficient. Similarly, in the studies of alleviating liver injury and anti-liver tumour activity, the active components of *H. cordata* are not clear, and the research on the metabolism of active components seems to be beneficial for *H. cordata* to exert its hepatoprotective activity in diet. Overall, according to the reported literature, *H. cordata* appears to demonstrate selectivity for lung tissue, alleviating the processes of pneumonia (Lee et al., 2015), lung injury (Shi et al., 2020), pulmonary fibrosis (Ng et al., 2007) and lung tumour (Lou et al., 2019), which might be supported by the theory of traditional Chinese medicine. In comparison, *H. cordata* shows a shortage in the treatment of digestive and cardiovascular diseases. In studies of LPS- and virus-induced inflammation *in vivo* and *in vitro*, water extract and ethanol extract of *H. cordata* and separated flavonoids, volatile oil, sodium houttuyfonate and polysaccharide components all showed effective inhibition of the release of inflammatory factors, which might play an important role in alleviating inflammation, acute lung injury, heart remodelling and other pathological changes in tissue. Moreover, the NF-κB and TGB-β1/Smad signalling pathways are involved in, and whether *H. cordata* regulates the inflammatory response through other signalling pathways, only a few studies have been performed. In antitumour research, inducing apoptosis and cell cycle arrest are important characteristics of *H. cordata* against lung tumour, liver tumour, gastric carcinoma, colon tumour, and breast tumour. However, we found that components of the extracts used in the studies are not clear, and it also have not been fully characterized, which is a common lack in the antitumour activity and even the whole pharmacological studies of *H. cordata*. The experiments of pharmacological research should be carefully designed, strictly carried out, detailed records, and appropriate models and accurate determination methods are necessary

TABLE 1 | Important chemical compositions of *Houttuynia cordata*.

Species	Serial number	Ingredients	Molecular formula	Molecular weight	References
Volatile oils	(a)	Houttuynin	C12H22O2	198.30	Chen et al. (2014)
	(b)	Bornyl acetate	C12H20O2	196.29	Lu et al. (2006)
	(c)	β-Myrcene	C10H16	136.23	Lu et al. (2006)
	(d)	Ethyl caprate	C12H24O2	200.32	Verma et al. (2017)
	(e)	Ethyl dodecanoate	C14H28O2	228.38	Verma et al. (2017)
Flavonoids	(f)	2-Undecanone	C11H22O	170.30	Verma et al. (2017)
	(g)	2-Tridecanone	C13H26O	198.35	Verma et al. (2017)
	(h)	Rutinum	C27H30O16	610.52	Xu et al. (2006)
	(i)	Hyperoside	C21H20O12	464.38	Xu et al. (2006)
	(j)	Quercitrin	C21H20O11	448.38	Lu et al. (2011)
	(k)	Quercetin	C15H10O7	302.24	WU et al. (2009)
	(l)	Houttynoid A	C33H38O13	665.22	Chen et al. (2012); Chen S. D. et al. (2013)
	(m)	Houttynoid B	C32H38O12	637.23	Chen et al. (2012); Chen S. D. et al. (2013)
	(n)	Houttynoid C	C33H38O13	665.22	Chen et al. (2012); Chen S. D. et al. (2013)
	(o)	Houttynoid D	C32H40O13	655.24	Chen et al. (2012); Chen S. D. et al. (2013)
	(p)	Houttynoid E	C32H40O13	655.24	Chen et al. (2012); Chen S. D. et al. (2013)
Phenolic acids	(q)	Linolenic acid	C18H32O2	1,280.44	Bauer et al. (1996)
	(r)	Linoleic acid	C18H32O2	280.44	Bauer et al. (1996)
	(s)	Oleic acid	C18H34O2	282.46	Bauer et al. (1996)
	(t)	Palmitic acid	C16H32O2	256.42	Bauer et al. (1996)
	(u)	Stearic acid	C18H36O2	284.48	Bauer et al. (1996)
	(v)	Neochlorogenic acid	C16H18O9	354.31	Nuengchamnong et al. (2009)
	(w)	Chlorogenic acid	C16H18O9	354.31	Nuengchamnong et al. (2009)
	(x)	4-Dicaffeoylquinic Acid	C16H18O9	354.31	Nuengchamnong et al. (2009)
Alkaloids	(y)	Aristololactam	C17H11NO4	293.27	Ma et al. (2017)
	(z)	Houttuynoside A	C21H22O11	450.40	Chou et al. (2009)
	(aa)	Houttuynamide A	C15H15NO2	273.10	Chou et al. (2009)

(Heinrich et al., 2020). Therefore, we suggest that the pharmacological experiments *in vivo* and *in vitro* of *H. cordata* should follow the qualitative standards, and more research in disease prevention and treatment in diet is needed.

H. cordata also shows potential in combination with other drugs. As a traditional basic antiviral and antibacterial Chinese medicine, research on *H. cordata* eye drops combined with olopatadine hydrochloride in the treatment of vernal keratoconjunctivitis revealed the synergistic effect of this combination (Xu and Cai, 2019). Additionally, a clinical trial involving *mangosteen*, *Lithospermum officinale*, *Tribulus terrestris* L., and *H. cordata* extracts in the treatment of mild to moderate acne showed that inflammation and noninflammatory skin lesion counts were significantly reduced (Yang et al., 2021). More importantly, the synergistic use of *H. cordata* shows effective activity in alleviating diabetes insulin resistance (Wang et al., 2017) and anticancer pain (Wan et al., 2016), anti-bacterial and auxiliary cell transplantation, which may be an important aspect of the research on the therapeutic activity of *H. cordata* in the future. It is noteworthy that the adverse event evaluation of *H. cordata* injection showed that anaphylactic shock caused in the treatment, and synergistic use of penicillins, cephalosporins and macrolides increases the risk of allergic reactions (Wang et al., 2010). Due to the various phytochemical components of *H. cordata*, its pharmacological activity seems too optimistic, and its potential risks should be carefully studied. A preparation technology that used macroporous resin to extract the essential oil of *H. cordata* and then embedded the microemulsion to improve its biological activity and safety suggests the direction of the future development of *H. cordata* and its derivative compounds as agents (Pang et al., 2017), and the combination of *H. cordata* and a drug delivery system is expected to further enhance its potential in treatment.

Furthermore, *H. cordata* showed effective mitigating activity in various diseases, but it cannot be ignored of its liver toxicity and nephrotoxicity, which seem to appear at a high dose; therefore, research based on toxicology and pharmacology still needs to be strengthened to promote its role as an agent in the treatment of diseases. In addition, the pharmacological studies of the compound *in vitro* and even *in vivo* are not evidence that it can be converted into a drug to play a therapeutic role. Structural modification based on effective natural ingredients is expected to help reduce toxicity and enhance therapeutic activity, such as the β-elemene component in the rhizome of Wenyujin has been approved by the China Food and Drug Administration (CFDA) to treat a variety of cancers (Bai et al., 2021). Therefore, in research on the bioactive components of *H. cordata*, structural modification may be an aspect that needs to be paid attention to. Overall, studying the components and its pharmacological, toxicological activity of *H. cordata*, and to provide more data to eliminate potential risks; and also reasonable synergistic use and structural modification of compounds are still important topics for future research.

AUTHOR CONTRIBUTIONS

ZW is the major contributor to this manuscript. ZW conducted the analytical part, wrote the first version of the manuscript, and Deng et al. finalized the manuscript. ZW downloaded the

reference and processed the graph and the table in the manuscript. QH, XX, and JJ collected the data. XM and MW conceived and coordinated the study, and critically evaluated the data. All authors read and approved the final manuscript.

FUNDING

This work was supported by National Natural Science Foundation of China (81874365, 81703725), Sichuan Science and Technology Program (2019YJ0492), Beijing Medical and Health Foundation (YWJKJJHKYJJ-B20645FN), Chengdu University of TCM Grant (QNXZ2018025).

ACKNOWLEDGMENTS

The authors would like to thank the reviewers and also the authors of all references. The reviewer's advice greatly improved this paper.

REFERENCES

Adhikari, B., Marasini, B. P., Rayamajhee, B., Bhattarai, B. R., Lamichhane, G., Khadayat, K., et al. (2020). Potential Roles of Medicinal Plants for the Treatment of Viral Diseases Focusing on COVID-19: A Review. *Phytother Res.* 35, 1298–1312. doi:10.1002/ptr.6893

Ahn, J., Chae, H. S., Chin, Y. W., and Kim, J. (2017). Alkaloids from Aerial Parts of Houttuynia Cordata and Their Anti-inflammatory Activity. *Bioorg. Med. Chem. Lett.* 27, 2807–2811. doi:10.1016/j.bmcl.2017.04.072

Bai, Z., Yao, C., Zhu, J., Xie, Y., Ye, X. Y., Bai, R., et al. (2021). Anti-Tumor Drug Discovery Based on Natural Product β-Elemene: Anti-tumor Mechanisms and Structural Modification. *Molecules* 26, 1499. doi:10.3390/molecules26061499

Bauer, R., Pröbstle, A., Lotter, H., Wagner-Redecker, W., and Matthiesen, U. (1996). Cyclooxygenase Inhibitory Constituents from Houttuynia Cordata. *Phytomedicine* 2, 305–308. doi:10.1016/s0944-7113(96)80073-0

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* 68, 394–424. doi:10.3322/caac.21492

Cai, D. S., Zhou, H., Liu, W. W., and Pei, L. (2013). Protective Effects of Bone Marrow-Derived Endothelial Progenitor Cells and Houttuynia Cordata in Lipopolysaccharide-Induced Acute Lung Injury in Rats. *Cell. Physiol. Biochem.* 32, 1577–1586. doi:10.1159/000356594

Chang, S. Y., Weber, E. J., Sidorenko, V. S., Chapron, A., Yeung, C. K., Gao, C., et al. (2017). Human Liver-Kidney Model Elucidates the Mechanisms of Aristolochic Acid Nephrotoxicity. *JCI insight* 2, 1–14. doi:10.1172/jci.insight.95978

Chen, X., Wang, Z., Yang, Z., Wang, J., Xu, Y., Tan, R. X., et al. (2011). Houttuynia Cordata Blocks HSV Infection through Inhibition of NF-Kb Activation. *Antivir. Res* 92, 341–345. doi:10.1016/j.antiviral.2011.09.005

Chen, S. D., Gao, H., Zhu, Q. C., Wang, Y. Q., Li, T., Mu, Z. Q., et al. (2012). Houttuynoids A-E, Anti-herpes Simplex Virus Active Flavonoids with Novel Skeletons from Houttuynia Cordata. *Org. Lett.* 14, 1772–1775. doi:10.1021/ol300017m

Chen, J., Wang, W., Shi, C., and Fang, J. (2014). A Comparative Study of Sodium Houttuyfonate and 2-undecanone for Their *In Vitro* and *In Vivo* Anti-inflammatory Activities and Stabilities. *Int. J. Mol. Sci.* 15, 22978–22994. doi:10.3390/ijms151222978

Chen, M. Y., Li, H., Lu, X. X., Ling, L. J., Weng, H. B., Sun, W., et al. (2019). Houttuynia Cordata Polysaccharide Alleviated Intestinal Injury and Modulated Intestinal Microbiota in H1N1 Virus Infected Mice. *Chin. J. Nat. Med.* 17, 187–197. doi:10.1016/S1875-5364(19)30021-4

Chen, H., Sha, X., Luo, Y., Chen, J., Li, X., Wang, J., et al. (2021). Acute and Subacute Toxicity Evaluation of Houttuynia Cordata Ethanol Extract and Plasma Metabolic Profiling Analysis in Both Male and Female Rats. *J. Appl. Toxicol.*, 1–15. doi:10.1002/jat.4198

Chen, S. D., Li, T., Gao, H., Zhu, Q. C., Lu, C. J., Wu, H. L., et al. (2013). Anti HSV-1 Flavonoid Derivatives Tethered with Houttuynin from Houttuynia Cordata. *Planta Med.* 79, 1742–1748. doi:10.1055/s-0033-1351051

Chen, X., Yue, W., Tian, L., Li, N., Chen, Y., Zhang, L., et al. (2021). A Plant-Based Medicinal Food Inhibits the Growth of Human Gastric Carcinoma by Reversing Epithelial-Mesenchymal Transition via the Canonical Wnt/β-Catenin Signaling Pathway. *BMC Complement. Med. Ther.* 21, 1–16. doi:10.1186/s12906-021-03301-6

Chen, Y. F., Yang, J. S., Chang, W. S., Tsai, S. C., Peng, S. F., and Zhou, Y. R. (2013). Houttuynia Cordata Thunb Extract Modulates G0/G1 Arrest and Fas/CD95-Mediated Death Receptor Apoptotic Cell Death in Human Lung Cancer A549 Cells. *J. Biomed. Sci.* 20, 18–8. doi:10.1186/1423-0127-20-18

Cheng, B. H., Chan, J. Y., Chan, B. C., Lin, H. Q., Han, X. Q., Zhou, X., et al. (2014). Structural Characterization and Immunomodulatory Effect of a Polysaccharide HCP-2 from Houttuynia Cordata. *Carbohydr. Polym.* 103, 244–249. doi:10.1016/j.carbpol.2013.12.048

Chiow, K. H., Phoon, M. C., Putti, T., Tan, B. K., and Chow, V. T. (2016). Evaluation of Antiviral Activities of Houttuynia Cordata Thunb. Extract, Quercetin, Quercetrin and Cinanserin on Murine Coronavirus and Dengue Virus Infection. *Asian Pac. J. Trop. Med.* 9, 1–7. doi:10.1016/j.apjtm.2015.12.002

Choi, H. J., Song, J. H., Park, K. S., and Kwon, D. H. (2009). Inhibitory Effects of Quercetin 3-rhamnoside on Influenza A Virus Replication. *Eur. J. Pharm. Sci.* 37, 329–333. doi:10.1016/j.ejps.2009.03.002

Chou, S. C., Su, C. R., Ku, Y. C., and Wu, T. S. (2009). The Constituents and Their Bioactivities of Houttuynia Cordata. *Chem. Pharm. Bull. (Tokyo)* 57, 1227–1230. doi:10.1248/cpb.57.1227

Das, S. K., Mahanta, S., Tanti, B., Tag, H., and Hui, P. K. (2021). Identification of Phytocompounds from Houttuynia Cordata Thunb. As Potential Inhibitors for SARS-CoV-2 Replication Proteins through GC-MS/LC-MS Characterization, Molecular Docking and Molecular Dynamics Simulation. *Mol. Divers.*, 1–24. doi:10.1007/s11030-021-10226-2

Dawood, S., Broglio, K., Buzdar, A. U., Hortobagyi, G. N., and Giordano, S. H. (2010). Prognosis of Women with Metastatic Breast Cancer by HER2 Status and Trastuzumab Treatment: An Institutional-Based Review. *J. Clin. Oncol.* 28, 92–98. doi:10.1200/JCO.2008.19.9844

de la Cruz-Merino, L., Chiesa, M., Caballero, R., Rojo, F., Palazón, N., Carrasco, F. H., et al. (2017). Breast Cancer Immunology and Immunotherapy: Current Status and Future Perspectives. *Int. Rev. Cel Mol. Biol.* 331, 1–53. doi:10.1016/bs.ircmb.2016.09.008

Drasar, P. B., and Khripach, V. A. (2020). Growing Importance of Natural Products Research. *Molecules* 25, 14–15. doi:10.3390/molecules25010006

Du, S., Li, H., Cui, Y., Yang, L., Wu, J., Huang, H., et al. (2012). Houttuynia Cordata Inhibits Lipopolysaccharide-Induced Rapid Pulmonary Fibrosis by Up-Regulating IFN-γ and Inhibiting the TGF-β1/Smad Pathway. *Int. Immunopharmacol.* 13, 331–340. doi:10.1016/j.intimp.2012.03.011

Fu, R., Li, J., Yu, H., Zhang, Y., Xu, Z., and Martin, C. (2021). The Yin and Yang of Traditional Chinese and Western Medicine. *Med. Res. Rev*, 1–19. doi:10.1002/med.21793

Gao, J. P., Chen, C. X., Wang, Y., Lü, J., and Gu, W. L. (2009). Effect of Sodium Houttuyfonate on Myocardial Hypertrophy in Mice and Rats. *J. Pharm. Pharmacol.* 61, 677–683. doi:10.1211/jpp/61.05.0018

Gao, J. P., Chen, C. X., Wu, Q., Gu, W. L., and Li, X. (2010). Effect of Sodium Houttuyfonate on Inhibiting Ventricular Remodeling Induced by Abdominal Aortic Banding in Rats. *Can. J. Physiol. Pharmacol.* 88, 693–701. doi:10.1139/Y10-049

Gong, L., Pan, Q., and Yang, N. (2020). Autophagy and Inflammation Regulation in Acute Kidney Injury. *Front. Physiol.* 11, 576463. doi:10.3389/fphys.2020.576463

Han, E. H., Park, J. H., Kim, J. Y., and Jeong, H. G. (2009). Houttuynia Cordata Water Extract Suppresses Anaphylactic Reaction and IgE-Mediated Allergic Response by Inhibiting Multiple Steps of FcepsilonRI Signaling

in Mast Cells. *Food Chem. Toxicol.* 47, 1659–1666. doi:10.1016/j.fct.2009.04.025

Han, X., Zhang, D. K., Guo, Y. M., Feng, W. W., Dong, Q., Zhang, C. E., et al. (2016). Screening and Evaluation of Commonly-Used Anti-influenza Chinese Herbal Medicines Based on Anti-neuraminidase Activity. *Chin. J. Nat. Med.* 14, 794–800. doi:10.1016/S1875-5364(16)30095-4

Han, K., Jin, C., Chen, H., Wang, P., Yu, M., and Ding, K. (2018). Structural Characterization and Anti-a549 Lung Cancer Cells Bioactivity of a Polysaccharide from Houttuynia Cordata. *Int. J. Biol. Macromol.* 120, 288–296. doi:10.1016/j.ijbiomac.2018.08.061

Heinrich, M., Appendino, G., Efferth, T., Fürst, R., Izzo, A. A., Kayser, O., et al. (2020). Best Practice in Research - Overcoming Common Challenges in Phytopharmacological Research. *J. Ethnopharmacol.* 246, 112230. doi:10.1016/j.jep.2019.112230

Hsu, C. C., Yang, H. T., Ho, J. J., Yin, M. C., and Hsu, J. Y. (2016). Houttuynia Cordata Aqueous Extract Attenuated Glycative and Oxidative Stress in Heart and Kidney of Diabetic Mice. *Eur. J. Nutr.* 55, 845–854. doi:10.1007/s00394-015-0994-y

Huang, W., Duan, Q., Li, F., Shao, J., Cheng, H., and Wu, D. (2015). Sodium Houttuyfonate and EDTA-Na_2 in Combination Effectively Inhibits *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Candida Albicans *In Vitro* and *In Vivo*. *Bioorg. Med. Chem. Lett.* 25, 142–147. doi:10.1016/j.bmcl.2014.10.072

Hung, P. Y., Ho, B. C., Lee, S. Y., Chang, S. Y., Kao, C. L., Lee, S. S., et al. (2015). Houttuynia Cordata Targets the Beginning Stage of Herpes Simplex Virus Infection. *PLoS One* 10, e0115475–19. doi:10.1371/journal.pone.0115475

Jang, S. Y., Bae, J. S., Lee, Y. H., Oh, K. Y., Park, K. H., and Bae, Y. S. (2011). Caffeic Acid and Quercitrin Purified from Houttuynia Cordata Inhibit DNA Topoisomerase I Activity. *Nat. Prod. Res.* 25, 222–231. doi:10.1080/14786410903339044

Jiang, R., Hu, C., Li, Q., Cheng, Z., Gu, L., Li, H., et al. (2019). Sodium New Houttuyfonate Suppresses Metastasis in NSCLC Cells through the Linc00668/miR-147a/slug axis. *J. Exp. Clin. Cancer Res.* 38, 1–16. doi:10.1186/s13046-019-1152-9

Jones, G. S., B, D. R. B., Jones, G. S., and Baldwin, D. R. (2018). Recent Advances in the Management of Lung Cancer. *Clin. Med.* (Northfield, LI), 18, s41–s46.

Kang, H., and Koppula, S. (2014). Houttuynia Cordata Attenuates Lipid Accumulation via Activation of AMP-Activated Protein Kinase Signaling Pathway in HepG2 Cells. *Am. J. Chin. Med.* 42, 651–664. doi:10.1142/S0192415X14500426

Kim, I. S., Kim, J. H., Kim, J. S., Yun, C. Y., Kim, D. H., and Lee, J. S. (2007). The Inhibitory Effect of Houttuynia Cordata Extract on Stem Cell Factor-Induced HMC-1 Cell Migration. *J. Ethnopharmacol.* 112, 90–95. doi:10.1016/j.jep.2007.02.010

Kim, J. M., Hwang, I. H., Jang, I. S., Kim, M., Bang, I. S., Park, S. J., et al. (2017). Houttuynia Cordata Thunb Promotes Activation of HIF-1a-FOXO3 and MEF2A Pathways to Induce Apoptosis in Human HepG2 Hepatocellular Carcinoma Cells. *Integr. Cancer Ther.* 16, 360–372. doi:10.1177/1534735416670987

Kumar, M., Prasad, S. K., and Hemalatha, S. (2014). A Current Update on the Phytopharmacological Aspects of Houttuynia Cordata Thunb. *Pharmacogn. Rev.* 8, 22–35. doi:10.4103/0973-7847.125525

Lai, K. C., Chiu, Y. J., Tang, Y. J., Lin, K. L., Chiang, J. H., Jiang, Y. L., et al. (2010). Houttuynia Cordata Thunb Extract Inhibits Cell Growth and Induces Apoptosis in Human Primary Colorectal Cancer Cells. *Anticancer Res.* 30, 3549–3556.

Lau, K. M., Lee, K. M., Koon, C. M., Cheung, C. S., Lau, C. P., Ho, H. M., et al. (2008). Immunomodulatory and Anti-SARS Activities of Houttuynia Cordata. *J. Ethnopharmacol.* 118, 79–85. doi:10.1016/j.jep.2008.03.018

Lee, J. S., Kim, I. S., Kim, J. H., Kim, J. S., Kim, D. H., and Yun, C. Y. (2008). Suppressive Effects of Houttuynia Cordata Thunb (Saururaceae) Extract on Th2 Immune Response. *J. Ethnopharmacol.* 117, 34–40. doi:10.1016/j.jep.2008.01.013

Lee, H. J., Seo, H. S., Kim, G. J., Jeon, C. Y., Park, J. H., Jang, B. H., et al. (2013). Houttuynia Cordata Thunb Inhibits the Production of Pro-inflammatory Cytokines through Inhibition of the NFκB Signaling Pathway in HMC-1 Human Mast Cells. *Mol. Med. Rep.* 8, 731–736. doi:10.3892/mmr.2013.1585

Lee, J. H., Ahn, J., Kim, J. W., Lee, S. G., and Kim, H. P. (2015). Flavonoids from the Aerial Parts of Houttuynia Cordata Attenuate Lung Inflammation in Mice. *Arch. Pharm. Res.* 38, 1304–1311. doi:10.1007/s12272-015-0585-8

Li, J., and Zhao, F. (2015). Anti-inflammatory Functions of Houttuynia Cordata Thunb. And its Compounds: A Perspective on its Potential Role in Rheumatoid Arthritis. *Exp. Ther. Med.* 10, 3–6. doi:10.3892/etm.2015.2467

Li, G. Z., Chai, O. H., Lee, M. S., Han, E. H., Kim, H. T., and Song, C. H. (2005). Inhibitory Effects of Houttuynia Cordata Water Extracts on Anaphylactic Reaction and Mast Cell Activation. *Biol. Pharm. Bull.* 28, 1864–1868. doi:10.1248/bpb.28.1864

Li, W., Zhou, P., Zhang, Y., and He, L. (2011). Houttuynia Cordata, a Novel and Selective COX-2 Inhibitor with Anti-inflammatory Activity. *J. Ethnopharmacol.* 133, 922–927. doi:10.1016/j.jep.2010.10.048

Li, W., Fan, T., Zhang, Y., Fan, T., Zhou, P., Niu, X., et al. (2013). Houttuynia Cordata Thunb. Volatile Oil Exhibited Anti-inflammatory Effects *In Vivo* and Inhibited Nitric Oxide and Tumor Necrosis Factor-α Production in LPS-Stimulated Mouse Peritoneal Macrophages *In Vitro*. *Phytother Res.* 27, 1629–1639. doi:10.1002/ptr.4905

Li, J., Zhou, T., and Zhao, F. (2014). Inhibitory Effect of Sodium Houttuyfonate on Synovial Proliferation *In Vitro* in Cells from a Patient with Rheumatoid Arthritis. *Exp. Ther. Med.* 7, 1639–1642. doi:10.3892/etm.2014.1636

Li, J. J., Chen, G. D., Fan, H. X., Hu, D., Zhou, Z. Q., Lan, K. H., et al. (2017a). Houttuynoid M, an Anti-HSV Active Houttuynoid from Houttuynia Cordata Featuring a Bis-Houttuynin Chain Tethered to a Flavonoid Core. *J. Nat. Prod.* 80, 3010–3013. doi:10.1021/acs.jnatprod.7b00620

Li, T., Liu, L., Wu, H., Chen, S., Zhu, Q., Gao, H., et al. (2017b). Anti-herpes Simplex Virus Type 1 Activity of Houttuynoid A, a Flavonoid from Houttuynia Cordata Thunb. *Antivir. Res* 144, 273–280. doi:10.1016/j.antiviral.2017.06.010

Li, W., Yang, F., Zhan, H., Liu, B., Cai, J., Luo, Y., et al. (2020). Houttuynia Cordata Extract Ameliorates Bladder Damage and Improves Bladder Symptoms via Anti-inflammatory Effect in Rats with Interstitial Cystitis. *Evid. Based Complement. Altern. Med.* 2020, 9026901. doi:10.1155/2020/9026901

Ling, L. J., Lu, Y., Zhang, Y. Y., Zhu, H. Y., Tu, P., Li, H., et al. (2020). Flavonoids from Houttuynia Cordata Attenuate H1N1-Induced Acute Lung Injury in Mice via Inhibition of Influenza Virus and Toll-like Receptor Signalling. *Phytomedicine* 67, 153150. doi:10.1016/j.phymed.2019.153150

Liu, J., Zhu, X., Yang, D., Li, R., and Jiang, J. (2020). Effect of Heat Treatment on the Anticancer Activity of Houttuynia Cordata Thunb Aerial Stem Extract in Human Gastric Cancer SGC-7901 Cells. *Nutr. Cancer* 73, 1–9. doi:10.1080/01635581.2020.1737153

Lou, Y., Guo, Z., Zhu, Y., Kong, M., Zhang, R., Lu, L., et al. (2019). Houttuynia Cordata Thunb. And its Bioactive Compound 2-undecanone Significantly Suppress Benzo(a)pyrene-Induced Lung Tumorigenesis by Activating the Nrf2-HO-1/NQO-1 Signaling Pathway. *J. Exp. Clin. Cancer Res.* 38, 1–21. doi:10.1186/s13046-019-1255-3

Lowy, F. D. (1998). Staphylococcus aureusInfections. *N. Engl. J. Med.* 339, 520–532. doi:10.1056/nejm199808203390806

Lu, H., Wu, X., Liang, Y., and Zhang, J. (2006). Variation in Chemical Composition and Antibacterial Activities of Essential Oils from Two Species of Houttuynia THUNB. *Chem. Pharm. Bull. (Tokyo)* 54, 936–940. doi:10.1248/cpb.54.936

Lu, Y., Wang, X., Chen, D., and Chen, G. (2011). Polystyrene/graphene Composite Electrode Fabricated by *In Situ* Polymerization for Capillary Electrophoretic Determination of Bioactive Constituents in Herba Houttuyniae. *Electrophoresis* 32, 1906–1912. doi:10.1002/elps.201100162

Lu, X., Yang, X., Li, X., Lu, Y., Ren, Z., Zhao, L., et al. (2013). *In Vitro* Activity of Sodium New Houttuyfonate Alone and in Combination with Oxacillin or Netilmicin against Methicillin-Resistant *Staphylococcus aureus*. *PLoS One* 8, e68053. doi:10.1371/journal.pone.0068053

Lu, Y., Jiang, Y., Ling, L., Zhang, Y., Li, H., and Chen, D. (2018). Beneficial Effects of Houttuynia Cordata Polysaccharides on "Two-Hit" Acute Lung Injury and Endotoxic Fever in Rats Associated with Anti-complementary Activities. *Acta Pharm. Sin. B* 8, 218–227. doi:10.1016/j.apsb.2017.11.003

Lu, L., Li, W., Chen, L., Su, Q., Wang, Y., Guo, Z., et al. (2019). Radiation-induced Intestinal Damage: Latest Molecular and Clinical Developments. *Future Oncol.* 15, 4105–4118. doi:10.2217/fon-2019-0416

Luo, M. (2012). Influenza Virus Entry. *Adv. Exp. Med. Biol.* 726, 201–221. doi:10.1007/978-1-4614-0980-9_9

Ma, Q., Wei, R., Wang, Z., Liu, W., Sang, Z., Li, Y., et al. (2017). Bioactive Alkaloids from the Aerial Parts of Houttuynia Cordata. *J. Ethnopharmacol.* 195, 166–172. doi:10.1016/j.jep.2016.11.013

Ma, X., Jiang, Y., Wen, J., Liu, W., Zhao, Y., Zhao, J., et al. (2020a). A Comprehensive Review of Natural Products to Fight Liver Fibrosis: Alkaloids, Terpenoids, Glycosides, Coumarins and Other Compounds. *Eur. J. Pharmacol.* 5 (888), 173578. doi:10.1016/j.ejphar.2020.173578

Ma, X., Jiang, Y., Zhang, W., Wang, J., Wang, R., Wang, L., et al. (2020b). Natural Products for the Prevention and Treatment of Cholestasis: A Review. *Phytother Res.* 34 (6), 1291–1309. doi:10.1002/ptr.6621

Marshall, G. D. (2018). Challenges in Allergy Immunology Practice: Solutions Needed for Persistent Patient Problems. *Ann. Allergy Asthma Immunol.* 121, 647–648. doi:10.1016/j.anai.2018.10.018

Michl, J., Ingrouille, M. J., Simmonds, M. S., and Heinrich, M. (2014). Naturally Occurring Aristolochic Acid Analogues and Their Toxicities. *Nat. Prod. Rep.* 31, 676–693. doi:10.1039/c3np70114j

Mielko, K. A., Jabłoński, S. J., Milczewska, J., Sands, D., Łukaszewicz, M., and Młynarz, P. (2019). Metabolomic Studies of *Pseudomonas aeruginosa*. *World J. Microbiol. Biotechnol.* 35, 1–11. doi:10.1007/s11274-019-2739-1

Muluye, R. A., Bian, Y., and Alemu, P. N. (2014). Anti-inflammatory and Antimicrobial Effects of Heat-Clearing Chinese Herbs: A Current Review. *J. Tradit. Complement. Med.* 4, 93–98. doi:10.4103/2225-4110.126635

Ng, L. T., Yen, F. L., Liao, C. W., and Lin, C. C. (2007). Protective Effect of Houttuynia Cordata Extract on Bleomycin-Induced Pulmonary Fibrosis in Rats. *Am. J. Chin. Med.* 35, 465–475. doi:10.1142/S0192415X07004989

Ng, A. W. T., Poon, S. L., Huang, M. N., Lim, J. Q., Boot, A., Yu, W., et al. (2017). Aristolochic Acids and Their Derivatives Are Widely Implicated in Liver Cancers in Taiwan and throughout Asia. *Sci. Transl. Med.* 9, eaan6446. doi:10.1126/scitranslmed.aan6446

Nuengchamnong, N., Krittasilp, K., and Ingkaninan, K. (2009). Rapid Screening and Identification of Antioxidants in Aqueous Extracts of Houttuynia Cordata Using LC-ESI-MS Coupled with DPPH Assay. *Food Chem.* 117, 750–756. doi:10.1016/j.foodchem.2009.04.071

Pan, P., Wang, Y. J., Han, L., Liu, X., Zhao, M., and Yuan, Y. F. (2010). Effects of Sodium Houttuyfonate on Expression of NF-Kb and MCP-1 in Membranous Glomerulonephritis. *J. Ethnopharmacol.* 131, 203–209. doi:10.1016/j.jep.2010.06.020

Pang, J., Dong, W., Li, Y., Xia, X., Liu, Z., Hao, H., et al. (2017). Purification of Houttuynia Cordata Thunb. Essential Oil Using Macroporous Resin Followed by Microemulsion Encapsulation to Improve its Safety and Antiviral Activity. *Molecules* 22, 1–16. doi:10.3390/molecules22020293

Park, E., Kum, S., Wang, C., Park, S. Y., Kim, B. S., and Schuller-Levis, G. (2005). Anti-inflammatory Activity of Herbal Medicines: Inhibition of Nitric Oxide Production and Tumor Necrosis Factor-Alpha Secretion in an Activated Macrophage-like Cell Line. *Am. J. Chin. Med.* 33, 415–424. doi:10.1142/S0192415X05003028

Prôbstle, A., and Bauer, R. (1992). Aristolactams and a 4,5-dioxoaporphine Derivative from Houttuynia Cordata. *Planta Med.* 58, 568–569. doi:10.1055/s-2006-961554

Prommaban, A., Kodchakorn, K., Kongtawelert, P., and Banjerdpongchai, R. (2012). Houttuynia Cordata Thunb Fraction Induces Human Leukemic Molt-4 Cell Apoptosis through the Endoplasmic Reticulum Stress Pathway. *Asian Pac. J. Cancer Prev.* 13, 1977–1981. doi:10.7314/APJCP.2012.13.5.1977

Řebíčková, K., Bajer, T., Šilha, D., Houdková, M., Ventura, K., and Bajerová, P. (2020). Chemical Composition and Determination of the Antibacterial Activity of Essential Oils in Liquid and Vapor Phases Extracted from Two Different Southeast Asian Herbs-Houttuynia Cordata (Saururaceae) and Persicaria Odorata (Polygonaceae). *Molecules* 25, 1–11. doi:10.3390/molecules25102432

Ren, X., Sui, X., and Yin, J. (2011). The Effect of Houttuynia Cordata Injection on Pseudorabies Herpesvirus (PrV) Infection *In Vitro*. *Pharm. Biol.* 49, 161–166. doi:10.3109/13880209.2010.505242

Roe, K. (2021). An Inflammation Classification System Using Cytokine Parameters. *Scand. J. Immunol.* 93, 6–10. doi:10.1111/sji.12970

Satthakarn, S., Chung, W. O., Promsong, A., and Nittayananta, W. (2015a). Houttuynia Cordata Modulates Oral Innate Immune Mediators: Potential Role of Herbal Plant on Oral Health. *Oral Dis.* 21, 512–518. doi:10.1111/odi.12313

Satthakarn, S., Hladik, F., Promsong, A., and Nittayananta, W. (2015b). Vaginal Innate Immune Mediators Are Modulated by a Water Extract of Houttuynia Cordata Thunb. *BMC Complement. Altern. Med.* 15, 183–188. doi:10.1186/s12906-015-0701-9

Sattler, S. (2017). The Role of the Immune System beyond the Fight against Infection. *Adv. Exp. Med. Biol.* 1003, 3–14. doi:10.1007/978-3-319-57613-8_1

Sekita, Y., Murakami, K., Yumoto, H., Mizuguchi, H., Amoh, T., Ogino, S., et al. (2016). Anti-bacterial and Anti-inflammatory Effects of Ethanol Extract from Houttuynia Cordata Poultice. *Biosci. Biotechnol. Biochem.* 80, 1205–1213. doi:10.1080/09168451.2016.1151339

Sekita, Y., Murakami, K., Yumoto, H., Hirao, K., Amoh, T., Fujiwara, N., et al. (2017). Antibiofilm and Anti-inflammatory Activities of Houttuynia Cordata Decoction for Oral Care. *Evid. Based Complement. Altern. Med.* 2017, 1–10. doi:10.1155/2017/2850947

Shen, C. Y., Jiang, J. G., Yang, L., Wang, D. W., and Zhu, W. (2017). Anti-ageing Active Ingredients from Herbs and Nutraceuticals Used in Traditional Chinese Medicine: Pharmacological Mechanisms and Implications for Drug Discovery. *Br. J. Pharmacol.* 174, 1395–1425. doi:10.1111/bph.13631

Shi, C. C., Zhu, H. Y., Li, H., Zeng, D. L., Shi, X. L., Zhang, Y. Y., et al. (2020). Regulating the Balance of Th17/Treg Cells in Gut-Lung axis Contributed to the Therapeutic Effect of Houttuynia Cordata Polysaccharides on H1N1-Induced Acute Lung Injury. *Int. J. Biol. Macromol.* 158, 52–66. doi:10.1016/j.ijbiomac.2020.04.211

Shilovskiy, I. P., Yumashev, K. V., Nikolsky, A. A., Vishnyakova, L. I., and Khaitov, M. R. (2021). Molecular and Cellular Mechanisms of Respiratory Syncytial Viral Infection: Using Murine Models to Understand Human Pathology. *Biochem. Mosc.* 86, 290–306. doi:10.1134/S0006297921030068

Shim, S. Y., Seo, Y. K., and Park, J. R. (2009). Down-regulation of FcepsilonRI Expression by Houttuynia Cordata Thunb Extract in Human Basophilic KU812F Cells. *J. Med. Food* 12, 383–388. doi:10.1089/jmf.2007.0684

Shin, S., Joo, S. S., Jeon, J. H., Park, D., Jang, M. J., Kim, T. O., et al. (2010). Anti-inflammatory Effects of a Houttuynia Cordata Supercritical Extract. *J. Vet. Sci.* 11, 273–275. doi:10.4142/jvs.2010.11.3.273

Shingnaisui, K., Dey, T., Manna, P., and Kalita, J. (2018). Therapeutic Potentials of Houttuynia Cordata Thunb. Against Inflammation and Oxidative Stress: A Review. *J. Ethnopharmacol.* 220, 35–43. doi:10.1016/j.jep.2018.03.038

Subhawa, S., Chewonarin, T., and Banjerdpongchai, R. (2020). The Effects of Houttuynia Cordata Thunb and Piper Ribesioides Wall Extracts on Breast Carcinoma Cell Proliferation, Migration, Invasion and Apoptosis. *Molecules* 25, 1196. doi:10.3390/molecules25051196

Tang, Y. J., Yang, J. S., Lin, C. F., Shyu, W. C., Tsuzuki, M., Lu, C. C., et al. (2009). Houttuynia Cordata Thunb Extract Induces Apoptosis through Mitochondrial-dependent Pathway in HT-29 Human colon Adenocarcinoma Cells. *Oncol. Rep.* 22, 1051–1056. doi:10.3892/or.10.3892/or_00000535

Tian, L., Shi, X., Yu, L., Zhu, J., Ma, R., and Yang, X. (2012). Chemical Composition and Hepatoprotective Effects of Polyphenol-Rich Extract from Houttuynia Cordata tea. *J. Agric. Food Chem.* 60, 4641–4648. doi:10.1021/jf3008376

Tu, X., Deng, Y., Chen, J., Hu, Q., He, C., Jordan, J. B., et al. (2016). Screening Study on the Anti-angiogenic Effects of Traditional Chinese Medicine - Part I: Heat-Clearing and Detoxicating TCM. *J. Ethnopharmacol.* 194, 280–287. doi:10.1016/j.jep.2016.09.010

Verma, R. S., Joshi, N., Padalia, R. C., Singh, V. R., Goswami, P., Kumar, A., et al. (2017). Chemical Composition and Allelopathic, Antibacterial, Antifungal, and Antiacetylcholinesterase Activity of Fish-Mint (Houttuynia cordataThunb.) from India. *Chem. Biodivers.* 14, e1700189. doi:10.1002/cbdv.201700189

Wan, C. F., Zheng, L. L., Liu, Y., and Yu, X. (2016). Houttuynia Cordata Thunb Reverses Oxaliplatin-Induced Neuropathic Pain in Rat by Regulating Th17/Treg Balance. *Am. J. Transl. Res.* 8, 1609–1614.

Wang, L., Cui, X., Cheng, L., Yuan, Q., Li, T., Li, Y., et al. (2010). Adverse Events to Houttuynia Injection: A Systematic Review. *J. Evid. Based. Med.* 3, 168–176. doi:10.1111/j.1756-5391.2010.01091.x

Wang, J. H., Bose, S., Lim, S. K., Ansari, A., Chin, Y. W., Choi, H. S., et al. (2017). Houttuynia Cordata Facilitates Metformin on Ameliorating Insulin Resistance Associated with Gut Microbiota Alteration in OLETF Rats. *Genes (Basel)* 8, 239. doi:10.3390/genes8100239

Woranam, K., Senawong, G., Utaiwat, S., Yunchalard, S., Sattayasai, J., and Senawong, T. (2020). Anti-inflammatory Activity of the Dietary Supplement Houttuynia Cordata Fermentation Product in RAW264.7 Cells and Wistar Rats. *PLoS One* 15, e0230645–18. doi:10.1371/journal.pone.0230645

Wu, L.-S., Si, J.-P., Yuan, X.-Q., and Shi, X.-R. (2009). Quantitive Variation of Flavonoids in Houttuynia Cordata from Different Geographic Origins in China. *Chin. J. Nat. Med.* 7, 40–46. doi:10.1016/s1875-5364(09)60042-x10.3724/sp.j.1009.2009.00040

Wu, D., Huang, W., Duan, Q., Li, F., and Cheng, H. (2014). Sodium Houttuyfonate Affects Production of N-Acyl Homoserine Lactone and Quorum Sensing-Regulated Genes Expression in *Pseudomonas aeruginosa*. *Front. Microbiol.* 5, 1–10. doi:10.3389/fmicb.2014.00635

Wu, D. Q., Cheng, H., Duan, Q., and Huang, W. (2015). Sodium Houttuyfonate Inhibits Biofilm Formation and Alginate Biosynthesis-Associated Gene Expression in a Clinical Strain of *Pseudomonas aeruginosa In Vitro*. *Exp. Ther. Med.* 10, 753–758. doi:10.3892/etm.2015.2562

Wu, Z., Tan, B., Zhang, H., Guo, Y., Tu, Y., Qiu, F., et al. (2017). Effects of Sodium Houttuyfonate on Pulmonary Inflammation in COPD Model Rats. *Inflammation* 40, 2109–2117. doi:10.1007/s10753-017-0650-1

Xu, X., and Cai, Y. (2019). Therapeutic Effects of Houttuynia Eye Drops Combined with Olopatadine Hydrochloride Eye Drops on vernal Keratoconjunctivitis. *Exp. Ther. Med.* 17, 1224–1227. doi:10.3892/etm.2018.7079

Xu, X., Ye, H., Wang, W., Yu, L., and Chen, G. (2006). Determination of Flavonoids in Houttuynia Cordata Thunb. And Saururus Chinensis (Lour.) Bail. by Capillary Electrophoresis with Electrochemical Detection. *Talanta* 68, 759–764. doi:10.1016/j.talanta.2005.05.027

Yang, J. H., Hwang, E. J., Moon, J., Yoon, J. Y., Kim, J. W., Choi, S., et al. (2021). Clinical Efficacy of Herbal Extracts in Treatment of Mild to Moderate Acne Vulgaris: an 8-week, Double-Blinded, Randomized, Controlled Trial. *J. Dermatolog. Treat.* 32, 297–301. doi:10.1080/09546634.2019.1657792

Yin, J., Li, G., Li, J., Yang, Q., and Ren, X. (2011). *In Vitro* and *In Vivo* Effects of Houttuynia Cordata on Infectious Bronchitis Virus. *Avian Pathol.* 40, 491–498. doi:10.1080/03079457.2011.605107

You, Y., Lee, H., Yoon, H. G., Park, J., Kim, O. K., Kim, K., et al. (2018). A Blend of Extracts from Houttuynia Cordata, nelumbo Nucifera, and Camellia Sinensis Protects against Ethanol-Induced Liver Damage in C57BL/6 Mice. *J. Med. Food* 21, 203–206. doi:10.1089/jmf.2017.4043

Zhang, L., Lv, H., Li, Y., Dong, N., Bi, C., Shan, A., et al. (2020). Sodium Houttuyfonate Enhances the Intestinal Barrier and Attenuates Inflammation Induced by *Salmonella typhimurium* through the NF-Kb Pathway in Mice. *Int. Immunopharmacol.* 89, 107058. doi:10.1016/j.intimp.2020.107058

Zhao, Y., Mei, L., Si, Y., Wu, J., Shao, J., Wang, T., et al. (2020). Sodium New Houttuyfonate Affects Transcriptome and Virulence Factors of *Pseudomonas aeruginosa* Controlled by Quorum Sensing. *Front. Pharmacol.* 11, 1–13. doi:10.3389/fphar.2020.572375

Zheng, C., Lin, J. F., Lin, Z. H., Lin, W. Q., Thapa, S., Lin, Y. Z., et al. (2018). Sodium Houttuyfonate Alleviates Post-infarct Remodeling in Rats via AMP-Activated Protein Kinase Pathway. *Front. Pharmacol.* 9, 1–11. doi:10.3389/fphar.2018.01092

Zhou, N. N., Tang, J., Chen, W. D., Feng, G. K., Xie, B. F., Liu, Z. C., et al. (2012). Houttuyninum, an Active Constituent of Chinese Herbal Medicine, Inhibits Phosphorylation of HER2/neu Receptor Tyrosine Kinase and the Tumor Growth of HER2/neu-Overexpressing Cancer Cells. *Life Sci.* 90, 770–775. doi:10.1016/j.lfs.2012.03.035

Zhu, H., Lu, X., Ling, L., Li, H., Ou, Y., Shi, X., et al. (2018). Houttuynia Cordata Polysaccharides Ameliorate Pneumonia Severity and Intestinal Injury in Mice with Influenza Virus Infection. *J. Ethnopharmacol.* 218, 90–99. doi:10.1016/j.jep.2018.02.016

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 15 September 2021
doi: 10.3389/fphar.2021.713090

Zanthoxylum Species: A Review of Traditional Uses, Phytochemistry and Pharmacology in Relation to Cancer, Infectious Diseases and Sickle Cell Anemia

Innocent Uzochukwu Okagu[1], *Joseph Chinedum Ndefo*[2]*, *Emmanuel Chigozie Aham*[1,3] *and Chibuike. C. Udenigwe*[4]*

[1]Department of Biochemistry, University of Nigeria, Nsukka, Nigeria, [2]Department of Science Laboratory Technology, University of Nigeria, Nsukka, Nigeria, [3]Natural Science Unit, School of General Studies, University of Nigeria, Nsukka, Nigeria, [4]School of Nutrition Sciences, University of Ottawa, Ottawa, ON, Canada

OPEN ACCESS

Edited by:
Michael Heinrich,
UCL School of Pharmacy,
United Kingdom

Reviewed by:
Eugenia Teodor,
National Institute of Research and Development for Biological Sciences (NIRDBS), Romania
Yan-Hui Fu,
Hainan Normal University, China

***Correspondence:**
Joseph Chinedum Ndefo
joseph.ndefo@unn.edu.ng
Chibuike. C. Udenigwe
cudenigw@uottawa.ca

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 21 May 2021
Accepted: 31 August 2021
Published: 15 September 2021

Citation:
Okagu IU, Ndefo JC, Aham EC and Udenigwe CC (2021) Zanthoxylum Species: A Review of Traditional Uses, Phytochemistry and Pharmacology in Relation to Cancer, Infectious Diseases and Sickle Cell Anemia. Front. Pharmacol. 12:713090. doi: 10.3389/fphar.2021.713090

The health benefits and toxicity of plant products are largely dependent on their secondary metabolite contents. These compounds are biosynthesized by plants as protection mechanisms against environmental factors and infectious agents. This review discusses the traditional uses, phytochemical constituents and health benefits of plant species in genus *Zanthoxylum* with a focus on cancer, microbial and parasitic infections, and sickle cell disease as reported in articles published from 1970 to 2021 in peer-reviewed journals and indexed in major scientific databases. Generally, *Z.* species are widely distributed in Asia, America and Africa, where they are used as food and for disease treatment. Several compounds belonging to alkaloids, flavonoids, terpenoids, and lignans, among others have been isolated from *Z.* species. This review discusses the biological activities reported for the plant species and their phytochemicals, including anticancer, antibacterial, antifungal, antiviral, anti-trypanosomal, antimalarial and anti-sickling properties. The safety profiles and suggestions for conservation of the *Z.* species were also discussed. Taken together, this review demonstrates that *Z.* species are rich in a wide range of bioactive phytochemicals with multiple health benefits, but more research is needed towards their practical application in the development of functional foods, nutraceuticals and lead compounds for new drugs.

Keywords: Zanthoxylum, Ethnobotany, health benefits, phytochemicals, functional foods, Nutraceutials

INTRODUCTION

Humans have continually depended on plants for food and medicine. Plants produce secondary metabolites in response to infective agents and environmental factors. Consequently, efforts have been made to isolate, characterize and investigate the beneficial effects of plant-derived secondary metabolites on human health. Notably, several bioactive compounds from plants have provided inspirations for the synthesis of chemical drugs, such as artesunates from artemisinins and quinolone antimalarials from quinine (Karunamoorthi et al., 2013; Lifongo et al., 2014; Pawar 2014; Numonov et al., 2019). Natural product chemists often rely on traditional knowledge on plants with medicinal potentials

to produce crude extracts with biological activities. This process is followed by downstream processing to isolate the bioactive compounds, and structural characterization to identify them. In some cases, chemical modifications of the phytochemicals are used to produce more clinically effective and safer entities.

Zanthoxylum species, also known as *Fagara* species, have a long history of use as sources of food and drug by locals in different parts of Asia, America and Africa. In traditional medicine, many of the plant species are used in treating sickle cell anemia, trypanosomiasis, malaria and microbial infections, including tuberculosis and enteritis, with *Z. zanthoxyloides* Lam being the most reported species for these applications (Erichsen-Brown 1979; Burkill 1985). For example, fruits of *Z. lepreurii* Guill. and Perr. and Z. zanthoxyloides Lam are used in managing fever, malaria, tumors and sickle cell anemia (Tamdem 2019) while the stem bark, leaves, and roots are applied to suppress pain, and to treat arthritis, leprosy, stomachache and venereal diseases in Cameroon (Burkill 1998; Ngoumfo et al., 2010). Furthermore, different parts of *Z. lepreurii* are used to treat or manage tuberculosis, malaria, human immunodeficiency virus (HIV) and several types of bacterial infection in Uganda and other parts of Africa (Lamorde et al., 2010; Tabuti et al., 2010; Bunalema et al., 2014). In China and other parts of Eastern Asia, *Z. bungeanum* Maxim. (Syn. *Z. piperitum* Benn.) is widely used as a food condiment because of its perceived health benefits Hwang et al. (2008) and as cosmetics for maintaining skin quality (Hwang et al., 2020). In Chinese medicine, *Z. bungeanum* is used as spices and for treating infection and bone diseases (Lee and Lim 2008; Kim et al., 2017). The leaves, fruits and barks are used in treating bacterial and fungal infections, as spices, and for food preservation in Japan (Hatano et al., 2004). Similarly, different parts of *Z. schinifolium Siebold and Zucc.* are used as food condiments and for treating stomach pain, diarrhea, jaundice, and cold in Eastern Asia (Cui et al., 2009). Furthermore, herbal preparation from different parts of *Z. americanum* Mill. is traditionally used for treating tumors, fungal skin infections, respiratory, urinary, genital and gastrointestinal (GIT) diseases by herbal healers in Canada and United States (Moerman 1998). In Kanayatn Dayak Community, West Kalimantan, Indonesia, the stem and root of Z. bungeanum are consumed raw or after boiling in water to prevent alcohol intoxication and treat respiratory diseases (Sepsamli and Prihastanti 2019). Other traditional and ethnobotanical uses of *Z.* species have been discussed elsewhere (Patiño et al., 2012; Adewole 2020; Lu et al., 2020a; Obakiro et al., 2020; Okagu et al., 2021). The objectives of this review are to discuss (1) the potential of *Z.* species as sources of bioactive phytochemicals that can be applied in the management and treatment of cancer, microbial and parasitic infections, and sickle cell disease; (2) chemical constituents involved in these biological activities; and (3) safety issues and suggestions for conservation of the plant species.

LITERATURE SEARCH STRATEGY AND CRITERIA FOR SELECTION OF ARTICLES

This study used a strategy similar to that reported by Nigussie et al. (2021). From repositories and search engines (PubMed, ScienceDirect, and Google Scholar), information related to the health benefits of *Z.* species, with emphasis on anticancer, anti-trypanosomal, antimicrobial, antiviral, antimalarial and anti-sickling properties, in peer-reviewed journals and ethnobotanical surveys published from 1970-July 3, 2021 were retrieved. The titles and abstracts of the studies were scanned using the inclusion criteria for this study. The search terms included cytotoxicity, anticancer, antimicrobial, antibacterial, anti-mycobacterial, antimalarial, antiviral, larvicidal, trypanocidal, anti-sickling and antiproliferative effect of *Zanthoxylum* species, *Fagara* species, and medicinal plants. In some cases, articles citing older papers and references of recent papers were used to obtain additional articles of interest. Studies reporting the biological activities of interest on different parts of *Z.* species including seeds, fruits, stem/stem bark, fruits, and root/root bark were included. Biological activities of crude extracts, their fractions and isolated compounds were also included. Where available, the mechanisms of action of the extract or isolated compounds were retrieved. Reviews, newspaper and other non-peer-reviewed articles were excluded. Similarly, studies reporting biological activities of *Z.* species other than those under consideration and in languages other than English were excluded. In this review, a test substance is considered bioactive when the outcome of the substance-treated group was substantial when determined qualitatively or quantitatively compared to controls (untreated group or group that received a standard drug).

The correctness of the scientific/botanical names of the plants reported in the included studies were confirmed with names available in botanical databases, including www.theplantlist.org, https://www.ipni.org/, https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/, and https://www.tropicos.org. In cases where the plant name in the article was not the acceptable taxonomical nomenclature, the name in the botanical databases was used. A number of reviews have records of plant species in genus *Zanthoxylum*, including *Z. armatum* DC (Brijwal et al., 2013; Mukhtar and Kalsi 2018; Paul et al., 2018; Verma et al., 2021), *Z. limonella* (Supabphol and Tangjitjareonkun 2014), Z. nitidum (Roxb.) DC (Lu et al., 2020a), *Z. rhetsa* (Roxb.) DC (Maduka and Ikpa, 2021), and *Zanthoxylum bungeanum* Maxim (Zhang M. et al., 2017). Some of these reviews are not comprehensive, while others focused on health benefits related to metabolic diseases (Okagu et al., 2021) or the phytoconstituents such as alkaloids (Yuan et al., 2015; Wei et al., 2021), or were published in non-English languages (Zhang M. et al., 2017). In some previous reviews on traditional uses, only selected species were discussed with respect to a particular disease condition, e.g., Imaga (2010) on sickle cell anemia, Ochwang'i et al. (2014) on cancer, Sinan et al. (2019) on malaria, and Obakiro et al. (2020) on tuberculosis. These reviews were carefully analyzed and most of the reviewed studies were excluded from the present review. Hence, this review covers information on the phytochemistry and biological activities of interest for 25 plants species in Genus *Zanthoxylum*, namely Z. leprieurii Guill. and Perr., Z. bungeanum Maxim. (Syn. Z. nitidum Bunge; Z. piperitum Benn.; Z. bungeanum var. bungeanum; Z. simulans Hance); Z. schinifolium Siebold and Zucc., Z. clava-herculis L., Z. heitzii (Aubrév. and Pellegr.) P.G.Waterman, Z. chalybeum Engl., Z. ailanthoides

Siebold and Zucc., Z. acanthopodium DC., Z. zanthoxyloides (Lam.) Zepern. and Timler, Z. paracanthum Kokwaro, Z. riedelianum Engl., Z. americanum Mill, Z. armatum DC. (Syn. Z. alatum Roxb.), Z. rhetsa DC., Z. buesgenii (Engl.) P.G.Waterman, Z. madagascariense Baker, Z. austrosinense C.C. Huang, Z. schreberi (J.F.Gmel.) Reynel ex C. Nelson (Syn. Z. monophyllum (Lam.) P. Wilson), Z. rhoifolium Lam., Z. davyi Waterm., Z. ovalifolium Tutcher, Z. fagara (L.) Sarg., Z. tingoassuiba A. St.-Hil., Z. gilletii (De Wild.) P.G.Waterman, and Z. poggei (Engl.) P.G.Waterman.

ZANTHOXYLUM SPECIES AS POTENTIAL SOURCES OF ANTICANCER AGENTS

Cancer is a disease that is characterized by uncontrolled cell division and loss of contact inhibition, leading to formation of tumors. Cancers are resistant to apoptosis and develop angiogenic and metastatic potentials. Prevalence of cancer is rising worldwide, even in developing countries where the rise is partly due to adoption of the Western diet and sedentary lifestyle, and increase in the aging population, among other factors (Morounke et al., 2017). Cancer-related deaths are higher in economically poor countries due to late detection and poor access to treatment and support (India State-Level Disease Burden Initiative Cancer Collaborators, 2018). The number of cancer cases and cancer deaths globally are projected to increase geometrically in the future (Smittenaar et al., 2016), thus placing cancer as a major global health issue. A number of approaches, such as radiation therapies, chemotherapies and surgeries, or their combination, are available for cancer management and treatment. Radiation therapies and surgeries are effective but present cancer patients with discomfort. In most cases, chemotherapies are linked with side effects and some cancers are resistant to chemotherapy. Development of clinically potent cancer drugs that are selectively toxic to cancer cells without harming normal cells has become a public health priority. One promising strategy is to search for natural products with cancer cell-specific cytotoxicity. Some natural compounds from plants and marine organisms have promising applications as anticancer agents (Lichota and Gwozdzinski 2018). Traditionally, medicinal plants have been used for treating cancer in different parts of the world (Abubakar et al., 2020; Adewole 2020). Medicinal plants belonging to *Z.* species and their phytochemicals with anticancer properties are discussed below.

The potential of *Z.* species in the treatment of cancer has been assessed using both drug-sensitive and drug-resistant cancer cells. For example, antiproliferative activities have been demonstrated using cell culture studies for extracts of *Z. clava-herculis* L. stem bark against lung cancer (A549) (Wansi et al., 2009), *Z. ailanthoides* Seibold. and Zucc. stem against human colon cancer cell line (Colo 205) (Chou et al., 2011), *Z. heitzii* (Aubrév. and Pellegr.) P.G.Waterman fruits and barks against cervical cancer (HeLa), breast cancer (MCF-7), acute monocytic leukemia (THP-1) and human Caucasian prostate cancer (PC-3) (Dzoyem et al., 2013), *Z. leprieuii* and *Z. zanthoxyloides* fruits against PC-3, MCF-7, liver (WRL-68), and colon (Caco-2) (Misra et al., 2013), *Z. rhetsa* DC. stem bark and root bark against human stomach-cancer cell lines, SCL, SCL-6, SCL-3706, SCL-9, Kato-3, and NUGC-4 (Ahsan et al., 2014), *Z. chalybeum* Engl. and *Z. parachanthum* Kowaro stem bark against drug-sensitive and multidrug-resistant leukemia cell lines (CCRF-CEM and CEM/ADR5000) (Omosa et al., 2019), *Z. acanthopodium* DC. seed against MCF-7 cell line (Arsita et al., 2019), *Z. zanthoxyloides* roots against liver cancer (HCC), larynx cancer (HEp2) and breast cancer (BT549) (Andima et al., 2020), and *Z. paracanthum* root bark against human breast cancer (HCC 1395) and human prostate cancer (DU 145) cell lines (Kaigongi et al., 2020). Most of these reports are inspired by the traditional uses of the plant in managing health conditions, including cancer. Specifically, decoctions of different parts of *Zanthoxylum poggei* (Engl.) P. G. Waterman are ingested to treat tumors, among other health issues, in Cameroon and Congo (Wouatsa et al., 2013). Using a combination of chromatographic and spectrophotometric techniques, acridone and indoloquinazoline alkaloids, poggeicridone and 2-methoxy-7,8-dehydroruteacarpine, respectively were isolated from stem bark of the plant. On exposure to cultured PC-3 cells, the two alkaloids elicited significant cytotoxic effects with IC_{50} values of 15.8 and 22.1 μM, respectively compared to IC_{50} value of 0.9 μM for doxorubicin, the reference anticancer drug (Wansi et al., 2016).

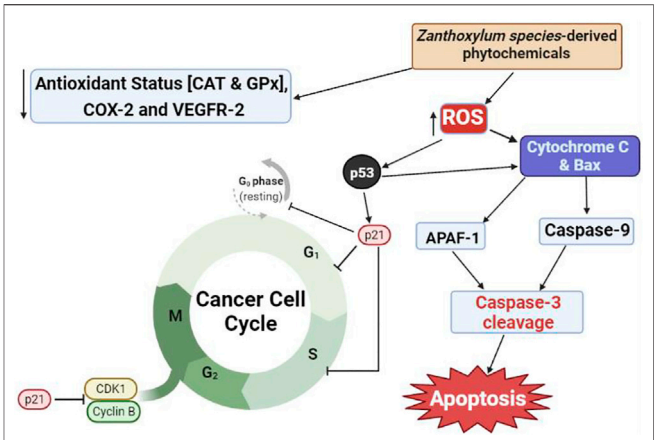


FIGURE 1 | Proposed mechanism of anticancer activities of natural products from *Zanthoxylum* species. CAT, catalase; GPx, glutathione peroxidase; COX-2, cyclooxygenase-2; VEGFR-2, vascular endothelial growth factor receptor-2; CDK1, cyclin-dependent kinase-1; ROS, reactive oxygen species; Bax, Bcl-2-associated X protein; APAF-1, apoptosis protein activating factor-1.

These reports have provided support to the traditional uses of decoctions of *Z.* species alone or as a cocktail with other plant species for managing cancer. Most anticancer natural products act by blocking different mechanisms through which cancer cells grow, multiply and invade other cells, as well as resist the immune system. These mechanisms include the induction of cell cycle arrest, apoptosis and oxidative stress, as well as inhibition of angiogenesis, metastasis and growth signaling pathways in cancer cells (Ahmad et al., 2003; Michalkova et al., 2021). As shown in **Figure 1**, extracts of *Z.* species have been demonstrated to elicit anticancer properties via inhibition of angiogenesis Harahap et al. (2018) and DNA synthesis,

TABLE 1 | Summary of *in vitro* anticancer properties of *Z.* species extracts.

Extract of *Z.* species	Range of concentration tested	Cancer model	Pharmacological effects	References
Ethanol extract of *Z. ailanthoides* stem	31.25–500 μg/ ml	Colo 205, Hep G2, B16-F1 and WEHI-3 cells	Suppressed cell viability by 46.4, 18.1, 9.2, and 5.2%, respectively. Extract induced apoptosis and cell cycle arrest at G2/M phase and increased ROS and Ca^{2+} levels, leading to cell damage.	Chou et al. (2011)
Ethyl acetate extract of *Z. acanthopodium* fruits	31.25–500 μg/ ml	4T1 breast cancer cells	Cytotoxic against cancer cells with IC_{50} value of 48.1 μg/ ml. Induced cell cycle arrest at G2/M phase and inhibited angiogenesis via suppression of gene expression of COX-2 and VEGFR-2.	Harahap et al. (2018)
Saponin-rich ethanol extract of *Z. armatum* DC fruit, bark and leaves	10–500 μg/ ml	Human breast cancer (MDA-MB-468 and MCF-7) and colorectal cancer (Caco-2) cells	At 200 μg/ ml, fruit, bark and leaf extracts inhibited the proliferation of MDA MB-468 by 95, 94.5 and 85.3%, MCF-7 by 79.8, 9.43 and 49.08%, and Caco-2 by 75.8, 61.8 and 68.62%, respectively. Inhibited DNA synthesis and induced apoptosis of the cancer cells.	Alam et al. (2017)
Methanol extracts of fruits and barks of *Z. heitzii*	10–100 μg/ ml	Human leukemia (HL-60) cells	Cytotoxic against cancer cells with IC_{50} values of 20 and 12 μg/ ml, respectively for fruit and bark extracts. Acted by generating mitochondrial-dependent apoptosis and Go/G1 phase arrest of the cancer cell cycle.	Pieme et al. (2014)
Methanol extract of *Z. heitzii* bark and fruits	1–100 μg/ml	Cervical cancer (HeLa), breast cancer (MCF-7), acute monocytic leukemia (THP-1) and prostate cancer (PC-3) cells	Bark extract showed antiproliferative effects with IC_{50} values of 66, 76, 8.4, and 42 μg/ ml, respectively while the fruit extract was only active against PC-3 and MCF-7 cells with IC_{50} values of 56 and 26 μg/ ml, respectively.	Dzoyem et al. (2013)
Methanol extract of *Z. leprieuii* fruits	1–100 mg/ ml	PC-3, MCF-7, liver (WRL-68), and colon (Caco-2) cells	Cytotoxic against the cancer cells with IC_{50} values of 88, >200, 17 and 60 μg/ml, respectively relative to doxorubicin with IC_{50} values of 5.0, 2.1, 0.85 and 3.3 μg/ml, respectively	Misra et al. (2013)
Methanol extract of *Z. zanthoxyloides* fruits	1–100 mg/ ml	PC-3, MCF-7, WRL-68, and Caco-2 cells	Antiproliferative against the cancer cells with IC_{50} values of 59, 55, 17 and 66 μg/ ml, respectively	Misra et al. (2013)
Dichloromethane/methanol extract of *Z. paracanthum* root bark	0.14–100 μg/ ml	Human breast cancer (HCC 1395) and human prostate cancer (DU 145) cells	Cytotoxic against cancer cells with IC_{50} values of 28.28 and 7.27 μg/ ml, respectively	Kaigongi et al. (2020)

induction of apoptosis Alam et al. (2017) and cell cycle arrest at Go/G1 Pieme et al. (2014) and G2-M phases, and suppression of cyclooxygenase (COX)-2 and vascular endothelial growth factor receptor type-2 (VEGFR-2) expression (Harahap et al., 2018); **Table 1** presents the anticancer properties of crude extracts of *Z.* species whose anticancer constituents are unknown.

Several studies have isolated the *Z.* species phytochemicals that may be responsible for their *in vitro* anticancer properties. Based on their classes, chemical compounds derived from *Z.* species with substantial inhibitory activities against cultured cancer cells are discussed below. An orbitide, [1-8-NαC]-zanriorb A1, isolated from *Z. riedelianum* Engl. leaves inhibited the proliferation of Jurkat leukemia T cells (IC_{50} 218 nM) by inducing apoptosis (Beirigo et al., 2016). In addition, phenolic compounds isolated from *Z. ailanthoides* stem, chlorogenic acid, flavone and isoflaxidin, were shown to suppress Colo 205 viability and induce apoptosis and cell cycle arrest at the G2/M-phase via upregulation of the expression of apoptosis-inducing factor, endonuclease G, and caspases 3, 7, and 9 while suppressing fatty acid synthase (FAS) (Chou et al., 2011). FAS is a multifunctional enzyme complex that is gaining attention as a target for cancer management. The inhibition of FAS activity in many cancer cells induce restimulation-induced cell death, one of the notable apoptotic pathways (Fhu and Ali 2020). Similarly, an alkamide, 4-(isoprenyloxy)-3-methoxy-3,4-deoxymethylenedioxyfagaramide, isolated from *Z. chalybeum* stem bark was moderately cytotoxic against CCRF-CEM and CEM/ADR5000 cells with IC_{50} values of 29.13 and 31 μM, respectively (Omosa et al., 2019), although the bioactivity mechanism is unknown. Such mechanistic information may facilitate the identification of specific molecular targets and derivatives of the compound with enhanced potency.

Furthermore, *Z.* species have been reported to contain several coumarins with broad-spectrum anticancer activities. For example, coumarins from *Z. schinifolium* stem, collinin, 8-methoxyanisocoumarin H and acetoxyschinifolin, significantly halted the proliferation of PC-3, HL-60, and colorectal (SNUC5) cancer cells with respective IC_{50} values of 4.62, 4.39 and 6.26 μM by collinin, IC_{50} of 5.02, 12.22 and 33.5 by 8-methoxyanisocoumarin H, and IC_{50} values of 5.12, 33.81 and 35.11 μM by acetoxyschinifolin. The coumarins acted by inducing apoptosis and suppression of the expression of genes (p-ERK1/2 MAPK, p-AKT, and c-myc) involved in cancer development and progression (Li et al., 2013). Furthermore, a pyranocoumarin from *Z. ailanthoides* stem bark, luvangetin exhibited weaker cytotoxic activity against human lung cancer

(A-549) cells with an IC_{50} value of 4.28 μg/ ml compared to 5-fluorouracil, a known anticancer agent (IC_{50} of 0.6 μg/ ml) (Cao et al., 2013). Nonetheless, the potential of luvangetin can be further explored by structural modification to possibly obtain more potent anticancer derivatives.

Zanthoxylum-isolated lignans have also been reported to have anticancer activities. Sesamin from *Z. parachanthum* demonstrated cytotoxic activity against CCRF-CEM and CEM/ADR5000 cancer cells with IC_{50} values of 40.74 and 30.70 μM, respectively (Omosa et al., 2019). In addition, (-)-xanthoxylol-3,3-dimethylallyl ether from *Z.* bungeanum Maxim stem bark was cytotoxic against MCF-7 cancer cells with an IC_{50} of 18.65 μg/ ml (Yang et al., 2009) while asarinin from *Z. americanum* stem suppressed the proliferation of HL-60 cancer cells with IC_{50} of 11.64 μM (Ju Y et al., 2001). Interestingly, the cytotoxicity of kobusin from *Z. rhetsa* bark against mouse melanoma (B16-F10) cells was weaker (IC_{50} values of 112.2 μg/ ml) Santhanam et al. (2016) than the activity of kobusin from *Z. armatum* bark against human lung (A549) and pancreatic (MIA-PaCa) cancer cells (IC_{50} values of 34.71 and 32.86 μg/ ml, respectively) (Mukhija et al., 2014). This demonstrates that activity is dependent on the cancer cell type, possibly because of differences in bioaccessibility of the compounds, their molecular targets and anticancer mechanisms. In general, the lignans acted by inducing apoptosis and cell cycle arrest, and inhibiting DNA synthesis in the cancer cells. In addition to the broad-spectrum antiproliferative properties, the potential of these lignans as anticancer agents is strengthened by the absence of cytotoxicity to human dermal fibroblasts and peripheral blood mononuclear cells.

In many studies, *Zanthoxylum* alkaloids were reported to exhibit cytotoxic activity against cancer cells *in vitro* (Wei et al., 2021). Specifically, a furoquinoline alkaloid, skimmianine, demonstrated cytotoxic activity against MCF-7 cancer cells with an IC_{50} value of 8.03 μg/ ml while an aporphine alkaloid, liriodenine, was cytotoxic against MCF-7, NCI-H460, and SF-268 cancer cells with IC_{50} values of 3.19, 2.38 and 2.19 μg/ ml, respectively (Andima et al., 2020). Recently, acridone alkaloids, fabiocinine and arborinine, and skimmianine from *Z. leprieu*rii Guill. and Perr. root bark were reported to exhibit selective cytotoxicity against HeLa cells with IC_{50} values of 28.49, 62.71 and 12.8 μg/ ml, respectively, which were lower than the activity of anticancer agent, emetine (IC_{50} values of 0.026 μg/ ml against HeLa cells) (Eze et al., 2020). Similarly, an indole alkaloid, canthin-6-one from *Z. parachanthum* inhibited CCRF-CEM and CEM/ADR5000 cancer cell proliferation with IC_{50} values of 15.82 and 10.52 μM, respectively (Omosa et al., 2019). Unlike doxorubicin, canthin-6-one demonstrated selective cytotoxicity against the drug-resistant cell line without affecting normal human peripheral blood mononuclear cells (Omosa et al., 2019). In other cancer cells (HCC 1395 and DU 145), canthin-6-one and its derivative, 10-methoxycanthin-6-one, from the same plant were strongly cytotoxic with IC_{50} values of 8.12, and 9.43 μg/ ml, and 14.70 and 1.58 μg/ ml, respectively (Kaigongi et al., 2020). Despite its lower cytotoxicity against cancer cells compared to doxorubicin, the better selectivity/nontoxicity to normal cells positions canthin-6-one as a promising candidate with a broad-spectrum anticancer activity.

Furthermore, benzophenanthridine alkaloid, 1-methoxy-12-methyl-12,13-dihydro-(1,3) dioxolo (4′,5′:4,5) benzo (1,2-c) phenanthridine-2,13-diol, from the aerial parts of *Z. buesgenii* (Engl.) P.G.Waterman showed moderate to strong cytotoxicity against sensitive and multidrug resistant cancer cells (CCRF-CEM, CEM/ADR5000, MDA-MB231, MDA-MB231/BCRP, HCT116 ($p53^{+/+}$), HCT116 ($p53^{-/-}$), U87MG, U87MG.ΔEGFR, and HepG2) with IC_{50} values of 0.24, 31.58, 30.14, 65.01, 42.46, 62.34, 60.55, 61.84, and 22.37, respectively, while sparing normal human liver (AML12) cells (Sandjo et al., 2014). Due to the broad-spectrum anticancer activity of the benzophenanthridine and aporphine alkaloids, further studies are required to understand the molecular mechanism of action against the cancer cells. Benzophenanthridine alkaloid from another species (Z. madagascariense Baker), rutaceline, showed inhibitory activity against Caco-2 cells by inducing apoptosis, cell cycle arrest at the G0/G1 phase and DNA fragmentation, and by inhibiting DNA synthesis (Pachón et al., 2007). Acting via similar mechanisms (induction of apoptosis and cell cycle arrest by strong binding to cyclin-dependent kinases (CDK2 and CDK6) and caspases 3 and 8), the ability of a benzophenanthridine alkaloid from *Z. zanthoxyloides* roots, dihydrochelerythrine to exhibit significant cytotoxicity against HCC and BT549 cancer cells Andima et al. (2020) demonstrates the strong anticancer potential of the benzophenanthridine alkaloids from *Z.* species. Through unknown mechanisms, other alkaloids such as isoquinoline alkaloids (e.g. nitidine, fagaronine chelerythridine and sanguinarine) from *Z. bungeanum* elicited selective DNA damage and cytotoxicity against mouse lymphocytic leukemia cells *in vitro* (Kaminskyy et al., 2008; Liao et al., 2013; Tian et al., 2017). Similarly, *Z. austrosinense* C.C. Huang root-derived carbazole alkaloids, zanthoaustrones A–C, exhibited strong antiproliferative activities against human leukemia (HL-60), liver (SMMC-7721), lung (A-549), breast (MCF-7) and colon (SW480) cancer cell lines (Fu et al., 2020). Despite the promising *in vitro* anticancer activities reported, the experimental designs often did not include appropriate positive controls. This is needed for validation of anticancer activities prior to *in vivo* studies, considering potential differences in assay conditions that may influence cellular activities.

Redox imbalance in cancer cells caused by reduction in antioxidant status and elevation of ROS production and lipid peroxidation has been targeted as a major mechanism through which some plant-derived compounds induce apoptosis (Redza-Dutordoir and Averill-Bates 2016). Other mechanisms include caspase-mediated signaling, which induces apoptosis, and p53-mediated cell cycle arrest (Zhang Y. et al., 2017). For example, an acridone alkaloid derivative (2-aminoacetamido-10-(3,5-dimethoxy)-benzyl-9(10H)-acridone hydrochloride) was shown to kill leukemia cells by decreasing mitochondrial transmembrane potential while increasing the expression of Bax, cytochrome C and apoptosis protein activating factor-1 to form an apoptosome (Wang et al., 2013). Formation of apoptosome activates caspase-9 with concomitant activation of

TABLE 2 | Chemical compounds isolated from *Zanthoxylum* species with *in vitro* anticancer properties.

Compounds	Class of compound	*Z.* species of source	Cancer cell model	Mechanism of action	Reference
[1-8-NαC]-zanriorb A1	Orbitide	*Z. riedelianum* leaves	Jurkat leukemia T cells	Induced apoptosis	Beirigo et al. (2016)
Chlorogenic acid	Phenolics	*Z. ailanthoides* stem	Human colon cancers (Colo 205)	Induced apoptosis and cell cycle arrest at the G2/M-phase	Chou et al. (2011)
Flavone					
Isoflaxidin					
4-(isoprenyloxy)-3-methoxy-3,4-deoxymethylenedioxyfagaramide	Alkamide	*Z. chalybeum* stem bark	CCRF-CEM and CEM/ADR5000 cancer cells	NR	Omosa et al. (2019)
Collinin	Coumarin	*Z. schinifolium* stem	PC-3, HL-60 and colorectal (SNUC5) cells	Induced apoptosis and suppressed the expression of p-ERK1/2 MAPK, p-AKT, and c-myc genes	Li et al. (2013)
8-methoxyanisocoumarin H					
Acetoxyschinifolin					
Luvangetin	Coumarin	*Z. ailanthoides* stem bark	A-549 cells	NR	Cao et al. (2013)
Sesamin	Lignan	*Z. parachanthum*	CCRF-CEM and CEM/ADR5000 cells	NR	Omosa et al. (2019)
(-)- xanthoxylol-3,3-dimethylallyl ether	Lignan	*Z. nitidum* stem bark	MCF-7 cells	NR	Yang et al. (2009)
Asarinin	Lignan	*Z. americanum* stem	HL-60 cancer cells	NR	Ju Y et al. (2001)
Kobusin	Lignan	*Z. armatum* bark and *Z. rhetsa* bark	mouse melanoma (B16-F10) cells	NR	Mukhija et al., 2014; Santhanam et al., 2016
Skimmianine	Alkaloid	*Z. bungeanum* root	MCF-7 cells	NR	Andima et al. (2020)
Liriodenine	Alkaloid	*Z. zanthoxyloides* root	MCF-7, NCI-H460, and SF-268 cancer cells	NR	Andima et al. (2020)
1-methoxy-12-methyl-12,13-dihydro-[1,3]dioxolo [4′,5′:4,5]benzo [1,2-c] phenanthridine-2,13-diol	Alkaloid	*Z. buesgenii* aerial parts	CCRF-CEM, CEM/ADR5000, MDA-MB231, MDA-MB231/BCRP, HCT116 ($p53^{+/+}$), HCT116 ($p53^{-/-}$), U87MG, U87MG.ΔEGFR, and HepG2 cells	NR	Sandjo et al. (2014)
Rutaceline	Alkaloid	*Z. madagascariense* bark	Caco-2 cells	NR	Pachón et al. (2007)
Dihydrochelerythrine	Alkaloid	*Z. zanthoxyloides* roots	HCC and BT549 cells	NR	Andima et al. (2020)
Nitidine	Alkaloid	*Z. bungeanum* stem	Mouse lymphocytic leukemia cells	Induced apoptosis and DNA damage	Liao et al., 2013; Tian et al., 2017
Fagaronine					
Chelerythridine					
Sanguinarine					
Zanthoaustrones A–C	Alkaloid	*Z. austrosinense* root	HL-60 cells	NR	Fu et al. (2020)
Canthin-6-one	Alkaloid	*Z. parachanthum* bark	CCRF-CEM, CEM/ADR5000, HCC 1395 and DU 145 cells	NR	Omosa et al., 2019; Kaigongi et al., 2020
10-methoxycanthin-6-one	Alkaloid	*Z. parachanthum* bark	HCC 1395 and DU 145 cells	NR	Kaigongi et al. (2020)
hyperoside	flavonol glycoside	*Z. bungeanum* leaves	Human colorectal cancer cells (SW620)	Induced cell cycle arrest at G2/M phase and apoptosis	Zhang et al. (2017b)

NR, Not reported.

caspase-3, the final inducer of apoptosis. The increase in intracellular ROS production induced by natural products also alters membrane phospholipid composition and integrity, all of which contribute to cancer cell death (Rahman et al., 2021).

Taken together, *Z.* species contain a repertoire of phytochemicals with promising application in the treatment of cancer. However, the reviewed studies were conducted in cell cultures *in vitro*, without validation of physiological anticancer effects of the extracts or isolated compounds using model organisms or in humans. This is a major limitation of the studies because of pharmacokinetic and pharmacodymanic considerations, which influence the bioaccessibility, bioavailability and target binding/sensitivity of the compounds. Furthermore, some cancer cells have developed mechanisms for resisting the cytotoxic actions of some anticancer agents, such as reduced expression of drug targets while upregulating the

expression of alternative survival pathways (Pistritto et al., 2016; Bukowski et al., 2020). Consequently, the multiple target mechanisms of anticancer activities identified *in vitro* for some *Z.* species-derived compounds make them strong candidates for *in vivo* studies and human clinical trials, and further development as anticancer agents. Chemical compounds isolated from *Z.* species with anticancer properties and their molecular mechanisms are presented in **Table 2**; **Figure 1**.

ZANTHOXYLUM SPECIES AS POTENTIAL SOURCES OF ANTIMICROBIAL AGENTS

Microorganisms play many roles essential for human survival and are used as sources of drugs such as antibiotics. However, many strains of bacteria and fungi, such as *Streptococcus mutans*, *S. aereus*, *Mycobacterium tuberculosis*, *K. pneumoniae, Candida* species, and *Escherichia coli* are causative agents of many diseases of clinical importance. Several classes of antimicrobial drugs are used to control microbial infections, by suppressing microbial growth or killing them. However, survival pressure has led to the emergence and spread of antibiotics-resistant strains of many microorganisms, including those that are resistant to multiple drugs of the same or different classes (multi-drug resistant strains). These drug resistant strains have led to prolonged treatment duration, frequent hospitalization, increased healthcare cost and mortality from treatable microbial infections (Naylor et al., 2018). This necessitates the urgent search for clinically effective antimicrobial agents against these "superbugs".

Antimicrobial agents from herbs that are traditionally used in treating microbial infections are being isolated and assessed for activities against drug-resistant microbial strains. Many natural products derived from genus *Zanthoxylum* show promising antimicrobial activities against bacteria and fungi of public health importance. Investigations for antimicrobial activities are mostly guided by traditional uses of the plant species in the treatment of infectious diseases. Among the *Z.* species, *Z. zanthoxyloides* is well known for its use in the management of microbial infections in China and Korea and other parts of Asia as well as in Uganda, Nigeria and Ghana (Anokbonggo et al., 1990; Ngono-Ngane et al., 2000; Imaga 2010; Ynalvez et al., 2012; Ouédraogo et al., 2019; Adeeyo et al., 2020). Additionally, *Z. zanthoxyloides* is used as chewing stick and to treat oral infections and toothaches (Anyanwu and Okoye 2017), thus suggesting that the plant species may have antimicrobial activities against oral pathogens. Similarly, *Z. rhetsa* has been used against urogenital microbial infections and in disinfection of contaminated surfaces in Bangladesh (Yusuf et al., 1994), while *Z. lemairei* (De Wild) P.G. Waterman is used against malaria and diarrhea (Adesina et al., 1997), *Z. chalybeum* stem bark against malaria in Rwanda and Ivory Coast (Kamanzi Atindehou et al., 2002), and *Z.* schreberi (J.F.Gmel.) Reynel ex C. Nelson in treating eye infections in the Caribbean, Venezuela, Colombia and Costa Rica (Rodríguez-Guzmán et al., 2011).

Instead of the whole plant parts, solvent extracts of different parts of *Z.* species have been investigated for antimicrobial activities. As shown in **Table 3**, the potency of antimicrobial activity [expressed as minimum inhibitory concentration (MIC) or inhibition zone diameter (IZD)] varies with the different plant species, part used (fruits, leaves, root bark, stem bark), microorganism tested, solvent used for extraction, or type of assay used in the studies. Moderately polar solvents appear to be the best medium for extraction of the antimicrobial compounds compared with highly polar and non-polar solvents (Gonçalves et al., 2019). It is also worth noting that most of the microorganisms studied are drug-sensitive species and only a few studies tested the extracts on drug-resistant microorganisms. Thus, it is challenging to assess and conclude on the potential of individual plant extracts as presented in the literature. It is apparent that the *Z.* species crude extracts contain antimicrobial principles, which may need to be purified to enhance the activity or for elucidating molecular mechanisms. Nonetheless, the combination of several potentially active principles in the crude extracts may present an opportunity. For instance, aqueous-methanol extract of *Z. zanthoxyloides* root bark showed antibacterial activity against *Streptococcus mutans, Sarcina lutea,* and *Lactobacillus* sp. at IZD of 20, 32, and 56 mm, respectively, at 100 mg/ ml compared to the stronger but limited effect of antibiotic drug, amoxicillin, which was active (IZD of 22 mm at 10 μg/ ml) only against *Lactobacillus* sp. (Okafor et al., 2017). Multi-component extracts with such promising antimicrobial activity can be further investigated for safety and pharmacological effects as low-cost alternatives to purified compounds or drugs. Aside from solvent extracts, the antimicrobial activities of multi-component essential oils derived from *Z.* species have been reported (**Table 3**). In one study, bioautophagy-directed fractionation of essential oil from Z. armatum leaves led to the isolation of β-fenchol and linalool, which had antifungal activities against *A. alternata* and *C. lunata* (Guleria et al., 2013).

In general, studies on the *Z.* species crude extracts and essential oils reported antimicrobial activity as IZD or MIC, and seldom investigated their molecular mechanisms. In one study, n-hexane extract of *Z. acanthopodium* fruits that was active against *M. smegmatis* was reported to induce loss of intracellular sodium and potassium ion concentration, suggesting that the extract acted by damaging the bacterial cell wall (Julistiono et al., 2018). Hong et al. (2017) also reported that essential oil from *Z. bungeanum* fruits, containing 6,9,12,15-hexadeca-tetraenoic acid-methyl ester, 4-terpinenylacetate, D-limonene, eucalyptol, α-terpineol, β-linalool, δ-cadinene and β-pinene, caused lysis of cultured *E. coli* membrane. This mechanism was supported by the high amount of bacterial intracellular (nucleic acids and proteins) and cell membrane components in the culture medium. *In vivo* evaluation in a mouse model of enteritis demonstrated that *Z. bungeanum* fruit-derived essential oil downregulated the expression of pro-inflammatory cytokines (Hong et al., 2017); this indicates that anti-inflammatory mechanism played a role in host protection by the essential oil against *E. coli* infection. Indeed, elucidation of molecular mechanisms would be more logical for isolated compounds with defined molecular targets in the microorganisms or host. Nonetheless, knowledge of the molecular basis of antimicrobial effect would enhance the direct utilization of crude extracts of *Z.* species for pharmacological applications.

TABLE 3 | Summary of the antimicrobial activities of solvent extracts and essential oils from different part of *Zanthoxylum* species.

Plant species and part used	Test substance	Microorganism targeted	Activity	References
Z. zanthoxyloides fruits	Crude methanol extract	*P. aeruginosa*	IZD of 15 mm	Misra et al. (2013)
	Essential oil	*K. pneumonia*, *P. aeruginosa* and *S. typhimurium*.	IZD of 12, 11, and 9 mm, resp.	
Z. leprieurii fruits	Crude methanol extract	*P. aeruginosa*	IZD of 15 mm	Misra et al. (2013)
Z. zanthoxyloides root bark	Aqueous-methanol extract	*S. mutans, S. lutea, C. albicans,* and *A. niger*	IZD of 20–32 mm	Okafor et al. (2017)
Z. zanthoxyloides fruits	Essential oil	*S. aureus, E. coli, E. faecalis,* and *C. albicans*	IZD of 8.6–18.8 mm	Tine et al. (2017)
Z. leprieurii root bark	Methanol extract	Pan sensitive (H37rv), rifampicin resistant (TMC 331) and isoniazid resistant (TMC 301) strains of *M. tuberculosis*	MIC of 47.3, 75.3 and 125 μg/ ml	Bunalema et al. (2017)
Z. zanthoxyloides	Ethylacetate and chloroform extracts	*E. coli, P. aeruginosa, Klebsiella sp, S. pneumoniae* and *B. cereus*) and fungal species (*A. niger, A. flavus, Trichoderma sp* and *Candida sp*.	IZD of 7.5–16 mm at 25 mg/ ml	Adeeyo et al. (2020)
F. heitzii fruits and root bark	Ethanol extract	*E.coli, P. aeruginosa, C. albicans,* and *A. fumigatis*	MIC of 500 μg/ ml (fruit) and 1,000 μg/ ml (root; for only *A. fumigates*)	Dzoyem et al. (2013)
Z. clava-herculis leaves and stem bark	Methanol extract	*E. coli* (AG102), *E. aerogenes* (EA27), *K. pneumonia* (KP63) and *Providencia stuartii* (NEA16)	MIC of 64–512 μg/ ml	Seukep et al. (2015)
Z. bungeanum fruits	Essential oil	*E. coli*	MIC of 24 mg/ ml (cell wall lysis *in vitro*)	Hong et al. (2017)
Z. chalybeum stem bark	Dichloromethane and ethanol extracts	*S. aureus, S. typhi* and *P. aeruginosa*	Dichloromethane extract, MIC of 32 μg/ml (*S. aureus*); ethanol extract, MIC values of 32, 250 and 500 μg/ml (*S. aureus, S. typhi* and *P. aeruginosa*, resp.)	Mugiraneza et al. (2013)
Z. chalybeum stem bark	Dichloromethane, ethylacetate and methanol extracts	Isoniazid-resistant strains of *M. madagascariense* and *M. indicus pranii*	Dichloromethane extract, MIC of 1.25 mg/ ml (both); methanol extract, MIC of 1.25 and 2.5 mg/ ml, resp.	Chrian et al. (2011)
Z. ovalifolium fruit	Hexane, ethylacetate and methanol extracts	*K. pneumonia* and *S. aureus*	Ethyl acetate extract, IZD of 15 and 16 mm, resp.; n-hexane extract, IZD of 14 and 10 mm, resp.; methanol extract, IZD of 13 and 14 mm, resp. at 100 μg/ ml	Pavani and Naika (2020)
Z. paracanthum root bark	Chloroform-ethanol extract	Methicillin-resistant *S. aureus* (MRSA), *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213) and *C. albicans* (ATCC 10231)	MIC of 3.91, 0.98, 1.95 and 7.81 μg/ml, resp.	Kaigongi et al. (2020)
Z. bungeanum leaves	Essential oil	*S. aureus, B. cereus, B. subtilis*, *L. monocytogenes*, *S. choleraesuis, V. parahaemolyticus, A. hydrophila, S. sonnei, V. vulnificus* and *S. enterica*	MIC of 1.25 μg/ ml	Lee et al. (2012)
Z. tingoassuiba roots	Methanol and dichloromethane extracts	*S. aureus* ATCC 25923 and four multidrug resistant strains of the bacterium	Methonol extract, IZD of 18.3–23.3; dichloromethane extract, IZD of 13.3–20.3 mm	Costa et al. (2017)
Z. armatum leaves	Crude methanol extract and essential oil	*Alternaria alternata*, and *Curvularia lunata*.	MIC of 1,071 and 948 μg/ ml, resp.	Guleria et al. (2013)
Z. leprieurii fruits	Methanol extract	*S. aereus and S. saprophyticus*	MIC of 2 and 7 μg/ ml, resp.	Njimoh et al., 2015
Z. acanthopodium fruits		*M. smegmatis*	MIC value of 64 μg/ ml	Julistiono et al. (2018)
Z. armatum seeds and fruits	Crude methanol extract	*S. aureus, B. subtilis, E. faecalis,* MRSA, and *S. epidermidis*	IZD of 11.73–20.72 mm	Phuyal et al. (2020)

IZD, inhibition zone diameter; MIC, minimum inhibitory concentration.

Efforts have been made to isolate compounds that are responsible for the reported antimicrobial activities of *Z.* species extracts. Most of the compounds are alkaloids and some possess antimicrobial activity against both drug-sensitive and drug-resistant species of public health importance. For example, alkaloids (6-acetonyldihydronitidine, 6-acetonyldihydroavicine and 6-acetonyldihydrochelerythrine) from the stem bark of *Z. rhoifolium* strongly inhibited the growth of *S. aureus, S. epidermidis, K. pneumonie, S. Setubal,* and *E. coli* with respective MIC values of 1.06, 1.06, 3.12, 3.12, and 1.06 μg/ ml (6-acetonyldihydronitidine), 1.06, 3.12, 1.06, 3.12, and 3.12 μg/ ml (6-acetonyldihydroavicine), and 12.5, 6.25,

6.25, 6.25, and 12.5 μg/ ml (6-acetonyldihydrochelerythrine) (Gonzaga et al., 2003). Other antimicrobial alkaloids isolated from *Z.* species include dihydrochelerythrine from *Z. rhetsa* roots and stem (Tantapakul et al., 2012); *bis*-[6-(5,6-dihydro-chelerythrinyl)] ether, 6-ethoxy-chelerythrine and 4-methoxy-N-methyl-2-quinolone from *Z.* schreberi leaves and bark (Rodríguez-Guzmán et al., 2011); β-carboline alkaloids (10-methoxycanthin-6-one and canthin-6-one) and phenanthridine alkaloids (8-acetonyldihydrochelerythrine and 8-oxochelerythrine) from *Z. paracanthum* root bark (Kaigongi et al., 2020); N-methylcanadine from *Z. tingoassuiba* A. St.-Hil. roots (Costa et al., 2017); and acridone alkaloids (hydroxy-1,3-dimethoxy-10-methyl-9-acridone and 3-hydroxy-1,5,6-trimethoxy-9-acridone) from *Z. leprieurii* stem bark (Bunalema et al., 2017). Similar to its broad-spectrum anticancer activity, canthin-6-one was strongly active against several bacteria (*S. aureus, E. coli*, *Proteus vulgaris* and *Klebsiella aerogenes*) and fungi (*A. niger* and *C. albicans*), with corresponding MIC values of 0.227, 0.114, 0.114, 0.227, and 0.114 μM, respectively. Furthermore, the acridone alkaloids strongly inhibited first line drug-resistant (H37rv), rifampicin-resistant (TMC 331) and isoniazid-resistant (TMC 301) strains of *M. tuberculosis* with MIC values of 1.5–8.3 μg/ ml (Bunalema et al., 2017), which positions *Z. leprieurii* as a potential source of anti-tuberculosis agents. Structure-activity relationship studies are needed to identify the pharmacophores of the alkaloids against specific microorganisms or their molecular targets. Likewise, antimicrobial mechanisms of the alkaloids are largely unknown, although Wouatsa et al. (2013) reported that acridone alkaloids, 3-hydroxy-1,5,6-trimethoxy-9-acridone and 2,4′-hydroxyzanthacridone oxide, from *Z. leprieurii* fruit extract acted by inhibition of aromatase and glycosyltransferase, which are involved in biosynthesis of bacterial lipopolysaccharides and cell wall.

Apart from alkaloids, other antimicrobial compounds isolated from different *Z.* species include lignans (e.g., sesamin and syringaresinol) (Rahman et al., 2008), tetraflavonoids (e.g., lemairones A and B) (Bitchagno et al., 2015), and phytosterol (e.g., stigmasterol) (Kaigongi et al., 2020). Lemairones A and B from *Z. lemairei* leaves moderately inhibited multidrug-resistant bacteria, *K pneumoniae* KP55 and *E. coli* AG100 with MIC values of 128 mg/ ml and 64 mg/ ml, respectively against *E. coli* AG100, and MIC value of 128 mg/ ml against *K. pneumoniae* KP55 (Bitchagno et al., 2015). Furthermore, a polymeric procyanidin from *Z. bungeanum* fruit exhibited cytotoxicity against drug resistant strains of *S. aureus* with an MIC value of 128 μg/ ml; the compound acted by inhibiting β-lactamase activity and by inducing cell wall damage (Kusuda et al., 2006). This study also showed that the isolated compound has potential for further development as an adjuvant of antibacterial drugs for mitigating the burden of drug-resistant microbial infections. Taken together, it is recommended that future studies should use drug resistant microorganisms since the ultimate goal is to overcome antimicrobial resistance. Findings from the studies reviewed suggest that some of the compounds isolated from *Z.* species have promising future as source of new antibiotics. **Figure 2** shows representative *Z.* species-derived compounds with anticancer, antiparasitic, antimicrobial or anti-sickling activities.

ANTIVIRAL POTENTIALS OF *ZANTHOXYLUM* SPECIES

The emergence and spread of viruses such as SARS-CoV-2, HIV and hepatitis B have led to heightened efforts in search for effective remedies. These efforts include repurposing of drugs developed for other viral diseases as well as exploring for new drug candidates in medicinal plants used in treating viral infections by traditional medicine practitioners. A few studies have reported antiviral activities for extracts from *Z.* species. Following the folkloric use in treating oral pathogens and symptoms related to picornaviridae infection, Choi (2016) found that the methanol extract of *Z. piperitum* leaves were cytotoxic to human rhinoviruses - HRVs (HRV2 and HRV3) and enteroviruses (coxsackie A16, B3, and B4 viruses, and human enterovirus71) of picornaviridae virus family with IC_{50} values of 59, 39, 45, 68, 93, and 4.4 μg/ml, respectively. As the extract had low toxicity against human cells (Hela and Vero cells), the active ingredients, if isolated, can serve as bioactive candidates against viral diseases caused by members of the picornaviridae family. Moreover, leaves of *Z. bungeanum* are used in Korea and Japan for treating respiratory diseases. To support this use, Ha et al. (2014) reported the anti-influenza virus A/NWS/33 (H1N1) effects of flavonol glycosides, quercetin-3-O-β-D-galactopyranoside, quercetin-3-O-α-L-rhamnopyranoside and kaempferol-3-O-α-L-rhamnopyranoside isolated from the *Z. bungeanum* leaves; the flavonol glycosides also inhibited influenza A virus neuraminidase activity with IC_{50} values of 434, 211, and 273 μg/ ml, respectively. Influenza A virus neuraminidase is involved in the release of newly made virus particle from infected cells, making it a good target for reducing the spread in host cells. Considering the multiple molecular targets of polyphenols, it is also possible that the antiviral activity was mediated via additional unknown mechanisms.

In addition to influenza virus, the antiviral potentials of *Z.* species-derived phytochemicals have also been reported against hepatitis B virus. A coumarin, collinin, from chloroform extract of *Z. schionifolium* bark exhibited anti-hepatitis B virus activity (ED_{50} of 68.3 μg/ ml) and also inhibited HBV-DNA replication (IC_{50} of 17.1 μg/ ml) (Chang et al., 1997). Using a different assay, two alkaloids, 5,6-dihydro-6-methoxynitidine and 5-methoxydictamnine, from Z. bungeanum Maxim roots at 0.2 μM showed higher anti-HBV activities by respectively inhibiting 43.3 and 49.3% of viral multiplication than 10 μM of standard antiviral drug, lamivudine with 29.6% inhibition (Yang and Chen 2008). In addition, a benzophenanthridine alkaloid (decarine), a furoquinoline alkaloid (γ-fagarine), and an amino alcohol derivative (+)-tembamide) from Z. *ailanthoides* root bark showed anti-HIV activities (EC_{50} values <0.05 μg/ ml) with no cytotoxicity against normal H9 lymphocyte cells (Cheng et al., 2005). It is challenging to compare the antiviral potential of the *Zanthoxylum* compounds because of differences in the structural type of

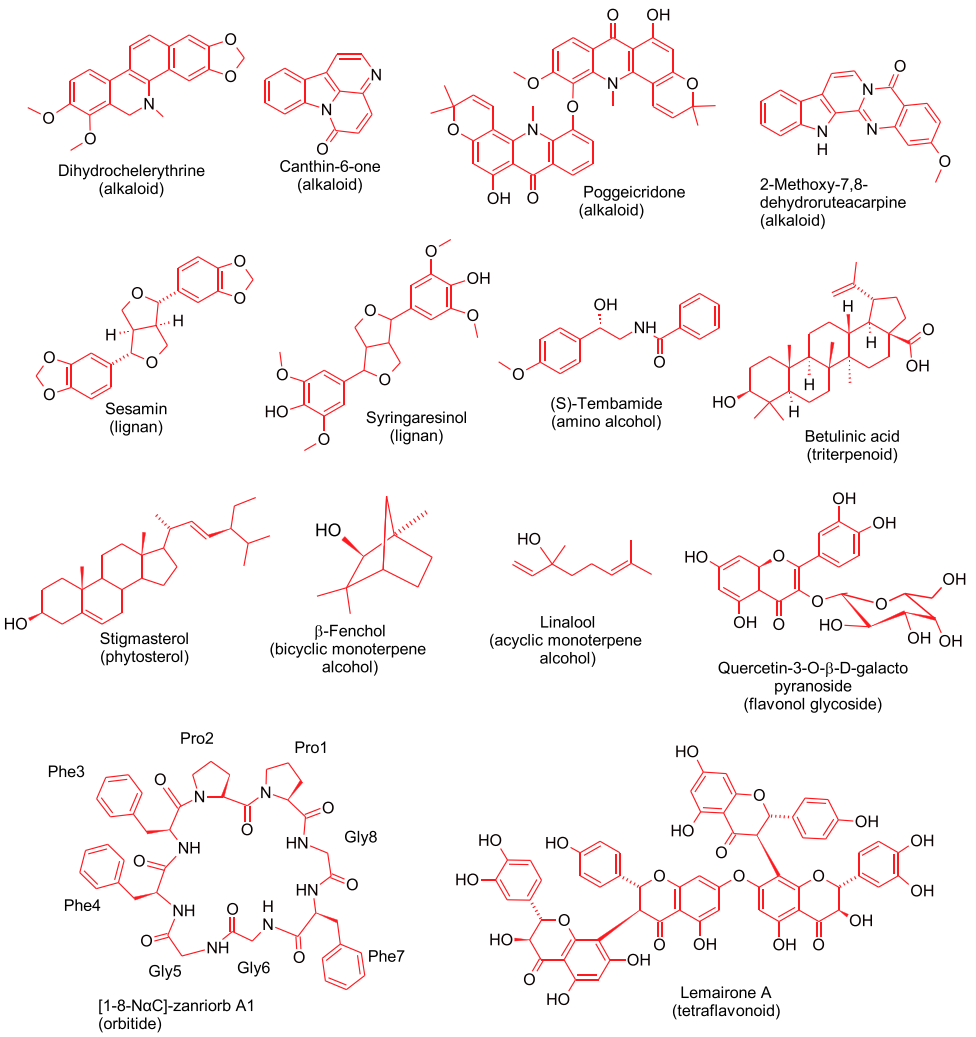


FIGURE 2 | Representative bioactive compounds, from different phytochemical classes, isolated from *Zanthoxylum* species.

the compounds, target virus, and assay method used in these studies. Nonetheless, due to their selected toxicity to viruses, the *Z.* species-derived alkaloids warrant further investigation for potential use in treating viral infections.

ZANTHOXYLUM SPECIES AS POTENTIAL SOURCE OF ANTI-PARASITIC AGENTS

Human African trypanosomiasis (sleeping sickness), a neglected tropical disease, is a *Trypanosoma* species-caused parasitic disease that is endemic in sub-Saharan Africa, where majority of the victims are poor rural dwellers. *Trypanosoma* species is transmitted by tsetse flies and cause a fatal disease if not properly treated. A few *T.* species, such as *T. brucei gambiense,* is responsible for the vast majority of *Trypanosoma* infection in West and Central Africa while *T. rhodesiense* is mostly responsible for infections in East Africa where less than 10% of the infection exists. The initial (hematolymphatic) stage of infection is asymptomatic and, if detected early, is treatable with pentamidine or suramin while the second (meningo-encephalitic) stage, which is characterized by the invasion of the central nervous system by the parasite, is treatable with melarsoprol or eflornithine (Simarro et al., 2011). Unfortunately, these drugs do not guarantee total recovery as the success rate in most cases is less than 90%. In addition to the growing drug resistance of the parasites, some devastating toxicities accompy the use of these drugs (Shaw et al., 2010). This presents the need for safer and potent alternatives, especially from natural products and medicinal plants that have a history of application in treating the disease. Medicinal plants are used for treating trypanosomiasis in Nigeria (Atawodi et al., 2003; Bulus et al., 2016; Okwor et al., 2020); thus, they have strong prospects for use as sources of clinically relevant anti-trypanosoma agents. In Nigeria and Ghana, roots, stem and leaves of *Z. zanthoxyloides* are used in treating trypanosomiasis (Mann et al., 2011). When cultured with the root extract, the viability of *T. brucei* was demonstrated to be suppressed (IC_{50} = 3.41 μg/ ml) by

induction of apoptosis and cell cycle arrest at G0/G1 phase (Dofuor et al., 2019). Subsequent investigation by the same group resulted in the isolation of an alkaloid, skimmianine, and an oxylipin, 9-oxo-10,12-octadecadienoic acid, from *Z. zanthoxyloides* root as the cytotoxic principles against *T. brucei* (GUTat 3.1 strains; EC_{50} values of 1.7 and 1.2 μM, respectively) (Dofuor et al., 2020). Although less active than diminazene aceturate, an antitrypanosomal drug (EC_{50} of 0.5 μM), the alkaloids acted by inducing cell cycle arrest at G0-G1 and (G2-M) phases and by inhibiting DNA synthesis of the parasite. Similarly, an acridone alkaloid, arborinine, derived from *Z. leprieurii* stem bark exhibited anti-trypanosomal activity in cultured *Trypanosoma brucei* (s427) cells with an IC_{50} of 13.2 μg/ ml by unknown mechanisms (Eze et al., 2020). There is a need to test the clinical effectiveness of these isolated compounds in trypanosoma infection to clarify if their *in vitro* activities can translate into clinically relevant *in vivo* effects. This is because some therapeutic agents that are active in culture studies are not biostable in the gastrointestinal tract or may face transport barriers during transepithelial transport when orally ingested (Udenigwe et al., 2021).

Apart from trypanosomiasis, malaria is another parasitic disease targeted with some *Z.* species. Malaria is caused by *Plasmodium* species and is transmitted by *Anopheles* species through the blood of an infected human. An estimate of over 200 million people die from malaria-related events and a vast majority of these deaths occur in sub-Saharan Africa with Nigeria bearing the highest burden (WHO, 2019). Majority of those who contract and die from malaria are poor rural dwellers who resort to cheap and ineffective drugs that relieve the symptoms, leading to relapses and increase in the development of resistant strains of the parasite (Karunamoorthi et al., 2013). In addition, some individuals are sensitive to some prescription antimalarial drugs (Haakenstad et al., 2019). Consequently, traditional medicine practitioners harness the therapeutic potentials of medicinal plants to treat malaria. Many *Zanthoxylum* species have been investigated as sources of antimalarial agents. For example, an *in vitro* study by Mofor et al. (2017) reported that extract of *Z. clava-herculis* stem bark inhibited multidrug resistant strain of *P.* species with IC_{50} of 4.94 μg/ ml and with low toxicity against monkey kidney epithelial cell line. Despite the prospects, the phytochemicals responsible for the antimalarial activity and their mechanism of action are unknown. Other studies have attempted to isolate some antimalarial principles from *Z.* species. Among the compounds, alkaloids, lignans and amides dominated as active compounds. For instance, sesamine from *Z. gilletii* stem bark showed significant anti-plasmodial activities against chloroquine-sensitive Sierra Leone (D6), chloroquine-resistant Indochina (W2), and artemisinin-resistant strain (3D7) of *P. falciparum* with IC_{50} of 1.92, 3.23, and 2.94 μg/ ml, respectively (Masinde 2014). Secondary metabolites such as syncarpamide and decarine from *Z. syncarpum* stem also significantly inhibited both chloroquine-sensitive and chloroquine-resistant strains of malaria parasite; IC_{50} values of 2.04 and 1.44 μM were recorded against *P. falciparum* D6 strain and 3.06 and 0.88 μM against *P. falciparum* W2 strain (Ross et al., 2004). Syncarpamide was cytotoxic against African green monkey kidney (VERO) fibroblast cell line only at high concentration of 56 μM, outside the range of bioactivity concentrations. This suggests that the *Z. syncarpum* compounds can potentially exhibit anti-plasmodial effect with low toxicity to the host.

Based on the use of different *Z. zanthoxyloides* parts for treating malaria (Adesina 2005; Enechi et al., 2019), Goodman et al. (2019) that four alkaloids, bis-dihydrochelerythrinyl ether, skimmianine, buesgenine and chelerythrine, isolated from roots, root-bark and stem-bark exhibited anti-plasmodial activity against chloroquine-sensitive (3D7) strains of *P. falciparum* (IC_{50} values of 4.3, 0.7, 2.0, and 0.4 μg/ ml, respectively). Previous studies on other alkaloids showed that nitidine from *Z. gilletii* stem bark exhibited anti-plasmodial activity against *P. falciparum* strain FcB1 with $IC_{50} < 5$ μg/ml by halting DNA synthesis in the parasite (Zirihi et al., 2005; Zirihi et al., 2009). Moreover, 8-acetonyldihydrochelerythrine from *Z. gilletii* stem bark inhibited D6, W2, and 3D7 strains of *P. falciparum* with IC_{50} values of 4.06, 4.02, and 3.37 μg/ ml, respectively through unknown mechanisms (Masinde, 2014). Apart from alkaloids, an amide, fagaramide, isolated from *Z. gilletii* stem bark was moderately active (IC_{50} of 7.73, 15.15, and 7.72 μg/ ml) against D6, W2, and 3D7 strains of *P. falciparum*, respectively (Masinde, 2014). Another amide, pellitorine, and a furanoquinolines, γ-fagarine, from *Z. zanthoxyloides* roots, root-bark and stem-bark also inhibited 3D7 strains of *P. falciparum* with IC_{50} values of 2.2 and 2.0 μg/ ml, respectively (Goodman et al., 2019). It is possible that the anti-plasmodial compounds may have acted alone or together if present in the plant extracts used in treating malaria (Enechi et al., 2019; Amah et al., 2021). Despite the promising results, the mechanism of action of the isolated compounds are unknown and the research design in some cases did not include reference antimalarial agents for comparison. Future studies need to evaluate the clinical efficacy of the isolated anti-plasmodial compounds in susceptible populations as a treatment option for combatting resistant species of the malarial parasites.

POTENTIAL APPLICATION OF *ZANTHOXYLUM* SPECIES IN SICKLE CELL DISEASE

Sickle cell disease (SSD) is a group of genetic diseases resulting from inheritance of two abnormal copies of hemoglobin genes. The most common among them is sickle cell anemia. This disease is characterized by hemolytic anemia and occlusion of the blood vessels that reoccurs often. This occlusion is the cause of the excruciating crisis in the joints, a common occurrence in people with SSD. Upon hemolysis, hemoglobin in the erythrocytes is released as free heme (which is pro-inflammatory) and free iron (which by Fenton-type reaction interacts with hydrogen peroxide to form reactive oxygen species). These two components collectively worsen the complications associated with SSD.

Consequently, there has been increased and continuous awareness on the prevention of SSD, and improvement in treatment regimen and other intervention strategies. People who cannot afford anti-sickling drugs like hydroxyurea, nitric oxide, purified poloxamer 118 and piracetam resort to medicinal plants with history of use in subsiding crisis associated with SSD (Okpuzor et al., 2008; Amujoyegbe et al., 2016). Medicinal plants have been investigated for anti-sickling activities and several have shown promising results. Notably, some members of genus *Zanthoxylum* (e.g., *Z. zanthoxyloides*, *Z. leprieuri* and *Z. gilletii*) are among the plants with history of traditional use in managing SSD (Akakpo-Akue et al., 2020). Some studies have reported the anti-sickling activities of *Z.* species and some anti-sickling compounds in these plant species have been isolated and characterized.

Among the species, *Z. zanthoxyloides*, *Z. lemairei, Z. leprieurii, Z. tessmannii* and *Z. gilletii* have been investigated for anti-sickling activity *in vitro* (Egunyomi et al., 2009; Ouattara et al., 2009). Moreover, the specific compounds responsible for anti-sickling activity of the plant extracts were scarcely reported. Particularly, three divallinoylquinic acids (burkinabins A, B, and C) isolated from *Z. zanthoxyloides* root bark at 1.964 mg/ml inhibited sickling of deoxygenated erythrocytes by 77, 78.6 and 82.5% for burkinabins A, B, and C, respectively, which were similar to the effect of sodium chromoglycate, a reference anti-sickling agent (Ouattara et al., 2009). In addition, phenolic acids, such as syringic acid, vanillic acid, proto-catechuic acid, and *p*-hydroxy-benzoic acid, have been documented to play major roles in the anti-sickling activities of *Z. zanthoxyloides* (Nurain et al., 2017). However, this conclusion was only based on their identification in high amount in the active plant root extracts. Hence, bioassay-guided fractionation studies are needed to confirm the bioactivity of the phenolic acids, and to isolate other active compounds in the *Z.* species that showed anti-sickling activities. Generally, the ability of burkinabins A-C to demonstrate good anti-sickling properties has positioned the *Z.* species as promising sources of therapeutic agents for managing SSD. Clinical trials with the isolated anti-sickling compounds from *Z.* species are recommended and further studies are needed to modify the compounds to more potent and safer derivatives.

SAFETY OF *ZANTHOXYLUM* SPECIES

Knowing that not all things natural is safe, there is a need to be cautious in the use of natural products for food and drug. Many natural products have exhibited different levels of toxicity, including lethality at high doses (Al-Nuaimi 2018). For instance, *Z. chalybeum* root bark extract at 4,000 mg/ kg elevated serum creatinine, sodium and potassium levels in rats, as well induced histomorphological deterioration of the intestine in a manner consistent with tumor formation (Engeu et al., 2008). In addition, *in vivo* and *in vitro* studies showed that *Z. chalybeum* leaves, stem bark and root bark (2000 mg/ kg) caused mortality of mice and elicited toxicity against normal human renal epithelium cells. However, after solvent fractionation, no sign of toxicity was recorded at a maximum dose of 5,000 mg/ kg, suggesting that the toxic compounds might be acting in synergy. Furthermore, *Z. gilletii* stem bark applied in treating erectile dysfunction in South Africa and Peru was also reported to elicit histological changes in the reproductive system of male rat after oral administration daily for 14 days. Similarly, at high concentrations, *Z. zanthoxyloides* stem bark extract was genotoxic and cytotoxic against human leukocytes (Ogunbolude et al., 2014), while *Z. lepreurii* and *Z. zanthoxyloides* roots extracts were cytotoxic against normal human prostate epithelium cells (Tamdem 2019). Furthermore, *Z. zanthoxyloides* root bark induced seizure and substantial damage to the liver and kidney, resulting in mortality in mice that received large doses of the herbal materials; LD_{50} was recorded to be 5 g/ kg (Ogwal-Okeng et al., 2003). *Z. zanthoxyloides* stem bark was also reported to decrease bile release, affecting negatively the serum lipid levels of rats fed the extract (Umaru et al., 2019). In Ugandan folkloric practice, overdose of *Z. zanthoxyloides* has been recorded to cause short-duration and self-healing stomach disturbances (Anokbonggo et al., 1990). Lastly, *Z. heitzii* stem bark at doses higher than 6 g/ kg elicited toxicity and organ damage in rats (Ntchapda et al., 2015). Collectively, toxicities of the *Z.* species vary based on species, plant part, extraction solvent, dosage, level of phytochemical fractionation, and animal model studied. It is worth noting that the doses of the plant extracts that caused toxicity far exceed those that led to the desirable bioactivity. Nonetheless, caution should be taken to avoid consumption of high doses of the plant preparations to avoid deleterious effects. Furthermore, safe doses of some *Z.* species are yet to be reported and this information is necessary for traditional medicine practitioners to properly administer the natural products and for the safety of consumers.

CONSERVATION OF *ZANTHOXYLUM* SPECIES FOR FUTURE APPLICATIONS

Considering the wide use of plants in the genus *Zanthoxylum* and the risk of extinction, conservationists have been advocating for measures to minimize overexploitation, especially for the species whose roots are the most commonly used part (Mbinile et al., 2020). For example, Ouédraogo et al. (2019) showed that the chemical constituents of the stem bark and root bark of *Z. zanthoxyloides* are similar; hence, extracts from these parts are likely to have similar biological activities. This observation needs to be confirmed by comparative phytochemomics and assessment of biological activities. If the relatedness is established, scientists and traditional practitioners would have sustainable alternatives and thus minimize overharvesting of the plant root. In addition, reforestation of medicinal plants and maintenance of their cell cultures for reuse should be emphasized (Li et al., 2020). Furthermore, genetic modification of medicinal plants to become more resilient to environmental threats, such as drought, will also help to make the species more sustainable.

It is worth noting that the names of some of the plants used in the studies reviewed were the "synonyms" as shown in plant

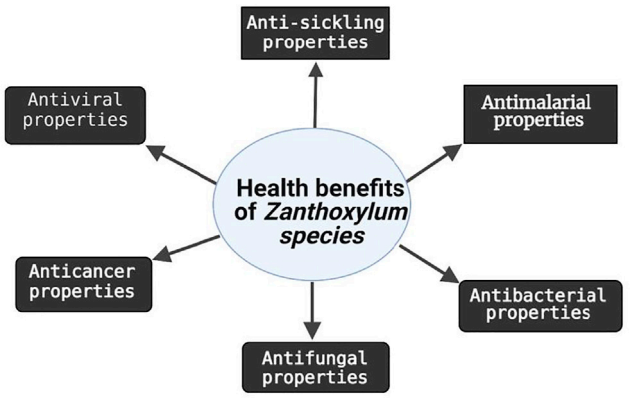


FIGURE 3 | Summary of biological activities of extracts or compounds from different parts of *Zanthoxylum* species.

databases. For example, some studies like Wu et al. (2007) and Fan et al. (2019) reported on *Zanthoxylum simulans* while Yang and Chen (2008), Yang et al. (2009), Chakthong et al. (2019), Sepsamli and Prihastanti, (2019), Lu et al. (2020a) and Lu et al. (2020b) reported on *Zanthoxylum nitidum* instead of the accepted nomenclature, "Zanthoxylum bungeanum Maxim". This issue was noted in several other papers, such as Chrian et al. (2011), Rodriguez-Guzman et al. (2011), Okafor et al. (2017), and Wansi et al. (2009). To address this issue, a recent study applied DNA barcoding for correct identification of plant species that were incorrectly named in previous studies (Veldmana et al., 2020). The authors noted that some medicinal plant researchers do not consult plant taxonomy experts and others do not confirm the plant identity by comparing their features with those available in reputable plant databases. Another possible source of this confusion is the local assignment of arbitrary names to many plant species by herbalists. This could also be associated with intraspecies and interspecies genetic diversity, and variance development because of changes in the environment (Feng et al., 2020). The issue could also be linked to the dependence of researchers in some countries on traditional medicine practitioners to provide and identify therapeutic plants. This system is not reliable. In one instance, some *Zanthoxylum* species with different chemical constituents and biological activities (*Z. bungeanum*, *Z. schinifolium*, and *Z. piperitum*) were distributed and intermixed as "*Zanthoxyli pericarpium*" by traditional medicine practitioners in Korea (Jang et al., 2020). Without proper identification, the outcome of research conducted with incorrectly labelled plant samples will be misleading. It is recommended that experts in phytotaxonomy should validate the identity of the plant species prior to further research and development. In addition, molecular characterization of the plant and the use of specific biomarkers may be helpful in ensuring that names given to plants used for phytopharmacological research are credible.

CONCLUSION

Zanthoxylum species are reservoir of phytochemicals with health-promoting properties, such as anti-sickling, anticancer and anti-infectious disease activities (**Figure 3**). The majority of the biological properties reported for *Z.* species were inspired by their traditional uses as therapeutic agents. Considering that the roots of *Z.* species are the most sourced parts in trado-medicinal uses, reforestation of the plants is highly recommended to avoid overharvesting. Similarly, the plant culture can be utilized instead of the freshly harvested plants. In many studies, drug-sensitive strains of infectious agents and cancer cells were used to assess bioactivity. Hence, future research should target the activity of *Z.* species against drug-resistant species or strains. This is important because one of the ultimate goals of new drug development is to curb drug resistance. Furthermore, several *Z.* species phytochemicals with strong bioactivities against infectious microorganisms, especially against drug-resistant strains of malarial parasites, viruses and other microbes, as well as drug-resistant cancer cells should be subjected to clinical trials as potential natural alternatives to synthetic drugs. In addition, since many studies were conducted *in vitro*, there is a dearth of information on the intestinal transport, biostability, bioaccessibility, and bioavailability of many of the active compounds. Additional research is needed to clarify the specific chemical compounds responsible for the promising biological activities of some of the plant extracts as well as the bioactivity and mechanisms of action of some of the isolated compounds. Future research should also confirm bioactivities and safety of the compounds *in vivo* using animal models and humans. Finally, researchers should endeavor to mimic the traditional methods used in preparation of the plant extract to ensure the preservation of the bioactive principles of interest.

AUTHOR CONTRIBUTIONS

IOU, JCN, and CCU contributed to conception and design of the study. EA conducted the literature search. IOU, JCN, and ECA wrote sections of the first draft of the manuscript. IOU and CCU revised the manuscript. All authors read and approved the submitted version.

REFERENCES

Abubakar, I. B., Ukwuani-Kwaja, A. N., Garba, A. D., Singh, D., Malami, I., Salihu, T. S., et al. (2020). Ethnobotanical Study of Medicinal Plants Used for Cancer Treatment in Kebbi State, North-west Nigeria. *Acta Ecologica Sinica* 40, 306–314. doi:10.1016/j.chnaes.2020.02.007

Adeeyo, A. O., Odelade, K. A., Msagati, T. A. M., and Odiyo, J. O. (2020). Antimicrobial Potencies of Selected Native African Herbs against Water Microbes. *J. King Saud Univ. - Sci.* 32, 2349–2357. doi:10.1016/j.jksus.2020.03.013

Adesina, S. K., Olugbade, T. A., Akinwusi, D. D., and Bergenthal, D. (1997). Extractives from *Zanthoxylum Lemairei* Root and Stem. *Pharmazie* 52, 720–724.

Adesina, S. (2005). The Nigerian Zanthoxylum: Chemical and Biological Values. *Afr. J. Trad. Compl. Alt. Med.* 2 (3), 282–301. doi:10.4314/ajtcam.v2i3.31128

Adewole, K. E. (2020). Nigerian Antimalarial Plants and Their Anticancer Potential: A Review. *J. Integr. Med.* 18, 92–113. doi:10.1016/j.joim.2020.01.001

Ahmad, M. U., Rahman, M. A., Huq, E., and Chowdhury, R. (2003). Alkaloids of *Zanthoxylum Budrunga*. *Fitoterapia* 74, 191–193. doi:10.1016/s0367-326x(02)00286-1

Ahsan, M., Haque, M. R., Hossain, M. B., Islam, S. N., Gray, A. I., and Hasan, C. M. (2014). Cytotoxic Dimeric Quinolone-Terpene Alkaloids from the Root Bark of *Zanthoxylum Rhetsa*. *Phytochemistry* 103, 8–12. doi:10.1016/j.phytochem.2014.03.008

Ahsan, M., Zaman, T. A., Hasan, C. M., Ito, C., and Islam, S. K. (2000). Constituents and Cytotoxicity of *Zanthoxylum Rhesta* Stem Bark. *Fitoterapia* 71, 697–700. doi:10.1016/s0367-326x(00)00214-8

Al-Nuaimi, A. A. H. D. (2018). Extracts of Plants Used as Traditional Medicines Have Toxic Effect on the Liver and Kidney. *Mojap* 5 (1), 32–41. doi:10.15406/mojap.2018.05.00161

Alam, F., Najum Us Saqib, Q., and Waheed, A. (2017). Cytotoxic Activity of Extracts and Crude Saponins from *Zanthoxylum Armatum* DC. Against Human Breast (MCF-7, MDA-MB-468) and Colorectal (Caco-2) Cancer Cell Lines. *BMC Complement. Altern. Med.* 17, 368. doi:10.1186/s12906-017-1882-1

Amah, C. C., Enechi, O. C., Ekpo, D. E., Okagu, I. U., Ononiwu, C. P., and Joshua, P. E. (2021). Safety Assessment and Antimalarial Property of Methanol Extract of *Fagara Zanthoxyloides* Root-Bark on *Plasmodium Berghei*-infected Mice. *Comp. Clin. Pathol.* 30 (1), 217–228. doi:10.1007/s00580-021-03202-7

Ameh, S. J., Tarfa, F. D., and Ebeshi, B. U. (2012). Traditional Herbal Management of Sickle Cell Anemia: Lessons from Nigeria. *Anemia*. 2012, 1–9. doi:10.1155/2012/607436

Amujoyegbe, O. O., Idu, M., Agbedahunsi, J. M., and Erhabor, J. O. (2016). Ethnomedicinal Survey of Medicinal Plants Used in the Management of Sickle Cell Disorder in Southern Nigeria. *J. Ethnopharmacol* 185, 347–360. doi:10.1016/j.jep.2016.03.042

Andima, M., Coghi, P., Yang, L. J., Wai Wong, V. K., Mutuku Ngule, C., Heydenreich, M., et al. (2019). Antiproliferative Activity of Secondary Metabolites from *Zanthoxylum Zanthoxyloides* Lam: *In Vitro* and *In Silico* Studies. *Pc* 10 (1), 44–51. doi:10.5530/pc.2020.1.8

Anokbonggo, W. W., Odoi-Adome, R., and Oluju, P. M. (1990). Traditional Methods in Management of Diarrhoeal Diseases in Uganda. *Bull. World Health Organ.* 68 (3), 359–363.

Anyanwu, M. U., and Okoye, R. C. (2017). Antimicrobial Activity of Nigerian Medicinal Plants. *J. Intercult Ethnopharmacol* 6 (2), 240–259. doi:10.5455/jice.20170106073231

Arsita, E. V., Saragih, D. E., and Aldrin, K. (2019). Anticancer Potential from Ethanol Extract of *Zanthoxylum Acanthopodium* DC. Seed to against MCF-7 Cell Line. *IOP Conf. Ser. Earth Environ. Sci.* 293, 012016. doi:10.1088/1755-1315/293/1/012016

Atawodi, S. E., Bulus, T., Ibrahim, S., Ameh, D. A., Nok, A. J., Mamman, M., et al. (2003). *In Vitro* trypanocidal Effect of Methanolic Extract of Some Nigerian savannah Plants. *Afr. J. Biotech.* 2 (9), 317–321.

Bitchagno, G. T. M., Tankeo, S. B., Tsopmo, A., Mpetga, J. D. S., Tchinda, A. T., Fobofou, S. A. T., et al. (2015). Lemairones A and B: Two New Antibacterial Tetraflavonoids from the Leaves of *Zanthoxylum Lemairei* (Rutaceae). *Phytochemistry Lett.* 14, 1–7. doi:10.1016/j.phytol.2015.08.012

Brijwal, L., Pandey, A., and Tamta, S. (2013). An Overview on Phytomedicinal Approaches of *Zanthoxylum Armatum* DC.: An Important Magical Medicinal Plant. *J. Med. Plants Res.* 7 (8), 366–370. doi:10.5897/JMPR12.743

Bukowski, K., Kciuk, M., and Kontek, R. (2020). Mechanisms of Multidrug Resistance in Cancer Chemotherapy. *Int. J. Mol. Sci.* 21 (9), 3233. doi:10.3390/ijms21093233

Bulus, T., Ahmed, A. B., Aboi, T. Y., and Danbaki, D. A. (2016). Determination of IC_{50} and IC_{90} = Ethanolic Extracts of Some Medicinal Plants against *Trypanosoma Brucei Brucei*. *Arch. Clin. Microbiol.* 7, 3. doi:10.4172/1989-8436.100048

Bunalema, L., Fotso, G. W., Waako, P., Tabuti, J., and Yeboah, S. O. (2017). Potential of *Zanthoxylum Leprieurii* as a Source of Active Compounds against Drug Resistant *Mycobacterium tuberculosis*. *BMC Complement. Altern. Med.* 17, 89. doi:10.1186/s12906-017-1602-x

Bunalema, L., Obakiro, S., Tabuti, J. R., and Waako, P. (2014). Knowledge on Plants Used Traditionally in the Treatment of Tuberculosis in Uganda. *J. Ethnopharmacol* 151, 999–1004. doi:10.1016/j.jep.2013.12.020

Burkill, H. M. (1998). *The Useful Plants of West Tropical Africa, Families M-R*. 2nd edition, Vol. 4. Kew: The Royal Botanic Garden, 1–969.

Burkill, H. M. (1985). *The Useful Plant of West Tropical Africa*. Kew-UK: Royal Botanic Gardens, 4–6.

Cao, X. L., Xu, J., Bai, G., Zhang, H., Liu, Y., Xiang, J. F., et al. (2013). Isolation of Anti-tumor Compounds from the Stem Bark of Zanthoxylum Ailanthoides Sieb. & Zucc. By Silica Gel Column and Counter-current Chromatography. *J. Chromatogr. B Analyt Technol. Biomed. Life Sci.* 929, 6–10. doi:10.1016/j.jchromb.2013.04.006

Chakthong, S., Ampaprom, R., Inparn, S., Phetkul, U., Chusri, S., Limsuwan, S., et al. (2019). New Alkylamide from the Stems of *Zanthoxylum Nitidum*. *Nat. Prod. Res.* 33 (2), 153–161. doi:10.1080/14786419.2018.1440218

Chang, C.-T., Doong, S.-L., Tsai, I.-L., and Chen, I.-S. (1997). Coumarins and Anti-HBV Constituents from Zanthoxylum Schinifolium. *Phytochemistry* 45, 1419–1422. doi:10.1016/S0031-9422(97)89023-1

Cheng, M. J., Lee, K. H., Tsai, I. L., and Chen, I. S. (2005). Two New Sesquiterpenoids and Anti-HIV Principles from the Root Bark of *Zanthoxylum Ailanthoides*. *Bioorg. Med. Chem.* 13 (21), 5915–5920. doi:10.1016/j.bmc.2005.07.050

Choi, H. J. (2016). Evaluation of Antiviral Activity of *Zanthoxylum* Species against Picornaviruses. *Osong Public Health Res. Perspect.* 7 (6), 400–403. doi:10.1016/j.phrp.2016.11.003

Chou, S. T., Peng, H. Y., Chang, C. T., Yang, J. S., Chung, H. K., Yang, S. T., et al. (2011). *Zanthoxylum Ailanthoides Sieb And Zucc*. Extract Inhibits Growth and Induces Cell Death through G2/M-phase Arrest and Activation of Apoptotic Signals in Colo 205 Human colon Adenocarcinoma Cells. *Anticancer Res.* 31, 1667–1676.

Chrian, M., Erasto, P., and Otieno, J. (2011). Antimycobacterial Activity and Cytotoxicity Effect of Extracts of Hallea Rubrostipulata and Zanthoxylum Chalybeum. *Spatula DD* 1, 147–152. doi:10.5455/spatula.20110811025348

Costa, R. S., Lins, M. O., Le Hyaric, M., Barros, T. F., and Velozo, E. S. (2017). *In Vitro* antibacterial Effects of *Zanthoxylum Tingoassuiba* Root Bark Extracts and Two of its Alkaloids against Multiresistant *Staphylococcus aureus*. *Revista Brasileira de Farmacognosia* 27, 195–198. doi:10.1016/j.bjp.2016.11.001

Cui, H. Z., Choi, H. R., Choi, D. H., Cho, K. W., Kang, D. G., and Lee, H. S. (2009). Aqueous Extract of Zanthoxylum Schinifolium Elicits Contractile and Secretory Responses via Beta1-Adrenoceptor Activation in Beating Rabbit Atria. *J. Ethnopharmacol* 126, 300–307. doi:10.1016/j.jep.2009.08.025

de A Gonzaga, W., Weber, A. D., Giacomelli, S. R., DalcolII, Hoelzel, S. C., and Morel, A. F. (2003). Antibacterial Alkaloids from *Zanthoxylum Rhoifolium*. *Planta Med.* 69, 371–374. doi:10.1055/s-2003-38882

Dofuor, A. K., Djameh, G. I., Ayertey, F., Bolah, P., Amoa-Bosompem, M., Kyeremeh, K., et al. (2019). Antitrypanosomal Effects of Zanthoxylum Zanthoxyloides (Lam.) Zepern. & Timler Extracts on African Trypanosomes. *Evid. Based Complement. Alternat Med.* 2019, 1730452. doi:10.1155/2019/1730452

Dofuor, A. K., Ayertey, F., Bolah, P., Djameh, G. I., Kyeremeh, K., Ohashi, M., et al. (2020). Isolation and Antitrypanosomal Characterization of Furoquinoline and Oxylipin from *Zanthoxylum Zanthoxyloides*. *Biomolecules* 10, 1670. doi:10.3390/biom10121670

Dos S Beirigo, P. J., Torquato, H. F., dos Santos, C. H., Carvalho, M. G., Castro, R. N., Paredes-Gamero, E. J., et al. (2016). [1-8-NαC]-Zanriorb A1, a Proapoptotic Orbitide from Leaves of Zanthoxylum Riedelianum. *J. Nat. Prod.* 79 (5), 1454–1458. doi:10.1021/acs.jnatprod.5b00177

Dzoyem, J. P., Guru, S. K., Pieme, C. A., Kuete, V., Sharma, A., Khan, I. A., et al. (2013). Cytotoxic and Antimicrobial Activity of Selected Cameroonian Edible Plants. *BMC Complement. Altern. Med.* 13, 78. doi:10.1186/1472-6882-13-78

Egunyomi, A., Moody, J. O., and Eletu, O. M. (2009). Antisickling Activities of Two Ethnomedicinal Plant Recipes Used for the Management of Sickle Cell Anaemia in Ibadan, Nigeria. *Afr. J. Biotech.* 8 (1), 20–25.

Enechi, O. C., Amah, C. C., Okagu, I. U., Ononiwu, C. P., Azidiegwu, V. C., Ugwuoke, E. O., et al. (2019). Methanol Extracts of Fagara Zanthoxyloides Leaves Possess Antimalarial Effects and Normalizes Haematological and

Biochemical Status of Plasmodium Berghei-Passaged Mice. *Pharm. Biol.* 57 (1), 577–585. doi:10.1080/13880209.2019.1656753

Engeu, P. E., Tumusiime, R., Agwaya, M., Mugisha, G., Grace, N., Galiwango, B., et al. (2008). Repeat-dose Effects of *Zanthoxylum Chalybeum* Root Bark Extract: A Traditional Medicinal Plant Used for Various Diseases in Uganda. *Afr. J. Pharm. Pharmacol.* 2 (6), 101–105.

Erichsen-Brown, C. (1979). *Use of Plants for the Past 500 Years*. Aurora, ON: Breezy Creeks Press.

Eze, F. I., Siwe-Noundou, X., Isaacs, M., Patnala, S., Osadebe, P. O., and Krause, R. W. M. (2020). Anti-cancer and Anti-trypanosomal Properties of Alkaloids from the Root Bark of *Zanthoxylum Leprieurii* Guill and Perr. *Trop. J. Pharm. Res.* 19 (11), 2377–2383. doi:10.4314/tjpr.v19i11.19

Fan, M., Tian, Y., Chen, G., Sarker, S. D., Nahar, L., Wu, J., et al. (2019). Enrichment and Analysis of Quaternary Alkaloids from Zanthoxylum Simulans Using Weak Cation Exchange Solid-phase Extraction Coupled with LC-MS. *Phytochem. Anal.* 30, 727–734. doi:10.1002/pca.28602019

Feng, S., Liu, Z., Hu, Y., Tian, J., Yang, T., and Wei, A. (2020). Genomic Analysis Reveals the Genetic Diversity, Population Structure, Evolutionary History and Relationships of Chinese Pepper. *Hortic. Res.* 7, 158. doi:10.1038/s41438-020-00376-z

Fhu, C. W., and Ali, A. (2020). Fatty Acid Synthase: An Emerging Target in Cancer. *Molecules* 25 (17), 3935. doi:10.3390/molecules25173935

Fu, Y. H., Guo, J. M., Xie, Y. T., Hua, J., Dai, Y. Y., Zhang, W., et al. (2020). Structural Characterization, Antiproliferative and Anti-inflammatory Activities of Alkaloids from the Roots of *Zanthoxylum Austrosinense*. *Bioorg. Chem.* 102, 104101. doi:10.1016/j.bioorg.2020.104101

Gbadamosi, I. T. (2015). An Inventory of Ethnobotanicals Used in the Management of Sickle Cell Disease in Oyo State, Nigeria. *Bot. Res Int* 8 (4), 65–72.

Gonçalves, M., Margarida Madureira, A., Duarte, A., Catarino, L., Monteiro, A., and Teixeira, G. (2019). *Zanthoxylum Zanthoxyloides* and *Zanthoxylum Leprieurii*: a Micromorphological, Phytochemical and Antibacterial Evaluation. *Ann. Med.* 51 (Suppl. 1), 81. doi:10.1080/07853890.2018.1561716

Goodman, C. D., Hoang, A. T., Diallo, D., Malterud, K. E., McFadden, G. I., and Wangensteen, H. (2019). Anti-plasmodial Effects of *Zanthoxylum Zanthoxyloides*. *Planta Med.* 85, 1073–1079. doi:10.1055/a-0973-0067

Guleria, S., Tiku, A. K., Koul, A., Gupta, S., Singh, G., and Razdan, V. K. (2013). Antioxidant and Antimicrobial Properties of the Essential Oil and Extracts of *Zanthoxylum Alatum* Grown in North-Western Himalaya. *ScientificWorldJournal* 2013, 790580. doi:10.1155/2013/7905802013

Ha, S. Y., Youn, H., Song, C. S., Kang, S. C., Bae, J. J., Kim, H. T., et al. (2014). Antiviral Effect of Flavonol Glycosides Isolated from the Leaf of *Zanthoxylum Piperitum* on Influenza Virus. *J. Microbiol.* 52 (4), 340–344. doi:10.1007/s12275-014-4073-5

Haakenstad, A., Harle, A. C., Tsakalos, G., Micah, A. E., Tao, T., Anjomshoa, M., et al. (2019). Tracking Spending on Malaria by Source in 106 Countries, 2000-16: an Economic Modelling Study. *Lancet Infect. Dis.* 19 (7), 703–716. doi:10.1016/S1473-3099(19)30165-3

Harahap, U., Hasibuan, P. A. Z., Sitorus, P., Arfian, N., and Satria, D. (2018). Antimigration Activity of an Ethylacetate Fraction of *Zanthoxylum Acanthopodium* DC. Fruits in 4T1 Breast Cancer Cells. *Asian Pac. J. Cancer Prev.* 19, 565–569. doi:10.22034/APJCP.2018.19.2.565

Hatano, T., Inada, K., Ogawa, T. O., Ito, H., and Yoshida, T. (2004). Aliphatic Acid Amides of the Fruits of *Zanthoxylum Piperitum*. *Phytochemistry* 65, 2599–2604. doi:10.1016/j.phytochem.2004.08.018

Hong, L., Jing, W., Qing, W., Anxiang, S., Mei, X., Qin, L., et al. (2017). Inhibitory Effect of *Zanthoxylum Bungeanum* Essential Oil (ZBEO) on *Escherichia coli* and Intestinal Dysfunction. *Food Funct.* 8, 1569–1576. doi:10.1039/C6FO01739H

Hwang, K. A., Kwon, J. E., Noh, Y., Park, B., Jeong, Y. J., Lee, S. M., et al. (2008). Effects of *Zanthoxylum Piperitum* Ethanol Extract on Osteoarthritis Inflammation and Pain. *Biomed. Pharmacother.* 105, 481–490. doi:10.1016/j.biopha.2018.05.109

Hwang, W., Kim, D., Kwon, O. S., Kim, Y. S., Ahn, B., and Kang, N. G. (2020). Topical Application of *Zanthoxylum Piperitum* Extract Improves Lateral Canthal Rhytides by Inhibiting Muscle Contractions. *Sci. Rep.* 10 (1), 21514. doi:10.1038/s41598-020-78610-w

Imaga, N. O. A., Shaire, E. A., Ogbeide, S., and Samuel, K. A. (2011). *In Vitro* biochemical Investigations of the Effects of *Carica Papaya* and *Fagara Zanthoxyloides* on Antioxidant Status and Sickle Erythrocytes. *Afr. J. Biochem. Res.* 5 (8), 226–236.

Imaga, N. O. A. (2010). The Use of Phytomedicines as Effective Therapeutic Agents in Sickle Cell Anemia. *Sci. Res. Essays* 5 (24), 3803–3807.

India State-Level Disease Burden Initiative Cancer Collaborators (2018). The burden of Cancers and Their Variations across the States of India: the Global Burden of Disease Study 1990-2016. *Lancet Oncol.* 19, 1289–1306. doi:10.1016/S1470-2045(18)30447-9

J, A.-A., T.K.M, K., Kiyinlma, C., Gnamien Marcel, A., Yvette, F., A, Y.-C., et al. (2020). Ethnobotanical Study of Medicinal Plants Used against Sickle Cell Anaemia in the Eastern Part of the Côte d'Ivoire. *J.Anim.Plant Sci.* 45 (1), 7839–7852. doi:10.35759/JAnmPlSci.v45-1.7

Jang, H. S., Jeong, B., Choi, S. Y., Lee, J., Kwon, Y. S., and Yang, H. (2020). The Rapid Discrimination and Quality Assessment of Three *Zanthoxylum* Species Using 1H NMR Spectrometry. *Int. J. Anal. Chem.* 2020, 1–7. doi:10.1155/2020/3830258

Ju, Y., Still, C. C., Sacalis, J. N., Li, J., and Ho, C. T. (2001). Cytotoxic Coumarins and Lignans from Extracts of the Northern Prickly Ash (Zanthoxylum Americanum). *Phytother Res.* 15 (5), 441–443. doi:10.1002/ptr.686

Julistiono, H., Lestari, F. G., Iryanto, R., and Lotulung, P. D. (2018). Antimycobacterial Activity of Fruit of Zanthoxylum Acanthopodium DC against M Ycobacterium Smegmatis. *Avicenna J. Phytomed* 8 (5), 432–438.

Kaigongi, M. M., Lukhoba, C. W., Yaouba, S., Makunga, N. P., Githiomi, J., and Yenesew, A. (2020). *In Vitro* antimicrobial and Antiproliferative Activities of the Root Bark Extract and Isolated Chemical Constituents of *Zanthoxylum Paracanthum* Kokwaro (Rutaceae). *Plants (Basel)* 9, 920. doi:10.3390/plants9070920

Kaminskyy, V., Lin, K. W., Filyak, Y., and Stoika, R. (2008). Differential Effect of Sanguinarine, Chelerythrine and Chelidonine on DNA Damage and Cell Viability in Primary Mouse Spleen Cells and Mouse Leukemic Cells. *Cell Biol Int* 32 (2), 271–277. doi:10.1016/j.cellbi.2007.09.004

Kamanzi Atindehou, K., Kone, M., Terreaux, C., and Traore, D. (2002). Evaluation of the Antimicrobial Potential of Medicinal Plants From the Ivory Coast. *Phytother. Res.* 16 (5), 497–502. doi:10.1002/ptr.970

Karunamoorthi, K., Sabesan, S., Jegajeevanram, K., and Vijayalakshmi, J. (2013). Role of Traditional Antimalarial Plants in the Battle against the Global Malaria burden. *Vector Borne Zoonotic Dis.* 13 (8), 521–544. doi:10.1089/vbz.2011.0946

Kim, M. H., Lee, H. J., Park, J. C., Hong, J., and Yang, W. M. (2017). *Zanthoxylum Piperitum* Reversed Alveolar Bone Loss of Periodontitis via Regulation of Bone Remodeling-Related Factors. *J. Ethnopharmacol* 195, 137–142. doi:10.1016/j.jep.2016.10.057

Kusuda, M., Inada, K., Ogawa, T. O., Yoshida, T., Shiota, S., Tsuchiya, T., et al. (2006). Polyphenolic Constituent Structures of *Zanthoxylum Piperitum* Fruit and the Antibacterial Effects of its Polymeric Procyanidin on Methicillin-Resistant *Staphylococcus aureus*. *Biosci. Biotechnol. Biochem.* 70 (6), 1423–1431. doi:10.1271/bbb.50669

Lamorde, M., Tabuti, J. R., Obua, C., Kukunda-Byobona, C., Lanyero, H., Byakika-Kibwika, P., et al. (2010). Medicinal Plants Used by Traditional Medicine Practitioners for the Treatment of HIV/AIDS and Related Conditions in Uganda. *J. Ethnopharmacol* 130, 43–53. doi:10.1016/j.jep.2010.04.004

Lassané, O., Aminata, P. N., Moussa, C., Latifou, L., and Martin, K. (2019). Stem Bark of Zanthoxylum Zanthoxylodes a Possible Substitute of Root Bark for the Conservation of the Species in Burkina Faso. *Afr. J. Biotechnol.* 18 (9), 197–205. doi:10.5897/AJB2019.16743

Lee, J.-H., Jang, M., Seo, J., and Kim, G. H. (2012). Antibacterial Effects of Natural Volatile Essential Oil Fromzanthoxylum Piperitum A.P. Dc. Against Foodborne Pathogens. *J. Food Biochem.* 36 (6), 667–674. doi:10.1111/j.1745-4514.2011.00581.x

Lee, S. J., and Lim, K. T. (2008). Glycoprotein of *Zanthoxylum Piperitum* DC Has a Hepatoprotective Effect via Anti-oxidative Character *In Vivo* and *In Vitro*. *Toxicol. Vitro* 22, 376–385. doi:10.1016/j.tiv.2007.10.002

Li, W., Sun, Y. N., Yan, X. T., Yang, S. Y., Kim, E. J., Kang, H. K., et al. (2013). Coumarins and Lignans from *Zanthoxylum Schinifolium* and Their Anticancer Activities. *J. Agric. Food Chem.* 61, 10730–10740. doi:10.1021/jf403479c

Li, Z., Tariq, A., Pan, K., Graciano, C., Sun, F., Song, D., et al. (2020). Role of glycine max in Improving Drought Tolerance in *Zanthoxylum Bungeanum*. *Peer J.* 8, e9040. doi:10.7717/peerj.9040

Liao, J., Xu, T., Zheng, J. X., Lin, J. M., Cai, Q. Y., Yu, D. B., et al. (2013). Nitidine Chloride Inhibits Hepatocellular Carcinoma Cell Growth *In Vivo* through the Suppression of the JAK1/STAT3 Signaling Pathway. *Int. J. Mol. Med.* 32 (1), 79–84. doi:10.3892/ijmm.2013.1358

Lichota, A., and Gwozdzinski, K. (2018). Anticancer Activity of Natural Compounds from Plant and marine Environment. *Int. J. Mol. Sci.* 19, 3533. doi:10.3390/ijms19113533

Lifongo, L. L., Simoben, C. V., Ntie-Kang, F., Babiaka, S. B., and Judson, P. N. (2014). A Bioactivity versus Ethnobotanical Survey of Medicinal Plants from Nigeria, West Africa. *Nat. Prod. Bioprospect* 4, 1–19. doi:10.1007/s13659-014-0005-7

Lu, Q., Li, C., and Wu, G. (2020b). Insight into the Inhibitory Effects of *Zanthoxylum Nitidum* against *Helicobacter pylori* Urease and *jack Bean* Urease: Kinetics and Mechanism. *J. Ethnopharmacol* 249, 112419. doi:10.1016/j.jep.2019.112419

Lu, Q., Ma, R., Yang, Y., Mo, Z., Pu, X., and Li, C. (2020a). *Zanthoxylum Nitidum* (Roxb.) DC: Traditional Uses, Phytochemistry, Pharmacological Activities and Toxicology. *J. Ethnopharmacol.* 260, 112946. doi:10.1016/j.jep.2020.112946

Maduka, T. O., and Ikpa, C. B. C. (2021). *Zanthoxylum Rhetsa* (Roxb.) DC.: a Systemic Review of its Ethnomedicinal Properties, Phytochemistry and Pharmacology. *WNOFNS* 37, 41–57.

Mallya, R., Malim, F., Naik, A., and Bhitre, M. (2019). Evaluation of Anthelmintic Potential of Leaves and Fruits of *Zanthoxylum Rhetsa*. *Pj* 11 (3), 475–478. doi:10.5530/pj.2019.11.75

Mann, A., Ifarajimi, O. R., Adewoye, A. T., Ukam, C., Udeme, E. E., Okorie, II, et al. (2011). *In Vivo* antitrypanosomal Effects of Some Ethnomedicinal Plants from Nupeland of North Central Nigeria. *Afr. J. Tradit Complement. Altern. Med.* 8 (1), 15–21. doi:10.4314/ajtcam.v8i1.60486

Masinde, W. R. G. (2014). *Phytochemical Investigation of* Zanthoxylum Gilletii *(Rutaceae) for Antiplasmodial Biomolecules*. Kenya: University of Nairobi.

Mbinile, S. D., Munishi, L. K., Ngondya, I. B., and Ndakidemi, P. A. (2020). Conservation and Management Challenges Facing a Medicinal Plant *Zanthoxylum Chalybeum* in Simanjiro Area, Northern Tanzania. *Sustainability* 12, 4140. doi:10.3390/su12104140

Michalkova, R., Mirossay, L., Gazdova, M., Kello, M., and Mojzis, J. (2021). Molecular Mechanisms of Antiproliferative Effects of Natural Chalcones. *Cancers (Basel)* 13, 2730. doi:10.3390/cancers13112730

Misra, L. N., Wouatsa, N. A., Kumar, S., Venkatesh Kumar, R., and Tchoumbougnang, F. (2013). Antibacterial, Cytotoxic Activities and Chemical Composition of Fruits of Two Cameroonian *Zanthoxylum* Species. *J. Ethnopharmacol.* 148, 74–80. doi:10.1016/j.jep.2013.03.069

Moerman, D. E. (1998). *Native American Ethnobotany*. Portland, OR: Timber Press.

Mofor, C. T., Zofou, D., Nangmo, L., Boyom, F. F., Fokou, E., Titanji, V. P. K., et al. (2017). Phytochemical Screening, Antiplasmodial Activity against Multi-Drug Resistant Parasites, Cytotoxicity and Antioxidant Profiling of Extracts from *Fagara Macrophylla* and *Eremomastax Speciosa*. *Int. J. Ind. Herbs Drugs* 2 (2), 1–8.

Morounke, S. G., Ayorinde, J. B., Benedict, A. O., Adedayo, F. F., Adewale, F. O., Oluwadamilare, I., et al. (2017). Epidemiology and Incidence of Common Cancers in Nigeria. *J. Cancer Biol. Res.* 5 (3), 1105.

Mugiraneza, P. J., Munyabuhoro, S., and Bajyana, S. E. (2013). *In Vitro* antimicrobial Investigation of *Zanthoxylum Chalybeum* Stem Bark. *J. Med. Plants Res.* 7 (21), 1577–1579. doi:10.5897/JMPR12.1231

Mukhija, M., Lal Dhar, K., and Nath Kalia, A. (2014). Bioactive Lignans from Zanthoxylum Alatum Roxb. Stem Bark with Cytotoxic Potential. *J. Ethnopharmacol* 152 (1), 106–112. doi:10.1016/j.jep.2013.12.039

Mukhtar, H. M., and Kalsi, V. (2018). A Review on Medicinal Properties of *Zanthoxylum Armatum* DC. *Rese. Jour. Pharm. Technol.* 11 (5), 2131–2138. doi:10.5958/0974-360X.2018.00395.5

Naylor, N. R., Atun, R., Zhu, N., Kulasabanathan, K., Silva, S., Chatterjee, A., et al. (2018). Estimating the burden of Antimicrobial Resistance: a Systematic Literature Review. *Antimicrob. Resist. Infect. Control.* 7, 58. doi:10.1186/s13756-018-0336-y

Ngane, A. N., Biyiti, L., Zollo, P. H., and Bouchet, P. (2000). Evaluation of antifungal activity of extracts of two Cameroonian Rutaceae: *Zanthoxylum leprieurii* Guill. et Perr. and *Zanthoxylum xanthoxyloides* Waterm. *J. Ethnopharmacol* 70 (3), 335–342. doi:10.1016/S0378-8741(99)00188-9

Ngoumfo, R. M., Jouda, J. B., Mouafo, F. T., Komguem, J., Mbazoa, C. D., Shiao, T. C., et al. (2010). *In Vitro* cytotoxic activity of isolated acridones alkaloids from Zanthoxylum leprieurii Guill. et Perr. *Bioorg. Med. Chem.* 18, 3601–3605. doi:10.1016/j.bmc.2010.03.040

Nigussie, D., Davey, G., Tufa, T. B., Brewster, M., Legesse, B. A., Fekadu, A., et al. (2021). Antibacterial and Antifungal Activities of Ethiopian Medicinal Plants: a Systematic Review. *Front. Pharmacol.* 12, 633921. doi:10.3389/fphar.2021.633921

Nogueira, J., Vinturelle, R., Mattos, C., Tietbohl, L. A., Santos, M. G., Junior, I. S., et al. (2014b). Acaricidal Properties of the Essential Oil from *Zanthoxylum Caribaeum* against *Rhipicephalus Microplus*. *J. Med. Entomol.* 51 (5), 971–975. doi:10.1603/me13236

Ntchapda, F., Maguirgue, K., Kemeta, A. D. R., Jean, M., Djedouboum, A., and Dimo, T. (2015). Acute Toxicity Studies of the Stem Bark Extract of *Zanthoxylum Heitzii* A. & P. (Rutaceae) on Haematological Parameters, and Body Temperature. *Int. J. Pharma Res. Health Sci.* 3 (2), 630–642.

Numonov, S., Sharopov, F., Salimov, A., Sukhrobov, P., Atolikshoeva, S., Safarzoda, R., et al. (2019). Assessment of Artemisinin Contents in Selected *Artemisia* Species from Tajikistan (Central Asia). *Medicines (Basel)* 6, 23. doi:10.3390/medicines6010023

Nurain, I. O., Bewaji, C. O., Johnson, J. S., Davenport, R. D., and Zhang, Y. (2017). Potential of Three Ethnomedicinal Plants as Antisickling Agents. *Mol. Pharm.* 14 (1), 172–182. doi:10.1021/acs.molpharmaceut.6b00767

Obakiro, S. B., Kiprop, A., Kowino, I., Kigondu, E., Odero, M. P., Omara, T., et al. (2020). Ethnobotany, Ethnopharmacology, and Phytochemistry of Traditional Medicinal Plants Used in the Management of Symptoms of Tuberculosis in East Africa: A Systematic Review. *Trop. Med. Health* 48, 68. doi:10.1186/s41182-020-00256-1

Ochwang'i, D. O., Kimwele, C. N., Oduma, J. A., Gathumbi, P. K., Mbaria, J. M., and Kiama, S. G. (2014). Medicinal Plants Used in Treatment and Management of Cancer in Kakamega County, Kenya. *J. Ethnopharmacol* 151 (3), 1040–1055. doi:10.1016/j.jep.2013.11.051

Ogunbolude, Y., Ibrahim, M., Elekofehinti, O. O., Adeniran, A., Abolaji, A. O., Rocha, J. B., et al.(2014). Effects of *Tapinanthus Globiferus* and *Zanthoxylum Zanthoxyloides* Extracts on Human Leukocytes *In Vitro*. *J. Intercult Ethnopharmacol* 3 (4), 167–172. doi:10.5455/jice.20140826110059

Ogwal-Okeng, J. W., Obua, C., and Anokbonggo, W. W. (2003). Acute Toxicity Effects of the Methanolic Extract of *Fagara Zanthoxyloides* (Lam.) Root-Bark. *Afr. Health Sci.* 3 (3), 124–126.

Okafor, S. N., Chibueze, G. C., Obonga, W. O., Ezeokonkwo, M. A., and Muogbo, C. C. (2017). Aqueous Methanol Extract of the Root Bark of *Zanthoxylum Zanthoxyloides* Provides Natural Remedy for Dental Caries and Toothache. *World J. Pharm. Res.* 6 (7), 336–349.

Okagu, I. U., Ndefo, J. C., Aham, E. C., and Udenigwe, C. C. (2021). *Zanthoxylum* Species: a Comprehensive Review of Traditional Uses, Phytochemistry, Pharmacological and Nutraceutical Applications. *Molecules* 26 (13), 4023. doi:10.3390/molecules26134023

Okpuzor, J., Adebesin, O., Ogbunugafor, H., and Amadi, I. (2008). The Potential of Medicinal Plants in Sickle Cell Disease Control: a Review. *Int. J. Biomed. Health Sci.* 4 (2), 47–55.

Okwor, O. H., Ogugua, V. N., and Okagu, I. U. (2020). Therapeutic Evaluation of Anti-trypanosoma Activity of Ethanol Extracts of *Jatropha Curcas* Roots in Comparison with Diminazene Aceturate in *Trypanosoma Brucei Brucei*-Parasitized Rats. *Comp. Clin. Pathol.* 29 (6), 1189–1198. doi:10.1007/s00580-020-03171-3

Omara, T. (2020). Antimalarial Plants Used across Kenyan Communities. *Evid. Based Complement. Alternat Med.* 2020, 4538602. doi:10.1155/2020/4538602

Omosa, L. K., Mbogo, G. M., Korir, E., Omole, R., Seo, E.-J., Yenesew, A., et al. (2019). Cytotoxicity of Fagaramide Derivative and Canthin-6-One from *Zanthoxylum* (Rutaceae) Species against Multidrug Resistant Leukemia Cells. *Nat. Product. Res.* 35, 579–586. doi:10.1080/14786419.2019.1587424

Ouattara, B., Jansen, O., Angenot, L., Guissou, I. P., Frédérich, M., Fondu, P., et al. (2009). Antisickling Properties of Divanilloylquinic Acids Isolated from *Fagara Zanthoxyloides* Lam. (Rutaceae). *Phytomedicine* 16 (2-3), 125–129. doi:10.1016/j.phymed.2008.10.013

Pachón, G., Rasoanaivo, H., Azqueta, A., Rakotozafy, J. C., Raharisololalao, A., de Cerain, A. L., et al. (2007). Anticancer Effect of a New Benzophenanthridine Isolated from *Zanthoxylum Madagascariense* (Rutaceline). *In Vivo* 21, 417–422.

Patiño, L. O. J., Prieto, R. J. A., and Cuca, S. L. E. (2012). "*Zanthoxylum* Genus as Potential Source of Bioactive Compounds," in *Bioactive Compounds in Phytomedicine. Rasooli I* (Croatia: InTech Open), 185–218. doi:10.5772/26037

Paul, A., Kumar, A., Singh, G., and Choudhary, A. (2018). Medicinal, Pharmaceutical and Pharmacological Properties of *Zanthoxylum Armatum*: a Review. *J. Pharmacogn Phytochem.* 7 (4), 892–900.

Pavani, P., and Naika, R. (2020). Evalution of Anti-bacterial Activity of *Zanthoxylum Ovalifolium* Wight (Rutaceae) against Selected Pathogenic Bacteria. *Plant Arch.* 20 (1), 2591–2594.

Pawar, H. A. (2014). Natural Product as a Source of lead to the Design of New Drugs. *Nat. Prod. Chem. Res. 2014* 2, 156. doi:10.4172/2329-6836.1000156

Phuyal, N., Jha, P. K., Raturi, P. P., and Rajbhandary, S. (2020). *In Vitro* antibacterial Activities of Methanolic Extracts of Fruits, Seeds, and Bark of *Zanthoxylum Armatum* DC. *J. Trop. Med.* 2020, 2803063. doi:10.1155/2020/2803063

Pieme, C. A., Santosh, G. K., Tekwu, E. M., Askun, T., Aydeniz, H., Ngogang, J. Y., et al. (2014). Fruits and Barks Extracts of Zanthozyllum Heitzii a Spice from Cameroon Induce Mitochondrial Dependent Apoptosis and Go/G1 Phase Arrest in Human Leukemia HL-60 Cells. *Biol. Res.* 47, 54. doi:10.1186/0717-6287-47-54

Pistritto, G., Trisciuoglio, D., Ceci, C., Garufi, A., and D'Orazi, G. (2016). Apoptosis as Anticancer Mechanism: Function and Dysfunction of its Modulators and Targeted Therapeutic Strategies. *Aging (Albany NY)* 8 (4), 603–619. doi:10.18632/aging.100934

Rahman, M. A., Hannan, M. A., Dash, R., Rahman, M. H., Islam, R., Uddin, M. J., et al. (2021). Phytochemicals as a Complement to Cancer Chemotherapy: Pharmacological Modulation of the Autophagy-Apoptosis Pathway. *Front. Pharmacol.* 12, 639628. doi:10.3389/fphar.2021.639628

Rahman, M. M., Gray, A. I., Khondkar, P., and Islam, M. A. (2008). Antimicrobial Activities of Alkaloids and Lignans from *Zanthoxylum Budrunga*. *Nat. Prod. Comm.* 3 (1), 45–47. doi:10.1177/1934578x0800300110

Redza-Dutordoir, M., and Averill-Bates, D. A. (2016). Activation of Apoptosis Signalling Pathways by Reactive Oxygen Species. *Biochim. Biophys. Acta* 1863 (12), 2977–2992. doi:10.1016/j.bbamcr.2016.09.012

Rodríguez-Guzmán, R., Fulks, L. C., Radwan, M. M., Burandt, C. L., and Ross, S. A. (2011). Chemical Constituents, Antimicrobial and Antimalarial Activities of *Zanthoxylum Monophyllum*. *Planta Med.* 77, 1542–1544. doi:10.1055/s-0030-1270782

Ross, S. A., Sultana, G. N., Burandt, C. L., ElSohly, M. A., Marais, J. P., and Ferreira, D. (2004). Syncarpamide, a New Antiplasmodial (+)-norepinephrine Derivative from *Zanthoxylum Syncarpum*. *J. Nat. Prod.* 67, 88–90. doi:10.1021/np030417t

Sandjo, L. P., Kuete, V., Tchangna, R. S., Efferth, T., and Ngadjui, B. T. (2014). Cytotoxic Benzophenanthridine and Furoquinoline Alkaloids from *Zanthoxylum Buesgenii* (Rutaceae). *Chem. Cent. J.* 8, 61. doi:10.1186/s13065-014-0061-4

Santhanam, R. K., Ahmad, S., Abas, F., Safinar Ismail, I., Rukayadi, Y., Tayyab Akhtar, M., et al. (2016). Bioactive Constituents of *Zanthoxylum Rhetsa* Bark and its Cytotoxic Potential against B16-F10 Melanoma Cancer and normal Human Dermal Fibroblast (HDF) Cell Lines. *Molecules* 21, 652. doi:10.3390/molecules21060652

Sepsamli, L., and Prihastanti, J. E. (2019). **Ethnobotany of Balimo (*Zanthoxylum Nitidum)* in the Kanayatn Dayak Community in Tapakng, West Kalimantan**. *Biosaintifika* 11 (3), 318–324. doi:10.15294/biosaintifika.v11i3.20688

Seukep, J. A., Ngadjui, B., and Kuete, V. (2015). Antibacterial Activities of *Fagara Macrophylla, Canarium Schweinfurthii, Myrianthus Arboreus, Dischistocalyx Grandifolius* and *Tragia Benthamii* against Multi-Drug Resistant Gram-Negative Bacteria. *SpringerPlus* 4, 567. doi:10.1186/s40064-015-1375-y

Shaw, A., Robays, J., Fèvre, E. M., Lutumba, P., and Boelaert, M. (2010). "The Burden of Human African Trypanosomiasis," in *The Burden of Human African Trypanosomiasis. Handbook of Disease Burdens and Quality of Life Measures*. Editors V. R. Preedy and R. R. Watson (New York, NY: Springer), 1433–1442. doi:10.1007/978-0-78665-0_8310.1007/978-0-387-78665-0_83

Simarro, P. P., Diarra, A., Ruiz Postigo, J. A., Franco, J. R., and Jannin, J. G. (2011). The Human African Trypanosomiasis Control and Surveillance Programme of the World Health Organization 2000-2009: the Way Forward. *Plos Negl. Trop. Dis.* 5 (2), e1007. doi:10.1371/journal.pntd.0001007

Sinan, K. I., Zengin, G., Bene, K., and Mahomoodally, M. F. (2019). Chemistry and Pharmacology of Three Antiplasmodial Traditional Medicinal Plants from Tropical Africa - A Review. *South Afr. J. Bot.* 126, 265–276. doi:10.1016/j.sajb.2019.04.024

Smittenaar, C. R., Petersen, K. A., Stewart, K., and Moitt, N. (2016). Cancer Incidence and Mortality Projections in the UK until 2035. *Br. J. Cancer* 115, 1147–1155. doi:10.1038/bjc.2016.304

Supabphol, R., and Tangjitjareonkun, J. (2014). Chemical Constituents and Biological Activities of *Zanthoxylum Limonella* (Rutaceae): A Review. *Trop. J. Pharm. Res.* 13 (12), 2119–2130. doi:10.4314/tjpr.v13i12.25

Tabuti, J. R., Kukunda, C. B., and Waako, P. J. (2010). Medicinal Plants Used by Traditional Medicine Practitioners in the Treatment of Tuberculosis and Related Ailments in Uganda. *J. Ethnopharmacol* 127, 130–136. doi:10.1016/j.jep.2009.09.035

Tamdem, S. B. G. (2019). *Phytochemical, Chemopreventive and Antimalarial Activity Evaluation of Five Selected Medicinal Plants from the Cameroonian flora*. UK: Liverpool John Moores University.

Tantapakul, C., Phakhodee, W., Ritthiwigrom, T., Yossathera, K., Deachathai, S., and Laphookhieo, S. (2012). Antibacterial Compounds from *Zanthoxylum Rhetsa*. *Arch. Pharm. Res.* 35 (7), 1139–1142. doi:10.1007/s12272-012-0703-9

Tian, Y., Zhang, C., and Guo, M. (2017). Comparative Study on Alkaloids and Their Anti-proliferative Activities from Three Zanthoxylum Species. *BMC Complement. Altern. Med.* 17 (1), 460. doi:10.1186/s12906-017-1966-y

Tine, Y., Diop, A., Diatta, W., Desjobert, J. M., Boye, C. S., Costa, J., et al. (2017). Chemical Diversity and Antimicrobial Activity of Volatile Compounds from *Zanthoxylum Zanthoxyloides* Lam. According to Compound Classes, Plant Organs and Senegalese Sample Locations. *Chem. Biodivers* 14 (1), e1600125. doi:10.1002/cbdv.201600125

Udenigwe, C. C., Abioye, R. O., Okagu, I. U., and Obeme-Nmom, J. I. (2021). Bioaccessibility of Bioactive Peptides: Recent Advances and Perspectives. *Curr. Opin. Food Sci.* 39, 182–189. doi:10.1016/j.cofs.2021.03.005

Umaru, H. A., Clarkson, W. P., and Christopher, K. (2019). Effect of Stem Bark Extract of *Fagara Zanthoxyloides* on Bile Secretion in Rats. *Am. J. Res. Comm.* 7 (7), 1–9.

Veldman, S., Ju, Y., Otieno, J. N., Abihudi, S., Posthouwer, C., Gravendeel, B., et al. (2020). DNA Barcoding Augments Conventional Methods for Identification of Medicinal Plant Species Traded at Tanzanian Markets. *J. Ethnopharmacol* 250, 112495. doi:10.1016/j.jep.2019.112495

Verma, K. K., Kumar, B., Raj, H., and Sharma, A. (2021). A Review on Chemical Constituents, Traditional Uses, Pharmacological Studies of *Zanthoxylum Armatum* (Rutaceae). *J. Drug Deliv. Ther.* 11 (2-S), 136–142. doi:10.22270/jddt.v11i2-S.4786

Wang, Y., Gao, D., Chen, Z., Li, S., Gao, C., Cao, D., et al. (2013). Acridone Derivative 8a Induces Oxidative Stress-Mediated Apoptosis in CCRF-CEM Leukemia Cells: Application of Metabolomics in Mechanistic Studies of Antitumor Agents. *PLoS One* 8 (5), e63572. doi:10.1371/journal.pone.0063572

Wang, Y., Zhao, C. M., Guo, T., Zhu, Y. L., and Zhao, P. (2015). Preliminary Screening of 44 Plant Extracts for Anti-tyrosinase and Antioxidant Activities. *Pak J. Pharm. Sci.* 28 (5), 1737–1744.

Wansi, J. D., Nwozo, S. O., Mbaze, L. M., Devkota, K. P., Donkwe Moladje, S. M., Fomum, Z. T., et al. (2009). Amides from the Stem Bark of *Fagara Macrophylla*. *Planta Med.* 75, 517–521. doi:10.1055/s-0029-1185327

Wansi, J. D., Tadjong Tcho, A., Toze, F. A. A., Nahar, L., Martin, C., and Sarker, S. D. (2016). Cytotoxic Acridone and Indoloquinazoline Alkaloids from *Zanthoxylum Poggei*. *Phytochemistry Lett.* 17, 293–298. doi:10.1016/j.phytol.2016.08.010

Wei, W. J., Chen, X. H., Guo, T., Liu, X. Q., Zhao, Y., Wang, L. L., et al. (2021). A Review on Classification and Biological Activities of Alkaloids from the Genus *Zanthoxylum* Species. *Mini Rev. Med. Chem.* 21 (3), 336–361. doi:10.2174/1389557520666200910091905

World Health Organization (Who) (2019). World Malaria Report 2019 Availableat: https://www.who.int/publications/i/item/world-malaria-report-2019/ Accessedon.October 2, 2020 .

Wouatsa, V. N., Misra, L., Kumar, S., Prakash, O., Khan, F., Tchoumbougnang, F., et al. (2013). Aromatase and Glycosyl Transferase Inhibiting Acridone Alkaloids from Fruits of Cameroonian *Zanthoxylum* Species. *Chem. Cent. J.* 7, 125. doi:10.1186/1752-153X-7-125

Wu, J., Mei, W. L., and Dai, H. F. (2007). A New Monoterpenoid Glycoside from Roots of *Zanthoxylum Simulans*. *Chin. Tradit Herb Drug* 38 (4), 488–490. doi:10.1002/chin.200713185

Yang, C. H., Cheng, M. J., Lee, S. J., Yang, C. W., Chang, H. S., and Chen, I. S. (2009). Secondary Metabolites and Cytotoxic Activities from the Stem Bark of *Zanthoxylum Nitidum*. *Chem. Biodivers* 6, 846–857. doi:10.1002/cbdv.200800107

Yang, G., and Chen, D. (2008). Alkaloids from the Roots of *Zanthoxylum Nitidum* and Their Antiviral and Antifungal Effects. *Chem. Biodivers* 5, 1718–1722. doi:10.1002/cbdv.200890160

Ynalvez, R. A., Cardenas, C., Addo, J. K., Adukpo, G. E., Dadson, B. A., and Addo-Mensa, A. (2012). Evaluation of the Antimicrobial Activity of *Zanthoxylum Zanthoxyloides* Root Bark Extracts. *Res. J. Med. Plant* 6 (2), 149–159. doi:10.3923/rjmp.2012.149.159

Yuan, H. M., Qiu, L., Xie, Z. J., Zou, L., Zheng, J., and Fu, Q. (2015). Research Progress on Alkaloids Constituents from Zanthoxylum and Their Pharmacological Activities. *Zhongguo Zhong Yao Za Zhi* 40 (23), 4573–4584. doi:10.4268/cjcmm20152309

Yusuf, M., Chowdhury, J. U., Wahab, M. A., and Begum, J. (1994). *Medicinal Plants of Bangladesh*. Chittagong, Bangladesh: BCSIR Laboratories.

Zhang, M., Wang, J., Zhu, L., Li, T., Jiang, W., Zhou, J., et al. (2017a). *Zanthoxylum Bungeanum* Maxim. (Rutaceae): A Systematic Review of its Traditional Uses, Botany, Phytochemistry, Pharmacology, Pharmacokinetics, and Toxicology. *Int. J. Mol. Sci.* 18 (10), 2172. doi:10.3390/ijms18102172

Zhang, Y., Dong, H., Zhang, J., and Zhang, L. (2017b). Inhibitory Effect of Hyperoside Isolated from *Zanthoxylum Bungeanum* Leaves on SW620 Human Colorectal Cancer Cells via Induction of the P53 Signaling Pathway and Apoptosis. *Mol. Med. Rep.* 16 (2), 1125–1132. doi:10.3892/mmr.2017.6710

Zirihi, G. N., Mambu, L., Guédé-Guina, F., Bodo, B., and Grellier, P. (2005). *In Vitro* antiplasmodial Activity and Cytotoxicity of 33 West African Plants Used for Treatment of Malaria. *J. Ethnopharmacol* 98, 281–285. doi:10.1016/j.jep.2005.01.004

Zirihi, G. N., N'guessan, K., Etien, D. T., and Serikouassi, B. P. H. (2009). Evaluation *In Vitro* of Antiplasmodial Activity of Ethanolic Extracts of *Funtumia Elastica, Rauvolfia Vomitoria* and *Zanthoxylum Gilletii* on *Plasmodium Falciparum* Isolates from Côted'Ivoire. *J. Anim. Plant Sci.* 5 (1), 406–413.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 27 September 2021
doi: 10.3389/fphar.2021.739758

The Potential Benefits of Using Garlic Oil and Its Active Constituent, Dially Disulphide, in Combination With Carvedilol in Ameliorating Isoprenaline-Induced Cardiac Damage in Rats

Syed Mohammed Basheeruddin Asdaq[1]*, *Obulesu Challa*[2], *Abdulhakeem S. Alamri*[3,4], *Walaa F. Alsanie*[3,4], *Majid Alhomrani*[3,4] and *Mohammed Asad*[5]

[1]Department of Pharmacy Practice, College of Pharmacy, AlMaarefa University, Riyadh, Saudi Arabia, [2]Department of Pharmacology, Krupanidhi College of Pharmacy, Bangalore, India, [3]Department of Clinical Laboratory Sciences, the Faculty of Applied Medical Sciences, Taif University, Taif, Saudi Arabia, [4]Centre of Biomedical Sciences Research (CBSR), Deanship of Scientific Research, Taif University, Taif, Saudi Arabia, [5]College of Applied Medical Sciences, Shaqra University, Shaqra, Saudi Arabia

OPEN ACCESS

Edited by:
Alessandra Durazzo,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Roisin Kelly-Laubscher,
University College Cork, Ireland
Mohamed Fizur Nagoor Meeran,
United Arab Emirates University, United Arab Emirates
Tarique Mahmood,
Integral University, India

***Correspondence:**
Syed Mohammed Basheeruddin Asdaq
sasdaq@gmail.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 11 July 2021
Accepted: 20 August 2021
Published: 27 September 2021

Citation:
Asdaq SMB, Challa O, Alamri AS, Alsanie WF, Alhomrani M and Asad M (2021) The Potential Benefits of Using Garlic Oil and Its Active Constituent, Dially Disulphide, in Combination With Carvedilol in Ameliorating Isoprenaline-Induced Cardiac Damage in Rats. Front. Pharmacol. 12:739758. doi: 10.3389/fphar.2021.739758

Garlic oil and its primary component, diallyl disulphide (DADS), were tested in rats with isoprenaline (ISO) induced myocardial infarction for cardioprotective benefits when combined with carvedilol. Garlic oil (GO) was administered to rats (Sprague-dawley strain) at two doses of 50 and 100 mg/kg body weight, whereas DADS was given in two doses of 4.47 and 8.94 mg/kg, respectively. The animals were given oral doses of garlic oil and DADS on alternate days for 3 weeks, either alone or in combination with carvedilol (2 mg/kg). Cardiac injury was done by administering two doses of isoprenaline (150 mg/kg, sc) to all treated groups except the first, which served as a control. Biomarkers of cardiac injury and histological investigations were studied for their potential in reducing ISO-induced myocardial damage. Animals pretreated with GO, DADS, and carvedilol had significantly ($p < 0.01$) lowered heart weight and heart to body weight ratio. In rats treated with carvedilol plus high dosages of garlic oil (100 mg/kg, p.o) and DADS (8.94 mg/kg, p.o) compared to the ISO control and carvedilol group, the activities of SOD and Catalase were enhanced in cardiac tissue homogenate. When compared to ISO control and carvedilol group, the activities of LDH and CK-MB were elevated in heart tissue homogenate with a simultaneous reduction in their serum levels in animals treated with a combination of carvedilol with high doses of garlic oil (100 mg/kg, p.o) and DADS (8.94 mg/kg, p.o). Overall, combining garlic oil or DADS with carvedilol improved the cardioprotective effect of carvedilol and protected rats from ISO-induced myocardial infarction. However, more research is needed to establish the mechanism of garlic oil and DADS interaction with carvedilol.

Keywords: GC-MS, antioxidants, garlic oil, diallyl disulphide, herbal supplements, carvedilol cellular damage

INTRODUCTION

Consumption of herbal medicines or nutritional supplements along with modern medicine is a common practice throughout the world. This is done with the belief that the addition of herb(s) or nutrient(s) to drug regimen may provide extra beneficial effects and/or reduce adverse effects of the drugs (Shaikh et al., 2020). The effect of many herbs and nutrients on the pharmacological effects of drugs is unknown, though some herbs and nutrients have been reported to affect the overall pharmacological effects of drugs due to pharmacodynamic and/or pharmacokinetic interactions (Ronis et al., 2018).

Myocardial infarction (MI), generally known as a heart attack, is one of the most dreadful disorders. According to estimates, MI will kill approximately 23.3 million individuals by 2030. Mortality due to MI is increasing at an alarming rate in Asian countries such as India and Malaysia (Xu et al., 2016; Rosello et al., 2017). Atherosclerosis is the most prevalent cause of MI, which generates a discrepancy in production and consumption in the myocardium, leading to hypoxic and waste product accumulation, that eventually leads to mycoyte mortality (Kumar et al., 2016). Despite this, the pathophysiology of MI remains a mystery. Inflammation and necrosis, on the other hand, have been identified as key factors in MI in a number of investigations (Goyal et al., 2015; Othman et al., 2017).

Isoprenaline (ISO) is a non-selective agonist for β adrenergic receptors. Administration of ISO at a higher concentration leads to a fall in myocardial compliance due to ischemic damage, and it is one of the most widely used models for assessing the cardioprotective efficacy of new drugs and studying the pathological consequences of human myocardial impairment (Zhang et al., 2005).

Garlic bulbs from the plant *Allium sativum* (family-Amaryllidaceae) are common flavouring agent added to different food recipes. Garlic is also known to possess excellent medicinal properties. The garlic bulb and its different preparations, such as garlic oil, garlic powder, and various garlic extracts, are mentioned for their therapeutic benefits in different traditional systems of medicine (Adaki et al., 2014). Further, the pharmacological and therapeutic properties of garlic and its constituents have been investigated by several authors earlier (Chang et al., 2011; Lissiman et al., 2014; Hou et al., 2015; Nicastro et al., 2015; El-Saber Batiha et al., 2015). We have earlier reported cardiovascular actions and interactions of garlic and its different preparations with several drugs. Garlic showed antihypertensive effect and enhanced bioavailability and antihypertensive effects of propranolol and hydrochlorthiazide in our earlier studies (Asdaq and Inamdar, 2009; Asdaq and Inamdar, 2011a; Asdaq and Inamdar, 2011b). Garlic and its active constituent s-allyl cysteine also enhanced the cardioprotective and antihypertensive effects of captopril (Asdaq and Inamdar, 2010). We also reported that garlic, aged garlic extract and s-allyl cysteine showed antioxidant and hypolipidemic effects that were influenced by the administration of conventional antihypertensives (Asdaq et al., 2009b; Asdaq, 2015). Recently, we reported the interaction of aged garlic extract and its constituent s-allyl csyteine on isoprenaline induced myocardial infarction in rats (Asdaq et al., 2021).

The active constituents of garlic and the most effective garlic preparation with cardiovascular benefits are not correctly known. Different studies have given conflicting reports on the active constituents and the best garlic preparation for cardiovascular benefits. Garlic constituents with potent antioxidant action such as s-allylcysteine and s-allylmercaptocysteine are reported as the constituents responsible for the beneficial effect of garlic on the cardiovascular system. Earlier, allicin (allyl 2-propenethiosulfinate) was believed to be the constituent with a cardioprotective effect, but it is a highly unstable compound and it gets converted to s-allylcysteine and s-allylmercaptocysteine in the body. Garlic oil is a typical garlic product that has been shown to improve anti-oxidant enzyme activity (Ko et al., 2016). Active components of garlic oil, such as diallyl sulphide (DAS), and diallyl disulphide (DADS), have been found to protect and treat oxidative damage (Guan et al., 2018). Garlic oil has been demonstrated to aid weight loss by reducing LDL cholesterol levels (Yang et al., 2018). Because hyperlipidemia is a known cause of MI, we want to learn more about the role of garlic oil in preventing ischemia damage, as well as whether its main active ingredient, diallyl disulphide (DADS), has similar or different cardioprotective properties.

Beta-adrenergic blockers are a class of drugs with multiple pharmacological actions. Carvedilol is a non-selective beta-blocker that also blocks alpha$_1$-adrenergic receptors, providing cardioprotective action with vasodilation. It is used in the treatment of hypertension, angina pectoris, cardiac arrhythmias, and also as an antioxidant and antiproliferative agent. Carvedilol is used in the treatment of left ventricular dysfunction and congestive heart failure. Apart from these, it also has a lipid lowering effect and augments renal dysfunction, indicating wide spread use of this non-selective beta-blocker (Singh and Preuss, 2021).

Continuing our efforts to determine the beneficial effects of garlic preparations and their influence on the effects of drugs affecting cardiovascular functions, the present study determined the effect of garlic oil and diallyl disulphide (DADS) on the cardioprotective effect of carvedilol on isoprenaline induced myocardial infarction in rats.

MATERIALS AND METHODS

Experimental Animals

Female Sprague-Dawley rats weighing 150–200 g were housed at 25 ± 5 °C in a well-ventilated animal house under 12:12 h light dark cycle. The rats had free access to standard rat chow (Amrut Laboratory Animal Feed, Maharashtra, India) containing (% w/w) protein 22.10, oil 4.13, fiber 3.15, ash 5.15, sand (silica) 1.12 and water *ad libitum*. The institutional animal ethics committee of Krupanidhi College of Pharmacy (KCP/IAEC-27) approved the experimental protocol and animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

Materials

Sigma Aldrich (United States) provided the garlic oil, which was GC-MS standardized for the presence of DADS. The CK-MB and LDH

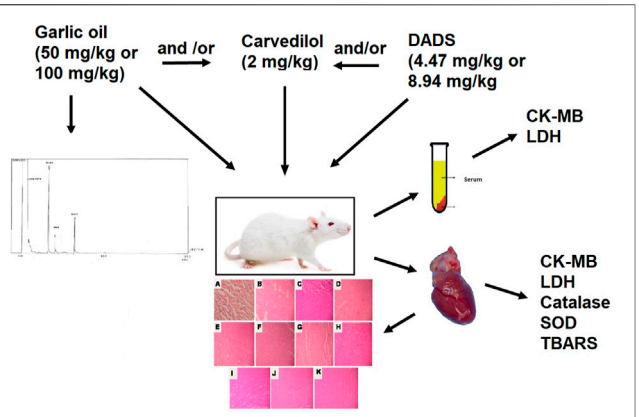


FIGURE 1 | Schematic representation of procedure followed.

kits were provided by Crest Biosystems and Coral Clinical Systems in Goa, India. All of the compounds utilized in this investigation were analytical grade and came from a standard source.

Quantification of DADS in Garlic Oil by GC-MS

A Hewlett Packard 5890 II GC with an HP 5972 Mass selective detector and HP-5ms capillary column (30 m 0.25 mm, film thickness 0.25 m) was used to assay the garlic oil sample for DADS. The injector and detector were adjusted to 220 and 290°C, respectively. The temperature of the column was raised from 50 to 220°C at a rate of 3°C/min for 10 min. At a flow rate of 1 ml/min, helium was used as the carrier gas. In the splitless method, 1 µL (1.0 L) of the concentrate was manually injected into 10 ml of each organic extract. An electron ionization device with a 70 eV ionization energy was employed for GC/MS detection. Temperatures for the injector and MS transfer line were set at 220 and 290°C, respectively. The chemicals were tentatively identified by comparing their mass spectra to those of the NIST 98 and Wiley 275 library data of the GC-MS system (Kimbaris et al., 2006).

Dose Selection

Garlic Oil (50/100 mg/kg, *p.o*) (Kuo et al., 2011) and carvedilol (2 mg/kg, *p.o*) (Watanabe et al., 2002) doses were selected from previous studies. The required doses of DADS (4.47/8.94 mg/kg) were selected based on GC-MS peaks of garlic oil (**Figure 1** and **Figure 2**) Experimental protocol.

Experimental Protocol

There were eleven groups of experimental animals used in this study, each having six rats. Group I was kept as normal control and given a vehicle (1 ml/kg, *p.o*) on alternate days for 3 weeks. The vehicle used was acacia (2% w/v). Groups 2 to 11 received the following treatments on alternate days for 3 weeks, followed by administration of two doses of isoprenaline (ISO) at a dose of 150 mg/kg subcutaenously for two consecutive days (Pipaliya et al., 2012). Group 2 was named ISO control and received vehicle (1 ml/kg, *p.o*) while Group 3 was administered with carvedilol (2 mg/kg, *p.o*). Groups 4 and 5 received garlic oil orally at a dose of 50 mg/kg (low dose) and 100 mg/kg (high dose) respectively, while groups 6 and 7 received a combination of carvedilol with a low dose and high dose of garlic oil respectively. Similarly, groups 8 and 9 received a low dose of DADS (4.47 mg/kg) and a high dose of DADS (8.94 mg/kg) and groups 10 and 11 received a combination of carvedilol with a low dose of DADS and a high dose of DADS respectively. A schematic diagram of the procedure is given in **Figure 1**.

As mentioned above, animals in all groups except group-1 received ISO (150 mg/kg, s.c) for two consecutive days. After 48 h of the first dose of ISO, blood was withdrawn from animals under anesthesia induced by a combination of ketamine hydrochloride (75 mg/kg, i.p) and xylazine (10 mg/kg, i.p) (Wellington et al., 2013). Serum levels of lactate dehydrogenase (LDH), and creatinine kinase-MB (CK-MB) were determined using commercially available biochemical kits. Following blood removal, a thoracic incision was performed, and the hearts were split open, flushed with saltwater (0.9 percent NaCl), and dried. The weight of the heart was assessed (Buerke et al., 1998) and heart tissue homogenate (HTH) was prepared separately for three hearts in an ice-cold 0.25 M sucrose solution using a mortar and pestle. The homogenate was then centrifuged at 5000 rpm for 15 min. After draining the supernatant, biochemical and molecular studies were performed (Lee et al., 2012). The LDH and CK-MB were estimated using commercially available kits. The estimation of superoxide dismutase (SOD) (Elstner and Heupel, 1976), catalase (Link, 1988) and thiobarbituric acid reactive substances (TBARS) (Niehaus and Samuelsson, 1968) were done in heart tissue homogenate. Slides for microscopic examination were made from the remaining hearts of three animals in each group for histological studies. Five micrometer sections were cut and stained using H and E stain. Depending on the severity of cardiac damage, scores were assigned between 0 and 3 with 0 being no damage, one- mild damage as indicated by degenerations at different foci and slight inflammation. A score of two was given when severe degeneration of myofibrils was observed with or without diffuse inflammation, while a score of three was assigned to slides that showed necrosis along with diffuse inflammation (Karthikeyan et al., 2007).

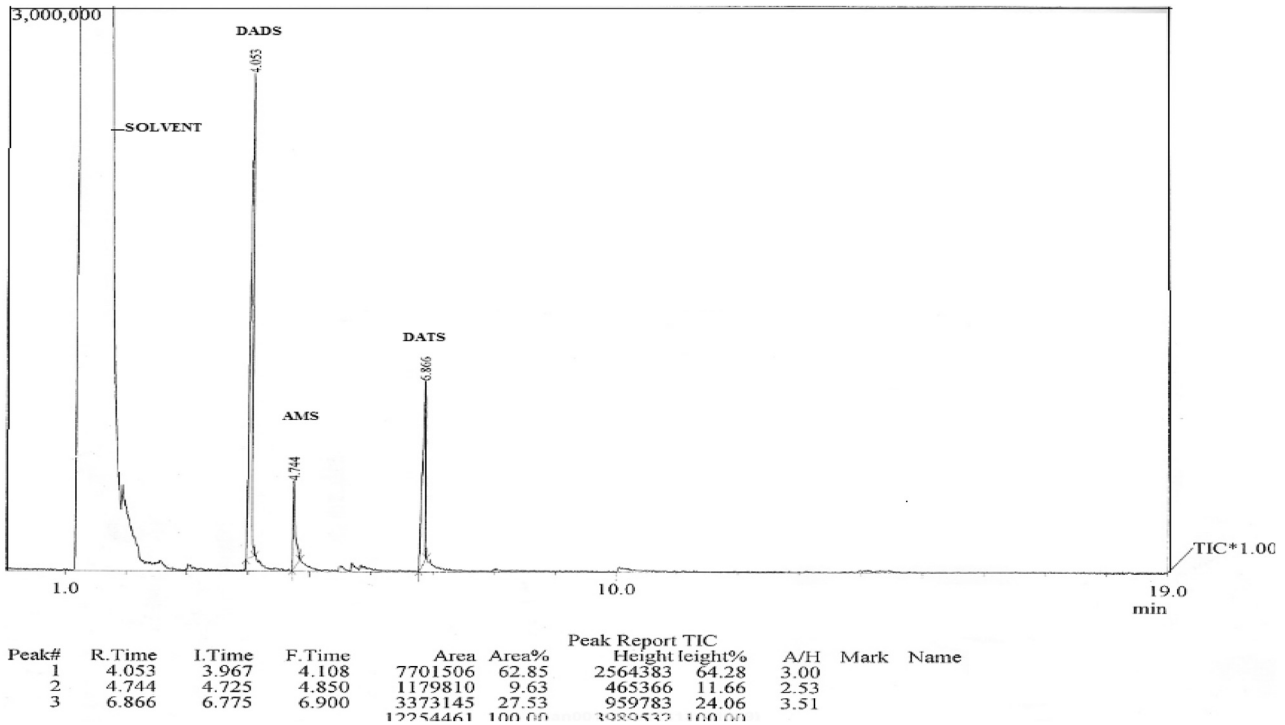
Data Analysis

One-way analysis of variance (ANOVA) was used to determine statistical significance, followed by Tukey's multiple comparison tests using the GraphPad Prism 8.0 computer software kit. The data was presented as mean ± SEM, with a $p < 0.05$ significance level chosen. Power analysis was carried out using G power application, keeping 0.05 as the alpha error probability in One-was ANOVA and post-hoc tests for parameters with low sample size (n = 3).

RESULTS

GC-MS Analysis of DADS in Garlic Oil

Analysis of garlic oil showed that it contained 8.94% w/w of DADS. On the basis of GC-MS peaks of garlic oil, the DADS doses were determined as 4.47 and 8.94 mg/kg (**Figure 2** and **Figure 3**). The other compounds detected in the oil were allyl methyl sulphide (AMS) and diallyl trisulphide (DATS).



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	4.053	3.967	4.108	7701506	62.85	2564383	64.28	3.00		
2	4.744	4.725	4.850	1179810	9.63	465366	11.66	2.53		
3	6.866	6.775	6.900	3373145	27.53	959783	24.06	3.51		
				12254461	100.00	3989533	100.00			

FIGURE 2 | GC-MS profile of garlic oil The GC-MS profile of garlic oil shows different peaks. Peak observed at retention time (RT) of 1 min represents the solvent. The DADS was observed at an RT of 4 min while peaks for allyl methyl sulphide (AMS) and diallyl trisulphide (DATS) was observed at 5 and 7 min respectively.

Effect on Heart Weight and Heart to Body Weight Ratio

The heart weight and the ratio of heart weight to body weight at the end of treatment are shown in **Table 1**. When compared to the normal control group, animals given two doses of isoprenaline (isoprenaline control) had a substantial ($p < 0.01$) increase in heart weight and heart weight to body weight ratio. When compared to the isoprenaline control, all animals pretreated with garlic oil, diallyl disulphide, and carvedilol had significantly ($p < 0.01$) lower heart weight and heart to body weight ratio. Moreover, animals given garlic oil/diallyl disulphide plus carvedilol had a substantial ($p < 0.05$) improvement in heart weight and heart weight to body weight ratio as compared to the garlic oil and diallyl disulphide groups, respectively. The heart to body weight ratio was more towards the normal value with high doses of garlic oil and diallyl disulphide than with their respective low doses.

Effect on Biomarkers of Cardiac Damage

The administration of ISO produced significant changes in different biomarkers of cardiac damage. There was no mortality in animals treated with ISO.

Effect of CK-MB Activity

Administration of ISO induced myocardial infarction as indicated by an increase in cardiac specific serum CK-MB levels and a decrease in CK-MB activity in HTH of ISO treated animals when compared to normal controls ($p < 0.001$). Carvedilol, garlic at both doses and DADS at both doses showed cardioprotective effects. The serum levels of CK-MB were reduced and an increase in CK-MB levels in HTH was observed in these groups when compared to the ISO control. Animals treated with a low dose of DADS (4.47 mg/kg, *p.o*) showed a significant increase in serum CK-MB compared to the normal group. Both doses of DADS in combination with carvedilolproduced a significant fall in serum CK-MB level compared to the carvedilol treated group. Both doses of DADS in combination with carvedilolshowed a significantly lower serum CK-MB activity compared to DADS treatment alone (**Figure 4**).

In HTH, animals treated with a high dose of garlic oil (100 mg/kg, *p.o*) showed a significant decrease in the CK-MB while animals treated with a combination of high dose of DADS (8.94 mg/kg, *p*.o) and carvedilol showed a moderately significant increase in the CK-MB, but the group treated with carvedilol group produced a moderate fall of CK-MB than normal. All treated groups except the low dose of garlic oil (50 mg/kg, *p.o*) and the combination of both doses of DADS with carvedilol showed a significant fall in the CK-MB level in HTH as compared to the ISO group. Furthermore, in groups treated with the low dose of garlic oil (50 mg/kg, *p.o*) and the combination of both doses of DADS with carvedilol, a significant rise in the CK-MB levels was observed compared to the carvedilol alone group. Group treated with a low dose of garlic oil (50 mg/kg, *p.o*) in combination with carvedilol showed a rise in the CK-MB activity compared to low dose of garlic oil (50 mg/kg, *p.o*) alone. Animals treated with a combination of carvedilol with either dose of

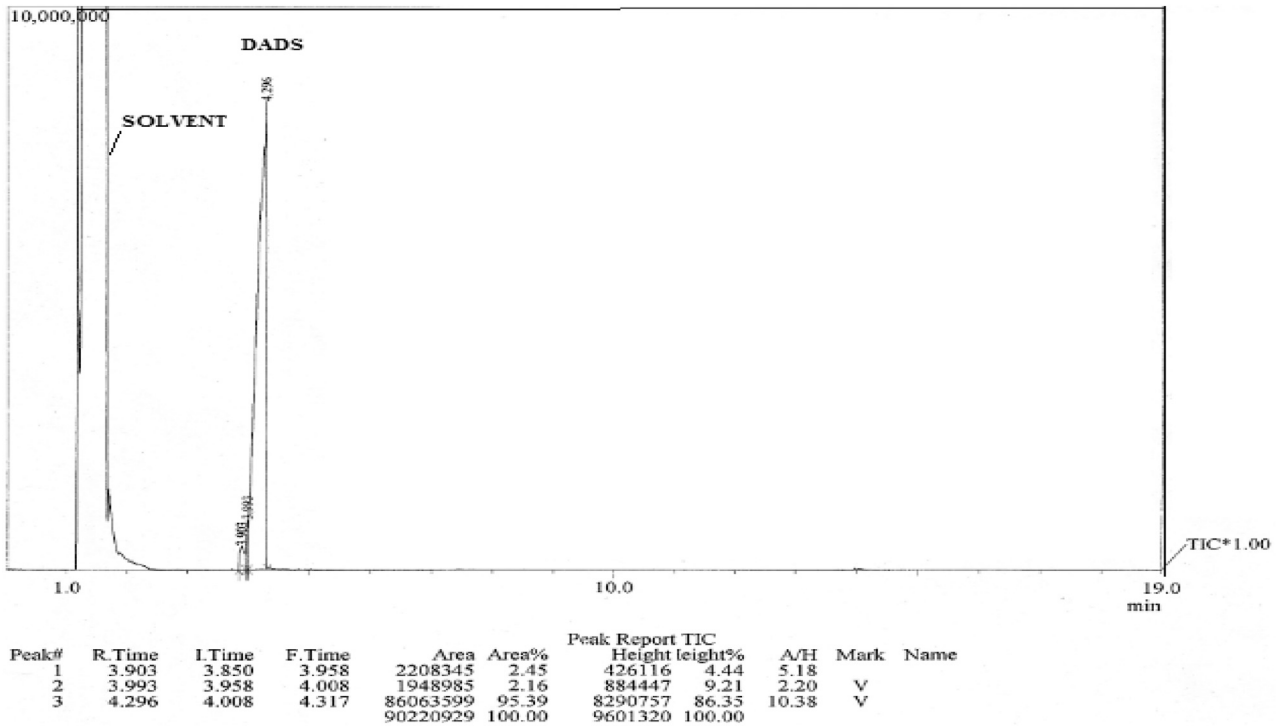


FIGURE 3 | GC-MS profile of diallyl disulphide The GC-MS profile of DADS shows two peaks. At an RT of 1 min, a peak for solvent was observed and DADS peak was observed at an RT of 4 min.

TABLE 1 | Effect on heart weight and heart/body weight ratio.

Groups	Heart weight (g)	Body weight (g)	Heart to body weight ratio (percentage)
Normal control	0.422 ± 0.06	177.34±1.31	0.237 ± 0.01
ISO control	0.690 ± 0.07***	172.32±4.81	0.400 ± 0.01***
Carvedilol	0.472 ± 0.05●●	173.56±5.31	0.271 ± 0.01●●●
GOLD	0.524 ± 0.06●●	171.45±6.21	0.305 ± 0.01●●
GOHD	0.484 ± 0.08●●	173.55±7.21	0.278 ± 0.01●●
DADSLD	0.532 ± 0.07●●	174.21±8.36	0.305 ± 0.01●●
DADSHD	0.498 ± 0.10●●	172.28±9.36	0.289 ± 0.01●●
GOLD + CAR	0.447 ± 0.08●●●*	175.89±1.69	0.254 ± 0.01●●●*
GOHD + CAR	0.436 ± 0.11●●●*	174.29±2.39	0.250 ± 0.01●●●*
DADSLD + CAR	0.431 ± 0.04●●●*	173.37±4.55	0.248 ± 0.01●●●*
DADSHD + CAR	0.426 ± 0.12●●●*	172.89±4.35	0.246 ± 0.01●●●*

Values are given as mean ± Standard error of mean for six rats in each group; ***p < 0.001 when compared to normal control;* ●●p < 0.01, ●●●p < 0.001 *compared to Isoprenaline control;* *p < 0.05, *compared to Garlic oil/Diallyl dilsulphide respective groups. CAR: Carvedilol (2 mg/kg,* p.o*); GOLD: Garlic oil (50 mg/kg,* p.o*); GOHD: Garlic oil (100 mg/kg,* p.o*); DADSLD: diallyl disulphide (4.47 mg/kg,* p.o*); DADSHD: diallyl disulphide (8.94 mg/kg,* p.o*).*

DADS were observed to have significantly more CK-MB activity compared to either dose of DADS alone (**Figure 5**).

Effect on LDH Activity

Similar to the CK-MB activity, ISO administration for two consecutive days produced an increase in serum levels of LDH and reduced its activity in HTH. However, the activity of LDH was reduced by all the treatments irrespective of dose and combinations when compared to ISO control (**Table 1**). Animals treated with a higher dose of garlic oil (100 mg/kg, *p*.o) showed a significant increase in the serum LDH, whereas animals treated with DADS (8.94 mg/kg, *p*.o) and carvedilol showed a significant decrease in the serum LDH when compared to normal control animals. In the group of animals treated with a combination of higher dose of garlic oil (100 mg/kg, *p*.o) and carvedilol, a slight decrease in LDH activity was observed, whereas the combination of both doses of DADS with carvedilol showed a significant fall in LDH level compared to the group treated with carvedilol alone. The group of rats treated with a higher dose of garlic oil (100 mg/kg, *p*.o) and carvedilol had significantly higher LDH activity in the HTH

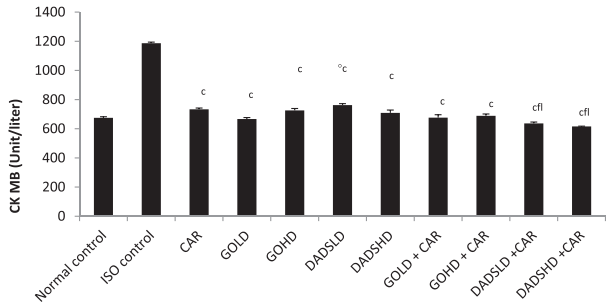


FIGURE 4 | Effect on serum CK-MB levels All values are mean ± SEM, n = 6, $^{\circ}p < 0.01$when compared to normal control; ^{c}P <0.001 compared to ISO control^{f}P<0.001compared to carvedilol; ^{l}P<0.001 when compared to respective DADS dose; CAR: Carvedilol (2 mg/kg, *p.o*); GOLD: Garlic oil (50 mg/kg, *p.o*); GOHD: Garlic oil (100 mg/kg, *p.o*); DADSLD: diallyl disulphide (4.47 mg/kg, *p.o*); DADSHD: diallyl disulphide (8.94 mg/kg, *p.o*).

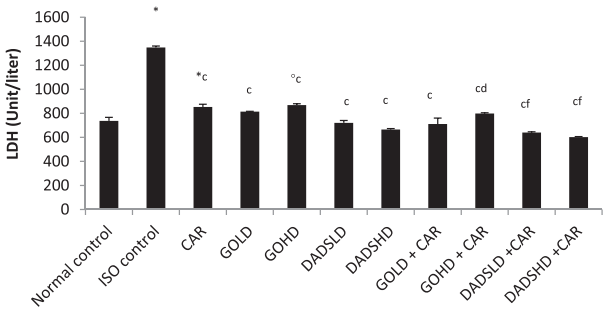


FIGURE 6 | Effect on serum LDH levels All values are mean ± SEM, n = 6, $^{\circ}p < 0.01$, $^{*}p < 0.001$ when compared to normal control; ^{c}P <0.001 compared to ISO control; ^{d}P<0.05, ^{f}P<0.001compared to carvedilol CAR: Carvedilol (2 mg/kg, *p.o*); GOLD: Garlic oil (50 mg/kg, *p.o*); GOHD: Garlic oil (100 mg/kg, *p.o*); DADSLD: diallyl disulphide (4.47 mg/kg, *p.o*); DADSHD: diallyl disulphide (8.94 mg/kg, *p.o*).

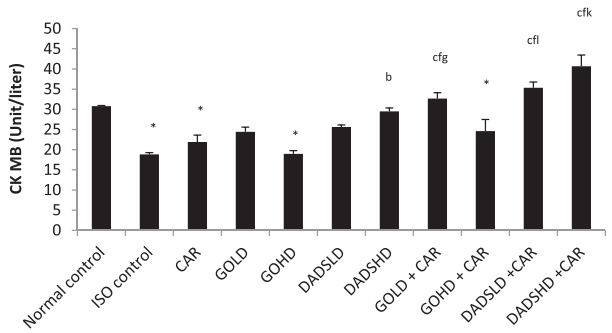


FIGURE 5 | Effect of CK-MB levels in the HTH All values are mean ± SEM, n = 6, $^{*}p < 0.001$ when compared to normal control; ^{b}P<0.01, ^{c}P <0.001 compared to ISO control; ^{d}P<0.05, ^{f}P<0.001compared to carvedilol; ^{g}P <0.05 when compared to respective garlic oil dose; ^{l}P<0.05, ^{k}P<0.01, ^{l}P<0.001 when compared to respective DADS dose; CAR: Carvedilol (2 mg/kg, *p.o*); GOLD: Garlic oil (50 mg/kg, *p.o*); GOHD: Garlic oil (100 mg/kg, *p.o*); DADSLD: diallyl disulphide (4.47 mg/kg, *p.o*); DADSHD: diallyl disulphide (8.94 mg/kg, *p.o*).

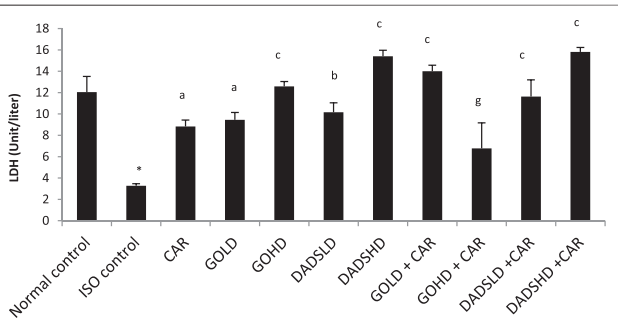


FIGURE 7 | Effect on LDH levels in the HTH All values are mean ± SEM, n = 3, $^{*}p < 0.001$ when compared to normal control; ^{a}P<0.05, ^{b}P<0.01, ^{c}P <0.001 compared to ISO control; ^{g}P <0.05 when compared to respective garlic oil dose; CAR: Carvedilol (2 mg/kg, *p.o*); GOLD: Garlic oil (50 mg/kg, *p.o*); GOHD: Garlic oil (100 mg/kg, *p.o*); DADSLD: diallyl disulphide (4.47 mg/kg, *p.o*); DADSHD: diallyl disulphide (8.94 mg/kg, *p.o*).

compared to the carvedilol treated group. Combined therapy of garlic oil high dose (100 mg/kg, *p.*o) and carvedilol produced a moderately significant increase in LDH in HTH compared to garlic oil high dose (100 mg/kg, *p.*o) alone ((**Figure 6** and **Figure 7**).

Effect on SOD and Catalase

The Administration of ISO caused a significant fall in the activities of antioxidant enzymes; SOD and catalase in HTH. Garlic oil (50 mg/kg, *p.*o) and its combination with carvedilol and the combination of both doses of DADS with carvedilol produced a significant increase in the SOD activity compared to the ISO control. Administration of carvedilol along with either dose of garlic oil or DADS was more effective in increasing SOD activity compared to administration of carvedilol alone. The SOD activity was higher in the group treated with a higher dose of garlic oil (100 mg/kg, *p.o*) with carvedilol compared to garlic oil (100 mg/kg, *p.o*) alone. Similarly, the group treated with a combination of carvedilol with DADS (4.47 mg/kg, *p.o*) or DADS (8.94 mg/kg, *p.o*) had significantly higher SOD activity in HTH compared to DADS treatment alone (**Figure 8**).

For catalase activity, the group treated with a high dose of DADS (4.47 mg/kg, *p.o*) in combination with carvedilol showed a moderate increase in catalase activity, while DADS (8.94 mg/kg, *p.o*) treatment alone showed a profound rise in catalase value compared to the normal control group. Animals treated with DADS (8.94 mg/kg, *p.o*) alone and a combination of either dose of DADS with carvedilol produced a significant rise in catalase, whereas DADS (4.47 mg/kg, *p.o*) and garlic oil (100 mg/kg, *p.o*) with carvedilol showed a slight increase in catalase level compared to the ISO group. Both doses of DADS in combination with carvedilol produced a significant rise in catalase levels compared to carvedilol treatment alone. The combination of garlic oil (100 mg/kg, *p.o*) with carvedilol showed a moderate increase in catalase value compared to garlic oil (100 mg/kg, *p.o*) alone (**Figure 9**).

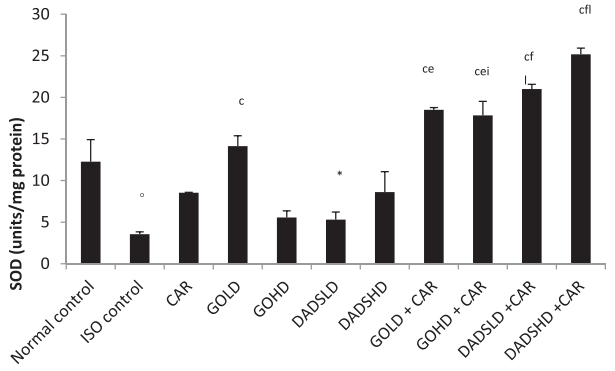


FIGURE 8 | Effect on SOD activity in the HTH All values are mean ± SEM, n = 3, °p < 0.01, *p < 0.001 when compared to normal control; [c]P <0.001 compared to ISO control; [e] p < 0.01, [f]P<0.001compared to carvedilol; [i]P <0.001 when compared to respective garlic oil dose; [l]P<0.001 when compared to respective DADS dose; CAR: Carvedilol (2 mg/kg, *p.o*); GOLD: Garlic oil (50 mg/kg, *p.o*); GOHD: Garlic oil (100 mg/kg, *p.o*); DADSLD: diallyl disulphide (4.47 mg/kg, *p.o*); DADSHD: diallyl disulphide (8.94 mg/kg, *p.o*).

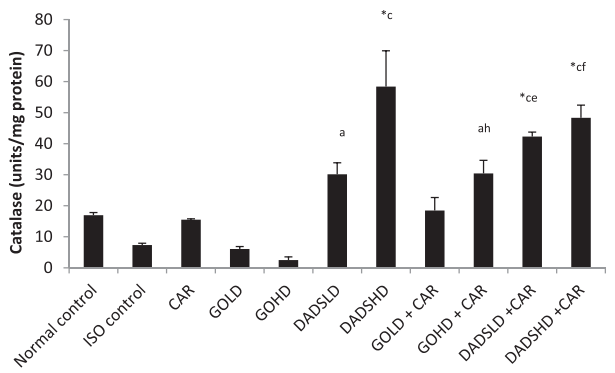


FIGURE 9 | Effect on catalase activity in the HTH All values are mean ± SEM, n = 3, *p < 0.001 when compared to normal control; [a]P<0.05, [c]P <0.001 compared to ISO control; [e] p < 0.01, [f]P<0.001compared to carvedilol; [h] p < 0.01 when compared to respective garlic oil dose; CAR: Carvedilol (2 mg/kg, *p.o*); GOLD: Garlic oil (50 mg/kg, *p.o*); GOHD: Garlic oil (100 mg/kg, *p.o*); DADSLD: diallyl disulphide (4.47 mg/kg, *p.o*); DADSHD: diallyl disulphide (8.94 mg/kg, *p.o*).

Effect on TBARS

Similar to other biomarkers, the TBARS levels increased significantly upon ISO administration compared to normal control. Animals treated with garlic oil (100 mg/kg, *p.o*) showed a significant increase, whereas those administered DADS (8.94 mg/kg, *p.*o) along with carvedilol showed a moderate decrease in the TBARS compared to normal control. All groups except the high dose of garlic oil (100 mg/kg, *p.o)* produced a significant fall in the TBARS value compared to the ISO control group. The group treated with garlic oil (100 mg/kg, *p.o)* together with carvedilol showed a moderate fall in TBARS value compared to the garlic oil (100 mg/kg, *p.o)* treated group. The combination of DADS (8.94 mg/kg, *p.*o) and carvedilol reduced TBARS values compared to the DADS (8.94 mg/kg, *p.*o) group (**Figure 10**).

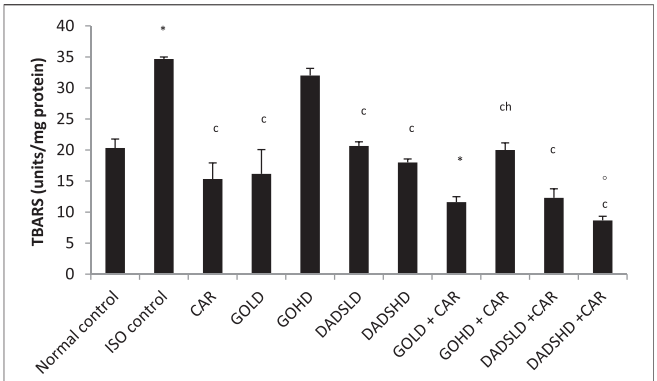


FIGURE 10 | Effect on TBARS in the HTH All values are mean ± SEM, n = 3, °p < 0.01, *p < 0.001 when compared to normal control; [c]P <0.001 compared to ISO control [h] p < 0.01when compared to respective garlic oil dose; CAR: Carvedilol (2 mg/kg, *p.o*); GOLD: Garlic oil (50 mg/kg, *p.o*); GOHD: Garlic oil (100 mg/kg, *p.o*); DADSLD: diallyl disulphide (4.47 mg/kg, *p.o*); DADSHD: diallyl disulphide (8.94 mg/kg, *p.o*).

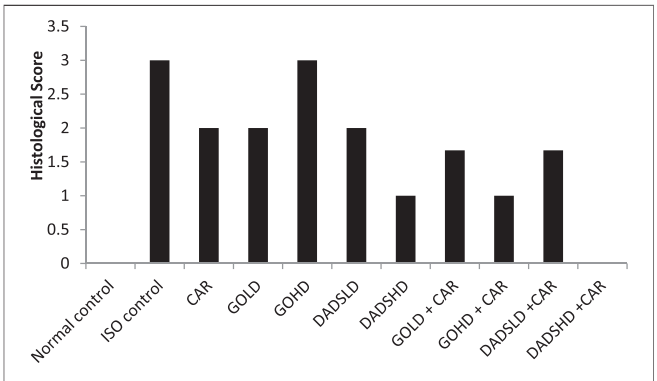


FIGURE 11 | Histological scores of the cardiac tissue.

Effect on Histological Score

An average histological score from three animals in each group. Severe necrosis with diffused inflammation was seen after administration of ISO. Pretreatment with DADSHD (8.94 mg/kg, *p.o*) and the combination of both doses of DADS or garlic oil with carvedilol offered protection against ISO induced damage by preventing severe cellular damage. In sections prepared from the hearts of animals of the above mentioned group, only reversible cellular degeneration and slight inflammation were observed. Garlic oil at both doses (50 mg/100 mg/kg, *p.o*) and DADS (4.47 mg/kg, *p.o*) offered less protection against ISO induced cellular damage (**Figure 11** and **Figure 12**).

Power Analysis

Because there were only three samples available for the parameters tested in heart tissue homogenate (HTH), a power analysis was performed to see if the required power of 80% was achieved in all of the observations. The results of the analysis showed 86.9, 89.1, 99.2%, 96.2, and 82.8 percent statistical power

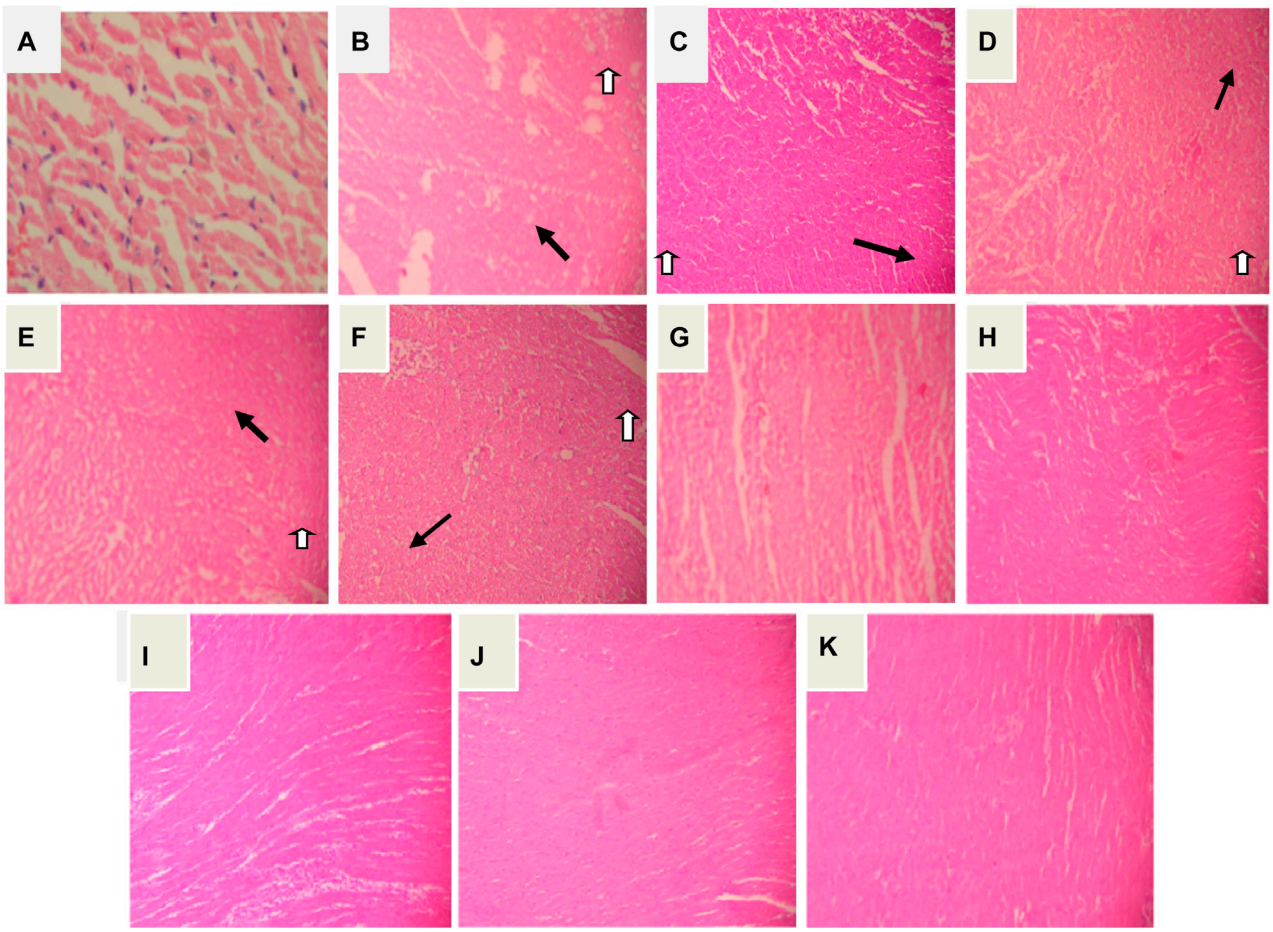


FIGURE12 | Haematoxylin and eosin (H&E) stained section of heart in isoprenaline induced myocardial damage. Photographed at magnification ×400 **(A)** Heart from normal control group **(B)** Iso control (150 mg/kg, s.c), **(C)** Carvedilol (2 mg/kg) group,**(D)** garlic oil low dose (50 mg/kg), **(E)** garlic oil high dose (100 mg/kg), **(F)** DADS low dose (4.47 mg/kg), **(G)** DADS high dose (8.94 mg/kg), **(H)** garlic oil low dose + CAR, **(I)** garlic oil high dose + CAR, **(J)** DADS low dose + CAR and **(K)** DADS high dose + CAR. A and K are hematoxylin and eosin (400×) stained microscopic sections of Normal control and DADSHD + CAR respectively shown histopathalogical score one i.e. mild necrosis, B and E are hematoxylin and eosin (400×) stained microscopic sections of ISO control and GOHD group respectively have a histopathalogical scoreof four i.e. Severe necrosis, inflammation and fibrosis. C, D and F were hematoxylin and eosin (400×) stained microscopic sections of ATN, GOLD and DADSLD respectively have a histopathalogical scoreof three i.e. Moderate diffuse necrosis, mild inflammation and fibrosis (necrosis shown with bold arrows and fibrosis with hollow arrows). G,H,I and J werehematoxylin and eosin (400×) stained microscopic sections of DADSHD, GOLD + CAR, GOHD + CAR and DADSLD + CAR groups respectively have histopathalogical scoreof 2–2.5 i.e.Mild diffuse, moderate necrosis and mild inflammation.

to the outcomes obtained in estimates of CK-MB, LDH, SOD, Catalase, and TBARS levels, respectively, because the variance within the group was minimum due to the calibrated experimental set up (**Figure 13**).

DISCUSSION

The results of the current study showed varying effects of DADS, garlic oil and their combination with carvedilol on different indicators of cardiac damage in isoprenaline induced myocardial infarction in rats. As mentioned earlier, we have evaluated different preparations of garlic for their effect on the pharmacological activity of commonly used cardiovascular drugs. Our aim has been to confirm the health-promoting effects of garlic supplementation and to identify the best garlic preparation and the chemical constituent(s) responsible for the beneficial cardioavasuclar actions. Since, garlic supplementation is used by many patients using modern medicine, we also determined the interaction between garlic preparations and their active constituents with different drugs.

The present study was done using garlic oil because of earlier reports on the cardiovascular benefits of this oil (Kuo et al., 2011; Seki et al., 2015). The active constituent; DADS, was also selected based on earlier studies on its effect on the cardiovascular system (Khatua et al., 2016) and its dose was selected as equivalent to that present in the garlic oil used in the study through GC-MS analysis.

High doses of isoprenaline (ISO) cause a cascade of events that culminate in ventricular hypertrophy, with disruption and

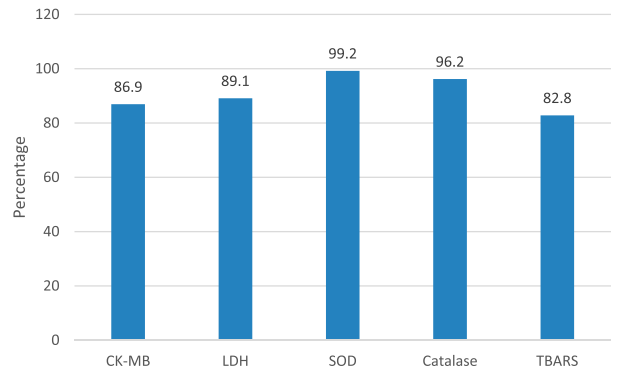


FIGURE 13 | Power Analysis of parameters with low sample size Values are given in percentage, analyzed using G power application, One way ANOVA and Post-hoc test with α error probability of 0.05 and a total sample size of 33 in eleven groups.

rupturing of the cardiac muscle resulting in extraceullar fluid flow and an increase in heart weight (Liu et al., 2013; Sagor et al., 2015). According to this, in isoprenaline-injected rats, heart weight and the ratio of heart weight to body weight were abnormally high, which was successfully mitigated in rats that received garlic oil, diallyl disulphide, or carvedilol treatment, either separately or jointly. This means that our therapies effectively protect the cardiac muscle against ISO-induced rupture by reducing oxidative stress and limiting water migration to the cardiomyocytes, hence maintaining cardiac homeostasis (Goyal et al., 2010). Carvedilol, like garlic oil and DADS, showed antioxidant properties, albeit the significance of this characteristic is unknown (Ayashi et al., 2016).

The cardiac myocardial infarction was induced using ISO, a catecholamine that increases myocardial function. Administration of ISO for two consecutive days at 150 mg/kg subcutaneously produce severe increase in myocardial function leading to myocardial infarction (Jiang et al., 2019; Feriani et al., 2020). The ISO induced myocardial infarction is one of the most widely used and accepted models of myocardial infarction and is used for evaluation of the cardioprotective effects of various agents. The events of myocardial damage follow the same pattern that is observed with pathological myocardial damage, which starts with biochemical changes followed by ultrastructural to microscopic changes. All these events occur within 48 h after the injection of isoprenaline (Dudnakova et al., 2002). The myocardial damage is also mediated through the generation of oxygen free radicals due to oxidation of ISO, leading to the formation of superoxide anions that ultimately result in the formation of hydrogen peroxide. These oxidative species increase the permeability of microsomes and calcium uptake in the mitochondria. They also reduce ATP formation and damage cellular proteins, lipids, and DNA (Jiang et al., 2019; Feriani et al., 2020). To assess the protection offered by pretreatment of garlic oil, DADS, carvedilol and their combinations, biochemical estimation of enzymes released from cardiac cells; CK-MB and LDH, levels of antioxidant enzymes; SOD and catalase, level of TBARS and histological scores were determined. Elevated serum levels of CK-MB and LDH with a simultaneous decrease in these enzymes in HTH indicates damage to the myocardium. The toxic free radicals and other reactive oxygen species (ROS) are scavenged by endogenous antioxidant enzymes, which include SOD that dismutates superoxide anion and catalase that reduces peroxides (Borek, 2001). Higher enzyme activities suggest enhanced protection against oxidative damage.

Garlic and its different preparations are used traditionally for the treatment of various diseases. Garlic preparations may be classified as allicin-rich and non-allicin preparations (Haina Wang et al., 2013). Allicin-rich preparations are made using raw garlic, while processed garlic is used for the preparation of non-allcin products (Sharifi-Rad et al., 2019). Other than allicin, these two preparations contain several other constituents that are different from each other (Kasuga et al., 2001). Of these different chemicals, organosulfur compounds (OSCs) such as DADS and dially trisulfidehave been reported for several pharmacological actions, including cardiovasulcular effects (Abe et al., 2019). These compounds are also known to modulate activities of different drug metabolism enzymes, especially those involved in phase II metabolism by unknown mechanisms (Zhao et al., 2013).

Carvedilol, a beta-adrenergic blocker, is used as an antianginal, antiarrhythmic, and antihypertentve agent. It is also used for treating both idiopathic and ischemic congestive heart failure. It prevents ISO induced myocardial infarction by antagonising the effect of ISO on the cardiac $beta_1$-adrenergic receptors (Elshourbagy, 2016). Carvedilol also displays antioxidant action, although the relevance of this property remains uncertain (Ayashi et al., 2016). However, antioxidant properties that some of these compounds appear to possess have previously been linked to a number of the positive cardiovascular benefits that this group of compounds has been linked to in the literature (Mak and Weglicki, 1988). The findings of this study corroborate prior observations with atenolol, another beta blocker (Asdaq and Avula, 2015). Garlic oil and DADS improved both atenolol and carvedilol's cardioprotective capacity during ISO-induced myocardial stress in experimental rats. Both beta blockers effects are well-known and have been proven in prior studies (Jonsson et al., 2005).

The garlic oil contains soluble organosulphur compounds called allyl sulphides, which imparts characteristic flavour to the oil. Diallyl disulphide (DADS) is a type of allyl sulphide that is found in garlic oil but is not found in garlic cloves. As mentioned above, DADS is reported to possess several pharmacological activities. It is formed as a product of allicin by the action of the allicinase enzyme during garlic cutting or crushing (Abe et al., 2019).

The ISO administration also produced an increase in the TBARS in the cardiac cells. Antioxidants reduce oxidative stress and reduce both the initiation and propagation of the lipid peroxidation process. Administration of carvedilol along with high dose garlic oil (100 mg/kg, *p.o*) or DADS (8.94 mg/kg, *p.*o) showed a slight fall in TBARS compared to ISO control, indicating a cardioprotective effect. To ascertain the extent of damage to myocardium, a histological examination was carried out along with biochemical estimations. Pretreatment with high doses of garlic oil and DADS alone or with carvedilol substantially

maintained the myocardial cellular integrity and decreased the pathological scores when compared to the ISO treated group. The number of samples for determination of different parameters in the HTH was only three. The sample size could not be increased due to ethical issues. Though the sample size was small, it was enough to draw conclusions as the LDH and CK-MB levels were simultaneously determined in the serum.

The results of the present study showed varying effects. Nevertheless, the results suggest that garlic oil or DADS at two different doses augment the cardioprotective action of carvedilol. A comparison of different groups showed a dose dependent effect of garlic oil and DADS, though a significant effect was not observed between the effects produced by the lower dose compared to the higher dose. The comparison of effects produced by the combination of carvedilol with either dose of garlic oil or DADS with individual treatments showed varying effects, with some of them being significant, while many other comparisons were not significant.

The antioxidant property of garlic oil and its constituent diallyl disulphide have been reported earlier (Chiang et al., 2006). The antioxidant action is due to organosulphurous compounds. but the exact mechanism is still not clear. It is apparent that allyl sulphur constituents of these preparations are responsible for accelerated antioxidant enzyme synthesis at times of ISO induced stress to the myocardium (Kim et al., 1997).

CONCLUSION

The findings of the present study showed a dose dependent increase in the cardioprotective efficacy of garlic oil and its active constituent, diallyl disulphide (DADS), during isoprenaline induced myocardial damage. Additionally, this study reiterates the role of garlic oil and DADS in amplifying the cardioprotective efficacy of carvedilol in ISO induced ischemic damage in experimental animals. Despite the fact that beta blockers are well-known for their cardioprotective properties, this work was the first to find the cardioprotective interaction of carvedilol with garlic oil and DADS during myocardial stress in rats. The findings of this study will pave the way for further research into the role of garlic oil and DADS in the therapeutic regimen, potentially lowering the dosage of traditional cardioprotective substances like carvedilol.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Krupanidhi College of Pharmacy animal ethical committee (KCP/IAEC-27).

AUTHOR CONTRIBUTIONS

Under the supervision of SA, OC carried out the research methodology. AA was responsible for formal analysis of the work while WA participated in writing original draft of the manuscript. MA administered the project and MA was instrumental in review and editing of the manuscript.

FUNDING

AA would like to acknowledge Taif university for support (No. TURSP (2020/288)).

ACKNOWLEDGMENTS

The authors would like to thank the management of the Krupanidhi College of Pharmacy for providing the necessary facilities to carry out this research. The authors are also thankful to AlMaarefa University in Riyadh for providing support to do this research.

REFERENCES

Abe, K., Hori, Y., and Myoda, T. (2019). Volatile compounds of fresh and processed garlic. *Exp. Ther. Med.* 19, 1585–1593. doi:10.3892/etm.2019.8394

Adaki, S., Adaki, R., Shah, K., and Karagir, A. (2014). Garlic: Review of literature. *Indian J. Cancer* 51, 577–581. Medknow Publications. doi:10.4103/0019-509x.175383

Asdaq, S. M. (2015). Antioxidant and hypolipidemic potential of aged garlic extract and its constituent, s-allyl cysteine, in rats. *Evid. Based. Complement. Alternat. Med.* 2015, 328545. doi:10.1155/2015/328545

Asdaq, S. M., and Avula, P. R. (2015). Interaction of garlic oil and its constituent diallyl disulfide with atenolol in isoproterenol induced acute myocardial injury in rats. *J. Pharm. Biol.* 5 (1), 44–52.

Asdaq, S. M., Inamdar, M. N., and Asad, M. (2009b). Effect of conventional antihypertensive drugs on hypolipidemic action of garlic in rats. *Indian J. Exp. Biol.* 47 (3), 176–181. PMID: 19405382.

Asdaq, S. M., and Inamdar, M. N. (2011a). Pharmacodynamic and Pharmacokinetic Interactions of Propranolol with Garlic (Allium sativum) in Rats. *Evid. Based. Complement. Alternat. Med.* 2011, 824042. doi:10.1093/ecam/neq076

Asdaq, S. M., and Inamdar, M. N. (2010). Potential of garlic and its active constituent, S-allyl cysteine, as antihypertensive and cardioprotective in presence of captopril. *Phytomedicine* 17, 1016–1026. doi:10.1016/j.phymed.2010.07.012

Asdaq, S. M., and Inamdar, M. N. (2011b). The potential benefits of a garlic and hydrochlorothiazide combination as antihypertensive and cardioprotective in rats. *J. Nat. Med.* 65, 81–88. doi:10.1007/s11418-010-0467-9

Asdaq, S. M., and Inamdar, M. N. (2009). The potential for interaction of hydrochlorothiazide with garlic in rats. *Chem. Biol. Interact.* 181, 472–479. doi:10.1016/j.cbi.2009a.07.02210.1016/j.cbi.2009.07.022

Asdaq, S. M. B., Challa, O., Alamri, A. S., Alsanie, W. F., Alhomrani, M., Almutiri, A. H., et al. (2021). Cytoprotective Potential of Aged Garlic Extract (AGE) and its Active Constituent, S-allyl-l-cysteine, in Presence of Carvedilol during

Isoproterenol-Induced Myocardial Disturbance and Metabolic Derangements in Rats. *Molecules* 26, 3203. doi:10.3390/molecules26113203

Ayashi, S., Assareh, A. R., Jalali, M. T., Olapour, S., and Yaghooti, H. (2016). Role of antioxidant property of carvedilol in mild to moderate hypertensive patients: A prospective open-label study. *Indian J. Pharmacol.* 48, 372–376. doi:10.4103/0253-7613.186206

Borek, C. (2001). Antioxidant health effects of aged garlic extract. *Am. Inst. Nutr.* 131, 1010S–1015S. doi:10.1093/jn/131.3.1010s

Buerke, M., Prüfer, D., Dahm, M., Oelert, H., Meyer, J., and Darius, H. (1998). Blocking of classical complement pathway inhibits endothelial adhesion molecule expression and preserves ischemic myocardium from reperfusion injury. *J. Pharmacol. Exp. Ther.* 286, 429–438.

Chang, S. H., Liu, C. J., Kuo, C. H., Chen, H., Lin, W. Y., Teng, K. Y.., et al. (2011). Garlic Oil Alleviates MAPKs- and IL-6-mediated Diabetes-related Cardiac Hypertrophy in STZ-induced DM Rats. *Evid. Based Complement. Alternat Med.* 2011, 950150. doi:10.1093/ecam/neq075

Chiang, Y. H., Jen, L. N., Su, H. Y., Lii, C. K., Sheen, L. Y., and Liu, C. T. (2006). Effects of garlic oil and two of its major organosulfur compounds, diallyl disulfide and diallyl trisulfide, on intestinal damage in rats injected with endotoxin. *Toxicol. Appl. Pharmacol.* 213, 46–54. doi:10.1016/j.taap.2005.08.008

Dudnakova, T. V., Lakomkin, V. L., Tsyplenkova, V. G., Shekhonin, B. V., Shirinskiĭ, V. P., and Kapel'ko, V. I. (2002). Isoproterenol induced changes of protein expression and myocardial ultrastructure. *Kardiologiia* 42 (11), 57–63. Russian. PMID: 12494039.

El-Saber Batiha, G., Magdy Beshbishy, A., G Wasef, L., Elewa, Y. H. A., A Al-Sagan, A., Abd El-Hack, M. E., et al. (2020). Chemical constituents and pharmacological activities of garlic (Allium sativum L.): A review. *Nutrients* 12, 12. doi:10.3390/nu12030872

Elshourbagy, N. (2016). The Effects of Carvedilol and q10 on Isoprenaline-Induced Cardiotoxicity: Electrocardiographic, Biochemical and Histopathological Evaluation in Rats. *Al-AzharJ. Pharm. Sci.* 54 (1), 148–158. doi:10.21608/AJPS.2018.6640

Elstner, E. F., and Heupel, A. (1976). Inhibition of nitrite formation from hydroxylammoniumchloride: A simple assay for superoxide dismutase. *Anal. Biochem.* 70, 616–620. doi:10.1016/0003-2697(76)90488-7

Feriani, A., Khdhiri, E., Tir, M., Elmufti, A., Tlili, N., Hajji, R.., et al. (2020). (E)-N'-(1-(7-Hydroxy-2-Oxo-2H-Chromen-3-Yl) Ethylidene) Benzohydrazide, a Novel Synthesized Coumarin, Ameliorates Isoproterenol-Induced Myocardial Infarction in Rats through Attenuating Oxidative Stress, Inflammation, and Apoptosis. *Oxid. Med. Cel. Longev.* 2020, 2432918. doi:10.1155/2020/2432918

Goyal, S. N., Arora, S., Sharma, A. K., Joshi, S., Ray, R., Bhatia, J., et al. (2010). Preventive effect of crocin of Crocus sativus on hemodynamic, biochemical, histopathological and ultrastuctural alterations in isoproterenol-induced cardiotoxicity in rats. *Phytomedicine* 17, 227–232. doi:10.1016/j.phymed.2009.08.009

Goyal, S. N., Sharma, C., Mahajan, U. B., Patil, C. R., Agrawal, Y. O., Kumari, S., et al. (2015). Protective effects of cardamom in isoproterenol-induced myocardial infarction in rats. *Int. J. Mol. Sci.* 16, 27457–27469. doi:10.3390/ijms161126040

Guan, M. J., Zhao, N., Xie, K. Q., and Zeng, T. (2018). Hepatoprotective effects of garlic against ethanol-induced liver injury: A mini-review. *Food Chem. Toxicol.* 111, 467–473. doi:10.1016/j.fct.2017.11.059

Haina Wang, X. J., Wang, W., Gao, C., Jiang, X., and Wang, H. (2013). Drug Metabolism and Pharmacokinetics of Organosulfur Compounds from Garlic. *J. Drug Metab. Toxicol.* 04, 159. doi:10.4172/2157-7609.1000159

Hou, L. Q., Liu, Y. H., and Zhang, Y. Y. (2015). Garlic intake lowers fasting blood glucose: meta-analysis of randomized controlled trials. *Asia Pac. J. Clin. Nutr.* 24, 575–582. doi:10.6133/apjcn.2015.24.4.15

Jiang, T., Zhang, L., Ding, M., and Li, M. (2019). Protective effect of vasicine against myocardial infarction in rats via modulation of oxidative stress, inflammation, and the PI3K/AKT pathway. *Drug Des. Devel. Ther.* 13, 3773–3784. doi:10.2147/DDDT.S220396

Jonsson, G., Abdelnoor, M., Müller, C., Kjeldsen, S. E., Os, I., and Westheim, A. (2005). A comparison of the two beta-blockers carvedilol and atenolol on left ventricular ejection fraction and clinical endpoints after myocardial infarction. a single-centre, randomized study of 232 patients. *Cardiology* 103 (3), 148–155. doi:10.1159/000084584

Karthikeyan, K., Bai, B. R., and Devaraj, S. N. (2007). Cardioprotective effect of grape seed proanthocyanidins on isoproterenol-induced myocardial injury in rats. *Int. J. Cardiol.* 115, 326–333. doi:10.1016/j.ijcard.2006.03.016

Kasuga, S., Uda, N., Kyo, E., Ushijima, M., Morihara, N., and Itakura, Y. (2001). Pharmacologic activities of aged garlic extract in comparison with other garlic preparations. *Am. Inst. Nutr.* 131, 1080S-4S. doi:10.1093/jn/131.3.1080s

Khatua, T. N., Dinda, A. K., Putcha, U. K., and Banerjee, S. K. (2016). Diallyl disulfide ameliorates isoproterenol induced cardiac hypertrophy activating mitochondrial biogenesis via eNOS-Nrf2-Tfam pathway in rats. *Biochem. Biophys. Rep.* 5, 77–88. doi:10.1016/j.bbrep.2015.11.008

Kim, S. M., Kubota, K., and Kobayashi, A. (1997). Antioxidative activity of sulfur-containing flavor compounds in garlic. *Biosci. Biotechnol. Biochem.* 61, 1482–1485. doi:10.1271/bbb.61.1482

Kimbaris, A. C., Siatis, N. G., Pappas, C. S., Tarantilis, P. A., Daferera, D. J., and Polissiou, M. G. (2006). Quantitative analysis of garlic (Allium sativum) oil unsaturated acyclic components using FT-Raman spectroscopy. *Food Chem.* 94, 287–295. doi:10.1016/j.foodchem.2005.01.017

Ko, J.-W., Park, S.-H., Lee, I.-C., Lee, S.-M., Shin, I.-S., Kang, S.-S., et al. (2016). Protective effects of garlic oil against 1,3-dichloro-2-propanol-induced hepatotoxicity: role of CYP2E1 and MAPKs. *Mol. Cel. Toxicol.* 12, 185–195. [CrossRef]. doi:10.1007/s13273-016-0023-0

Kumar, M., Kasala, E. R., Bodduluru, L. N., Dahiya, V., and Lahkar, M. (2016). Baicalein protects isoproterenol induced myocardial ischemic injury in male Wistar rats by mitigating oxidative stress and inflammation. *Inflamm. Res.* 65, 613–622. doi:10.1007/s00011-016-0944-z

Lee, D. Y., Li, H., Lim, H. J., Lee, H. J., Jeon, R., and Ryu, J. H. (2012). Anti-inflammatory activity of sulfur-containing compounds from garlic. *J. Med. Food* 15, 992–999. [CrossRef] [PubMed]. doi:10.1089/jmf.2012.2275

Link, E. M. (1988). The mechanism of pH-dependent hydrogen peroxide cytotoxicity *in vitro*. *Arch. Biochem. Biophys.* 265, 362–372. doi:10.1016/0003-9861(88)90139-7

Lissiman, E., Bhasale, A. L., and Cohen, M. (2014). Garlic for the common cold. *Cochrane Database Syst. Rev.* 2014, CD006206. doi:10.1002/14651858.CD006206.pub4

Liu, Y. T., Jia, H. M., Chang, X., Ding, G., Zhang, H. W., and Zou, Z. M. (2013). The metabolic disturbances of isoproterenol induced myocardial infarction in rats based on a tissue targeted metabonomics. *Mol. Biosyst.* 9, 2823–2834. doi:10.1039/c3mb70222g

Mak, I. T., and Weglicki, W. B. (1988). Protection by beta-blocking agents against free radical-mediated sarcolemmal lipid peroxidation. *Circ. Res.* 63, 262–266. doi:10.1161/01.res.63.1.262

Nicastro, H. L., Ross, S. A., and Milner, J. A. (2015). Garlic and onions: Their cancer prevention properties. *Cancer Prev. Res. (Phila)* 8, 181–189. doi:10.1158/1940-6207.CAPR-14-0172

Niehaus, W. G., and Samuelsson, B. (1968). Formation of Malonaldehyde from Phospholipid Arachidonate during Microsomal Lipid Peroxidation. *Eur. J. Biochem.* 6, 126–130. doi:10.1111/j.1432-1033.1968.tb00428.x

Othman, A. I., Elkomy, M. M., El-Missiry, M. A., and Dardor, M. (2017). Epigallocatechin-3-gallate prevents cardiac apoptosis by modulating the intrinsic apoptotic pathway in isoproterenol-induced myocardial infarction. *Eur. J. Pharmacol.* 794, 27–36. doi:10.1016/j.ejphar.2016.11.014

Pipaliya, H., and Vaghasiya, J. (2012). Cardio protective effect of vitamin A against isoproterenol-induced myocardial infarction. *J. Nutr. Sci. Vitaminol (Tokyo)* 58, 402–407. doi:10.3177/jnsv.58.402

Ronis, M. J. J., Pedersen, K. B., and Watt, J. (2018). Adverse Effects of Nutraceuticals and Dietary Supplements. *Annu. Rev. Pharmacol. Toxicol.* 58, 583–601. doi:10.1146/annurev-pharmtox-010617-052844

Rosselló, X., Huo, Y., Pocock, S., Van de Werf, F., Chin, C. T., Danchin, N., et al. (2017). Global geographical variations in ST-segment elevation myocardial infarction management and post-discharge mortality. *Int. J. Cardiol.* 245, 27–34. doi:10.1016/j.ijcard.2017.07.039

Sagor, M. A. T., Tabassum, N., Potol, M. A., Alam, M. A., and Alam, M. (2015). Xanthine Oxidase Inhibitor, Allopurinol, Prevented Oxidative Stress, Fibrosis, and Myocardial Damage in Isoproterenol Induced Aged Rats. *Oxidative Med. Cell Longevity* 2015, 1–9. doi:10.1155/2015/478039

Saikh, A. S., Thomas, A. B., and Chitlange, S. S. (2020). Herb–drug interaction studies of herbs used in treatment of cardiovascular disorders—A narrative review of preclinical and clinical studies. *Phyther. Res.* 34, 1008–1026.

Seki, T., and Hosono, T. (2015). Prevention of cardiovascular diseases by garlic-derived sulfur compounds. *J. Nutr. Sci. Vitaminol (Tokyo)* 61 Suppl, S83–S85. doi:10.3177/jnsv.61.S83

Sharifi-Rad, J., Cristina Cirone Silva, N., Jantwal, A., D. Bhatt, I., Sharopov, F., C. Cho, W., et al. (2019). Therapeutic Potential of Allicin-Rich Garlic Preparations: Emphasis on Clinical Evidence toward Upcoming Drugs Formulation. *Appl. Sci.* 9, 5555. doi:10.3390/app9245555

Singh, S., and Preuss, C. V. (2021). *Carvedilol. [Updated 2021 Feb 17]. in: StatPearls [Internet]. Treasure Island (FL).* Treasure Island, FL: StatPearls Publishing. Available from: https://www.ncbi.nlm.nih.gov/books/NBK534868/(accessed on July 11, 2021).

Watanabe, K., Ohta, Y., Nakazawa, M., Higuchi, H., Hasegawa, G., Naito, M.., et al. (2000). Low dose carvedilol inhibits progression of heart failure in rats with dilated cardiomyopathy. *Br. J. Pharmacol.* 130, 1489–1495. doi:10.1038/sj.bjp.0703450

Wellington, D., Mikaelian, I., and Singer, L. (2013). Comparison of ketamine-xylazine and ketamine-dexmedetomidine anesthesia and intraperitoneal tolerance in rats. *J. Am. Assoc. Lab. Anim. Sci.* 52, 481–487.

Xu, H., Li, W., Yang, J., Wiviott, S. D., Sabatine, M. S., Peterson, E. D., et al. (2016). The China Acute Myocardial Infarction (CAMI) registry: A national long-term registry-research-education integrated platform for exploring acute myocardial infarction in China. *Am. Heart J.* 175, 193–e3. doi:10.1016/j.ahj.2015.04.014

Yang, C., Li, L., Yang, L., Lǚ, H., Wang, S., and Sun, G. (2018). Anti-obesity and Hypolipidemic effects of garlic oil and onion oil in rats fed a high-fat diet. *Nutr. Metab. (Lond)* 15, 43. doi:10.1186/s12986-018-0275-x

Zhang, G., Kimura, S., Nishiyama, A., Shokoji, T., Rahman, M., and Yao, L. (2005). Cardiac oxidative stress in acute and chronic isoproterenol-infused rats. *Cardiovasc. Res.* 65, 230–238.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 September 2021
doi: 10.3389/fphar.2021.713414

Medicinal Plants in Traditional Herbal Wines and Liquors in the East of Spain and the Balearic Islands

V. Martínez-Francés[1]*, *D. Rivera*[2], *C. Obon*[3], *F. Alcaraz*[2] *and S. Ríos*[1]

[1]*Biological Research Station-Botanical Garden of Torretes, Institute of Biodiversity CIBIO, University of Alicante, Alicante, Spain,* [2]*Departamento de Biología Vegetal, Facultad de Medicina, Universidad de Murcia, Murcia, Spain,* [3]*Centro de Investigación e Innovación Agroalimentaria y Agroambiental (CIAGRO), EPSO, Universidad Miguel Hernández de Elche, Alicante, Spain*

Homemade herbal preparations from the East of Spain are the witness of traditional medicine inherited from the ancient complex formulas of herbal teas and medicinal wines. In this study, we document the use of traditional alcoholic beverages, identify their ingredients, almost exclusively botanical, record the local medicinal uses of these mixtures, and discuss patterns of distribution of this knowledge in regions of eastern Spain, the Balearic Islands and Andorra. We determine marker species and relevant patterns of herbal formulas in the different regions of the territory. Homemade liquors and liqueurs are consumed for their digestive and tonic-restorative properties but they also play in some cases an important social role. The elderly remember other medicinal uses such as aperitif, emmenagogue, or antidiarrheal, for some of the most popular preparations. The herbal liqueur formulas include predominantly Lamiaceae, Asteraceae, Rosaceae, Rutaceae, and Apiaceae species. Herbs (58%), fruits (28%), and mixtures of both (12%) are ingredients of liquors and wines, being the aerial parts the most frequent in terms of species (30%) and records (49%). *Dictamnus hispanicus, Santolina villosa, Salvia blancoana* subsp. *mariolensis*, *Rosmarinus officinalis, Thymus vulgaris,* and *Clinopodium serpyllifolium* subsp. *fruticosum* are the species most frequently used. Others species used to a lesser extent as *Polygonatum odoratum, Thymus moroderi,* and *Saxifraga longifolia* are restricted to locally homemade preparations because their collection and uses require special knowledge of the rare or endemic flora. Sustainability of these practices is strongly limited by the overall loss of local traditional knowledge and by the limited availability of most of the wild species; some of them are endangered or threatened mainly by the loss of their natural habitats. Cultivation and domestication are a promising alternative to collecting from wild populations. The cultivation of *Thymus moroderi* in the province of Alicante and *Polygonatum odoratum* in the province of Teruel are good examples. There is a notable decrease in the complexity of the formulas registered throughout the nearly 15 years of the study. This is interpreted as a consequence of a loss of knowledge, less accessibility to wild resources, and changes in traditions and preferences.

Keywords: appetite stimulant, digestive, emmenagogue, ethnobotany, medicinal wines, medicinal liquor, tonic

OPEN ACCESS

Edited by:
Massimo Lucarini,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Marina Silalahi,
Universitas Kristen Indonesia, Indonesia
Songül Karakaya,
Atatürk University, Turkey
Yeter Yeşil,
Istanbul University, Turkey
Emin Ugurlu,
Bursa Technical University, Turkey

***Correspondence:**
V. Martínez-Francés
vanessa.martinez@ua.es

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *22 May 2021*
Accepted: *09 August 2021*
Published: *29 September 2021*

Citation:
Martínez-Francés V, Rivera D, Obon C, Alcaraz F and Ríos S (2021) Medicinal Plants in Traditional Herbal Wines and Liquors in the East of Spain and the Balearic Islands. Front. Pharmacol. 12:713414. doi: 10.3389/fphar.2021.713414

INTRODUCTION

Hippocrates is credited for the sentence "let thy food be thy medicine and thy medicine be thy food." This phrase is unlikely from Hippocrates; however, yet in line with the above philosophy, we are currently witnessing a reappraisal of the complementarity of nutrition and pharmacology (Witkamp and van Norren, 2018). The ethnobotany of wine has been studied recently, and ancient Egyptian and Phoenician wine residues reveal that these peoples added spices and healing herbs. Some healing plants whose remains were found in ancient Egyptian wines were coriander, lemon balm, peppermint, rosemary, sage, savory, senna, and thyme (Plotkin, 2021).

Soaking herbs for several days, weeks, or years in wine or distillates with different alcohol concentrations are a common practice in different cultures all over the world (Min and Jeong, 1995; Hwang et al., 2005; Wujisguleng et al., 2012; Egea et al., 2015; Egea et al., 2016). Their product is a wide range of extracts known as herbal wines and liquors (Egea et al., 2015).

The origin of liquors with medicinal plants, obtained by maceration in alcohols or by distilling wines, is difficult to determine but in any case it is the result of the development of distillation techniques (Forbes, 2009). The first trustworthy references for distilled alcohol date back to 1130 and 1160 CE at the Salerno School, where a certain Master Salernus distilled alcohol to make perfumes and medicines (Cierbide, 2007; Forbes, 2009). The first herbal liquors known as "*aqua ardens compositae*" appeared between the 13th and 14th centuries in the works of Salernitan scholars natives of the kingdom of Aragon such as Joanis de Rupescissa, Arnau de Villanova, and Raimond Llull (or their disciples) (Loring, 1993; Bueno and Alegre, 2001; Cierbide, 2007; Forbes, 2009; Cierbide, 2013; López-Pérez, 2016).

Spirits, often named liquor, contain no added sugar and at least 20% alcohol by volume, while liquors with added sugar and often herbs or flavorings are best known as liqueurs (Jurado, 2004; Egea et al., 2015).

The reasons for considering alcohol as medicinal are rooted in Galenism. According to the galenic principle "*contraria contrariis curantur*," spirits which are hot and dry in third grade became a true panacea to cure all cold and wet diseases, especially gynecological disorders. Galenic "composite waters" combined the value of "hot" plants with alcohol (*aquae ardens* or *quinta esencia*, Rupecissa, 1346) as a remedy *per se* and solvent, vehicle, and preservative (García-Ballester, 2001; Cierbide, 2007). This *aqua vitae* was perceived closest to the *Elixir of life* so sought by alchemy (Martín-Reyes, 2004; López-Pérez, 2016). These liqueurs were called spirits, derived from the Greek concept of *pneuma* applied to medicine by Galen and assimilated to the product obtained from wine distillation as its spirit (Cierbide, 2007).

Antidotarium Arnaldi and *De Vinis,* both of Arnau de Villanova (McVaugh, 1993; Simó-Santonja, 2007; Gil-Sotres, 2011), recommended the use of herbal macerates, spiced wines, and spirits for gynecological disorders.

Traditional medical and alchemical knowledge in Spain is influenced by the Andalusian Muslim medicine (García-Ballester, 1984, 2001). This process was greater in the Kingdom of Valencia (García-Ballester, 1989; García-Ballester and McVaugh, 1996) through popularization of knowledge (García-Ballester, 1982; García-Ballester, 1984; García-Ballester, 2001) before the final expulsion of the Moors from Spain.

The greatest development of distillation and new medicines occurs in Spain from the second half of the 16th century, due to the support of King Felipe II and his court (Loring, 1993; Puerto, 1993; Bueno and Alegre, 2001; Martín-Reyes, 2004; López-Pérez, 2016). The greatest advances were made on this subject through the so-called "royal distillers" who worked in three great alchemy laboratories created and protected by the Crown in Aranjuez, Madrid, and El Escorial. One of them, Diego de Santiago, wrote a distillation manual *Arte separatoria*, and many distillation devices that were exported to other European courts (Bueno and Alegre, 2001). In 1592, Francisco Vallés following the orders of Felipe II tried to regulate the distillation of spirits throughout Spain, putting it exclusively in the hands of the apothecaries (Bueno and Alegre, 2001; Martín-Reyes, 2004). This law, which was a pioneer in Europe, implicitly recognizes that there were outside the court and its area of influence numerous particular industries that manufactured spirits to satisfy the great demand for distilled waters that existed among the population (Bueno and Alegre, 2001; Martín-Reyes, 2004).

Complex classical medical wines evolved into complex spirits or, as they are more popularly known, medicinal liquors. This is evidenced in the Castilian translations of Dioscorides by Laguna (1555), Laguna (1566) and in the first Catalan edition of 1617 from the *Libro de los Secretos de Agricultura, Casa de Campo y Pastoril* of Fray Miquel Agustín (Agustín, 1722).

This conceptual change will already remain invariant until the beginning of the 20th century. Today homemade and informal preparations produced locally at family level or small industries are known as traditional alcoholic beverages (Egea et al., 2016). The World Health Organization includes these traditional drinks in the so-called "unrecorded alcohol," highlighting its cultural, social, and economic importance around the world, and the risk of their consumption. It has been estimated that almost one-quarter of all the alcohol consumed worldwide is drunk in the form of unrecorded alcohol (Rehm et al., 2014; WHO, 2018; Okaru et al., 2019). For many decades, home distillations have been disappearing. Currently, homemade liquor makers buy the liquor from local distilleries that already sell the legalized solvent to them.

In the East of Spain, most of these homemade liquors are elaborated, macerating some wild or cultivated plants in anise-flavored alcohol (Martínez-Francés and Ríos, 2005; Martínez-Francés et al., 2012; Martínez-Francés et al., 2015; Martínez-Francés et al., 2017). They are part of the traditional medicinal recipe repertory, inherited from the ancient complex formulas of herbal teas and medicinal wines (Martínez-Francés et al., 2015). The know-how linked to the elaboration of herbal liquors is part of the Traditional Knowledge System. This knowledge evolves usually over long periods of time and it is transmitted from one generation to the next (Hunn, 1998). The importance of this type of knowledge, defined as Intangible Cultural Heritage, was recognized by UNESCO in 2003 (Savo et al., 2011). The essence of Traditional Knowledge is not the mere product or use of a plant but includes a great sum of

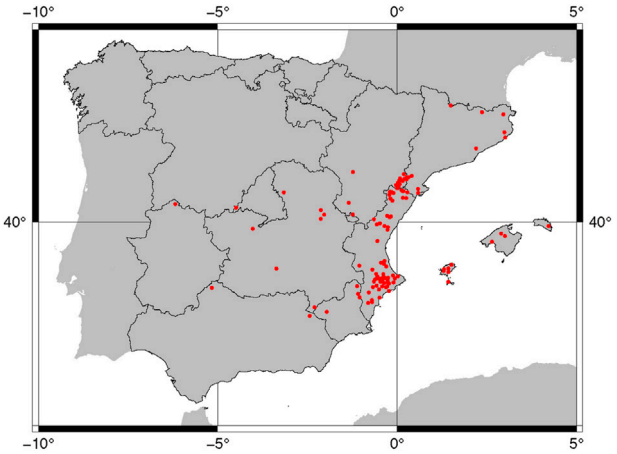


FIGURE 1 | Location map of the studied areas.

knowledge about the local environment and its ecological rules embracing culture, history, and symbolism. Nowadays, it is disappearing in industrialized countries as a consequence of different economic trends, food, and drug availability and changes in communication, culture, and values (Savo et al., 2011).

There are various general ethnobotanical studies in the different regions covered in this work with some partial references to traditional homemade herbal liquors: Aragon (Villar et al., 1992), Balearic Islands (Carrió and Vallès, 2012; Moll, 2003, 2005), Castilla-La Mancha (Fajardo et al., 2007; Verde et al., 2008), Catalonia (Agelet and Vallès, 2001; Bonet and Vallès, 2002; Parada et al., 2011; Figueras and Castelló, 2012), Murcia (Rivera et al., 2008), and Valencia (Mulet, 1991; Pellicer, 2001). But there are no works that cover the whole of the territory and are specifically dedicated to this matter that is on the interface on food and medicine.

The specific aims of the present research are as follows.

To document the formulation and uses, particularly those medicinal, of traditional homemade alcoholic beverages and the related knowledge in eastern Spain. For this, we intend to identify the different ingredients and their relative importance in the whole as well as determining the types and styles of these drinks and their geographic patterns if they exist.

It is also intended to analyze the evolution in the complexity of the registered formulas over the almost 15 years of study, discuss the factors that determine the patterns of geographic distribution of this knowledge, and, finally compare our results with available evidence from western Mediterranean areas on similar formulations reputed as nutraceuticals.

MATERIALS AND METHODS

Study Area

The study area extends along eastern Spain including the Balearic Islands (Mallorca, Menorca, Ibiza, and Formentera Islands) and Andorra (**Figure 1**). The main sampling activities were developed within the Comunidad Valenciana (provinces of Alicante, Castellón, and Valencia), followed by southern Aragon (Teruel province) and southern Catalonia (Tarragona province). Castilla-La Mancha, Murcia, Madrid, Extremadura, and Andalusia region were less intensively sampled. We paid special attention to obtaining ethnobotanical data in the Valencian region and the Aragon border (**Table 1**). The rest of the areas have also prospected, although to a lesser extent, to follow our study methodology and to be able to compare the data.

The provinces of Alicante, Valencia, and Castellón offer a homogeneous physiognomy from the general ethnological point of view. Murcia is a transition zone between Andalusia and the Levant, but socially and economically, Murcia is a Levantine land with Castilian influences. In both territories, the Moorish cultural footprint has persisted, especially in the garden areas along the rivers subject to traditional irrigation (Caro-Baroja, 1981). This Moorish persistence can also be seen in the Lower Aragón territories and the Teruel mountain ranges, singularly where fertile orchards are cultivated along the rivers (Caro-Baroja, 1981). Traditional housing is based on the use of local resources and is oriented to cope with extreme temperatures and the impact of torrential rains in the form of floods. Especially in the Valencian territory, the festivals are characterized by their ostentation and luxury and offer an exceptional occasion for the shared consumption of different herbal liqueurs or "herberos." The population structure of the Balearic Islands differs from that of the Levantine coasts of the peninsula. However, the Valencian and Catalan influence, and even Aragonese, from the linguistic, social, and economic points of view, has been intensely felt in more modern times (Caro-Baroja, 1981). The Catalan territories are defined mainly by the Catalan language, which philologically differs from those of Valencia and the Balearic Islands, with which it shares a common substratum. Within the sampled area, the eastern Catalan dialect predominates in the provinces of Gerona and Barcelona and the western Catalan dialect in the south of Tarragona and the neighboring territories of Aragon (Caro-Baroja, 1981).

TABLE 1 | Geographical provenance of the formulas and samples analyzed.

Autonomous communities and countries	Provinces	Samples
Andalucia	(total)	2
Andorra[a]	(total)	3
Aragón	Teruel	56
	(total)	56
Baleares	(total)	26
Castilla-La Mancha	(total)	20
Cataluña	Tarragona	37
	(total)	55
Com. Valenciana	Alicante	239
	Castellón	105
	Valencia	48
	(total)	392
Extremadura	(total)	1
Madrid	(total)	1
Reg. Murcia	(total)	13

[a]*Andorra is an independent country of the Pyrenees.*

The whole of the study area lies within the Mediterranean Region, characterized by summer drought, which in the mountains is eventually attenuated by occasional storms (Peinado-Lorca and Rivas-Martínez, 1987). Most of the sampled localities fall, in terms of biogeography and vegetation, within the Valencian-Provençale-Balearic province and, especially, within the different sectors of the Valencian subprovince (57%) (Rivas-Martinez et al., 2014). However, other territories sampled in some detail belong to the Balearic Islands biogeographical subprovince (10%), Oro Iberian subprovince (c. 12%), and the Alicantese-Murcian sector (c. 9%) of the Murcian Almeriese province. Bioclimatically, most are, in terms of temperatures, Mesomediterranean (with average annual temperature 13–17°C) or Thermomediterranean (17–19°C) and in terms of rainfall are semiarid (200–350 mm), dry (350–600 mm), or subhumid (600–1,000 mm) (Peinado-Lorca and Rivas-Martínez, 1987).

The calcicolous thyme scrub, rosemary and *Cistus* garrigue, and holm oaks or junipers woodlands are the main formations in the Valencian subprovince (Folch, 1981; Folch et al., 1984; Costa, 1999) and where our informants obtain most of the ingredients along with fields and orchard gardens.

The geological and climatological diversity of this territory has generated numerous habitats, multiplying the plant diversity that has conditioned the population's way of life. Mountain areas acquired a great reputation in terms of medicinal plant richness becoming centers of herbs collection and also as the most important places for peregrination (De-Terán, 1949).

Data Collection and Plant Specimens Identification

Ethnobotanical fieldwork for this study was conducted along two main periods: the first, more intensive between 2005 and 2011, and a second period with less intensity later until 2018. Prior informed consent was obtained verbally before each interview, following the ISE Code of Ethics (ISE, 2006). We used a combined sampling strategy starting by defining a sampling frame focusing on inns, distilleries, and traditional "herberos" fairs in the main area of study and, then, recurred to the snowball sampling strategy when it was possible (Lambert, 1990; Turner, 2003).

Local knowledge for medicinal plants used in liquors and wines was collected from 83 female (170 records) and 194 male informants (412 records), using semistructured, focalized interviews (details are available in **Supplementary Table S1**). From the point of view of the languages and dialects (Burgueño, 2002; García-Mouton, 2007; Vales, 2012) spoken by our informants and in which the recipes were collected, Valencian prevails, followed by Spanish, Catalan of southern Catalonia, and Balearic. The occupations of our informants were varied, ranging from innkeeper (56%) or distiller (14%) to official or pharmacist (6% each), shepherd (4%), craft seller, farm shop keeper, honey seller, or folk group member (2% each) and forest ranger, tobacconist, rural tourism manager, healer, or priest (1% each).

It is worth noting that thirteen records were obtained in interviews that involved the joint participation of men and women from the same family unit. The estimated age of the interviewees at the interview moment ranged from 23 to 93. Based on the estimated age, only one informant was under 25; approximately 10% were between 25 and 39; the maximum number of informants occurred in the age ranges 40–54 (31%) and 55–69 (39%). Approximately 19% of our informants were 70–84 and only four were within the 85–99 age range.

Information on the herbs used and the way these were combined in each particular case was obtained from three main sources: first, and more often, the informants supplied us with the recipe or formula including the herbal ingredients and accompanied us to the field in order to verify the botanical identity of these when this was possible and to collect voucher specimens; alternatively, they gave us the bottle containing the liquor and the herbs and finally, which for us was the optimum, but more difficult to achieve, we obtained both recipe and liquor sample.

Plant specimens were collected from field surveys, given, dried, by the informants, or obtained by the analyses of samples (bottles with medicinal wines or liquors) (**Figure 2**; **Table 2**). They were identified using the Flora dels Països Catalans (Bolòs and Vigo 1984; Bolòs and Vigo, 1989; Bolòs and Vigo, 1996; Bolòs and Vigo, 2001) and, in some cases, with the help of specialists in floristic and systematic botany. Voucher specimens were deposited in the Herbarium of the Barcelona University (BCN) and the Herbarium of the Valencia University (Jari Botanic) (VAL) (details are available in **Supplementary Table S2**). The material obtained alive in good condition for cultivation was introduced in the living collections of the Torretes Botanical Garden and used to prepare voucher specimens. Plant nomenclature is in accordance with POWO (POWO, 2021). For foreign or cultivated species determination, we followed Rivera et al. (1996); Rivera et al. (1998) and actualized their nomenclature according to GRIN (2021). The species were identified by Martinez-Frances and Rios.

Data Management and Analysis

The data collected during the fieldwork refer to the preparation and consumption of any type of homemade herbal alcoholic beverage. These data have been entered in a database generated with Microsoft Excel® 2007. These include, among others, information on plant ingredients (scientific name, family, vernacular name/s, and plant parts used), type of solvent (distillate, anise-flavored alcohol, and wine), type of beverage (distillate, liquor, and aromatized wine), main herbal types (fruits, herbs, mixture of fruits and herbs, fungus, and animal), processing and main consumption contexts and patterns, current usage, and medicinal local uses (digestive, aperitif, tonic-restorative, antidiarrheal, emmenagogue, and preservative-wine). Each elemental record represents one citation, defined as a single reported use for a single plant by a single informant. Different records are considered those that differ from each other in at least one of the following data: species, plant parts used, informant, type of beverage, and use category. We used for the analysis of data the set of functions and formulas available in Microsoft Excel® 2007. These include the creation of thesaurus to standardize scientific names of plants and names of regions and provinces using VLOOKUP. Among other

FIGURE 2 | Main types of herbal liquors analyzed. **(A)** Herbero from Valencia region. **(B)** Beatamaria from Tarragona. **(C)** Ratafia from Girona. **(D)** Beatamaria from Teruel. **(E)** Cantueso from South of Alicante province. **(F)** Herbero from Formentera island. **(G)** Salvieta from North of Alicante province. **(H)** Gitam and fruit spirit from Castellon province. **(I)** Fruit spirit from Albacete province. **(J)** Collection of voucher specimens in the field accompanied with the informants. **(K,L)** Identification of bottle contents in the laboratory by a member of the research team.

functions frequently used for this study are SUMIF and COUNTIF.

Given the extraordinary diversity of the sample in terms of informants (277), localities (126), different formulas (533), and identified ingredients (215 species), the dissimilarities calculated through the Sokal-Sneath index (Perrier et al., 2003), based on the presence/absence using Darwin 6 (Perrier and Jacquemoud-Collet, 2006), resulted in non-Euclidean distances, with low resolution. In the factorial analysis, only 7% of inertia was explained by the first axis. Therefore, we considered that the use of multivariate analysis techniques would not be appropriate for the study of the group that is the object of this work.

Maps have been created with The Generic Mapping Tools© 1991–2020, version 6.1.1 [64-bit] (GMT, 2021).

TABLE 2 | Main types of information recorded and their regional frequency.

Region	Only formulas	Only liquor samples	Formula and sample	Totals
Castilla-La Mancha	8	2	10	20
Com. Valenciana	268	54	78	400
Andorra[a]	3	0	0	3
Cataluña	28	1	18	47
Extremadura	0	0	1	1
Andalucia	2	0	0	2
Baleares	17	5	4	26
Madrid	1	0	0	1
Reg. Murcia	11	0	2	13
Aragón	49	2	5	56
Total	377	64	118	569

[a]*Andorra is an independent country of the Pyrenees.*

TABLE 3 | Ethanol contents of the bases used for macerating the plant ingredients and sources for this alcohol.

A

Alcohol contents in percentage	Formulas and liquor samples
Wine (16–20%)	63
Low alcohol spirit (20–34%)	312
Aniseed spirit and others (35–70%)	194

B

Source and type	Formulas and samples
Wine/distilled wine alcohol	289
Honey	9
Distilled molasses alcohol	298
Spirit aromatized with "*anís de matalauva*" or star anise	278

Often the final liquor is elaborated by mixing different bases; hence, the total can exceed the number of 569 formulas studied.

Main differences in the type of homemade beverages taken into account in this study consist in the ingredients macerated (species and part of the plant) and the type of solvent used (**Table 3**). For liquors, anise-flavored alcohol (Martínez-Francés and Ríos, 2005; Martínez-Francés et al., 2012; Martínez-Francés et al., 2017) is usual. The alcoholic concentration of spirits has been noted and classified considering two categories: one between 20 and 35° and the other between 36 and 70°. In many cases, this is the difference between spirits bought in warehouses, the former, or made in their own alembics, the latter. The origin of alcohol (*Vitis vinifera* L., *Beta vulgaris* L. subsp. *vulgaris* Sugar beet group, and *Saccharum officinarum* L.), the sweetener (*B. vulgaris* subsp. *vulgaris*, *S. officinarum*, and honey), and the anise flavoring (*Illicium verum* Hook.f. and *Pimpinella anisum* L.) has also been identified wherever possible. However, these were not included in our analysis since they were not part of the macerate mixtures and, therefore, did not yielded voucher specimens. In the case of aromatized wines, the most common base used is red wine, although in some cases, white or rose wines have also been used.

Those species that serve as a base for the alcoholic beverage, as sweeteners, or as flavorings and are previous to the addition of the herbs to macerate are excluded from the total species count of each recipe.

RESULTS

General Results

We interviewed 277 informants (194 men; 83 women) whose average age was 56 years. Men account for 70% of those interviewed, highlighting the importance of this aspect in the preparation of medicinal liquors, compared to the usual role of women in family care.

In the first interview period, until 2011, older people were prioritized, reaching 23% of those interviewed, with an age between 70 and 93 years. The most numerous age group studied is between 40 and 70 years, representing 66% of the total. In the second period, until 2018, the interviews with younger people were intensified. This was the period when some of them replaced, in the liquor preparation activities, the older informants of their families.

The approximate age of the informants is relevant for the number of formulas recorded, with a maximum of 227 records

TABLE 4 | Medicinal and other uses of the studied herbal wines and spirits.

Main use	Formulas and samples	%
Medicinal		
• Digestive	493	86.6
• Tonic (restorative)	178	31.3
• Aperitif	78	13.7
• Antidiarrheal	53	9.3
• Emmenagogue	49	8.6
Others		
• Social	440	77.3
• Preservative (wine)	26	4.6

Liquors are often consumed for more than one purpose (cf. ***Table 6****); hence, the totals can exceed the number of 569 formulas studied.*

TABLE 5 | Frequency of plant parts among the analyzed liquors.

Parts	Species	%	Records[a]	%
Aerial parts	70	30.0	1,279	49.3
Leaves	45	19.3	423	16.3
Flowers/inflorescences	35	15.0	250	9.6
Fruits	31	13.3	257	9.5
Tender shoots	22	9.4	53	2.0
Roots	7	3.0	46	1.8
Seeds	5	2.1	37	1.4
Stems	4	1.7	118	4.6

[a]*Note that one single sample with different plant species can contain more than once the different recognized parts.*

from informants between 40 and 54 years, followed by 166 from those between 55 and 69 and 113 from those aged 70 to 84.

The bases used for macerating the plant ingredients are wines (usually red wine) and spirits of different ethanol concentrations (**Table 3**).

We compiled an inventory of 215 taxa from 56 families. Most, 128 (60%), are wild species, followed by 74 cultivated (34%), few are imported (12, 6%), and one is feral. The proportion of imported species differs along the types and styles recorded. Vascular plant taxa are 212, two are fungi, and one is zoological.

From a phytogeographic perspective (POWO, 2021), Mediterranean species predominate (118, 55%) followed by those European (37, 17%) and widespread (30, 14%). Minor represented groups include Northern Hemisphere, Asia, America, Southern Hemisphere, and Pacific Islands.

Medicinal Uses of the Herbal Wines and Spirits

Almost 87% of the recorded formulations are reputed by our informants as being digestive and are consumed as such (**Table 4**). However, they recognized that also most herbal liquors were consumed in social events involving high food intake or, especially by the younger population, merely "per se" no matter the supposed health benefits of their consumption. The use as a general tonic or to improve their mood along with appetite stimulant follows in importance (**Table 4**). Their uses as antidiarrheal (especially Balearic *herberos*) or as emmenagogue (notably *beatamaria*) are worthy of notice, although in terms of percentage they are low (below 10% each).

The eldest informants are those who have described a greater diversity of medicinal uses for wines and liquors, also referring to the use of the same formulas in herbal teas on a regular basis. For them, some liquors are multipurpose, reporting even four or five different uses for one single liquor (twenty-nine and seven formulas, respectively). However, the commonest figure recorded is two or three different uses (340 and 147 formulas, respectively). Therefore, the largest number of references recorded present two uses: to be consumed in social events combined with a single medicinal purpose. Only forty-five formulas were reported to have a single one use (medicinal or not).

TABLE 6 | Plant families more often present in the analyzed liquors.

Plant family	Samples	%	Species	%
Rutaceae	244	42.9	11	5.1
Lamiaceae	240	42.2	60	27.9
Asteraceae	177	31.1	20	9.3
Apiaceae	154	27.1	11	5.1
Rosaceae	86	15.1	20	9.3
Verbenaceae	86	15.1	1	0.5
Rubiaceae	57	10.0	5	2.3
Lauraceae	53	9.3	2	0.9
Juglandaceae	44	7.7	1	0.5
Malvaceae	38	6.7	4	1.9
Myrtaceae	28	4.9	3	1.4
Vitaceae	26	4.6	1	0.5
Hypericaceae	24	4.2	3	1.4
Asparagaceae	22	3.9	1	0.5
Cistaceae	9	1.6	7	3.3

Among the 215 species identified, two are fungi, one is Animalia, and 212 are Plantae. Of the 56 families of plants, animals or fungi identified, only the fifteen with more than five species or that appear in more than 20 samples are represented in this table and they are all plants.

The Ingredients

Aerial parts are the most frequently used plant parts (**Table 5**) followed by leaves, flowers, and fruits.

Lamiaceae is by far the most widely represented plant family in terms of species (c. 28%) but slightly surpassed by Rutaceae in the percentage of samples (**Table 6**). Asteraceae follows in the percentage of samples notwithstanding its high number of species in the Spanish flora (Mateo and Crespo, 2014; Anthos, 2021). We must underline the overrepresentation of Rutaceae in the formulas that are due in the case of cultivated *Citrus* species to their aromatic leaves, flowers, and fruits and the abundance of orange, lemon, and tangerine orchards in, notably, Valencia and Murcia regions. Wild Rutaceae species are noticeable because nevertheless their relative scarcity in the area, they are highly sought for with the purpose of elaborating liqueurs (**Table 6**).

Among the 215 species identified, only 21 were recorded as ingredients of more than 40 formulas (**Table 7**). Given the high number of *herberos* analyzed, their contribution to the list of relevant species is significant.

TABLE 7 | Most frequently species in the liquors analyzed from E Spain.

Species	Frequency (samples)	%
Dictamnus hispanicus Webb ex Willk.	183	32.2
Santolina villosa Mill.	114	20.0
Salvia blancoana subsp. *mariolensis* Figuerola	103	18.1
Rosmarinus officinalis L.	100	17.6
Thymus vulgaris L. subsp. *vulgaris*	97	17.0
Clinopodium serpyllifolium subsp. *fruticosum* (L.) Bräuchler	90	15.8
Aloysia citriodora Palau	86	15.1
Foeniculum vulgare Mill. subsp. *piperitum* (Ucria) Cout.	81	14.2
Sideritis hirsuta L.	79	13.9
Sideritis tragoriganum Lag. subsp. *tragoriganum*	74	13.0
Citrus x *limon* (L.) Osbeck	67	11.8
Eryngium campestre L.	63	11.1
Teucrium capitatum subsp. *gracillimum* (Rouy) Valdés Berm.	59	10.4
Melissa officinalis L.	54	9.5
Thymus moroderi Pau ex Martínez	52	9.1
Cinnamomum verum J.Presl	46	8.1
Stachys heraclea All.	46	8.1
Coffea arabica L.	45	7.9
Mentha pulegium L.	45	7.9
Juglans regia L.	44	7.7
Chiliadenus glutinosus (L.) Fourr.	41	7.2

Among the 215 recorded species, those 21 species found in more than 40 samples are only represented.

TABLE 8 | Species furnishing more than two ingredients.

Standardized species	Number of ingredients	Ingredients
Citrus x *limon* (L.) Osbeck	5	Tender shoots, flowers/inflorescences, fruit rind, leaves, and extract (juice)
Foeniculum vulgare Mill. subsp. *piperitum* (Ucria) Cout.	4	Aerial parts, leaves, stems, and seeds
Citrus x *sinensis* (L.) Osbeck	4	Flowers/inflorescences, fruits, fruit rind, and leaves
Dictamnus hispanicus Webb ex Willk.	3	Aerial parts, leaves, and roots
Artemisia absinthium L.	3	Aerial parts, tender shoots, and extract (essential oil)
Juniperus oxycedrus L.	3	Tender shoots, leaves, and cones
Rubus ulmifolius Schott	3	Tender shoots, fruits and flowers/inflorescences
Pinus halepensis Mill.	3	Tender shoots, leaves, and flowers/inflorescences

Among the 36 species furnishing more than one ingredient (depending on the plant part used), only those furnishing three or more ingredients are represented.

Lamiaceae is not only the most relevant plant family in the number of species but also in the frequency they are used. Among the 21 most commonly used plant species, over one-half are Lamiaceae (**Table 7**). Among these, *Salvia blancoana* subsp. *mariolensis, Rosmarinus officinalis, Thymus vulgaris*, and *Clinopodium serpyllifolium* subsp. *fruticosum* are present in more than 15% of the formulas.

A few species furnish more than one ingredient which are separately used by our informants. Those supplying three or more ingredients are listed in **Table 8**. Three Rutaceae are included, *Citrus* x *limon*, *C.* x *sinensis*, and *Dictamnus hispanicus*. These three species, together with *Foeniculum vulgare* subsp. *piperitum*, stand out for their popular role as flavoring in foods.

Regarding the number of ingredients macerated in the base alcohol for the elaboration of the liquors, it varies from one to more than 30 (**Figure 3**). The maximum diversity records for this study are 48 species in a ratafia recipe from Girona province and 47 in one herbero from Mallorca Island.

Main Types and Styles Based on the Mixture of Herbs

We have recorded 569 formulas and/or samples of wines, spirits, and liquors (**Table 2**). Among these, 423 are unique differing in their combination of ingredients. These were classified according to their solvent and herbal ingredient composition into the following main types: absinthe, *beatamaria*, *cantueso*, cucumber spirit, fruit spirit, fruit wine, *gitam*, herbal spirit, herbal wine, *herbero*, honey spirit, mushroom spirit, *ratafia, resoli, salvieta*, spirit, vermouth and walnut wine, and Benedictine-like and digestive wine. Their relative frequencies and distribution in the different regions studied show peculiar patterns that are summarized in **Figure 4**. The most relevant species and their combinations allow defining these peculiar types of herbal liquors (**Table 9**).

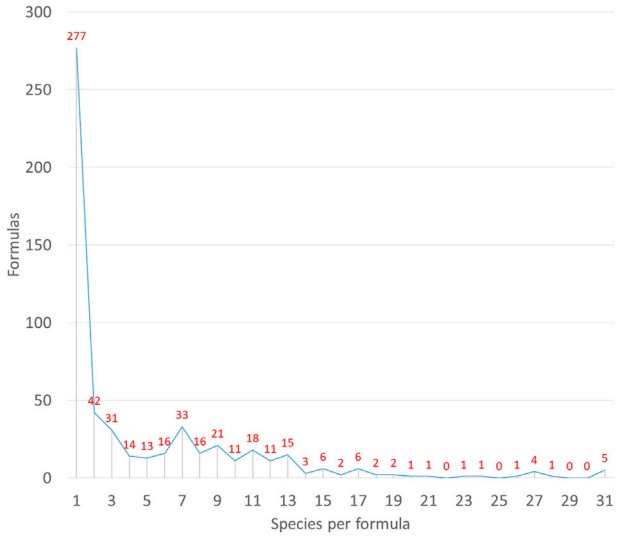


FIGURE 3 | Complexity of formulations in terms of number of species entering the formulas.

The complexity of the formulations recorded is extremely variable between the different types and even within each type. *Herbero* with 47 and *ratafia* with 48 present the maximum number of ingredients within a single formulation. However, within these same types, we have found simpler formulations with five or fewer ingredients. The rest of the types have a number of ingredients oscillating between one and nine. *Absenta, beatamaria, cantueso, gitam,* and *salvieta* are noteworthy herbal liquors simple or with lesser ingredient diversity.

Polygonatum odoratum characterizes the *beatamaria* formulations of Aragon and Catalonia (**Table 9**). It is possible to find simple and complex variants of the main formula, being always the core species *Polygonatum odoratum* (**Figure 5**). *beatamaria* is elaborated in a small area where coincide three provinces, Tarragona (Catalonia), Teruel (Aragon), and Castellón (Valencia region).

From the same area but extending widely to the south reaching Murcia (**Table 9**), the *gitam* stands out (**Figure 6**). It is made with *Dictamnus hispanicus* and the distribution of this liquor coincides practically with that of the species in the East of Spain. In the upper part of the production territory, these two liquors are made with high-alcohol content spirits.

In South of Valencia close to the province of Alicante, *salvieta* liquor becomes noticeable, both in its simple formula with *Salvia blancoana* subsp. *mariolensis* and in the complex formulas where other sage species are mixed such as *S. microphylla* var. *microphylla, S. officinalis* subsp. *lavandulifolia*, *S. officinalis* subsp. *officinalis*, *S.* x *auriculata*, and *S.* x *hegelmaieri* (Martínez-Francés et al., 2017).

In the South of Alicante province, *Dictamnus* disappears and *Thymus moroderi* becomes the main species. This single liquor is called *cantueso* and it is made and also commercialized in a very small area between Alicante and Murcia.

In the area described so far, from Tarragona to Murcia, excluding Aragon, complex herbal liquors are made that we have typified as *herbero*. We collected 186 formulas, of which 88% are from the different mountainous areas of the Valencian region. In this herbero type, complex formulas of Balearic Islands are included (**Table 9**). The Balearic formulas lack *Dictamnus* as an essential plant, because it does not exist on the islands, but it is replaced by rue. But there is a much greater difference between the composition of these liquors between the main island, Mallorca, and the rest. In Mallorca, whose main function was to combat recurrent diarrhea, *herbero* is made mainly with Rosaceae species, and with *Tanacetum balsamita*, a species considered a panacea, which also stands out. In Menorca, Ibiza, and Formentera, their liquors include both cultivated and wild plants and the *herbero* type is more similar to the peninsular ones. The Balearic Islands local variants of *herbero* are characterized by the use of *Santolina magonica* (local endemic species), *Aloysia citriodora*, and *Rosmarinus officinalis* among others (**Table 9**).

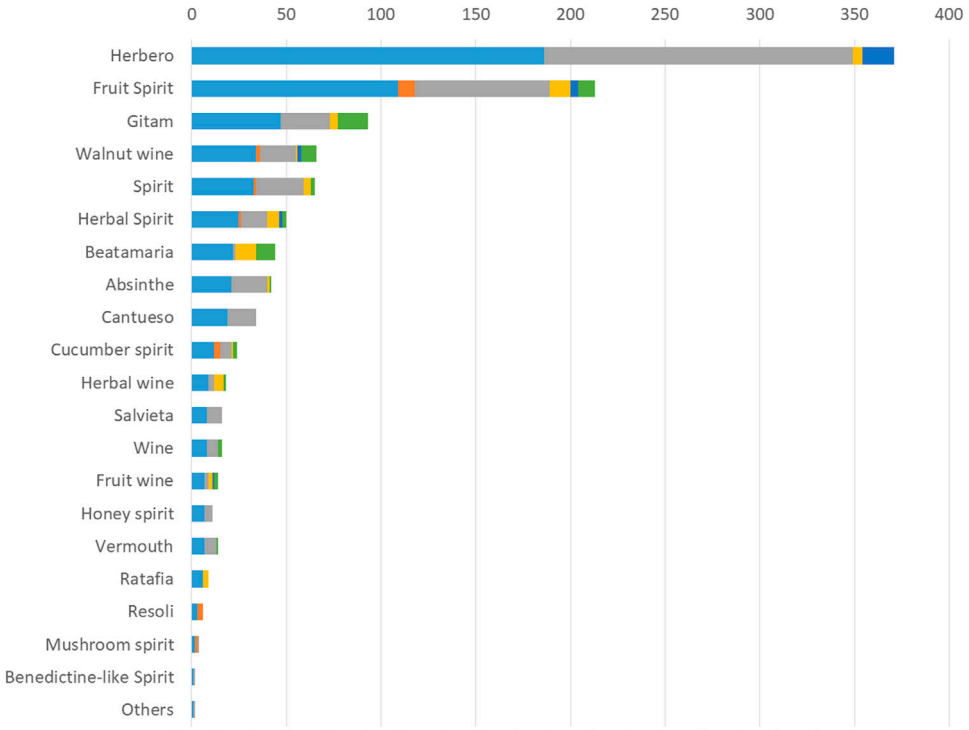
Dictamnus hispanicus and *Santolina villosa* are present in over 60% of the 186 *herbero* formulations analyzed, mainly from Valencia and Balearic Islands (**Figure 6**). Other species often used in herbero are *Salvia blancoana* subsp. *mariolensis* Figuerola and *Thymus vulgaris* L.

Vermouth and absinthe, though underrepresented in our samples, are both characterized by the use of *Artemisia absinthium.* Absinthe and vermouth are linked and although there have been crops and distilleries in the interior of Castellón province, their consumption was mainly restricted to the coastal areas where it was also produced at a popular and commercial level. Both sum 28 records, belonging c 90%, to the Valencia region.

The infrequent Benedictine-like liquor, which is no longer produced, is characterized by the use of *Angelica archangelica* and a mixture of spices. Walnut wine is made, especially in Valencia but also in Aragon, Castilla-La Mancha, and Murcia regions, with *Juglans regia* tender fruits that enter also in the ratafia formulations of Catalonia. Fruit wines are often aromatized with cinnamon bark.

Cucumber spirit is a fruit liquor made in the past throughout the territory, being used as a powerful digestive, but many of the bottles found were very old. Honey spirit is also elaborated in different regions of Spain. We reported formulas of *resoli* mainly from the northeast of Castilla-La Mancha, and also a mushroom spirit is elaborated there and in Castellón (Valencia region). All of them are used as digestives.

Data collected before 2007 yielded an average number of herbal ingredients of c. 10. This allowed us to establish a preliminary geographical differentiation of formulas according to the dominance of different specific groups of plants. An obvious generational rupture, away from rural and traditional customs and a subsequent interest of return to natural lifestyles, led to homemade globalization of this type of medicinal beverage. Accordingly, formulas recorded were drastically simplified with an average number of ingredients varying between 3.2 and 5.5 depending on the 4 years period considered. Presently, they can be seen as merely "author elaborations" as digestive liquors. The



	Others	Benedictine-like Spirit	Mushroom spirit	Resoli	Ratafia	Vermouth	Honey spirit	Fruit wine	Wine	Salvieta	Herbal wine	Cucumber spirit	Cantueso	Absinthe	Beatamaria	Herbal Spirit	Spirit	Walnut wine	Gitam	Fruit Spirit	Herbero
Total	1	1	2	3	6	7	7	7	8	8	9	12	19	21	22	25	33	34	47	109	186
Castilla-La Mancha	0	0	1	3	0	0	0	0	0	0	0	3	0	0	0	1	1	2	0	9	0
Com. Valenciana	1	1	1	0	0	6	4	2	6	8	3	6	15	19	1	14	25	19	26	71	163
Cataluña	0	0	0	0	3	0	0	2	0	0	5	1	0	1	11	6	4	1	4	11	5
Baleares	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	0	2	0	4	17
Aragón	0	0	0	0	0	1	0	2	2	0	1	2	0	1	10	2	2	8	16	9	0

FIGURE 4 | Main types of herbal liquors analyzed and their frequencies.

average number of herbal ingredients of the formulas recorded after January 1 of 2016 is 2.9. Therefore, in almost 20 years of study, we have recorded an impoverishment of the use of plant species and, also, the medicinal uses for this type of preparation.

DISCUSSION

Relevant Species

Dictamnus hispanicus (**Figures 5**, **6**) is present in one-third of the formulas recorded during our study (**Table 7**). The species grows in the study area, nowadays, among the unmanaged scrub or in inaccessible areas of the Iberian mountains. Its distribution area fits with the elaboration area of the characteristic *gitam* and *herberos*. In Spain, leaves of *D. hispanicus* (Font-Quer, 1985; Mulet, 1991; Ríos and Martínez-Francés, 2003; Martínez-Francés and Ríos, 2005; Merle et al., 2006; Popović et al., 2014; Martínez-Francés et al., 2015) were used instead of the rhizome, contrary to what is commonly used in Central Europe (Fournier, 1947; Ivanova et al., 2004; Panesar et al., 2009; Tiță et al., 2009). The parts most used in the spirits studied in eastern Spain are leaves (173), while the aerial parts including stems and flowers (if any) appear in nine and rhizomes only in two. The almost exclusive use of leaves is clearly related to the sustainability of the resource from the point of view of our informants. *D. hispanicus* aerial parts were used in teas as digestive and emmenagogue (Martínez-Francés et al., 2015; Martínez-Francés et al., 2018a).

This species has multiple medicinal uses that are treated in detail in Martínez-Francés et al. (2015). The use of *D. hispanicus* for dyspepsia and other diseases of the digestive system involves the maceration in alcoholic beverages, steeping fresh leaves, or flowering tops into spirits of 25–40% of ethanol, in simple *gitam* formulas but often in other more complex named *beatamaria* and *herberet* or *herbero*, which in recent decades have gained popularity as digestive and tonics and are used within a broad social context (Martínez-Francés et al., 2015).

The use of *Dictamnus* in herbal liquors recalls that of Alpine wormwood (*Artemisia genipi* and other spp.) and

TABLE 9 | Regional styles based on ingredient frequencies in the percentage of regional samples.

Standardized species	Baleares	Com. Valenciana	Aragón	Cataluña	Castilla-La Mancha	Reg. Murcia
Dictamnus hispanicus Webb ex Willk.	0.00	37.56	28.57	18.18	0.00	15.38
Santolina villosa Mill.	23.08	26.42	0.00	10.91	0.00	0.00
Salvia blancoana subsp. *mariolensis* Figuerola	0.00	24.35	0.00	0.00	0.00	0.00
Thymus vulgaris L. subsp. *vulgaris*	3.85	23.83	0.00	5.45	0.00	7.69
Clinopodium serpyllifolium subsp. *fruticosum* (L.) Bräuchler	0.00	21.50	0.00	7.27	5.00	0.00
Sideritis hirsuta L.	0.00	20.47	0.00	0.00	0.00	0.00
Rosmarinus officinalis L.	50.00	19.69	0.00	16.36	0.00	0.00
Sideritis tragoriganum Lag. subsp. *tragoriganum*	0.00	19.17	0.00	0.00	0.00	0.00
Aloysia citriodora Palau	53.85	15.80	0.00	14.55	0.00	0.00
Teucrium capitatum subsp. *gracillimum* (Rouy) Valdés Berm.	0.00	15.28	0.00	0.00	0.00	0.00
Foeniculum vulgare Mill. subsp. *piperitum* (Ucria) Cout.	7.69	15.03	0.00	0.00	0.00	0.00
Thymus moroderi Pau ex Martínez	0.00	12.69	0.00	0.00	0.00	23.08
Juglans regia L.	11.54	5.96	14.29	5.45	10.00	15.38
Pimpinella anisum L.	7.69	5.18	0.00	5.45	15.00	15.38
Cinnamomum verum J.Presl	11.54	6.99	3.57	12.73	20.00	15.38
Vitis vinifera L.[a]	0.00	3.63	3.57	3.64	20.00	7.69
Syzygium aromaticum (L.) Merr. and L.M.Perry	0.00	1.81	0.00	10.91	15.00	7.69
Citrus x *limon* (L.) Osbeck	46.15	9.33	1.79	7.27	0.00	0.00
Santolina magonica (O.Bolòs, Molin. and P.Monts.) Romo	38.46	0.00	0.00	0.00	0.00	0.00
Foeniculum vulgare Mill. subsp. *piperitum* (Ucria) Cout.	38.46	0.26	0.00	10.91	0.00	0.00
Thymbra capitata (L.) Cav.	34.62	0.52	0.00	7.27	0.00	0.00
Citrus x *sinensis* (L.) Osbeck	23.08	2.85	0.00	5.45	15.00	7.69
Ruta chalepensis L.	23.08	0.00	0.00	7.27	0.00	0.00
Melissa officinalis L.	15.38	11.40	0.00	5.45	0.00	0.00
Coffea arabica L.	15.38	8.29	0.00	1.82	20.00	7.69
Juniperus oxycedrus L.	19.23	0.00	0.00	7.27	0.00	0.00
Mentha spicata L.	19.23	5.18	3.57	3.64	0.00	0.00
Salvia microphylla Kunth	19.23	3.37	0.00	0.00	0.00	0.00
Citrus x *sinensis* (L.) Osbeck	19.23	0.78	0.00	3.64	0.00	0.00
Citrus x *limon* (L.) Osbeck	15.38	1.55	1.79	12.73	0.00	7.69
Laurus nobilis L.	15.38	0.52	0.00	3.64	0.00	0.00
Pinus halepensis Mill.	15.38	0.00	0.00	0.00	0.00	0.00
Polygonatum odoratum (Mill.) Druce	0.00	0.26	17.86	20.00	0.00	0.00

Ingredients with a frequency of 15% or higher within at least one regional ensemble are only represented here.
[a]*Raisins.*

gentian (*Gentiana lutea* and other spp.) in medicinal homemade digestive liquors in the Alps reputed as panaceas but used especially against coughs and as digestive and depurative (Pieroni and Giusti, 2009). Alpine *Artemisia* species and *G. lutea* have a crucial role in the Alpine folk medicine (Mattalia et al., 2012).

Salvia blancoana subsp. *mariolensis* (**Figure 5**) is an endemic plant taxon with an area restricted to a small region in the North of Alicante province and the South of Valencia province which is present in the 20% of the formulas registered (**Table 7**). It has the same traditional medicinal uses as *S. officinalis* subsp. *lavandulifolia* whose distribution is much wider (Anthos, 2021), this last species being the accepted source for the Spanish sage oil (Martínez-Francés et al., 2012; Martínez-Francés et al., 2017). *S. blancoana* subsp. *mariolensis* is used to prepare a digestive liquor called "*salvieta*," alone or mixed with other wild and cultivated sage species. Its consumption in local festivities is consolidating its social use among the youngest, becoming a potential commercial product. This species is used in herbal teas as hypotensive, detoxifying, antitussive, anticold, against gum inflammation, digestive, emmenagogue, sedative and antipyretic, and the hydroalcoholic extract as tonic and digestive (Martinez-Francés et al., 2017). There are no data on the cultivation of this plant outside the botanical gardens (Reales et al., 2004; Martinez–Francés et al., 2017).

Clinopodium serpyllifolium subsp. *fruticosum*, with a discontinuous area, grows on the eastern coast of the Mediterranean, present in the East of Spain and in the Italian and Balkan Peninsulas and Israel (Bolòs and Vigo, 1996). In the limestone and stony rocks where it appears, it is frequently collected both to consume in teas and to make digestive liquors. In every place it grows, it is an appreciated plant, taken for relieving stomach aches and also for the treatment of nervous system disorders (Mulet, 1991; Azab, 2016).

Thymus moroderi (**Figure 5**) is an endemic plant of southeastern Spain that it is also cultivated and used to make an industrial liquor marketed since 1867 named *cantueso* (Díaz-García et al., 2014; Marco-Medina and Casas, 2015). *T. moroderi* is a potential source of anthocyanins as food additives, with both high polyphenols content and high antioxidant activity (Díaz-García et al., 2014). Due to its wide popularity and its scarcity, it is susceptible to being adulterated with other species, such as *T. longiflorus*, *T. antoninae*, and *T. granatensis* subsp. *micranthus* between others (Díaz-García et al., 2014); however, in the present study, such type of adulteration was not recorded. Long journeys are frequent in the western and southern Alicante area during *T.*

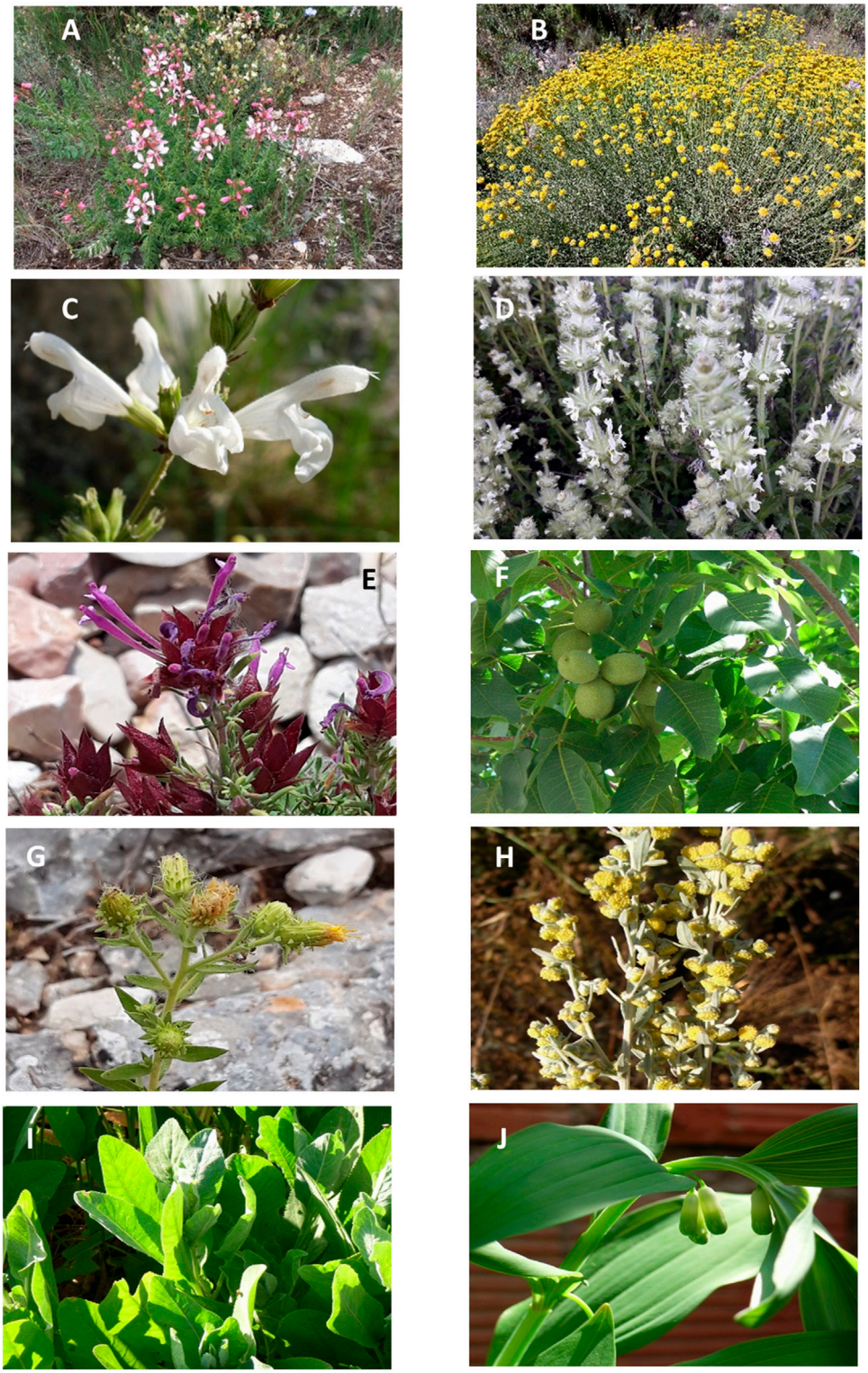


FIGURE 5 | Plant species characteristic of the different types and styles of liquors. **(A)** *Dictamnus hispanicus* (herbero and gitam). **(B)** *Santolina villosa* (herbero). **(C)** *Salvia blancoana* subsp. *mariolensis* (salvieta). **(D)** *Sideritis hirsuta* (herbero). **(E)** *Thymus moroderi* (cantueso). **(F)** *Juglans regia* (Walnut wine). **(G)** *Chiliadenus glutinosus* (Herbal wines). **(H)** *Artemisia absinthium* (Absinthe and Vermouth). **(I)** *Tanacetum balsamita* (herbero). **(J)** *Polygonatum odoratum* (beatamaria).

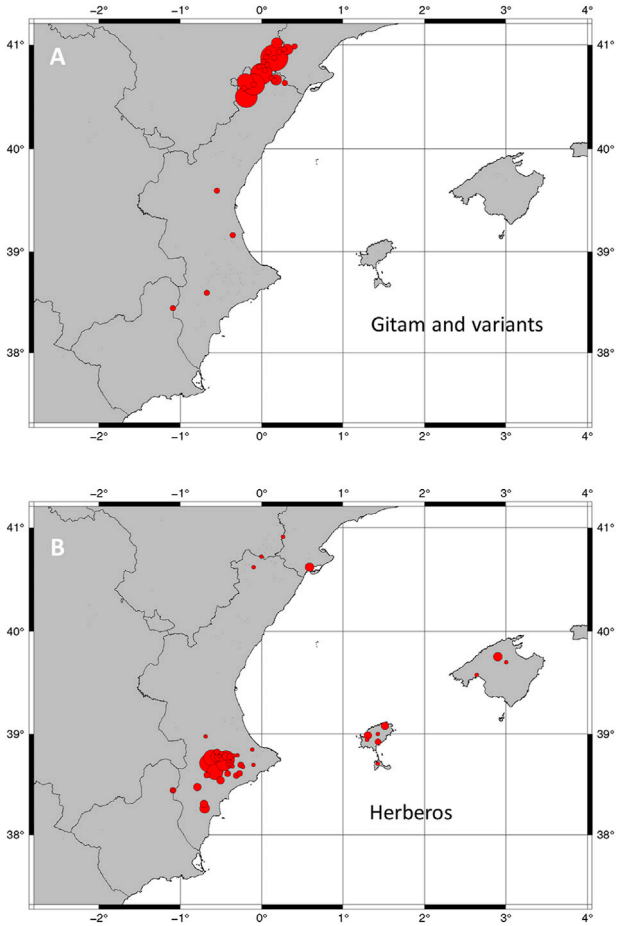


FIGURE 6 | Geographical distribution of the two major types of liquors. **(A)** Gitam, *Dictamnus hispanicus* liquors. **(B)** Herberos. Note: dimensions of circles are proportional to the number of formulas recorded in the locality.

moroderi flowering season, in which the population collects the plant for consumption. In Elche *Thymus moroderi*, it is also linked to one of the most deeply rooted traditions (although in evident decline, as the festival itself is suppressed), such as that of "*fer herbetes*" in the mountains that surround the Pantano de Elche on Ascension Day. In addition to being marketed for infusion, it is also the base of the most typical liqueur from Elche (Marco-Medina, 2010; Maciá, 2016).

It should be noted that in Cuenca province a liquor also known as *cantueso* is made using *Lavandula pedunculata* instead of *Thymus moroderi*; in this case, it is macerated in spirit, and later water and sugar are added (Fajardo et al., 2007).

Another highly valued endemic thyme species is *T. piperella*. Thyme species are characterized by a strong penetrating odor, but these two species are exceptional as a seasoning in meals and as an aromatizing in spirits for their pronounced balsamic and spicy flavor (Ruiz-Navajas et al., 2012).

Polygonatum odoratum (**Figure 5**) extends along temperate regions of Eurasia and N Africa (POWO, 2021). The species is scarce in the southern half of the Iberian Peninsula (Anthos, 2021). It is cultivated as an ornamental plant with numerous varieties (RHS, 2021). In southern Aragon and the south of Tarragona, a homemade liquor is made: beatamaria, which is used in menopause-associated disorders (Martínez-Francés et al., 2018b). This plant is called beatamaria only in this area, while in the rest of Spain, the Spanish translation of Salomon's Seal is the common name. In China, the rhizomes of *P. odoratum* are usually soaked with liquor and sugar for 6 months. Before being used, the drink is strained with gauze. When drinking frequently, it is supposed to eliminate fatigue, moisten skin, and make one beautiful. The rhizomes are also used as herbal tea (Wujisguleng et al., 2012). The rhizome of *P. odoratum* is known as "*Yuzhu*." It has the functions of nourishing Yin, clearing heat, and helping produce saliva. It can be used for the treatment of lung diseases, cough, diabetes, and indigestion. Different types of compounds have been isolated from the rhizome cholestanol saponins, furostanol saponins, spirostanol saponins, triterpenoid saponins, polysaccharides, and homoisoflavanones (Zhao et al., 2017). Pharmacological studies validate its antihyperglycemic activity, anti-inflammatory and expectorant properties in the respiratory system, immunostimulant activity, its induction to osteoclast differentiation, and the apoptosis in some human cancer (Xiao et al., 1990; Guan-Fu, 1991; Lin et al., 1994; Youn et al., 2008; Shu et al., 2009; Tai et al., 2016).

Wormwood species are native from warm regions of Europe, Asia, and North Africa (Obón et al., 2018a). In our study area, two of them, *Artemisia absinthium* and *A. arborescens*, are used in absinthe liquor and vermouth but also as an ingredient of herbero and ratafia. Absinthe has been commonly used in coastal areas as a malaria remedy, macerating wormwood in highly concentrated alcohol, in some cases near to 80% in volume (Pielech-Przybylska and Balcerek, 2019). But a complex absinthe preparation, called Vermouth, a wine-derived aperitif widely consumed (Morata et al., 2019), is also elaborated in some local distilleries of the studied territory. This aromatized liquor with a bitter taste is prepared from a base of white wine, fortified with wine spirit, colored by caramel, and aromatized with several dried herbs, spices, or their extracts (Panesar et al., 2009; Morata et al., 2019). Locally, *Centaurium quadrifolium* subsp. *barrelieri*, from Gentianaceae family, is also used in Alicante in liquors and vermouths as quina (*Cinchona officinalis*) preparations in Balearic Islands.

Aloysia citriodora is a widely cultivated species in our territory, although its American origin. It is an excellent organoleptic aroma and flavor corrector and also used for relieving mental stress, to aid sleep and for symptomatic treatment of digestive complaints (Vanaclocha and Cañigueral, 2019), being used in teas and liquors frequently in this area and, for instance, in Cuenca province (Fajardo et al., 2007).

Sideritis hirsuta (**Figure 5**) and *S. tragoriganum* subsp. *tragoriganum* are the most used species of this genus in herbal formulas. Highlighting the high number of genus *Sideritis* endemic species in the Southeast of Spain (Rivera and Obón, 1997), only the use of four species and one hybrid was recorded in this study. These are often used in teas for their medicinal properties (Obón et al., 2018b; 2018c).

Eryngium campestre roots and aerial parts are collected for use mainly in *herberos*. It is considered an essential plant, along with

D. hispanicus. According to popular knowledge, it is added to liquors to counteract the toxicity of the Rutaceae plant. When *Dictamnus* cannot be found, it is usually replaced by another *Ruta* species and the same happens for *E. campestre*, which can be replaced by other Umbelliferae species, such as *Foeniculum vulgare* subsp. *piperitum*, *Ferula hispanica, F. tingitana,* and *Thapsia villosa*.

Although irrelevant in terms of frequency, the presence of two fungi species (*Cantharellus cibarius* Fr. and *Tuber melanosporum* Vittad.) and hen eggs (*Gallus gallus* (Linnaeus, 1758)) in the samples is worthy of mention. Mushrooms are used in liquors within the context of Traditional Chinese Medicine (Mizuno et al., 1995) and in other cultures such as in traditional medicine of Madeira (Portugal) (Rivera and Obón, 1995).

Plant Collection and Conservation Issues

There is a relevant aspect regarding the collection of herbs. Those liquors that are made from species living far from their homes and in almost inaccessible places require our informants to travel great distances and time to get them. In *herberos* formulations, *Dictamnus hispanicus* is the most sought for, but the presence of other species (**Table 7**) such as *Asperula aristata*, *A. cynanchica*, *Centaurea boissieri* subsp. *mariolensis*, *Centaurium quadrifolium* subsp. *linariifolium*, *Chiliadenus glutinosus*, *Clinopodium alpinum*, *Inula montana*, *Leucanthemopsis pallida*, *Mentha aquatica*, *Saxifraga longifolia*, *Stachys heraclea*, or *S. ocymastrum* (Menéndez et al., 2018) depended on the exhaustive search in the field. Thus, the presence/absence of such a rare species inform us of the age of liquor since nowadays they are infrequent and thus no longer available.

Usually, women have been in charge of family health care, and their presence at home was continuously required, so they could not be absent from home for a long journey. That is why men are responsible for making these herbal liquors and therefore, the transmission of this knowledge is patrilineal, from father to son (Agelet and Vallès, 2001) involving some type of restrictions for the transmission outside this circuit. For this reason, data collection has not been easy, because formulas are a familiar secret and also men refused to give the recipe to a woman. In other places, such as the Balearic Islands, where the collection of herbs is limited to family gardens and nearby areas, these medicinal liquors are made by women, and the transmission is from mother to daughter.

In different parts of the studied area, we find the same problems of sustainability for some herbal resources, especially those from the most endemic and sparse species such as *Dictamnus*, *Polygonatum*, *Saxifraga*, and *Sideritis* species, similarly occurring with Alpine wormwood (*Artemisia genipi, A. glacialis,* and *A. umbelliformis*) (Pieroni and Giusti, 2009; Cornara et al., 2014).

Dictamnus hispanicus is distributed on the eastern side of the Iberian Peninsula, but it apparently exhibits a very irregular abundance, being a globally rare autochthonous species. According to Anthos (2021), its western Iberian limit reaches the provinces of Granada, Jaén, Ciudad Real, Cuenca, and Guadalajara, progressively approaching the sea towards the N, where it reaches those of Huesca, Lleida, and Girona (Belda et al., 2017). However, the presence of the species in one territory does not suppose the use. In the exhaustive ethnobotanical research in the upper Guadiana River area (Ciudad Real and Albacete provinces), *D. hispanicus* uses were not recorded, neither as a medicine nor in herbal liquors despite being a relatively abundant species (Rivera et al., 2019). The elaboration of the herbero has led to an overcollection of certain species that have seen their biology modified by a change in the use of the territory. This is the case of *timó real*, *Dictamnus hispanicus,* a species with which the Torretes Research Station is carrying out a recovery project (Ríos and Martínez-Francés, 2008).

Thymus moroderi appears in the 2008 Red List of the Spanish vascular flora as "near threatened species" (NT) (Moreno 2008; Moreno, 2011). The main threat to this species is its collection for the manufacture of liquor and the preparation of herbal teas, as well as other local uses. Fortunately, it is a good first colonizer, like all the thyme in the Pseudothymbra section, which invades abandoned crops. Flora Protegida (2021) suggests that collection should be strictly regulated and the diversification of this activity with other species (*T. funkii* and *T. membranaceus*) (thus adulteration!), as well as establishing a minimum protected area. Since 2013, some "*in vitro*" or nursery reproduction projects have been developed, as it has been the case of Raúl Agulló from Elche. Cultivation trials are being carried out at the Camp d'Elx, promoted by the *Associació per al Desenvolupament del Camp d'Elx* (ADR) with the support of the Valencian Institute of Agrarian Research (IVIA) of the Generalitat. Three farmers initially offered to plant cantueso, but after the initiative spread, there are currently seven experimental plantations that exist in Camp d'Elx, including some 1,500 plants. The project has also been joined by the company Salas y Sirvent (SYS), which markets cantueso liquor, and which also has its own plantations (Marco-Medina, 2010; Maciá, 2016).

The Solvents

Currently, the most common maceration bases used are spirits flavored with aniseed (*Pimpinella anisum*) or star anise (*Illicium verum*) and marketed in local warehouses. There is a multitude of traditional aniseed preparations distributed throughout the European continent (France, Italy, Turkey, Greece, etc.) and the areas under its cultural influence (Central and South America) (Pielech-Przybylska and Balcerek, 2019).

The Valencian region also participates in this tradition and there are numerous distilleries that distribute aniseed of various kinds to herbal liquor makers. These distilleries, almost all, are located within the point of maximum production of homemade and traditional *herberos* (Martínez-Francés and Ríos, 2005). Before the proliferation of distilleries, the use of homemade alcoholic distillates was very common in mountainous regions. People took advantage of winemaking residues or the entire harvest in the highest areas with low-quality wines to produce their spirits with high ethanol (between 36 and 70°).

The production technology of these spirits is widespread in the Mediterranean area. It is based on the maceration and/or distillation or redistillation of alcohol in the presence of seeds or other plant parts. Natural distilled extracts of anise seeds may also be added (Pielech-Przybylska and Balcerek, 2019). Currently,

the origin of the alcohol of this aniseed spirit is molasses (*Beta vulgaris* subsp. *rapa* and *Saccharum officinarum*) and contains between 35 and 50% of ethanol in volume (Jurado, 2004) (although after soaking the herbs this content slightly decreases).

Levantine vineyards are mainly modern. Jumilla or Utiel and Requena wine regions hardly produced wines for self-sufficiency until the second half of the 19th century when they became large wine-producing areas. But there were important and deep-rooted liquor factories in the Valencian and Murcia regions (Plasencia and Villalón, 1999) despite the shortage of vineyards. The prohibition of this activity in the first half of the 20th century throughout Spain brought with it the dependence of herbero makers on legalized distilleries, which probably reduced the initial diversity of this product. Because home distillation has almost completely disappeared, many of the samples studied belong to older people or their descendants, who keep these spirits and formulas.

The Origins of Liquors Diversity in the Area

The origin of these medicinal wines and liquors is based on the adaptation of the old herbal teas used to maintain family health to an extracting and preservative solvent such as wine and spirits. Better accessibility to medical care and medicines is the turning point for the abandonment of these practices. In many other traditional resources, their demise is imminent. This practice of making medicinal alcoholic beverages has been maintained over time thanks to its social use in popular festivals and festivities, although both the formulas complexity and their medicinal application have been distorted (Martínez-Francés and Ríos, 2009). However, Plotkin (2021) in his review underlines the ancient tradition of macerating herbs in wine for confectioning medicines that were externally applied or orally administered by ancient Mesopotamians, Egyptians, or Phoenicians; thus, part of these formulations may be independent of herbal teas. Thus, likely herbal teas do not always preced medicinal wines and liquors, and often it was the contrary (Obón et al., 2021). Data compiled by Plotkin (2021) suggest the antiquity of the discovery that hydroalcoholic extraction improves the extraction of active compounds and thus the effectiveness of the medicine and it should have been the main reason for macerating herbs in wines and beers, no matter these were topically or orally administered.

The daily intake of alcohol among the population increased associated with greater availability of distillates. At the end of the 19th century and the beginning of the 20th century, wine was a cheap product and its consumption was within the reach of any economy (Beneito-Lloris, 2003). The workers drank large amounts of wine during meals and drank also herbal liqueurs before and after work. Many mothers had some homemade healing and restorative remedies that were often given to children when they were ill, which were made with an alcoholic base. The population drank spirits or other alcoholic beverages while fasting, practice to which preventive and purifying powers were attributed (Beneito-Lloris, 1993, 2003).

In Alpine regions, we find similarities in this resource and its evolution. There, these homemade liqueurs reflect an ancient use of these products that shifted from medicine towards liquors when improved economic conditions enabled rural communities to have at their disposal larger amounts of industrial alcohol (Pieroni and Giusti, 2009).

Liquor Types and Relationships With Industry and Other Traditions

Wine preparations are the first step between tisanes and herbal hydroalcoholic macerates. Presently, recipes with wine are rare in folk medicine, mainly in areas where homemade liquor production predominates. But some essential species remain in medicinal wine preparations, both in simple formulas with *Aloysia citriodora*, *Artemisia absinthium*, *A. arborescens* (De Natale and Pollio, 2007; Fajardo et al., 2007; Obón et al., 2018a), *Cinchona officinalis*, *Centaurium quadrifolium* subsp. *linariifolium*, *Cydonia oblonga*, *Foeniculum vulgare, Juglans regia*, *Prunus* sp. pl., *Rubus ulmifolius*, *Sambucus nigra*, and *Sorbus domestica* or complex formulas (Pollio et al., 2008), many of them, flavored with spices. In the present study, we have compiled 64 recipes involving maceration in wine. More than 50% correspond to well-known walnut wine (*J. regia*), distributed practically throughout the territory studied.

The production of homemade spirits has practically disappeared, but the older informants keep their copper alembics, the witness bottles, and the knowledge about their elaboration (**Figure 2**). They have been made throughout the territory. The accessibility to buy these spirits in nearby warehouses and the state persecution given its illegality have favored this abandonment. We recorded 33 samples of these homemade spirits of high graduation. These have been made mainly with the remains of the pressing of grapes (*Vitis vinifera*) or with the whole harvest due to its low production and quality in mountainous areas. Special mention for spirits made with surpluses from other crops such as *Cucurbita moschata* and *Ficus carica*. Due to the harsh taste of these spirits, in many cases, they have been rectified with anise-like flavorings as *Dictamnus hispanicus*, *Foeniculum vulgare*, *Pimpinella anisum*, and *Ruta* sp. pl. and with citral-like flavorings as *Aloysia citriodora*, *Citrus* x *limon*, *C.* x *sinensis*, and *Melissa officinalis*. Other simple liquors, generally with less alcoholic and aniseed spirits, are made with species of genres such as *Clinopodium, Mentha, Myrtus, Rosa, Rosmarinus, Salvia, Thymus*, and *Syringa*.

In Mallorca (Balearic Islands), a myrtle liquor (*Myrtus communis*) is done (Tardío et al., 2018), sometimes with flowers and leaves and others with fruits and taken as antidiarrheal and digestive. Berries of *M. communis* are used to produce a sweet myrtle liqueur in Sardinia through their infusion in spirit during 2 weeks (with 40% of ethanol in volume) (Correddu et al., 2019).

Ratafia is a liquor that follows the preparations mentioned in Tarragona and that extends to the north of Catalonia and Andorra (Table 11). It seems to be the union of two herbal traditions: on the one hand, the walnut fruit liquor accompanied by spices and other species included in the treatise of

Charlemagne (closer to monastic medicine) and on the other, the herbs used in popular medicine, some of them endemic. Recipes vary from simple (one to five plants) to very complex (up to 80 plants) and are claimed to be digestive and useful in gynecological disorders (Agelet and Vallès, 2001; Bonet and Vallès, 2002; Parada et al., 2011).

Other traditional aniseed spirit base elaborations, mainly with fruits, share space with herbal wines and liquors, although in this area, they are relegated to the background. More than one hundred formulas of fruit spirit type have been collected, of which 65% belong to Valencia region, 10% to Catalonia, and 8% to Castilla-La Mancha and Aragon, respectively. The main fruits used belong to the following species: *Arbutus unedo*, *Coffea arabica*, *Cucumis sativus*, *Cucumis melo* subsp. *flexuosus*, *Juniperus communis*, *Malus domestica*, *Prunus avium*, *P. cerasus*, *P. domestica*, *P. dulcis*, *P. persica*, *P. spinosa*, and *Rubus ulmifolius*. One of the most popular fruit spirits is that obtained with *P. spinosa,* and also its Navarrese name "patxaran" is well-known everywhere (Cavero et al., 2011). Liquors made with fruits actually are increasing between nouvelle makers in most of the regions where it grows.

In other areas of Spain, such as Cuenca, the inhabitants of the sierra collected various types of wild fruits to sell them to liquors industries, especially juniper (*J. communis*) and blackthorn (*Crataegus*). In parallel, the preparation of *Pacharan* with the fruits of *Prunus spinosa* is common. In this area, homemade liqueurs are prepared with a spirit base in which various herbs, spices, and/or fruits are macerated (Fajardo et al., 2007). The most characteristic liquor of Cuenca is the *resolí* that has different forms of preparation and formulas, often secret; one of them is with orange peel, aniseed, cinnamon bark, clove, river tea (*Mentha aquatica*), pennyroyal (*M. pulegium*), coffee grains, sugar, and grape pomace (Fajardo et al., 2007). The use of these liquors is as a digestive. Liqueurs made with various fruits, plums, cherries, blackberries, or walnuts, are common in Albacete. They are usually prepared with wine spirit and sugar and it is common to let them marinate for a minimum of 6 months (Rivera et al., 2006).

The number of ingredients is extremely variable, under the type of liquor known as *ratafia*, and similarly, in the *herberos* type, we can find simple formulas with three ingredients and others with increasing complexity, with over 40 ingredients. Bonet and Vallès (2002) recorded 80 ingredients at Montseny in Barcelona province within a *ratafia* type. In our study of herbal liquors, the most frequent is the single ingredient one, followed at a distance by that of two (**Figure 3**). Then there is a decrease in frequency as the number of ingredients increases, but presenting saw teeth that coincide with the odd numbers.

The odd numbers' superstition presents different forms; in Europe, a tradition that goes back to the Roman authors Virgil and Pliny and to the Pythagorean doctrine says that odd numbers bring good fortune (Schimmel, 1993). For ancient Romans, odd numbers are finite, complete and absolute, and opposed to even numbers that are imperfect and unlimited (Schimmel, 1993). From the data collected during the fieldwork, the persistence of this traditional belief that associates luck to odd numbers is evident. This concept, which is widespread in the folk medical practices of the Mediterranean area, is transferred in this case to the number of plants or the number of countable parts of the plants in the herbal formulas.

The Medicinal Role of Liquors

Diseases associated with high morbidity and mortality rates marked the public health history of eastern Spain. Between the 17th and 19th centuries, epidemics (tertian fevers, typhus, cholera, plague, and yellow fever) caused high mortality in eastern Spain. During the 20th century, influenza and tuberculosis, typhoid fever, and malaria were common. Infectious, parasitic, and digestive diseases were the main cause of mortality followed by respiratory diseases, all this associated with hygienic, sanitary, and, on many occasions, poor nutritional conditions (Beneito-Lloris, 1993; Fresquet et al., 1994; Bernabeu-Mestre, 2004).

There is fragmentary evidence that suggests a direct relationship between specific locally prevalent pathologies and the elaboration of determined herbal mixtures to be consumed in form of teas, decoctions, or hydroalcoholic extracts obtained by maceration. This connection is attributed to the different active compounds provided by the herbal ingredients (Atoui et al., 2005; Carmona et al., 2005; Egea et al., 2015; Egea et al., 2016; Obón et al., 2014; Obón et al., 2021).

Traditional *herbero* complex formulations try to respond to these pathologies, in such a form that they can be geographically grouped by territories (**Table 9**) with a relationship with the locally prevalent diseases (Martínez-Francés and Ríos, 2009).

Cinchona and *Artemisia* preparations (absinthe and vermouth) have been widely recorded until today in lowland areas with wetlands where malaria was prevalent until 1964, as being commonly consumed, while other herbal liquor and wine preparations are more common in mountain areas where malaria was less frequent. Absinthe and vermouth have been consumed in areas near the mouths of large rivers, inland deltas, small lagoons, and marshes, mainly located in Tarragona province (Catalonia) and Castellón and Valencia provinces (Valencia region) (Fresquet et al., 1994; Martínez-Francés and Ríos, 2005).

Complex herbal and cinchona preparations in Balearic Islands have been consumed to combat recurrent diarrhea and dysentery, due to the biological contamination of their drinking waters, and also malaria. It is important to note that these islands have not permanent watercourses and the water for consumption and irrigation comes from groundwater because the predominance of limestone favors infiltration (Carrió et al., 2012a; 2012b).

In the Ports Mountains (Tarragona) and overlapping with southeastern Aragon (Teruel province), two particular liquors, *gitam* and *beatamaria*, have been recorded as used in the treatment of women disorders. The first is elaborated with *Dictamnus hispanicus* and the second is with *Polygonatum odoratum*. These two plants, of scattered and sparse distribution, but with numerous active substances (Lin et al., 1994; Martínez-Francés et al., 2015; Martínez-Francés et al., 2018a; Martínez-Francés et al., 2018b), are praised by the locals. In the case of *P. odoratum*, bottles are transmitted in

families to the next generation as a heritage that is linked to their identity and popular wisdom (Martínez-Francés et al., 2018b).

CONCLUSION

Most of the 569 formulas recorded are recognized in the communities to be medicinal. Almost 87% of the recorded formulations are reputed as digestive and are consumed as such. However, most herbal liquors are mainly consumed in social events. Other medicinal uses recorded are as a tonic or to improve their mood, appetite stimulant, antidiarrheal (especially Balearic *herberos*), or emmenagogue (notably *beatamaria*).

We compiled an inventory of 215 taxa from 56 families, where 212 are plants, two are fungi, and two are animals. Most are local wild species (60%), followed by cultivated and only a few are imported (6%). The proportion of imported species differs along the types and styles recorded.

Aerial parts are the most frequently used plant parts followed by leaves, flowers, and fruits. Lamiaceae is by far the most widely represented plant family in terms of species but slightly surpassed by Rutaceae in the percentage of samples. This is due to the regional relevance of *Dictamnus hispanicus, Ruta* sp. pl., and *Citrus* species.

Among the 569 formulas and/or samples of wines, spirits, and liquors studied, 423 are uniquely differing in their combination of ingredients. However, these could be classified, according to their solvent and herbal ingredient composition into twenty main types among which for their cultural relevance can be highlighted: *beatamaria, cantueso, gitam, herbero, ratafia, resoli,* and *salvieta*. Their relative frequencies and distribution in the different regions show peculiar patterns linked to the different cultural traditions and diverse resources availability.

Complex formulations with over 40 ingredients coexist with others simplest with only one single herbal ingredient. But, in the almost 15 years of this study, we have recorded an impoverishment of a 70% in the diversity of plant ingredients of the formulas and, also, the loss of medicinal uses. The main causes of the partial loss of this knowledge are diverse: improvement of hygiene, nutrition, and the health system; change or loss of rural exchanges; production of homemade distillates illegalization; intergenerational transmission of medicinal knowledge failure; and overexploitation of some resources making their collection difficult.

We collected 186 complex herbal formulas mainly in Valencia region and Balearic Islands, typified as *herbero*, of which 88% are from the different mountainous areas of the Valencian region where Dictamnus hispanicus is prevalent. The Balearic formulas lack *Dictamnus* because it does not exist on the islands, but it is replaced by rue. The different historically prevalent diseases and the associated herbal remedies together with the differential availability of resources seem to have been the main conditioning factors for this high herbal liquor diversity.

This herbal liquor diversity is extraordinary when compared not only with those of the adjacent areas but also with those of the Apennines and Alpine region.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

VM-F, DR, and SR were responsible for fieldwork, interviews, collection, and identification of samples and ethnopharmacological analysis. VM-F, CO, and SR carried out the bibliographic search. VM-F, DR, CO, FA, and SR are responsible for writing the manuscript. All authors proofread the manuscript and made contributions to the final version.

ACKNOWLEDGMENTS

We deeply acknowledge the help in collecting samples and information offered by all our interviewees.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.713414/full#supplementary-material

REFERENCES

Agelet, A., and Vallès, J. (2001). Studies on Pharmaceutical Ethnobotany in the Region of Pallars (Pyrenees, Catalonia, Iberian Peninsula). Part I. General Results and New or Very Rare Medicinal Plants. *J. Ethnopharmacol.* 77, 57–70. doi:10.1016/s0378-8741(01)00262-8

Agustín, M. (1722). *Libro de los Secretos de Agricultura, Casa de Campo y Pastoril.* Barcelona: Juan Piferrer.

Anthos (2021). Anthos. Sistema de información sobre las plantas de España. Available at: http://www.anthos.es/.

Atoui, A., Mansouri, A., Boskou, G., and Kefalas, P. (2005). Tea and Herbal Infusions: Their Antioxidant Activity and Phenolic Profile. *Food Chem.* 89 (1), 27–36. doi:10.1016/j.foodchem.2004.01.075

Azab, A. (2016). Micromeria: Chemistry and Medicinal Activities. *Eur. Chem. Bull.* 5 (7), 300–307.

Belda, A., Belenguer, R., Zaragozí, B., and Laguna, E. (2017). Situación actual de Dictamnus hispanicus Webb (Rutaceae) en la provincia de Alicante: distribución y aspectos etnobotánicos. *Flora Montiberica* 68, 52–60.

Beneito-Lloris, A. (1993). *Comportamiento epidémico y evolución de las causas de defunción en la comarca de l'Alcoià-Comtat. (PhD) Facultad de Filosofía y Letras*. Alicante: Universidad de Alicante.

Beneito-Lloris, A. (2003). *Condicions de vida i salut a Alcoi durant el procés d'industrialització*. València: Universitat Politècnica de València, 293.

Bernabeu-Mestre, J. (2004). *La topografía médica de Ontinyent*. Onteniente: Ayuntamiento de Onteniente. Servicio de Publicaciones.

Bolòs, O., and Vigo, J. (1984). *Flora Dels Paisos Catalans. Vol. 1*. Barcelona: Barcino.

Bolòs, O., and Vigo, J. (1989). *Flora Dels Paisos Catalans. Vol. 2*. Barcelona: Barcino.

Bolòs, O., and Vigo, J. (1996). *Flora Dels Paisos Catalans. Vol. 3*. Barcelona: Barcino.

Bolòs, O., and Vigo, J. (2001). *Flora Dels Paisos Catalans. Vol. 4*. Barcelona: Barcino.

Bonet, M. A., and Vallès, J. (2002). Use of Non-crop Food Vascular Plants in Montseny Biosphere reserve (Catalonia, Iberian Peninsula). *Int. J. Food Sci. Nutr.* 53, 225–248. doi:10.1080/09637480220132841

Bueno, M. R., and Alegre, M. E. (2001). Los destiladores de Su Majestad. Destilación, espagiria y paracelsismo en la corte de Felipe II. *Dynamis. Acta Hisp. Med. Sci. Hist. Illust.* 21, 323–350.

Burgueño, J. (2002). El mapa escondido: las lenguas de España. *Boletín de la Asociación de Geógrafos Españoles, núm.* 34, 171–192.

Carmona, M. D., Llorach, R., Obon, C., and Rivera, D. (2005). "Zahraa", a Unani Multicomponent Herbal tea Widely Consumed in Syria: Components of Drug Mixtures and Alleged Medicinal Properties. *J. Ethnopharmacol* 102 (3), 344–350. doi:10.1016/j.jep.2005.06.030

Caro-Baroja, J. (1981). *Los Pueblos de España. Vol. 2*. Madrid: Istmo.

Carrió, E., Rigat, M., Garnatje, T., Mayans, M., Parada, M., and Vallès, J. (2012b). Plant Ethnoveterinary Practices in Two Pyrenean Territories of Catalonia (Iberian Peninsula) and in Two Areas of the Balearic Islands and Comparison with Ethnobotanical Uses in Human Medicine. *Evid. Based Complement. Alternat. Med.* 2012, 896295. doi:10.1155/2012/896295

Carrió, E., and Vallès, J. (2012). Ethnobotany of Medicinal Plants Used in Eastern Mallorca (Balearic Islands, Mediterranean Sea). *J. Ethnopharmacol.* 141, 1021–1040. doi:10.1016/j.jep.2012.03.049

Carrió, E., Mayans, M., and Xirau, J. V. (2012a). Abans que es pins facin magranes i ses figueres melons: primeros datos de dos investigaciones etnobotánicas en Formentera y en Mallorca. *Mètode Sci. Stud. J. Annu. Rev.* (72), 66–70.

Cavero, R. Y., Akerreta, S., and Calvo, M. I. (2011). Pharmaceutical Ethnobotany in the Middle Navarra (Iberian Peninsula). *J. Ethnopharmacol.* 137, 844–855. doi:10.1016/j.jep.2011.07.001

Cierbide, R. (2007). El aguardiente y el vino como componentes de remedios medicinales según los textos medievales. *Rev. Int. Estu. Vascos.* 52 (1), 11–79.

Cierbide, R. (2013). *Remedios médicos medievales basados en el aguardiente, el vino y otros ingredientes*. Álava: Guías docentes EHU/UPV.

Cornara, L., La Rocca, A., Terrizzano, L., Dente, F., and Mariotti, M. G. (2014). Ethnobotanical and Phytomedical Knowledge in the North-Western Ligurian Alps. *J. Ethnopharmacol.* 155, 463–484. doi:10.1016/j.jep.2014.05.046

Correddu, F., Maldini, M., Addis, R., Petretto, G. L., Palomba, M., Battacone, G., et al. (2019). Myrtus Communis Liquor Byproduct as a Source of Bioactive Compounds. *Foods* 8 (7), 237. doi:10.3390/foods8070237

Costa, M. (1999). *La Vegetación y el Paisaje en las Tierras Valencianas*. Madrid: Editorial Rueda.

De Natale, A., and Pollio, A. (2007). Plants Species in the Folk Medicine of Montecorvino Rovella (Inland Campania, Italy). *J. Ethnopharmacol* 109, 295–303. doi:10.1016/j.jep.2006.07.038

De-Terán, M. (1949). "La genialidad geográfica de la península ibérica," in *Geografía Universal. V. IX* (Barcelona: Montaner y Simón).

Díaz-García, M. C., Castellar, M. R., Obón, J. M., Obón, C., Alcaraz, F., and Rivera, D. (2014). Production of an Anthocyanin-Rich Food Colourant from Thymus Moroderi and its Application in Foods. *J. Sci. Food Agric.* 95, 1283–1293. doi:10.1002/jsfa.6821

Egea, T., Signorini, M. A., Bruschi, P., Rivera, D., Obón, C., Alcaraz, F., et al. (2015). Spirits and Liqueurs in European Traditional Medicine: Their History and Ethnobotany in Tuscany and Bologna (Italy). *J. Ethnopharmacol.* 175, 241–255. doi:10.1016/j.jep.2015.08.053

Egea, T., Signorini, M. A., Ongaro, L., Rivera, D., Obón de Castro, C., and Bruschi, P. (2016). Traditional alcoholic beverages and their value in the local culture of the Alta Valle del Reno, a mountain borderland between Tuscany and Emilia-Romagna (Italy). *J. Ethnobiol. Ethnomed* 12, 27. doi:10.1186/s13002-016-0099-6

Fajardo, J., Verde, A., Rivera, D., and Obón, C. (2007). *Etnobotánica en la Serranía de Cuenca. Las plantas y el hombre*. Cuenca: Diputación Provincial de Cuenca.

Figueras, A., and Castelló, M. T. (2012). "Licors Tradicionals, Aiguardents I Vins Generosos," in *Priorat, Terra Alta, Ribera d'Ebre*. (Barcelona: Editor Farell).

Flora Protegida (2021). Flora Protegida. Available at: http://www.floraprotegida.es/flora-vulnerable.php/Thymus-moroderi-122/(Accessed 03 30, 2021).

Folch, R., Franquesa, T., and Camarasa, J. (1984). *Vegetació. (Vol. 7 of Història Natural Dels Països Catalans)*. Barcelona: Fundació Enciclopèdia Catalana.

Folch, R. (1981). *La Vegetació Dels Països Catalans*. Barcelona: Ketres Editora.

Font-Quer, P. (1985). *Plantas Medicinales. El Dioscórides Renovado*. 9th ed. Labor: Barcelona.

Forbes, R. J. (2009). *A Short History of the Art of Distillation: From the Beginnings up to the Death of Cellier Blumenthal*. Portland: White Mule Press.

Fournier, P. (1947). *Le livre des plantes médicinales et vénéuses de France: 1.500 espèces par le texte et par l'image, d'après l'ensemble de nos connaissances actuelles*. Paris: Lechevalier.

Fresquet, J. L., Tronchoni, J. A., Ferrer, F., and Bordallo, A. (1994). *Salut malatia i terapèutica popular. Els municipis riberencs de l'Albufera. Col·lecció Josep Servés, de documentació i recerca*. Catarroja: Servei de publicacions de l'Ajuntament de Catarroja.

García-Ballester, L. (1982). Arnau de Vilanova (c. 1240-1311) y la reforma de los estudios médicos en Montpellier (1309): El Hipócrates latino y la introducción del nuevo Galeno. *Acta Hispanica ad Medicina Scientiarumque Historiam Illustrandam* 2, 97–158.

García-Ballester, L. (2001). *La búsqueda de la salud. Sanadores y enfermos en la España medieval*. Barcelona: Península/HCS.

García-Ballester, L. (1989). *La Medicina a la València medieval*. Valéncia: Alfons el Magnànim IVDEI.

García-Ballester, L. (1984). *Los moriscos y la medicina. Un capítulo de la medicina y la ciencia marginadas en la España del siglo XVI*. Barcelona: Labor Universitaria.

García-Ballester, L., and McVaugh, M. R. (1996). *Arnaldi Villanova Opera Medica Omnia. X.I. Regimen Sanitatis Ad Regem Aragonum*. Barcelona: Fundació Noguera/Universitat de Barcelona.

García-Mouton, P. (2007). *Lenguas y Dialectos de España*. 5th edition. Madrid: Arco Libros SL.

Gil-Sotres, P. (2011). El Antidotario de Arnau de Villanova: A vueltas con la autenticidad. *Arxiu de textos catalans antics*. 30, 239–253.

GMT (2021). The Generic Mapping Tools, Version 6.1.1 [64-bit] [MP] [8 Cores] © 1991-2020 the GMT Team. Available at: https://www.generic-mapping-tools.org/team.html.

GRIN (2021). GRIN Taxonomy. Available at: https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysearch.

Guan-Fu, H. (1991). Resources of Medicinal Plants in China. *Mem. Inst. Oswaldo Cruz* 86 (Suppl. II), 9–12.

Hunn, E. S. (1998). "What Is Traditional Ecological Knowledge?," in *Traditional Ecological Knowledge: Wisdom for Sustainable Development, Centre for Resource and Environmental Studies*. Editors N.M. Williams and G. Baines (Canberra: Australian National University), 13–15.

Hwang, I. S., Kim, S. J., Park, I. B., Park, Y. M., Park, J. W., Song, H. W., et al. (2005). Physiological Activities of Liquors Prepared with Medicinal Plants. *Korean J. Food Preservation* 12 (3), 282–286.

ISE (2006). *International Society of Ethnobiology Code of Ethics (With 2008 Additions)*. Available at: http://ethnobiology.net/code-of-ethics/(Accessed April 14, 2020).

Ivanova, A., Kostova, I., Mikhova, B., and Vitkova, A. (2004). Chemical Components of Dictamnus Albus L. *Dokl.Bulg.Akad.Nauk.* 57 (11), 45–48.

Jurado, J. M. (2004). "Caracterización analítica de aguardientes anisados," (Sevilla: Universidad de Sevilla). Ph.D. Thesis.

Laguna, A. (1555). *Pedacio Dioscórides Anazarbeo acerca de la Materia Medica*. Anwerpen: Iuan Latio.

Laguna, A. (1566). *Pedacio Dioscórides Anazarbeo acerca de la Materia Medica*. Salamanca: Mathias Gast.

Lambert, E. (1990). *The Collection and Interpretation of Data from Hidden Populations*. Rockville, MD: U.S. Department of Health and Human Services, Public Health Service, Alcohol, Drug Abuse, and Mental Health Administration, National Institute on Drug Abuse.

Lin, H. W., Han, G. Y., and Liao, S. X. (1994). Studies on the Active Constituents of the Chinese Traditional Medicine Polygonatum odoratum (Mill.) Druce. *Yao Xue Xue Bao* 29 (3), 215–222.

López-Pérez, M. (2016). Paracelso en España. *Stud. Hermetica J.* 6, 3–22.

Loring, J. M. (1993). "Aportación de los destiladores de El Escorial a la fabricación de quintaesencias: material vegetal empleada en dichas técnicas y un tratado anónimo de destilación (s.XVI) en el ámbito escurialense," in *Campos, F.J. (Coord.). La ciencia en el Monasterio del Escorial* (Madrid: Real Centro Universitario del Escorial/María Cristina), 585–616.

Maciá, G. (2016). Cantueso con I+D+I incorporado. Las Provincias 22 mayo 2016. Available at: https://www.lasprovincias.es/elche/201605/22/cantueso-incorporado-20160522003416-v.html (Accessed 04 30, 2021).

Marco-Medina, A. (2010). "Aplicación de técnicas *In Vitro* a la conservación de Thymus moroderi," in *Tesis Doctoral* (Alicante: Universidad de Alicante).

Marco-Medina, A., and Casas, J. L. (2015). *In Vitro* multiplication and Essential Oil Composition of Thymus Moroderi Pau Ex Martinez, an Endemic Spanish Plant. *Plant Cel Tiss Organ. Cult* 120, 99–108. doi:10.1007/s11240-014-0583-4

Martín-Reyes, G. (2004). *Breve historia de la Alquimia*. Tenerife: Fundación Canaria Orotava.

Martínez-Francés, V., Hahn, E., Ríos, S., Rivera, D., Reich, E., Vila, R., et al. (2017). Ethnopharmacological and Chemical Characterization of Salvia Species Used in Valencian Traditional Herbal Preparations. *Front. Pharmacol.* 8, 467. doi:10.3389/fphar.2017.00467

Martínez-Francés, V., Rivera, D., Heinrich, M., Obón, C., and Ríos, S. (2015). An Ethnopharmacological and Historical Analysis of "Dictamnus", a European Traditional Herbal Medicine. *J. Ethnopharmacol.* 175, 390–406. doi:10.1016/j.jep.2015.09.011

Martínez-Francés, V., Hahn, E., Juan-Vicedo, J., Vila, R., Ríos, S., and Cañigueral, S. (2012). Ethnobotanical Study of the Sages Used in Traditional Valencia Medicine and Their Essential Oils Characterization of an Endemic Salvia and its Contribution to Local Development. *Contrib. Sci.* 8, 77–84. doi:10.2436/20.7010.01.137

Martínez-Francés, V., and Ríos, S. (2005). "Lesser Known Herbal Spirits of Valencia and Alicante Provinces of Southeastern Spain," in *Industrial Crops and Rural Development*. Editors M.J. Villalobos, F.S. Nakayama, C.A. Bailey, E. Correal, and W.W. Schloman (Murcia: IMIDA), 417–426.

Martínez-Francés, V., Ríos, S., Obón, C., and Verde, A. (2018a). "*Dictamnus Hispanicus* Webb Ex Willk.," in *Inventario Español de los Conocimientos Tradicionales relativos a la Biodiversidad. Fase II (2)*. Editors M. Pardo de Santayana, R. Morales, J. Tardío, L. Aceituno-Mata, and M. Molina (Madrid: Ministerio para la Transición Ecológica), 291–295.

Martínez-Francés, V., and Ríos, S. (2009). "Tissanes i altres preparats herbals emprats en la medicina popular de certes comarques d'interio valencianes (l'Alcoià, el Comtat i la Vall d'Albaida), durant els períodes epidèmics dels segles XIX-XX," in *Salut, alimentació i cultura popular al País Valencià: VI trobades del Seminari d'Estudis sobre la Ciència: "medicina rural i cultura popular al País Valencià: homenatge a Joan Pellicer i Bataller*. Editors X. Guillem-Llobat and G. García

Martínez-Francés, V., Ríos, S., Verde, A., Ferrer-Gallego, P. P., and Laguna, E. (2018b). "*Polygonatum Odoratum* (Mill.) Druce," in *Inventario Español de los Conocimientos Tradicionales relativos a la Biodiversidad. Fase II (3)*. Editors M. Pardo de Santayana, R. Morales, J. Tardío, L. Aceituno-Mata, and M. Molina (Madrid: Ministerio para la Transición Ecológica), 248–249.

Mateo, G., and Crespo, J. B. (2014). *Manual para la determinación de la flora valenciana, ed. 4 [Monogr. Flora Montiber 5]*. Alicante: Librería Compás.

Mattalia, G., Quave, C. L., and Pieroni, A. (2012). Traditional Uses of Wild Food and Medicinal Plants Among Brigasc, Kyé, and Provençal Communities on the Western Italian Alps. *Genet. Resour. Crop Evol.* 60 (2), 587–603. doi:10.1007/s10722-012-9859-x

McVaugh, M. R. (1993). *Medicine before the Plague. Practicioners and Their Patients in the Crown of Aragon 1285-1345*. Cambrige: Cambridge History of Medicine.

Menéndez, G., Morales, R., and Pardo de Santayana, M. (2018). "*Stachys Officinalis* (L.) Trevis," in *Inventario Español de los Conocimientos Tradicionales relativos a la Biodiversidad. Fase II (2)*. Editors M. Pardo de Santayana, R. Morales, J. Tardío, L. Aceituno-Mata, and M. Molina (Madrid: Ministerio para la Transición Ecológica), 123–128.

Merle, H., Ferriol, M., Boira, H., and Blázquez, A. (2006). Composition of the Essential Oil ofDictamnus Hispanicusfrom Spain. *J. Essent. Oil Res.* 18, 483–485. doi:10.1080/10412905.2006.9699148

Min, Y. K., and Jeong, H. S. (1995). Manufacture of Some Korean Medicinal Herb Liquors by Soaking. *Korean J. Food Sci. Technol.* 27 (2), 210–215.

Mizuno, T., Sakai, T., and Chihara, G. (1995). Health Foods and Medicinal Usages of Mushrooms. *Food Rev. Int.* 11 (1), 69–81. doi:10.1080/87559129509541020

Moll, M. (2005). *Les Plantes a Menorca. Noms I Usos*. Maó (Collecció Recerca, 10): Institut Menorquí d'Estudis.

Moll, M. (2003). *Medicina Popular Menorquina. Segles XVI-XXI. Plantes, Animals, Minerals I Altres Modalitats Curatives*. Palma de Mallorca: Documenta Balear.

Morata, A., Vaquero, C., Palomero, F., Loira, I., Bañuelos, M. A., and Suárez-Lepe, J. A. (2019). "Technology of Vermouth Wines," in *Alcoholic Beverages the Science of Beverages, V. 7*. Editors A.M. Grumezescu and A.M. Holban (Cambridge, MA: Woodhead Publishing), 35–63. doi:10.1016/B978-0-12-815269-0.00002-7

Moreno, J. C. (2008). *Lista Roja 2008 de la flora vascular española*. Madrid: Dirección General de Medio Natural y Política Forestal (Ministerio de Medio Ambiente, y Medio Rural y Marino, y Sociedad Española de Biología de la Conservación de Plantas).

Moreno, J. C. (2011). *Lista Roja de la Flora Vascular Española 2008. Actualización con los datos del Adenda 2010 al Atlas y Libro Rojo de la Flora Vascular Amenazada*. Madrid: Dirección General del Medio Natural y Política Forestal (Ministerio de Medio Ambiente y Medio Rural y Marino), Sociedad Española de Biología de la Conservación de Plantas y Tragsatec.

Mulet, L. (1991). *Estudio etnobotánico de la provincia de Castellón*. Castellón: Diputación de Castellón.

Obón, C., Rivera, D., Alcaraz, F., and Attieh, L. (2014). Beverage and Culture. "Zhourat", a Multivariate Analysis of the Globalization of a Herbal tea from the Middle East. *Appetite* 79, 1–10. doi:10.1016/j.appet.2014.03.024

Obón, C., Rivera, D., Fonollá, E., Alcaraz, F., and Attieh, L. (2021). A Comparison Study on Traditional Mixtures of Herbal Teas Used in Eastern Mediterranean Area. *Front. Pharmacol.* 12, 1–18. doi:10.3389/fphar.2021.632692

Obón, C., Rivera, D., Laguna, E., Ferrer-Gallego, P. P., and Verde, A. (2018b). "*Sideritis Hirsuta* L," in *Inventario Español de los Conocimientos Tradicionales relativos a la Biodiversidad. Fase II (3)*. Editors M. Pardo de Santayana, R. Morales, J. Tardío, L. Aceituno-Mata, and M. Molina (Madrid: Ministerio para la Transición Ecológica), 178–186.

Obón, C., Rivera, D., Laguna, E., Ferrer-Gallego, P. P., and Verde, A. (2018c). "*Sideritis Tragoriganum* Lag," in *Inventario Español de los Conocimientos Tradicionales relativos a la Biodiversidad. Fase II (3)*. Editors M. Pardo de Santayana, R. Morales, J. Tardío, L. Aceituno-Mata, and M. Molina (Madrid: Ministerio para la Transición Ecológica), 190–194.

Obón, C., Rivera, D., Verde, A., Martínez-Francés, V., Fajardo, J., Vallès, J., et al. (2018a). "*Artemisia Arborescens* L," in *Inventario Español de los Conocimientos Tradicionales relativos a la Biodiversidad. Fase II (2)*. Editors M. Pardo de Santayana, R. Morales, J. Tardío, L. Aceituno-Mata, and M. Molina (Madrid: Ministerio para la Transición Ecológica), 76–79.

Okaru, A. O., Rehm, J., Sommerfeld, K., Kuballa, T., Walch, S. G., and Lachenmeier, D. W. (2019). "The Threat to Quality of Alcoholic Beverages by Unrecorded Consumption," in *Alcoholic Beverages the Science of Beverages, V. 7*. Editors A.M. Grumezescu and A.M. Holban (Cambridge, MA: Woodhead Publishing), 35–63. doi:10.1016/b978-0-12-815269-0.00001-5

Panesar, P. S., Kumar, N., Marwaha, S. S., and Joshi, V. K. (2009). Vermouth Production Technology. An Overview. *Nat. Prod. Rad.* 8, 334–344.

Parada, M., Carrió, E., and Vallès, J. (2011). Ethnobotany of Food Plants in the Alt Empordà Region (Catalonia, Iberian Peninsula). *J. Appl. Bot. Food Qual.* 84, 11–25.

Peinado-Lorca, M., and Rivas-Martínez, S. (1987). *La Vegetación de España*. Alcalá de Henares: Universidad de Alcalá.

Pellicer, J. (2001). *Costumari Botànic*. València: Edicions del Bullent.

Perrier, X., Flori, A., and Bonnot, F. (2003). "Data Analysis Methods," in *Genetic Diversity of Cultivated Tropical Plants*. Editors P. Hamon, M. Seguin, X. Perrier, and J. C. Glaszmann (Montpellier: Enfield, Science Publishers), 43–76.

Perrier, X., and Jacquemoud-Collet, J. P. (2006). DARwin Software. Available at: http://darwin.cirad.fr/darwin (Accessed 04 30, 2021).

Pielech-Przybylska, K., and Balcerek, M. (2019). "New Trends in Spirit Beverages Production," in *Alcoholic Beverages the Science of Beverages, V. 7*. Editors A.M. Grumezescu and A.M. Holban (Cambridge, MA: Woodhead Publishing), 65–111. doi:10.1016/B978-0-12-815269-0.00002-710.1016/b978-0-12-815269-0.00003-9

Pieroni, A., and Giusti, M. E. (2009). Alpine Ethnobotany in Italy: Traditional Knowledge of Gastronomic and Medicinal Plants Among the Occitans of the Upper Varaita valley, Piedmont. *J. Ethnobiol. Ethnomed.* 5, 32–45. doi:10.1186/1746-4269-5-32

Plasencia, P., and Villalón, T. (1999). *Manual de los vinos de España*. Everest: León.

Plotkin, M. (2021). The Ethnobotany of Wine as Medicine in the Ancient Mediterranean World. *Herbalgram* 129, 56–71.

Pollio, A., De Natale, A., Appetiti, E., Aliotta, G., and Touwaide, A. (2008). Continuity and Change in the Mediterranean Medical Tradition: *Ruta* Spp. (Rutaceae) in Hippocratic Medicine and Present Practices. *J. Ethnopharmacol* 116, 469–482. doi:10.1016/j.jep.2007.12.013

Popović, Z., Smiljanić, M., Kostić, M., Nikić, P., and Janković, S. (2014). Wild flora and its Usage in Traditional Phytotherapy (Deliblato Sands, Serbia, South East Europe). *Indian J. Traditional Knowledge* 13 (1), 9–35.

POWO (2021). Plants of the World Online. Available at: http://www.plantsoftheworldonline.org/(Accessed 03 30, 2021).

Puerto, F. J. (1993). "La farmacia renacentista española y la botica del Escorial," in *Campos, F.J. (Coord.), La ciencia en el Monasterio del Escorial* (Madrid: Real Centro Universitario del Escorial/María Cristina), 73–132.

Reales, A., Rivera, D., Palazón, J. A., and Obón, C. (2004). Numerical Taxonomy Study of Salvia Sect. Salvia (Labiatae). *Salvia (Labiatae) Bot. J. Linn. Soc.* 145, 353–371. doi:10.1111/j.1095-8339.2004.00295.x

Rehm, J., Kailasapillai, S., Larsen, E., Rehm, M. X., Samokhvalov, A. V., Shield, K. D., et al. (2014). A Systematic Review of the Epidemiology of Unrecorded Alcohol Consumption and the Chemical Composition of Unrecorded Alcohol. *Addiction* 109 (6), 880–893. doi:10.1111/add.12498

RHS (2021). Royal Horticultura Society. Available at: https://www.rhs.org.uk/Plants/Search-Results?form-mode=trueandquery=Polygonatum%20odoratum (Accessed 03 30, 2021).

Ríos, S., and Martínez-Francés, V. (2003). Plantas de los herberos en la Sierra Mariola (SW de Valencia, N-NW de Alicante, España). *Flora Montibérica* 25, 42–51.

Ríos, S., and Martínez-Francés, V. (2008). Problemes en la conservació del timó real Dictamnus hispanicus Webb. *Ex. Wilk. IBERIS* 6 (2008), 53–64. Available at: https://www.alcoi.org/export/sites/default/es/areas/medi_ambient/cimal/publicacions/descargas/Iberis6.pdf (Accessed 03 30, 2021).

Rivas-Martinez, S., Penas, A., Diaz-González, T. E., Río, S. D., Cantó, P., Herrero, L., et al. (2014). Biogeography of Spain and Portugal. Preliminary Typological Synopsis. *Int. J. Geobotanical Res.* 4, 1–64. doi:10.5616/ijgr.140001

Rivera, D., and Obón, C. (1995). The Ethnopharmacology of Madeira and Porto Santo Islands, a Review. *J. Ethnopharmacol* 46 (2), 73–93. doi:10.1016/0378-8741(95)01239-a

Rivera, D., Verde, A., Fajardo, J., Obón, C., Consuegra, V., García-Botía, J., et al. (2019). Ethnopharmacology in the Upper Guadiana River Area (Castile-La Mancha, Spain). *J. Ethnopharmacol* 241, 111968. doi:10.1016/j.jep.2019.111968

Rivera, D., Alcaraz, F. J., Verde, A., Fajardo, J., and Obón, C. (2008). "Las plantas en la cultura popular," in *Enciclopedia divulgativa de la historia natural de Jumilla-Yecla. Vol. 9* (Jumilla: Obras Sociales Caja Mediterráneo).

Rivera, D., Obón, C., Ríos, S., Selma, C., Méndez, F., Verde, A., et al. (1996). "Las variedades tradicionales de frutales de la Cuenca del Río Segura," in *Catálogo Etnobotánico (1): Frutos secos, oleaginosos, frutales de hueso, almendros y frutales de pepita* (Murcia: Universidad de Murcia. Servicio de Publicaciones), 360. 84-7684-744-0.

Rivera, D., Obón, C., Ríos, S., Selma, C., Méndez, F., Verde, A., et al. (1998). "Las variedades tradicionales de frutales de la Cuenca del Río Segura," in *Catálogo Etnobotánico (2): Cítricos, Frutos Carnosos Y Vides* (Lleida: DM Editor, Lleida??), 264.

Rivera, D., and Obón, C. (1997). Study of the Biogeography and Centres of Diversity in Section Sideritis (Genus Sideritis, Labiatae). *Lagascalia* 19 (1-2), 865–872.

Rivera, D., Verde, A., Fajardo, J., Inocencio, C., Obón, C., and Heinrich, M. (2006). *Guía Etnobotánica de los alimentos locales Recolectados en la Provincia de Albacete*. Albacete: Instituto de Estudios Albacetenses.

Ruiz-Navajas, Y., Viuda-Martos, M., Sendra, E., Perez-Alvarez, J. A., and Fernández-López, J. (2012). Chemical Characterization and Antibacterial Activity of *Thymus Moroderi* and *Thymus Piperella* Essential Oils, Two *Thymus* Endemic Species from Southeast of Spain. *Food Control* 27, 294–299. doi:10.1016/j.foodcont.2012.04.005

Savo, V., Giulia, C., Maria, G. P., and David, R. (2011). Folk Phytotherapy of the Amalfi Coast (Campania, Southern Italy). *J. Ethnopharmacol* 135 (2), 376–392. doi:10.1016/j.jep.2011.03.027

Schimmel, A. (1993). *The Mistery of Numbers*. USA: Oxford University Press.

Shu, X. S., Lv, J. H., Tao, J., Li, G. M., Li, H. D., and Ma, N. (2009). Antihyperglycemic Effects of Total Flavonoids from Polygonatum Odoratum in STZ and Alloxan-Induced Diabetic Rats. *J. Ethnopharmacol* 124 (3), 539–543. doi:10.1016/j.jep.2009.05.006

Simó-Santonja, V. L. (2007). *De vinis. Arnau de Vilanova*. Valencia: L'Oronella Servicis Editorials Valencians.

Tai, Y., Sun, Y. M., Zou, X., Pan, Q., Lan, Y. D., Huo, Q., et al. (2016). Effect of Polygonatum odoratum Extract on Human Breast Cancer MDA-MB-231 Cell Proliferation and Apoptosis. *Exp. Ther. Med.* 12 (4), 2681–2687. doi:10.3892/etm.2016.3630

Tardío, J., Macía, M. J., and Morales, R. (2018). "*Myrtus Communis* L.," in *Inventario Español de los Conocimientos Tradicionales relativos a la Biodiversidad. Fase II (3)*. Editors M. Pardo de Santayana, R. Morales, J. Tardío, L. Aceituno-Mata, and M. Molina (Madrid: Ministerio para la Transición Ecológica), 267–272.

Tiță, I., Mogoșanu, G. D., and Tiță, M. G. (2009). Ethnobotanical Inventory of Medicinal Plants from the South-West of Romania. *Farmacia* 57 (2), 141–156.

Turner, A. (2003). Sampling Frames and Master Samples. United Nations Secretariat ESA/STAT/AC.93/3, Statistics Division, New York. Available at: https://unstats.un.org/unsd/demographic/meetings/egm/Sampling_1203/docs/no_3.pdf (Accessed 04 19, 2021).

Vales, M. (2012). *Lenguas de España y Variedades del Español. Technicka Univerzita v Liberci*. Liberec: Fakulta přírodovědně-humanitní a pedagogická Katedra románských jazyků.

Vanaclocha, B., and Cañigueral, S. (2019). *Fitoterapia. Vademecum de prescripción*. 5a ed. Barcelona: Elsevier.

Verde, A., Rivera, D., Fajardo, J., Obón, C., and Cebrian, F. (2008). *Guía de las Plantas Medicinales de Castilla-La Mancha (y otros recursos medicinales de uso tradicional)*. Albacete: Altabán Ediciones.

Villar, L., Palacín, J. M., Calvo, C., Gómez, D., and Monserrat, G. (1992). *Plantas medicinales del Pirineo aragonés y demás tierras oscenses*. Huesca: Diputación Provincial de Huesca.

WHO (2018). *Global Status Report on Alcohol and Health*. Available at: https://apps.who.int/iris/bitstream/handle/10665/274603/9789241565639-eng.pdf?ua=1 (Accessed 04 18, 2020).

Witkamp, R. F., and van Norren, K. (2018). Let Thy Food Be Thy medicine. . ..when Possible. *Eur. J. Pharmacol.* 836, 102–114. doi:10.1016/j.ejphar.2018.06.026

Wujisguleng, W., Liu, Y., and Long, C. (2012). Ethnobotanical Review of Food Uses of *Polygonatum* (Convallariaceae) in China. *Acta Soc. Bot. Pol.* 81 (4), 239–244. doi:10.5586/asbp.2012.045

Xiao, J., Cui, F., Ning, T., and Zhao, W. (1990). Effects of Alcohol Extract from Polygonatum Odoratum (Mill.) Druce and Cuscuta Australis R. Br. On Immunological Function of Mice Injured by burns. *Zhongguo Zhong Yao Za Zhi* 15 (9), 557–578.

Youn, Y. N., Lim, E., Lee, N., Kim, Y. S., Koo, M. S., and Choi, S. Y. (2008). Screening of Korean Medicinal Plants for Possible Osteoclastogenesis Effects *In Vitro*. *Genes Nutr.* 2 (4), 375–380. doi:10.1007/s12263-007-0062-1

Zhao, P., Zhao, C., Li, X., Gao, Q., Huang, L., Xiao, P., et al. (2017). The Genus Polygonatum: A Review of Ethnopharmacology, Phytochemistry and Pharmacology. *J. Ethnopharmacol* 214, 274–291. doi:10.1016/j.jep.2017.12.006

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 13 October 2021
doi: 10.3389/fphar.2021.741683

An *In Vitro* Anticancer Activity Evaluation of *Neolamarckia cadamba* (Roxb.) Bosser Leaves' Extract and its Metabolite Profile

Shakirah Razali[1], *Al'aina Yuhainis Firus Khan*[1], *Alfi Khatib*[2,3]*, *Qamar Uddin Ahmed*[2], *Ridhwan Abdul Wahab*[1] *and Zainul Amiruddin Zakaria*[4,5]*

[1]Department of Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Kuantan, Malaysia, [2]Pharmacognosy Research Group, Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy, International Islamic University Malaysia, Kuantan, Malaysia, [3]Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, [4]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia, [5]Laboratory of Halal Science Research, Halal Products Research Institute, Universiti Putra Malaysia, Serdang, Malaysia

OPEN ACCESS

Edited by:
Alessandra Durazzo,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Ali M. Alqahtani,
King Khalid University, Saudi Arabia
Muhammad Nadeem Akhtar,
Islamia University, Pakistan
Ahmed El-Sharkawy,
Institute of Genetics and Biophysics (CNR), Italy
Harika Atmaca,
Celal Bayar University, Turkey

***Correspondence:**
Alfi Khatib
alfikhatib@iium.edu.my
Zainul Amiruddin Zakaria
drzazakaria@gmail.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 15 July 2021
Accepted: 13 September 2021
Published: 13 October 2021

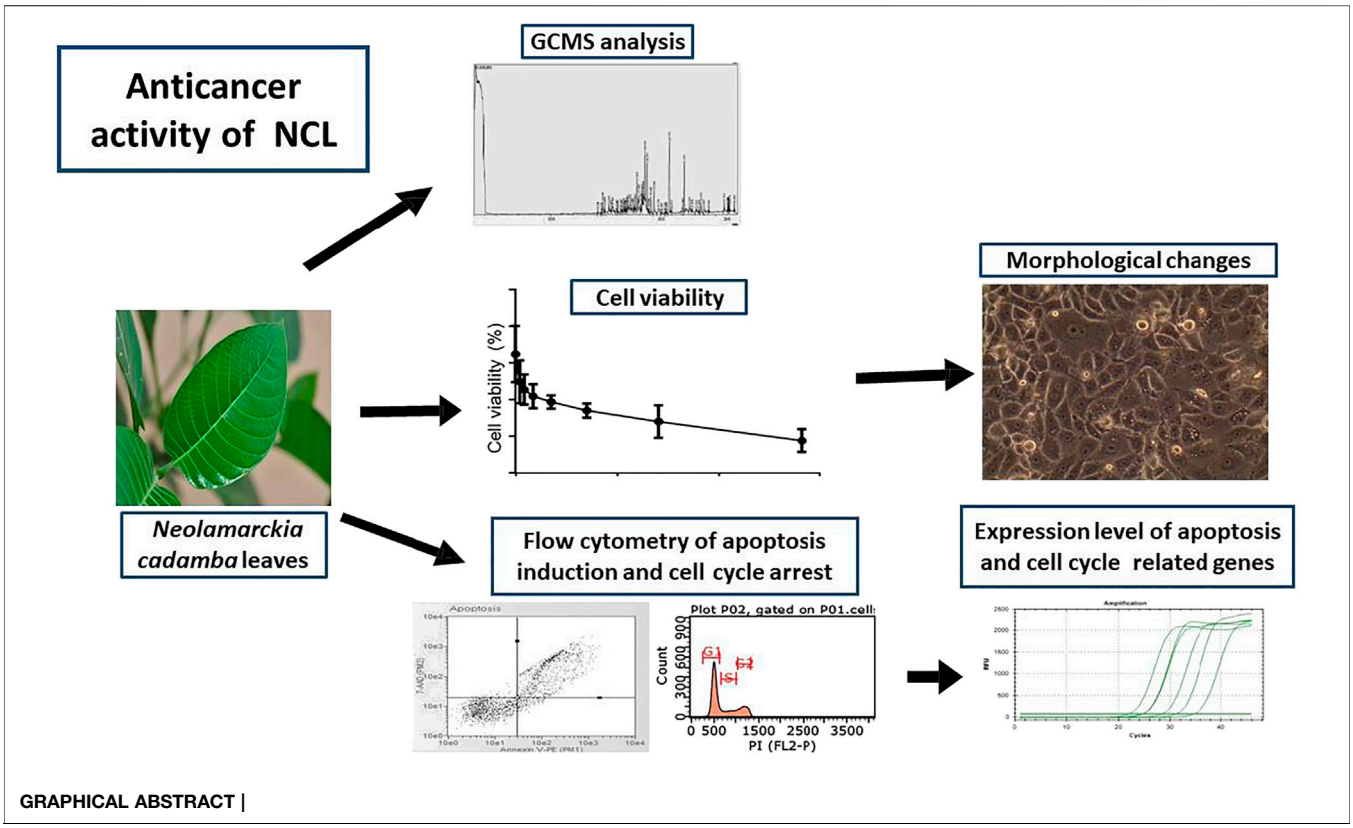
Citation:
Razali S, Firus Khan AY, Khatib A, Ahmed QU, Abdul Wahab R and Zakaria ZA (2021) An In Vitro Anticancer Activity Evaluation of Neolamarckia cadamba (Roxb.) Bosser Leaves' Extract and its Metabolite Profile. Front. Pharmacol. 12:741683. doi: 10.3389/fphar.2021.741683

The leaves of *Neolamarckia cadamba* (NC) (Roxb.) Bosser (family: Rubiaceae) are traditionally used to treat breast cancer in Malaysia; however, this traditional claim is yet to be scientifically verified. Hence, this study was aimed to evaluate the anticancer effect of NC leaves' ethanol extract against breast cancer cell line (MCF-7 cells) using an *in vitro* cell viability, cytotoxicity, and gene expression assays followed by the gas chromatography analysis to further confirm active principles. Results revealed 0.2 mg/ml as the half maximal inhibitory concentration (IC_{50}) against MCF-7. The extract exerted anticancer effect against MCF-7 cells in a dose- and time-dependent manner. The cell cycle assay showed that the extract arrested MCF-7 cells in the G0/G1 phase, and apoptosis was observed after 72 h by the Annexin-V assay. The gene expression assay revealed that the cell cycle arrest was associated with the downregulation of *CDK2* and subsequent upregulation of p21 and cyclin E. The extract induced apoptosis *via* the mediation of the mitochondrial cell death pathways. A chromatography analysis revealed the contribution of D-pinitol and myo-inositol as the two major bioactive compounds to the activity observed. Overall, the study demonstrated that NC leaves' ethanol extract exerts anticancer effect against MCF-7 human breast cancer cells through the induction of apoptosis and cell cycle arrest, thereby justifying its traditional use for the treatment of breast cancer in Malaysia.

Keywords: ***Neolamarckia cadamba*, breast cancer, gas chromatography, gene expression assay, mechanisms of action, *in vitro* anticancer**

INTRODUCTION

Breast cancer is the most commonly occurring cancer in women and the second most common cancer overall in terms of fatality after lung cancer (Bray et al., 2018). The rate of breast cancer patients' cases is increasing every year, both in developed and developing countries (Torre et al., 2015). It is estimated that 26 million new cancer cases and 17 million cancer deaths are likely to occur per year globally by 2030 (Thun et al., 2010). In Asia alone, the incident from 2008 to 2030 is estimated to increase from 6.1 to 10.7 million cancer cases and 4.1 to 7.5 million deaths (Sankaranarayanan et al., 2014). Malaysia is



GRAPHICAL ABSTRACT |

one of the Asian countries with an increased prevalence of breast cancer cases (Azizah et al., 2016), affecting both genders but increased exponentially with age in women and thus accounted for the leading cause of cancer-related deaths among women in Malaysia (Lukong, 2017). Surgery, radiotherapy, chemotherapy, hormonal therapy, and gene-targeted therapy have been greatly used in treating breast cancer patients. The advances of current treatments and detecting methods have contributed to the increased survival rate (Blumen et al., 2016). Unfortunately, these treatments cause short-term and long-term side effects for the patients. Moreover, studies have demonstrated that the burden of the cost related to cancer treatment is uncharacteristically rising (Blumen et al., 2016; Trogdon et al., 2017). Therefore, using natural sources as alternative, effective, and non-invasive entities to treat cancer is warranted.

According to the World Health Organization (WHO), many countries, including developing countries, still use plants and natural source–related products for therapeutic purposes (Rayan et al., 2017). About 60% of anticancer agents have been originated from the natural sources globally (Newman and Cragg, 2016). The nature-derived compounds are readily available, generally more tolerated, and considered non-toxic to normal human cells (Shah et al., 2013).

Neolamarckia cadamba (Roxb.) Bosser (family: Rubiaceae) is a tree that is traditionally used for the treatment of various illnesses. This underexplored evergreen tropical plant has been widely used in the Ayurvedic medicine system of India in treating eye infection, skin diseases, dyspepsia, gum-related troubles, stomatitis, cough, fever, anemia, blood disorders, and stomach ache (Dubey et al., 2011; Pandey and Negi, 2016). In addition, the leaves of this plant have been shown to possess several pharmacological properties, including antioxidant (Kaur and Kumar, 2011; Chandel et al., 2012), antidiabetic (Ahmed et al., 2011), antitumor (Dolai et al., 2012), anti-inflammatory, antipyretic, analgesic (Mondal et al., 2009), antimicrobial (Rafshanjani et al., 2014), and anticancer effects (Singh et al., 2013). Furthermore, the *N. cadamba* leaves have also been used as a topical application to treat breast cancer. However, this traditional application has never been scientifically investigated to verify its anticancer effect. Therefore, the main purpose of the study was to evaluate the anticancer effects of *N. cadamba* leaves' extract on breast cancer cell line (MCF-7) with emphasis on the mechanism of action, that is, apoptosis induction and cell cycle arrest. The characterization of *N. cadamba* leaves' extract was also carried out using a gas chromatography–mass spectrometry (GCMS) approach to identify bioactive compounds.

Novelty

1. It is the pioneer study highlighting the anticancer effect of *Neolamarckia cadamba* (Roxb.) Bosser leaves' ethanol extract on breast cancer cells for the first time.
2. *Neolamarckia cadamba* leaves' ethanol extract showed dose- and time-dependent anticancer effects.
3. Treated cells were arrested in the G1 phase and underwent apoptosis *via* the mitochondria pathway.
4. Findings of this study further support the traditional use of *Neolamarckia cadamba* (Roxb.) Bosser leaves as an anticancer agent in the treatment of breast cancer.

MATERIALS AND METHODS

Materials and Chemicals

The GCMS reagents were purchased from Sigma-Aldrich, United States (Pyridine and N-Methyl-N-(trimethylsilyl)trifluoroacetamide-MSTFA) and Supelco, United States (methoxyamine HCl). The normal human fibroblast (HDF) and human breast adenocarcinoma (MCF-7) cells were obtained from American Type Culture Collection (ATCC), United States. All cells were maintained in complete growth medium (CGM) consisting of Dulbecco's modified Eagle medium (DMEM) (Nacalei Tesque, Japan), 10% fetal bovine serum (Nacalei Tesque, Japan), and 1% of penicillin–streptomycin (Nacalei Tesque, Japan). The phosphate-buffered saline (PBS) from Gibco, United States, was used for cell-washing purposes. Reagents for apoptosis (Nexin reagent) and a cell cycle reagent were purchased from Merck Millipore, United States. Kits used for the mRNA analysis were purchased from Analytik Jena, Germany (InnuPREP DNA/RNA mini kit), BIOLINE, United Kingdom (SensiFAST cDNA synthesis and SensiFAST SYBR® No-ROX kits), and Integrated DNA technologies, Singapore (primer).

Preparation of *Neolamarckia cadamba* Leaves' 80% Ethanol Extract (NC)

Fresh leaves of *N. cadamba* were collected from Balok, 25200 Kuantan, Pahang DM, Malaysia, and deposited (voucher specimen #: PIIUM 0266) in the Herbarium, Kulliyyah of Pharmacy, IIUM, Malaysia. The leaves were prepared according to the previous method (Liu et al., 2017) with slight modification. The leaves were washed and air-dried. The dried leaves were pulverized and soaked in 80% ethanol three times using an ultrasonic sonicator. The extract was filtered, concentrated by a rotary evaporator, and freeze-dried. It was stored at −20°C until further usage. The extract was dissolved in 80% ethanol to obtain a stock solution of 100 mg/ml. Working solutions were prepared fresh from ethanol stock in the DMEM medium by serial dilutions. The solutions were then filtered with a sterilized 0.22 µl syringe filter before being used in an *in vitro* assay.

Gas Chromatography–Mass Spectrometry (GCMS) Analysis of NC

Derivatization of the samples was performed for the GCMS analysis following the method described (Liu et al., 2017). Briefly, about 50 µl pyridine, 100 µl of methoxyamine HCl (20 mg/ml in pyridine), and 300 µl of MSTFA were added to the extract. The extract solutions were filtered and covered with aluminum foil to be left overnight at room temperature before the analysis. The GCMS analysis was carried out using GCMS-TQ8040 (Shimadzu, Japan), where the analysis was carried out using a DB-5MS 5% phenyl methyl siloxane (30 mm × 0.25 mm, i.d. × 0.25 µm thickness). The initial oven temperature used was set to 50°C for 5 min and raised to 200°C at the rate of 5°C/min and held for 5 min before reaching the target temperature of 300°C at the same rate. The carrier gas applied was helium with a flow rate of 1.0 ml/min, while the injector and detector temperature were set up to 250 and 280°C, respectively. Full-scan mass spectra were retrieved by setting parameters ranging from 50 to 550 *m*/*z*. The chromatograms of the compounds were compared to the database of the National Institute of Standards and Technology (NIST) 2014.

Effect of NC on Cell Viability of MCF-7 and HDF Cell Growth

All cells were maintained in a sterilized environment set to 37°C with 5% CO_2 humidified atmosphere. The cell viability of NC-treated cells was measured using the trypan blue exclusion (TBE) method as previously described (Hsieh et al., 2005). In determining the median concentration of NC on MCF-7 cells, the cells were seeded at 4.7×10^4 cells/ml in 6-well plates. After overnight, different concentrations of NC prepared by serial dilution (500.0, 250.0, 125.0, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, and 0.9 µg/ml) were added to MCF-7 cells and kept for 72 h before performing cell density evaluation using TBE. MCF-7 cells were exposed to NC for 24, 48, 72, and 96 h prior evaluation with the TBE assay to determine the MCF-7 growth exposure with NC. The cytotoxicity of NC against normal HDF cells, which were cultured at 5.0×10^4 cells/ml in 6-well plates, was determined by examining the IC_{50} value of NC after 72 h. The untreated cells were not exposed to NC and were used as a negative control.

Cell Cycle Analysis

MCF-7 cells collected from the well were prepared in triplicate prior to staining with the cell cycle reagent (Guava Cell Cycle Reagent), following the manufacturer's instructions. The PI-stained cells were observed using a Guava EasyCyte flow cytometer system (Hsieh et al., 2005).

Apoptosis Analysis Using Annexin V

Briefly, MCF-7 cells were exposed to NC for 72 h. The collected cells were treated with the FITC Annexin-V Apoptosis Detection Kit I, following the manufacturer's instructions. The cells were treated with 1x binding buffer, FITC Annexin-V, and propidium iodide (PI) before their application to the Guava easyCyte flow cytometer system (EMD Millipore, Germany) (Hsieh et al., 2005).

Real-Time Quantitative PCR Analysis of Target Genes

The quantification of the mRNA expression of the targeted primer mRNA corresponding to apoptosis, cell cycle, and metastasis was carried out following our previously published study (Strober, 2015). Briefly, the RNA extraction kit was used to extract the total RNA from the plant treated and non-treated MCF-7 cells after 72 h. The RNA's purity and integrity were evaluated prior to complementary DNA (cDNA) synthesis. The cDNA was prepared using a cDNA synthesis kit with 200 ng of RNA template following the manufacturer's instructions.

TABLE 1 | Putative compounds in NC leaves' extract identified by GCMS.

No.	Retention time	Name of compound	Area %	Similarity index
1	14.25	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.21	93
2	16.08	Levoglucosan	0.23	91
3	20.36	β-D-(+)-Talopyranose	0.38	92
4	20.78	D-pinitol	14.18	86
5	20.98	Hexadecanoic acid	0.52	91
6	22.13	Myo-inositol	7.98	90
7	23.22	Oleic acid	0.66	89
8	23.48	Octadecanoic acid	0.43	90

Primers were selected from the National Centre for Biotechnology Information (NCBI) database based on the criteria listed below.

i. Melting temperature, Tm (59–65°C)
ii. Amplicon size (70–150 bases)
iii. Forward and reverse primers (spanning exon–exon junctions)
iv. Primer's 3′ end with a C or G residue
v. GC content (40–60%)
vi. Primer's sequence (18–25 nucleotides)

The PCR amplifications were conducted using SYBR green in CFX96 Touch™ Real-Time PCR (Bio-Rad, United States). The efficiency of the primer PCR was evaluated using 1:10 serial dilutions of the cDNA sample. The standard curve's amplification efficiency (E) range was set between 93.5 and 115.4%, while the correlation coefficients (R2) ranged from 0.984 to 0.998. The optimized parameters of the amplifications include 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 s at 95°C, and 1 min at 60°C. The CFX Manager Software was used to analyze the results obtained by applying threshold cycle (CT) values. The reference genes used were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB). The primers' detailed information is displayed in **Supplementary Table S1**.

Statistical Analysis

Statistical and the IC_{50} values' (half maximal inhibitory concentration) calculations were carried out using GraphPad Prism software version 6. The cytotoxicity data were analyzed using multiple *t*-tests. The flow cytometry analysis and gene expression data were determined using multiple *t*-tests (Holm–Sidak method). All data were displayed as mean ± SD (standard deviation). * Signifies the significant difference when $p < 0.05$.

RESULTS

Gas Chromatography–Mass Spectrometry Analysis of NC

Table 1 presents the putative compounds detected in the NC leaves' 80% ethanol extract. The metabolites with the similarity index (SI) of more than 85% based on the National Institute of Standards and Technology (NIST) 14 database can be regarded as the putative compounds. The chromatogram showed the highest peak area corresponding to D-pinitol, followed by myo-inositol, oleic acid, hexadecanoic acid, and octadecanoic acid.

Breast Cancer Cells (MCF-7) Viability After NC Treatment

The inhibitory activity of NC leaves' 80% ethanol extract was investigated against MCF-7 cells. The cell viability was directly tested using the trypan blue exclusion assay method where the dead cells were stained blue. **Figure 1A** shows the antiproliferative activity of NC leaves' 80% ethanol extract on MCF-7 cells after 72 h of incubation. The finding revealed an IC_{50} of 206.0 ± 3.4 μg/ml. Cell viability was found to decrease with the increasing concentration of the extract used. **Figure 1B** demonstrates the IC_{50} value of NC to inhibit MCF-7 growth with cell viability percentage difference between NC and UT by 6.3, 12.2, 23.1, and 35.4% at 24, 48, 72, and 96 h, respectively. From the findings, it can be seen that the NC inhibition effect on MCF-7 growth increased over time. **Figure 1C** depicts the image of MCF-7 cells treated with NC in comparison to the untreated as a control. The image shows the cells exhibiting a distinguished plasma membrane with intact nuclei, and cells were seen to grow adjacent to the neighboring cells in a monolayer. Most of the cells were attached to the tissue culture plate. In contrast, cells treated with NC extract for 72 h showed apoptotic-like features including cell rounding, loss of contact with the adjacent cells, cell shrinkage, and formation of apoptotic bodies. **Figure 1D** presents the viability of normal HDF cells exposed to NC leaves' 80% ethanol extract for 72 h. The graph reveals that NC leaves' 80% ethanol extract had slight toxicity on HDF cells by inhibiting cell growth by 19.5% when compared to UT. Overall, the NC ethanol extract exhibited a concentration and time inhibition pattern related to the MCF-7 cell growth and slight toxicity on normal HDF cells. IC_{50} obtained was used for further investigation in this study.

Cell Cycle Analysis of NC Against MCF-7 Cells

To understand the growth inhibition found earlier, NC leaves' 80% ethanol extract–treated MCF-7 cells were subjected to cell cycle analysis with UT serving as control. **Figure 2A** demonstrates MCF-7 cells treated with NC leaves' 80%

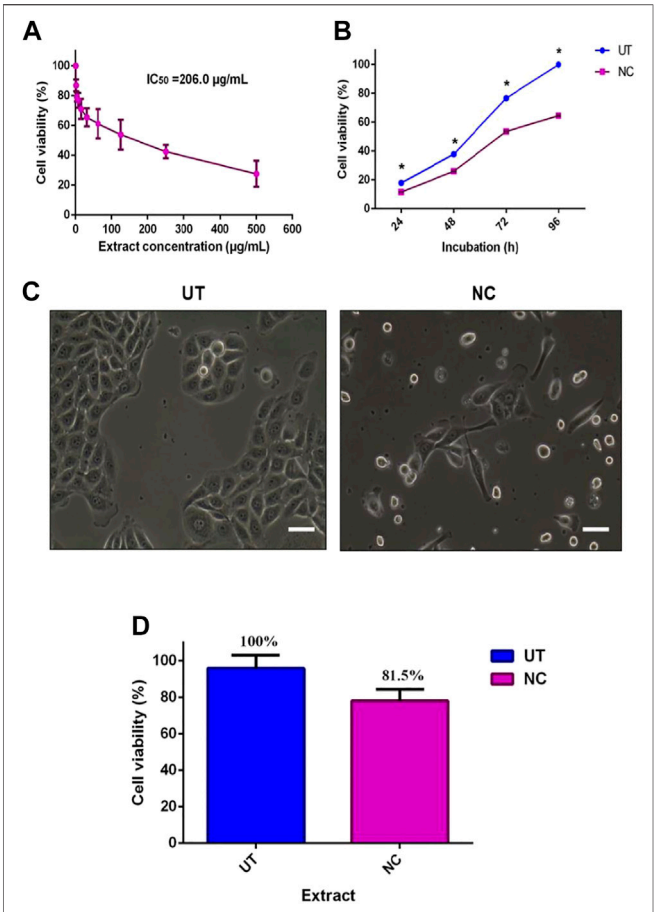


FIGURE 1 | Effect of NC leaves' 80% ethanol extract on MCF-7 cell viability. **(A)** The median concentration of MCF-7 treated with NC leaves' 80% ethanol extract for 72 h. **(B)** Antiproliferative effect of NC leaves' 80% ethanol extract at 24, 28, 72, and 96 h with untreated cells (UT) as controls. **(C)** Image of MCF-7 cells with NC leaves' 80% ethanol extract treated and untreated at 72 h. **(D)** Cytotoxic effect of NC leaves' 80% ethanol extract median concentration on normal human dermal fibroblast (HDF) cells at 72 h incubation. The results presented as mean ± SD (n = 3) (bar graph = 25 µm). * indicates $p < 0.05$.

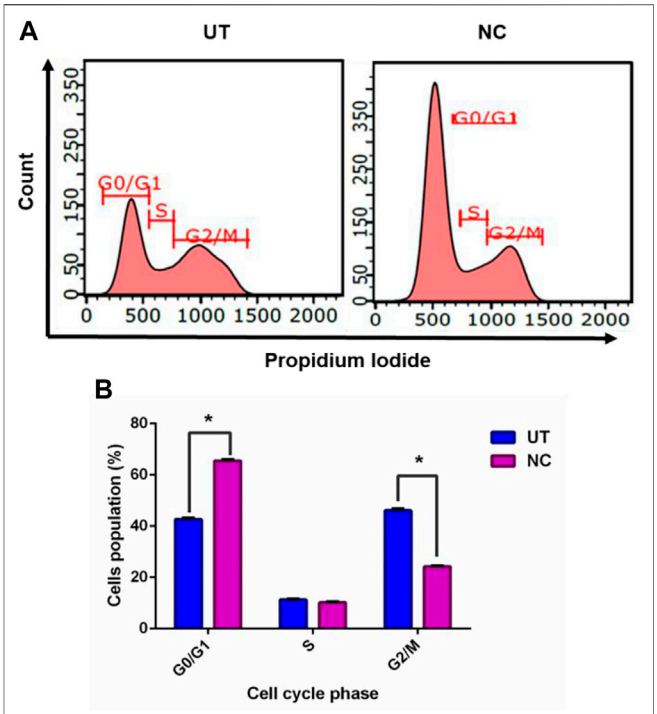


FIGURE 2 | Effect of NC leaves' 80% ethanol extract on the MCF-7 cell cycle. **(A)** Representation of DNA histogram of NC leaves' 80% ethanol extract–treated cells, with UT as control at 72 h of incubation. DNA histograms display cell cycle phases of treated cells, namely, G1/G0, S, and G2/M (n = 3). **(B)** The proportion of cells according to the cell cycle phase are presented in percentage mean ± SD (n = 3). * indicates $p < 0.05$ concerning untreated.

ethanol extract and UT DNA histogram at 72 h of incubation, which indicates that NC leaves' 80% ethanol extract exerted MCF-7 arrest in G1/G0 compared to UT. **Figure 2B** shows the proportion of cells according to cell cycle phase from **Figure 2A**. The graph reveals that NC leaves' 80% ethanol extract significantly induced G1/G0 arrest in MCF-7 cells evidenced by the increase of cell population by 22.9% in G1/G0 and simultaneous decrease of G2/M by 21.9% compared to UT.

Apoptosis Analysis of NC Leaves 80% Ethanol Extract Against MCF-7 Cells

As evidenced earlier, NC leaves' 80% ethanol extract showed growth inhibition and cell death; hence, further investigation was conducted to evaluate the possibility of cells to undergo apoptosis using flow cytometer analysis. The Annexin-V assay helped to differentiate individual cells in live, early apoptotic, late apoptotic, and necrotic quadrants following 72 h of NC leaves' 80% ethanol extract treatment to MCF-7 cells. **Figure 3A** shows MCF-7 cells, NC leaves' 80% ethanol extract-treated cells, and UT dot plot distribution in quadrants. The Annexin-V assay revealed that NC leaves' 80% ethanol extract–treated cells induces early and late apoptosis by 12.0 and 32.1% compared to UT. On the contrary, UT cells are majorly located in the live quadrant. **Figure 3B** demonstrates the proportion percentages of apoptotic (early and late apoptosis) and live cells of NC leaves' 80% ethanol extract–treated and UT MCF-7 cells. The proportion of apoptotic percentage from NC leaves' 80% of ethanol extract–treated cells was significantly higher by 44.3% compared to UT 8.6%, while the proportion of live cells percentage was higher in UT MCF-7 by 91.5% than in NC-treated cells by 65.7%.

Effects of NC Leaves' 80% Ethanol Extract on Gene Expression of MCF-7

A deeper investigation was further performed to investigate gene expression of NC leaves' 80% ethanol extract supplement to MCF-7 cells with regards to cell cycle arrest and apoptosis induction. Five genes related to apoptosis were measured for their expressions through the qPCR assay *viz.* Bax, Bcl-2,

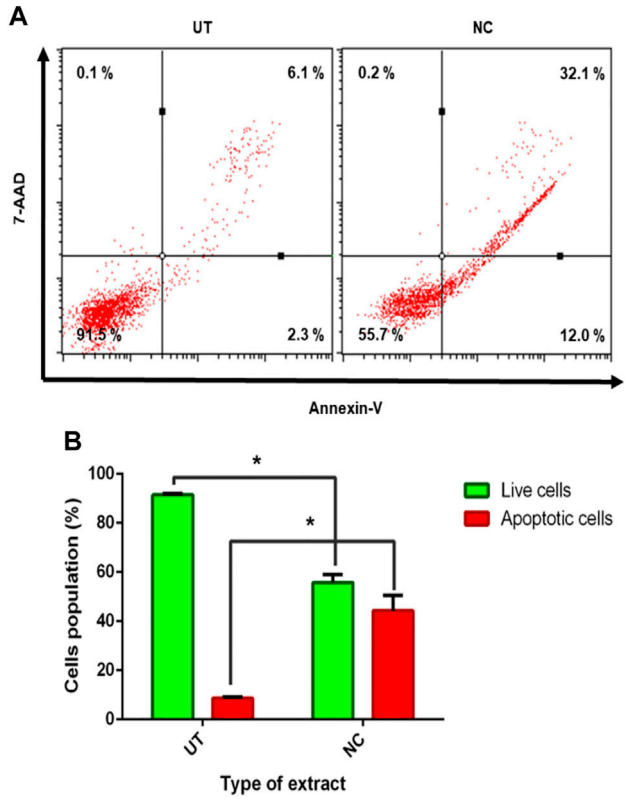


FIGURE 3 | Effect of NC leaves 80% ethanol extract on MCF-7 cells. **(A)** Representation of dot plot distribution of cells in living, death, and early and late apoptosis quadrants of MCF-7 cells treated with NC leaves 80% ethanol extract at 72 h with UT as control ($n = 3$). **(B)** The proportion percentages of apoptotic (early and late apoptosis) and live cells are presented as percentage mean ± SD ($n = 3$). * indicates $p < 0.05$ concerning untreated.

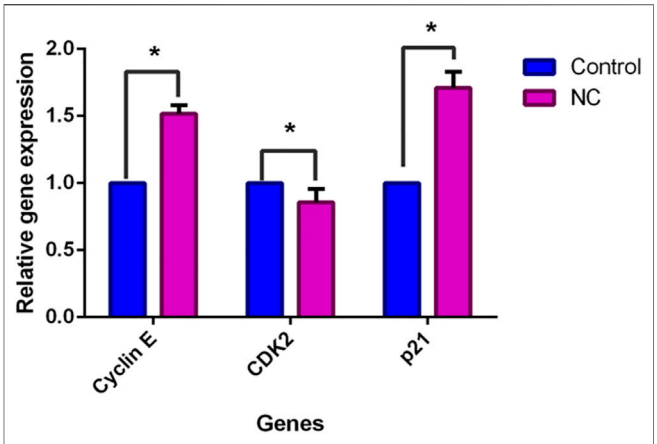


FIGURE 4 | Effect of NC leaves 80% ethanol extract on cell cycle-related gene expression upon treatment with IC_{50} for 72 h. Relative expression for cyclin E, CDK2, and p21 were calculated by measuring the fold change with GAPDH and β-actin as reference genes in untreated cells (the ratio was equal to 1). Data presented in mean ± standard deviation of triplicates of the experiment. * indicates a significant difference when $p < 0.05$.

caspase-9, caspase-7, and cytochrome c. The expression of cell cycle–related genes on MCF-7–treated cells with a growth arrest at the G0/G1 phase, cyclin E, CDK2, and p21 were studied. **Figure 4** shows that upon NC leaves' 80% ethanol extract treatment, the expression of cyclin *E* and *p21* were found to be upregulated with 1.6 ± 0.54 and 1.7 ± 0.02 folds, respectively. Meanwhile, CDK2 was downregulated with 0.9 ± 0.07 folds. From this result, the upregulated expression of *p21* in NC-treated cells indicates that the *p21* inhibits the activity of *CDK2* and cyclin E catalytic complex formation which blocks the progression to the next stage of the cell cycle.

In **Figure 5A**, Bax (proapoptotic) and cytochrome c were downregulated by NC leaves' 80% ethanol extract by 2.1 ± 0.07-fold and 5.1 ± 0.42-fold, respectively, whereas Bcl-2 was downregulated by 0.9 ± 0.04-fold. Meanwhile, in **Figure 5B** caspase family (caspase-9 and caspase-7) expressions were upregulated with NC leaves' 80% ethanol extract treatment by 3.1 ± 0.65 and 1.8 ± 0.19-fold, respectively. The upregulation of Bax, cytochrome c, and caspases family corresponded with the downregulation of *Bcl-2* gene, suggesting that NC extract treatment lowered cell survival and induced apoptosis *via* the intrinsic mitochondrial pathway.

DISCUSSION

Throughout the whole world, breast cancer is affecting women the most compared to other types of cancers with a high incidence and mortality rate (Harbeck et al., 2019; Lin et al., 2019). Additionally, there are some undocumented and unverified claims for *N. cadamba* leaves that it is used to treat breast cancer in different parts of Malaysia; however, no scientific study using breast cancer cell lines has ever been carried out to confirm its potential in the management of breast cancer. Hence, this study was performed to verify *N. cadamba* leaves' extract as a potential anticancer agent with minimal toxicities. In this study, NC leaves' 80% ethanol extract was initially characterized with GCMS and then assessed for its *in vitro* anticancer properties and mechanism of action using MCF-7 cells. Phytochemical characterization of NC leaves s 80% ethanol extract through the GCMS analysis identified eight putative bioactive compounds including D-pinitol, myo-inositol, oleic acid, hexadecanoic acid, octadecanoic acid, β-D-(+)-talopyranose, levoglucosan, and phenol, 2, 4-bis(1,1-dimethylethyl).

Among the compounds, D-pinitol (6-methoxycyclohexane-1,2,3,4,5-pentol) was found to be the most abundant one. D-pinitol is a natural, sugar-like cyclitol, a cyclic polyol found in a variety of plants including soybean and has been reported to exert diverse biological activities particularly antioxidant, antiviral, antidiabetic, anti-inflammatory, and anticancer. D-pinitol has been reported to inhibit prostate cancer metastasis through the inhibition of αVβ3 integrin by modulating FAK, c-Src, and NF-κB pathways (Lin et al., 2013). Furthermore, a study revealed that d-pinitol was able to induce apoptosis in MCF-7 by upregulating Bax and downregulating Bcl2 expressions. It was also demonstrated that D-pinitol promoted apoptosis in MCF-7 cells *via* induction of p53 and Bax and inhibition of Bcl-2 and NF-κB

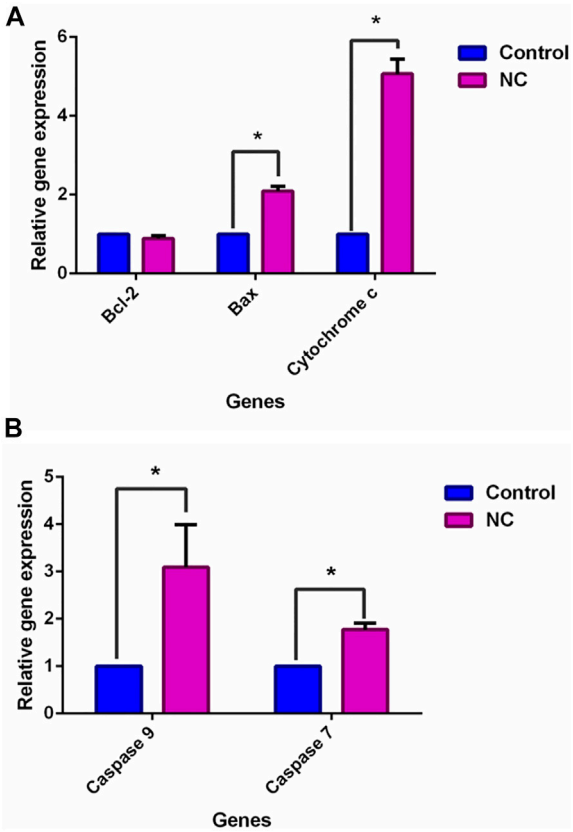


FIGURE 5 | Effect of NC leaves 80% ethanol extract on apoptosis-related gene expression upon treatment with IC_{50} for 72 h. Relative expression for **(A)** Bcl-2, Bax, and cytochrome c, and **(B)** caspase-9 and caspase-7 were calculated by measuring the fold change with GAPDH and β-actin as a reference gene in untreated cells, whose ratio was equal to 1. Data presented in mean ± standard deviation of triplicates of the experiment. * indicates a significant difference when $p < 0.05$.

(Rengarajan et al., 2014). Myo-inositol, the next most abundant bioactive compound in *N. cadamba* leaves' extract, has been earlier isolated from fresh samples of *Cosmos caudatus* Kunth (Javadi et al., 2015) and *Sapindus mukorossi* Gaertn (Liu et al., 2018). Myo-inositol has been currently used clinically to treat polycystic ovary syndrome (PCOS) (Artini et al., 2013).

Hexadecanoic acid was identified in our study as another bioactive compound, and identified earlier from methanol (44.88%) and hexane (17.96%) extracts of *N. cadamba* leaves. Additionally, octadecanoic acid was also found in methanol, ethyl acetate, chloroform, and hexane extract (Zayed et al., 2014). N-hexadecanoic acid found in *Kigelia africana* subsp. africana (syn. *Kigelia pinnata*) leaves had caused cytotoxicity in HCT-116 cell lines (Ravi and Krishnan, 2017). Moreover, octadecanoic acid has induced apoptosis in breast cancer cells (Evans et al., 2009). Oleic acid found in almond oil (*Prunus dulcis* (Mill.) D. A.Webb) has displayed antiproliferative and anticancer effects on colon carcinoma cells (Mericli et al., 2017). Meanwhile, the ability of phenol, 2,4-bis(1,1-dimethylethyl) compound docked with Bcl-2 showing low binding affinity (−7.4 kcal/mol) after caryophyllene compound (−7.5 kcal/mol) indicated the potential of *Solanum trilobatum* L. extract compounds as a potent anticancer agent (Xavier et al., 2013).

The results showed that the population of MCF-7 cell lines was reduced to 50% from 100% of viable cells. NC extract exhibited a growth inhibitory effect toward MCF-7 in a concentration- and time-dependent manner in which the percentage of viable cells decreased in correspondence with the increase of NC extract concentration. Different bioactive compounds were reported in *N. cadamba* leaves using different solvents and associated with various biological activities, including antioxidant, anticancer, or antitumor activity (Zayed et al., 2014). Sun and Hai Liu (2006) described that the existence of different components in the natural sources may produce additive and synergistic effects rather than a single effect. The biochemical and different morphological changes such as cell shrinkage, cell blebbing, the formation of apoptotic bodies, nuclear condensation, and DNA fragmentation are the hallmarks of apoptosis (Ouyang et al., 2012). The apoptotic-like features of MCF-7 cells were observed under a phase-contrast inverted microscope. Cell rounding, cell shrinkage, membrane blebs, loose contact with adjacent cells, the formation of apoptotic bodies, and cell vacuolization were seen in the treated MCF-7 cells. The appearance of apoptotic cell features has also been reported in MCF-7 cells treated with rapamycin under inverted microscope observation (Khairi et al., 2014). Alteration in the morphology of

MCF-7 cells following the NC extract treatment indicated that the cells experienced apoptosis (Wen et al., 2017). One of the main challenges with the current treatments is the capacity to hit cancer cells without killing normal cells surrounding the cancer cells. However, the findings in this study revealed that NC extract was slightly toxic toward normal cells.

Inhibition of proliferation and/or induction of apoptosis in cancer cells is considered one of the main important criteria for many anticancer agents (Barhoi et al., 2021; Orabi et al., 2021). The flow cytometric analysis in the present study revealed that MCF-7 cells underwent apoptosis upon treatment with NC, abundantly in late apoptosis. A similar finding was found on MCF-7 cells after the treatment with rapamycin in which late apoptosis up to 23.2% had been reported (Khairi et al., 2014). Early apoptosis was detected in the present study indicating the externalization of negatively charged phosphatidylserine (PS) (Riedl et al., 2011; Jamali et al., 2018), which is mainly localized in the inner layer of the membrane. Late apoptosis is also known as secondary necrosis, causing loss of membrane integrity (Poon et al., 2010).

The cell cycle consists of consecutive four phases that are important in cell division and cell proliferation. The unscheduled proliferation and genomic instability give rise to the accumulation of tumor cells (Hanahan and Weinberg, 2000). The finding in this study suggests that NC leaves 80% ethanol extract caused cell cycle arrest at the G0/G1 phase in MCF-7 cells after 72 h of exposure. The results clearly showed a significant increase of cell distribution in the G0/G1 phase accompanied by a decrease of cell distribution in phase S. Accumulation of cells in the G0/G1 phase has been reported in MCF-7 cancer cells (Sun and Hai Liu, 2006; Choi and Kim, 2008) and other cancer cell lines (Fan et al., 2015; Karade and Jadhav, 2018) after plant extract treatment. A similar finding was also reported in a study in which epirubicin caused the G0/G1 cell cycle arrest on MCF-7 and T47D cells (Xiong et al., 2016). Upon detecting the DNA damage, cell cycle checkpoints stop the progression of the cell cycle from one phase to the next phase to ensure the fidelity of genetic information (Vermeulen et al., 2003). The arrest of MCF-7 cells at the G0/G1 phase prevents the cells from entering the next phases of the cell cycle, the S and G2/M phases, which eventually inhibits the synthesis of DNA and suppresses proliferation of the cells. To find out the possible mechanism of the extract to induce apoptosis and cell cycle arrest in MCF-7 cells, the qPCR assay was performed. The upregulation of cytochrome c links with the upregulation of Bax expression and downregulation of Bcl-2 (Ilhan, 2020). Bax executes its action on mitochondria by promoting the release of cytochrome c, *via* increasing the permeability of mitochondria membrane, into the intracellular space (Kilbride and Prehn, 2013). The inhibition of *Bcl-2* activity involves with the heterodimer formation on *Bcl-2* by Bax gene to counteract the antiapoptotic effects of *Bcl-2* (Adams and Cory, 2007). The liberation of cytochrome c would activate apoptosome, which then activates caspase-9 and the effector caspases, caspase-3 and caspase-7 leading to cell death (Olsson & Zhivotovsky, 2011; Westphal et al., 2011; Looi et al., 2013). The morphological and biochemical changes are associated with caspase activation upon apoptotic stimuli (Duclos et al., 2017). The upregulation of caspase-9 and caspase-7 expressions was detected upon NC treatment on MCF-7 cells. Activation of the effector caspases *via* proteolytic cleavage by caspase-9 and cytochrome c is believed to play a crucial role in the execution of apoptosis (Hassan et al., 2014). These results might suggest that NC extract exerts apoptosis through the intrinsic pathway. The expression of *p21* (tumor suppressor gene) stops the cell cycle progression from dividing damaged cells. The binding of p21 on CDKs or cyclin-CDK complexes disrupts these interactions and inhibits cell cycle progression (Karimian et al., 2016; Kreis et al., 2019). Upregulated *p21* and downregulated *CDK2* support the G0/G1 cell cycle arrest in the previous data. Interestingly, cyclin *E* in the present study was found to be upregulated, which suggested that cyclin *E* independently binds to *CDK2* for halting the cell cycle. It was suggested that inhibition of breast cancer cell MCF-7 proliferation may be linked to the upregulation of cyclin E and p21 as well as downregulation of *CDK2* upon NC extract treatment. In addition, p21 was found to be associated with apoptosis. A study found the cytochrome c blockage and caspase-3 inactivation in HCT116 *p21−/−* but not in HCT *p53−/−* and HCT116 Bax−/− of colon cancer cells after treatment with curcumin. The study also found a reduced expression of *Apaf-1* in the HCT116 *p21−/−* cells, whereas it was not expressed in wild type (Gogada et al., 2011). Cytochrome c release and Apaf-1 are required for caspase-9 activation of the intrinsic apoptosis pathway (Zhou et al., 2015), which in turn activates executioner caspases, including caspase-3 and caspase-7. Based on the present findings, it is suggested that *p21* upregulated expressions may have been involved in apoptosis activities by promoting the cytochrome c release to inhibit the proliferation of MCF-7 cells upon treatment.

CONCLUSION

The present research data suggest that NC leaves ethanol extract inhibits cellular growth in MCF-7 cells by inducing apoptosis and cell cycle arrest. As supported by the flow cytometric analysis, upregulation of Bax, cytochrome c, and caspases (caspase-9 and -7) with downregulation of Bcl-2 in MCF-7 cells suggest that NC leaves ethanol extract promotes apoptosis *via* the intrinsic pathway. In addition, upregulation of p21 and cyclin E, and downregulation of CDK2 expressions suggest that cell cycle arrest occurs in MCF-7 cells upon NC leaves' ethanol extract treatment. Through this finding, the upregulation of cytochrome c may be associated with the upregulation of p21, which ultimately leads to apoptosis induction. Other than that, upregulation of cyclin E alone may have caused cell cycle arrest, in which overexpression may reduce the proliferation of MCF-7 cells. The GCMS analysis revealed that D-pinitol, myo-inositol, hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), oleic acid, and phenol, 2,4-bis(1,1-dimethylethyl) compounds may have contributed to the aforementioned anticancer effects of the NC leaves ethanol extract. Nevertheless, other compounds that could not be identified in the GCMS library might have also played some roles in the

anticancer effects of the NC leaves ethanol extract. Therefore, further research is still required to identify other possible bioactive compounds possessing anticancer effects and evaluate all the possible bioactive anticancer candidates. Additionally, studies involving an *in vivo* model are still necessary to further analyze NC anticancer effects and the mechanism of action.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AK and RW conceptualized the idea and outlined the research project. SR and AK performed all preliminary tests, experiments, and wrote the research manuscript. ZZ and QA provided research ideas and laboratory facilities, and edited the research manuscript. All authors equally participated in the preparation of the final research manuscript and approved it for publication without any conflict of interest.

FUNDING

This research was funded by the Malaysian Ministry of Education, grant number FRGS 16-046-0545 (Granted to AK) and IIUM Publication Research Initiative Grant fund, grant number PRIGS18-027-0027 (Granted to AK).

ACKNOWLEDGMENTS

We truly acknowledge technical assistance and equipment by the Molecular Laboratory staff of Kulliyyah of Medicine, Central Research and Animal Facility Cream (CREAM), and the Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy in International Islamic University Malaysia (IIUM). Thanks to Airlangga University for the invitation in Adjunct Professorship Program 2019.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.741683/full#supplementary-material

REFERENCES

Adams, J. M., and Cory, S. (2007). Bcl-2-Regulated Apoptosis: Mechanism and Therapeutic Potential. *Curr. Opin. Immunol.* 19, 488–496. doi:10.1016/j.coi.2007.05.004

Ahmed, F., Rahman, S., Ahmed, N., Hossain, M., Biswas, A., Sarkar, S., et al. (2011). Evaluation of Neolamarckia Cadamba (Roxb.) Bosser Leaf Extract on Glucose Tolerance in Glucose-Induced Hyperglycemic Mice. *Afr. J. Tradit Complement. Altern. Med.* 8, 79–81. doi:10.4314/ajtcam.v8i1.60549

Artini, P. G., Di Berardino, O. M., Papini, F., Genazzani, A. D., Simi, G., Ruggiero, M., et al. (2013). Endocrine and Clinical Effects of Myo-Inositol Administration in Polycystic Ovary Syndrome. A Randomized Study. *Gynecol. Endocrinol.* 29, 375–379. doi:10.3109/09513590.2012.743020

Azizah, A. M., Nor Saleha, I. T., Noor Hashimah, A., Asmah, Z. A., and Mastulu, W. (2016). *Malaysian National Cancer Registry Report 2007-2011: Malaysia Cancer Statistic, Data and Figure*. Malaysia: Malaysia Natl. Cancer Inst.

Barhoi, D., Upadhaya, P., Barbhuiya, S. N., Giri, A., and Giri, S. (2021). Aqueous Extract of Moringa Oleifera Exhibit Potential Anticancer Activity and Can Be Used as a Possible Cancer Therapeutic Agent: A Study Involving *In Vitro* and *In Vivo* Approach. *J. Am. Coll. Nutr.* 40, 70–85. doi:10.1080/07315724.2020.1735572

Blumen, H., Fitch, K., and Polkus, V. (2016). Comparison of Treatment Costs for Breast Cancer, by Tumor Stage and Type of Service. *Am. Health Drug Benefits* 9, 23–32.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* 68, 394–424. doi:10.3322/caac.21492

Chandel, M., Sharma, U., Kumar, N., Singh, B., and Kaur, S. (2012). Antioxidant Activity and Identification of Bioactive Compounds from Leaves of Anthocephalus Cadamba by Ultra-performance Liquid Chromatography/electrospray Ionization Quadrupole Time of Flight Mass Spectrometry. *Asian Pac. J. Trop. Med.* 5, 977–985. doi:10.1016/S1995-7645(12)60186-2

Choi, E. J., and Kim, G. H. (2008). Daidzein Causes Cell Cycle Arrest at the G1 and G2/M Phases in Human Breast Cancer MCF-7 and MDA-MB-453 Cells. *Phytomedicine* 15, 683–690. doi:10.1016/j.phymed.2008.04.006

Dolai, N., Karmakar, I., Suresh Kumar, R. B., Kar, B., Bala, A., and Haldar, P. K. (2012). Evaluation of Antitumor Activity and *In Vivo* Antioxidant Status of *Anthocephalus Cadamba* on Ehrlich Ascites Carcinoma Treated Mice. *J. Ethnopharmacol.* 142, 865–870. doi:10.1016/j.jep.2012.05.050

Dubey, A., Nayak, S., and Goupale, D. C. (2011). A Review on Phytochemical, Pharmacological and Toxicological Studies on Neolamarckia Cadamba. *Der. Pharm. Let.* 3, 45–54.

Duclos, C., Lavoie, C., and Denault, J. B. (2017). Caspases Rule the Intracellular Trafficking Cartel. *FEBS J.* 284, 1394–1420. doi:10.1111/febs.14071

Evans, L. M., Cowey, S. L., Siegal, G. P., and Hardy, R. W. (2009). Stearate Preferentially Induces Apoptosis in Human Breast Cancer Cells. *Nutr. Cancer* 61, 746–753. doi:10.1080/01635580902825597

Fan, W., Sun, L., Zhou, J. Q., Zhang, C., Qin, S., Tang, Y., et al. (2015). Marsdenia Tenacissima Extract Induces G0/G1 Cell Cycle Arrest in Human Esophageal Carcinoma Cells by Inhibiting Mitogen-Activated Protein Kinase (Mapk) Signaling Pathway. *Chin. J. Nat. Med.* 13, 428–437. doi:10.1016/S1875-5364(15)30036-4

Gogada, R., Amadori, M., Zhang, H., Jones, A., Verone, A., Pitarresi, J., et al. (2011). Curcumin Induces Apaf-1-dependent, P21-Mediated Caspase Activation and Apoptosis. *Cell Cycle* 10, 4128–4137. doi:10.4161/cc.10.23.18292

Hanahan, D., and Weinberg, R. A. (2000). The Hallmarks of Cancer. *Cell* 100, 57–70. doi:10.1016/s0092-8674(00)81683-9

Harbeck, N., Penault-Llorca, F., Cortes, J., Gnant, M., Houssami, N., Poortmans, P., et al. (2019). Breast Cancer. *Nat. Rev. Dis. Primers* 5, 66. doi:10.1038/s41572-019-0111-2

Hassan, M., Watari, H., AbuAlmaaty, A., Ohba, Y., and Sakuragi, N. (20142014). Apoptosis and Molecular Targeting Therapy in Cancer. *Biomed. Res. Int.* 2014, 150845. doi:10.1155/2014/150845

Hsieh, T. C., Wijeratne, E. K., Liang, J. Y., Gunatilaka, A. L., and Wu, J. M. (2005). Differential Control of Growth, Cell Cycle Progression, and Expression of NF-kappaB in Human Breast Cancer Cells MCF-7, MCF-10A, and MDA-MB-231 by Ponicidin and Oridonin, Diterpenoids from the Chinese Herb Rabdosia Rubescens. *Biochem. Biophys. Res. Commun.* 337, 224–231. doi:10.1016/j.bbrc.2005.09.040

Ilhan, S. (2020). Essential Oils from Vitex Agnus Castus L. Leaves Induces Caspase-dependent Apoptosis of Human Multidrug-Resistant Lung Carcinoma Cells through Intrinsic and Extrinsic Pathways. *Nutr. Cancer* 73, 1–9. doi:10.1080/01635581.2020.1823439

Jamali, T., Kavoosi, G., Safavi, M., and Ardestani, S. K. (2018). *In-vitro* Evaluation of Apoptotic Effect of OEO and Thymol in 2D and 3D Cell Cultures and the Study of Their Interaction Mode with DNA. *Sci. Rep.* 8, 15787. doi:10.1038/s41598-018-34055-w

Javadi, N., Abas, F., Mediani, A., Abd Hamid, A., Khatib, A., Simoh, S., et al. (2015). Effect of Storage Time on Metabolite Profile and Alpha-Glucosidase Inhibitory Activity of Cosmos Caudatus Leaves - GCMS Based Metabolomics Approach. *J. Food Drug Anal.* 23, 433–441. doi:10.1016/j.jfda.2015.01.005

Karade, P. G., and Jadhav, N. R. (2018). *In Vitro* Studies of the Anticancer Action of Tectaria Cicutaria in Human Cancer Cell Lines: G0/G1 P53-Associated Cell Cycle Arrest-Part I. *J. Tradit. Complement. Med.* 8, 459–464. doi:10.1016/j.jtcme.2017.07.003

Karimian, A., Ahmadi, Y., and Yousefi, B. (2016). Multiple Functions of P21 in Cell Cycle, Apoptosis and Transcriptional Regulation after DNA Damage. *DNA Repair (Amst)* 42, 63–71. doi:10.1016/j.dnarep.2016.04.008

Kaur, S., and Kumar, S. (2011). Studies on the Genoprotective/Antioxidant Potential of Methanol Extract of Anthocephalus Cadamba (Roxb.). *Miq. J. Med. Plants Res.* 5, 4764–4770. doi:10.5897/JMPR.9001158

Khairi, M., Shamsuddin, S., and Jaafar, H. (2014). Effects of Rapamycin on Cell Apoptosis in MCF-7 Human Breast Cancer Cells. *Asian Pac. J. Cancer Prev.* 15, 10659–10663. doi:10.7314/apjcp.2014.15.24.10659

Kilbride, S. M., and Prehn, J. H. (2013). Central Roles of Apoptotic Proteins in Mitochondrial Function. *Oncogene* 32, 2703–2711. doi:10.1038/onc.2012.348

Kreis, N. N., Louwen, F., and Yuan, J. (2019). The Multifaceted P21 (Cip1/Waf1/CDKN1A) in Cell Differentiation, Migration and Cancer Therapy. *Cancers (Basel)* 11, 1220. doi:10.3390/cancers11091220

Lin, L., Yan, L., Liu, Y., Yuan, F., Li, H., and Ni, J. (2019). Incidence and Death in 29 Cancer Groups in 2017 and Trend Analysis from 1990 to 2017 from the Global Burden of Disease Study. *J. Hematol. Oncol.* 12, 96. doi:10.1186/s13045-019-0783-9

Lin, T. H., Tan, T. W., Tsai, T. H., Chen, C. C., Hsieh, T. F., Lee, S. S., et al. (2013). D-pinitol Inhibits Prostate Cancer Metastasis through Inhibition of αVβ3 Integrin by Modulating FAK, C-Src and NF-Kb Pathways. *Int. J. Mol. Sci.* 14, 9790–9802. doi:10.3390/ijms14059790

Liu, J., Liu, Y., Wang, Y., Abozeid, A., Zu, Y. G., and Tang, Z. H. (2017). The Integration of GC-MS and LC-MS to Assay the Metabolomics Profiling in Panax Ginseng and Panax Quinquefolius Reveals a Tissue- and Species-specific Connectivity of Primary Metabolites and Ginsenosides Accumulation. *J. Pharm. Biomed. Anal.* 135, 176–185. doi:10.1016/j.jpba.2016.12.026

Liu, M., Chen, Y. L., Kuo, Y. H., Lu, M. K., and Liao, C. C. (2018). Aqueous Extract of Sapindus *Mukorossi* Induced Cell Death of A549 Cells and Exhibited Antitumor Property *In Vivo*. *Sci. Rep.* 8, 4831. doi:10.1038/s41598-018-23096-w

Looi, C. Y., Arya, A., Cheah, F. K., Muharram, B., Leong, K. H., Mohamad, K., et al. (2013). Induction of Apoptosis in Human Breast Cancer Cells via Caspase Pathway by Vernodalin Isolated from *Centratherum Anthelminticum* (L.) Seeds. *PloS one* 8, e56643. doi:10.1371/journal.pone.0056643

Lukong, K. E. (2017). Understanding Breast Cancer - the Long and Winding Road. *BBA Clin.* 7, 64–77. doi:10.1016/j.bbacli.2017.01.001

Mericli, F., Becer, E., Kabadayı, H., Hanoglu, A., Yigit Hanoglu, D., Ozkum Yavuz, D., et al. (2017). Fatty Acid Composition and Anticancer Activity in Colon Carcinoma Cell Lines of *Prunus Dulcis* Seed Oil. *Pharm. Biol.* 55, 1239–1248. doi:10.1080/13880209.2017.1296003

Mondal, S., Dash, G. K., and Acharyya, S. (2009). Analgesic, Anti-inflammatory and Antipyretic Studies of *Neolamarckia Cadamba* Barks. *J. Pharm. Res.* 2, 1133–1136.

Newman, D. J., and Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* 79, 629–661. doi:10.1021/acs.jnatprod.5b01055

Olsson, M., and Zhivotovsky, B. (2011). Caspases and Cancer. *Cell Death Differ* 18, 1441–1449. doi:10.1038/cdd.2011.30

Orabi, K. Y., Abaza, M. S., Luqmani, Y. A., and Al-Attiyah, R. (2021). Psiadin and Plectranthone Selectively Inhibit Colorectal Carcinoma Cells Proliferation via Modulating Cyclins Signaling and Apoptotic Pathways. *PLoS ONE* 16, e0252820. doi:10.1371/journal.pone.0252820

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., et al. (2012). Programmed Cell Death Pathways in Cancer: A Review of Apoptosis, Autophagy and Programmed Necrosis. *Cell Prolif* 45, 487–498. doi:10.1111/j.1365-2184.2012.00845.x

Pandey, A., and Negi, P. S. (2016). Traditional Uses, Phytochemistry and Pharmacological Properties of Neolamarckia Cadamba: A Review. *J. Ethnopharmacol.* 181, 118–135. doi:10.1016/j.jep.2016.01.036

Poon, I. K., Hulett, M. D., and Parish, C. R. (2010). Molecular Mechanisms of Late Apoptotic/Necrotic Cell Clearance. *Cel Death Differ* 17, 381–397. doi:10.1038/cdd.2009.195

Rafshanjani, M., Shuaib, A., Parvin, S., and Kader, M. (2014). Antimicrobial and Preliminary Cytotoxic Effects of Ethanol Extract and its Fractions of *Anthocephalus Cadamba* (Roxb.) Miqstem Bark. *Int. J. Pharm. Life Sci.* 5, 4038–4044. doi:10.7897/2230-8407.0511175

Ravi, L., and Krishnan, K. (2017). Cytotoxic Potential of N-Hexadecanoic Acid Extracted from Kigelia Pinnata Leaves. *Asian J. Cel Biol.* 12, 20–27. doi:10.3923/ajcb.2017.20.27

Rayan, A., Raiyn, J., and Falah, M. (2017). Nature Is the Best Source of Anticancer Drugs: Indexing Natural Products for Their Anticancer Bioactivity. *PLoS One* 12, e0187925. doi:10.1371/journal.pone.0187925

Rengarajan, T., Nandakumar, N., Rajendran, P., Haribabu, L., Nishigaki, I., and Balasubramanian, M. P. (2014). D-pinitol Promotes Apoptosis in MCF-7 Cells via Induction of P53 and Bax and Inhibition of Bcl-2 and NF-Kb. *Asian Pac. J. Cancer Prev.* 15, 1757–1762. doi:10.7314/apjcp.2014.15.4.1757

Riedl, S., Rinner, B., Asslaber, M., Schaider, H., Walzer, S., Novak, A., et al. (20111808). In Search of a Novel Target - Phosphatidylserine Exposed by Non-apoptotic Tumor Cells and Metastases of Malignancies with Poor Treatment Efficacy. *Biochim. Biophys. Acta* 1808, 2638–2645. doi:10.1016/j.bbamem.2011.07.026

Sankaranarayanan, R., Ramadas, K., and Qiao, Y. L. (2014). Managing the Changing Burden of Cancer in Asia. *BMC Med.* 12, 3. doi:10.1186/1741-7015-12-3

Shah, U., Shah, R., Acharya, S., and Acharya, N. (2013). Novel Anticancer Agents from Plant Sources. *Chin. J. Nat. Medicines* 11, 16–23. doi:10.1016/s1875-5364(13)60002-3

Singh, S., Ishar, M. P. S., Saxena, A. K., and Kaur, A. (2013). Cytotoxic Effect of Anthocephalus Cadamba Miq. Leaves on Human Cancer Cell Lines. *Pharmacognosy J.* 5, 127–129. doi:10.1016/j.phcgj.2013.04.005

Strober, W. (2015). Trypan Blue Exclusion Test of Cell Viability. *Curr. Protoc. Immunol.* 111, A3. doi:10.1002/0471142735.ima03bs111

Sun, J., and Hai Liu, R. (2006). Cranberry Phytochemical Extracts Induce Cell Cycle Arrest and Apoptosis in Human MCF-7 Breast Cancer Cells. *Cancer Lett.* 241, 124–134. doi:10.1016/j.canlet.2005.10.027

Thun, M. J., DeLancey, J. O., Center, M. M., Jemal, A., and Ward, E. M. (2010). The Global Burden of Cancer: Priorities for Prevention. *Carcinogenesis* 31, 100–110. doi:10.1093/carcin/bgp263

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Tieulent, J. L., and Jemal, A. (2015). Global Cancer Statistics, 2012. *CA Cancer J. Clin.* 65, 87–108. doi:10.3322/caac.21262

Trogdon, J. G., Ekwueme, D. U., Poehler, D., Thomas, C. C., Hayes, K. R., and Allaire, B. T. (2017). Medical Costs of Treating Breast Cancer Among Younger Medicaid Beneficiaries by Stage at Diagnosis. *Breast Cancer Res. Treat.* 166, 207–215. doi:10.1007/s10549-017-4386-2

Vermeulen, K., Van Bockstaele, D. R., and Berneman, Z. N. (2003). The Cell Cycle: A Review of Regulation, Deregulation and Therapeutic Targets in Cancer. *Cel Prolif* 36, 131–149. doi:10.1046/j.1365-2184.2003.00266.x

Wen, Y., Chen, Z., Lu, J., Ables, E., Scemama, J. L., Yang, L. V., et al. (2017). Quantitative Analysis and Comparison of 3D Morphology between Viable and Apoptotic MCF-7 Breast Cancer Cells and Characterization of Nuclear Fragmentation. *PLoS One* 12, e0184726. doi:10.1371/journal.pone.0184726

Westphal, D., Dewson, G., Czabotar, P. E., and Kluck, R. M. (2011). Molecular Biology of Bax and Bak Activation and Action. *Biochim. Biophys. Acta* 1813, 521–531. doi:10.1016/j.bbamcr.2010.12.019

Xavier, A. P., Santharam, L., Panigrahi, S., Muthuraman, M. S., and Pemiah, B. (2013). Antitumor Potential of Ethanolic Extract of *Solanum Trilobatum* against Ehrlich's Ascites Carcinoma. *Int. J. Pharm. Tech. Res.* 5, 1119–1125.

Xiong, J., Fan, F., Zhang, Y., Chen, W., and Mao, W. (2016). Epirubicin Inhibits Proliferation of Breast Cancer Cells through Upregulating P21 (Cip1) Expression. *Int. J. Clin. Exp. Med.* 9, 22764–22772.

Zayed, M. Z., Ahmad, F. B., Ho, W. S., and Pang, S. L. (2014). GC-MS Analysis of Phytochemical Constituents in Leaf Extracts of *Neolamarckia Cadamba* (Rubiaceae) from Malaysia. *Int. J. Pharm. Pharm. Sci.* 6, 123–127.

Zhou, M., Li, Y., Hu, Q., Bai, X. C., Huang, W., Yan, C., et al. (2015). Atomic Structure of the Apoptosome: Mechanism of Cytochrome C- and dATP-Mediated Activation of Apaf-1. *Genes Dev.* 29, 2349–2361. doi:10.1101/gad.272278.115

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Reviewer MA declared a past coauthorship with one of the author ZZ to the handling editor.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 21 October 2021
doi: 10.3389/fphar.2021.740302

Pharmacology of Natural Volatiles and Essential Oils in Food, Therapy, and Disease Prophylaxis

Nicholas John Sadgrove[1]*, *Guillermo Federico Padilla-González*[1], *Olga Leuner*[2], *Ingrid Melnikovova*[2] and *Eloy Fernandez-Cusimamani*[2]*

[1]Jodrell Science Laboratory, Royal Botanic Gardens, Kew, Richmond, United Kingdom, [2]Department of Crop Sciences and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, Prague, Czech Republic

This commentary critically examines the modern paradigm of natural volatiles in 'medical aromatherapy', first by explaining the semantics of natural volatiles in health, then by addressing chemophenetic challenges to authenticity or reproducibility, and finally by elaborating on pharmacokinetic and pharmacodynamic processes in food, therapy, and disease prophylaxis. Research over the last 50 years has generated substantial knowledge of the chemical diversity of volatiles, and their strengths and weaknesses as antimicrobial agents. However, due to modest *in vitro* outcomes, the emphasis has shifted toward the ability to synergise or potentiate non-volatile natural or pharmaceutical drugs, and to modulate gene expression by binding to the lipophilic domain of mammalian cell receptors. Because essential oils and natural volatiles are small and lipophilic, they demonstrate high skin penetrating abilities when suitably encapsulated, or if derived from a dietary item they bioaccumulate in fatty tissues in the body. In the skin or body, they may synergise or drive *de novo* therapeutic outcomes that range from anti-inflammatory effects through to insulin sensitisation, dermal rejuvenation, keratinocyte migration, upregulation of hair follicle bulb stem cells or complementation of anti-cancer therapies. Taking all this into consideration, volatile organic compounds should be examined as candidates for prophylaxis of cardiovascular disease. Considering the modern understanding of biology, the science of natural volatiles may need to be revisited in the context of health and nutrition.

Keywords: pharmacokinetics, pharmacodynamics, pathogen, antimicrobial, anti-inflammatory, gas chromatography, headspace, aromatherapy

OPEN ACCESS

Edited by:
Michael Heinrich,
UCL School of Pharmacy,
United Kingdom

Reviewed by:
Andre Luis Dias Araujo Mazzari,
University College Cork, Ireland
Namrita Lall,
University of Pretoria, South Africa
Shelini Surendran,
University of Surrey, United Kingdom

***Correspondence:**
Nicholas John Sadgrove
n.sadgrove@kew.org
Eloy Fernandez-Cusimamani
eloy@ftz.czu.cz

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *12 July 2021*
Accepted: *04 October 2021*
Published: *21 October 2021*

Citation:
Sadgrove NJ, Padilla-González GF, Leuner O, Melnikovova I and Fernandez-Cusimamani E (2021) Pharmacology of Natural Volatiles and Essential Oils in Food, Therapy, and Disease Prophylaxis. Front. Pharmacol. 12:740302. doi: 10.3389/fphar.2021.740302

INTRODUCTION: MEDICAL AROMATHERAPY

The modern culture of aesthetic aromas and volatile organic compounds in human health is polarised by controversiality, with the prevailing criticism being the lack of scientific credibility. Yet there is ample scientific evidence of modest to noteworthy biological effects from aromatic plant-based cosmetics, nutraceuticals, and therapies. Unfortunately, the subjectivity in the aromatherapeutic industry and the credulity of participants has attracted much negative attention.

Consequently, the Cambridge English Dictionary defines aromatherapy as . . .

'the treatment of worry or nervousness, or medical conditions that are not serious, by rubbing pleasant-smelling natural substances into the skin or breathing in their smell'

As exemplified above, the prevailing view in western societies is that aromatherapy is limited to either massaging with essential oils or the inhalation of plant-derived volatile organic compounds to achieve mood altering effects. However, a search of clinicaltrials.gov provides an alternative overview of the applications of essential oils: out of hundreds of studies less than a quarter were dedicated to mood altering effects, i.e., a search of 'essential oils' gave several results related to mouth washes, throat gargles, pain management, antiseptic applications, facial acne and muscle cramps.

Definitions of aromatherapy, as explained in dictionaries, encyclopaedias or portrayed in memes, do not acknowledge the diversity of techniques that are corroborated by empirical science. For example, the essential oil of *Cordia verbenacea* A. DC administered to rats, systemically or orally, confers noteworthy anti-inflammatory effects (Medeiros et al., 2007). It is marketed in Brazil as an active ingredient in the product Acheflan which is applied topically. The positive effects of Acheflan are achieved *via* the aroma molecules, *E*-caryophyllene and α-humulene (Fernandes et al., 2007).

The problem of correctly defining aromatherapy is also strained by a lack of cultural inclusiveness in the current definition (Sadgrove N. J., 2020). Under the umbrella of aromatherapy, aromatic extracts have been used in indigenous African cultures to alleviate foot odours (Hulley et al., 2019) and in steam/smoke inhalation therapies (Khumalo et al., 2019). Similarly, in Australian Aboriginal cultures aromatic plants are used successfully to treat fungal infections in the form of fat extracts (Sadgrove et al., 2011; Sadgrove and Jones, 2014b) and in smoke fumigation applications (Sadgrove and Jones, 2013; Sadgrove et al., 2014).

Research on volatile organic compounds is starting to convey that potentiation of other products is occurring more often than realised, such as in antimicrobial outcomes (Mikulášová et al., 2016) or other mainstream medicines. Immunomodulatory effects are also being observed in relation to cytokine release (Anastasiou and Buchbauer, 2017), T-cell proliferation (Anastasiou and Buchbauer, 2017), agonism of membrane receptors (toll-like (Amiresmaeili et al., 2018)) or nuclear receptors (PPAR (Goto et al., 2010)) and reduced mast cell degranulation (Anastasiou and Buchbauer, 2017).

With growing scientific validation there is a widening polarization of the schools of aromatherapeutic practice, with one side aligned to the supernatural, and the other on the more traditional medical ethos. However, a distinction clearly needs to be made. The author Kurt Schnaubelt successfully made this distinction by the use of the elaborated term 'medical aromatherapy' (Schnaubelt, 1999) to imply a more objective approach to therapy with essential oils and natural volatiles.

Thus, 'medical aromatherapy' can be defined as . . .

'the objective of achieving a health benefit from topical application, oral administration, or inhalation of a natural product mixture that includes at least one "active" or "coactive" volatile organic compound

In this definition it is explained that medical aromatherapy can also be achieved by using raw aromatic plants, as crushed leaves or extracts, to achieve therapeutic effects (Sadgrove, 2020b), as an alternative to hydrodistilled essential oils. Although the two are not mutually exclusive, medical aromatherapy practitioners are not restricted to the use of essential oils because volatile organic compounds are also present in aromatic extracts, such as supercritical CO_2 extracts of aromatic leaves (Damjanovic et al., 2006; Wenqiang et al., 2007), or aromatic fat extracts familiar to the French practice of enfleurage. In this scenario the volatile compounds are not necessarily the sole driver of efficacy because plant organs and extracts contain other families of metabolites, i.e., the chemical diversity of whole aromatic plants includes volatile and non-volatile ingredients that may achieve combined effects (potentiation, synergism, or additive) in the context of medical aromatherapy (Langat et al., 2021; Nsangou et al., 2021). In this latter hypothetical, volatile organic compounds are 'coactive', meaning that they contribute to efficacy but are not the only driver of efficacy.

Aromatic plants are rich in volatile organic compounds that can be distilled to produce essential oils, but it is not correct to call them essential oils prior to separation by distillation, i.e., aromatic plants do not contain essential oils, in the same way that milk does not contain cheese or wheat does not contain bread. According to the modern definition of an essential oil provided by the International Standards Organisation, a single organic compound, such as limonene, is not an essential oil, it is a volatile organic compound that is a common ingredient in an essential oil: it is an 'essential oil component', to use the expression coined by Adams (2007) to make this important distinction. Essential oils are mixtures of volatile organic compounds that have been separated by distillation from aromatic species, including bryophytes, such as liverworts (Asakawa and Ludwiczuk, 2013), and higher plants.

THE CURRENT RESEARCH PARADIGM

In recent decades, contingent with the increasing convenience of chemical analysis of volatiles, there has been an unprecedented number of publications reporting the chemistry of essential oils from the world's flora. This large body of data was born from the collective of laboratories equipped with the universal mass spectral detector at the end of a gas chromatography column (GC-MS) (Sadgrove NJ., 2020; Sadgrove N. J. et al., 2020). However, world experts in the field of natural volatiles and essential oils are now unanimously encouraging a shift of emphasis away from mere chemical reports.

For some time the superfluity of chemical reports, and competitiveness in publishing, were overcome by including results of *in vitro* antibacterial testing, to add value to the dataset (Guimarães et al., 2019). These *in vitro* studies use

micro-titre plate broth-dilution assays of whole essential oils to derive minimum inhibition concentration (MIC) values against pathogenic or model bacteria (Sadgrove and Jones, 2015; Van Vuuren and Holl, 2017). While such information is valuable, a pattern has emerged that makes further work predictable, and generally the MIC values are not regarded as pharmacologically interesting from a commercial perspective (Sadgrove and Jones, 2019). Furthermore, these methods omit the antimicrobial activity of volatile organic compounds that are in the vapour phase, which is more inhibitory compared to the liquid phase. The methods for determination of the antibacterial effect of volatile agents that are simultaneously in the liquid and vapor phase were developed recently (Houdkova et al., 2017; Houdkova et al., 2021). Nevertheless, with no consideration to the vapour phases of volatile organic compounds, high concentrations ($0.1–20\ mg.mL^{-1}$) are required to achieve antibacterial effects of essential oils. If some bactericidal essential oil components were to be present in human plasma at concentrations high enough to have antibacterial effects, they will enact toxic and potentially lethal effects to the person. Hence, antibacterial effects of essential oils are best represented in different contexts, in topical applications to treat odours and fungal infections, in sterilizing skin or surfaces, laundry and so forth (Jones and Sadgrove, 2015; Van Vuuren and Holl, 2017). Furthermore, volatile organic compounds present in edible aromatic species can influence the gut microbiota and attenuate fermentation or bacterial overgrowth in gastrointestinal pathologies (Li et al., 2018). High gastrointestinal concentrations will not lead to high systemic concentrations because metabolism of volatile organic compounds is generally efficient. Because *in vitro* MIC values for contact inhibition are not possible, it is better to consider the apparent immunomodulatory and gene regulatory effects (Sadgrove and Jones, 2019).

Unfortunately, complementary studies that translate phytochemical knowledge into pharmacological serendipity for wider human benefit are not being pursued outside of just a few laboratories. Pioneering new ways to appreciate essential oils and volatiles require pushing the boundaries of encapsulation methodology, extraction technology, food preservation, knowledge of synergistic activity or potentiation in the plight of resistance mechanisms in pathogenic bacteria and their effects in human physiology. Thus, since the dawn of the 21st century, particularly in the last few years, numerous research groups have shifted emphasis towards utilising the phytochemical information that has been amassed hitherto.

A BRIEF HISTORY OF ESSENTIAL OILS

Even without the integrated efforts of scientists, human interest in volatile organic compounds and distilled essential oils will continue to be independently motivated. This can be partly explained by the aesthetic experience in aroma, which reinvigorates the cultural and symbolic significance. Humans have utilised the aromatic principle of plants since before recorded history and contingent with the development of modern-type hydrodistillation technology essential oils *per se* were 'invented' in the 1200 AD by Arabic pioneers (Bauer and Garbe, 1985; Sadgrove and Jones, 2015). However, long before modern hydrodistillation methods low quality essential oils were captured using a primitive apparatus invented by the Persians, that was better for making floral waters. Remains of the Persian terracotta distillation apparatus are estimated to be approx. 3500 B.C. Something similar was also used by the ancient Egyptians, who packed the outlet pipe with rags to create a type of condenser that captured floral waters and some of the essential oil, which could be collected from the rags by compression.

The sophistication of modern technology means that essential oils can be produced in mass by hydrodistillation (plant material in boiling water), steam distillation (plant material placed in path of steam) and microwave assisted steam distillation. In this regard, the modern definition of an essential oil as dictated by the International Standards Organisation is a . . .

> "product obtained from natural raw material, either by distillation with water and steam, or from the epicarp of citrus fruits by mechanical processing, or by dry distillation" (Schnaubelt, 1999; ISO, 2015).

The etymological background of the term 'essential oil' is in the Latin expression '*quinta essentia*' which literally means 5th element. The essential oil was referred to as the soul or spirit of the plant, which is strongly related to the etymology of the term 'spirits' to mean alcohol or liqueur (Sell, 2010). Essential oils should never be referred to as 'extracts' or 'extracted', because they can only be produced by evaporation; essential oils are actually 'exorcized' from the plant, not extracted, which would require the use of solvents or mechanical pressing. The only exception to this is from the epicarp of citrus fruits, but this is due to the inertia of pre-established rural language.

Sometimes a single aromatic plant species can be divided according to distinct chemical groups called chemotypes. In rare cases, one species can be divided into as many as 10 or more chemotypes that have completely different chemical profiles (Sadgrove and Jones, 2014a). While chemotypes tend to be highly consistent in terms of chemistry (i.e., borneol type always has borneol), the chemistry can also change in response to environmental factors and seasonal variation, causing an effect called phenotypic plasticity (Sadgrove NJ., 2020). Phenotypic plasticity can create chemotypes within species, or it can occur on a spectrum, which involves many entities with chemistry that overlap between chemotypes. In the Australian flora, volatile compounds can appear or disappear from the chemical profile in response to wet and dry cycles of weather (Sadgrove NJ., 2020) or other factors.

CHEMOPHENETICS OF ESSENTIAL OILS AND SOLVENT EXTRACTS

It is often the case that the chemical profiles within species are 'flamboyant' (Sadgrove NJ., 2020; Sadgrove NJ. et al., 2020), i.e., highly variable, which can be caused by abiotic stressors

that change expression patterns of volatiles, known as 'phenoplasticity', as mentioned above. In these cases, a known plant species is not guaranteed to deliver the same essential oil chemical profile. It is therefore important to be cognisant of chemical variation that could be caused by chemotypes or environmental factors, particularly in the context of health claims for the essential oil components. For example, two chemotypes of oregano are known, the thymol type and the carvacrol type (Bedini et al., 2021). Hence, it is important to be aware of these differences if used in therapeutic or prebiotic applications.

The discipline that examines the potential chemical differences within species is formally known as 'chemophenetics'. This subject title is used today as a replacement for the old term 'chemotaxonomy' (Zidorn, 2019). The new name was necessary to avoid criticism because in classic chemotaxonomy it was imagined that chemical profiles could be used to fingerprint taxa with high reproducibility, but phenoplasticity and the existence of chemotypes within taxa antagonised reproducibility.

In the last 5 years chemophenetic research of volatile organic compounds has started to utilise solvent extracts, rather than hydrodistilled essential oils. This is both convenient and creates more detailed information. While hydrodistillation requires masses of leaves, energy input, time, and effort to produce essential oils, solvent extraction requires a small leaf and a small volume of solvent (DCM, Hexane). This method was used in a chemophenetic study of heterogeneous species aggregates in *Eucalyptus* (Collins et al., 2018), *Phebalium nottii* (Sadgrove N. J. et al., 2020) and *Eremophila* (Sadgrove et al., 2021), and in the former two the leaf samples were taken from herbarium voucher specimens. In the case of *Eucalyptus*, the sesquiterpene diol cryptomeridiol does not survive hydrodistillation and eliminates a hydroxyl group to randomly produce three eudesmols, either alpha (α-), beta (β-), or gamma (γ-). By using solvent extraction instead, cryptomeridiol is detected (Collins et al., 2018). In the pink flowered *Phebalium nottii* complex, putative new species were often in significant agreement with semi-volatile coumarins that have vapour pressures too low to be produced in hydrodistillation. The semi-volatile coumarins were easily detected by GC-MS if the column temperature was raised to 280–300°C and held for 20 min (Sadgrove N. J. et al., 2020). Species in *Eremophila* also express semi-volatiles that may have significance in taxonomic studies because of a reduced susceptibility to the effects of phenoplasticity (Sadgrove et al., 2021). In this latter study it was realized that the effects of phenoplasticity from contemporary weather changes, such as droughts or excessive wet periods, are more dramatic in leaf material than in timber. It was suggested that chemophenetic studies may yield more reproducible data if the timber volatiles are studied, rather than leaves.

FACTORS AFFECTING ESSENTIAL OIL COLLECTION

The amount of an essential oil in a species, as determined by the 'yield' from hydrodistillation, can vary considerably. The phenylpropanoid dominated essential oil from clove can yield as high as 7.4–11.5% $g.g^{-1}$ from dried cloves or 1.2% if fresh (Wenqiang et al., 2007; Murni et al., 2016). This contrasts with the oleo-resin made by supercritical CO_2 extraction which yields 15–20% $g.g^{-1}$ from dried clove buds, but this higher yield is related to the presence of non-volatile substances such as cuticular waxes (Wenqiang et al., 2007). Some Australian species also demonstrate very high yields, such as the monoterpene-rich isomenthone, or karahanenone diploid chemotypes of *Eremophila longifolia* (Smith et al., 2010; Sadgrove et al., 2011; Sadgrove and Jones, 2014a) yielding from 2–10% $g.g^{-1}$ of wet leaves, which varies yearly according to drought effects. A similar phenylpropanoid-rich safrole/methyl eugenol chemotype is known from the country's far west. Another high yielding genus includes the sesquiterpene-rich heterogeneous species aggregates of *Prostanthera* sp. aff. *ovalifolia* and *P.* sp. aff. *rotundifolia* (Sadgrove et al., 2015), which invariably give 1–2% $g.g^{-1}$ from fresh leaves.

Low yields can make it difficult to produce an essential oil, which makes them very expensive in the market, such as rose essential oil from *Rosa damascena* Mill., which yields only 0.03% $g.g^{-1}$ from fresh rose petals after steam or hydrodistillation (Dobreva et al., 2011). In this regard, careful temperature and hydrosol modulation is required to ensure successful condensation and collection, respectively. Gases need to be sufficiently cooled by the condenser to adequately capture the volatiles, returning them to the liquid phase. They are then pooled in a chamber that is less than 30°C, to prevent re-evaporation. The water phase, called the hydrosol, needs to be minimised because part of the oil is dissolved there. Although volatile organic compounds are only slightly aqueously soluble, high volumes of hydrosol and small quantities of essential oil can make the difference between successful collection and failure. Some distillers use cohobation, which is the process of returning the hydrosol to the boiler to ensure recovery of dissolved components. An even better design is the Clevenger apparatus, a near century old design (Clevenger, 1928) that captures only a small amount of hydrosol and returns the rest to the boiler in real time.

Acids are generally not volatile enough to be evaporated in hydrodistillation unless they are extremely small, but their small size means they are mostly dissolved into the hydrosol and phase separated from the oil, such as in the case of the boswellic acids of *Boswellia serrata* (Raman and Gaikar, 2003). Esterification of acids makes them more volatile. Hence, esters of acids are detected in essential oils, such as the C19-norditerpene 'gratissihalimanoic ester' from *Croton gratissimus* (Sadgrove et al., 2019). As previously mentioned, diterpene dominated essential oils are uncommon. It is also rare to find benzoic acid derivatives, such as *p*-methoxycinnamate identified in the essential oil of *Eriostemon obovalis* (now *Philotheca obovalis*) by the late Erich Lassak in 1974 (Lassak and Southwell, 1974).

ESSENTIAL OILS IN MEDICAL AROMATHERAPY

The European Pharmacopoeia lists 28 essential oils, defining them as safe (Pauli and Schilcher, 2010). Unfortunately there

are also many essential oils that have potential in human health but are rejected on the basis of poorly performed safety assays, such as thujone-rich oils (Németh and Nguyen, 2020). Essential oils and their components are pharmacologically versatile. As previously mentioned, they are lipophilic, which enables them to absorb into and interact with prokaryotic and eukaryotic cell membranes. They also affect neuronal and muscle ion channels, neurotransmitter receptors, G-protein coupled (odorant) receptors, second messenger systems and enzymes (Bowles, 2003; Buchbauer, 2010).

Pharmacokinetics of Volatile Organic Compounds

For any organic compound to be volatile it must have low molar mass and low polarity. Low polarity is also expressed as lipophilicity (fat solubility). As a progression, volatile organic compounds are dissolved into and transverse human skin layers (Cal, 2006), due to the phospholipid membranes of squamous cells and the phospholipid bilayer of the extracellular matrix. Lipophilic compounds with moderate polar head space, such as by having a keto or hydroxyl group, travel through the dermis faster than carbures (hydrocarbons), however even α-pinene can follow the transdermal route, albeit fluxing at a slower rate than components such as linalool or terpinen-4-ol (Cal, 2006). Nevertheless, because essential oil components are penetration enhancers of other drugs (Okabe et al., 1990; Chen et al., 2016), it is feasible that they are also enhancers of other components in an essential oil, meaning that carbures in combination with moderately polar components (i.e., terpinene-4-ol or linalool) will have more efficient transdermal penetration. Unfortunately, not much is known about the differences of absorption with whole essential oils compared to individual components.

Hence, topical application of essential oil components and transdermal penetration is more efficient than expected by non-specialists, but an encapsulation vehicle, such as a pressed oil (i.e., rosehip oil) is sometimes necessary to augment this effect, particularly to slow the rate of evaporation of the essential oil from the skin. For example, 97% of topical linalool was evaporated if applied with ethanol onto the skin (Green, 2007), but if mixed with a fixed oil 'carrier' most of it is absorbed (Jäger et al., 1992). Furthermore, East Indian Sandalwood essential oil (*Santalum album*) was topically applied onto candidates who wore a face mask to prevent inhalation of the aroma and resulted in statistically significant physiological changes, such as blood pressure, pulse rate and 'alertness' compared to the control (Hongratanaworakit et al., 2004). Inhaled essential oils can also become systemic and lead to changes in metabolic pathways associated with anxiety related behaviour, which has been demonstrated to occur in rats (Wu et al., 2012).

Topically applied, ingested or inhaled essential oils, or aromatic extracts, release components into the body that rapidly 'sink' into fat tissue, while some components are transported around the body in the vehicle of blood albumin. Compounds with keto groups (carbonyls) bind to blood albumin and are circulated throughout the body but are thereafter eliminated in metabolism or sunk into adipose tissue or the phospholipid membranes of some cells, like keratinocytes. Components that are *trans*-dermally absorbed (via lungs or skin) enter capillaries and the blood stream, where they are detected within 20 min and for as long as 90 min (Jäger et al., 1992) before sinking and/or eliminated in metabolism. Lipophilic compounds cross the blood brain barrier, and can create psychoactive effects, such as the phenylpropanoid elemicin (Beyer et al., 2006), the terpene incensole acetate (Moussaieff et al., 2008) or the phytocannabinoids (Griffin et al., 1999).

The transdermal route greatly slows the metabolism of compounds by avoidance of the 'first pass' effect that occurs in digestion of orally administered matter, where metabolites entering portal circulation from the intestines are circulated directly to the liver (Sadgrove and Jones, 2019). However, in some cases, the oral route to the absorption of volatile organic compounds is more convenient. For example, although D-limonene does not have a keto group, plasma levels reached as high as 1.65 μM with lemonade drinking and over the course of 4 weeks accumulated in adipose tissues to levels nearly 200 fold greater than maximum plasma concentration (Miller et al., 2010). Alternatively, via the oral route linalyl acetate is immediately converted into linalool in the digestive process (Nölder et al., 2011), and linalool concentrations peak in blood plasma at 1915 ng ml^{-1} (Shi et al., 2016). Thus, in the case of linalyl acetate, topical application is better, i.e., topical application of lavender essential oil to a human abdomen resulted in maximum plasma concentrations of >250 ng ml^{-1} essential oil, made up by 100 ng ml^{-1} linalool and 121 ng ml^{-1} linalyl acetate (Jäger et al., 1992). Linalool accumulates in organs and fat at concentrations many folds higher than peak plasma concentrations (Nölder et al., 2011). Similarly, in the 'Karoo' of South Africa a lamb that forages on *Pentzia incana* (Thunb.) Kuntze reputably acquires an artemisia flavour to its meat, known as the 'Karoo lamb', which is a consequence of volatile organic compounds accumulating in its adipose tissues (Hulley et al., 2018).

Essential oil components can also have prooxidant effects that are a negative consequence of higher than safe levels (Bakkali et al., 2008). This is of relevance to phenylpropanoids and other phenolics that demonstrate pronounced *in vitro* radical scavenging abilities. As previously mentioned, lipophilic compounds dissolve into the phospholipid walls of human cells. The concentration determines if a positive or negative effect occurs, wherein a wide concentration range for positive therapeutic effects is available. Volatile organic compounds increase the permeability of phospholipid membranes, not just in cell walls but also in the walls around organelles. Permeabilization of the mitochondrial membrane can potentially interfere with the electron transport chain, leading to the upregulation of radical oxygen species that oxidise cellular contents. If phenolic compounds are present, their oxidation will generate significantly more reactive species (Bakkali et al., 2008). However, studies that report on prooxidant effects are still describing concentrations that are high, such as 30–90 μg ml^{-1} (200–600 μM) of carvacrol (Liang and Lu, 2012). While such concentrations may seem unrealistic, they are frequently reported as peak plasma concentrations in mice studies. For example, a

pharmacokinetic study of borneol and menthol demonstrated peak plasma concentrations of 20 and 70 μg ml^{-1} respectively, which were metabolised in one and 3 hours respectively (Xu et al., 2011).

In considering pharmacokinetic studies wholistically, plasma concentrations peak instantly with intravenous administration, or after oral administration anywhere from 20 min to 3 h. The peak plasma concentrations are dependent upon dose, but *in vivo* rat models have demonstrated as high as 300 μg ml^{-1} in plasma, present mostly bound to plasma proteins (Dawidowics and Dybowski, 2014). Peak plasma concentrations are usually lower than tissue plasma concentrations, but tissue plasma concentrations are usually not measured in studies.

Essential oil components are either metabolised or absorbed into adipose tissues and organs, lowering plasma levels to baseline within 1–3 h for low doses, but at high doses plasma concentrations are maintained for several days as the components are buffered from the body's tissues. Hence, during metabolism they are slowly removed from adipose tissue and the organs, which is highest in fat, followed by the liver, then kidneys and lowest in cerebrospinal fluids and brain. After 24–72 h after a single dose the components are still present in adipose tissue, where they persist for some time. This is corroborated by evidence presented in animal studies (Serrano et al., 2007).

Metabolism and Safety of Volatile Organic Compounds

In metabolism, essential oil components are oxidised by phase 1 and 2 enzyme mediated reactions in the liver or other tissues (Zehetner et al., 2019) creating polar derivatives in phase 1, then sulphate, glutathione or glucuronide conjugates in phase 2. A list of metabolic derivatives of common essential oil components is given by Kohlert et al. (2000).

After the xenobiotics are metabolised by phase 1 or 2 processes they are then eliminated via urination or secreted into the bowel for microbial fermentation. For example, during a pharmacokinetic study of menthol, participants received an oral dose of >500 mg pure menthol, yet peak plasma concentrations did not exceed 160 ng ml^{-1} whereas menthol glucuronides were as high as 7 μg ml^{-1} (Valente et al., 2015). Although these glucuronides are created to facilitate the removal of menthol from the system, they may be recycled for therapeutic effects by deconjugation when in contact with the enzyme glucuronidase, which is upwardly expressed in the inflamed tissues of the body (Shimoi and Nakayama, 2005). This encourages us to think of the conjugated forms of essential oil components as quasi-bioavailable.

The phase 2 β-glucuronide metabolite is characterised by a glucuronide moiety O-linked to the xenobiotic (the compound). As previously mentioned, once these products reach this higher level of polarity, they have short half-lives because they are efficiently eliminated by the kidneys. However, high amounts of these polar conjugates can be dissolved in blood plasma and transported to any extracellular space in the body, reaching sites of infection or inflammation. Higher amount of β-glucuronidase alluded to above is common in cancers as well as inflamed tissues. When the glucuronide moiety is removed, the xenobiotic is returned to a much more hydrophobic intermediate, commonly a derivative that was formed in an earlier metabolic step, if not the pre-metabolised form. This causes the xenobiotic to lose solubility and accumulate on-site, potentially enacting biological effects locally (Sperker et al., 2001). Current research on the pharmacokinetics of natural products ignores this latter observation in the context of rational *in vivo* translation of *in vitro* outcomes (Sadgrove and Jones, 2019).

Glutathione conjugates are less commonly described as a metabolic product of essential oil components. When the glutathione conjugates were observed in earlier studies, they were thought to be non-enzymatic phase 2 reactions that were initiated by a phase 1 oxidation (Thompson et al., 1990). However, the understanding of glutathione S-transferases and their role in conjugation of glutathione to xenobiotics (Sheehan et al., 2001) changed this view. Several studies describe glutathione conjugates of essential oil components, such as cinnamaldehyde (Choi et al., 2001), pulegone (Lassila et al., 2016) and eugenol (Thompson et al., 1990), just to name a few. Conjugation by S-transferases typically creates an S-linked glutathione but in some cases N-linked conjugates are non-enzymatically formed, which can occur when furans form reactive aldehydes that react in a Schiff-base fashion with the free glutamyl amine on the glutathione reactant, which happens to menthofuran (Lassila et al., 2016). Essential oils are known to upregulate the expression of glutathione S-transferase in the liver (Banerjee et al., 1994; Abd El-Moneim et al., 2012), but minimal study has been dedicated to the P isoform that is upregulated in cancers (Tew et al., 2011). It is unclear if upregulation of glutathione S-transferase in cancers by essential oils is a positive or negative outcome because chemotherapeutic drugs are metabolised faster, which is a negative, but so are carcinogens, which is a positive. Furthermore, the biological effects of glutathione conjugates of essential oils have minimal research, but they should be examined in the context of cancers as part of the growing body of research dedicated to glutathione S-transferase prodrugs (Townsend and Tew, 2003). Finally, many xenobiotics are not conjugated to glutathione (Kohlert et al., 2000), and because there are minimal reports of this occurring in essential oil components, it may be considered less common.

While essential oil components are usually metabolised by both phase 1 and 2 processes in the liver, there is some evidence that more is 'sunk' into adipose tissues and organs than is eliminated, i.e., one study reported in humans that with 1 mg oral dose of thymol the peak plasma concentration reached 0.093 μg ml^{-1}, but only about 16% was eliminated as thymol sulphate or glucuronide, suggesting accumulation in organs and fat (Kohlert et al., 2002).

Minimal studies are available to determine peak plasma or organ concentrations before toxic effects may be considered in people. A single study was found that examined the human maximum tolerance dose of D-limonene and quantities administered ranged from 0.5 to 12 g m^2 orally. It was

determined that the safe dose was 8 g m^2 i.e., 12–16 g oral dose, which could be sustained for 11 months with no adverse effects. Despite such a high oral dose, the peak plasma concentrations were 2.12 μg ml^{-1} compared to tissue plasma concentration of 5.52 μg ml^{-1}, and the major phase 1 metabolic products are perillic acid, perillic acid isomers, perillyl alcohol and limonene-diol derivatives. Hence, peak plasma concentrations of limonene combined with its oxidised forms were >14 μg ml^{-1} (Vigushin et al., 1998). Some biological roles of these metabolic products have been demonstrated, including immunomodulation, anti-inflammatory and antiproliferative effects against pancreatic cancer cells (da Silveira e Sá et al., 2013).

In mice there are several studies that push the limits in terms of safety. For example, intravenous and oral doses of 12.5 mg geraniol in mice produced peak plasma concentrations of approximately 250–300 μg ml^{-1} by both routes, which was metabolised or 'sunk' within 2 h. To test for toxicity the authors then administered a 10-fold higher concentration of 120 mg day^{-1} for 4 weeks and demonstrated no apparent toxic effects (Pavan et al., 2018). This indicates that mammals can experience plasma concentrations familiar to many of the *in vitro* studies, whilst remaining several orders of magnitude below possibly toxic concentrations. Evidently, toxicity is dependent on the functional groups of individual essential oil components, so they will need to be considered on an individual basis.

Unfortunately, there are limited studies that focus on the possible biological effects of the metabolic conjugates or phase 1 metabolites of essential oil components, i.e., phase 1 oxidised or phase 2 conjugated sulphate, glutathione or glucuronide forms. Although this has been visited to an extent in the cases of limonene metabolites (da Silveira e Sá et al., 2013), it is worth considering other leads in future studies.

Volatile organic compounds can also influence the expression and activity of cytochrome P450 enzymes and transferases involved in metabolism (Sadgrove and Jones, 2019), which can influence the nature of its own metabolism or the metabolism of other drugs in the system, either slowing down or speeding up the rate of metabolism and changing the drug's half-life (Zehetner et al., 2019). There is a growing body of knowledge of the metabolism of essential oil components when administered in pure form (Zehetner et al., 2019), but less is known about component metabolism when ingested as part of parent plant material that also includes components that modulate cytochrome p450 (CYP) isozymes and change the rate of metabolism of specific components relative to others. In several cases plant material has CYP isozyme inhibitors that increase the peak plasma concentrations of the metabolite (Ashour et al., 2017). Furthermore, interactions of essential oil components with drugs should be taken into consideration if candidates use pharmaceuticals. A comprehensive guide to the safety of essential oils is given by Tisserand and Young (2013).

The ability of essential oil components to modulate CYP isozymes may in part be related to their affinity for the pregnane X receptor (Šadibolová et al., 2019), but recent evidence has not been conclusive. Nevertheless, a comprehensive summary of the enzymes that are modulated in relation to the essential oil component is provided by Zehetner et al. (2019), where induction, inhibition and metabolizing enzymes are listed.

Additive, Synergistic, Antagonistic or Potentiator

It is common for studies to demonstrate interesting biological effects from crude extracts of plant organs, but to fail to identify an active ingredient after following a bioassay guided fractionation approach (Sadgrove and Jones, 2019). In admitting defeat, authors of these types of studies often speculate that synergism is responsible for irreproducibility of their earlier outcome. Unsurprisingly, it is indeed true that synergisms occur, but research has only recently started to explain these synergisms and essential oil components are repeatedly demonstrated to be significantly involved (Sadgrove N. J., 2020; Langat et al., 2021; Nsangou et al., 2021). However, during the fractionation process volatile components are often lost when removing solvents, making it difficult for researchers to recognise synergistic effects.

The effects of drug or compound combinations are classified according to the four categories, additive, synergistic, antagonistic or potentiator. In the context of antimicrobial studies, these categories are usually determined by testing combinations and calculating summed fractional inhibitory concentrations (ΣFIC). This is calculated using the minimum inhibition concentration (MIC) of individual components or essential oils and comparing to combinations. The MIC assay for antimicrobial testing is elucidated by Sadgrove and Jones (Sadgrove and Jones, 2015), but briefly the protocol uses a serial two-fold dilution of the test substance in agar that is then inoculated with the bacterial organism, so that a range of concentrations are tested. The minimum concentration that can create inhibition is regarded as the MIC. Hence, to calculate the ΣFIC, several MIC values are required, then the calculations follow **Eqs. 1**–**3**.

In **Eq. 1** the concentration of A (Con.A) in the mixture of drugs A+ B at the combined MIC concentration, is divided by the MIC of drug A alone (MIC-A) to give FIC-α. In **Eq. 2** the concentration of B (Con.B) in MIC A+ B is divided by the MIC of drug B alone to give FIC-β. In **Eq. 3** FIC-α is combined with FIC-β (α+β) to give the ΣFIC value (Sueke et al., 2010). For example, if the MIC value of A + B is 0.5 mg ml^{-1} at a ratio of 1: 4 of A:B, then Con. A is 0.1 and Con. B is 0.4 mg ml^{-1}. If the MIC value of A alone is 1.5 mg ml^{-1} and B alone is 1.0 mg ml^{-1} then FIC-α is 0.1/1.5 = 0.07 and FIC-β is 0.4/1.0 = 0.4; then the ΣFIC value is 0.07 + 0.4 = 0.47.

$$FIC\text{-}\alpha = \frac{Con.A\ (in\ MIC\ A + B)}{MIC\text{-}A} \tag{1}$$

$$FIC\text{-}\beta = \frac{Con.B\ (in\ MIC\ A + B)}{MIC\text{-}B} \tag{2}$$

$$\Sigma FIC = FIC\text{-}\alpha + FIC\text{-}\beta \tag{3}$$

When the ΣFIC is ≤ 0.5 it is synergistic, if > 0.5–1.0 it is additive, if > 1.0 – ≤4.0 it is noninteractive and >4.0 makes it antagonistic (Van Vuuren and Viljoen, 2011).

The distinction between synergism and potentiation needs to be made. Synergism is defined by the increase of activity by a

combination of two 'active' compounds. In synergism, enhancement of the activity is greater than the sum of the two effectors (A+ B) giving 1 + 1 ≠ 2 or 1 + 1 = >2. Alternatively, a subset of synergism is potentiation, which occurs when a non-active compound enhances the activity of the active compound (i.e., 1 + 0 = >1). The distinction is usually only made in the context of defeating resistance mechanisms, i.e., if one component blocks an efflux channel to augment the effect of an antibiotic, it is regarded as the potentiator (Mandeville and Cock, 2018).

While *in vivo* synergism can be caused by a wider range of system interactions, such as by changing a xenobiotic's pharmacokinetics by slowing its metabolism, *in vitro* synergism has a narrower range, which is commonly the outcome of targeting two different mechanisms to achieve an enhanced outcome, including resistance mechanisms of pathogenic microbes. This means that by testing against a single target, such as a single enzyme, synergism is not possible, i.e., synergism requires at least a whole cell to manifest. Because most essential oil components confer effects to cell walls of bacteria and eukaryotes, their synergistic effects when combined with compounds that have specific targets, are caused by destabilising the walls of target cells.

In many synergism studies, essential oils and volatile organic compounds are regarded as non-active participants in combination with pharmaceuticals, so they are described as potentiators. While other researchers require stronger effects from antimicrobials, most researchers consider an MIC at <1 mg mL^{-1} as active (Van Vuuren and Holl, 2017), which is common in essential oils research. Consequently, the terms synergistic and potentiation are often used at the discretion of the authors in the published literature.

The most common potentiating effects described for volatile organic compounds or essential oils in the literature is focused on combinations with antibiotics from 'big pharma', i.e., essential oils from *Thymus vulgaris* L synergistically enhance the antibiotic cefixime (Jamali et al., 2017). In the pharmaceutical world the use of volatile organic compounds on their own to enact antimicrobial outcomes is not feasible for economic reasons. The concentrations must be many orders of magnitude higher to be comparable to microbially derived antibiotics (Sadgrove and Jones, 2019), which raises the cost of production to beyond reasonable, and limits the range of applications to topical use only (inhalation, topical dermal or gastro/intestinal epithelial). Hence, rather than being antimicrobial *per se*, volatile organic compounds are appropriately thought of as antiseptic compounds (Kon and Rai, 2012), with only general specificity in the mechanism of action. However, synergistic or potentiation effects are still of interest to pharma, by antagonising resistance mechanisms in pathogenic strains. The most commonly cited potentiation effect ascribed to plant metabolites is the attenuated effects of efflux 'pumps' (Khameneh et al., 2019). Prokaryotic efflux pumps are bacterial or viral membrane bound channels called 'transport proteins' that promote the disposal of cellular waste or toxins. Gene modulation effects by volatile organic compounds also occur in the prokaryotic cells of pathogenic microbes, which involves the downregulation of resistance associated genes (Chovanová et al., 2016), leading to the potentiation of other antimicrobial metabolites or antibiotics. Furthermore, volatile organic compounds have also shown the ability to downregulate expression of genes responsible for pathogen toxin secretion (Khoury et al., 2016), which attenuates virulence.

Normally the excretion of antimicrobial drugs via efflux pumps does not antagonise drug efficacy, but with the new trends involving overexpression of multidrug resistance efflux pump genes (Blanco et al., 2016), antibiotics are becoming less efficacious. Inhibiting this mechanism causes the accumulation of the antimicrobial drug in the bacteria's cytoplasm, which enables an active concentration of the drug to be reached (Bambeke et al., 2003). While there are no efflux pump inhibitors in wider clinical use, volatile organic compounds are known to have this effect (Mikulášová et al., 2016). For example, the sesquiterpenes *epi*-cubenol and 15-copaenol were able to produce ΣFIC values in the range of 0.03–0.26 in combination with standard antibiotics against strains of *Staphylococcus aureus* that overexpress the NorA gene for the NorA efflux pump. These potentiating effects are attributed to efflux pump inhibition at a concentration of sesquiterpene that is less than 0.25 μg ml^{-1} (Espinoza et al., 2019), a concentration that is feasible in blood plasma alone, discounting accumulating effects in the body's tissues.

Essential oil components may also augment the efficacy of other drugs by enhancement of their penetration (Aqil et al., 2007; Chen et al., 2015). The mechanism is thought to be related to disruption of the highly ordered structure of the stratus corneum lipid, leading to an increase in the intercellular diffusivity. This is established by the observation of a shift from '*trans* to *gauche*' conformation in the methylene carbons along the alkyl chain of lipids (Chen et al., 2015). In the case of paracetamol, penetration enhancement values for each of the essential oils correlate to the skin permeation profiles of the individual essential oils. Hence, penetration enhancement can be predicted from the flux (μg.cm^{-2}. h^{-1}) or Q48 (μg.cm^{-2}) of the individual essential oil. Essential oils with pronounced permeation values were produced by clove, angelica and chuanxiong and the most represented components were ligustilide and eugenol (Chen et al., 2015), which have the character of delocalised electrons (aromatic rings).

Furthermore, the cell and organelle wall permeabilising effects of lipophilic compounds can potentiate the influx of other exogenous compounds that confer gene modulatory effects. Although not a great deal of research has focused on this, the whole range of compounds in extracts from aromatic species may have synergistic or potentiation effects. Extracts from aromatic species have a range of compounds in their extracts, which include fixed (non-volatile) and volatile metabolites. As an example, the aromatic species *Elytropappus rhinocerotis* (L.f) Less. is used in South Africa as a treatment against foot odours and infections. The extract includes volatile organic compounds and non-volatile labdane diterpenes, both of which antagonised the growth of food fungal pathogens and odour causing bacteria (i.e., *Brevibacterium agri*) (Hulley et al., 2019).

The possibility for synergism between essential oil components and cannabinoids has been explored in theory (Russo, 2011). However, limited research has been conducted to specifically address these questions. The dominant sesquiterpene β-caryophyllene has been the focus of many studies, including as a synergist in antimicrobial outcomes. A recent study of *Vepris gossweileri* I. Verd., discovered several antimicrobial synergisms with β-caryophyllene in an extract of the leaves, which was defined as a 'multi-layered' synergism (Langat et al., 2021). Synergism against a model yeast and Gram-positive organism was demonstrated between β-caryophyllene and minor alkaloids with a ΣFIC value of 0.02. However, the synergistic effects were augmented by the chlorophyll derivative pheophytin A. In a follow-up study (results unpublished) it was realized that chlorophyll, pheophytins or pheophorbides are potent antimicrobial synergists in combination with β-caryophyllene. Hence, antimicrobial effects of green plant extracts, such as cannabis or CBD oil, are likely to be the result of a synergism between β-caryophyllene and the chlorophyll derivatives.

Another study of synergism argued that essential oil components in roots of *Citrus* x *limon* (L.) Osbeck synergised with specific methoxylated flavonoids released out of decomposing leaf litter at the base of the tree, providing protective effects against pathogenic root fungi (Nsangou et al., 2021). A hypothetical that is inspired by this research outcome involves antimicrobial synergisms against ruminant organisms that reside in the gut of herbivorous insects. This has not yet been examined in detail but stands out as a high possibility. Otherwise, antimicrobial synergisms may be considered as an important part of plant defence and the wider scientific community is encouraged to examine this in more detail, because there are both ecological and health-related implications from such research, i.e., there are many ways that such synergisms can be utilised in human health as prebiotics for the gastrointestinal tract.

Mammalian Gene Regulation and Immunomodulation

While it is necessary for volatile organic compounds to reach high concentrations to confer 'contact' antimicrobial effects, gene-modulation can occur at concentrations that are many folds' lower. Mammalian cells have diverse super-families of transcription factors that have lipid binding domains that inevitably become the target of lipophilic compounds. One study that focused on the dermal fibroblast demonstrated upregulation of hundreds of genes associated with (anti) inflammation, metabolism and proliferation, which occurs at a concentration of less than 100 μg g^{-1} (0.01%) by a variety of volatile compounds (Han and Parker, 2017). The genes are modulated uniquely by each of the compounds tested, at concentrations that are low enough to be feasible in topical applications or tissue bioaccumulation, without cytotoxic effects (2017).

In the context of human (eukaryotic) cells, modulation of the expression of peroxisome proliferator-activated receptor (PPAR) genes can occur in response to essential oil components (Goto et al., 2010). For example, limonene at 10 μM (1.5 μg g^{-1}) increased phosphorylation of Akt leading to enhanced glucose uptake in adipocytes, promoted adipocyte differentiation and also allegedly the expression of PPAR-γ genes (Soundharrajan et al., 2018). Linalool appears to do the opposite, by inhibiting adipocyte differentiation (Cheng et al., 2018), which may be useful in obesity control. Nevertheless, both compounds can reach systemic concentrations of 2 μg ml^{-1} in humans, by topical and oral application, with no adverse effects.

PPAR agonism is also associated with anti-inflammatory effects (immunomodulation) by inhibition of interleukin-1 induced cyclooxygenase-2 expression (Hotta et al., 2010). However, immunomodulation by essential oil components is not limited to this effect. The literature describes the modifying effects on secretion of a wide diversity of cytokines other than interleukin-1 (Valdivieso-Ugarte et al., 2019), which can occur by agonism of nuclear or membrane receptors, such as the cannabinoid receptors, but the details are often not explained. For example, systemic treatment using 50 mg kg^{-1} essential oil from *Cordia verbenacea* A. DC reduced tumour necrosis factor (TNF-α) production, which interrupted the inflammatory cascade induced by carrageenan (Passos et al., 2007). Ingestion of 50 mg kg^{-1} of the two main volatile organic compounds, α-humulene and *E*-caryophyllene, also reduced inflammation. *E*-Caryophyllene only diminished TNF-α release whereas α-humulene also interrupts interleukin-1β, cyclooxygenase-2, nitric oxide and prostaglandin E-2 (PGE(2)) (Fernandes et al., 2007). Furthermore, inflammation was greatly attenuated by oral treatment an hour before lipopolysaccharide (LPS) was used as an inducer, evidently by the same mechanism as above (Medeiros et al., 2007). In topical applications a much lower concentration is required. Hence, a commercial product named Acheflan with *C. verbenacea* essential oil as an active ingredient is available in Brazil as a topical anti-inflammatory. Pharmacokinetic studies of the main sesquiterpene, α-humulene, using oral and intravenous doses of 1,000 mg kg^{-1} mouse, demonstrated that peak plasma concentrations can reach from 2–20 μg ml^{-1} without adverse effects in the short term (Chaves et al., 2008).

Before the term 'potentiator' came into practice, researchers used the former term 'entourage effect' to describe the potentiating effects of volatile organic compounds from the marijuana variety of *Cannabis sativa*. The psychoactive effects from marijuana are caused by tetrahydrocannabinol, which is a potent agonist for cannabinoid receptor-1. However, as previously mentioned, the headspace of marijuana also includes the volatile sesquiterpene β-caryophyllene, which is regarded as a phytocannabinoid that is not psychoactive because it is a selective agonist of cannabinoid receptor-2 (CB2), a receptor in immune cells (Gonçalves et al., 2020). Caryophyllene is the most publicised example of a specific CB2 agonist (Francomano et al., 2019). It is known to promote wound healing in dermal skin models by following multiple routes, but the anti-inflammatory effects are likely to be the most important in this outcome. Concomitant with higher rates of re-epithelialization is the upregulated expression of hair follicle bulge stem cells, which has strong implications to hair health (Koyama et al., 2019).

But the effects of β-caryophyllene are not restricted to cannabinoid receptors. β-Caryophyllene positively regulates the pI3K/Akt/mTOR signalling pathway in tissues that express Akt3, a protein kinase B isoform important for the regulation of neuronal development. Alternatively, in liver cells and T lymphocytes this pathway is negatively regulated by the same treatment but upregulated in neuronal cells, indicating a role in tissue-specific inflammation treatment. Regulation of the pI3K/Akt/mTOR pathway is entirely dependent on Akt3, meaning that it makes sense that the JAK/STAT signalling pathway is upregulated independently. Hence, the essential oil of copaiba that is rich in β-caryophyllene confers gene regulatory effects that differ according to the tissue (Urasaki et al., 2020), i.e., copaiba essential oil can confer anti-inflammatory effects without dulling the immune response. Furthermore, it was demonstrated that by upregulation of the pI3k/Akt/mTOR pathways in the dermis, promotion of reepithelization of superficial wounds occurs (Koyama et al., 2019).

In contrast, the gingerols from *Zingiber officinale* allegedly downregulate the pI3k/Akt/mTOR pathway (Wang et al., 2016). The gingerols are also potent antioxidants (Mao et al., 2019). Together these effects confer protection against oxidative species generation from mitochondrial respiration. Previously it was thought that anecdotal accounts of use of ginger for hair restoration, in men living with androgenetic alopecia, were a contradiction because it slowed the growth of dermal papilla cells *in vitro* (Miao et al., 2013), however evidence now indicates the Akt-mTOR pathway is overactive in bald scalps and this process is associated with overproduction of reactive oxygen species (Sadgrove, 2021).

A bit like CBD oil, the anti-inflammatory effects of essential oil components may also play a role in aiding sleep. A famous sleep-inducing herbal tea is chamomile (*Matricaria recutita* L., Asteraceae). The blue colour of the essential oil is caused by chamazulene, which is an anti-inflammatory component that is active at 10–60 μg ml^{-1} *in vitro* (Ma et al., 2020). However, chamazulene is a derivative produced in hydrodistillation by conversion of the precursor matricine, which is the version that is present in chamomile tea. Matricine is active at a lower concentration, inhibiting NF-KB activation within the margin of 3–22 μg ml^{-1} (Flemming et al., 2015). Because NF-KB activation is associated with sleep deprivation (Irwin and Wang, 2008), the link between chamomile tea and restful sleep may be in the anti-inflammatory effects of matricine, or its combined effects with the non-volatile flavonoid component quercetin (Kambe et al., 2010). Alternatively, mice treated with essential oil from chamomile were observed to have lower plasma histamine levels than control after challenging with 2,4-dinitrochlorobenzene (Lee et al., 2010), which conveys that the essential oil may be an antihistamine and antihistamines are known to induce sleep or drowsiness.

Other essential oil components that have been demonstrated as anti-inflammatory by *in vitro* assay of lipopolysaccharide induced cytokine release include the santalol isomers from sandalwood (Sharma et al., 2014), eugenol from clove (Saad et al., 2013), and carvacrol from thyme (Hotta et al., 2010) among others. The numbers of essential oil components associated with anti-inflammatory effects are numerous and mechanisms are often not explained but it is reasonable to hypothesise a role for PPARS.

Areas for Further Research

Cardiovascular Disease

Essential oil components are worthy of further consideration in the context of cardiovascular disease prophylaxis. It is the contention of this narrative that aromatic foods that are included in the diet in the long term enact positive effects that interrupt the aetiological progression of many forms of disease, particularly cardiovascular diseases. However, prophylactic effects are only realised over the course of decades, so it is difficult to prove *in vivo*. Nevertheless, there is mounting indirect evidence to support this hypothesis. For example, dietary D-limonene has demonstrated insulin sensitising effects and reduced oxidative stress in rats fed on an obesogenic diet (Santiago et al., 2012). Because insulin resistance is regarded as a risk factor (Petrie et al., 2018), then it is feasible that attenuation of negative effects associated with insulin resistance is prophylactic for cardiovascular disease.

There are many essential oil components that confer insulin sensitising effects alone (Sebai et al., 2013; Hasanein and Riahi, 2015) or as part of a combination of fatty oils and plant extracts (Talpur et al., 2004). Authors of these types of studies offer the explanation that essential oil components help the body's cells to cope with oxidative stress, either by direct radical quenching or modulation of antioxidant genes (Liu et al., 2013; Mohamed et al., 2016), and further to confer anti-inflammatory effects, all of which attenuate insulin resistance.

According to the modern paradigm of cardiovascular disease, chronic inflammation is considered as the root of its pathogenesis. One group of authors argue that the comorbidities of cardiovascular disease are characterised by chronic systemic inflammation and propose that if untreated will lead to heart disease (Bigeh et al., 2020). Chronic systemic inflammation has two main dietary triggers, with the first being obesogenic eating (de Luca and Olefsky, 2008), leading into high caloric loading and reactive oxygen species generation, mitochondrial burnout and activation of the polyol pathway (Johnson et al., 2017).

Considering the strong link between inflammation and the eventual development of cardiovascular diseases, dietary inclusion of anti-inflammatory phytochemicals over a long period of time may be considered prophylactic. However, it must be considered if volatile organic compounds can be raised to high enough concentrations in plasma to achieve the anti-inflammatory effects demonstrated *in vitro*. Fortunately, it has already, been demonstrated in rats that many of the anti-inflammatory essential oil components are feasibly raised to the required plasma concentrations by dietary application at quantities present in a serving of aromatic food, but the mechanism as explained by *in vitro* studies are not necessarily the actual mechanisms *in vivo*. For example, *in vitro* inflammation in macrophages stimulated by TNF-α and nitric oxide was attenuated by the essential oil components of *Cinnamomum zeylanicum* Blume at concentrations of

7.5–8.6 μg ml^{-1} for *E*-cinnamaldehyde or 5.7–12.6 μg ml^{-1} for O-methoxycinnamaldehyde (Gunawardena et al., 2015). With consideration to the cytochrome P450 inhibiting effects of *E*-cinnamaldehyde (Chan et al., 2016), these concentrations may be more easily met in blood plasma than other types of monoterpenes, however it is unclear if these plasma concentrations can be feasibly met in humans (Zhu et al., 2017), or if the metabolic products cinnamic acid, cinnamyl alcohol or methyl cinnamate also enact anti-inflammatory effects. Nevertheless, *in vivo* effects are achievable in male Wistar rats at an oral dose of 143.8 μmol kg^{-1} daily (Farrokhfall et al., 2010). Generally *in vivo* studies that demonstrate positive outcomes followed a repeated dosing regime, rather than a single oral dose. Hence, the effects may be related to accumulation of essential oil components and their respective metabolites in tissues and changes to the expression of metabolising enzymes in liver and the dermis.

As mentioned earlier, the mechanism of anti-inflammatory effects of essential oil components may be enacted by agonism of peroxisome proliferator activated receptors (PPAR) (Goto et al., 2010; Hotta et al., 2010; Katsukawa et al., 2010; Li et al., 2015), because PPARS are important modulators of inflammation (Daynes and Jones, 2002). The concentrations required to achieve agonism of PPARS are similar to the concentrations in studies describing anti-inflammation in macrophages, i.e., cinnamaldehyde activated PPARS at 1.3–6.6 μg ml^{-1} (Li et al., 2015). However, because the PPARS are concentrated in adipose tissues and liver, then the concentrations of xenobiotic essential oil components will be many folds higher in the vicinity of PPARS. Hence, these effects are feasible *in vivo* with moderate consumption of aromatic foods, i.e., rats fed D-limonene demonstrated significant upregulation of PPARα genes (Jing et al., 2013). Because PPARS are also important in the action of insulin signalling and blood glucose control (Leonardini et al., 2009) this may also explain the mechanism of diabetic control by oral essential oil in rat studies.

The second leading cause of systemic inflammation is gastrointestinal bacterial dysbiosis (Jin et al., 2018). The problem starts with 'leaky gut', which results from intestinal inflammation as a response to bacterial overgrowth. Due to damage to the mucosal or epithelial barrier bacterial lipopolysaccharides enter into the lining and cross in portal circulation (Onal et al., 2019). In cases of more severe disturbance to the intestinal epithelial barrier function, live bacteria escape the gut lumen and translocate into systemic circulation, contributing to atherosclerotic symptoms and myocardial infarction (Zhou et al., 2018). The key to attenuating this problem lies in strengthening the intestinal epithelial barrier via the nurturing of commensal gut bacteria and attenuation of bacterial overgrowth (Ohland and Macnoughton, 2010).

Hence, the use of aromatic plant foods as prebiotics may be considered prophylactic for cardiovascular disease. As previously mentioned, synergisms between essential oil components and chlorophyll or the derivatives, pheophytin or pheophorbide, is a worthy research undertaking. The possibility of controlling bacterial overgrowth in the intestinal space is a neglected but important vision in the prebiotic initiative (Zhong et al., 2017). In this regard, controlling bacterial overgrowth attenuates or prevents inflammation, enhance re-epithelialization, and closes the barrier between portal circulation and bacterial lipopolysaccharide.

Safety and Chemoprevention With Volatile Organic Compounds

Because essential oil components accumulate in the body's tissues, the obstacle of bioavailability may be overcome, particularly in cancers. As previously mentioned, metabolite conjugation reduces a compound's bioavailability and prevents it from reaching a potentially toxic concentration in normal tissue, but in cancerous tissue deconjugation reverses the phase 2 metabolism and causes a localised build-up of preconjugated xenobiotics. The prooxidant effects (Burt, 2004) that are normally not occurring in healthy tissue are enabled by this localised concentration of xenobiotics, which include a host of ingested plant-derived secondary metabolite, including essential oil components.

Generally, phase 1 metabolism makes oxidised derivatives of essential oil components and in phase 2 metabolism they are conjugated to either a glucuronide, glutathione or a sulphate moiety (Sadgrove and Jones, 2019). Although this process is thought to make the respective xenobiotic completely unavailable, it is now known that deconjugation processes return xenobiotics to their active pre-conjugated forms. These effects are well known for non-volatile plant compounds, such as curcumin, which is rapidly metabolised into a glucuronide that is regarded as unavailable, but is transformed back into the aglycone by β-glucuronidase activity in bone tissue undergoing osteoclastogenesis (Kunihiro et al., 2019). Previously several essential oils were discovered as preventors of bone reabsorption (osteoclast breakdown) (Mühlbauer et al., 2003), and the modern realization of the β-glucuronidase activity clarifies how the essential oil components can meet the required concentrations. A similar scenario was observed with the non-volatile metabolite resveratrol, which is quickly metabolised into a sulphate conjugate that is reversed by steroid sulphatases in cancer cells, inducing autophagy (Andreadi et al., 2014).

Cancers express high levels of β-glucuronidase (Su et al., 2014) and steroid sulphatases (Foster, 2021). Hence, conjugated xenobiotics may be regarded as glucuronide or sulphate prodrugs that are activated on-site to enact therapeutic effects (de Graaf et al., 2002). Although this type of research has not focused on essential oil components, the anticancer effects of phase 1 metabolites are sometimes examined by forward thinking researchers, which are the likely forms that appear in tumours or cancers when deconjugation occurs. For example, D-limonene was previously considered a worthy candidate in chemotherapy, and the phase 1 metabolites were also of interest in this context (da Silveira e Sá et al., 2013).

Research on the anticancer effects of essential oil components generally focuses on cytotoxic effects caused by various mechanisms, including overexpression of liver detoxification enzymes, changes to the membrane potential of mitochondria

(causing leakage of free radicals) and whole cancer cells, prooxidant effects (mainly by arenes), inhibition of angiogenesis, and modification of tumour-inducing genes (Bhalla et al., 2013; Legards et al., 2014). Complementary, additive or synergistic effects, where a supportive role to the more conventional treatment, is also a point of interest in modern research (Legards et al., 2014). However, the insulin sensitising effect of essential oil components (Talpur et al., 2004) is a worthy consideration, because modulation of glucose metabolism in cancers has also become a point of interest in modern research (Hay, 2016).

Alternatively, lifestyles that include diets fortified with aromatic species may be preventative or antagonistic of oncogenesis. This school of thought is known widely as a chemoprevention strategy. Hence, the powers of plant-derived xenobiotics, such as essential oil components, are more than likely realized as preventative because of the anticancer subtly by comparison with conventional chemotherapy drugs. Milder nature-based chemotherapy metabolites, such as essential oil components, can be endured by the human body over the long term, meaning that cancerous cells can be antagonised before they establish as larger resilient cysts. Furthermore, anticancer research of essential oils should focus on the possible antagonism of cancer metastasis during the remission period between conventional anticancer treatments. This is a feasible initiative because it requires following cancer survivors and quantifying those who stay in remission whilst incorporating a plant-based health regime.

AUTHOR CONTRIBUTIONS

NS and GP-G conceptualized and wrote the manuscript. OL, IM and EF-C motivated the article and provided funds for open access.

FUNDING

This research was funded by the Internal Grant Agency of FTA, grant number 20213105 Czech University of Life Sciences Prague, the Czech Republic.

REFERENCES

Abd El-Moneim, M. R., Fatma, S. A., and Turky, A. F. (2012). Control of Tetranychus Urticae Koch by Extracts of Three Essential Oils of Chamomile, Marjoram and Eucalyptus. *Asian Pac. J. Trop. Biomed.* 2, 24–30. doi:10.1016/S2221-1691(11)60184-6

Adams, R. P. (2007). *Identification of Essential Oil Components by Gas Chromatography/mass Spectrometry*. Carol Stream, IL: Allured Publishing Corporation. ISBN 978-1-932633-21-4.

Amiresmaeili, A., Roohollahi, S., Mostafavi, A., and Askari, N. (2018). Effects of Oregano Essential Oil on Brain TLR4 and TLR2 Gene Expression and Depressive-like Behavior in a Rat Model. *Res. Pharm. Sci.* 13, 130–141. doi:10.4103/1735-5362.223795

Anastasiou, C., and Buchbauer, G. (2017). Essential Oils as Immunomodulators: Some Examples. *Open Chem.* 15, 352–370. doi:10.1515/chem-2017-0037

Andreadi, C., Britton, R. G., Patel, K. R., and Brown, K. (2014). Resveratrol-sulfates Provide an Intracellular Reservoir for Generation of Parent Resveratrol, Which Induces Autophagy in Cancer Cells. *Autophagy* 10, 524–525. doi:10.4161/auto.27593

Aqil, M., Ahad, A., Sultana, Y., and Ali, A. (2007). Status of Terpenes as Skin Penetration Enhancers. *Drug Discov. Today* 12, 1061–1067. doi:10.1016/j.drudis.2007.09.001

Asakawa, Y., and Ludwiczuk, A. (2013). "Bryophytes: Liverworts, Mosses, and Hornworts: Extraction and Isolation Procedures," in *Metabolomics Tools for Natural Product Discovery*. Editors U. Roessner and D. A. Dias (Totowa, NJ: Humana Press), 1–20. doi:10.1007/978-1-62703-577-4_1

Ashour, M. L., Youssef, F. S., Gad, H. A., and Wink, M. (2017). Inhibition of Cytochrome P450 (CYP3A4) Activity by Extracts from 57 Plants Used in Traditional Chinese Medicine (TCM). *Pharmacogn Mag.* 13, 300–308. doi:10.4103/0973-1296.204561

Bakkali, F., Averbeck, S., Averbeck, D., and Idaomar, M. (2008). Biological Effects of Essential Oils-Aa Review. *Food Chem. Toxicol.* 46, 446–475. doi:10.1016/j.fct.2007.09.106

Banerjee, S., Sharma, R., Kale, R. K., and Rao, A. R. (1994). Influence of Certain Essential Oils on Carcinogen-Metabolizing Enzymes and Acid-Soluble Sulfhydryls in Mouse Liver. *Nutr. Cancer* 21, 263–269. doi:10.1080/01635589409514324

Bauer, K., and Garbe, D. (1985). *Common Fragrance and Flavor Materials. Preparation, Properties and Uses*. Weinheim: VCH Verlagsgesellschaft.

Bedini, S., Farina, P., Napoli, E., Flamini, G., Ascrizzi, R., Verzera, A., et al. (2021). Bioactivity of Different Chemotypes of Oregano Essential Oil against the Blowfly *Calliphora Vomitoria* Vector of Foodborne Pathogens. *Insects* 12, 52. doi:10.3390/insects12010052

Beyer, J., Ehlers, D., and Maurer, H. H. (2006). Abuse of Nutmeg (Myristica Fragrans Houtt.): Studies on the Metabolism and the Toxicologic Detection of its Ingredients Elemicin, Myristicin, and Safrole in Rat and Human Urine Using Gas Chromatography/mass Spectrometry. *Ther. Drug Monit.* 28, 568–575. doi:10.1097/00007691-200608000-00013

Bhalla, Y., Gupta, V. K., and Jaitak, V. (2013). Anticancer Activity of Essential Oils: a Review. *J. Sci. Food Agric.* 93, 3643–3653. doi:10.1002/jsfa.6267

Bigeh, A., Sanchez, A., Maestas, C., and Gulati, M. (2020). Inflammatory Bowel Disease and the Risk for Cardiovascular Disease: Does All Inflammation lead to Heart Disease? *Trends Cardiovasc. Med.* 30, 463–469. doi:10.1016/j.tcm.2019.10.001

Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., et al. (2016). Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. *Microorganisms* 4, 14. doi:10.3390/microorganisms4010014

Bowles, J. E. (2003). *The Chemistry of Aromatherapeutic Oils*. Crows Nest, NSW Australia: Allen & Unwin.

Brain, L. J., Green, D. M., and Api, A. M. (2007). *In Vitro* Human Skin Penetration of the Fragrance Material Linalool. *Toxicologist* 102 (1), 319.

Buchbauer, G. (2010). "Chapter 9. Biological Activities of Essential Oils," in *Handbook of Essential Oils: Science, Technology and Applications*. Editors K. H. C. Başer and G. Buchbauer (London: CRC Press, Taylor and Francis Group).

Burt, S. (2004). Essential Oils: Their Antibacterial Properties and Potential Applications in Foods-Aa Review. *Int. J. Food Microbiol.* 94, 223–253. doi:10.1016/j.ijfoodmicro.2004.03.022

Cal, K. (2006). Skin Penetration of Terpenes from Essential Oils and Topical Vehicles. *Planta Med.* 72, 311–316. doi:10.1055/s-2005-916230

Chan, J., Oshiro, T., Thomas, S., Higa, A., Black, S., Todorovic, A., et al. (2016). Inactivation of CYP2A6 by the Dietary Phenylpropanoid Trans-cinnamic Aldehyde (Cinnamaldehyde) and Estimation of Interactions with Nicotine and Letrozole. *Drug Metab. Dispos* 44, 534–543. doi:10.1124/dmd.115.067942

Chaves, J. S., Leal, P. C., Pianowisky, L., and Calixto, J. B. (2008). Pharmacokinetics and Tissue Distribution of the Sesquiterpene Alpha-Humulene in Mice. *Planta Med.* 74, 1678–1683. doi:10.1055/s-0028-1088307

Chen, J., Jiang, Q. D., Chai, Y. P., Zhang, H., Peng, P., and Yang, X. X. (2016). Natural Terpenes as Penetration Enhancers for Transdermal Drug Delivery. *Molecules* 21, 1709. doi:10.3390/molecules21121709

Chen, J., Jiang, Q. D., Wu, Y. M., Liu, P., Yao, J. H., Lu, Q., et al. (2015). Potential of Essential Oils as Penetration Enhancers for Transdermal Administration of Ibuprofen to Treat Dysmenorrhoea. *Molecules* 20, 18219–18236. doi:10.3390/molecules201018219

Cheng, B. H., Sheen, L. Y., and Chang, S. T. (2018). Hypolipidemic Effects of S-(+)-linalool and Essential Oil from *Cinnamomum Osmophloeum* Ct. Linalool Leaves in Mice. *J. Tradit Complement. Med.* 8, 46–52. doi:10.1016/j.jtcme.2017.02.002

Choi, J., Lee, K. T., Ka, H., Jung, W. T., Jung, H. J., and Park, H. J. (2001). Constituents of the Essential Oil of the Cinnamomum cassia Stem Bark and the Biological Properties. *Arch. Pharm. Res.* 24, 418–423. doi:10.1007/BF02975187

Chovanová, R., Mikulášová, M., and Vaverková, Š. (2016). Modulation of mecA Gene Expression by Essential Oil from Salvia Sclarea and Synergism with Oxacillin in Methicillin Resistant Staphylococcus Epidermidis Carrying Different Types of Staphylococcal Chromosomal Cassette Mec. *Int. J. Microbiol.* 2016, 6475837. doi:10.1155/2016/6475837

Clevenger, J. F. (1928). Apparatus for the Determination of Volatile Oil*. *J. Am. Pharm. Assoc. (1912)* 17, 345–349. doi:10.1002/jps.3080170407

Collins, T. L., Andrew, R. L., Greatrex, B. W., and Bruhl, J. J. (2018). Reliable Analysis of Volatile Compounds from Small Samples of *Eucalyptus Magnificata* (Myrtaceae). *Aust. Syst. Bot.* 31, 232–240. doi:10.1071/sb18005

Damjanovic, B., Skala, D., Baras, J., and Petrovic-Djakov, D. (2006). Isolation of essential oil and supercritical carbon dioxide extract of Juniperus communis L. fruits from Montenegro. *Flavour Fragr. J.* 21, 875–880.

Dawidowics, A. L., and Dybowski, M. P. (2014). Simple SPE–GC Method for Anethole Determination in Human Serum. *J. Sep. Sci.* 37, 393–397.

Daynes, R. A., and Jones, D. C. (2002). Emerging Roles of PPARS in Inflammation and Immunity. *Nat. Rev. Immunol.* 2, 748–759. doi:10.1038/nri912

de Cássia da Silveira e Sá, R., Andrade, L., and De Sousa, D. (2013). A Review on Anti-inflammatory Activity of Monoterpenes. *Molecules* 18, 1227–1254. doi:10.3390/molecules18011227

De Graaf, M., Boven, E., Scheeren, H. W., Haisma, H. J., and Pinedo, H. M. (2002). Beta-Glucuronidase-Mediated Drug Release. *Curr. Pharm. Des.* 8, 1391–1403. doi:10.2174/1381612023394485

De Luca, C., and Olefsky, J. M. (2008). Inflammation and Insulin Resistance. *FEBS Lett.* 582, 97–105. doi:10.1016/j.febslet.2007.11.057

Dobreva, A., Kovatcheva, N., Astatkie, T., and Zheljazkov, V. D. (2011). Improvement of Essential Oil Yield of Oil-Bearing (*Rosa Damascena* Mill.) Due to Surfactant and Maceration. *Ind. Crops Prod.* 34, 1649–1651. doi:10.1016/j.indcrop.2011.04.017

El Khoury, R., Atoui, A., Verheecke, C., Maroun, R., El Khoury, A., and Mathieu, F. (2016). Essential Oils Modulate Gene Expression and Ochratoxin A Production in *Aspergillus carbonarius*. *Toxins (Basel)* 8, 242. doi:10.3390/toxins8080242

Espinoza, J., Urzúa, A., Sanhueza, L., Walter, M., Fincheira, P., Muñoz, P., et al. (2019). Essential Oil, Extracts, and Sesquiterpenes Obtained from the Heartwood of Pilgerodendron Uviferum Act as Potential Inhibitors of the *Staphylococcus aureus* NorA Multidrug Efflux Pump. *Front. Microbiol.* 10, 337. doi:10.3389/fmicb.2019.00337

Farrokhfall, K., Khoshbaten, A., Zahediasl, S., Mehrani, H., and Karbalaei, N. (2010). Improved Islet Function Is Associated with Antiinflammatory, Antioxidant and Hypoglycemic Potential of Cinnamaldehyde on Metabolic Syndrome Induced by High Tail Fat in Rats. *J. Funct. Foods* 10, 397–406.

Fernandes, E. S., Passos, G. F., Medeiros, R., Da Cunha, F. M., Ferreira, J., Campos, M. M., et al. (2007). Anti-inflammatory Effects of Compounds Alpha-Humulene and (-)-Trans-Caryophyllene Isolated from the Essential Oil of *Cordia Verbenacea*. *Eur. J. Pharmacol.* 569, 228–236. doi:10.1016/j.ejphar.2007.04.059

Flemming, M., Kraus, B., Rascle, A., Jürgenliemk, G., Fuchs, S., Fürst, R., et al. (2015). Revisited Anti-inflammatory Activity of Matricine *In Vitro*: Comparison with Chamazulene. *Fitoterapia* 106, 122–128. doi:10.1016/j.fitote.2015.08.010

Foster, P. A. (2021). Steroid Sulphatase and its Inhibitors: Past, Present, and Future. *Molecules* 26, 2852. doi:10.3390/molecules26102852

Francomano, F., Caruso, A., Barbarossa, A., Fazio, A., La Torre, C., Ceramella, J., et al. (2019). β-Caryophyllene: A Sesquiterpene with Countless Biological Properties. *Appl. Sci.* 9, 5420. doi:10.3390/app9245420

Gonçalves, E. C. D., Baldasso, G. M., Bicca, M. A., Paes, R. S., Capasso, R., and Dutra, R. C. (2020). Terpenoids, Cannabimimetic Ligands, beyond the *Cannabis* Plant. *Molecules* 25, 1567. doi:10.3390/molecules25071567

Goto, T., Takahashi, N., Hirai, S., and Kawada, T. (2010). Various Terpenoids Derived from Herbal and Dietary Plants Function as PPAR Modulators and Regulate Carbohydrate and Lipid Metabolism 2010, 9. *PPAR Res.* doi:10.1155/2010/483958

Griffin, G., Wray, E. J., Martin, B. R., and Abood, M. E. (1999). Cannabinoid Agonists and Antagonists Discriminated by Receptor Binding in Rat Cerebellum. *Br. J. Pharmacol.* 128, 684–688. doi:10.1038/sj.bjp.0702806

Guimarães, A. C., Meireles, L. M., Lemos, M. F., Guimarães, M. C. C., Endringer, D. C., Fronza, M., et al. (2019). Antibacterial Activity of Terpenes and Terpenoids Present in Essential Oils. *Molecules* 24, 2471. doi:10.3390/molecules24132471

Gunawardena, D., Karunaweera, N., Lee, S., Van Der Kooy, F., Harman, D. G., Raju, R., et al. (2015). Anti-inflammatory Activity of Cinnamon (C. Zeylanicum and C. cassia) Extracts - Identification of E-Cinnamaldehyde and O-Methoxy Cinnamaldehyde as the Most Potent Bioactive Compounds. *Food Funct.* 6, 910–919. doi:10.1039/c4fo00680a

Han, X., and Parker, T. L. (2017). Essential Oils Diversely Modulate Genome-wide Gene Expression in Human Dermal Fibroblasts. *Cogent Med.* 4, 1307591. doi:10.1080/2331205x.2017.1307591

Hasanein, P., and Riahi, H. (2015). Antinociceptive and Antihyperglycemic Effects of *Melissa Officinalis* Essential Oil in an Experimental Model of Diabetes. *Med. Princ Pract.* 24, 47–52. doi:10.1159/000368755

Hay, N. (2016). Reprogramming Glucose Metabolism in Cancer: Can it Be Exploited for Cancer Therapy? *Nat. Rev. Cancer* 16, 635–649. doi:10.1038/nrc.2016.77

Hongratanaworakit, T., Heuberger, E., and Buchbauer, G. (2004). Evaluation of the Effects of East Indian Sandalwood Oil and Alpha-Santalol on Humans after Transdermal Absorption. *Planta Med.* 70, 3–7. doi:10.1055/s-2004-815446

Hotta, M., Nakata, R., Katsukawa, M., Hori, K., Takahashi, S., and Inoue, H. (2010). Carvacrol, a Component of Thyme Oil, Activates PPARalpha and Gamma and Suppresses COX-2 Expression. *J. Lipid Res.* 51, 132–139. doi:10.1194/jlr.M900255-JLR200

Houdkova, M., Chaure, A., Doskocil, I., Havlik, J., and Kokoska, L. (2021). Broth Macrodilution Volatilization Method for Antibacterial Susceptibility Testing of Volatile Agents and Evaluation of Their Toxicity Using Modified MTT Assay *In Vitro*. *Molecules* 26.

Houdkova, M., Rondevaldova, J., Doskocil, I., Havlik, J., and Kokoska, L. (2017). Evaluation of Antibacterial Potential and Toxicity of Plant Volatile Compounds Using New Broth Microdilution Volatilization Method and Modified MTT Assay. *Fitoterapia* 118, 56–62.

Hulley, I. M., Van Vuuren, S. F., Sadgrove, N. J., and Van Wyk, B. E. (2019). Antimicrobial Activity of *Elytropappus Rhinocerotis* (Asteraceae) against Micro-organisms Associated with Foot Odour and Skin Ailments. *J. Ethnopharmacol* 228, 92–98. doi:10.1016/j.jep.2018.09.014

Hulley, I. M., Sadgrove, N. J., Tilney, P. M., Özek, G., Yur, S., Özek, T., et al. (2018). Essential Oil Composition of *Pentzia Incana* (Asteraceae), an Important Natural Pasture Plant in the Karoo Region of South Africa. *Afr. J. Range Forage Sci.* 35, 137–145. doi:10.2989/10220119.2018.1495265

Irwin, M. R., Wang, M., Ribeiro, D., Cho, H. J., Olmstead, R., Breen, E. C., et al. (2008). Sleep Loss Activates Cellular Inflammatory Signaling. *Biol. Psychiatry* 64, 538–540. doi:10.1016/j.biopsych.2008.05.004

Iso (2015). *International Standards Organisation - Home Page*. [Online]. Available: http://www.iso.org/iso/home.htm (Accessed, , 2015).

Jäger, W. R., Buchbauer, G., Jirovetz, L., and Fritzer, M. (1992). Percutaneous Absorption of Lavender Oil from a Massage Oil. *J. Soc. Cosmet. Chemists* 43, 49–54.

Jamali, C. A., Kasrati, A., Fadli, M., Hassani, L., Leach, D. N., and Abbad, A. (2017). Synergistic Effects of Three Moroccan Thyme Essential Oils with Antibiotic Cefixime. *Phytothérapie*, 1–6. doi:10.1007/s10298-017-1107-2

Jin, M., Qian, Z., Yin, J., Xu, W., and Zhou, X. (2018). The Role of Intestinal Microbiota in Cardiovascular Disease. *J. Cel Mol Med* 23, 2343–2350. doi:10.1111/jcmm.14195

Jing, L., Zhang, Y., Fan, S., Gu, M., Guan, Y., Lu, X., et al. (2013). Preventive and Ameliorating Effects of Citrus D-Limonene on Dyslipidemia and Hyperglycemia in Mice with High-Fat Diet-Induced Obesity. *Eur. J. Pharmacol.* 715, 46–55. doi:10.1016/j.ejphar.2013.06.022

Johnson, R. J., Sánchez-Lozada, L. G., Andrews, P., and Lanaspa, M. A. (2017). Perspective: A Historical and Scientific Perspective of Sugar and its Relation with Obesity and Diabetes. *Adv. Nutr.* 8, 412–422. doi:10.3945/an.116.014654

Jones, G. L., and Sadgrove, N. J. (2015). "Ethnopharmacology in Australia and Oceania," in *Ethnopharmacology - A Reader*. Editors M. Heinrich and A. K. Jäger (West Sussex, UK: John Wiley & Sons).

Kambe, D., Kotani, M., Yoshimoto, M., Kaku, S., Chaki, S., and Honda, K. (2010). Effects of Quercetin on the Sleep-Wake Cycle in Rats: Involvement of Gamma-Aminobutyric Acid Receptor Type A in Regulation of Rapid Eye Movement Sleep. *Brain Res.* 1330, 83–88. doi:10.1016/j.brainres.2010.03.033

Katsukawa, M., Nakata, R., Takizawa, Y., Hori, K., Takahashi, S., and Inoue, H. (2010). Citral, a Component of Lemongrass Oil, Activates PPARα and γ and Suppresses COX-2 Expression. *Biochim. Biophys. Acta* 1801, 1214–1220. doi:10.1016/j.bbalip.2010.07.004

Khameneh, B., Iranshahy, M., Soheili, V., and Fazly Bazzaz, B. S. (2019). Review on Plant Antimicrobials: a Mechanistic Viewpoint. *Antimicrob. Resist. Infect. Control.* 8, 118. doi:10.1186/s13756-019-0559-6

Khumalo, G. P., Sadgrove, N. J., Van Vuuren, S., and Van Wyk, B.-E. (2019). Antimicrobial Activity of Volatile and Non-volatile Isolated Compounds and Extracts from the Bark and Leaves of *Warburgia Salutaris* (Canellaceae) against Skin and Respiratory Pathogens. *South Afr. J. Bot.* 122, 547–550. doi:10.1016/j.sajb.2018.10.018

Kohlert, C., Schindler, G., März, R. W., Abel, G., Brinkhaus, B., Derendorf, H., et al. (2002). Systemic Availability and Pharmacokinetics of Thymol in Humans. *J. Clin. Pharmacol.* 42, 731–737. doi:10.1177/009127002401102678

Kohlert, C., Van Rensen, I., März, R., Schindler, G., Graefe, E. U., and Veit, M. (2000). Bioavailability and Pharmacokinetics of Natural Volatile Terpenes in Animals and Humans. *Planta Med.* 66, 495–505. doi:10.1055/s-2000-8616

Kon, K. V., and Rai, M. K. (2012). Plant Essential Oils and Their Constituents in Coping with Multidrug-Resistant Bacteria. *Expert Rev. Anti Infect. Ther.* 10, 775–790. doi:10.1586/eri.12.57

Koyama, S., Purk, A., Kaur, M., Soini, H. A., Novotny, M. V., Davis, K., et al. (2019). Beta-caryophyllene Enhances Wound Healing through Multiple Routes. *PLoS one* 14, e0216104. doi:10.1371/journal.pone.0216104

Kunihiro, A. G., Luis, P. B., Brickey, J. A., Frye, J. B., Chow, H. S., Schneider, C., et al. (2019). Beta-Glucuronidase Catalyzes Deconjugation and Activation of Curcumin-Glucuronide in Bone. *J. Nat. Prod.* 82, 500–509. doi:10.1021/acs.jnatprod.8b00873

Langat, M. K., Mayowa, Y., Sadgrove, N., Danyaal, M., Prescott, T. A. K., Kami, T., et al. (2021). Multi-layered Antimicrobial Synergism of (E)-caryophyllene with Minor Compounds, Tecleanatalensine B and Normelicopine, from the Leaves of Vepris Gossweileri (I. Verd.) Mziray. *Nat. Product. Res.*, 1–11. doi:10.1080/14786419.2021.1899176

Lassak, E., and Southwell, I. (1974). Occurrence of Some Unusual Compounds in the Leaf Oils of *Eriostemon Obovalis* and *Phebalium Glandulosum* Subsp. *Glandulosum*. *Aust. J. Chem.* 27, 2703–2705. doi:10.1071/ch9742703

Lassila, T., Mattila, S., Turpeinen, M., Pelkonen, O., and Tolonen, A. (2016). Tandem Mass Spectrometric Analysis of S- and N-Linked Glutathione Conjugates of Pulegone and Menthofuran and Identification of P450 Enzymes Mediating Their Formation. *Rapid Commun. Mass. Spectrom.* 30, 917–926. doi:10.1002/rcm.7518

Lee, S. H., Heo, Y., and Kim, Y. C. (2010). Effect of German Chamomile Oil Application on Alleviating Atopic Dermatitis-like Immune Alterations in Mice. *J. Vet. Sci.* 11, 35–41. doi:10.4142/jvs.2010.11.1.35

Leonardini, A., Laviola, L., Perrini, S., Natalicchio, A., and Giorgino, F. (2009). Cross-Talk between PPARgamma and Insulin Signaling and Modulation of Insulin Sensitivity. *PPAR Res.* 2009, 818945. doi:10.1155/2009/818945

Lesgards, J. F., Baldovini, N., Vidal, N., and Pietri, S. (2014). Anticancer Activities of Essential Oils Constituents and Synergy with Conventional Therapies: A Review. *Phytother Res.* 28, 1423–1446. doi:10.1002/ptr.5165

Li, J. E., Futawaka, K., Yamamoto, H., Kasahara, M., Tagami, T., Liu, T. H., et al. (2015). Cinnamaldehyde Contributes to Insulin Sensitivity by Activating PPARδ, PPARγ, and RXR. *Am. J. Chin. Med.* 43, 879–892. doi:10.1142/S0192415X15500512

Li, Y., Fu, X., Ma, X., Geng, S., Jiang, X., Huang, Q., et al. (2018). Intestinal Microbiome-Metabolome Responses to Essential Oils in Piglets. *Front. Microbiol.* 9, 1988. doi:10.3389/fmicb.2018.01988

Liang, W. Z., and Lu, C. H. (2012). Carvacrol-induced [Ca2+]i Rise and Apoptosis in Human Glioblastoma Cells. *Life Sci.* 90, 703–711. doi:10.1016/j.lfs.2012.03.027

Liu, J., Zhu, Y., Du, G., Zhou, J., and Chen, J. (2013). Response of *Saccharomyces cerevisiae* to *D*-Limonene-Induced Oxidative Stress. *Appl. Microbiol. Biotechnol.* 97, 6467–6475. doi:10.1007/s00253-013-4931-9

Ma, D., He, J., and He, D. (2020). Chamazulene Reverses Osteoarthritic Inflammation through Regulation of Matrix Metalloproteinases (MMPs) and NF-Kβ Pathway in *In-Vitro* and *In-Vivo* Models. *Biosci. Biotechnol. Biochem.* 84, 402–410. doi:10.1080/09168451.2019.1682511

Mandeville, A., and Cock, I. E. (2018). *Terminalia Chebula* Retz. Fruit Extracts Inhibit Bacterial Triggers of Some Autoimmune Diseases and Potentiate the Activity of Tetracycline. *Indian J. Microbiol.* 58, 496–506. doi:10.1007/s12088-018-0754-9

Mao, Q. Q., Xu, X. Y., Cao, S. Y., Gan, R. Y., Corke, H., Beta, T., et al. (2019). Bioactive Compounds and Bioactivities of Ginger (*Zingiber Officinale* Roscoe). *Foods* 8, 185. doi:10.3390/foods8060185

Medeiros, R., Passos, G. F., Vitor, C. E., Koepp, J., Mazzuco, T. L., Pianowski, L. F., et al. (2007). Effect of Two Active Compounds Obtained from the Essential Oil of *Cordia Verbenacea* on the Acute Inflammatory Responses Elicited by LPS in the Rat Paw. *Br. J. Pharmacol.* 151, 618–627. doi:10.1038/sj.bjp.0707270

Miao, Y., Sun, Y., Wang, W., Du, B., Xiao, S. E., Hu, Y., et al. (2013). 6-Gingerol Inhibits Hair Shaft Growth in Cultured Human Hair Follicles and Modulates Hair Growth in Mice. *PLoS one* 8, e57226. doi:10.1371/journal.pone.0057226

Mikulášová, M., Chovanová, R., and Vaverková, Š. (2016). Synergism between Antibiotics and Plant Extracts or Essential Oils with Efflux Pump Inhibitory Activity in Coping with Multidrug-Resistant Staphylococci. *Phytochemistry Rev.* 15, 651–662.

Miller, J. A., Hakim, I. A., Chew, W., Thompson, P., Thomson, C. A., and Chow, H. H. (2010). Adipose Tissue Accumulation of *D*-Limonene with the Consumption of a Lemonade Preparation Rich in *D*-Limonene Content. *Nutr. Cancer* 62, 783–788. doi:10.1080/01635581003693066

Mohamed, O. I., El-Nahas, A. F., El-Sayed, Y. S., and Ashry, K. M. (2016). Ginger Extract Modulates Pb-Induced Hepatic Oxidative Stress and Expression of Antioxidant Gene Transcripts in Rat Liver. *Pharm. Biol.* 54, 1164–1172. doi:10.3109/13880209.2015.1057651

Moussaieff, A., Rimmerman, N., Bregman, T., Straiker, A., Felder, C. C., Shoham, S., et al. (2008). Incensole Acetate, an Incense Component, Elicits Psychoactivity by Activating TRPV3 Channels in the Brain. *FASEB J.* 22, 3024–3034. doi:10.1096/fj.07-101865

Mühlbauer, R. C., Lozano, A., Palacio, S., Reinli, A., and Felix, R. (2003). Common Herbs, Essential Oils, and Monoterpenes Potently Modulate Bone Metabolism. *Bone* 32, 372–380. doi:10.1016/s8756-3282(03)00027-9

Murni, V. W., Saepudin, E., Cahyana, A. H., Rahayu, D. U. C., Hastuti, L. T., and Haib, J. (2016). Effect of Oven Drying and Storage on Essential Oil Composition of Clove (*Syzygium Aromaticum*) from Toli-Toli. *Int. Symp. Curr. Prog. Math. Sci.* 1862, 030081–030084, .

Nölder, M., Germer, S., and Koch, E. (2011). Pharmacokinetics of Linalool and Linalyl Acetate, the Two Main Constituents of Silexan, an Essential Oil from *Lavandula Angustifolia* Flowers, in Rats. *Planta Med.* 77, PM44.

Nsangou, M. F., Happi, E. N., Fannang, S. V., Atangana, A. F., Waffo, A. F. K., Wansi, J. D., et al. (2021). Chemical Composition and Synergistic Antimicrobial Effects of a Vegetatively Propagated Cameroonian Lemon, *Citrus X limon* (L.) Osbeck. *ACS Food Sci. Technol.* 1, 354–361. doi:10.1021/acsfoodscitech.0c00071

Ohland, C. L., and Macnaughton, W. K. (2010). Probiotic Bacteria and Intestinal Epithelial Barrier Function. *Am. J. Physiol. Gastrointest. Liver Physiol.* 298, G807–G819. doi:10.1152/ajpgi.00243.2009

Okabe, H., Obata, Y., Takayama, K., and Nagai, T. (1990). Percutaneous Absorption Enhancing Effect and Skin Irritation of Monocyclic Monoterpenes. *Drug Des. Deliv.* 6, 229–238.

Onal, E. M., Afsar, B., Covic, A., Vaziri, N. D., and Kanbay, M. (2019). Gut Microbiota and Inflammation in Chronic Kidney Disease and Their Roles in the Development of Cardiovascular Disease. *Hypertens. Res.* 42, 123–140. doi:10.1038/s41440-018-0144-z

Passos, G. F., Fernandes, E. S., Da Cunha, F. M., Ferreira, J., Pianowski, L. F., Campos, M. M., et al. (2007). Anti-inflammatory and Anti-allergic Properties of the Essential Oil and Active Compounds from *Cordia Verbenacea*. *J. Ethnopharmacol* 110, 323–333. doi:10.1016/j.jep.2006.09.032

Pauli, A., and Schilcher, H. (2010). "*In Vitro* Antimicrobial Activities of Essential Oils Monographed in the European Pharmacopoeia 6th Edition," in *Handbook of Essential Oils: Science, Technology, and Applications*. Editors K. H. C. Başer and G. Buchbauer (Boca Raton, FL: CRC Press), 353–547. doi:10.1201/9781420063165-c12

Pavan, B., Dalpiaz, A., Marani, L., Beggiato, S., Ferraro, L., Canistro, D., et al. (2018). Geraniol Pharmacokinetics, Bioavailability and its Multiple Effects on the Liver Antioxidant and Xenobiotic-Metabolizing Enzymes. *Front. Pharmacol.* 9, 18. doi:10.3389/fphar.2018.00018

Petrie, J. R., Guzik, T. J., and Touyz, R. M. (2018). Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms. *Can. J. Cardiol.* 34, 575–584. doi:10.1016/j.cjca.2017.12.005

Raman, G., and Gaikar, V. G. (2003). Hydrotropic Solubilization of Boswellic Acids from *Boswellia Serrata* Resin. *Langmuir* 19, 8026–8032. doi:10.1021/la034611r

Russo, E. B. (2011). Taming THC: Potential Cannabis Synergy and Phytocannabinoid-Terpenoid Entourage Effects. *Br. J. Pharmacol.* 163, 1344–1364. doi:10.1111/j.1476-5381.2011.01238.x

Saad, N. Y., Muller, C. D., and Lobstein, A. (2013). Major Bioactivities and Mechanism of Action of Essential Oils and Their Components. *Flavour Fragr. J.* 28, 269–279. doi:10.1002/ffj.3165

Sadgrove, N. J. (2020a). Comparing Essential Oils from Australia's 'Victorian Christmas Bush' (Prostanthera Lasianthos Labill., Lamiaceae) to Closely Allied New Species: Phenotypic Plasticity and Taxonomic Variability. *Phytochemistry* 176, 112403. doi:10.1016/j.phytochem.2020.112403

Sadgrove, N. J., and Jones, G. L. (2013). A Possible Role of Partially Pyrolysed Essential Oils in Australian Aboriginal Traditional Ceremonial and Medicinal Smoking Applications of *Eremophila longifolia* (R. Br.) F. Muell (*Scrophulariaceae*). *J. Ethnopharmacol* 147, 638–644. doi:10.1016/j.jep.2013.03.060

Sadgrove, N. J., and Jones, G. L. (2014a). Cytogeography of Essential Oil Chemotypes of *Eremophila longifolia* F. Muell (Scrophulariaceae). *Phytochemistry* 105, 43–51. doi:10.1016/j.phytochem.2014.05.005

Sadgrove, N. J., and Jones, G. L. (2019). From Petri Dish to Patient: Bioavailability Estimation and Mechanism of Action for Antimicrobial and Immunomodulatory Natural Products. *Front. Microbiol.* 10, 2470. doi:10.3389/fmicb.2019.02470

Sadgrove, N. J., Jones, G. L., and Greatrex, B. W. (2014). Isolation and Characterisation of (-)-genifuranal: The Principal Antimicrobial Component in Traditional Smoking Applications of *Eremophila longifolia* (Scrophulariaceae) by Australian Aboriginal Peoples. *J. Ethnopharmacol* 154, 758–766. doi:10.1016/j.jep.2014.05.003

Sadgrove, N. J., Madeley, L. G., and Van Wyk, B. E. (2019). Volatiles from African Species of *Croton* (Euphorbiaceae), Including New Diterpenes in Essential Oil from *Croton Gratissimus*. *Heliyon* 5, e02677. doi:10.1016/j.heliyon.2019.e02677

Sadgrove, N. J., Padilla-González, G. F., Green, A., Langat, M. K., Mas-Claret, E., Lyddiard, D., et al. (2021). The Diversity of Volatile Compounds in Australia's Semi-desert Genus Eremophila (Scrophulariaceae). *Plants (Basel)* 116, 1170–1178. doi:10.3390/plants10040785

Sadgrove, N. J., Padilla-González, G. F., Telford, I. R. H., Greatrex, B. W., Jones, G. L., Andrew, R., et al. (2020a). Prostanthera (Lamiaceae) as a 'Cradle of Incense': Chemophenetics of Rare Essential Oils from Both New and Forgotten Australian 'Mint Bush' Species. *Plants (Basel)* 9, 1570. doi:10.3390/plants9111570

Sadgrove, N. J., Greatrex, B. W., and Jones, G. L. (2015). α-Cyclodextrin Encapsulation Enhances Antimicrobial Activity of Cineole-Rich Essential Oils from Australian Species of Prostanthera (Lamiaceae). *Nat. Volatiles Essent. OIls* 2, 30–38.

Sadgrove, N. J., and Jones, G. L. (2014b). Reviewing the Importance of Aromatic Medicinal Plants in the Traditional Pharmacopoeia of Australian Aboriginal People. XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014). *V World* 1125, 297–302.

Sadgrove, N., and Jones, G. (2015). A Contemporary Introduction to Essential Oils: Chemistry, Bioactivity and Prospects for Australian Agriculture. *Agriculture* 5, 48–102. doi:10.3390/agriculture5010048

Sadgrove, N. J. (2020b). Southern Africa as a 'cradle of Incense' in Wider African Aromatherapy. *South. Africa as a 'cradle incense' wider Scientific Afr.* 9, e00502. doi:10.1016/j.sciaf.2020.e00502

Sadgrove, N. J., Telford, I. R. H., Padilla-González, G. F., Greatrex, B. W., and Bruhl, J. J. (2020b). GC-MS 'chemophenetics' on Australian Pink-Flowered Phebalium (Rutaceae) Using Herbarium Leaf Material Demonstrates Phenetic Agreement with Putative New Species. *Phytochemistry Lett.* 38, 112–120. doi:10.1016/j.phytol.2020.05.014

Sadgrove, N. J. (2021). The 'bald' Phenotype (Androgenetic Alopecia) Is Caused by the High Glycaemic, High Cholesterol and Low mineral 'western Diet'. *Trends Food Sci. Tech.* doi:10.1016/j.tifs.2021.06.056

Sadgrove, N., Mijajlovic, S., Tucker, D. J., Watson, K., and Jones, G. L. (2011). Characterization and Bioactivity of Essential Oils from Novel Chemotypes of *Eremophila longifolia* (F. Muell) (Myoporaceae): a Highly Valued Traditional Australian Medicine. *Flavour Fragrance J.* 26, 341–350. doi:10.1002/ffj.2062

Šadibolová, M., Zárybnický, T., Smutný, T., Pávek, P., Šubrt, Z., Matoušková, P., et al. (2019). Sesquiterpenes Are Agonists of the Pregnane X Receptor but Do Not Induce the Expression of Phase I Drug-Metabolizing Enzymes in the Human Liver. *Int. J. Mol. Sci.* 20, 4562.

Schnaubelt, K. (1999). *Medical Aromatherapy: Healing with Essential Oils*. Bombay, India: Frog Books.

Sebai, H., Selmi, S., Rtibi, K., Souli, A., Gharbi, N., and Sakly, M. (2013). Lavender (*Lavandula Stoechas* L.) Essential Oils Attenuate Hyperglycemia and Protect against Oxidative Stress in Alloxan-Induced Diabetic Rats. *Lipids Health Dis.* 12, 189. doi:10.1186/1476-511X-12-189

Sell, C. (2010). "Chapter 5. Chemistry of Essential Oils," in *Handbook of Essential Oils: Science, Technology, and Applications*. Editors K. H. C. Başer and G. Buchbauer (London: CRC Press, Taylor and Francis Group).

Serrano, E., Cornu, A., Kondjoyan, N., Figueredo, G., Agabriel, J., and Micol, D. (2007). Terpene Accumulation in Muscle and Fatty Tissues of Calves Supplemented with Essential Oils. *J. Anim. Feed Sci.* 16, 168–179. doi:10.22358/jafs/66736/2007

Sharma, M., Levenson, C., Bell, R. H., Anderson, S. A., Hudson, J. B., Collins, C. C., et al. (2014). Suppression of Lipopolysaccharide-Stimulated Cytokine/chemokine Production in Skin Cells by Sandalwood Oils and Purified α-santalol and β-santalol. *Phytother Res.* 28, 925–932. doi:10.1002/ptr.5080

Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001). Structure, Function and Evolution of Glutathione Transferases: Implications for Classification of Non-mammalian Members of an Ancient Enzyme Superfamily. *Biochem. J.* 360, 1–16. doi:10.1042/0264-6021:3600001

Shi, F., Zhao, Y., Firempong, C. K., and Xu, X. (2016). Preparation, Characterization and Pharmacokinetic Studies of Linalool-Loaded Nanostructured Lipid Carriers. *Pharm. Biol.* 54, 2320–2328. doi:10.3109/13880209.2016.1155630

Shimoi, K., and Nakayama, T. (2005). Glucuronidase Deconjugation in Inflammation. *Methods Enzymol.* 400, 263–272. doi:10.1016/S0076-6879(05)00015-7

Smith, J., Tucker, D., Alter, D., Watson, K., and Jones, G. (2010). Intraspecific Variation in Essential Oil Composition of *Eremophila longifolia* F. Muell. (Myoporaceae): Evidence for Three Chemotypes. *Phytochemistry* 71, 1521–1527. doi:10.1016/j.phytochem.2010.06.006

Soundharrajan, I., Kim, D. H., Srisesharam, S., Kuppusamy, P., and Choi, K. C. (2018). R-limonene Enhances Differentiation and 2-Deoxy-D-Glucose Uptake in 3T3-L1 Preadipocytes by Activating the Akt Signaling Pathway. *Evid. Based Complement. Alternat Med.* 2018, 4573254. doi:10.1155/2018/4573254

Sperker, B., Tomkiewicz, C., Burk, O., Barouki, R., and Kroemer, H. K. (2001). Regulation of Human Beta-Glucuronidase by A23187 and Thapsigargin in the Hepatoma Cell Line HepG2. *Mol. Pharmacol.* 59, 177–182. doi:10.1124/mol.59.2.177

Su, Y. C., Cheng, T. C., Leu, Y. L., Roffler, S. R., Wang, J. Y., Chuang, C. H., et al. (2014). PET Imaging of β-glucuronidase Activity by an Activity-Based 124I-Trapping Probe for the Personalized Glucuronide Prodrug Targeted Therapy. *Mol. Cancer Ther.* 13, 2852–2863. doi:10.1158/1535-7163.MCT-14-0212

Sueke, H., Kaye, S. B., Neal, T., Hall, A., Tuft, S., and Parry, C. M. (2010). An *In Vitro* Investigation of Synergy or Antagonism between Antimicrobial Combinations against Isolates from Bacterial Keratitis. *Invest. Ophthalmol. Vis. Sci.* 51, 4151–4155. doi:10.1167/iovs.09-4839

Talpur, N., Echard, B., Ingram, C., Bagchi, D., and Preuss, H. (2004). Effects of a Novel Formulation of Essential Oils on Glucose-Insulin Metabolism in Diabetic and Hypertensive Rats: a Pilot Study. *Diabetes Obes. Metab.* 7, 193–199. doi:10.1111/j.1463-1326.2004.00386.x

Tew, K. D., Manevich, Y., Grek, C., Xiong, Y., Uys, J., and Townsend, D. M. (2011). The Role of Glutathione S-Transferase P in Signaling Pathways and

S-Glutathionylation in Cancer. *Free Radic. Biol. Med.* 51, 299–313. doi:10.1016/j.freeradbiomed.2011.04.013

Thompson, D., Constantin-Teodosiu, D., Egestad, B., Mickos, H., and Moldéus, P. (1990). Formation of Glutathione Conjugates during Oxidation of Eugenol by Microsomal Fractions of Rat Liver and Lung. *Biochem. Pharmacol.* 39, 1587–1595. doi:10.1016/0006-2952(90)90525-p

Tisserand, R., and Young, R. (2013). *Essential Oil Safety: A Guide for Health Care Professionals*, 2e. Elsevier.

Townsend, D. M., and Tew, K. D. (2003). The Role of Glutathione-S-Transferase in Anti-cancer Drug Resistance. *Oncogene* 22, 7369–7375. doi:10.1038/sj.onc.1206940

Urasaki, Y., Beaumont, C., Talbot, J. N., Hill, D. K., and Le, T. T. (2020). Akt3 Regulates the Tissue-specific Response to Copaiba Essential Oil. *Int. J. Mol. Sci.* 21, 2851. doi:10.3390/ijms21082851

Valdivieso-Ugarte, M., Gomez-Llorente, C., Plaza-Díaz, J., and Gil, Á. (2019). Antimicrobial, Antioxidant, and Immunomodulatory Properties of Essential Oils: A Systematic Review. *Nutrients* 11, 2786. doi:10.3390/nu11112786

Valente, A., Carrillo, A. E., Tzatzarakis, M. N., Vakonaki, E., Tsatsakis, A. M., Kenny, G. P., et al. (2015). The Absorption and Metabolism of a Single L-Menthol Oral versus Skin Administration: Effects on Thermogenesis and Metabolic Rate. *Food Chem. Toxicol.* 86, 262–273. doi:10.1016/j.fct.2015.09.018

Van Bambeke, F., Glupczynski, Y., Plésiat, P., Pechère, J. C., and Tulkens, P. M. (2003). Antibiotic Efflux Pumps in Prokaryotic Cells: Occurrence, Impact on Resistance and Strategies for the Future of Antimicrobial Therapy. *J. Antimicrob. Chemother.* 51, 1055–1065. doi:10.1093/jac/dkg224

Van Vuuren, S., and Holl, D. (2017). Antimicrobial Natural Product Research: A Review from a South African Perspective for the Years 2009-2016. *J. Ethnopharmacol* 208, 236–252. doi:10.1016/j.jep.2017.07.011

Van Vuuren, S., and Viljoen, A. (2011). Plant-based Antimicrobial Studies-Mmethods and Approaches to Study the Interaction between Natural Products. *Planta Med.* 77, 1168–1182. doi:10.1055/s-0030-1250736

Victor Antony Santiago, J., Jayachitra, J., Shenbagam, M., and Nalini, N. (2012). Dietary *D*-Limonene Alleviates Insulin Resistance and Oxidative Stress-Induced Liver Injury in High-Fat Diet and L-NAME-Treated Rats. *Eur. J. Nutr.* 51, 57–68. doi:10.1007/s00394-011-0182-7

Vigushin, D. M., Poon, G. K., Boddy, A., English, J., Halbert, G. W., Pagonis, C., et al. (1998). Phase I and Pharmacokinetic Study of D-Limonene in Patients with Advanced Cancer. Cancer Research Campaign Phase I/II Clinical Trials Committee. *Cancer Chemother. Pharmacol.* 42, 111–117. doi:10.1007/s002800050793

Wang, S., Sun, X., Jiang, L., Liu, X., Chen, M., Yao, X., et al. (2016). 6-Gingerol Induces Autophagy to Protect HUVECs Survival from Apoptosis. *Chem. Biol. Interact* 256, 249–256. doi:10.1016/j.cbi.2016.07.020

Wenqiang, G., Shufen, L., Ruixiang, Y., Shaokun, T., and Can, Q. (2007). Comparison of Essential Oils of Clove Buds Extracted with Supercritical Carbon Dioxide and Other Three Traditional Extraction Methods. *Food Chem.* 101, 1558–1564.

Wu, Y., Zhang, Y., Xie, G., Zhao, A., Pan, X., Chen, T., et al. (2012). The Metabolic Responses to Aerial Diffusion of Essential Oils. *PLoS one* 7, e44830. doi:10.1371/journal.pone.0044830

Xu, X., Li, Y., Hou, J., Zhang, S., Xu, Y., Wang, Y., et al. (2011). Pharmacokinetic Study of Borneol and Menthol in Rats after Oral Administration of Qingyan Drop Pills. *Planta Med.* 77, 1600–1604. doi:10.1055/s-0030-1270998

Zámboriné Németh, É., and Thi Nguyen, H. (2020). Thujone, a Widely Debated Volatile Compound: what Do We Know about it? *Phytochem. Rev.* 19, 405–423. doi:10.1007/s11101-020-09671-y

Zehetner, P., Höferl, M., and Buchbauer, G. (2019). Essential Oil Components and Cytochrome P450 Enzymes: a Review. *Flavour Fragr J.* 34, 223–240. doi:10.1002/ffj.3496

Zhong, C., Qu, C., Wang, B., Liang, S., and Zeng, B. (2017). Probiotics for Preventing and Treating Small Intestinal Bacterial Overgrowth: A Meta-Analysis and Systematic Review of Current Evidence. *J. Clin. Gastroenterol.* 51, 300–311. doi:10.1097/MCG.0000000000000814

Zhou, X., Li, J., Guo, J., Geng, B., Ji, W., Zhao, Q., et al. (2018). Gut-dependent Microbial Translocation Induces Inflammation and Cardiovascular Events after ST-Elevation Myocardial Infarction. *Microbiome* 6, 66. doi:10.1186/s40168-018-0441-4

Zhu, R., Liu, H., Liu, C., Wang, L., Ma, R., Chen, B., et al. (2017). Cinnamaldehyde in Diabetes: A Review of Pharmacology, Pharmacokinetics and Safety. *Pharmacol. Res.* 122, 78–89. doi:10.1016/j.phrs.2017.05.019

Zidorn, C. (2019). Plant Chemophenetics - A New Term for Plant Chemosystematics/plant Chemotaxonomy in the Macro-Molecular Era. *Phytochemistry* 163, 147–148. doi:10.1016/j.phytochem.2019.02.013

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 27 October 2021
doi: 10.3389/fphar.2021.757090

Antidiabetic Medicinal Plants Used in Democratic Republic of Congo: A Critical Review of Ethnopharmacology and Bioactivity Data

Félicien Mushagalusa Kasali[1,2,3], Justin Ntokamunda Kadima[2,4], Emanuel L. Peter[1,3,5], Andrew G. Mtewa[1,3,6], Clement Olusoji Ajayi[1,3,7], Jonans Tusiimire[3], Casim Umba Tolo[1], Patrick Engeu Ogwang[1,3], Anke Weisheit[1] and Amon Ganafa Agaba[8]*

[1]Pharm-Bio Technology and Traditional Medicine Center, Mbarara University of Science and Technology, Mbarara, Uganda, [2]Department of Pharmacy, Faculty of Pharmaceutical Sciences and Public Health, Official University of Bukavu, Bukavu, Democratic Republic of Congo, [3]Department of Pharmacy, Faculty of Medicine, Mbarara University of Science and Technology, Mbarara, Uganda, [4]Department of Pharmacology, School of Medicine and Pharmacy, University of Rwanda, Huye, Rwanda, [5]Department of Innovation, Technology Transfer and Commercialization, National Institute for Medical Research, Dar es Salaam, Tanzania, [6]Chemistry Section, Department of Applied Studies, Institute of Technology, Malawi University of Science and Technology, Limbe, Malawi, [7]Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria, [8]Department of Pharmacology and Therapeutics, Faculty of Medicine, Mbarara University of Science and Technology, Mbarara, Uganda

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Annah Moteetee, University of Johannesburg, South Africa
Roodabeh Bahramsoltani, Tehran University of Medical Sciences, Iran
Da-Cheng Hao, Dalian Jiaotong University, China
Fikri-Benbrahim Kawtar, Sidi Mohamed Ben Abdellah University, Morocco

***Correspondence:**
Félicien Mushagalusa Kasali
felicienkasali@gmail.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *11 August 2021*
Accepted: *08 October 2021*
Published: *27 October 2021*

Citation:
Kasali FM, Kadima JN, Peter EL, Mtewa AG, Ajayi CO, Tusiimire J, Tolo CU, Ogwang PE, Weisheit A and Agaba AG (2021) Antidiabetic Medicinal Plants Used in Democratic Republic of Congo: A Critical Review of Ethnopharmacology and Bioactivity Data. Front. Pharmacol. 12:757090. doi: 10.3389/fphar.2021.757090

Several studies have been conducted and published on medicinal plants used to manage Diabetes Mellitus worldwide. It is of great interest to review available studies from a country or a region to resort to similarities/discrepancies and data quality. Here, we examined data related to ethnopharmacology and bioactivity of antidiabetic plants used in the Democratic Republic of Congo. Data were extracted from Google Scholar, Medline/PubMed, Scopus, ScienceDirect, the Wiley Online Library, Web of Science, and other documents focusing on ethnopharmacology, pharmacology, and phytochemistry antidiabetic plants used in the Democratic Republic of Congo from 2005 to September 2021. The Kew Botanic Royal Garden and Plants of the World Online web databases were consulted to verify the taxonomic information. CAMARADES checklist was used to assess the quality of animal studies and Jadad scores for clinical trials. In total, 213 plant species belonging to 72 botanical families were reported. Only one plant, *Droogmansia munamensis,* is typically native to the DRC flora; 117 species are growing in the DRC and neighboring countries; 31 species are either introduced from other regions, and 64 are not specified. Alongside the treatment of Diabetes, about 78.13% of plants have multiple therapeutic uses, depending on the study sites. Experimental studies explored the antidiabetic activity of 133 plants, mainly in mice, rats, guinea pigs, and rabbits. Several chemical classes of antidiabetic compounds isolated from 67 plant species have been documented. Rare phase II clinical trials have been conducted. Critical issues included poor quality methodological protocols,

Abbreviations: 11ß-HSD1, 11β-hydroxysteroid dehydrogenase type 1; BG, blood glucose; DM, diabetes mellitus; DMT2, Diabetes mellitus type 2; DPP-4, dipeptidyl peptidase-4; DRC, Democratic Republic of Congo; HIV/AIDS, human immunodeficiency *virus*/Acquired immunodeficiency syndrome; GLP-1, glucagon-like peptide- 1; GLUT, Glucose Transport; HbA1c, Glycosylated hemoglobin; LD_{50}, lethal dose 50; mRNA, Messenger ribonucleic acid; OGTT, Oral Glucose Tolerance Test; PPAR, peroxisome proliferator-activated receptor; PTP1B, Protein tyrosine phosphatase 1B.

author name incorrectly written (16.16%) or absent (14.25%) or confused with a synonym (4.69%), family name revised (17.26%) or missing (1.10%), voucher number not available 336(92.05%), ecological information not reported (49.59%). Most plant species have been identified and authenticated (89.32%). Hundreds of plants are used to treat Diabetes by traditional healers in DRC. However, most plants are not exclusively native to the local flora and have multiple therapeutic uses. The analysis showed the scarcity or absence of high-quality, in-depth pharmacological studies. There is a need to conduct further studies of locally specific species to fill the gap before their introduction into the national pharmacopeia.

Keywords: antidiabetic plants, ethnopharmacology, phytochemicals, bioactivity, Democratic Republic of Congo

1 INTRODUCTION

Most African traditional healers who detain ancestral heritages are illiterate, and their knowledge transmitted verbally from generation to generation is at risk of disappearing. To minimize such risk, the World Health Organization (WHO) recommends scientists carry out ethnopharmacological and experimental studies to record folk knowledge, create databases, and validate scientifically traditional claims from the perspective of developing improved medications (WHO, 2013). WHO estimates that 80% of people rely on conventional medicine to meet primary health care needs, and most of them use remedies from plants (Surya et al., 2014). Ethnopharmacological surveys help gather holistic knowledge and practices of conventional healthcare systems. Experimental investigations evaluate efficacy and safety by developing suitable standardized pharmaceutical dosage forms that can complement, if not replace, current modern medicines. Medicinal plants used as complementary/alternative medicines (CAM) to manage various diseases provide a real opportunity in developed and developing societies. In this sense, herbal medications appear to offer readily available means of managing metabolic disorders by minimizing the risk of side effects and sometimes potentiating the treatment outcomes of modern drugs (Etxeberria et al., 2012). Medicinal plants are also used as food and contain several healthy dietary compounds. For example, some flavonoids interfere with metabolic events and play a crucial role in preventing and managing metabolic disorders through different pathways (Farzaei et al., 2019).

One of the most explored diseases is diabetes mellitus (DM). Over 800 plant species showing hypoglycemic activities can be essential sources for discovering and developing new types of antidiabetic molecules (Patel et al., 2012). The magnitude justifies this craze that Diabetes is gaining more and more globally, making it a severe public health problem. Not long ago, the disease was associated with industrialization. DM is no longer a disease of high-income countries but a global health pandemic. In 2013, according to the International Diabetes Federation, the global population of adults with both type-1(DMT1) and type-2(DMT2) was projected to increase from 382 million to 592 million by 2035, with DMT2 accounting for 90–95% of cases (Glezeva et al., 2017). In Africa, the number was expected to double from 14 million in 2015 to 34 million by 2040. With its continuous and rapid increase in its prevalence worldwide, it should be one of the leading causes of morbidity and mortality in the coming years (Glezeva et al., 2017).

Recent data show about 1.7 million people suffer from DM in the Democratic Republic of Congo (DRC), ranking fourth in the top ten countries by diabetes cases in Africa (Zhivov et al., 2015; Kasangana et al., 2018). Like other African countries, and not withdrawing modern medicines, 80% of people rely on traditional medicine to meet primary health care needs (Mahomoodally, 2013). Ethnopharmacological and pharmacological studies have been conducted globally; however, the related data are disparate and uncontrolled. A preliminary review reported vernacular names, parts used, and the formulation of 70 medicinal plants used to treat DM in DRC. A few phytoconstituents and antidiabetic mechanisms are also mentioned (Jacques et al., 2015).

This review aimed to describe what is known hitherto about ethnopharmacological, pharmacological, and clinical studies embracing medicinal plants used to manage DM in the traditional medicine of the DRC, to highlight which plants are native or introduced, how they are formulated and used, what valid experimental studies have been conducted in preclinical and clinical phases. A critical analysis is made to assess the quality of studies carried inside DRC and resort similarities/discrepancies with studies conducted outside.

2 METHODS

2.1 Literature Search Process

The review was an Internet search on Google Scholar, Medline, PubMed, Scopus, ScienceDirect, the Wiley Online Library, Web of Science, and other documents focusing on ethnopharmacology, pharmacology phytochemistry of antidiabetic plants used in the Democratic Republic of Congo from 2005 to September 2021. The review was conducted following Preferred Regulatory Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines 2009. A total of 34 studies were included. Ethnopharmacological/Ethnobotanical/Ethnomedicinal (n = 24), preclinical bioactivity (n = 9); and one clinical trial (n = 1) studies. One paper includes both an *in vivo* study and an Ethnobotanical survey.

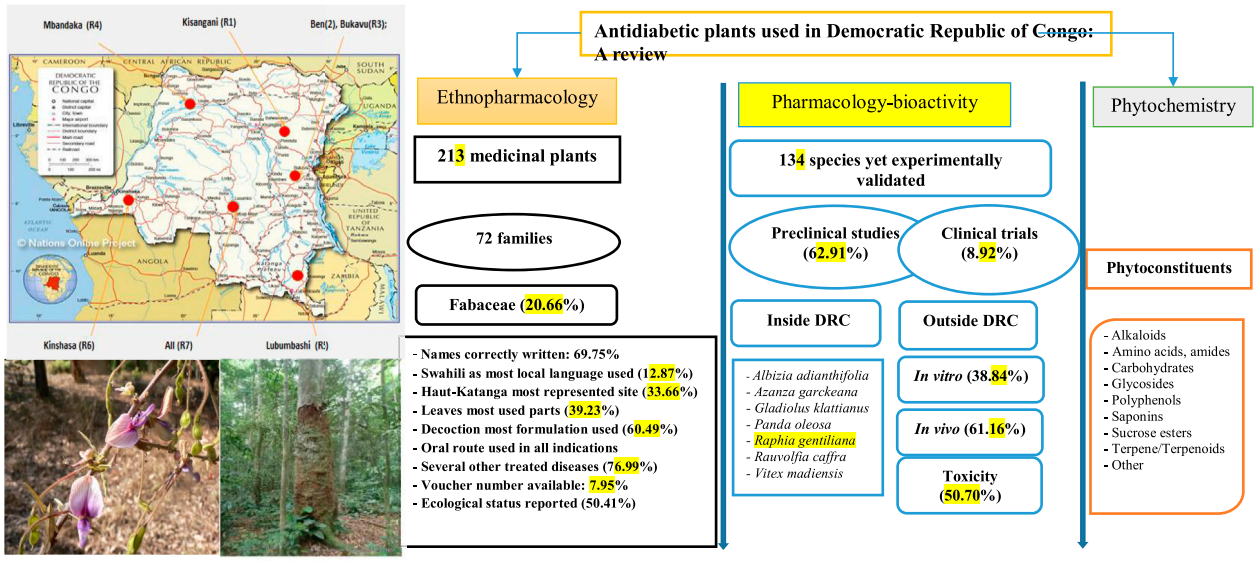


FIGURE 1 | Graphical Abstract.

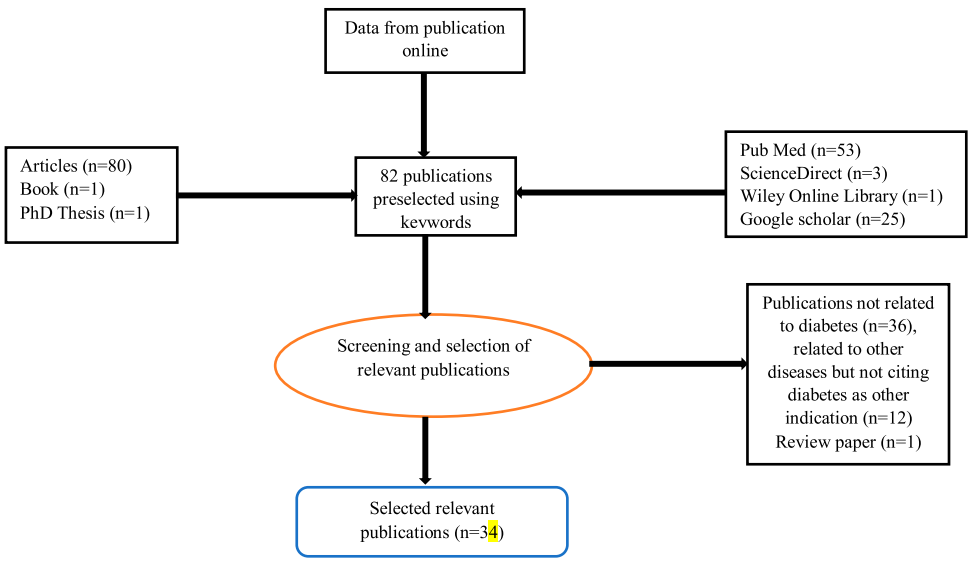


FIGURE 2 | Flowchart for the selection of relevant publications.

2.2 Quality Critical Assessment

Studies that reported ethnopharmacology, phytochemistry, experimental pharmacology, and related clinical data were assessed for eligibility. The Kew Botanic Royal Garden and Plants of the World Online web databases were consulted to verify the taxonomic information on the species mentioned. All species names were checked at the UOB University herbarium. The quality of animal experiments reported was evaluated by examining the peer-reviewed publication, statement of control of temperature, appropriate animal model, compliance with animal welfare regulations for preclinical experiments, random allocation to treatment or management, blinded assessment of outcome, allocation sample size calculation, statement of potential conflict of interests, concealment, use of co-interventions/co-morbid. We used a CAMARADES checklist to assess the quality. Each task was given a quality score out of a possible total of 10 points. Thus, studies were categorized into low quality for mean score 1–5 and high quality for mean score 6–10 (Hooijmans et al., 2014; Auboire et al., 2018). The quality assessment of clinical trials has been evaluated using the Jadad

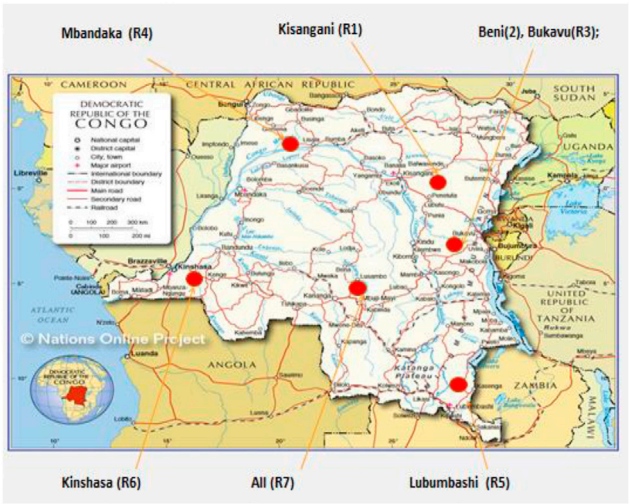


FIGURE 3 | Study sites in the Democratic Republic of Congo.

scale for reporting randomized controlled trials based on randomization, blinding, withdrawals, and dropouts methods (Halpern and Douglas, 2007).

2.3 Statistical Values of Plant Species

Some indexes often express the frequency of quoting for botanical families and plant species. In the present review, the following indexes have been used: *Frequency of citation* (*FC* = Number of times a particular species was mentioned/Total number of times that all species were mentioned x 100); *Relative Frequency of Citation (RFC = FC/N; 0<RFC<1)*: index, where FC is the number of informants who mentioned the use of the species and N, is the total number of informants (Tardío and Pardo-De-Santayana, 2008); *Use Value (UV = ΣU/n)* where U is the number of usable reports for a given plant species cited by each informant and n is the total number of informants interviewed for a given plant (Bano et al., 2014). The *Relative Importance Index (RII)* of each plant species was calculated based on the normalized number of pharmacological properties attributed to it and the normalized number of body systems (BS) it affects (Bennett and Prance, 2000).

2.4 Study Sites

Figure 3 shows different locations where 24 Ethnopharmacological/Ethnobotanical/Ethnomedicinal studies were conducted on the DRC map. The studies were done in Kisangani (R1) by Katemo et al. (2012), Mpiana et al. (2015); in Beni and Lubero (R2) by (Kasika et al., 2015); in Bukavu (R3) by (Karhagomba et al., 2013; Kasali et al., 2013, 2021; Chiribagula et al., 2020; Manya et al., 2020); in Mbandaka, Bagdolite, and Kungu (R4) by (Mongeke et al., 2018) ; in Lubumbashi, Kafubu, Kasumbalesa, Kipushi, Likasi and Sambwa (R5) by (Muya et al., 2014; Mbayo et al., 2016; Amuri et al., 2017, 2018; Bashige-Chiribagula et al., 2017; Mbuyi et al., 2019; Valentin et al., 2020) (Amuri et al., 2017); in Kinshasa, Kwango and Kongo central (R6) by (Ngbolua et al., 2016a, 2016b, 2019; Latham and Mbuta, 2017; Masunda et al., 2019; Pathy et al., 2021); in non-specified sites (R7) by (Moswa et al., 2005; Manzo, 2012).

3 RESULTS

3.1 Ethnopharmacological Data

Table 1 describes the names, parts, forms used, locations, and some statistical values of plants cited. From 24 reviewed papers, we identified 213 plant species belonging to 72 botanical families.

As shown in **Figure 4**, the most frequent botanical families were Fabaceae with 44(20.66%) species, Asteraceae 10(4.69%), Phyllanthaceae 9(4.23%), Malvaceae 8(3.76%), Solanaceae 8(3.76%), Euphorbiaceae 7(3.29%), Rubiaceae 7(3.29%), Apocynaceae 6(2.82%), and Lamiaceae 6(2.82%). Most plants were found at the site R5(33.66%) and R6(27.78%). The distribution varied from study to study. *Catharanthus roseus* was found in almost all locations (6/7 sites) and *Allium cepa* in 5 zones. The vernacular names were linked or not to ethnic dialects. Swahili is the most reported language 48(12.87%), followed by Kongo 46(12.33%), Luba 36(9.65%), and Bemba 32(8.58%). In most cases, the vernacular name is not specified 47(12.60%) or not reported 8(2.14%). The formulations prepared consisted more often of decoction 173(60.49%), maceration 31(10.84%), and infusion 29(10.14%). The leaf is the most used part 122(39.23%), followed by roots 73(23.47%), and stem bark 43(13.83%).

3.2 Pharmacological Investigations Inside Democratic Republic of Congo

3.2.1 Preclinical Pharmacological and Toxicological Investigations Inside Democratic Republic of Congo

Only seven plants presented in **Figure 5** were exclusively studied in experimental animals inside DRC; *Albizia adianthifolia*, *Azanza garckeana*, *Gladiolus klattianus*, *Panda oleosa*, *Raphia gentiliana*, *Rauvolfia caffra*, and *Vitex madiensis*; five studied in DRC. It also reported the only plant species native exclusively from DRC.

3.2.2 Preclinical Toxicological Investigations Inside Democratic Republic of Congo

Many toxicological studies have been carried out in animals (rodents) using plant extracts. Some studies have been undertaken in mice, guinea pigs, and rabbits to explore the acute toxicity of *Panda oleosa*. Endpoints consisted mainly of mortality, pathophysiological syndromes, and microscopic examination of the pancreas and other vital organs pathological changes. The sub-chronic evaluation focused on assessing biochemical, hematological, and histopathological markers after a relatively long period (14 days and sometimes 90 days). The level of exposure to different organs, including the fetus, liver, kidney, heart, etc., of different doses of plant extracts was also determined. Thus, most plant extracts produce a toxic effect in specific organs or systems at high doses.

TABLE 1 | Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Acanthaceae									
Brillantaisia patula T.Anderson	Muleta (Zela), Lembalemba (Kongo),Lesongo (Swahili)	Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Justicia flava (Forssk.) Vahl	Luhe (Luba)	Sb	D	R5Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Amaranthaceae									
Chenopodium ambrosioides L., *Dysphania ambrosioides* (L.) Mosyakin & Clemants (Synonym)	Kulamoka (Kongo), Dikanga (Tshiluba)	Wp	D	R6 Masunda et al. (2019) and R7 Moswa et al. (2005)	2	0.0054	0.0016	0.0008	0.029
Amaryllidaceae									
Allium cepa L.	Itunguru (Swahili) Ditungulu (Tshiluba)	Bk, Sd,Ap	D,M,I	R1 Katemo et al. (2012), R3 (Kasali et al. (2013), R5 Amuri et al. (2018); Mbuyi et al. (2019), R6 Masunda et al. (2019), and R7 Moswa et al. (2005)	6	0.0165	0.0047	0.0016	0.059
Allium sativum L.	Itungurusumu (Mashi), Hayi (Tshiluba)	Bk	D,P,F	R3 Kasali et al. (2013), R5 Amuri et al. (2018), R6 Masunda et al., (2019), and R7 Moswa et al., (2005)	4	0.0110	0.0032	0.0008	0.029
Crinum ornatum (Aiton) Herb.	Munsele bende (Kongo)	Lf	D	R1Katemo et al. (2012)	1	0.0027	0.0008	0.0008	0.029
Anacardiaceae									
Anacardium occidentale L.	Nkasu, diboto (Kongo)	Lf	N	R6 Latham and Mbuta (2017)	1	0.0027	0.0008	0.0016	0.059
Mangifera indica L.	Mutshiwa mangaya (Tshiluba), Hembe (Swahili), Mwembe (Mashi)	Sb, Ro,Lf	D	R1 Katemo et al. (2012), R3 Kasali et al. (2013); Chiribagula et al. (2020), R6 Masunda et al. (2019), and R7 Moswa et al. (2005)	5	0.0137	0.0039	0.0071	0.160
Spondias mombin L.	Mingenge (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Anisophylleaceae									
Anisophyllea boehmii Engl.	Fungo (Sanga), Lufunga (Tabwa)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Annonaceae									
Annona senegalensis Pers. *Annona arenaria* Thonn (Synonym)	Kilolo (Kongo), Bomengo na esobe (Lingala), Lobo (not specified), Lomboloka (not specified)	Ro, Bk,Lf	D,N	R6 Ngbolua et al. (2016b); (2019); Latham and Mbuta (2017); Masunda et al. (2019); Pathy et al. (2021), and R7 (Moswa et al. (2005)	6	0.0165	0.0047	0.0110	0.229
Monodora myristica (Gaertn.) Dunal	Mpei (Lingala)	Fr, Sd	D	R6 Ngbolua et al. (2016a)	1	0.0027	0.0008	0.0016	0.059
Xylopia aethiopica (Dunal) A.Rich.	Nsombo (Not specified), Nkuya nkuya (Not specified)	Bb,Bk	D	R6 Masunda et al. (2019); Pathy et al. (2021)	2	0.0055	0.0016	0.0047	0.151
Apocynaceae									
Catharanthus roseus (L.) G.Don	Pervanche de Madagascar (French), Fulele (Ngwaka), Mtunda (Swahili)	Lf, Ro	D,M,N	R1 Katemo et al. (2012), R2 Kasika et al. (2015), R3 Kasali et al. (2013), R5 Amuri et al. (2018), R6 Latham and Mbuta (2017); Masunda et al. (2019), and R7 Moswa et al. (2005)	7	0.0192	0.0055	0.0063	0.183
Diplorhynchus condylocarpon (Müll.Arg.) Pichon	Mwenge (Swahili)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Rauvolfia caffra Sond.	Mutalala (Bemba)	Fr, Sb	D,M,N	R5 Amuri et al. (2017), (2018)	2	0.0055	0.0016	0.0008	0.029
Rauvolfia obscura K.Schum.	Mudisi (Kbla), Kilungu (Kongo)	Lf	D	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0000	0.000
Rauvolfia vomitoria Wennberg	Pandanganga (Luba)	Ro	D	R1 Katemo et al. (2012), R5 Amuri et al. (2018), and R6 Masunda et al. (2019)	3	0.0082	0.0024	0.0032	0.118
Vinca minor L.	Fololo (Lingala), Vinka nyeupe (Swahili)	Lf, Ro	D,M	R1 Katemo et al. (2012) and R3 Kasali et al. (2013)	2	0.0055	0.0016	0.0079	0.137
Arecaceae									
Elaeis guineensis Jacq.	Ba di ngasi (Kongo), Ngaji(Tshiluba), Palmier à huile (French)	Ro, Lt	D,N	R2 Kasika et al. (2015), R5 Amuri et al. (2018), and R6 Pathy et al. (2021)	3	0.0082	0.0024	0.0221	0.325
Raphia gentiliana De Wild.	Makeke (Not specified), BalempâBakulu (Lingala)	Lf, Sb	M	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0008	0.029
Aristolochiaceae									
Aristolochia hockii De Wild.	Kapanganganga (Bemba)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0024	0.088
Asparagaceae									
Asparagus africanus Lam.	Mukoma wa kanyengelele (Luba)	Lf, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Asphodelaceae									
Aloe congolensis De Wild. & T.Durand	Bà di nseki (not specified)	Lf	D	R6 Pathy et al. (2021)	1	0.0027	0.0008	0.0016	0.059
Aloe vera (L.) Burm.f.	Subiri (Swahili), Kizimia Muliro (Mashi)	Lf, Lt	M,Pr, P	R1 Katemo et al. (2012), R3 Kasali et al. (2013), and R5 Amuri et al. (2018)	3	0.0082	0.0024	0.0008	0.029
Asteraceae									
Ageratum conyzoides L.	Mpala kasakula (Kongo),	Lf	D	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0000	0.000
Artemisia absinthium L.	Kanyambuba kalume (Mashi)	Lf, Sd	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0024	0.088
Artemisia annua L.	Armoise annuelle (French), Sweet Annie(English)	Lf, Sd	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0047	0.151
Bidens pilosa L.	Mpotayambwa (Luba), Kashisha (Swahili) Kokoyalimo (Lokele)	Lf, Sd, Ro	D	R1 Katemo et al. (2012), R3 Kasali et al. (2013), and R5 Amuri et al. (2018)	3	0.0082	0.0024	0.0055	0.206
Calendula officinalis L.	Mundudi ndudi (Not specified)	Bk	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Crassocephalum picridifolium (DC.)	Cifula (Mashi), Bupamba (Kibembe), Anatta (Kibembe)	Lf	D	R3 Chiribagula et al. (2020)	1	0.0027	0.0008	0.0000	0.000
Gymnanthemum coloratum (Willd.) H.Rob. & B.Kahn	Kilulukunju (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Tithonia diversifolia (Hemsl.) A.Gray.	Bilombalomba (Lélé), Mubirizi (Mashi); Mululuca (Bembe)	Lf	M,C	R3 Kasali et al. (2013) and R5 Amuri et al. (2018)	2	0.0055	0.0016	0.0055	0.127
Vernonia amygdalina Delile	Nyata sololo, Mukari kari (Kongo), Mubirizi (Mashi), Mukadi kadi (Kiyanzi), Mindudi mintenla (Kiyombe)	Lf	D	R1 Katemo et al. (2012), R3 Karhagomba et al. (2013); Kasali et al. (2013), R6 Masunda et al. (2019), and R7 Moswa et al. (2005)	5	0.0137	0.0039	0.0095	0.249
Vernonia shirensis Oliv. & Hiern.	Kilulukunja (Swahili)	Lf, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0039	0.095
Basellaceae									
Basella alba L.	Nderema (Mashi), Ndelema (Kilega), Epinard Indien (French)	Lf	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0016	0.033

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Betulaceae									
Betula pendula Roth		Lf	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Bignoniaceae									
Kigelia africana (Lam.) Benth.	Kivungu (Luba)	Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.033
Spathodea campanulata P.Beauv.	Cifulula, Langalanga (Mashi), Mbalimbali (Swahili)	Sb	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0016	0.033
Brassicaceae									
Brassica juncea (L.) Czern.	Ndunda (Kisoko), Nkofi (Kongo), Chou vert (French)	Lf	D	R1 Katemo et al. (2012)	1	0.0027	0.0008	0.0008	0.029
Brassica oleracea L.	Chou (French), Shu (Swahili), Nkofi nkolula (Kongo)	Lf	D,I	R1 Katemo et al. (2012), R5 Amuri et al. (2018), and R6(Masunda et al. (2019)	3	0.0082	0.0024	0.0024	0.088
Bromeliaceae									
Ananas comosus (L.) Merr.	Nanasi (Swahili) Ananas (French), Cikaka (Tshiluba)	Fr	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Burseraceae									
Canarium schweinfurthii Engl.	Mpashi (Bemba), Mpafu (Luba)	Lf	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0024	0.062
Cactaceae									
Opuntia ficus-indica (L.) Mill.	Cactus (French)	Lf	C	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Caricaceae									
Carica papaya L.	Kipawo (Sanga), Papai (Swahili), Ipapayi (Mashi)	Lf, Fr, Ro	D,I,M	R3 Kasali et al. (2013), R5 Amuri et al. (2018); Mbuyi et al. (2019), and 6 Masunda et al. (2019)	4	0.0110	0.0032	0.0095	0.196
Celastraceae									
Maytenus senegalensis (Lam.) Exell	Tshingala mutshi (Luba)	Lf, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Salacia pynaertii De Wild	Mbondi (Not specified)	Lf	Rw	R6 Pathy et al. (2021)	1	0.0027	0.0008	0.0000	0.000
Chrysobalanaceae									
Parinari capensis Harv.	Nsudi funi (Not specified)	Lf	D	R6 Pathy et al. (2021)	1	0.0027	0.0008	0.0008	0.029
Clusiaceae									
Garcinia huillensis Welw. ex Oliv.	Mungindu (Tchokwe), Kisima (Not specified)	Ro, Lf, Fr	D,P	R5 Amuri et al. (2018) and R6 Pathy et al. (2021)	2	0.0055	0.0016	0.0063	0.157
Garcinia kola Heckel	Ngadiadia (Not specified)	Sd	D,N	R6 Ngbolua et al. (2016b), (2019)	2	0.0055	0.0016	0.0032	0.092
Combretaceae									
Combretum celastroides Welw. ex M.A.Lawson	Lukondambo (Luba), Mwina kyulu (Sanga)	Lf, Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Terminalia catappa L.	Madame (Lingala), Kalanga ya Wazungu (Swahili)	Lf	D	R1 Katemo et al. (2012)	1	0.0027	0.0008	0.0000	0.000
Terminalia chebula Retz.	Madame (Not specified)	Lf	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Terminalia mollis M.A.Lawson	Kianga (Hemba), Tshibangu Mutshi (Tshiluba)	Lf, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0032	0.092
Commelinaceae									
Palisota schweinfurthii C.B.Clarke	Mabongu-bongu (Kiyanzi), Bunda-bunda (Kongo)	Lf, Wp	D	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0000	0.000

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Convolvulaceae									
Ipomoea mauritiana Jacq.	Not reported	Tb	D	R1 Katemo et al. (2012)	1	0.0027	0.0008	0.0000	0.000
Ipomoea spathulata Hallier f.	Mulapa (Sanga)	Lf	C	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Costaceae									
Costus lucanusianus J.Braun & K.Schum.	Boso boso, musanga vulu, ngo n'keni (Kongo)	Lf	N	R6 Latham and Mbuta (2017)	1	0.0027	0.0008	0.0016	0.059
Costus phyllocephalus K.Schum	Mafulungu (Kongo), Musangala (Kimbala)	Lf	D	R6 Masunda et al. (2019) and R7 Moswa et al. (2005)	2	0.0055	0.0016	0.0008	0.029
Cucurbitaceae									
Cucumis sativus L.	Concombre (French)	Fr	F	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Momordica charantia L.	Lumbusu (Not specified)	Lf, Fr	D,I	R6 Masunda et al. (2019) and R7 Manzo (2012)	2	0.0055	0.0016	0.0055	0.127
Cyperaceae									
Cyperus alternifolius R.Br.	Ndao (Luba), Nsaku (Kongo)	Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0024	0.062
Dilleniaceae									
Tetracera poggei Gilg	Mudia-ngulungu (Tshiluba)	Lf	D	R1 (Katemo et al. (2012) and R7 Moswa et al. (2005)	2	0.0055	0.0016	0.0047	0.124
Dioscoreaceae									
Dioscorea bulbifera L.	Nsoko ngamba, kimasoko (Not specified)	Tb	D	R6 Pathy et al. (2021)	1	0.0027	0.0008	0.0000	0.000
Dioscorea dumetorum (Kunth) Pax	Nsemi nsemi, ngamba (Kongo), kiazi kikuu (Swahili)	Tb	D	R6 Latham and Mbuta (2017)	1	0.0027	0.0008	0.0000	0.000
Dioscorea praehensilis Benth.	Bandindi (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Ebenaceae									
Diospyros heudelotii Hiern	Mulolo kongolo (Kyzi), Lufwa lu ndomba (Kongo)	Ro	D	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0000	0.000
Euphorbiaceae									
Alchornea cordifolia (Schumach. & Thonn.) Müll.Arg.	Ditokoto (Tshiluba) Mambunzila (Kongo)	Ro	D,N	R2 Kasika et al. (2015), R6 Masunda et al. (2019), and R7 Moswa et al. (2005)	3	0.0082	0.0024	0.0063	0.183
Croton macrostachyus Hochst. ex Delile	Mutara mutshi (Bemba)	Lf	D	R5 Mbayo et al. (2016); Amuri et al. (2018)	2	0.0055	0.0016	0.0024	0.062
Euphorbia prostrata Aiton	Kapalatonvitonvi (Bemba)	Wp	D	R5 Mbayo et al. (2016)	1	0.0027	0.0008	0.0047	0.124
Jatropha curcas L.	Mbono (Swahili), Kilembelembe (Luba)	Lf, Sd, Ro	D,M,P	R1 Katemo et al. (2012) and R5 Mbayo et al. (2016); Amuri et al. (2018)	3	0.0082	0.0024	0.0126	0.287
Maprounea africana Müll.Arg.	Kafulumume (Bemba), Kazembezembe (Luba)	Ro, Sb	D	R5 Mbayo et al. (2016); Amuri et al. (2018), R6 Masunda et al. (2019) and R7 Moswa et al. (2005)	4	0.0110	0.0032	0.0079	0.137
Ricinus communis L.	Lundimba ndimba (Luba), Mubalika (Bemba)	Lf, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0039	0.095
Tetrorchidium didymostemon (Baill.) Pax & K.Hoffm.	bosefo, didi (Kilulua)	Lf	D	R1 Mpiana et al. (2015)	1	0.0027	0.0008	0.0000	0.000
Fabaceae									
Abrus precatorius L.	Kansengulu kandindi (Tshiluba), Abrus (French)	Sb	P	R3 Karhagomba et al. (2013) and R7 Moswa et al. (2005)	2	0.0055	0.0016	0.0032	0.065

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Acacia karroo Hayne	Munga (Luba), Mutonge (Sanga), Mugunga (Hemba)	Lf, Sb	D	R5Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Acacia polyacantha Willd.	Kibimbo, hibomo (hemba), Kimungamunga (Luba), Kashia (Swahili), Irangi (Kihavu)	Rb,Lf	D,I	R3 Chiribagula et al. (2020) and R5 Bashige-Chiribagula et al. (2017); Mbuyi et al. (2019)	3	0.0082	0.0024	0.0071	0.213
Afrormosia angolensis (Baker) Harms	Mubanga (Bemba), Mubanga kyulu (Luba)	Ro,Sb	D	R5 Amuri et al. (2018); Mbuyi et al. (2019)	2	0.0055	0.0016	0.0008	0.029
Albizia adianthifolia (Schumach.) W.Wight	Mulu (Kongo), Kampetanzevu(Tshiluba) Murunda (Swahili), Kapentazovu (Bemba)	Lf, Ro	D,N	R5 Amuri et al. (2017), (2018); Bashige-Chiribagula et al. (2017); Mbuyi et al. (2019); Valentin et al. (2020), R6 Latham and Mbuta (2017); Masunda et al. (2019), and R7 Moswa et al. (2005)	8	0.0220	0.0063	0.0055	0.154
Albizia grandibracteata Taub.	Mushebeye (Mashi) Kahunda (Kibembe)	Sb	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0024	0.088
Arachis hypogaea L.	Mwema (Bembe) Nguba (Lingala), kalanga (Swahili)	Lf	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0024	0.088
Caesalpinia bonduc (L.) Roxb.	Not reported	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Caesalpinia decapetala (Roth) Alston	Lurhe (Mashi)	Lf	D	R3Kasali et al. (2013)	1	0.0027	0.0008	0.0024	0.062
Cassia alata L. *Senna alata* (L.) Roxb. (Synonym)	Lukunda bajanyi (Tshiluba), Mbaw-mbaw (Kongo)	Lf, Ro, Sd	D,M,N	R1 Katemo et al. (2012) and R6 Masunda et al. (2019)	2	0.0055	0.0016	0.0024	0.088
Cassia occidentalis L.	Lukunda bajanyi (Tshiluba), Mbaw-mbaw (Kongo), Mushigemanjoka (Mashi), Mujangajanga (Fuliru)	Lf, Ro, Sd	D,M,N	R1 Katemo et al. (2012), R3 Kasali et al. (2013); Chiribagula et al. (2020), R5 Amuri et al. (2017), (2018), and R7 Moswa et al. (2005)	6	0.0165	0.0047	0.0055	0.075
Cassia petersiana Bolle	Kafunga nashya (Bemba)	Ro	M	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Cassia sieberiana DC.	Kandungandunga (Tshiluba), Mugunga (Hemba)	Lf	D,I,N	R5 Amuri et al. (2017), (2018)	2	0.0055	0.0008	0.0000	0.000
Crotalaria spinosa Hochst. ex Benth.	Kabalala (Sanga)	Ro, Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Cyamopsis tetragonoloba (L.) Taub.	Not reported	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Dalbergia boehmii Taub.	Katembo mutshi (Luba), Katembo (Sanga)	Lf, Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0039	0.095
Droogmansia munamensis De Wild.	Mununganunga (Bemba), Mulundeni (Lala)	Lf, Sb	D	R7 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Eminia polyadenia Hauman	-	Ro	M	R5 Muya et al. (2014)	1	0.0027	0.0008	0.0024	0.062
Entada abyssinica Steud. ex A.Rich.	Kipungu (Sanga)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0032	0.065
Erythrina abyssinica Lam.	Kisongwa (Hemba), Katshiyitshiyi (Luba), Kikumbu, kikumbu ki nzambi (Kongo)	Ro,Lf,Bk	D,N	R5 Amuri et al. (2017), (2018), and R6 Latham and Mbuta (2017); Masunda et al. (2019)	4	0.0110	0.0032	0.0032	0.118
Erythrophleum africanum (Benth.) Harms	Kayimbi (Tshiluba)	Lf, Sb	D,M	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Glycine max (L.) Merr.	Soja (Swahili)	Lf	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Indigofera arrecta Hochst. ex A.Rich	Abwebwe (Kibembe), Musholotsi (Kihavu)	Ro	C	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0016	0.059

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Indigofera capitata Kotschy	Nkeka za ngo (Not specified)	Wp	P	R6 Pathy et al. (2021)	1	0.0027	0.0008	0.0000	0.000
Isoberlinia tomentosa (Harms) Craib & Stapf	Mbaru (Mashi)	Lf	D	R3 Chiribagula et al. (2020)	1	0.0027	0.0008	0.0087	0.193
Lonchocarpus katangensis De Wild.	Chuya (Bemba)	Sb	M	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Millettia drastica Welw. ex Baker	Mwengeti (Kongo), Nsiengieri (Kiyanzi)	Ro	D	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0039	0.068
Millettia eetveldeana (Micheli) Hauman	Mbwenge (Not specified)	Ro	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Millettia laurentii De Wild.	Kiboto (Not specified)	Bk	D	R6 Masunda et al. (2019); Pathy et al. (2021)	2	0.0055	0.0016	0.0000	0.000
Mucuna poggei Taub.	Mpesa (Tshiluba)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Pentaclethra macrophylla Benth.	Mutie nzama (Kongo), Tshengesha (Tshiluba), Ngansi (Luba)	Sb	D	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0063	0.131
Phaseolus lunatus L.	Haricot (French), Maharagi (Swahili)	Lf, Ro	D,I	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Phaseolus vulgaris L.	Cishimbo, mukenji (Mashi), Madesu (Lingala)	Gp	D,Tr	R3 Kasali et al. (2013), R5 Amuri et al. (2018), and R6 Masunda et al. (2019); Pathy et al. (2021)	4	0.0110	0.0032	0.0024	0.088
Piliostigma thonningii (Schumach.) Milne-Redh.	Kifumbe (Bemba, Luba)	Ro	M	R5Amuri et al. (2018)	1	0.0027	0.0008	0.0032	0.092
Pterocarpus angolensis DC.	Mukundambazu (Tabwa), Muyanga (Bemba), Sokosoko (Not specified)	Sb,Bk	D,P	R5 Amuri et al. (2018) and R6 Masunda et al. (2019); Pathy et al. (2021)	3	0.0082	0.0024	0.0039	0.095
Pterocarpus marsupium Roxb.	Nkila (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Pterocarpus tinctorius Welw.	Mukula (Chokwe)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Rhynchosia insignis (O.Hoffm.) R.E.Fr.	Munkoyo (Swahili)	Ro	M	R5Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Scorodophloeus zenkeri Harms	Kiwaya (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Senna timoriensis (D.C.) H.S.Irwin & Barneby	Mapalata (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0008	0.029
Swartzia madagascariensis Desv.	Munienze (Luba), Mpampi (Tshiluba)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0039	0.147
Tephrosia vogelii Hook.f.	Uleku (Kongo), Kai-kaya (Kybe), Bubawu (Tshiluba)	Lf	D	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0008	0.003
Trigonella foenum-graecum L.	Kiwaya (Not specified)	Lf	M	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0024	0.062
Vigna sinensis (L.) Savi ex Hausskn.	Lukunde (kikabinda)	Lf, Ro	D,M	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Gnetaceae									
Gnetum africanum Welw	Fumbwa (Lingala)	Lf	D,P	R1 Katemo et al. (2012) and R6 Masunda et al. (2019); Pathy et al. (2021)	3	0.0082	0.0024	0.0000	0.000
Hypericaceae									
Harungana madagascariensis Lam. ex Poir.	Mukuta (Tshiluba), Kadwamuko (Mashi), Ndura (Swahili)	Lf, Ro, Sb	D	R3 Kasali et al. (2013), R5 Amuri et al. (2018), and R7 Moswa et al. (2005)	3	0.0082	0.0024	0.0079	0.190

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Psorospermum corymbiferum Hochr.	Munkubagwa (Mashi)	Rb	M	R3 Chiribagula et al. (2020)	1	0.0027	0.0008	0.0055	0.127
Iridaceae									
Gladiolus gregarius Welw. Ex Baker	Litungulu ya zamba (Not specified)	Bb	D,N	R6 Ngbolua et al. (2019)	1	0.0027	0.0008	0.0008	0.029
Gladiolus klattianus Hutch	Kitala (Bemba), Kitokatoka (Luba)	Bk	D,M,N	R5 Amuri et al. (2017), (2018)	1	0.0027	0.0008	0.0016	0.059
Coleus kilimandschari Gürke	Mcubya (Bemba), Mutozo (Mashi), Mulavumba (Swahili)	Lf, Ro	D,I,M	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0047	0.124
Leucas martinicensis (Jacq.) R.Br.	Kanyamafundwe (Mashi), Namafundo (Fuliru)	Wp	D	R3 Chiribagula et al. (2020)	1	0.0027	0.0008	0.0039	0.095
Ocimum gratissimum L.	Malumba-lumba (Luba), Dinsusu-nsunsu (Kongo), Kitungu (Swahili), mayuyu (Kiyanzi), Dikondi, mazulu (Not specified)	Lf, Ro	D,I	R6 Masunda et al. (2019); Pathy et al. (2021), and R7 Moswa et al. (2005); Manzo (2012)	4	0.0110	0.0032	0.0150	0.271
Ocimum minimum L.	Dinsunsu nsusu Difioti (Not specified)	Lf	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Salvia officinalis L.	Sauge (French)	Lf	I	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0039	0.095
Vitex madiensis Oliv.	Mufutu (Luba)	Lf, Ro	D,N	R5 Amuri et al. (2017), (2018), R6 Masunda et al. (2019), and R7 Moswa et al. (2005)	4	0.0110	0.0032	0.0047	0.124
Lauraceae									
Persea americana Mill.	Ikipapai (Lamba), Avocatier (French), Ivoka (Mashi)	Lf, Sb, Fr	D	R1 Katemo et al. (2012), R3 Kasali et al. (2013), R5 Amuri et al. (2018), and R6 Ngbolua et al. (2016a); Masunda et al. (2019)	5	0.0137	0.0039	0.0047	
Loganiaceae									
Strychnos cocculoides Baker.	Katongatonga (Luba), Bukoke (Hemba), Nzanza (Bemba)	Ro,Lf	D,I	R5 Amuri et al. (2018); Valentin et al. (2020)	2	0.0055	0.0016	0.0032	0.065
Strychnos innocua Delile.	Kakomekone (Swahili)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0024	0.062
Strychnos spinosa Lam.	Kisongole (Bemba), Nsansa (Swahili)	Ro, Sb	D,N	R5 Amuri et al. (2017), (2018)	2	0.0055	0.0016	0.0047	0.098
Strychnos stuhlmannii Gilg.	Mubanga Kyilu (Bemba), Nkanga kyulu (Zela)	Ro	D	R5 Amuri et al. (2018); Valentin et al. (2020)	2	0.0055	0.0016	0.0016	0.059
Lythraceae									
Punica granatum L.	Not reported	Fw	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Malvaceae									
Adansonia digitata L.	Mululu punga (Bemba)	Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Azanza garckeana (F. Hoffm.) Excell & Hillc	Muti ya makamashi (Swahili)	Lf, Sb	D,I,N	R5 Amuri et al. (2017), (2018)	2	0.0055	0.0016	0.0016	0.059
Cola acuminata (P. Beauv.) Schott & Endl.	Makasu (Not specified)	Lf, Sd	D,N	R6 Masunda et al. (2019); Ngbolua et al. (2019)	2	0.0055	0.0016	0.0008	0.029
Cola nitida (Vent.) Schott & Endl.	Mapio (Bambenga)	Fr	N	R4 Mongeke et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Grewia flava DC.	Bungwe (Luba)	Lf, Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Hibiscus esculentus L., *Abelmoschus esculentus* (L.) Moench (Synonym)	Dongodongo (Lingala)	Fr	D,M,P	R1 Katemo et al. (2012) and R6 Masunda et al. (2019); Pathy et al. (2021)	3	0.0082	0.0024	0.0016	0.033
Sida acuta Burm.f.	Mudundu (Mashi)	Sb	D,N	R2 Kasika et al. (2015) and R3 Kasali et al. (2013)	2	0.0055	0.0016	0.0063	0.131

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Urena lobata L.	Pungala (Not specified), Mpungala (Not specified)	Lf, Ro,Bb	D	R6 Masunda et al. (2019); Pathy et al. (2021)	2	0.0055	0.0016	0.0000	0.000
Meliaceae									
Azadirachta indica A.Juss	Nime (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0016	0.059
Menispermaceae									
Penianthus longifolius Miers	Not reported	Sb	M	R1 Katemo et al. (2012)	1	0.0027	0.0008	0.0032	0.092
Moraceae									
Ficus benghalensis L.	Nsanda (Not specified)	Lf, Bk	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Ficus exasperata Vahl	Kikuya (Kongo)	Lf	D	R1 Katemo et al. (2012)	1	0.0027	0.0008	0.0032	0.065
Ficus sycomorus L.	Mukunyu (Swahili), Tshikuyi (Luba)	Lf, Sb, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Moringaceae									
Moringa oleifera Lam	Moringa (Not specified), Mti maria (Mashi), Mlongelonge (Swahili)	Lf, Fw	I,Tr,D	R3 Kasali et al. (2013) and R6 Masunda et al. (2019); Pathy et al. (2021)	3	0.0082	0.0024	0.0032	0.065
Musaceae									
Musa x sapientum L.	Bananier (French)	Bb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0032	0.092
Myrtaceae									
Eucalyptus globulus Labill.	Bikalubitus (Not specified)	Lf	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0024	0.062
Psidium guajava L.	Lipela (Swahili), Ngalafua (Tshiluba), Ngoyavi (Kongo)	Lf, Ro	D,M	R5 Amuri et al. (2018), R6 Masunda et al. (2019), and R7 Moswa et al. (2005)	3	0.0082	0.0024	0.0039	0.068
Syzygium cumini (L.) Skeels	Telezia (Swahili)	Fr	D	R1 Katemo et al. (2012) and R6 (Masunda et al. (2019)	2	0.0055	0.0016	0.0000	0.000
Syzygium guineense (Willd.) DC.	Musanfwa (Bemba)	Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0032	0.092
Nyctaginaceae									
Bougainvillea spectabilis Willd	Bougainvillé (French)	Fw	M	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Ochnaceae									
Ochna schweinfurthiana F.Hoffm.	Not reported	Ro	M	R5 Muya et al. (2014)	1	0.0027	0.0008	0.0024	0.062
Olacaceae									
Olax obtusifolia De Wild.	Kulokumo (Bemba)	Ro	D	R5Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Oleaceae									
Olea europaea L.	Olivier (French)	Lf	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Pandaceae									
Panda oleosa Pierre	Okali (Lingala)	Sb	D	R1 Katemo et al. (2012)	1	0.0027	0.0008	0.0008	0.029
Passifloraceae									
Adenia gummifera (Harv.) Harms	Komboponoke (Lamba), Kimboyi (Lala)	Sb	I	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Adenia venenata Forssk.	Mafula (Luba)	Lf, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Pedaliaceae									
Sesamum angolense Welw.	Kipalabwengo (Bemba)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Sesamum indicum L.	Wangila (Not specified)	Sd	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Phyllanthaceae									
Antidesma membranaceum Müll.Arg. *Antidesma meiocarpum* J.Léonard (Synonym)	Tshilumba mutshi (Tshiluba), Mulambabwato (Bemba)	Lf, Sb	D,I	R5 Mbayo et al. (2016)	1	0.0027	0.0008	0.0024	0.062
Antidesma venosum E.Mey. ex Tul.	Kifubia (Luba), Misengo (Kongo), Nalushushwa (Fuliru)	Ro, Sb,Lf	D	R3 Manya et al. (2020) and R5 (Mbayo et al., (2016); Amuri et al. (2018)	3	0.0082	0.0024	0.0142	0.189
Bridelia ferruginea Benth	Kimwindu ki nseke (Kongo), Kindundu (Kintandu), Kimwindu (not specified)	Sb, Ro	D	R6 Masunda et al. (2019); Pathy et al. (2021) and R7 Moswa et al. (2005)	3	0.0082	0.0024	0.0118	0.232
Hymenocardia acida Tul.	Kapembe (Bemba), Lupep (Tchokwe), Kigeti (Kongo)	Ro	D	R5 Amuri et al. (2018) and R7 Moswa et al. (2005)	2	0.0055	0.0016	0.0063	0.157
Phyllanthus amarus Schumach. & Thonn.	Not reported	Lf	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Phyllanthus muellerianus (Kuntze) Exell	Mupetwalupe (Bemba), Lulembalemba, Ludimba, lundimba, Kajimbajimba lujimba (Luba), Lulembalemba, Mulembalemba (Hemba)	Lf, Ro,Fr	D,Rw	R5 Mbayo et al. (2016); Bashige-Chiribagula et al. (2017); Mbuyi et al. (2019)	3	0.0082	0.0024	0.0102	0.173
Phyllanthus niruri L.	Kahungahunga (Tshiluba), Kapondo (Songye)	Wp	D	R5 Mbayo et al. (2016) and R6 Masunda et al. (2019)	2	0.0055	0.0016	0.0079	0.163
Pseudolachnostylis maprouneifolia Pax.	Musangati (Swahili), Musangali (Bemba)	Lf, Ro, Sd	D,C	R5 Mbayo et al. (2016); Amuri et al. (2018)	2	0.0055	0.0016	0.0102	0.173
Uapaca kirkiana Müll.Arg.	Masuku (Bemba, Luba)	Sb	D	R5 Mbayo et al. (2016); Amuri et al. (2018)	2	0.0055	0.0016	0.0079	0.163
Piperaceae									
Piper guineense Schumach. & Thonn.	Kapindi (Kongo), Ketshu (Luba), Nketu (Tshiluba)	Fr	P	R3 Kasali et al. (2013) and R7 Moswa et al. (2005)	2	0.0055	0.0016	0.0055	0.101
Poaceae									
Cymbopogon citratus (DC.) Stapf	Majani tshai (Swahili), Sinda (Kongo), Citronelle (French), Lemongrass (English)	Lf	D	R1 Katemo et al. (2012), R3 Karhagomba et al. (2013), and R6 Masunda et al. (2019)	3	0.0082	0.0024	0.0024	0.062
Cymbopogon densiflorus (Steud.) Stapf	Lusangu sangu (Not specified)	Lf	I,D	R6 Latham and Mbuta (2017); Masunda et al. (2019)	2	0.0055	0.0016	0.0071	0.160
Oryza sativa L.	Loso (Not specified)	Lf	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Zea mays L.	Muyindi (Swahili), Cigonji (Mashi)	Sp	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0055	0.180
Polygalaceae									
Polygala acicularis Oliv.	Lunsambi nsambi (Not specified)	Lf,Bk	D	R6 Masunda et al. (2019); Pathy et al. (2021)	2	0.0055	0.0016	0.0016	0.059
Proteaceae									
Faurea saligna Harv.	Mulemu (Sanga)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Protea obtusifolia Engl.	Mwinkala nikata (Tabwa)	Ro, Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Rhamnaceae									
Maesopsis eminii Engl.	Ndunga (Luba)	Lf, Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0024	0.088
Ziziphus mucronata Willd.	Kankona (Luba, Bemba, Sanga)	Ro, Sb	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.033

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Rubiaceae									
Crossopteryx febrifuga (Afzel. ex G.Don) Benth.	Mutoshi (Tshiluba), Konsekonse (Bemba), Mvala (Kongo)	Lf, Ro	D,M	R5 Amuri et al. (2018) and R7 (Moswa et al. (2005)	2	0.0055	0.0016	0.0032	0.065
Mitragyna stipulosa (DC.) Kuntze Hallea stipulosa (DC.) J.-F.Leroy (Synonym)	Liluku (Lingala), Tshindubula, Mutoshi (Tshiluba), Longwa, nlongu (Kongo),	Sb,Bk	D	R6 Latham and Mbuta (2017) and R7 Moswa et al. (2005)	2	0.0055	0.0016	0.0008	0.029
Morinda citrifolia L.	Nsiki (Not specified)	Bk	D	R7 Manzo (2012)	1	0.0027	0.0008	0.0024	0.062
Morinda lucida Benth	Nsiki (Kongo), Indombe (Lingala), Isuku (Swahili)	Lf, Sb	D,M	R1 Katemo et al. (2012), R6 Masunda et al. (2019), and R7 (Moswa et al. (2005)	3	0.0082	0.0024	0.0079	0.137
Morinda morindoides (Baker) Milne-Redh.	Kileso nkama (Kongo), Nkonga bululu (Tshiluba), Kongo bololo (Not specified)	Lf	D	R1 Katemo et al. (2012), R6 Ngbolua et al. (2016b); Masunda et al. (2019); Pathy et al. (2021), and R7 Moswa et al. (2005)	5	0.0137	0.0039	0.0102	0.173
Nauclea latifolia Sm. *Sarcocephalus latifolius* (Sm.) E.A.Bruce (Synonym)	Lolo kienga (Kongo), Bungondo (Tshiluba)	Ro, Sd	D	R6 Ngbolua et al. (2016b), (2019); Masunda et al. (2019) and R7 Moswa et al. (2005)	4	0.0110	0.0032	0.0047	0.098
Sarcocephalus pobeguinii Hua ex Pobég.	Kenga kimansa (Not specified)	Lf	D,N	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Rutaceae									
Citrus limon (L.) Osbeck	Citronier (French), Indimu (Mashi), Chunghwa kali (Swahili),	Fr, Ro	D,Pr	R1 Katemo et al. (2012), R3 Kasali et al. (2013), and R5 Amuri et al. (2018)	3	0.0082	0.0024	0.0095	0.249
Citrus x aurantium L., *Citrus sinensis* (L.) Osbeck.(Synonym)	N'lala (Kongo), Dingama (Kongo), Ndimu (Swahili), Lala (Kongo), Oranger doux(French)	Lf, Ro, Fr	D,M	R5 Amuri et al. (2018), R6 Latham and Mbuta (2017), and R7 (Moswa et al. (2005)	3	0.0082	0.0024	0.0047	0.177
Zanthoxylum chalybeum Engl.	Mpupwe kiulu (Luba), Pupwe (Bemba)	Lf, Sb, Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0039	0.147
Santalaceae									
Viscum album L.	Not reported	Lf	I	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000
Simaroubaceae									
Quassia africana (Baill.) Baill.	Mupeshipe (Not specified), Munkadi nkadi (Not specified)	Lf,Ro	M,D,N	R6 Ngbolua et al. (2019); Pathy et al. (2021)	2	0.0055	0.0016	0.0118	0.232
Solanaceae									
Physalis angulata L.	Ndimba, lumbundu (Not specified)	Wp, Lf, Fr	D	R7Manzo (2012)	1	0.0027	0.0008	0.0047	0.124
Physalis peruviana L.	Mbuma, Mpuhuhu (Mashi), Mbupuru (Kinande)	Lf	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0024	0.088
Schwenckia americana L.	Lunzila nzila, Yabala mbula, Tumpa di nkombo (Kongo)	Wp	D	R6 Masunda et al. (2019) and R7 (Moswa et al. (2005)	2	0.0055	0.0016	0.0087	0.166
Solanum aethiopicum L., *Solanum gilo* Raddi (Synonym), *Solanum subsessile* De Wild. (Synonym)	Nyanya (Swahili), Mutete (Luba)	Ro,Fr, Lf, Sd	D,F	R1 Katemo et al. (2012), R5 Amuri et al. (2018), and R6 Masunda et al. (2019)	3	0.0082	0.0024	0.0008	0.029
Solanum americanum Mill., *Solanum nigrum* L. (Synonym)	Makeke (Swahili), Mulunda (Mashi)	Lf	D	R1 Katemo et al. (2012) and R3 Kasali et al. (2013)	2	0.0055	0.0016	0.0032	0.092
Solanum melongena L.	Mbolongo (Not specified)	Fr	D	R6 Masunda et al. (2019)	1	0.0027	0.0008	0.0000	0.000

(Continued on following page)

TABLE 1 | (*Continued*) Ethnopharmacological specifications of plant species used to treat diabetes in DRC.

Family Scientific Name	Vernacular name	Part	Form	Site (References)	NC	FC	RFC	UV	RII
Solanum seretii De Wild.	Impwa (Bemba)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0016	0.059
Solanum tuberosum L.	Pomme de terre (French), Birai (Swahili)	Tb	F	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0008	0.029
Thomandersiaceae									
Thomandersia hensii De Wild. & T.Durand	Ikoka (Turumbu), Liowa (Topoke)	Lf	D	R1 Katemo et al. (2012)	1	0.0027	0.0008	0.0071	0.186
Urticaceae									
Musanga cecropioides R.Br. ex Tedlie	Nsanga (Kongo), Mulombele (Tshiluba)	Lf	I	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0039	0.095
Myrianthus arboreus P.Beauv.	-	Ro	D	R6 Latham and Mbuta (2017)	1	0.0027	0.0008	0.0063	0.131
Urtica dioica L.	Chachingi (Mashi)	Lf	T,I	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0039	0.147
Verbenaceae									
Lantana camara L.	Mavi ya kuku (Swahili)	Lf	D,I	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0071	0.160
Lippia multiflora Moldenke	Bulukuti (Kongo), Filia m'filu filu (Kiyombe), Bulukutu, mbulunkutu (Kongo)	Lf	I,D,P	Latham and Mbuta (2017); Masunda et al. (2019); Pathy et al. (2021), and 7 Moswa et al. (2005)	4	0.0110	0.0032	0.0039	0.121
Stachytarpheta indica (L.) Vahl	Telezia (Swahili)	Lf	D	R3 Kasali et al. (2013)	1	0.0027	0.0008	0.0008	0.029
Vitaceae									
Vitis vinifera L.	Raisin (French)	Lf	D	R5 Amuri et al. (2018)	1	0.0027	0.0008	0.0000	0.000
Zingiberaceae									
Aframomum melegueta K. Schum.	Mundongo (Lingala), Ndungu zi nzo (Kongo)	Sd	P	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0024	0.088
Zingiber officinale Roscoe	Tangawisi (Swahili, Luba, Kongo), Nungu zikanda (Kybe), Tangawiwi (Lingala), Nunguzikanda (Kiyombe)	Rz	P	R7 Moswa et al. (2005)	1	0.0027	0.0008	0.0071	0.160
Zygophyllaceae									
Balanites aegyptiaca (L.) Delile	Mubambangoma (Swahili), Mbambangoma (Luba)	Ro	D	R5 Amuri et al. (2018)	1	0.0027	0.0016	0.0032	0.092

Legend: The parts: Ap(aerial part); Bb(bulb); Bk(bark); Fr(fruit); Fw(flower); Gp(green pods); Lf(leaf); Lt(Latex); Ro(root); Rb(Root bark); Rz(rhizome); Sd(seed); Sb(stem bark); Sp(spathe); Tb(tuber); and Wp(whole plant). Forms: D(decoction), I(infusion), M(maceration), T(Tincture), Tr(Trituration), N(Not specified), Pr(Pression), C(Chewing), P(Powder), Rw(Raw) Regions: R1(Kisangani), R2(Beni and Lubero), R3(Bukavu), R4(Bagdolite and Kungu), R5(Lubumbashi, Kafubu, Kasumbalesa, Kipushi, Likasi and Sambwa), R6(Kinshasa, Kwango and Kongo central). Quantitavive Ethnopharmacology: FC(Frequency of citation), NC(Number of citations), RFC(Relative Frequency of Citation), RII(Relative Imprtance Index), UV(Use value).

3.2.3 Clinical Trials Inside Democratic Republic of Congo

Data from the present study showed the lack of local clinical trials of antidiabetic plants used to manage Diabetes in the DRC. Of seven native herbals, only *Raphia gentiliana* fruit extract was given to 25 males and 20 females, aged 18–50 years old, with normal blood sugar levels (Mpiana et al., 2013). Thirty persons were submitted to the fruits of *R. gentiliana* as food (0.14 g/kg), while fifteen were introduced to the glucose solution (0.07 g/kg) (standard). The glycemia was measured by spectrophotometry, and the triangle surface area ratio method was used to calculate the glycemic and load index. The observed values of glycemic index and load were −3.1% and −1.36%. The approach followed by the authors did not comply with any clinical trial requirements, and instead, their behavior went like traditional healers themselves.

3.3 Phytochemical Investigations

Some studies have been undertaken to explore the chemical composition of *Panda oleosa*, *Physalis peruviana*, and *Vernonia amygdalina*.

4 DISCUSSION

4.1 Ethnopharmacological Data

4.1.1 Ethnobotanical Information Reported

The analysis presented in **Table 2** showed that the ecological status was reported for 185(50.41%) plants and not for

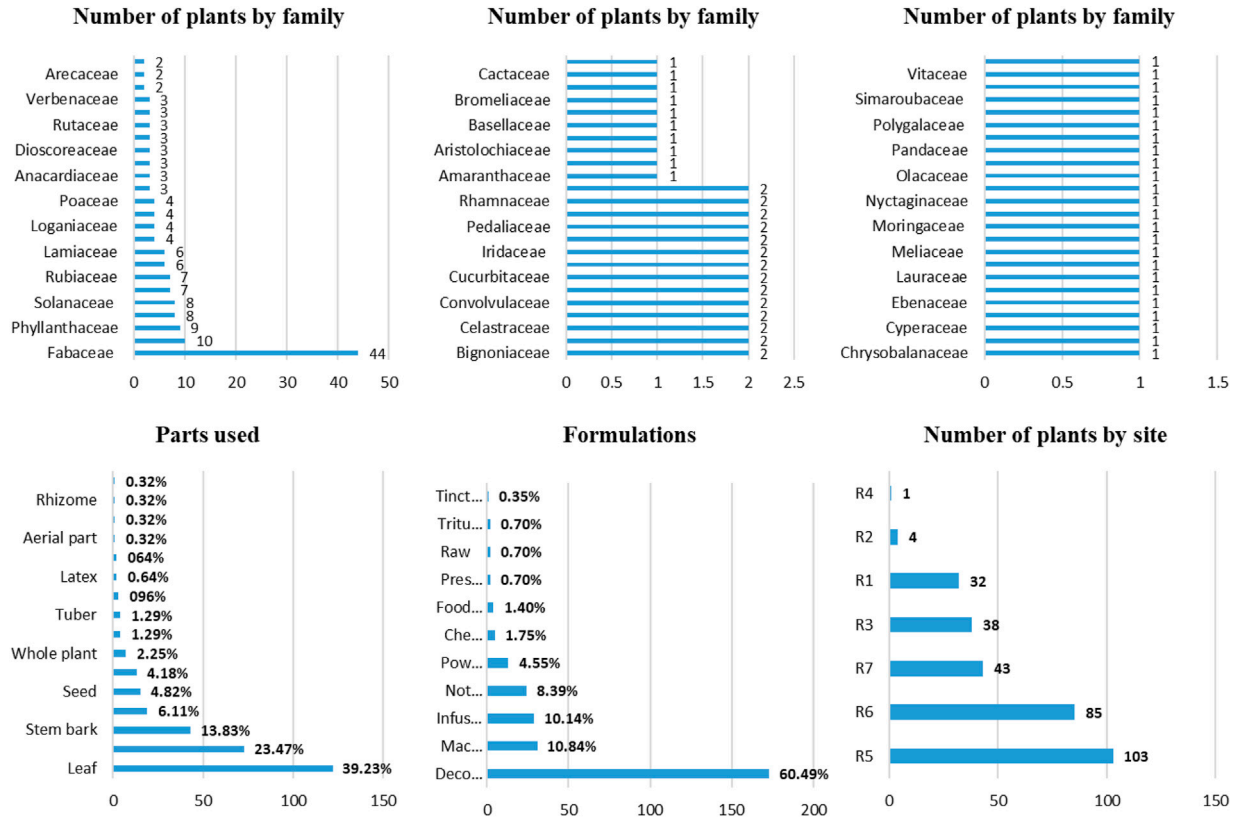


FIGURE 4 | Frequencies of antidiabetic plants in DRC by botanical families, parts used, formulations, and sites.

FIGURE 5 | Plants that underwent experimental pharmacology inside DRC.

TABLE 2 | Quality analysis of ethnobotany information.

Ecological source	%	Errors detected	%
Ecological status reported	50.41	Author name correct	69.59
Ecological status not reported	49.59	Author name incorrect	16.16
Common plants to DRC and Africa	54.93	Author name absent	14.25
Introduced from the Americas and Europe	14.55	Family name unchanged	81.64
Exclusively native to DRC (*D. munamensis*)	0.47	Family name revised	17.23
Origin not mentioned	30.05	Family name absent	1.10
Voucher number reported	7.95	Plant name confused with its synonym	4.69
Voucher number not reported	92.05	Plant identification reported	89.32
		Plant identification not reported	10.68

182(49.59%). On the other hand, plant identification was reported in 326(89.32%) cases and not 39(10.68%).

The errors in plant authors included entirely different authors, spelling mistakes, inappropriate use of the period, improper use of bracket, and incomplete author name.

The origin of plants was specified in 69.95% and not in 30.05% of species. However, 54.93% of plants with known origin were native to Africa, 14.55% species were introduced, and *Droogmansia munamensis* was the only species exclusively native to DRC flora ("Haut-Katanga"). Concerning the data quality, the author names of plant species were correctly written in 69.59% of cases, not correctly registered in 16.16%, or absent in 14.25%. Furthermore, 17.26% of plants had family names changed, and 81.64% not changed. In few cases (n = 10), the main plant was confused with its synonym. For example, *Antidesma metacarpus* (*A. membranaceum*), *Annona senegalensis* (*A. arenaria*), *Cassia alata* (*Senna alata*), *Chenopodium ambrosioides* (*Dysphania ambrosioides*), *Citrus x aurantium* (*C. sinensis*), *Hibiscus esculentus* (*Abelmoschus esculentus*), *Mitragyna stipulosa* (*Hallea stipulosa*), *Nauclea latifolia* (*Sarcocephalus latifolius*), *Solanum americanum* (*S. nigrum*), and *Solanum gilo* (*S. aethiopicum* and *S. subsessile*). Most species (89.32%) from different sites were identified and authenticated in other herbariums or laboratories of ecology, but only a few (7.95%) had a voucher number published. This situation implicates the responsibilities of publishers and reviewers. **Table 2** shows the quality analysis of findings compared to data from Plants of the World Online web (http://powo.science.kew.org) database and http://plantsoftheworldonline.org via the Royal Botanic Garden Kew database.

4.1.2 Ethnopharmacological Data Reported

The country is home to different ethnic groups, making it one of the most diverse countries globally, with more than 200 other ethnic groups speaking an estimated 213 native languages. Sometimes referred to as the Baluba, the Luba people are the largest ethnic group. The community is native to the Kasai, Maniema, and Katanga regions. The Mongo people comprise several smaller constituent groups, including the Mbole, Ekonda, Boyela, Bolia, and Nkutu. The Kongo ethnic group is native to DRC and Angola, speaking Kongo alongside Lingala, Kyanzi, and Kintandu. The Mangbetu ethnicity is concentrated within the Orientale Province (Kisangani). The Zande people reside in the tropical rainforest and the savanna and speak nearly five dialects of the Azande language. The Pygmies are considered to have been some of the earliest peoples to inhabit the Congo River Basin. Their short stature characterizes them, they are mainly hunters and gatherers, and they occupy the rainforest. The plants are distributed within tropical and subtropical ecological regions, flooded grasslands, moist broad-leaf forests, savannas, and mangroves.

Swahili is the most reported language 48(12.87%), followed by Kongo 46(12.33%), Luba 36(9.65%), Bemba 32(8.58%), Tshiluba 29(7.77%), Mashi 26(6.97%), French 21(5.63%), and Lingala 14(3.75%). After le French, which is the official language, there are four regionally distributed national languages, including Ciluba (Tshiluba), Kongo, Lingala, and Swahili, among 213 native languages identified in DRC. Those four languages are used in out-group communication, in lower primary school years (mainly in rural and semi-urban areas), cultural and religious purposes, etc. (Kasanga, 2012).

Fabaceae was the most representative family, consistent with other studies that showed this family is commonly found in tropical rain and dry forests in the Americans and Africa (Burnham and Johnson, 2004). Around 60% of the Congo-basin lies in the DRC, the second-largest contiguous tract of tropical forests globally, and the greatest extent of tropical rainforests in Africa. It covers more than 100 million hectares (Abernethy et al., 2016).

The leaf was the most used part 122(39.23%), followed by roots 73(23.47%), and stem bark 43(13.83%). According to (Moshi et al., 2012), the frequent use of leaves is associated with ease of accessibility among the aboveground parts of plants in natural ecosystems. The formulations prepared consisted more often of decoction for 173(60.49%), followed by maceration 31(10.84%) and infusion 29(10.14%). However, in 24 cases (8.39%), the formulation has not been reported. Decoction has often been the effective formulation of herbal remedies as it is easy to prepare by mixing a drug with boiling water (Mahomoodally et al., 2016).

Out of 213 plants listed, 103(33.66%) were found at site R5 and 85(27.78%) at R6. The majority of plants had local vernacular names, except in few cases where the author did not mention the names. For instance, *Catharanthus roseus* was found at almost all locations (except site R4) and *Allium cepa* at five sites. However, *A. sativum, Cassia alata, C. occidentalis, Mangifera indica, Persea americana*, and *Vernonia amygdalina* were quoted at four locations.

TABLE 3 | Antidiabetic plants used locally for the treatment of other various disorders.

Disorders/effect	Plants used
Abdominal pain	*Afrormosia angolensis; Ageratum conyzoides; Allium sativum; Anisophyllea boehmii; Coleus kilimandschari; Cymbopogon densiflorus; Cyperus alternifolius; Dalbergia boehmii; Nauclea latifolia; Phaseolus lunatus; Psidium guajava; Solanum aethiopicum; Solanum seretii; Strychnos cocculoides; Strychnos spinosa; Uapaca kirkiana; Tithonia diversifolia; Ziziphus mucronata; Zingiber officinale; Pseudolachnostylis maprouneifolia; Maprounea africana; Acacia polyacantha*
Abdominal cramps	*Antidesma venosum; Cymbopogon densiflorus; Piper guineense*
Abortions repeated	*Musanga cecropioides; Antidesma venosum; Brillantaisia patula; Dalbergia boehmii; Schwenckia americana*
Abscess	*Antidesma venosum; Aloe vera; Annona senegalensis; Bidens pilosa; Chenopodium ambrosioides*
Amoebiasis	*Elaeis guineensis; Cassia occidentalis; Morinda lucida; Cymbopogon densiflorus; Morinda morindoides; Bridelia ferruginea; Caesalpinia decapetala; Carica papaya; Crossopteryx febrifuga; Garcinia huillensis; Hymenocardia acida; Garcinia kola; Harungana madagascariensis; Jatropha curcas; Justicia flava; Myrianthus arboreus; Alchornea cordifolia;* Psorospermum corymbiferum*; Pentaclethra macrophylla; Strychnos cocculoides; Tetracera poggei; Uapaca kirkiana; Tithonia diversifolia; Vinca minor; Vitex madiensis; Psidium guajava; Nauclea latifolia; Mangifera indica; Maprounea africana*
Anemia	*Annona senegalensis,* Isoberlinia tomentosa; *Phyllanthus muellerianus; Alchornea cordifolia; Hymenocardia acida; Ocimum gratissimum; Ficus sycomorus; Ochna schweinfurthiana; Persea americana; Piliostigma thonningii; Vitex madiensis; Momordica charantia*
Angina	*Coleus kilimandschari;* Isoberlinia tomentosa; *Morinda lucida*
Anorexia	*Ananas comosus; Tithonia diversifolia; Zingiber officinale*
Aphrodisiac	*Albizia adianthifolia; Antidesma venosum; Phyllanthus muellerianus; Uapaca kirkiana; Zingiber officinale*
Ascites	*Schwenckia americana; Xylopia aethiopica*
Asthenia	*Tithonia diversifolia*
Asthma	*Antidesma membranaceum; Catharanthus roseus; Cymbopogon densiflorus; Cyperus alternifolius; Elaeis guineensis; Lantana camara; Costus phyllocephalus; Ocimum gratissimum; Phyllanthus niruri; Schwenckia americana; Vitex madiensis*
Arthritis	*Allium cepa; Phaseolus vulgaris; Zea mays*
Backache	*Aframomum melegueta; Chenopodium ambrosioides; Cola acuminata; Gladiolus gregarious; Nauclea latifolia; Ocimum gratissimum; Zingiber officinale*
Birth troubles	*Adenia gummifera*
Bleunorrhagia	*Carica papaya; Citrus limon; Croton macrostachyus; Diplorhynchus condylocarpon; Ficus exasperata; Strychnos innocua; Strychnos spinosa; Tetracera poggei; Zingiber officinale*
Bronchitis	*Allium cepa*
Bronchopneumonia	*Ocimum gratissimum; Quassia Africana; Alchornea cordifolia*
Burns	*Aloe vera; Brassica oleracea*
Buruli ulcer	*Elaeis guineensis*
Cancer	*Brassica oleracea; Antidesma venosum; Catharanthus roseus; Chenopodium ambrosioides; Erythrina abyssinica; Erythrophleum africanum; Urtica dioica; Ageratum conyzoides; Aloe vera; Harungana madagascariensis; Zea mays; Vinca minor*
Cancer (prostate)	*Ageratum conyzoides*; *Arachis hypogaea; Bidens pilosa; Sida acuta*
Cataract Eye	*Moringa oleifera; Thomandersia hensii;* Crassocephalum picridifolium*; Euphorbia prostrata*
Chest pain	*Schwenckia americana*
Cholera	*Phyllanthus muellerianus*
Cold	*Cymbopogon densiflorus; Lantana camara; Morinda citrifolia, Ocimum gratissimum; Tithonia diversifolia*
Colitis	*Ageratum conyzoides, Carica papaya; Citrus limon; Morinda morindoides; Schwenckia americana; Vinca minor; Pseudolachnostylis maprouneifolia; Physalis peruviana; Mangifera indica*
Conjunctivitis	*Moringa oleifera; Mangifera indica*
Constipation	*Lantana camara; Ageratum conyzoides; Bridelia ferruginea; Carica papaya; Cassia occidentalis; Pentaclethra macrophylla; Persea Americana; Phyllanthus niruri; Jatropha curcas; Artemisia annua;* Leucas martinicensis; *Tithonia diversifolia; Rauvolfia vomitoria; Mangifera indica; Maprounea africana; Momordica charantia*

(Continued on following page)

TABLE 3 | (*Continued*) Antidiabetic plants used locally for the treatment of other various disorders.

Disorders/effect	Plants used
Convulsions	*Bridelia ferruginea; Vigna sinensis*
Cough	*Abrus precatorius*, *Aframomum melegueta*; *Aloe vera; Artemisia annua; Bidens pilosa; Carica papaya; Catharanthus roseus; Citrus limon; Citrus x aurantium; Coleus kilimandschari; Elaeis guineensis; Garcinia huillensis;* Isoberlinia tomentosa; *Jatropha curcas; Lantana camara; Myrianthus arboreus; Piliostigma thonningii; Zanthoxylum chalybeum; Zingiber officinale; Vitex madiensis; Piper guineense; Ocimum gratissimum; Lippia multiflora;* Crassocephalum picridifolium.
Delirium	*Ageratum conyzoides*
Dermatitis	*Abrus precatorius; Costus phyllocephalus*
Dehydration	Isoberlinia tomentosa
Diarrhea	*Cassia occidentalis; Balanites aegyptiaca; Annona senegalensis; Antidesma membranaceum; Bridelia ferruginea; Ficus exasperata; Ficus sycomorus;* Isoberlinia tomentosa; Leucas martinicensis; Psorospermum corymbiferum*; Persea americana; Sida acuta; Albizia adianthifolia; Dalbergia boehmii; Psidium guajava; Quassia Africana; Phyllanthus muellerianus; Acacia polyacantha; Antidesma venosum; Bidens pilosa; Phyllanthus niruri; Entada abyssinica; Syzygium guineense; Terminalia mollis; Uapaca kirkiana; Momordica charantia; Zea mays; Vinca minor; Pterocarpus angolensis; Piper guineense; Nauclea latifolia; Millettia drastica; Maytenus senegalensis*
Dizziness	*Vinca minor*
Dysentery	*Canarium schweinfurthii; Carica papaya; Droogmansia munamensis; Euphorbia prostrata; Strychnos cocculoides; Strychnos spinosa; Thomandersia hensii; Tetracera poggei; Uapaca kirkiana; Vernonia amygdalina; Xylopia aethiopica; Ziziphus mucronata; Psidium guajava; Pseudolachnostylis maprouneifolia*
Dysmenorrhea	*Aristolochia hockii; Artemisia absinthium; Carica papaya; Cassia alata; Balanites aegyptiaca; Citrus x aurantium; Croton macrostachyus; Antidesma venosum; Justicia flava; Phyllanthus muellerianus; Salvia officinalis; Artemisia annua; Maprounea africana*
Dyspepsia	*Artemisia absinthium*
Dystocia	*Bridelia ferruginea*
Edema	*Jatropha curcas; Syzygium guineense; Tetracera poggei; Urtica dioica; Zea mays*
Edema of the lower extremities	*Azanza garckeana*
Elephantiasis	*Crinum ornatum*
Emphysema	*Quassia Africana*
Enuresis	*Caesalpinia decapetala*
Epilepsy	*Annona senegalensis; Azanza garckeana;* Costus lucanusianus; *Elaeis guineensis; Lippia multiflora; Solanum americanum*
Erectile malfunction	*Garcinia huillensis*
Eye troubles	*Maesopsis eminii*
Female infertility	*Ageratum conyzoides; Carica papaya; Elaeis guineensis; Musanga cecropioides; Antidesma venosum; Costus phyllocephalus; Phyllanthus muellerianus; Hymenocardia acida; Tephrosia vogelii; Psidium guajava*
Fever	*Phyllanthus niruri; Alchornea cordifolia; Citrus limon; Citrus x aurantium; Cymbopogon densiflorus; Elaeis guineensis; Gladiolus klattianus;* Isoberlinia tomentosa; *Lantana camara ;Morinda morindoides;* Leucas martinicensis; *Myrianthus arboreus ;Ocimum gratissimum; Persea americana; Physalis angulata; Penianthus longifolius; Tetracera poggei; Mangifera indica; Morinda citrifolia; Momordica charantia*
Filariasis	*Albizia grandibracteata; Tephrosia vogelii*
Fractures	*Ageratum conyzoides; Euphorbia prostrata; Hibiscus esculentus; Indigofera arrecta; Pentaclethra macrophylla; Ocimum gratissimum; Sida acuta*
Frigidity and narrowing of the vagina	*Elaeis guineensis*
Gallbladder disorders	*Artemisia absinthium*
Gallstone	*Vernonia amygdalina*
Gangrene	*Strychnos stuhlmannii*
Gastric hypoacidity	*Artemisia annua; Caesalpinia decapetala*
Gastroenteritis	*Vinca minor*

(Continued on following page)

TABLE 3 | (*Continued*) Antidiabetic plants used locally for the treatment of other various disorders.

Disorders/effect	Plants used
Gastrointestinal disorders	*Alchornea cordifolia; Ananas comosus; Annona senegalensis; Garcinia huillensis; Pseudolachnostylis maprouneifolia; Piper guineense; Physalis angulata*
Gastric ulcer	*Momordica charantia*
Gastritis	*Cassia occidentalis; Bridelia ferruginea; Brillantaisia patula; Elaeis guineensis;* Isoberlinia tomentosa; *Salvia officinalis; Sida acuta; Antidesma venosum; Jatropha curcas; Citrus limon; Myrianthus arboreus; Vernonia amygdalina; Quassia Africana; Solanum tuberosum; Zanthoxylum chalybeum; Pseudolachnostylis maprouneifolia*
Goiter ringworm	*Jatropha curcas;* Crassocephalum picridifolium
Gonorrhea	*Quassia Africana; Albizia adianthifolia; Bridelia ferruginea; Cassia alata; Citrus x aurantium; Croton macrostachyus;* Costus lucanusianus; *Gladiolus klattianus; Morinda morindoides; Spathodea campanulata; Phyllanthus niruri; Antidesma venosum; Ricinus communis; Jatropha curcas;* Crassocephalum picridifolium*; Maprounea africana; Phyllanthus muellerianus; Strychnos spinosa; Uapaca kirkiana; Pseudolachnostylis maprouneifolia.*
Gout	*Jatropha curcas; Garcinia kola; Phaseolus vulgaris*
Headache	*Ageratum conyzoides; Catharanthus roseus; Elaeis guineensis; Phyllanthus muellerianus; Ocimum gratissimum; Artemisia annua; Mangifera indica; Schwenckia americana; Solanum seretii; Uapaca kirkiana; Vernonia amygdalina; Vernonia shirensis; Vigna sinensis; Vinca minor; Morinda citrifolia*
Helminthiasis	*Phyllanthus niruri; Thomandersia hensii ; Vernonia amygdalina ; Ocimum gratissimum; Quassia africana; Sida acuta; Morinda lucida; Morinda morindoides; Antidesma venosum*
Hemorrhoids	*Annona senegalensis; Bridelia ferruginea; Elaeis guineensis;* Crassocephalum picridifolium*; Nauclea latifolia; Quassia africana; Alchornea cordifolia; Asparagus africanus; Canarium schweinfurthii;* Isoberlinia tomentosa; *Chenopodium ambrosioides; Coleus kilimandschari; Crossopteryx febrifuga; Cyperus alternifolius; Ageratum conyzoides;* Crassocephalum picridifolium*; Ficus exasperata; Hymenocardia acida; Morinda morindoides; Entada abyssinica; Myrianthus arboreus; Ocimum gratissimum; Polygala acicularis; Gladiolus gregarius; Pentaclethra macrophylla; Sida acuta; Phyllanthus muellerianus; Vernonia shirensis; Zingiber officinale; Pterocarpus angolensis; Monodora myristica; Millettia drastica*
Hemorrhage	*Bidens pilosa; Bridelia ferruginea; Citrus limon; Opuntia ficus-indica; Vinca minor*
Hepatitis	*Erythrina abyssinica;* Crassocephalum picridifolium*; Vernonia amygdalina Aloe vera Physalis angulata; Tetracera poggei; Urtica dioica; Mangifera indica*
Hernia	*Aloe congolensis, Annona senegalensis, Antidesma membranaceum; Elaeis guineensis; Erythrina abyssinica; Grewia flava; Harungana madagascariensis; Hymenocardia acida; Morinda lucida; Musa x sapientum; Pentaclethra macrophylla; Phyllanthus niruri;* Leucas martinicensis; *Quassia Africana; Schwenckia americana; Pterocarpus angolensis; Xylopia aethiopica*
Hiccup	*Albizia adianthifolia*
Hip pains	*Zanthoxylum chalybeum*
HIV/Aids	*Panda oleosa*
Hypertension	*Allium cepa; Allium sativum; Catharanthus roseus; Citrus limon;* Isoberlinia tomentosa; Leucas martinicensis; *Pentaclethra macrophylla;* Anacardium occidentale; *Quassia Africana; Harungana madagascariensis; Zea mays*
Hypotension	*Allium sativum; Acacia polyacantha;* Psorospermum corymbiferum
Indigestion	*Albizia adianthifolia, Anana comesus; Cassia occidentalis*
Infected wounds	*Ochna schweinfurthiana*
Infections	*Adenia gummifera; Allium sativum; Antidesma venosum; Arachis hypogaea; Cymbopogon densiflorus; Gongronema latifolium; Morinda lucida; Nauclea latifolia; Rauvolfia caffra; Vernonia amygdalina; Zingiber officinale; Moringa oleifera*
Infertility	*Uapaca kirkiana; Millettia drastica*; *Musa x sapientum; Zanthoxylum chalybeum, Pseudolachnostylis maprouneifolia*
Inflammation	*Ageratum conyzoides; Raphia gentiliana; Physalis angulata; Physalis peruviana*
Influenza	*Chenopodium ambrosioides; Ocimum gratissimum*
Insomnia	*Catharanthus roseus*
Intercostal (or chest) pain	*Elaeis guineensis*
Interruption of the menstruation without being pregnant	*Bridelia ferruginea*
Intestinal worms	*Allium sativum; Antidesma venosum; Bridelia ferruginea; Carica papaya; Catharanthus roseus; Chenopodium ambrosioides; Entada abyssinica; Garcinia huillensis; Garcinia kola; Ipomoea spathulata; Jatropha curcas; Morinda*

(Continued on following page)

TABLE 3 | (*Continued*) Antidiabetic plants used locally for the treatment of other various disorders.

Disorders/effect	Plants used
	morindoides; Penianthus longifolius; Strychnos spinosa; Syzygium guineense; Tephrosia vogelii; Vernonia shirensis; Zingiber officinale; Musa x sapientum; Millettia drastica; Maprounea africana
Irritable bowel	*Carica papaya*
Jaundice	*Acacia karroo; Carica papaya; Eminia polyadenia; Harungana madagascariensis; Jatropha curcas; Musanga cecropioides; Rhynchosia insignis; Thomandersia hensii; Terminalia mollis.*
Joint pain	*Aloe congolensis, Annona senegalensis; Costus phyllocephalus; Morinda morindoides; Lippia multiflora*
Kidney stone	*Phaseolus vulgaris; Zea mays*
Laryngitis	*Bridelia ferruginea*
Leishmaniasis	*Morinda lucida*
Lice	*Rauvolfia vomitoria*
Lumbago	*Elaeis guineensis*
Madness	*Elaeis guineensis; Polygala acicularis*
Malaria	*Crossopteryx febrifuga; Alchornea cordifolia; Acacia polyacantha; Albizia adianthifolia; Antidesma venosum; Artemisia annua; Azadirachta indica; Cassia occidentalis; Cymbopogon citratus; Catharanthus roseus; Jatropha curcas; Lantana camara; Morinda lucida; Morinda morindoides; Citrus x aurantium; Coleus kilimandschari; Cymbopogon densiflorus; Eucalyptus globulus; Garcinia kola; Indigofera arrecta; Musanga cecropioides; Myrianthus arboreus; Ocimum gratissimum;* Parinari capensis*; Pentaclethra macrophylla; Phyllanthus niruri; Rauvolfia caffra; Thomandersia hensii; Vernonia amygdalina; Harungana madagascariensis; Momordica charantia; Penianthus longifolius; Vernonia shirensis; Rauvolfia vomitoria; Piliostigma thonningii; Piper guineense; Physalis angulata; Physalis peruviana; Moringa oleifera; Monodora myristica; Eucalyptus globulus; Quassia africana*
Male impotence	*Elaeis guineensis; Phyllanthus muellerianus; Cassia petersiana; Citrus limon; Sida acuta; Balanites aegyptiaca; Cola nitida; Kigelia africana; Penianthus longifolius; Schwenckia americana; Lippia multiflora; Xylopia aethiopica*
Mastitis	*Aloe congolensis; Ocimum gratissimum, Pterocarpus angolensis*
Measles	*Aristolochia hockii; Cymbopogon citratus; Costus phyllocephalus; Thomandersia hensii*
Migraine	*Elaeis guineensis; Ocimum gratissimum; Vinca minor*
Mycosis	*Cassia alata; Stachytarpheta indica*
Nephritis	*Zea mays*
Neuralgia	*Musa x sapientum*
Oligospermia	*Phyllanthus muellerianus*
Oliguria	*Maprounea africana*
Oral cavity	*Euphorbia prostrata*
Oropharyngeal diseases	*Salvia officinalis; Lantana camara*
Otitis	*Citrus x aurantium; Ocimum gratissimum;* Crassocephalum picridifolium*; Zanthoxylum chalybeum; Tephrosia vogelii*
Oxytocic	*Sida acuta*
Pain	*Quassia Africana; Phyllanthus niruri; Persea americana*
Paralysis	*Brassica juncea; Olax obtusifolia*
Pneumonia	*Acacia polyacantha; Elaeis guineensis;* Psorospermum corymbiferum*; Pseudolachnostylis maprouneifolia*
Poisoning antidote	Isoberlinia tomentosa; *Gongronema latifolium; Vernonia amygdalina; Pseudolachnostylis maprouneifolia*
Poliomyelitis	*Xylopia aethiopica*
Premature ejaculation	*Elaeis guineensis*
Psychosomatic disorders	*Solanum americanum*
Premature delivery	*Cymbopogon citratus*
Prevention of tetanus	*Pseudolachnostylis maprouneifolia*
Pruritus	*Jatropha curcas*

(Continued on following page)

TABLE 3 | (*Continued*) Antidiabetic plants used locally for the treatment of other various disorders.

Disorders/effect	Plants used
Rashes with itching	*Abrus precatorius; Solanum americanum; Vernonia amygdalina*
Rheumatism	*Allium cepa; Bridelia ferruginea; Dalbergia boehmii; Elaeis guineensis; Quassia africana; Morinda morindoides; Costus phyllocephalus; Erythrophleum africanum; Garcinia huillensis; Ocimum gratissimum; Pentaclethra macrophylla; Harungana madagascariensis; Urtica dioica; Lantana camara; Xylopia aethiopica; Schwenckia americana.*
Scabies	*Elaeis guineensis; Vernonia amygdalina; Quassia africana; Jatropha curcas*
Schistosomiasis	*Annona senegalensis; Balanites aegyptiaca; Citrus limon; Cymbopogon densifloru; Eminia polyadenia; Entada abyssinica; Garcinia huillensis; Harungana madagascariensis; Hymenocardia acida; Strychnos innocua; Strychnos spinosa; Syzygium guineense; Terminalia mollis; Vernonia shirensis; Piliostigma thonningii; Pterocarpus angolensis; Ochna schweinfurthiana; Maytenus senegalensis; Maprounea africana*
Sciatic neuralgia	*Elaeis guineensis; Ocimum gratissimum; Schwenckia americana*
Sickle cell disease	*Adansonia digitate*; *Annona senegalensis; Bridelia ferruginea; Carica papaya; Coleus kilimandschari; Combretum celastroides; Costus phyllocephalus; Cymbopogon densiflorus; Jatropha curcas; Terminalia ivorensis; Mitragyna stipulosa; Persea americana; Thomandersia hensii; Bougainvillea spectabilis; Morinda lucida; Hymenocardia acida; Harungana madagascariensis; Vigna sinensis; Maesopsis eminii.*
Sinusitis	*Erythrina abyssinica*
Skin infections	*Albizia grandibracteata; Allium cepa; Brassica oleracea*
Skin rash	*Acacia polyacantha; Tephrosia vogelii*
Smallpox	*Morinda morindoides*
Snakebites	*Thomandersia hensii ; Euphorbia prostrata; Rauvolfia caffra*
Sore throat	*Aframomum melegueta; Citrus limon; Euphorbia prostrata; Ficus exasperata; Piper guineense*
Spasms	*Acacia polyacantha;* Psorospermum corymbiferum
Splenomegaly	*Aloe congolensis; Annona senegalensis; Elaeis guineensis; Tithonia diversifolia*
Sprain	*Hibiscus esculentus*
Stomach pain	*Antidesma venosum; Basella alba; Crossopteryx febrifuga; Physalis angulata; Lantana camara; Phyllanthus niruri; Citrus limon; Quassia africana; Jatropha curcas; Phyllanthus muellerianus; Ageratum conyzoides;* Crassocephalum picridifolium*; Solanum americanum*
Sweating	*Salvia officinalis*
Swollen breasts	*Morinda lucida*
Swollen gums	*Ricinus communis*
Swollen testicles	*Pseudolachnostylis maprouneifolia; Ricinus communis*
Syphilis	*Albizia adianthifolia; Antidesma venosum; Aristolochia hockii; Asparagus africanus;* Isoberlinia tomentosa; *Pseudolachnostylis maprouneifolia; Ricinus communis; Strychnos innocua; Strychnos spinose; Strychnos stuhlmannii; Terminalia mollis; Lonchocarpus katangensis; Maprounea africana*
Tachycardia	*Musanga cecropioides; Thomandersia hensii*
Testicular disappearance	*Annona senegalensis; Elaeis guineensis*
Tiredness	*Costus phyllocephalus*
Tooth decay	*Ageratum conyzoides; Antidesma venosum; Dalbergia boehmii; Elaeis guineensis; Lonchocarpus katangensis; Myrianthus arboreus; Phyllanthus muellerianus; Ricinus communis; Swartzia madagascariensis;* Psorospermum corymbiferum;*Uapaca kirkiana;* Anacardium occidentale; *Pseudolachnostylis maprouneifolia; Mangifera indica; Millettia drastica; Maprounea africana; Acacia polyacantha*
Trypanosomiasis	*Annona senegalensis; Morinda lucida*
Tuberculosis	*Abrus precatorius; Azadirachta indica; Bridelia ferruginea; Canarium schweinfurthii; Citrus limon; Eucalyptus globulus; Hymenocardia acida; Myrianthus arboreus; Ocimum gratissimum; Chenopodium ambrosioides; Costus phyllocephalus; Garcinia huillensis; Schwenckia americana; Rauvolfia caffra; Vernonia amygdalina; Vitex madiensis; Rauvolfia vomitoria; Momordica charantia; Lippia multiflora*
Typhoid fever	*Antidesma venosum; Arachis hypogaea; Morinda morindoides*
Urinary infections	*Albizia grandibracteata; Alchornea cordifolia; Bidens pilosa; Eminia polyadenia; Maesopsis eminii; Mangifera indica; Spathodea campanulata; Strychnos cocculoides; Vitex madiensis.*

(Continued on following page)

TABLE 3 | (*Continued*) Antidiabetic plants used locally for the treatment of other various disorders.

Disorders/effect	Plants used
Uterine bleeding	*Urtica dioica*
Uterine contraction	*Uapaca kirkiana*
Vaginal infections	*Acacia karroo*; *Kigelia Africana; Acacia polyacantha; Salvia officinalis*
Venereal diseases	*Crotalaria spinosa*
Verminous	*Cassia sieberiana*
Vitiligo	*Elaeis guineensis*
Vomitings	*Basella alba; Cassia occidentalis; Piper guineense; Vinca minor*
Weak immunity system	*Allium sativum*
Whitlow	*Elaeis guineensis*
Wounds	*Morinda morindoides; Annona senegalensis; Bidens pilosa; Quassia africana; Jatropha curcas*
Yellow fever	*Elaeis guineensis; Harungana madagascariensis*

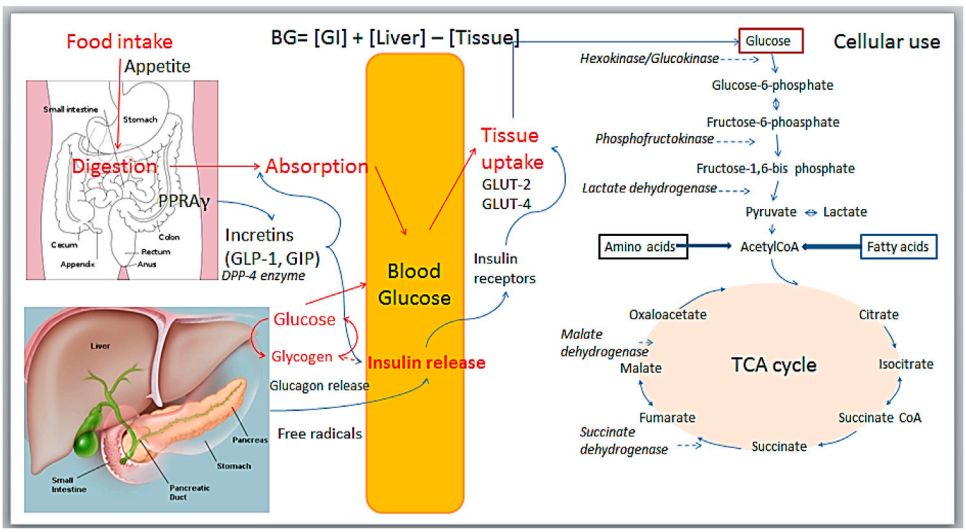


FIGURE 6 | Illustrative sites and pathways of antidiabetic bioactivity.

4.1.3 Validation of Ethnopharmacological Data

Studies undertook outside DRC confirmed the use of the majority of plants cited as antidiabetic remedies. *Albizia adianthifolia* was the most reported antidiabetic with eight citations representing an RFC of 0.0063, followed by *Catharanthus roseus* 7(RFC = 0.0055). However, *Allium cepa*, *Annona senegalensis*, and *Cassia occidentalis* were reported six times (RFC = 0.0047), followed by *Mangifera indica, Morinda morindoides*, *Phaseolus lunatus,* and *Vernonia amygdalina* with five citations (RFC = 0.0039).

Comparatively, *Elaeis guineensis* was endorsed by 28 uses; the (UV score of 0.0221 was the highest compared to *Ocimum gratissimum* (0.0150), *Antidesma membranaceum* (0.0142), *Jatropha curcas* (0.0126), *Bridelia ferruginea* (0.0118), and *Quassia africana* (0.0118). Also, *Balanites aegyptiaca*, which is employed to treat nine body systems, showed the highest Relative Importance Index (32.5%), compared to *Vitis vinifera* (28.7%), *Zingiber officinale* (27.1%), *Solanum seretii* (24.9%), *Thomandersia hensii* (24.9%), *Lippia multiflora* (23.2%) and *Stachytarpheta indica* (23.2%). The use-value indicators (**Table 1**) are relative and susceptible to changing since, in the methodology, many authors generally limit themselves to the total number of participants in the studies. The lack of information on the number of informants interviewed was commonly observed in the reviewed studies. According to the Declaration of Helsinki, the direct consequence is that it is no longer possible to analyze the quantitative aspects of these studies (The World Medical Association, 2001).

TABLE 4 | Local plants studied for antidiabetic effect in animals.

Plant	Ecology	Form	Part	Animal	Model	Dose range	Quality score	References
Albizia adianthifolia	T, Fo, At	D	Stem bark	Guinea pig	OGTT	500 mg/kg[a]	5/10 low	Amuri et al. (2017)
Azanza garckeana	Tp/Sh, Cult, SA.	D	Leaf	Guinea pig	OGTT	500 mg/kg[a]	5/10 low	Amuri et al. (2017)
Gladiolus klattianus	T, Cult, Sarc	M	Bulb	Guinea pig	OGTT	500 mg/kg[a]	5/10 low	Amuri et al. (2017)
Panda oleosa	Anh, Cult, Cosm	D	Bark	Rabbit	OGTT	25, 50 and 100 mg/kg	5/10 low	Muhoya et al. (2017)
Raphia gentiliana	Sh, Cult, At	M	Fruit	Mouse	OGTT	200 mg/kg	5/10 low	Mpiana et al. (2013)
Rauvolfia caffra	T, Fo, Pan	D	Root	Guinea pig	OGTT	500 mg/kg[a]	5/10 low	Amuri et al. (2017)
Vitex madiensis	T,GSZ,Sav	D	Leaves	Guinea pig	OGTT	500 mg/kg[a]	5/10 low	Amuri et al. (2017)

T(tree); Fo(forest); At(Afro-tropical); Tp(perennial-tree); Sh (Shrub), Cult(Cultivar); SA(South Africa); Sarc(Sarcochores); Anh(annual herb); Cosm(cosmopolitan); Pan(pantropical); Sav(savannah); GSZ(Guinean and Zambian); D(Decoction), M(Maceration); OGTT (oral glucose tolerance test).
[a]*Justification of dose.*

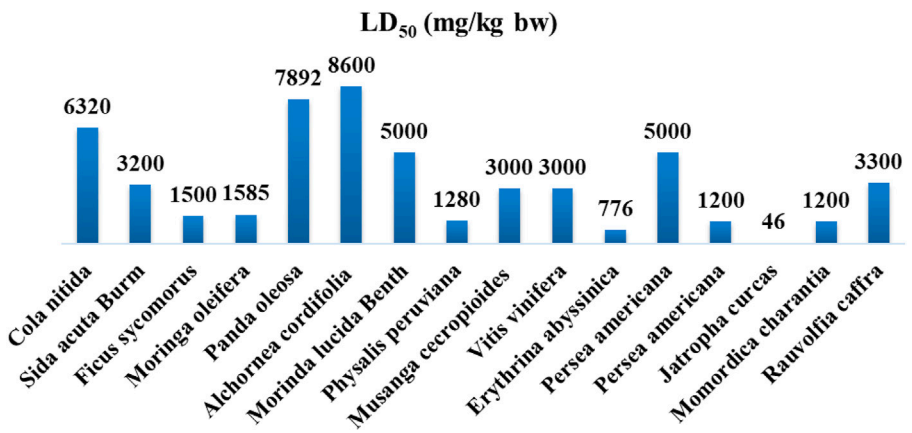


FIGURE 7 | Comparative range of LD_{50} for some plants.

Consequently, it is not possible to quantify certain vital indexes such as the Cultural Importance Index (CII), Fidelity Level of Citation (FL), Family Use Value, Importance Consensus Factor (ICF), etc. It should be noted that some rare studies make an effort to investigate these parameters, although the information on the number of respondents remains a challenge. One of the weaknesses of ethnopharmacological surveys is that the respondents are often the healers themselves and not or seldom the users. The questionnaires do not scrutinize evidence on the number of people treated and outcomes.

Table 3 illustrates the information gathered through the literature for some plants that can manage Diabetes and other comorbidities and complications. Among the plants listed as antidiabetic, 164(76.99%) species are being used locally in the treatment of several other diseases, mainly infections (bacterial, parasitic, viral, fungal), gastrointestinal and abdominal disorders, cardiac and neurological diseases, gynecological disorders, sexual problems, wounds, dermatological, hematological and metabolic diseases. Commonly, no one plant holds only one indication due to the complexity of the chemical content. The data combine both inside and outside studies.

4.2 Analysis of Pharmacological Data

4.2.1 Preclinical Studies

Different strategies and pathways are used to determine the mechanism of antidiabetic agents, as shown in **Figure 6**. No study explored in-depth pharmacological mechanisms of action, but all speculated over different modulating metabolic pathways, including 1) Reducing food intake; 2) Reducing carbohydrate digestion and absorption (alpha-amylase, alpha-glucosidase inhibition); 3) Increasing glycogenesis or reducing glycogenolysis and cholesterol synthesis; 4) Free radical scavenging action; 5) Insulin release and pancreas β-cells regeneration; 6) Enhancing glucose transport GLUT4 translocation; 7) dipeptidyl peptidase-4 (DPP-4) inhibition; 8) (PPARs); 9) Insulin-mimetic activity; 10) Modulation on Krebs cycle enzymes.

The analysis of the accurate data for all 213 plants listed showed that most studies used rats and mice, and in a few cases, guinea pigs and rabbits. Both streptozocin (35.55%) and alloxan (24.64%) represented 60.19% of all *in vivo* reported study models. Streptozotocin presents many advantages over alloxan, including its longer half-life, more productive, stable, and selective to islet beta cells, less toxic, and causing less mortality in animal models

TABLE 5 | The interpretation of Jadad score on Clinical trials of antidiabetic plants found in DRC.

Plant used	Used part/Preparation	Author and year	Randomization	Blinding	Withdrawals and dropouts	Total
Allium cepa	Fresh pods	Jafarpour-Sadegh et al. (2017)	2	2	1	5
Allium sativum	Pods	Ashraf et al. (2011)	1	2	0	3
Aloe vera	High molecular weight fractions	Yagi et al. (2009)	0	0	0	0
Balanites aegyptiaca	Fruits	Rashad et al. (2017)	2	2	1	5
Carica papaya	Fermented papaya	Raffaelli et al. (2015)	0	0	0	0
Elaeis guineensis	Standardized leaf extract	Kalman et al. (2013)	1	2	0	3
Laurus nobilis	Ground leaves	Khan et al. (2009)	1	0	0	1
Momordica charantia	Fruit powder	Kim et al. (2020)	1	1	1	3
Morinda cordifolia	Juice from fermented fruit puree	Algenstaedt et al. (2018)	0	0	0	0
Moringa oleifera	Leaf powder	Leone et al. (2018)	0	2	0	2
Rauvolfia-Citrus	Leaf powder	Campbell-Tofte et al. (2011)	1	2	1	4
Raphia gentiliana	Fruits	Mpiana et al. (2013) [a]	0	0	0	0
Salvia officinalis	Leave powder	Kianbakht and Dabaghian, (2013)	1	2	1	4
Terminalia chebula	Fruit aqueous extract	Pingali et al. (2020)	2	2	1	5
Trigonella foenum-graecum	Seed powder	Hadi et al. (2020)	2	1	1	4
Urtica dioica	Leaf extract	Kianbakht et al. (2013)	2	2	1	5
Vernonia amygdalina	Leaf juice	Okolie et al. (2008)	2	0	0	2
Zea mays	Maize starch	Sands et al. (2009)	1	0	0	1
Zingiber officinale	Rhizome powder	Shidfar et al. (2015)	1	2	0	3

[a]*Only study carried out in DRC; Score ≥ 3(Good quality); Score < 3 (Poor quality).*

(Lenzen, 2008; Wang-fischer and Garyantes, 2018). The majority of the bioactivity investigations link the antioxidant or free radical-scavenging activity with the pathophysiology of Diabetes. However, currently, the *in vitro* antioxidant model using, for example, DPPH and the others, is not pharmacologically relevant. It can be used as a chemical screening tool. Only *in vivo* or cell-based models remain relevant (Heinrich et al., 2020). Enzymes are a frequent pharmacological target for establishing the mechanism of action of new drugs. Upon *in vitro* studies, alpha-glucosidase activity inhibition was the most common investigation (45.3%), followed by inhibition of PTP1B (13.8%), alpha-amylase (9.7%), DPP-4 (1.4%), and 11ß-HSD1 activity (1.0%). Additionally, in cell lines studies, glucose uptake (28.0%) was to be the most commonly used, followed by glucose uptake regulation markers such as GLUT4 translocation and expression levels (9.7%) and PPAR (9.6%) (Munhoz and Fröde, 2017). Some examples of studies are given below.

Azadirachta indica aqueous leaf extract (400 mg/kg bw) improved levels of BG, serum insulin, lipid profile, insulin signaling molecules, and GLUT4 proteins in the tissue of high-fat fructose-induced type-2 diabetic male rat after 30 days of treatment, compared to the control group. In Goto-Kakizaki rats, the acetone extract of *Syzygium cumini* seed was a potent inhibitor of alpha-glucosidase hydrolysis of maltose compared to untreated control animals (Shinde et al., 2008). Moreover, hepatic tissue demonstrated increased PPARɣ and PPARα protein expressions (Sharma et al., 2012). *Oryza sativa* extracts significantly elevated glucose uptake, GLUT1, and GLUT4, mRNA levels (Boue et al., 2016). Ethanol extract induced a significant gain in GLUT4 on plasma membranes of L6-GLUT4myc muscle cells at no cytotoxic concentrations (Kadan et al., 2013). Choosing an experimental model is not easy and usually depends on many factors. Ideally, the experiments should be carried out in several different models, considering that none of them ultimately reflects the complexity of human diabetes mellitus type DMT2 and that precautions should be taken to extrapolate the findings to the clinical practice (Arias-Díaz and Balibrea, 2007).

A. adianthifolia is also used to treat syphilis, hiccups, diarrhea, malaria, indigestion, blueness, and an aphrodisiac. Oral administration of 500 mg/kg of plant extract reduced hyperglycemia by 57% in guinea pigs subject to OGTT (Amuri et al., 2017). *Albizia grandibracteata* is used in filariasis and skin and urinary tract infections. *A. garckeana* is used in epilepsy and edema of the lower limbs. Oral administration of 500 mg/kg bw aqueous extract under OGTT conditions reduced fasting blood sugar to 36.9% compared to 49.6% of glibenclamide as the reference medicine (Amuri et al., 2017). Certain parts of the plant may be toxic or contain cytotoxic compounds, particularly with gossypol for non-ruminant animals (Randel et al., 1992). *Gladiolus gregarius* is used to treat hemorrhoids and back pain. *Gladiolus klattianus* is used for gonorrhea and fever. Under OGTT conditions, the aqueous extract of *G. klattianus* reduced 35% of blood sugar after 60 min (Amuri et al., 2017). *Panda oleosa* Pierre has been proposed for HIV/AIDS. The aqueous extract of *P. oleosa* (25–100 mg/kg) significantly reduced glucose levels in a dose-dependent manner in rabbits under OGTT conditions (Muhoya et al., 2017). *Vitex madiensis* is used in asthma, anemia, diarrhea, tuberculosis, cough, urinary tract infections, and intestinal amebiasis. The aqueous extract of *V. madiensis* (500 mg/kg bw) reduced hypoglycemia to 43% compared to 55% obtained with glibenclamide (Amuri et al., 2017). *Raffia gentiliana* is used for inflammation. Oral administration of aqueous fruit extracts in mice under OGTT conditions demonstrated 27 and 56% reduction after one and 2 hours (Mpiana et al., 2013).

TABLE 6 | Major phytochemicals of each plant with demonstrated antidiabetic activity.

Scientific Names	**Antidiabetic compounds**
Abrus precatorius	Luteolin, lupenone, 24-methylnecycloartenone, and luteolin Vadivel et al. (2011); Yonemoto et al. (2014)
Aframomum melegueta	Arylalkanes, 6-paradol, 6-shogaol, 6-gingerol, 6-gingeredione, a pentacyclic triterpene, oleanolic acid isolated from the fruit Sugita et al. (2013); Mohammed et al. (2017).
Ageratum conyzoides	Precocene II Adebayo et al. (2010), and Kaempferol Tahora et al. (2018),
Allium cepa	Ferulic acid, alliin Tang et al. (2008), agavasaponin C Tang et al. (2008), flavonoid alliuocide G Mohamed (2008), quercetin, sulfur compounds, alcohols, aldehydes, esters, and other chemical groups. S-methyl cystein sulfoxide, S-allyl cysteine and diallyl thiosulfanate Kim et al. (2010), Bakhshaeshi et al. (2012), Noor et al. (2013), Cepadial D ; 1,3,11a-trihydroxy-9-(3,5,7-trihydroxy-4H-1-benzopyran-4-on-2-yl)-5a-[4-(β-D-glucopyranosyloxy)-3-hydroxyphenyl]-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one ; and 1,3,11a-trihydroxy-9-(3,5,7-trihydroxy-4H-1-benzopyran-4-on-2-yl)-5a-[1,3,11a-trihydroxy-5a-(3,4-dihydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12- on-9-yl]-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-on Vu et al. (2020).
Allium sativum	S-allylcysteine sulfoxide, alliin, diallyl trisulfide Liu et al. (2007); Mikaili et al. (2013), isoeruboside B, agavasaponin C, proto-iso-erubisoide B, 2-Vinyl-4H-1,3-dithiin Tang et al. (2008), allicin, diallyl disulfide, diallyl sulfide, ajoene, and allyl mercaptan Bayan et al. (2014).
Aloe vera	Lophenol, 24-methyl-lophenol, 24-ethyl-lophenol, cycloartanol and cycloartanol Misawa et al. (2012), aloeresin A Chang et al. (2013b), aloerisin Jong-anurakkun et al. (2008), aloe-emodin-8-O-glucoside, polysaccharides Salehi et al. (2018), aloin, barbaloin, isobarbaloine, aloetic acid, emodin, cinnamic acid, crysophanic acidleucine, isoleucine, alanin, glucomannan, cellulose, mannose, zinc, glucosamines Bharti et al. (2018).
Anacardium occidentale	Anacardic acid Tedong et al. (2010), lectin Maclel et al. (2012)
Arachis hypogaea	Leucocyanidin, stigmasterol Tang et al. (2008), resveratrol Gothai et al. (2016) phenolic compounds such as catechin, caffeic acid, epicatechin, p-coumaric acid, rutin, trans-ferulic acid, isoquercitri, resveratrol, luteolin, quercetin, trans-cinnamic acid, chrysoeriol Park et al. (2017).
Artemisia absinthium	α-and ß-thujones Daradka et al. (2014)
Azadirachta indica	3-Deacetyl-3-cinnamoyl-azadirachtin Jalil et al. (2013), 4'-methyl-quercetin-7-*O*-β-D-glucuronopyranoside, 2,3-hexahydroxydiphenoyl-(α/β)-D-(4)C1-glucopyranose, avicularin, castalagin, quercetin-3-O-glucoside Abdelhady et al. (2016) sistosterol, stigmasterol, campestrol, squalene, nimbiol and others Sanni et al. (2019).
Balanites aegyptiaca	Furostanol saponins Ezzat et al. (2017), balanitin 1 and 2, diosgenin, stigmast-4-en-3-ol, pure saponins Hassanina et al. (2018)
Bidens pilosa	Cytopiloyne, 2- β -D-Glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne Chang et al. (2013a), polyynes Bartolome et al. (2013), 3-β-D-glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne; 2-β-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne Chang et al. (2013b).
Bougainvillea spectabilis	Pinitol, βsitosterol, quercetin, quercetin 3-O-α-L-rhamnopyranoside Jawlal et al. (2013).
Brassica juncea	Cinnamic acid Guzman (2014), Kaempferol Gothai et al. (2016), aniline Sundowo et al. (2018)
Brassica oleracea	Cinnamic acid Guzman (2014), kaempferol Gothai et al. (2016)
Bridelia ferruginea	Epigallocatechin, epigallocatechin gallate Bakoma et al. (2018)
Caesalpinia decapetala	Apigenin-7-rhamnoside, astragalin, 6-hydroxy kaempferol, quercitrin Parveen et al. (2017)
Calendula officinalis	Caffeic acid, aesculetin, quercetin and isorhamnetin Olennikov and Kashchenko (2014).
Carica papaya	Flavonoids, alkaloids, saponin, and tannin Chang et al. (2013b)
Cassia alata	Emodin Uwazie et al. (2020)
Cassia occidentalis	Flavonoids Gupta et al. (2017)
Catharanthus roseus	Gallic acid, chlorogenic acid, flavonoids Rianika and Robert (2007), vindoline I, vindolidine II, vindolicine III and vindolinine Tiong et al. (2013), catharanthine, vindoline, vindolinene vinblastine, vincristine Bharti et al. (2018).
Citrus x aurantium	Narigin Pu et al. (2012), neohesperidin Osfor et al. (2013), Jia et al. (2015), epigallocatechin 3-gallate Chang et al. (2013b), diosmin, hesperetin Gothai et al. (2016), *p*-synerphine Suntar et al. (2018), N-acyl-2-aminothiazoles fused (+)-nootkatone Guo et al. (2020).
Citrus limon	Diosmin, eriodictyol, naringenin, hesperetin Gothai et al. (2016)
Cola nitida	caffein-rich Erukainure et al. (2017), caffeine and theobromine Erukainure et al. (2019).
Cucumis sativus	Kaempferol Ibitoye et al. (2017)
Cyamopsis tetragonoloba	Polyphenols-rich Gandhi et al. (2014)
Erythrina abyssinica	Benzofurans, coumestans Nguyen et al. (2010), flavonoids Ndinteh (2018)
Eucalyptus globulus	Euglobals, essential oils, macrocarpals Dey and Mitra (2013)
Ficus exasperata	α-amyrin acetate Nnamonu et al. (2016)
Garcinia kola	Kolaviron, a biflavonoid complex Adaramoye and Adeyemi (2006)
Glycine max	Daidzein, genistein, glycitein, beta-Sitosterol, Soyasaponin A1-A6, soyasaponin V, stigmasterol Tang et al. (2008), anthocyanins Nizamutdinova et al. (2009), lyceollin I-II Chang et al. (2013b), kaempferol glycoside rich fraction, kaempferol Zang et al. (2014), stigmasterol Wang et al. (2017b), soy isoflavones (genistein, diadzein) Bharti et al. (2018).
Harungana madagascariensis	Harunganols, kenganthranol A, harunganin, ferruginin A Johnson et al. (2015).
Hibiscus esculentus	Polysaccharide "rhamnogalacturonan" Liu et al. (2017).
Jatropha curcas	Flavonoid glycosides (rhiofolin, isoorientin, and isoquercetrin) El-baz et al. (2014)
Lantana camara	Stearoyl glucoside of ursolic acid (urs-12-en-3β-ol-28-oic acid 3β-D-glucopyranosyl-4′- octadecanoate) Kazmi et al. (2012)
Mangifera indica	The mangiferin Cruz-Vega et al. (2009), 1,2,3,4,6 penta-*O*-galloyl-β-d-glucose Mohan et al. (2013); curcumin, morin Gothai et al. (2016), gallic acid, 3,4-dihydroxy benzoic acid (Protocatechuic acid), kaempferol Ediriweera et al. (2017), flavonoids Pan et al. (2018); 1,2,3,4,6-penta-O-galloyl-β-D-glucoside, and 1,2,3,4,6-penta-O-galloyl-α-D-glucoside Yang et al. (2020).
Momordica charantia	

(Continued on following page)

TABLE 6 | (*Continued*) Major phytochemicals of each plant with demonstrated antidiabetic activity.

Scientific Names	Antidiabetic compounds
	Saponins Keller et al. (2011), cucurbitane triterpenoids Harinantenaina et al. (2006), Han et al. (2018), polysaccharide Xu et al. (2015), cucurbitane saponins Yue et al. (2017), saponins and polysaccharides Wang et al. (2019), insulin-like peptide, charantin, alkaloid vicine Pahlavani et al. (2019), 3β,7β,25-trihydroxycucurbita-5,23(E)-dien-19-al, charantal, charantoside XI, and 25ξ-isopropenylchole-5, 6-ene-3-*O*-D-glucopyranoside Shivanagoudra et al. (2019), polysaccharide-chromium (III) complex Zhang et al. (2019), saponins and polysaccharides Wang et al. (2019), momordicinin Kulkarni et al. (2021), Karaviloside VI and VIII Perera et al. (2021), 3β,7β,25-trihydroxycucurbita-5,23(*E*)-dien-19-al Noruddin et al. (2021), yeojoosides G-H, momordicoside U, karavilagenin A, goyaglycoside d, momordicoside F_1, momordicoside L, momordicoside K, and 68 (3β,7β,23S)-3,7,23-trihydroxycucurbita-5,24-dien-19-al 7-β-D-glucopyranose Lee et al. (2021).
Moringa oleifera	Isothiocyanate-rich Waterman et al. (2016), protein (Mo-LPI) Paula et al. (2017), phenolic glycosides Wang et al. (2017b), 4-hydroxyphenylacetonitrite, fluoropyrazine, methyl-4-hydroxybenzoate, vanillin Hafizur et al. (2018)
Musa x sapientum	Rutin Kappel et al. (2013), syringin Sundaram et al. (2014)
Ocimum gratissimum	Chicoric acid Casanova et al. (2014)
Olea europaea	Oleuropein, oleanolic acid Sato et al. (2007), luteolin Dekdouk et al. (2015)
Opuntia ficus-indica	Polysaccharides El-mostafa et al. (2014), polyphenols, dietary minerals, betalains, gallic acid, vanillic acid, catechins Gupta et al. (2017), mucopolysaccharide Bharti et al. (2018)
Oryza sativa	γ-oryzanol Burlando and Cornara (2014), ferulic acid, *p*-coumaric Aalim et al. (2019), cyanidin 3-glucoside, and (2*R*,3*R*)-taxifolin Yoon et al. (2020).
Phaseolus vulgaris	Stigmasterol Tang et al. (2008), catechin Gothai et al. (2016), flavonoids and their glucosides of delphinidin, petunidin, and malvidin, anthocyanins, catechin, myricetin 3-O-arabinoside, epicatechin, vanillic acid, syringic acid, and O-coumaric acid Ganesan and Xu (2017), and triacylglycerols Sutedja et al. (2020).
Phyllanthus amarus	Oleanolic acid and ursolic acid (2:1) mixture Ali et al. (2006)
Phyllanthus niruri	Ellagic acid and its derivatives Bharti et al. (2018)
Physalis angulata	Withangulatin-A Raju and Mamidala (2015)
Physalis peruviana	Peruvioses A,B,C,D,E,F Bernal et al. (2018)
Psidium guajava L.	Quercetin, kaempferol, myricetin , Strictinin, isostrictinin Wang et al. (2010), pedunculagin, glycoprotein Chauhan et al. (2010); Singab et al. (2014), and polysaccharides Zhang et al. (2016).
Pterocarpus marsupium	Phenolic-C-glycosides Mishra et al. (2013)
Punica granatum	gallic acid Huang et al. (2005), valoneic acid dilactone Jain et al. (2012), ursolic and oleanolic Salah El Dine et al. (2014), polyphenols Tang et al. (2018), rutin, gallic acid, nictoflorin, and tulipanin El Deeb et al. (2021).
Salvia officinalis	Essential oils with 71.3% of monoterpenes Belhadj et al. (2018)
Sesamum indicum	(+)-Pinoresinol Wikul et al. (2012), furofuran lignans Worawalai et al. (2016)
Solanum americanum	Amide alkaloids Silva et al. (2017)
Solanum melongena	Phenylethyl cinnamides Liu et al. (2011)
Spondias mombin	3b-olean-12-en-3-yl (9Z)-hexadec-9-enoate Fred-Jaiyesimi et al. (2009)
Syzygium cumini	Gallic acid, umbelliferone, ellagic acid Perera et al. (2017), mallic acid, chlorogenic acid Bharti et al. (2018).
Terminalia chebula	Chebulagic acid Huang et al. (2012); Shyni et al. (2014), hydrolyzable tannins Lee et al. (2017).
Trigonella foenum-graecum	GII Puri et al. (2011), galactomannan Anwar et al. (2011), 4-hydroxyisoleucine Rangari et al. (2014); Naicker et al. (2016), diosgenin, galactomannan, flavonoids, trigonelline Zameer et al. (2017); isonarthogenin, 22β-acetoxyolean-12-ene- 3β, 24-diol, and soyasapogenol B Zhang et al. (2020).
Urtica dioica	Quercetin, quercetrin, apigenin, rutin, apigenin-7-*O*-glucoside Bharti et al. (2018).
Vernonia amygdalina	Sesquiterpenes Zhao et al. (2012), monoterpenes, sobrerol Li et al. (2013b), vernoamyoside E Anh et al. (2021)
Vitis vinifera	Cinnamic acid Guzman (2014), resveratrol, naringenin Gothai et al. (2016), proanthocyanidin, raisin Bharti et al. (2018).
Xylopia aethiopica	Oleanolic acid Mohammed et al. (2021).
Zanthoxylum chalybeum	Chaylbemides A-C, fagaramide, skimmianine, norchelerythrine, 6-acetonyldihydrochelerythrine, and 6-hydroxy-N-methyl decarine Ochieng et al. (2020).
Zea mays	Hirsutrin Kim et al. (2013), anthocyanins Hong et al. (2013), phenolics compounds Nile and Park (2014)
Zingiber officinale	Gingerol Sekiya et al. (2004), aframodial, camphene, 6-shogaol Tang et al. (2008), β-bisabolol Le (2014), 6-shogaol Fajrin et al. (2020).

Of the 213 plant species listed, 134(62.91%) underwent experimental studies in animals or *in vitro*, while only 8.92% reached the clinical trial phase. Inside DRC, only seven plants shown in **Table 4** have been studied in animals. A critical analysis of the seven studies carried out inside DRC showed low quality (grade = 4–5). The majority (85.71%) used a single dose in antidiabetic evaluation. However, the *Panda oleosa* study used three-dose rages (25, 50, and 100 mg/kg body weight). Overall, it is not easy to define an exact upper cut-off dose. In most cases, an oral dose range of 100–200 mg/kg body weight for plant extracts *in vivo* investigations should be considered the upper limit (Heinrich et al., 2020). Experiments on *Albizia adianthifolia, Azanza garckeana, Gladiolus klattianus*, and *Rauvolfia caffra* extracts used the highest dose (500 mg/kg bw) calculated from the human patients of 60 kg treated with 750 ml of plant extract (corresponding to 250 g of dried herbal material per day). Par the way, differences in doses that normalize interspecies variation should be taken into account (Nair and Jacob, 2016).

Temperature and humidity were not reported. The effeteness of the treatment was based on the capacity of the extract to reduce baseline glycemia (hypoglycemia effect) or the capacity to reduce induced hyperglycemia; this varied between 25 and 75%, compared to reference drugs (glibenclamide and metformin). According to (Baker et al., 2014), over 85% of published animal

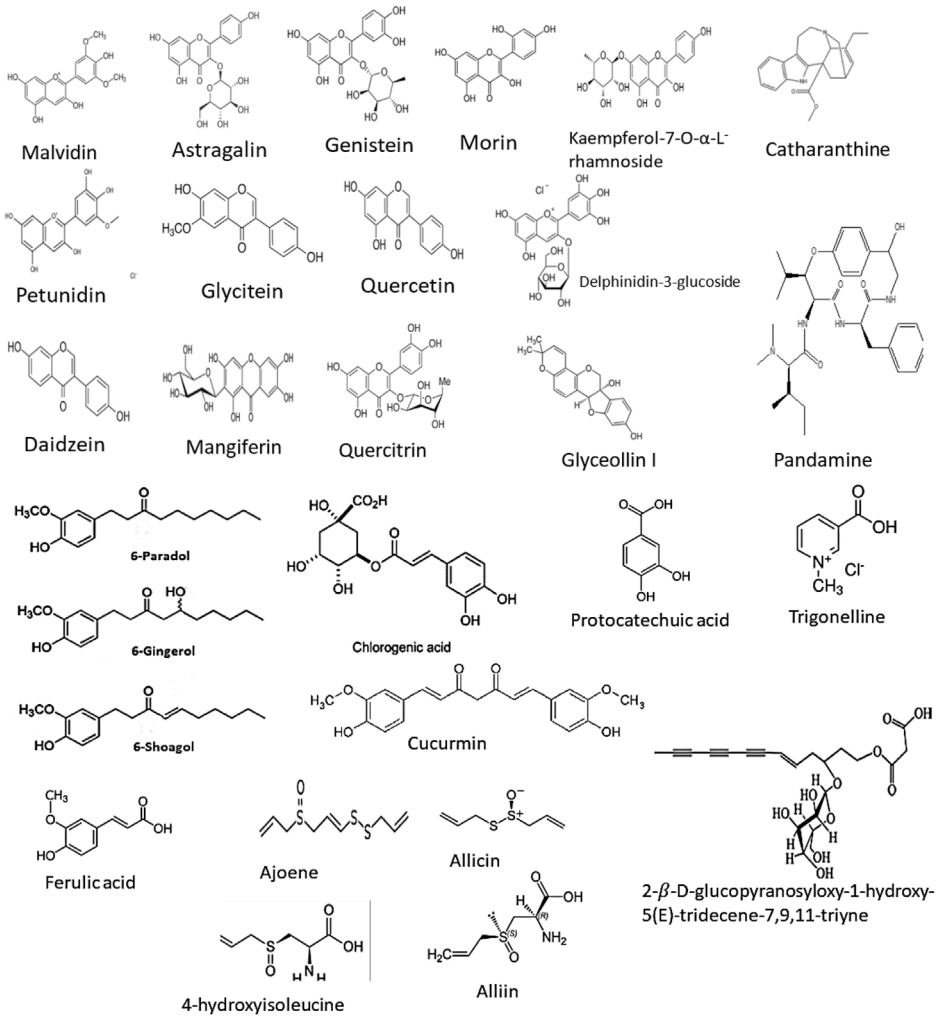


FIGURE 8 | Some bioactive components isolated.

studies do not describe randomization or blinding, and over 95% lack the estimation of sufficient sample size needed for detecting actual effects.

4.2.2 Toxicological Data

For acute toxicity, **Figure 7** shows comparative values of LD_{50} reported for *Cola nitida, Sida acuta, Ficus sycomorus*, *Moringa oleifera, Panda oleosa, Alchornea cordifolia, Morinda lucida, Physalis peruviana, Musanga cecropioides, Vitis vinifera, Erythrina abyssinica, Persea americana, Jatropha curcas, Momordica charantia,* and *Rauwolfia caffra*. Almost all plants but *Jatropha curcas* are relatively non-toxic (LD_{50} > 500 mg/kg).

The highest value of LD_{50} was observed at 8,600 mg/kg bw in rodents with an oral administration of *Alchornea cordifolia.* The bark extract of Panda oleosa was practically non-toxic in guinea pigs with an LD_{50} of approximately 7,892 mg/kg bw; no signs of intoxication were observed with oral doses less than 2,000 mg/kg. However, at doses above 6,000 mg/kg, poor mobility, poor appetite, anuria, and death have been noted in animals (Katemo et al., 2018). The administration of the aqueous extract from the bark of *Ficus benghalensis* suggested an LD_{50} > 5,000 mg/kg. In some cases, the toxic effects depended on sex, like *Alchornea cordifolia*, which showed different values of LD_{50} in mice male compared to female animals (8,600 mg/kg in male and 3,800 mg/kg female) (Djimeli et al., 2017). Despite low acute toxicity, many plants exhibit some significant sub-chronic toxicity. *Caesalpinia bonduc* extract showed hematological changes after a subchronic study for 28 days at a dose up to 400 mg/kg bw in rats (Ogunlana et al., 2013). Except for the ripe fruit, solanine and other alkaloids present in all parts of Solanum americanum are toxic (Kuete, 2014). Aloe-emodin (anthraquinone) from *Aloe species* could be mutagenic or/and genotoxic in organs (Lynch et al., 2011). A daily and prolonged administration (28 days) of resveratrol in *Vitis vinifera* exhibited nephrotoxicity in the rat at the high dose (3000 mg/kg bw). Extracts of *Aframomum melegueta* (286–345 mg/kg bw) and *Artemisia annua* (300 mg/kg bw) produced a toxic effect on the development of fetus by

discontinuation of first trimester pregnancies in rats (Inegbenebor et al., 2009; Abolaji et al., 2012). Leaf methanol extract of *Jatropha curcas* decreased the number of live fetuses and increased placental weight (Teixeira et al., 2017). Bulbs' aqueous extract of *Crinum ornatum* had caused significant effects (Central Nervous System), including losing appetite, slow movement, depression, less aggression, and lying at the corners of the cage (Lawal and Dangoggo, 2014). *Erythrina abyssinica* showed similar nervous effects in mice, significantly decreasing motility, sedation, frequent urination, and tremors during the first 6 h after drug administration at different doses (Bunalema et al., 2011). Some compounds in *Salvia officinalis* (Camphor, thujone, and terpene ketones) are considered the most toxic. Their consumption is not recommended in pregnancy and lactation because they are harmful to the fetus and newborn (Ghorbani and Esmaeilizadeh, 2017). A methanol extract (500 and 1,000 mg/kg/day) of *Catharanthus roseus* in the subacute investigation for 14 days showed inevitable mortality and presented some of the signs of intoxication on the study of the liver and kidney rats (Kevin et al., 2012). Sometimes, there are some contradictions in findings from different authors on toxicological studies in animals. In *Cassia occidentalis*, Lagarto et al. (2011) and Mishra et al. (2018) are contradictory. The first group did not report any toxicological signs in biochemical, hematological, and morphological markers, while the second group noticed some changes.

4.2.3 Clinical Trials

Data from the present study showed the lack of local clinical trials of antidiabetic plants used to manage Diabetes in the DRC. Of seven native herbals, only *Raphia gentiliana* fruit extract was given to 25 males and 20 females, aged 18–50 years old, with normal blood sugar levels (Mpiana et al., 2013). The approach followed by the authors did not comply with any clinical trial requirements, and instead, their behavior went like traditional healers themselves.

Table 5 illustrates the assessment of the quality of clinical trials of antidiabetic plants using the Jadad scale for reporting randomized controlled trials based on randomization, blinding, withdrawals, and dropout methods.

In general, out of 213 plants censored, approximately 8.92% (n = 19) have been validated by clinical evidence. These are *Allium cepa* (Jafarpour-Sadegh et al., 2017), *Allium sativum* (Ashraf et al., 2011), *Balanites aegyptiaca* (Rashad et al., 2017), *Citrus aurantium* (Campbell-Tofte et al., 2011), *Elaeis guineensis* (Kalman et al., 2013), *Laurus nobilis* (Khan et al., 2009), *Momordica charantia* (Kim et al., 2020), *Morinda cordifolia* (Algenstaedt et al., 2018), *Moringa oleifera* (Leone et al., 2018), *Rauvolfia-Citrus* (Campbell-Tofte et al., 2011), *Salvia officinalis* (Kianbakht and Dabaghian, 2013), *Terminalia chebula* (Pingali et al., 2020), *Trigonella foenum-graecum* (Hadi et al., 2020); *Urtica dioica* (Kianbakht et al., 2013), *Vernonia amygdalina* (Okolie et al., 2008), *Zea mays* (Sands et al., 2009), and *Zingiber officinale* (Shidfar et al., 2015).

For example, a double-blind, placebo-controlled, randomized clinical trial conducted on 20–60 year-old DMT2 patients who did not receive insulin showed that 3 months supplementation of 3 g of ginger (*Zingiber officinale*) improved glycemic indices, total antioxidant capacity, malondialdehyde, C-reactive protein, serum paraoxonase, dietary intake, and physical activity, measured at the beginning and end of the study, and after 12 h fasting compared to control groups. A randomized, placebo-controlled, parallel-group study with 42 treated patients treated with leaf hydroethanolic extract (500 mg/8 h for 3 months) and 44 as placebo groups showed that the *Salvia officinalis* leaves lowered fasting glucose and HbA1c the baseline at the endpoint with no adverse effects reported. A clinical trial on a juice extract from the fruit of *Morinda cordifolia* (2 ml/kg bw once a day) in patients with DMT2, after 90 days of treatment, presented a significant reduction of morning BG in several cases, an improvement of hyperglycemia status. In a prospective, randomized, double-blind, placebo-controlled clinical investigation, the administration of *Terminalia chebula* (250 and 500 mg/kg bw, for 12 weeks) in 60 diabetic patients significantly improved the endothelial function (reflection index) compared to placebo (−2.55 ± 1.82%, and −5.21 ± 2.41%, respectively). In an 8-weeks randomized controlled clinical trial study of the effect of *Trigonella foenum-graecum* intake seed in 50 patients with T2DM, the plant significantly reduced fasting blood glucose. It improved some liver and kidney function compared with control interventions.

A randomized, double-blind, placebo-controlled clinical trial of *Urtica dioica* leaf extract (500 mg/8 h, 3 months) combined with conventional oral antihyperglycemic drugs was conducted in 46 treated patients vs 46 placebo groups. At the endpoint, the extract significantly lowered the blood levels of fasting glucose, 2 h postprandial glucose, and HbA1c, without significant effects on other hepatic or cardiovascular parameters, vs the placebo. All considered these results demonstrated that nettle is safe and may have a beneficial effect on glycemic control in patients with advanced DMT2 needing insulin therapy. *Vernonia amygdalina* elicited a significant reduction in BG levels at the most postprandial time points and area-under-curve.

Unfortunately, many studies were carried out in poor quality conditions, with unclear randomization methods, threats to blinding, and lack of baseline demographics (Rios et al., 2015). The interpretation of Jadad score on clinical trials reviewed showed that studies conducted on *A. cepa, A. sativum, B.* aegyptiaca, *E. guineensis, M. charantia, R. vomitoria, S. officinalis, U. dioica,* and *Z. officinale* presented excellent quality (Jadad score ≥ 3)(Hartling et al., 2011). In addition to the effectiveness of the plant materials (extracts, isolated compounds), clinical trials must include other vital parameters to an antidiabetic evaluation in particular glycosylated hemoglobin A1c (HbA1c), personal medication, insulin, glycogen, lipid and protein profiles, and severity of adverse effects, patient's risk factors, ease of use, patient's financial situation, etc. (Chaudhury et al., 2017).

4.3 Analysis of Phytochemical Data

Various second metabolites have been identified and isolated, as shown in **Table 6** and **Figure 8**. Qualitative and

quantitative content may vary with the soil where the plants are growing.

To illustrate, *Allium cepa* contains ferulic acid, alliin (Tang et al., 2008), agavasaponin C (Tang et al., 2008), flavonoid alliuocide G (Mohamed, 2008), quercetin, sulfur compounds, S-methyl cystein sulfoxide, S-allyl cysteine and diallyl thiosulfanate (Bakhshaeshi et al., 2012; Noor et al., 2013). *Allium sativum* contains S-allylcysteine sulfoxide, alliin, diallyl trisulfide (Liu et al., 2007; Mikaili et al., 2013), isoeruboside B, agavasaponin C, proto-iso-erubisoide B, 2-Vinyl-4H-1,3-dithiin (Tang et al., 2008), allicin, diallyl disulfide, diallyl sulfide, ajoene, and allyl mercaptan (Bayan et al., 2014). In *mangifera indica*, one found mangiferin (Cruz-Vega et al., 2009), 1,2,3,4,6 Penta-O-galloyl-β-d-glucose (Mohan et al., 2013), curcumin, morin (Gothai et al., 2016), gallic acid, protocatechuic acid, kaempferol (Ediriweera et al., 2017). *Catarantus* has gallic acid, chlorogenic acid (Rianika and Robert, 2007), vindoline I, vindolidine II, vindolicine III and vindolinine (Tiong et al., 2013), catharanthine, vindoline, vindolinene, vinblastine, vincristine (Bharti et al., 2018). *Brassica juncea* (L.) Czern has cinnamic acid (Guzman, 2014), kaempferol (Gothai et al., 2016), aniline. *Bidens pilosa* has cytopiloyne, 2-β-D-Glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (Chang et al., 2013a), polyynes (Bartolome et al., 2013), 3-β-D-glucopyranosyl-1-hydroxy-6(E)-tetradecene-8,10,12-triyne; 2-β-D-glucopyranosyloxy-1-hydroxy-5(E)-tridecene-7,9,11-triyne (Chang et al., 2013b). *Caesalpinia decapetala* has apigenin-7-rhamnoside, astragalin, 6-hydroxy kaempferol, quercitrin (Parveen et al., 2017). *Erythrina abyssinica* contains daidzein, genistein, glycitein, beta-Sitosterol, Soyasaponin A1-A6, soyasaponin V, stigmasterol (Tang et al., 2008), anthocyanins (Nizamutdinova et al., 2009), lyceollin I-II (Chang et al., 2013b), kaempferol glycoside, kaempferol (Zang et al., 2014), stigmasterol (Wang F. et al., 2017), genistein, diadzein) (Bharti et al., 2018). *Phaseolus vulgaris* contains stigmasterol (Tang et al., 2008), catechin (Gothai et al., 2016), flavonoids and their glucosides of delphinidin, petunidin, and malvidin, anthocyanins, catechin, myricetin 3-O-arabinoside, epicatechin, vanillic acid, syringic acid, and O-coumaric acid (Ganesan and Xu, 2017). *Tephrosia vogelii* has galactomannan (Anwar et al., 2011), 4-hydroxyisoleucine (Rangari et al., 2014; Naicker et al., 2016), diosgenin, galactomannan, flavonoids, trigonelline (Zameer et al., 2017). *Syzygium guineense* contains pinitol, β-sitosterol, quercetin, quercetin 3-O-α-L-rhamnopyranoside (Jawlal et al., 2013). *Aframomum melegueta* has 3 arylalkanes, 6-paradol, 6-shogaol, 6-gingerol, 6-gingeredione, a pentacyclic triterpene, oleanolic acid isolated from the fruit (Sugita et al., 2013; Mohammed et al., 2017).

4.3.1 Alkaloids

The sulfur compounds present in the onion can significantly control the blood glucose and lipids in serum and tissues and normalize liver hexokinase, glucose 6-phosphatase and HMG CoA reductase (Akash et al., 2014). It was shown that vindoline I, vindolidine II, vindolicine III, and vindolinine improve the hyperglycemia condition of type 2 diabetes by enhancing glucose uptake in pancreatic or muscle cells. In addition, they can inhibit *in vitro* PTP-1B, which lessens insulin resistance. Vindolicine III was the most potent (Tiong et al., 2013). Catharanthine, vindolinene, vinblastine, vincristine lower blood sugar levels through free radical scavenging action (Bharti et al., 2018). On the other hand, p-synephrine increased the glucose output concentration and ameliorated glycolysis and glycogenolysis (Suntar et al., 2018). *N-trans-p*-coumaroyloctopamine, *N-trans-p*-feruloyl-octopamine, *N-trans-p*-coumaroyltyramine, and *N-trans-p*-feruloyltyramine, amide alkaloids, showed alpha-glucosidase effect and free radicals inhibitions (Silva et al., 2017).

4.3.2 Amino Acids, Amines, and Carboxylic Acid Derivatives

Alliin offered protection against glucose or methylglyoxal-induced glycation of superoxide dismutase (Anwar and Younus, 2017). S-allyl cystein sulfoxide (SACS), allicin, and garlic oil precursor stimulated *in vitro* insulin secretion from beta cells isolated from normal rats (Kodera et al., 2017). It restored erectile function in diabetic rats (Yang et al., 2013). Unique and repeated intraperitoneal administrations of a protein (Mo-LPI) decreased blood glucose concentration at different times in rats. 2S, 3R,4S hydroxy isoleucine, an amino acid considered an insulinotropic agent, possesses antidiabetic potential by several mechanisms, including regulating glucose metabolism, lipid profile, and uric acid (Rangari et al., 2014).

4.3.3 Carbohydrates and Sucrose Esters

Peruvioses A,B,C,D,E, and F possess antidiabetic potential by alpha-amylase inhibition activity (Bernal et al., 2018). In the Streptozotocin-induced diabetic mice group, rhamnogalacturonan (a polysaccharide) decreased blood glucose level and glucose tolerance and slightly improved blood glucose within 30 min (Liu et al., 2017). Polysaccharides repaired the pancreatic β cells damages in a high-fat diet STZ-induced type 2 diabetic mice by improvement of SOD concentration and the reduction of MDA level and restoration of kidney and pancreas tissues (Wang et al., 2019). Furthermore, a water-soluble polysaccharide significantly lowered fasting blood glucose level and improved glucose tolerance and weight loss in alloxan-induced diabetic mice compared to the diabetic control group (Xu et al., 2015).

4.3.4 Glycosides

Cytopiloyne, a polyacetylene glucoside, reduced postprandial blood glucose levels, increased blood insulin, improved glucose tolerance, suppressed HbA1c level, and protected pancreatic islets in diabetic db/db mice (Chang et al., 2013b).

Supplementation of *Naringin* improved glucose intolerance and insulin resistance in a model of high-fat-diet–fed mice (Pu et al., 2012). Naringin (together with Neohesperidin, *h*esperidin, and nobiletin) significantly inhibited amylase-catalyzed starch digestion and played roles in hyperglycemia management by increasing hepatic glycolysis and glycogen concentration and lowering hepatic gluconeogenesis. Furthermore, hesperidin, naringin, and nobiletin reduced hepatic gluconeogenesis and improved insulin sensitivity in animal models (Lv et al., 2015).

Neohesperidin significantly decreased fasting glucose, serum glucose, and glycosylated serum protein in mice. In addition, this compound significantly reduced serum triglycerides, total cholesterol, leptin level, and liver index; it inhibited lipid accumulation in the liver and decreased the size of epididymal adipocytes in the KK-Ay mice (Osfor et al., 2013; Jia et al., 2015).

Some phenolic glycosides, including niazirin A, S-Methyl-N-{4-[(α-l-rhamnosyloxy)benzyl]}thiocarbamate, reduced blood glucose levels in STZ-induced diabetic mice and promoted the glucose consumption of IR cells (Wang F. et al., 2017).

Isothiocyanates inhibited gluconeogenesis and hepatic glucose-6-phosphatase (G6P) expression in hepatoma cells and improved glucose tolerance and insulin signaling sensitivity (Waterman et al., 2016).

Galactomannan showed significant dose-related hypoglycaemic and antihyperglycaemic effects; the obtained results were better than glibenclamide used as reference (Anwar et al., 2011).

Aloe-emodin-8-*O*-glycoside enhanced glucose transport through proximal and distal marker modulation involved in glucose uptake and its transformation into glycogen (Salehi et al., 2018).

Syringin, a phenylpropanoid glucoside, indicated a significant reduction of blood glucose and HbA1c levels and improved transaminase enzymes, plasma protein, blood urea, serum creatinine, and uric acid levels. Inversely, it increased plasma insulin and hemoglobin levels in diabetic rats (Sundaram et al., 2014).

Rutin (a flavonol glycoside) significantly increased *in vivo* glucose-induced insulin secretion and acted as an insulin secretagogue in the management of glucose homeostasis (Kappel et al., 2013). Hirsutrin was suggested to prevent osmotic stress in hyperglycemia conditions by inhibiting RLAR activity and galactitol formation in rat lenses (Kim et al., 2013).

According to Fernando et al. (2019), three flavone C-glycosides, vicenin-1, isoschaftoside, and schaftoside, respectively, inhibited 60.3, 33.8, and 95.5% of pancreatic lipase enzyme, which plays a vital role in obesity (as a crucial factor in the occurrence of DMT2). Phenolic-C-glycosides enhanced and stimulated the glucose update process in mouse skeletal muscle cells (Mishra et al., 2013).

A stearoyl glucoside of ursolic acid (urs-12-en-3β-ol-28-oic acid 3β-D-glucopyranosyl-4′- octadecanoate) demonstrated an antidiabetic property by lowering sugar blood in rats from the 8th day to the 21st day of the experiment (Kazmi et al., 2012).

4.3.5 Phytosterols

Sanni et al. (2019) suggested that sitosterol, stigmasterol, campesterol, squalene, and nimbiol might have antidiabetic potential through their molecular docking with AMP-activated protein kinase (α-AMPK) and alpha-amylase and alpha-glucosidase inhibitions. Stigmasterol increased GLUT4 translocation and expression *in vitro*. In mice, it alleviated insulin resistance, glucose tolerance by reducing fasting blood glucose levels and blood lipid (triglyceride and cholesterol) (Wang F. et al., 2017).

4.3.6 Polyphenols

Quercetin and its glycosides protected β-cell mass and function under high-fructose induction (Li et al., 2013). 4'-methyl-quercetin-7-*O*-β-D-glucuronopyranoside enzymes, quercetin-3-*O*-glucoside, avicularin, castalagin, and 2,3-hexahydroxydiphenoyl-(α/β)-D-(4)C1-glucopyranose showed inhibition capacity of sucrase (Abdelhady et al., 2016). Moreover, they exhibited significant inhibition of alpha-glucosidase and alpha-amylase enzymes compared to acarbose (Wang et al., 2010; Olennikov and Kashchenko, 2014). A flavonoid named alliuocide G showed *in vitro* alpha-amylase inhibitory activity and radical scavenging potency (Mohamed, 2008).

Cinnamic acid and its derivatives (caffeic acid, ferulic acid, isoferulic acid, and *p*-hydroxycinnamic acid) are associated with a beneficial influence on Diabetes and its complications through many mechanisms. The most well-known are: stimulation of insulin secretion, improvement of pancreatic β-cell functionality, inhibition of hepatic gluconeogenesis, enhanced glucose uptake, increased insulin signaling pathway, delay of carbohydrate digestion and glucose absorption, and inhibition of protein glycation (Adisakwattana, 2017). Ferulic acid regenerated pancreatic beta-cells, reduced the risk of high-fat diet-induced hyperglycemia via insulin secretion and hepatic glucose-regulating enzyme activities, and regulated blood glucose levels by elevating glucokinase activity and production of glycogen (Silva and Batista, 2017). Caffeic acid produced a significant alpha-glucosidase inhibition comparing with acarbose (Olennikov and Kashchenko, 2014). In addition, together with chlorogenic acid and chicoric acid, it increased glucose uptake in muscle cells and stimulated insulin secretion from an insulin-sensitizing and insulin-secreting cell line and islets (Tousch et al., 2008; Ferrare et al., 2017). Caffeoylquinic acid derived from caffeic acid showed high inhibitory activity against digestive enzymes, exceptionally higher against alpha-amylase and alpha-glucosidase (Olennikov et al., 2018).

A study indicated that epigallocatechin and epigallocatechin gallate reduced fasting blood glucose levels, triglycerides, and total cholesterol in streptozotocin-induced diabetic mice (Bakoma et al., 2018). Also, apigenin-7-rhamnoside, astragalin, 6-hydroxy kaempferol, quercitrin exhibited significant activity against alpha-glucosidase enzyme (Parveen et al., 2017). Kaempferol (Fraction B) lowered blood glucose of alloxan-induced diabetic rats. It also inhibited alpha-amylase and alpha-glucosidase and reversed altered lipid profile and oxidative stress biomarkers in diabetic rats (Ibitoye et al., 2017). Kaempferol and myricetin showed high inhibitory activities against alpha-amylase and alpha-glucosidase (Wang et al., 2010).

Compared with the reference compound acarbose, Aesculetin and isorhamnetin demonstrated significantly higher inhibitory activity (Olennikov and Kashchenko, 2014). Polyphenols

compounds such as proanthocyanidins and anthocyanins showed as potential natural alpha-glucosidase inhibitors (Dey and Mitra, 2013).

Anthocyanins efficiently protected pancreatic beta-cells from cell death in HIT-T15 cell culture and db/db mice (Hong et al., 2013). Johnson et al. (2015) demonstrated that prenylated anthranols possess an alpha-glucosidase inhibitory potential. According to their findings, the most antidiabetic activity was found with harunganol compared to acarbose.

Kolaviron, a bioflavonoid complex, demonstrated a significant reduction of glycemia in normoglycemic rats. Moreover, kolaviron showed a significant antidiabetic potential in streptozotocin-induced rats (Adaramoye and Adeyemi, 2006).

Ellagic acid and its derivatives act as a hypoglycaemic agent on carbohydrate digestion and absorption, insulin secretion (Bharti et al., 2018). Hydrolyzable tannins including 1,2,3,6-tetra-*O*-galloyl-4-*O*-cinnamoyl-b-D-glucose and 4-*O*-(200,400-di-*O*-gal-loyl-a-L-rhamnosyl) ellagic acid showed significant alpha-glucosidase inhibitory efficacy with IC_{50} values of 2.9 and 6.4 mM, respectively (Lee et al., 2017).

Chicoric acid lowered the glycaemic levels of diabetic mice (Casanova et al., 2014) significantly. Valoneic acid dilactone, a hydrolyzable tannin, showed a potential antidiabetic effect alpha-amylase enzyme activity compared to the value obtained by acarbose. In the same way, it significantly inhibited aldose reductase enzyme activity and PTP1B enzyme activity. However, *in vivo* evaluation, it reduced the BGL considerably in acute evaluation for 4 h. Furthermore, oral administration of the compound for 21 days significantly decreased BGL and improved the tolerance to glucose compared to control groups (Jain et al., 2012).

Gingerols demonstrated antidiabetic potential by enhancing glucose uptake. Primarily, (S)-[8]-Gingerol was found to be the most potent on glucose uptake and increase in the surface distribution of GLUT4 protein on the L6 myotube plasma membrane (Noipha and Ninla-aesong, 2018).

ρ-Coumaric acid exhibited higher inhibition activity against alpha-glucosidase (98.8%) than acarbose (62.5%). However, acarbose showed the most potent inhibition against alpha-amylase (98.6 vs 66.8%) (Aalim et al., 2019).

Luteolin showed significant alpha-glucosidase and alpha-amylase inhibitory activities (Dekdouk et al., 2015). Chebulagic acid (a benzopyran tannin) reduced maltose-hydrolysis and sucrose-hydrolysis activities. Meanwhile, it induced a decrease at 11.1% of postprandial blood sugar value in maltose-loaded Sprague-Dawley rats (Huang et al., 2012).

Furofuran lignans with a free hydroxyl synthesized from herein demonstrated an inhibition potential against alpha-glucosidase and free radicals (Worawalai et al., 2016). Previously, Wikul et al. (2012) conducted bio-guided isolation and showed that (+)-pinoresinol, a lignan, had inhibitory activity against rat intestinal maltase. Also, *N-trans*-feruloyl tyramine, *N-trans-p*-coumaroyl tyramine, and *N-cis-p*-coumaroyl tyramine (Phenylmethyl cinnamates) showed inhibitory activity against alpha-glucosidase (Liu et al., 2011).

4.3.7 Saponins

Pseudoprototinosaponin AIII and prototinosaponins AIII produced a hypoglycaemic effect on glucose uptake and insulin release due to their actions on hepatic gluconeogenesis or glycogenolysis (Patel et al., 2012). Furostanol saponin showed significant antidiabetic potential *in vitro* by reducing the fasting plasma glucose level by 46.14% and increasing insulin and C-peptide levels (Ezzat et al., 2017). [3β,7β,25- trihydroxycucurbita-5,23(E)-dien-19-al, momordicine I, momordicine II, 3-hydroxycucurbita-5,24-dien-19-al-7,23-di-*O*-β-glucopyranoside, and kuguaglycoside G] were potent in the β-cell insulin secretion evaluation. Momordicine II and kuguaglycoside have stimulated insulin secretion 7.3 and 7.1 times and 8.1 and 7.8 times more, respectively, than the control group (Keller et al., 2011).

25-O-methylkaraviagein D, karaviloside II, and (19R,23E)-5b,19-epox y-19,25-dimethoxycucurbita-6,23-dien-3b-ol, cucurbitane exhibited significant inhibitory activity on alpha-glucosidase with IC_{50} values of 10.19, 28.55, and 20.20 μM, respectively (Yue et al., 2017). Oral administration of saponins improved body weight and insulin resistance. There was an increase in fasting blood glucose concentration and the proportion of hepatic phosphorylated adenosine monophosphate-activated protein kinase (p-AMPK)/total protein (Wang et al., 2019).

4.3.8 Terpenoids

Oleanolic acid, a plant-derived triterpenoid, boosted insulin secretion *in vitro* and stimulated insulin secretion at both basal and stimulatory glucose concentrations in INS-1 832/13 cells, and enhanced acute glucose-stimulated insulin secretion cultured β-cells (Teodoro et al., 2008). Furthermore, it decreased serum glucose and insulin concentrations in mice fed with a high-fat diet and enhanced glucose tolerance (Sato et al., 2007). Oleanolic and ursolic acids showed potent alpha-glucosidase and alpha-amylase inhibition. Ursolic acid showed uncompetitive inhibition of alpha-glucosidase compared to acarbose as a competitive inhibitor (Ali et al., 2006; Salah El Dine et al., 2014).

Thujone, a monoterpene existing as two stereoisomers (α- and β-Thujone), is an ingredient of essential oils of many great different herbs; it can increase free insulin-stimulated glucose transporter by activation of adenosine monophosphate-activated protein kinase (Daradka et al., 2014).

α-amyrin acetate (a pentacyclic triterpenoid) lowered the blood glucose profile in STZ-induced diabetic rats and db/db mice at 50 mg kg1 dose level (Singh et al., 2009). Pahlavani et al. (2019) showed that some compounds like charantin (a triterpenoid phytoconstituent), possess antidiabetic potential by several mechanisms, including insulin secretion increase, insulin resistance decrease, skeletal muscle cell glucose utilization increase, and inhibition of intestinal enzymes.

Cucurbitane-type compounds (3β,7β,25-trihydroxycucurbita-5,23(E)-dien-19-al, charantal, charantoside XI, and 25ξ-isopropenylchole-5, 6-ene-3-O-D-glucopyranoside),

demonstrated an alpha-amylase and alpha-glucosidase inhibitory activities ranging from 56 to 79% (Shivanagoudra et al., 2019).

Two monoterpenes (1S,2R,3R,5S)-2-hydroxymethyl-6,6-dimethylbicyclo[3.1.1]heptane-2,3- diol, and sobrerol significantly increased glucose uptake in 3T3-L1 adipocytes (Li et al., 2013b). On the other hand, three germacrene sesquiterpenes increased glucose uptake substantially without significant toxic effects in 3T3-L1 adipocytes (Zhao et al., 2012).

5 CONCLUSION

Multiple investigations have been carried out on natural products, mainly plants used to treat Diabetes Mellitus worldwide. In DRC, a country with a high ecological, cultural and human diversity, traditional medicine through plants occupies an important place in the health system. Several ethnopharmacological and ethnobotanical studies have been conducted previously in this perspective, and various plant species have been identified. Contrary to the previous review, the present review assessed the quality of studies carried inside DRC and resorted similarities/discrepancies with studies conducted outside. The findings confirm the high diversity of the flora and the various ethnic groups in DRC. Most of the plants claimed as antidiabetic and used by traditional healers in the DRC are not specifically native to DRC. One hundred thirty-four native and introduced species have been experimentally validated by various pharmacological, toxicological, and phytochemical researches. Many plants are safe at doses < 500 mg/kg, but long-term use may trigger sub-chronic toxicity. Exclusively conducted in DRC, preclinical and clinical studies of some plant species demonstrated poor protocol quality. Locally specific species deserve in-depth investigations to meet scientific requirements for their introduction studies into the national pharmacopeia. Although a few plants reduced blood sugar levels, clinical data and antidiabetic studies of the isolated compounds remain limited to allow the availability and accessibility of standardized phytomedicines to Congolese. This review constitutes a primary database for further experimental studies, especially for unstudied species in the perspective of safe and efficient use of easily accessible natural resources.

AUTHOR CONTRIBUTIONS

FK conceived the manuscript, wrote the first draft, and analyzed data. JK analyzed data and rewrote the manuscript draft. All authors conducted the literature search. All authors read, corrected, and approved the final manuscript.

ACKNOWLEDGMENTS

The authors are grateful to the Mbarara University of Science and Technology (MUST), Pharm-Bio Technology and Traditional Medicine Centre (PHARMBIOTRAC), and The World Academy of Science (TWAS), International Center of Chemical and Biological Sciences (ICCBS) for providing Ph.D. Scholarships to FK.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.757090/full#supplementary-material

REFERENCES

Aalim, H., Belwal, T., Jiang, L., Huang, H., Meng, X., and Luo, Z. (2019). Extraction Optimization, Antidiabetic and Antiglycation Potentials of Aqueous Glycerol Extract from rice (*Oryza Sativa* L.) Bran. *Lwt* 103, 147–154. doi:10.1016/j.lwt.2019.01.006

Abdelhady, M. I., Shaheen, U., Bader, A., and Youns, M. A. (2016). A New Sucrase Enzyme Inhibitor from *Azadirachta indica*. *Pharmacogn. Mag.* 12, S293–S296. doi:10.4103/0973-1296.185705

Abernethy, K., Maisels, F., and White, L. J. T. (2016). Environmental Issues in Central Africa. *Annu. Rev. Environ. Resour.* 41, 1–33. doi:10.1146/annurev-environ-110615-085415

Abolaji, A. O., Eteng, M. U., Ebong, P. E., Brisibe, E. A., Dar, A., Kabir, N., et al. (2012). A Safety Assessment of the Antimalarial HerbArtemisia annuaDuring Pregnancy in Wistar Rats. *Phytother. Res.* 27, 647–654. doi:10.1002/ptr.4760

Adaramoye, O. A., and Adeyemi, E. O. (2006). Hypoglycaemic and Hypolipidaemic Effects of Fractions from Kolaviron, a Biflavonoid Complex from *Garcinia Kola* in Streptozotocin-Induced Diabetes Mellitus Rats. *J. Pharm. Pharmacol.* 58, 121–128. doi:10.1211/jpp.58.1.0015

Adebayo, A. H., Zeng, G. Z., Zhang, Y. M., Ji, C. J., Akindahunsi, A., and Tan, N. H. (2010). Toxicological Evaluation of Precocene II Isolated from *Ageratum Conyzoides* L. (Asteraceae) in Sprague Dawley Rats. *Afr. J. Biotechnol.* 9, 2938–2944.

Adisakwattana, S. (2017). Cinnamic Acid and its Derivatives: Mechanisms for Prevention and Management of Diabetes and its Complications. *Nutrients* 9, 163. doi:10.3390/nu9020163

Adrien Kasanga, L. (2012). English in the Democratic Republic of the Congo. *World Englishes* 31, 48–69. doi:10.1111/j.1467-971x.2011.01732.x

Agarwal, A., D'Souza, P., Johnson, T. S., Dethe, S. M., and Chandrasekaran, C. (2014). Use of *In Vitro* Bioassays for Assessing Botanicals. *Curr. Opin. Biotechnol.* 25, 39–44. doi:10.1016/j.copbio.2013.08.010

Akash, M. S. H., Rehman, K., and Chen, S. (2014). Spice Plant *Allium cepa*: Dietary Supplement for Treatment of Type 2 Diabetes Mellitus. *Nutrition* 30, 1128–1137. doi:10.1016/j.nut.2014.02.011

Algenstaedt, P., Stumpenhagen, A., and Westendorf, J. (2018). The Effect ofMorinda citrifoliaL. Fruit Juice on the Blood Sugar Level and Other Serum Parameters in Patients with Diabetes Type 2. *Evidence-Based Complement. Altern. Med.* 2018, 1–10. doi:10.1155/2018/3565427

Ali, H., Houghton, P. J., and Soumyanath, A. (2006). Alpha-Amylase Inhibitory Activity of Some Malaysian Plants Used to Treat Diabetes; with Particular Reference to Phyllanthus Amarus. *J. Ethnopharmacol.* 107, 449–455. doi:10.1016/j.jep.2006.04.004

Amuri, B., Maseho, M., Simbi, L., Duez, P., and Byanga, K. (2018). Ethnobotanical Survey of Herbs Used in the Management of Diabetes Mellitus in Southern Katanga Area/DR Congo. *Pan Afr. Med. J.* 30, 218. doi:10.11604/pamj.2018.30.218.11718

Amuri, B., Maseho, M., Simbi, L., Okusa, P., Duez, P., and Byanga, K. (2017). Hypoglycemic and Antihyperglycemic Activities of Nine Medicinal Herbs Used

as Antidiabetic in the Region of Lubumbashi (DR Congo). *Phytother Res.* 31, 1029–1033. doi:10.1002/ptr.5814

Anh, H. L. T., Vinh, L. B., Lien, L. T., Cuong, P. V., Arai, M., Ha, T. P., et al. (2021). *In Vitro* study on α-amylase and α-glucosidase Inhibitory Activities of a New Stigmastane-type Steroid Saponin from the Leaves of Vernonia Amygdalina. *Nat. Product. Res.* 35, 873–879. doi:10.1080/14786419.2019.1607853

Anwar, S., and Younus, H. (2017). Inhibitory Effect of Alliin from *Allium Sativum* on the Glycation of Superoxide Dismutase. *Int. J. Biol. Macromol.* 103, 182–193. doi:10.1016/j.ijbiomac.2017.05.043

Anwar, S., Desai, S., Eidi, M., and Eidi, A. (2011). Antidiabetic Activities of Fenugreek (Trigonella Foenum-Graecum) Seeds. *Nuts and Seeds in Health and Disease Prevention* 469, 478. doi:10.1016/B978-0-12-375688-6.10056-8

Arias-Díaz, J., and Balibrea, J. (2007). Animal Models in Glucose Intolerance and Type-2 Diabetes. *Nutr. Hosp.* 22, 160–168. doi:10.2478/cipms-2019-0036

Ashraf, R., Khan, R., and Ashraf, I. (2011). Effects of Garlic on Blood Glucose Levels and HbA1c in Patients with Type 2 Diabetes Mellitus. *J. Med. Plants Res.* 5, 2922–2928.

Auboire, L., Sennoga, C. A., Hyvelin, J. M., Ossant, F., Escoffre, J. M., Tranquart, F., et al. (2018). Microbubbles Combined with Ultrasound Therapy in Ischemic Stroke: A Systematic Review of *Iin-Vvivo* Preclinical Studies. *PLoS One* 13, e0191788. doi:10.1371/journal.pone.0191788

Baker, D., Lidster, K., Sottomayor, A., and Amor, S. (2014). Two Years Later: Journals Are Not yet Enforcing the ARRIVE Guidelines on Reporting Standards for Pre-clinical Animal Studies. *Plos Biol.* 12, e1001756. doi:10.1371/journal.pbio.1001756

Bakhshaeshi, M., Khaki, A., Fathiazad, F., Khaki, A. A., and Ghadamkheir, E. (2012). Anti-oxidative Role of Quercetin Derived from *Allium cepa* on Aldehyde Oxidase (OX-LDL) and Hepatocytes Apoptosis in Streptozotocin-Induced Diabetic Rat. *Asian Pac. J. Trop. Biomed.* 2, 528–531. doi:10.1016/S2221-1691(12)60090-2

Bano, A., Ahmad, M., Ben Hadda, T., Saboor, A., Sultana, S., Zafar, M., et al. (2014). Quantitative Ethnomedicinal Study of Plants Used in the Skardu valley at High Altitude of Karakoram-Himalayan Range, Pakistan. *J. Ethnobiol. Ethnomed.* 10, 43. doi:10.1186/1746-4269-10-43

Bartolome, A. P., Villaseñor, I. M., and Yang, W.-C. (2013). Bidens pilosaL. (Asteraceae): Botanical Properties, Traditional Uses, Phytochemistry, and Pharmacology. *Evidence-Based Complement. Altern. Med.* 2013, 1–51. doi:10.1155/2013/340215

Bashige Chiribagula Valentin, B. C., Alombong Alonie Gracia, A. A., Kamwimba Mahungala Arlette, K. M., Bakari Amuri Salvius, B. A., and Okusa Ndjolo philippe, O. N. (2020). Ethnobotanical Study of Medicinal Plants Used in the Treatment of Sexual Dysfunctions in Traditional Medicine in Kampemba-Lubumbashi, DR Congo. *World J. Adv. Res. Rev.* 7, 016–032. doi:10.30574/wjarr.2020.7.3.0328

Bashige-Chiribagula, V., Bakari-Amuri, S., Mbuyi-Kalonji, S., Kahumba-Byanga, J., Duez, P., and Lumbu-Simbi, J. B. (2017). Étude ethnobotanique, phytochimique et évaluation de l'activité antiplasmodiale de 13 plantes réputées antipaludéennes dans la commune du Kenya (Lubumbashi, RDC). *Phytothérapie*. doi:10.1007/s10298-017-1152-x

Batomayena, B., Benedicte, B., Aboudoulatif, D., Kwashie, E.-G., Kodjo, A., Messanvi, G., et al. (2018). Catechins as Antidiabetic Compounds of *Bridelia Ferruginea* Benth Root Bark Extract. *J. Pharmacognosy Phytother.* 10, 182–186. doi:10.5897/JPP2018.0528

Bayan, L., Koulivand, P. H., and Gorji, A. (2014). Garlic: A Review of Potential Therapeutic Effects. *Avicenna J. Phytomed* 4, 1–14.

Belhadj, S., Hentati, O., Hammami, M., Ben Hadj, A., Boudawara, T., Dammak, M., et al. (2018). Metabolic Impairments and Tissue Disorders in Alloxan-Induced Diabetic Rats Are Alleviated by *Salvia Officinalis* L. Essential Oil. *Biomed. Pharmacother.* 108, 985–995. doi:10.1016/j.biopha.2018.09.108

Bennett, B. C., and Prance, G. T. (2000). Introduced Plants in the Indigenous Pharmacopoeia of Northern South America. *Econ. Bot.* 54, 90–102. doi:10.1007/bf02866603

Bernal, C. A., Castellanos, L., Aragón, D. M., Martínez-Matamoros, D., Jiménez, C., Baena, Y., et al. (2018). Peruvioses A to F, Sucrose Esters from the Exudate of Physalis Peruviana Fruit as α-amylase Inhibitors. *Carbohydr. Res.* 461, 4–10. doi:10.1016/j.carres.2018.03.003

Bharti, S. K., Krishnan, S., Kumar, A., and Kumar, A. (2018). Antidiabetic Phytoconstituents and Their Mode of Action on Metabolic Pathways. *Ther. Adv. Endocrinol. Metab.* 9, 81–100. doi:10.1177/2042018818755019

Boue, S. M., Daigle, K. W., Chen, M. H., Cao, H., and Heiman, M. L. (2016). Antidiabetic Potential of Purple and Red rice (*Oryza Sativa* L.) Bran Extracts. *J. Agric. Food Chem.* 64, 5345–5353. doi:10.1021/acs.jafc.6b01909

Bunalema, L., Kirimuhuzya, C., Tabuti, J. R., Waako, P., Magadula, J. J., Otieno, N., et al. (2011). The Efficacy of the Crude Root Bark Extracts of Erythrina Abyssinica on Rifampicin Resistant *Mycobacterium tuberculosis*. *Afr. Health Sci.* 11, 587–593.

Burlando, B., and Cornara, L. (2014). Therapeutic Properties of rice Constituents and Derivatives (*Oryza Sativa* L.): A Review Update. *Trends Food Sci. Technology* 40, 82–98. doi:10.1016/j.tifs.2014.08.002

Burnham, R. J., and Johnson, K. R. (2004). South American Palaeobotany and the Origins of Neotropical Rainforests. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 359, 1595–1610. doi:10.1098/rstb.2004.1531

Campbell-Tofte, J. I., Mølgaard, P., Josefsen, K., Abdallah, Z., Hansen, S. H., Cornett, C., et al. (2011). Randomized and Double-Blinded Pilot Clinical Study of the Safety and Anti-diabetic Efficacy of the Rauvolfia-Citrus tea, as Used in Nigerian Traditional Medicine. *J. Ethnopharmacol.* 133, 402–411. doi:10.1016/j.jep.2010.10.013

Casanova, L. M., Da Silva, D., Sola-Penna, M., Camargo, L. M., Celestrini, Dde. M., Tinoco, L. W., et al. (2014). Identification of Chicoric Acid as a Hypoglycemic Agent from *Ocimum Gratissimum* Leaf Extract in a Biomonitoring *In Vivo* Study. *Fitoterapia* 93, 132–141. doi:10.1016/j.fitote.2013.12.024

Chang, C. L.-T., Liu, H.-Y., Kuo, T.-F., Hsu, Y.-J., Shen, M.-Y., Pan, C.-Y., et al. (2013a). Antidiabetic Effect and Mode of Action of Cytopiloyne. *Evidence-Based Complement. Altern. Med.* 2013, 1–13. doi:10.1155/2013/685642

Chang, C. L. T., Lin, Y., Bartolome, A. P., Chen, Y.-C., Chiu, S.-C., and Yang, W.-C. (2013b). Herbal Therapies for Type 2 Diabetes Mellitus: Chemistry, Biology, and Potential Application of Selected Plants and Compounds. *Evidence-Based Complement. Altern. Med.* 2013, 1–33. doi:10.1155/2013/378657

Chaudhury, A., Duvoor, C., Reddy Dendi, V. S., Kraleti, S., Chada, A., Ravilla, R., et al. (2017). Clinical Review of Antidiabetic Drugs: Implications for Type 2 Diabetes Mellitus Management. *Front. Endocrinol. (Lausanne)* 8, 6. doi:10.3389/fendo.2017.00006

Chauhan, A., Sharma, P., Srivastava, P., Kumar, N., and Dudhe, R. (2010). Plants Having Potential Antidiabetic Activity: a Review. *Der Pharm. Lett.* 2, 369–387. doi:10.5958/j.0975-4261.2.1.005

Cruz-Vega, D., Verde-Star, M. J., Salinas-Gonzalez, N. R., Rosales-Hernandez, B., Estrada-Garcia, I., Mendez-Aragon, P., et al. (2009). Review of Pharmacological Effects of *Glycyrrhiza Radix* and its Bioactive Compounds. *Zhongguo Zhong Yao Za Zhi* 22, 557–559. doi:10.1002/ptr

Daradka, H. M., Abas, M. M., Mohammad, M. A. M., and Jaffar, M. M. (2014). Antidiabetic Effect of *Artemisia Absinthium* Extracts on Alloxan-Induced Diabetic Rats. *Comp. Clin. Pathol.* 23, 1733–1742. doi:10.1007/s00580-014-1963-1

de Oliveira Silva, E., and Batista, R. (2017). Ferulic Acid and Naturally Occurring Compounds Bearing a Feruloyl Moiety: A Review on Their Structures, Occurrence, and Potential Health Benefits. *Compr. Rev. Food Sci. Food Saf.* 16, 580–616. doi:10.1111/1541-4337.12266

Dekdouk, N., Malafronte, N., Russo, D., Faraone, I., De Tommasi, N., Ameddah, S., et al. (2015). Phenolic Compounds fromOlea europaeaL. Possess Antioxidant Activity and Inhibit Carbohydrate Metabolizing EnzymesIn Vitro. *Evidence-Based Complement. Altern. Med.* 2015, 1–9. doi:10.1155/2015/684925

Dey, B., and Mitra, A. (2013). Chemo-profiling of eucalyptus and Study of its Hypoglycemic Potential. *World J. Diabetes* 4, 170–176. doi:10.4239/wjd.v4.i5.170

Djimeli, M. N., Fodouop, S. P. C., Njateng, G. S. S., Fokunang, C., Tala, D. S., Kengni, F., et al. (2017). Antibacterial Activities and Toxicological Study of the Aqueous Extract from Leaves of *Alchornea Cordifolia* (Euphorbiaceae). *BMC Complement. Altern. Med.* 17, 349. doi:10.1186/s12906-017-1854-5

Ediriweera, M. K., Tennekoon, K. H., and Samarakoon, S. R. (2017). A Review on Ethnopharmacological Applications, Pharmacological Activities, and Bioactive Compounds ofMangifera indica(Mango). *Evidence-Based Complement. Altern. Med.* 2017, 1–24. doi:10.1155/2017/6949835

El Deeb, K. S., Eid, H. H., Ali, Z. Y., Shams, M. M., and Elfiky, A. M. (2021). Bioassay-guided Fractionation and Identification of Antidiabetic Compounds from the Rind of *Punica granatum* Var. Nana. *Nat. Prod. Res.* 35, 2103–2106. doi:10.1080/14786419.2019.1655411

El-baz, F. K., Aly, H. F., Abd-alla, H. I., and Saad, S. A. (2014). Bioactive Flavonoid Glycosides and Antidiabetic Activity of *Jatropha Curcas* on Streptozotocin-Induced Diabetic Rats. *Int. J. Pharm. Sci. Rev. Res.* 29, 143–156.

El-mostafa, K., El Kharrassi, Y., Badreddine, A., Andreoletti, P., Vamecq, J., El Kebbaj, M. S., et al. (2014). Nopal Cactus (*Opuntia Ficus-indica*) as a Source of Bioactive Compounds for Nutrition, Health and Disease. *Molecules* 19, 14879–14901. doi:10.3390/molecules190914879

Erukainure, O. L., Oyebode, O. A., Sokhela, M. K., Koorbanally, N. A., and Islam, M. S. (2017). Caffeine - Rich Infusion from Cola Nitida (Kola Nut) Inhibits Major Carbohydrate Catabolic Enzymes; Abates Redox Imbalance; and Modulates Oxidative Dysregulated Metabolic Pathways and Metabolites in Fe2+-Induced Hepatic Toxicity. *Biomed. Pharmacother.* 96, 1065–1074. doi:10.1016/j.biopha.2017.11.120

Erukainure, O. L., Sanni, O., Ijomone, O. M., Ibeji, C. U., Chukwuma, C. I., and Islam, M. S. (2019). The Antidiabetic Properties of the Hot Water Extract of Kola Nut (Cola Nitida (Vent.) Schott & Endl.) in Type 2 Diabetic Rats. *J. Ethnopharmacol.* 242, 112033. doi:10.1016/j.jep.2019.112033

Etxeberria, U., de la Garza, A. L., Campión, J., Martínez, J. A., and Milagro, F. I. (2012). Antidiabetic Effects of Natural Plant Extracts via Inhibition of Carbohydrate Hydrolysis Enzymes with Emphasis on Pancreatic Alpha Amylase. *Expert Opin. Ther. Targets* 16, 269–297. doi:10.1517/14728222.2012.664134

Ezzat, S. M., Abdel Motaal, A., El Awdan, S. A. W., and El, W. (2017). *In Vitro* and *In Vivo* Antidiabetic Potential of Extracts and a Furostanol Saponin from *Balanites Aegyptiaca*. *Pharm. Biol.* 55, 1931–1936. doi:10.1080/13880209.2017.1343358

Fajrin, F. A., Nugroho, A. E., Nurrochmad, A., and Susilowati, R. (2020). Ginger Extract and its Compound, 6-shogaol, Attenuates Painful Diabetic Neuropathy in Mice via Reducing TRPV1 and NMDAR2B Expressions in the Spinal Cord. *J. Ethnopharmacol.* 249, 112396. doi:10.1016/j.jep.2019.112396

Farzaei, M. H., Singh, A. K., Kumar, R., Croley, C. R., Pandey, A. K., Coy-Barrera, E., et al. (2019). Targeting Inflammation by Flavonoids: Novel Therapeutic Strategy for Metabolic Disorders. *Ijms* 20, 4957. doi:10.3390/ijms20194957

Fernando, W. I. T., Attanayake, A. M. K. C., Perera, H. K. I., Sivakanesan, R., Jayasinghe, L., Araya, H., et al. (2019). Isolation, Identification and Characterization of Pancreatic Lipase Inhibitors from *Trigonella Foenum-graecum* Seeds. *South Afr. J. Bot.* 121, 418–421. doi:10.1016/j.sajb.2018.10.023

Ferrare, K., Bidel, L. P. R., Awwad, A., Poucheret, P., Cazals, G., Lazennec, F., et al. (2018). Increase in Insulin Sensitivity by the Association of Chicoric Acid and Chlorogenic Acid Contained in a Natural Chicoric Acid Extract (NCRAE) of Chicory (*Cichorium Intybus* L.) for an Antidiabetic Effect. *J. Ethnopharmacology* 215, 241–248. doi:10.1016/j.jep.2017.12.035

Fred-Jaiyesimi, A., Kio, A., and Richard, W. (2009). α-Amylase Inhibitory Effect of 3β-olean-12-en-3-yl (9Z)-Hexadec-9-Enoate Isolated from Spondias Mombin Leaf. *Food Chem.* 116, 285–288. doi:10.1016/j.foodchem.2009.02.047

Gandhi, G. R., Vanlalhruaia, P., Stalin, A., Irudayaraj, S. S., Ignacimuthu, S., and Paulraj, M. G. (2014). Polyphenols-rich Cyamopsis Tetragonoloba (L.) Taub. Beans Show Hypoglycemic and β-cells Protective Effects in Type 2 Diabetic Rats. *Food Chem. Toxicol.* 66, 358–365. doi:10.1016/j.fct.2014.02.001

Ganesan, K., and Xu, B. (2017). Polyphenol-rich Dry Common Beans (*Phaseolus vulgaris* L.) and Their Health Benefits. *Int. J. Mol. Sci.* 18. doi:10.3390/ijms18112331

Ghorbani, A., and Esmaeilizadeh, M. (2017). Pharmacological Properties of *Salvia Officinalis* and its Components. *J. Traditional Complement. Med.* 7, 433–440. doi:10.1016/j.jtcme.2016.12.014

Glezeva, N., Chisale, M., Mcdonald, K., Ledwidge, M., Gallagher, J., and Watson, C. J. (2018). Diabetes and Complications of the Heart in Sub-saharan Africa: An Urgent Need for Improved Awareness, Diagnostics and Management. *Diabetes Res. Clin. Pract.* 137, 10–19. doi:10.1016/j.diabres.2017.12.019

Gothai, S., Ganesan, P., Park, S. Y., Fakurazi, S., Choi, D. K., and Arulselvan, P. (2016). Natural Phyto-Bioactive Compounds for the Treatment of Type 2 Diabetes: Inflammation as a Target. *Nutrients* 8. doi:10.3390/nu8080461

Guo, Y., Hou, E., Ma, N., Liu, Z., Fan, J., and Yang, R. (2020). Discovery, Biological Evaluation and Docking Studies of Novel N-Acyl-2-Aminothiazoles Fused (+)-nootkatone from Citrus Paradisi Macf. As Potential α-glucosidase Inhibitors. *Bioorg. Chem.* 104, 104294. doi:10.1016/j.bioorg.2020.104294

Gupta, R. C., Chang, D., Nammi, S., Bensoussan, A., Bilinski, K., and Roufogalis, B. D. (2017). Interactions between Antidiabetic Drugs and Herbs: an Overview of Mechanisms of Action and Clinical Implications. *Diabetol. Metab. Syndr.* 9, 59. doi:10.1186/s13098-017-0254-9

Guzman, J. D. (2014). Natural Cinnamic Acids, Synthetic Derivatives and Hybrids with Antimicrobial Activity. *Molecules* 19, 19292–19349. doi:10.3390/molecules191219292

Hadi, A., Arab, A., Hajianfar, H., Talaei, B., Miraghajani, M., Babajafari, S., et al. (2020). The Effect of Fenugreek Seed Supplementation on Serum Irisin Levels, Blood Pressure, and Liver and Kidney Function in Patients with Type 2 Diabetes Mellitus: A Parallel Randomized Clinical Trial. *Complement. Ther. Med.* 49, 102315. doi:10.1016/j.ctim.2020.102315

Hafizur, R. M., Maryam, K., Hameed, A., zaheer, L., Bano, S., Sumbul, S., et al. (2018). Insulin Releasing Effect of Some Pure Compounds from *Moringa Oleifera* on Mice Islets. *Med. Chem. Res.* 27, 1408–1418. doi:10.1007/s00044-018-2157-1

Halpern, S. H., and Douglas, M. J. (2007). Appendix: Jadad Scale for Reporting Randomized Controlled Trials. *Evid Based. Obstet. Anesth.*, 237–238. doi:10.1002/9780470988343.app1

Han, J.-H., Tuan, N. Q., Park, M.-H., Quan, K. T., Oh, J., Heo, K.-S., et al. (2018). Cucurbitane Triterpenoids from the Fruits of *Momordica Charantia* Improve Insulin Sensitivity and Glucose Homeostasis in Streptozotocin-Induced Diabetic Mice. *Mol. Nutr. Food Res.* 62, 1700769. doi:10.1002/mnfr.201700769

Harinantenaina, L., Tanaka, M., Takaoka, S., Oda, M., Mogami, O., Uchida, M., et al. (2006). *Momordica Charantia* Constituents and Antidiabetic Screening of the Isolated Major Compounds. *Chem. Pharm. Bull. (Tokyo)* 54, 1017–1021. doi:10.1248/cpb.54.1017

Hartling, L., Bond, K., Vandermeer, B., Seida, J., Dryden, D. M., and Rowe, B. H. (2011). Applying the Risk of Bias Tool in a Systematic Review of Combination Long-Acting Beta-Agonists and Inhaled Corticosteroids for Persistent Asthma. *PLoS One* 6, e17242. doi:10.1371/journal.pone.0017242

Hassanin, K. M. A., Mahmoud, M. O., Hassan, H. M., Abdel-Razik, A. H., Aziz, L. N., and Rateb, M. E. (2018). Balanites Aegyptiaca Ameliorates Insulin Secretion and Decreases Pancreatic Apoptosis in Diabetic Rats: Role of SAPK/JNK Pathway. *Biomed. Pharmacother.* 102, 1084–1091. doi:10.1016/j.biopha.2018.03.167

Hong, S. H., Heo, J. I., Kim, J. H., Kwon, S. O., Yeo, K. M., Bakowska-Barczak, A. M., et al. (2013). Antidiabetic and Beta Cell-protection Activities of Purple Corn Anthocyanins. *Biomol. Ther. (Seoul)* 21, 284–289. doi:10.4062/biomolther.2013.016

Hooijmans, C. R., Rovers, M. M., de Vries, R. B., Leenaars, M., Ritskes-hoitinga, M., and Langendam, M. W. (2014). SYRCLE's Risk of Bias Tool for Animal Studies. *BMC Med. Res. Methodol.* 14, 43. doi:10.1186/1471-2288-14-43

Huang, T. H., Peng, G., Kota, B. P., Li, G. Q., Yamahara, J., Roufogalis, B. D., et al. (2005). Anti-diabetic Action of *Punica granatum* Flower Extract: Activation of PPAR-Gamma and Identification of an Active Component. *Toxicol. Appl. Pharmacol.* 207, 160–169. doi:10.1016/j.taap.2004.12.009

Huang, Y. N., Zhao, D. D., Gao, B., Zhong, K., Zhu, R. X., Zhang, Y., et al. (2012). Anti-hyperglycemic Effect of Chebulagic Acid from the Fruits of *Terminalia Chebula* Retz. *Int. J. Mol. Sci.* 13, 6320–6333. doi:10.3390/ijms13056320

Ibitoye, O. B., Uwazie, J. N., and Ajiboye, T. O. (2017). Bioactivity-guided Isolation of Kaempferol as the Antidiabetic Principle from *Cucumis Sativus* L. Fruits. *J. Food Biochem.* 42, e12479. doi:10.1111/jfbc.12479

Inegbenebor, U., Ebomoyi, M. I., Onyia, K. A., Amadi, K., and Aigbiremolen, A. E. (2009). Effect of alligator Pepper (Zingiberaceae Aframomum Melegueta) on First Trimester Pregnancy in Sprague Dawley Rats. *Niger. J. Physiol. Sci.* 24, 161–164. doi:10.4314/njps.v24i2.52901

Jafarpour-Sadegh, F., Montazeri, V., Adili, A., Esfehani, A., Rashidi, M. R., and Pirouzpanah, S. (2017). Consumption of Fresh Yellow Onion Ameliorates Hyperglycemia and Insulin Resistance in Breast Cancer Patients during Doxorubicin-Based Chemotherapy: A Randomized Controlled Clinical Trial. *Integr. Cancer Ther.* 16, 276–289. doi:10.1177/1534735416656915

Jain, V., Viswanatha, G. L., Manohar, D., and Shivaprasad, H. N. (2012). Isolation of Antidiabetic Principle from Fruit Rinds of *Punica granatum*. *Evidence-Based Complement. Altern. Med.* 2012, 1–11. doi:10.1155/2012/147202

Jalil, A., Ashfaq, U. A., Shahzadi, S., Rasul, I., Rehman, S. U., Shah, M., et al. (2013). Screening and Design of Anti-diabetic Compounds Sourced from the Leaves of Neem (*Azadirachta indica*). *Bioinformation* 9, 1031–1035. doi:10.6026/97320630091031

Jawla, S., Kumar, Y., and Khan, M. (2013). Isolation of Antidiabetic Principle from *Bougainvillea Spectabilis* Willd (Nyctaginaceae) Stem Bark. *Trop. J. Pharm. Res.* 12, 761–765. doi:10.4314/tjpr.v12i5.15

Jia, S., Hu, Y., Zhang, W., Zhao, X., Chen, Y., Sun, C., et al. (2015). Hypoglycemic and Hypolipidemic Effects of Neohesperidin Derived from Citrus Aurantium L. In Diabetic KK-A(y) Mice. *Food Funct.* 6, 878–886. doi:10.1039/C4FO00993B

Johnson, O. O., Zhao, M., Gunn, J., Santarsiero, B. D., Yin, Z.-Q., Ayoola, G. A., et al. (2016). α-Glucosidase Inhibitory Prenylated Anthranols from Harungana Madagascariensis. *J. Nat. Prod.* 79, 224–229. doi:10.1021/acs.jnatprod.5b00924

Jong-anurakkun, N., Bhandari, M. R., Hong, G., and Kawabata, J. (2008). Alpha-glucosidase Inhibitor from Chinese Aloes. *Fitoterapia* 79, 456–457. doi:10.1016/j.fitote.2008.02.010

Kadan, S., Saad, B., Sasson, Y., and Zaid, H. (2013). In VitroEvaluations of Cytotoxicity of Eight Antidiabetic Medicinal Plants and Their Effect on GLUT4 Translocation. *Evidence-Based Complement. Altern. Med.* 2013, 1–9. doi:10.1155/2013/549345

Kalman, D. S., Schwartz, H. I., Feldman, S., and Krieger, D. R. (2013). Efficacy and Safety of *Elaeis Guineensis* and *Ficus Deltoidea* Leaf Extracts in Adults with Pre-diabetes. *Nutr. J.* 12, 36. doi:10.1186/1475-2891-12-36

Kappel, V. D., Frederico, M. J., Postal, B. G., Mendes, C. P., Cazarolli, L. H., and Silva, F. R. (2013). The Role of Calcium in Intracellular Pathways of Rutin in Rat Pancreatic Islets: Potential Insulin Secretagogue Effect. *Eur. J. Pharmacol.* 702, 264–268. doi:10.1016/j.ejphar.2013.01.055

Karhagomba, I. B., Mirindi T, A., Mushagalusa, T. B., Nabino, V. B., Koh, K., and Kim, H. S. (2013). The Cultivation of Wild Food and Medicinal Plants for Improving Community Livelihood: The Case of the Buhozi Site, DR Congo. *Nutr. Res. Pract.* 7, 510–518. doi:10.4162/nrp.2013.7.6.510

Kasali, M. F., Mahano, A. O., Bwironde, F. M., Amani, A. C., Mangambu, J. D., Nyakabwa, D. S., et al. (2013). Ethnopharmacological Survey of Plants Used against Diabetes in Bukavu City (D.R. Congo). *J. Ethnobiol. Tradit. Med.* 119, 538–546.

Kasangana, P. B., Nachar, A., Eid, H. M., Stevanovic, T., and Haddad, P. S. (2018). Root Bark Extracts of *Myrianthus Arboreus* P. Beauv. (Cecropiaceae) Exhibit Anti-diabetic Potential by Modulating Hepatocyte Glucose Homeostasis. *J. Ethnopharmacol.* 211, 117–125. doi:10.1016/j.jep.2017.09.017

Kasika, E. L., Vasombolwa, V. K., and Lejoly, J. (2015). Contribution to the Knowledge of Plants Used by Bantu and Pygmy Healers in Beni and Lubero Territories (Democratic Republic of Congo). *Jps* 4, 157–176. doi:10.5539/jps.v4n2p157

Katemo, F. M., Kadima, J. N., and Marini, R. D. (2018). Determination of *In Vitro* Antioxidant Activity, Acute Toxicity in guinea-pig and TLC-Fingerprint of *Panda Oleosa* (Pierre) Bark Extract Used to Treat Diabetes in Folk Medicine. *Int. J. Pharm. Pharm. Res.* 13, 38–50.

Katemo, M., Mpiana, P. T., Mbala, B. M., Mihigo, S. O., Ngbolua, K. N., Tshibangu, D. S., et al. (2012). Ethnopharmacological Survey of Plants Used against Diabetes in Kisangani City (DR Congo). *J. Ethnopharmacol.* 144, 39–43. doi:10.1016/j.jep.2012.08.022

Kazmi, I., Rahman, M., Afzal, M., Gupta, G., Saleem, S., Afzal, O., et al. (2012). Anti-diabetic Potential of Ursolic Acid Stearoyl Glucoside: A New Triterpenic Gycosidic Ester from *Lantana Camara*. *Fitoterapia* 83, 142–146. doi:10.1016/j.fitote.2011.10.004

Keller, A. C., Ma, J., Kavalier, A., He, K., Brillantes, A. M., and Kennelly, E. J. (2011). Saponins from the Traditional Medicinal Plant *Momordica Charantia* Stimulate Insulin Secretion *In Vitro*. *Phytomedicine* 19, 32–37. doi:10.1016/j.phymed.2011.06.019

Kevin, L., Hussin, A., Zhari, I., and Chin, J. (2012). Sub-acute Oral Toxicity Study of Methanol Leaves Extract of Catharanthus Roseus in Rats. *J. Acute Dis.* 1, 38–41. doi:10.1016/s2221-6189(13)60009-8

Khan, A., Zaman, G., and Anderson, R. A. (2009). Bay Leaves Improve Glucose and Lipid Profile of People with Type 2 Diabetes. *J. Clin. Biochem. Nutr.* 44, 52–56. doi:10.3164/jcbn.08-188

Kianbakht, S., and Dabaghian, F. H. (2013). Improved Glycemic Control and Lipid Profile in Hyperlipidemic Type 2 Diabetic Patients Consuming *Salvia Officinalis* L. Leaf Extract: A Randomized Placebo. Controlled Clinical Trial. *Complement. Ther. Med.* 21, 441–446. doi:10.1016/j.ctim.2013.07.004

Kianbakht, S., Khalighi-Sigaroodi, F., and Dabaghian, F. H. (2013). Improved Glycemic Control in Patients with Advanced Type 2 Diabetes Mellitus Taking *Urtica Dioica* Leaf Extract: A Randomized Double-Blind Placebo-Controlled Clinical Trial. *Clin. Lab.* 59, 1071–1076. doi:10.7754/Clin.Lab.2012.121019

Kim, M.-H., Jo, S.-H., Jang, H.-D., Lee, M. S., and Kwon, Y.-I. (2010). Antioxidant Activity and α-glucosidase Inhibitory Potential of Onion (Allium cepa L.) Extracts. *Food Sci. Biotechnol.* 19, 159–164. doi:10.1007/s10068-010-0022-1

Kim, S. K., Jung, J., Jung, J. H., Yoon, N., Kang, S. S., Roh, G. S., et al. (2020). Hypoglycemic Efficacy and Safety of *Momordica Charantia* (Bitter Melon) in Patients with Type 2 Diabetes Mellitus. *Complement. Ther. Med.* 52, 102524. doi:10.1016/j.ctim.2020.102524

Kim, T. H., Kim, J. K., Kang, Y.-H., Lee, J.-Y., Kang, I. J., and Lim, S. S. (2013). Aldose Reductase Inhibitory Activity of Compounds from Zea maysL. *Biomed. Res. Int.* 2013, 1–8. doi:10.1155/2013/727143

Kodera, Y., Ushijima, M., Amano, H., Suzuki, J. I., and Matsutomo, T. (2017). Chemical and Biological Properties of S-1-Propenyl-L-Cysteine in Aged Garlic Extract. *Molecules* 22. doi:10.3390/molecules22040570

Kuete, V. (2014). "Health Effects of Alkaloids from African Medicinal Plants," in *Toxicological Survey of African Medicinal Plants*. Editor V. Kuete (Elsevier), 611–633. doi:10.1016/B978-0-12-800018-2.00021-2

Kulkarni, P., Lohidasan, S., and Mahadik, K. (2021). Isolation, Characterisation and Investigation of *In Vitro* Antidiabetic and Antioxidant Activity of Phytoconstituents from Fruit of *Momordica Charantia* Linn. *Nat. Product. Res.* 35, 1035–1037. doi:10.1080/14786419.2019.1613400

Latham, P., and Mbuta, K. ku. A. (2017). *Useful Plants of Kongo central Province,*. Third edition. Democratic Republic of Congo.

Lawal, A., and Dangoggo, S. (2014). Phytochemical, Proximate and Toxicity Studies of Aqueous Extract of *Crinum Ornatum* (Toad' S Onion). *Chemsearch J.* 5, 45–50.

Le, L. (2014). Computational Study of Antidiabetic Activities of Bioactive Compounds in *Zingiber Officinale*. *World J. Pharm. Pharm. Sci.* 3, 1995–2011.

Lee, D. Y., Kim, H. W., Yang, H., and Sung, S. H. (2017). Hydrolyzable Tannins from the Fruits of Terminalia Chebula Retz and Their α-glucosidase Inhibitory Activities. *Phytochemistry* 137, 109–116. doi:10.1016/j.phytochem.2017.02.006

Lee, Y. H., Yoon, S. Y., Baek, J., Kim, S. J., Yu, J. S., Kang, H., et al. (2021). Metabolite Profile of Cucurbitane-type Triterpenoids of Bitter Melon (Fruit of *Momordica Charantia*) and Their Inhibitory Activity against Protein Tyrosine Phosphatases Relevant to Insulin Resistance. *J. Agric. Food Chem.* 69, 1816–1830. doi:10.1021/acs.jafc.0c06085

Lenzen, S. (2008). The Mechanisms of Alloxan- and Streptozotocin-Induced Diabetes. *Diabetologia* 51, 216–226. doi:10.1007/s00125-007-0886-7

Leone, A., Bertoli, S., Di Lello, S., Bassoli, A., Ravasenghi, S., Borgonovo, G., et al. (2018). Effect of *Moringa Oleifera* Leaf Powder on Postprandial Blood Glucose Response: *In Vivo* Study on Saharawi People Living in Refugee Camps. *Nutrients* 10, 1494. doi:10.3390/nu10101494

Li, J.-M., Wang, W., Fan, C.-Y., Wang, M.-X., Zhang, X., Hu, Q.-H., et al. (2013). Quercetin Preservesβ-Cell Mass and Function in Fructose-Induced Hyperinsulinemia through Modulating Pancreatic Akt/FoxO1 Activation. *Evidence-Based Complement. Altern. Med.* 2013, 1–12. doi:10.1155/2013/303902

Li, X., Huang, G., Zhao, G., Chen, W., Li, J., and Sun, L. (2013). Two New Monoterpenes from *Tithonia Diversifolia* and Their Anti-hyperglycemic Activity. *Fitoterapia* 83. doi:10.1016/j.fitote.2012.09.007

Liu, C. T., Sheen, L. Y., and Lii, C. K. (2007). Does Garlic Have a Role as an Antidiabetic Agent? *Mol. Nutr. Food Res.* 51, 1353–1364. doi:10.1002/mnfr.200700082

Liu, J., Zhao, Y., Wu, Q., John, A., Jiang, Y., Yang, J., et al. (2018). Structure Characterisation of Polysaccharides in Vegetable "Okra" and Evaluation of Hypoglycemic Activity. *Food Chem.* 242, 211–216. doi:10.1016/j.foodchem.2017.09.051

Liu, X., Luo, J., and Kong, L. (2011). Phenylethyl Cinnamides as Potential Alpha-Glucosidase Inhibitors from the Roots of Solanum Melongena. *Nat. Prod. Commun.* 6, 851–853. doi:10.1177/1934578X1100600623

Lv, X., Zhao, S., Ning, Z., Zeng, H., Shu, Y., Tao, O., et al. (2015). *Citrus* Fruits as a Treasure Trove of Active Natural Metabolites that Potentially Provide Benefits for Human Health. *Chem. Cent. J.* 9, 68. doi:10.1186/s13065-015-0145-9

Lynch, B., Simon, R., and Roberts, A. (2011). *In Vitro* and *In Vivo* Assessment of the Genotoxic Activity of Aloesin. *Regul. Toxicol. Pharmacol.* 61, 215–221. doi:10.1016/j.yrtph.2011.07.011

MacIel, M. I., de Mendonça Cavalcanti, Mdo. S., Napoleão, T. H., Paiva, P. M., De Almeida Catanho, M. T., and Coelho, L. C. (2012). *Anacardium Occidentale* Bark Lectin: Purification, Immobilization as an Affinity Model and Influence in the Uptake of technetium-99M by Rat Adipocytes. *Appl. Biochem. Biotechnol.* 168, 580–591. doi:10.1007/s12010-012-9798-1

Mahomoodally, M. F., Mootoosamy, A., and Wambugu, S. (2016). Traditional Therapies Used to Manage Diabetes and Related Complications in Mauritius: A Comparative Ethnoreligious Study. *Evidence-Based Complement. Altern. Med.* 2016, 1–25. doi:10.1155/2016/4523828

Mahomoodally, M. F. (2013). Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants. *Evidence-Based Complement. Altern. Med.* 2013, 1–14. doi:10.1155/2013/617459

Manya, M. H., Keymeulen, F., Ngezahayo, J., Bakari, A. S., Kalonda, M. E., Kahumba, B. J., et al. (2020). Antimalarial Herbal Remedies of Bukavu and Uvira Areas in DR Congo: An Ethnobotanical Survey. *J. Ethnopharmacol.* 249, 112422. doi:10.1016/j.jep.2019.112422

Manzo, M. L. (2012). *Etude phytochimique et pharmacologique de plantes antipaludiques utilisées en médecine traditionnelle congolaise*. Liège: Université de Liège.

Mbayo, K. M., Kalonda, M. E., Tshisand, T. P., Kisimba, K. E., Mulamba, M., Richard, M. K., et al. (2016). Contribution to Ethnobotanical Knowledge of Some Euphorbiaceae Used in Traditional Medicine in Lubumbashi and its Surroundings (DRC). *J. Adv. Bot. Zool.* 4. doi:10.15297/JALS.V4I2.01

Mbuyi, K. S., Kalunga, M. R., Kalonda, M. E., Cimanga, C. C., Numbi, W. I. E., Kahumba, B. J., et al. (2019). Aperçu ethnobotanique de plantes réputées antipaludéennes utilisées dans la ville de Lubumbashi et ses environs, dans le Haut-Katanga en RD Congo. *Ethnopharmacologia* 61, 75–84.

Mikaili, P., Maadirad, S., Moloudizargari, M., Aghajanshakeri, S., and Sarahroodi, S. (2013). Therapeutic Uses and Pharmacological Properties of Garlic, Shallot, and Their Biologically Active Compounds. *Iran. J. Basic Med. Sci.* 16, 1031–1048.

Misawa, E., Tanaka, M., Nomaguchi, K., Nabeshima, K., Yamada, M., Toida, T., et al. (2012). Oral Ingestion of *Aloe Vera* Phytosterols Alters Hepatic Gene Expression Profiles and Ameliorates Obesity-Associated Metabolic Disorders in Zucker Diabetic Fatty Rats. *J. Agric. Food Chem.* 60, 2799–2806. doi:10.1021/jf204465j

Mishra, A., Srivastava, R., Srivastava, S. P., Gautam, S., Tamrakar, A. K., Maurya, R., et al. (2013). Antidiabetic Activity of Heart wood of Pterocarpus Marsupium Roxb. And Analysis of Phytoconstituents. *Indian J. Exp. Biol.* 51, 363–374.

Mohamed, G. A. (2008). Alliuocide G, a New Flavonoid with Potent α-amylase Inhibitory Activity from Allium cepa L. *Arkivoc* 2008, 202–209. doi:10.3998/ark.5550190.0009.b20

Mohammed, A., Gbonjubola, V. A., Koorbanally, N. A., and Islam, M. S. (2017). Inhibition of Key Enzymes Linked to Type 2 Diabetes by Compounds Isolated from *Aframomum Melegueta* Fruit. *Pharm. Biol.* 55, 1010–1016. doi:10.1080/13880209.2017.1286358

Mohammed, A., Victoria Awolola, G., Ibrahim, M. A., Anthony Koorbanally, N., and Islam, M. S. (2021). Oleanolic Acid as a Potential Antidiabetic Component of *Xylopia Aethiopica* (Dunal) A. Rich. (Annonaceae) Fruit: Bioassay Guided Isolation and Molecular Docking Studies. *Nat. Prod. Res.* 35, 788–791. doi:10.1080/14786419.2019.1596094

Mohan, C. G., Viswanatha, G. L., Savinay, G., Rajendra, C. E., and Halemani, P. D. (2013). 1,2,3,4,6 Penta-O-Galloyl-β-D-Glucose, a Bioactivity Guided Isolated Compound from Mangifera Indica Inhibits 11β-HSD-1 and Ameliorates High Fat Diet-Induced Diabetes in C57BL/6 Mice. *Phytomedicine* 20, 417–426. doi:10.1016/j.phymed.2012.12.020

Mongeke, M. M., Ngbolua, K., Bakola, R. D., Inkoto, C. L., Elikanpani, P. N., and Mouli, C. (2018). Enquête sur les plantes utilisées en médecine traditionnelle par les Bambenga: pygmées du secteur de Dongo en République Démocratique du Congo. *Rev. Mar. Sci. Agron. Vét* 6, 469–475.

Moshi, M. J., Otieno, D. F., Weisheit, A., Almeida, C., Amorim, E., Albuquerque, U., et al. (2012). Ethnomedicine of the Kagera Region, North Western Tanzania. Part 3: Plants Used in Traditional Medicine in Kikuku Village, Muleba District. *J. Ethnobiol. Ethnomed.* 8, 14. doi:10.1186/1746-4269-8-14

Moswa, J. L., Ciamala, C., Bongombola, B., Nzingula, N., Kapanda, N., Bokatshinde, N., et al. (2005). Plants Used for the Treatment of Diabetes Mellitus in the Democratic Republic of Congo. *Ann. Pharm.* 3, 87–93.

Mpiana, P., Masunda, T. A., Longoma, B. F., Tshibangu, D. S. T., and Ngbolua, K. N. (2013). Anti-hyperglycemic Activity of *Raphia Gentiliana* De Wild. (Arecaceae). *Ejmp* 3, 233–240. doi:10.9734/ejmp/2013/2459

Mpiana, P. T., Mbula, J. P., Kwembe, J. T. K., Tshilanda, D. D., Asimonio, J., Toegaho, A., et al. (2015). Ethnobotanical Survey of Aromatic Plants of Masako forest reserve (Kisangani, DR Congo). *J. Adv. Bot. Zool.* 2. doi:10.15297/JABZ.V2I3.04

Muhoya, F. K., Kadima, J. N., Ranarivelo, N., Frédérich, M., Hubert, P., and Djang'eing'a, R. M. (2017). Preliminary Phytochemical Content and Antidiabetic Potential Investigations of Panda Oleosa (Pierre) Used in Kisangani Areas. *Ajac* 08, 564–581. doi:10.4236/ajac.2017.89041

Munhoz, A. C. M., and Fröde, T. S. (2017). Isolated Compounds from Natural Products with Potential Antidiabetic Activity - A Systematic Review. *Cdr* 14, 36–106. doi:10.2174/1573399813666170505120621

Mushagalusa Kasali, F., Ahadi Irenge, C., Murhula Hamuli, P., Birindwa Mulashe, P., Murhula Katabana, D., Mangambu Mokoso, J. D. D., et al. (2021). Ethnopharmacological Survey on Treatment of Hypertension by Traditional Healers in Bukavu City, DR Congo. *Evidence-Based Complement. Altern. Med.* 2021, 1–10. doi:10.1155/2021/6684855

Mustafa, N. R., and Verpoorte, R. (2007). Phenolic Compounds in Catharanthus Roseus. *Phytochem. Rev.* 6, 243–258. doi:10.1007/s11101-006-9039-8

Muya, K., Tshoto, K., Cioci, C. C., Aseho, M. M., Kalonji, M., Byanga, K., et al. (2014). Survol ethnobotanique de quelques plantes utilisées contre la schistosomiase urogénitale à Lubumbashi et environs. *Phytothérapie* 12, 213–228. doi:10.1007/s10298-014-0877-z

Naicker, N., Nagiah, S., Phulukdaree, A., and Chuturgoon, A. (2016). *Trigonella Foenum-graecum* Seed Extract, 4-hydroxyisoleucine, and Metformin Stimulate Proximal Insulin Signaling and Increase Expression of Glycogenic Enzymes and GLUT2 in HepG2 Cells. *Metab. Syndr. Relat. Disord.* 14, 114–120. doi:10.1089/met.2015.0081

Nasser Singab, A., Youssef, F. S., and Mohamed, L. A. (2014). Medicinal Plants with Potential Antidiabetic Activity and Their Assessment. *Med. Aromat. Plants* 03, 1–12. doi:10.4172/2167-0412.1000151

Ndinteh, D. T. (2018). Antidiabetic Potential of *Erythrina Abyssinica* via Protein Tyrosine Phosphate 1B Inhibitory Activity. *Emerg. Trends Chem. Sci.*, 377–389. doi:10.1007/978-3-319-60408-4_22

Ngbolua, K., Inkoto, C., Mongo, N., Ashande, M. C., Masens, Y., and Mpiana, P. T. (2019). Étude ethnobotanique et floristique de quelques plantes médicinales commercialisées à Kinshasa, République Démocratique du Congo. *Rev. Marocaine Des Sci. Agron. Vétérinaires* 7, 118–128.

Ngbolua, K., Mandjo, B. L., Munsebi, J. M., Ashande, M. C., Moke, L. E., Asamboa, L. S., et al. (2016a). Etudes ethnobotanique et écologique des plantes utilisées en médecine traditionnelle dans le District de la Lukunga à Kinshasa (RD du Congo) [Ethno-botanical and ecological studies of medicinal plant species traditionally used in Lukunga district , Kinsh. *Int. J. Innov. Sci. Res.* 26, 612–633.

Ngbolua, K., Mihigo, S. O., Liyongo, C. I., Masengo, C., Tshibangu, D. S. T., Zoawe, B. G., et al. (2016b). Ethno-botanical Survey of Plant Species Used in Traditional Medicine in Kinshasa City (Democratic Republic of the Congo). *Trop. Plant Res.* 3, 413–427.

Nguyen, P. H., Nguyen, T. N., Dao, T. T., Kang, H. W., Ndinteh, D. T., Mbafor, J. T., et al. (2010). AMP-activated Protein Kinase (AMPK) Activation by Benzofurans and Coumestans Isolated from *Erythrina Abyssinica*. *J. Nat. Prod.* 73, 598–602. doi:10.1021/np900745g

Nile, S. H., and Park, S. W. (2014). Antioxidant, α-glucosidase and Xanthine Oxidase Inhibitory Activity of Bioactive Compounds from maize (Zea mays L.). *Chem. Biol. Drug Des.* 83, 119–125. doi:10.1111/cbdd.12205

Nizamutdinova, I. T., Jin, Y. C., Chung, J. I., Shin, S. C., Lee, S. J., Seo, H. G., et al. (2009). The Anti-diabetic Effect of Anthocyanins in Streptozotocin-Induced Diabetic Rats through Glucose Transporter 4 Regulation and Prevention of Insulin Resistance and Pancreatic Apoptosis. *Mol. Nutr. Food Res.* 53, 1419–1429. doi:10.1002/mnfr.200800526

Nnamonu, L., Tor-Anyiin, T., Ugbenyo, N., and Anyam, J. (2016). Isolation and Characterization of α -Amyrin from Stem Bark of Ficus Exasperata (Vahl). *Bji* 16, 1–7. doi:10.9734/BJI/2016/29737

Noipha, K., and Ninla-aesong, P. (2018). Antidiabetic Activity of *Zingiber Officinale* Roscoe Rhizome Extract: An *In Vitro* Study. *Hayati J. Biosci.* 25, 160–168. doi:10.4308/hjb.25.4.160

Noor, A., Bansal, V. S., and Vijayalakshmi, M. A. (2013). Current Update on Anti-diabetic Biomolecules from Key Traditional Indian Medicinal Plants. *Curr. Sci.* 104, 721–727.

Noruddin, N. A. A., Hamzah, M. F., Rosman, Z., Salin, N. H., Shu-Chien, A. C., and Muhammad, T. S. T. (2021). Natural Compound 3β,7β,25-trihydroxycucurbita-5,23(E)-dien-19-al from Momordica Charantia Acts as PPARγ Ligand. *Molecules* 26. doi:10.3390/molecules26092682

Ochieng, C. O., Nyongesa, D. W., Yamo, K. O., Onyango, J. O., Langat, M. K., and Manguro, L. A. O. (2020). α-Amylase and α-glucosidase Inhibitors from Zanthoxylum Chalybeum Engl. Root Bark. *Fitoterapia* 146, 104719. doi:10.1016/j.fitote.2020.104719

Ogunlana, O. O., Ogunlana, O. E., Adeneye, A. A., Udo-Chijioke, O., Dare-Olipede, T., Olagunju, J. A., et al. (2013). Evaluation of the Toxicological Profile of the Leaves and Young Twigs of *Caesalpinia Bonduc* (Linn) Roxb. *Afr. J. Tradit Complement. Altern. Med.* 10, 504–512. doi:10.4314/ajtcam.v10i6.20

Okolie, U. V., Okeke, C. E., Oli, J. M., and Ehiemere, J. (2008). Hypoglycemic Indices of *Vernonia Amygdalina* on Postprandial Blood Glucose Concentration of Healthy Humans. *Afr. J. Biotechnol.* 7, 4581–4585. doi:10.5897/AJB08.903

Olennikov, D. N., Chirikova, N. K., Kashchenko, N. I., Nikolaev, V. M., Kim, S. W., and Vennos, C. (2018). Bioactive Phenolics of the Genus Artemisia (Asteraceae): HPLC-DAD-ESI-TQ-MS/MS Profile of the Siberian Species and Their Inhibitory Potential against α-Amylase and α-Glucosidase. *Front. Pharmacol.* 9, 756. doi:10.3389/fphar.2018.00756

Olennikov, D. N., and Kashchenko, N. I. (2014). Componential Profile and Amylase Inhibiting Activity of Phenolic Compounds fromCalendula officinalisL. Leaves. *Scientific World J.* 2014, 1–9. doi:10.1155/2014/654193

Osfor, M. M. H., Hegazy, A., Abd El-moaty, M., Elmadbouly, M., Afify, A., and Elbahnasawy, A. S. (2013). Hypo-cholesterolemic and Hypoglycemic Effects of orange Albedo Powder (*Citrus Aurantium* L.) on Male Albino Rats. *Ijnfs* 2, 70–76. doi:10.11648/j.ijnfs.20130202.17

Pahlavani, N., Roudi, F., Zakerian, M., Ferns, G. A., Navashenaq, J. G., Mashkouri, A., et al. (2019). Possible Molecular Mechanisms of Glucose-Lowering Activities of *Momordica Charantia* (Karela) in Diabetes. *J. Cel. Biochem.*, 1–9. doi:10.1002/jcb.28483

Pan, J., Yi, X., Zhang, S., Cheng, J., Wang, Y., Liu, C., et al. (2018). Bioactive Phenolics from Mango Leaves (*Mangifera Indica* L.). *Ind. Crops Prod.* 111, 400–406. doi:10.1016/j.indcrop.2017.10.057

Park, S. H., Do, M. H., Lee, J. H., Jeong, M., Lim, O. K., and Kim, S. Y. (2017). Inhibitory Effect of *Arachis hypogaea* (Peanut) and its Phenolics against Methylglyoxal-Derived Advanced Glycation End Product Toxicity. *Nutrients* 9. doi:10.3390/nu9111214

Parveen, A., Z, Z., Farooqi, M. Q., Kyunn, W. W., Arshad, M., and Farooqi, Q. (2017). Phytochemical Screening and Content Determination of Different Species of Genus *Caesalpinia* Belonging to Different Origin with Antidiabetic Activity. *Pj* 9, 743–749. doi:10.5530/pj.2017.6.117

Patel, D. K., Prasad, S. K., Kumar, R., and Hemalatha, S. (2012). An Overview on Antidiabetic Medicinal Plants Having Insulin Mimetic Property. *Asian Pac. J. Trop. Biomed.* 2, 320–330. doi:10.1016/S2221-1691(12)60032-X

Pathy, K. K., Flavien, N. B., Honoré, B. K., Vanhove, W., and Van Damme, P. (2021). Correction to: Ethnobotanical Characterization of Medicinal Plants Used in Kisantu and Mbanza-Ngungu Territories, Kongo-Central Province in DR Congo. *J. Ethnobiol. Ethnomed.* 17, 8. doi:10.1186/s13002-021-00436-1

Paula, P. C., Sousa, D. O., Oliveira, J. T., Carvalho, A. F., Alves, B. G., Pereira, M. L., et al. (2017). A Protein Isolate from *Moringa Oleifera* Leaves Has Hypoglycemic and Antioxidant Effects in Alloxan-Induced Diabetic Mice. *Molecules* 22. doi:10.3390/molecules22020271

Perera, P. R. D., Ekanayake, S., and Ranaweera, K. K. D. S. (2017). Antidiabetic Compounds in Syzygium Cumini Decoction and Ready to Serve Herbal Drink. *Evidence-Based Complement. Altern. Med.* 2017, 1–5. doi:10.1155/2017/1083589

Perera, W. H., Shivanagoudra, S. R., Pérez, J. L., Kim, D. M., Sun, Y., K. Jayaprakasha, G., et al. (2021). Anti-inflammatory, Antidiabetic Properties and *In Silico* Modeling of Cucurbitane-type Triterpene Glycosides from Fruits of an Indian Cultivar of *Momordica Charantia* L. *Molecules* 26, 1038. doi:10.3390/molecules26041038

Pingali, U., Sukumaran, D., and Nutalapati, C. (2020). Effect of an Aqueous Extract of *Terminalia Chebula* on Endothelial Dysfunction, Systemic Inflammation, and Lipid Profile in Type 2 Diabetes Mellitus: A Randomized Double-Blind, Placebo-Controlled Clinical Study. *Phytother Res.* 34, 3226–3235. doi:10.1002/ptr.6771

Pu, P., Gao, D. M., Mohamed, S., Chen, J., Zhang, J., Zhou, X. Y., et al. (2012). Naringin Ameliorates Metabolic Syndrome by Activating AMP-Activated Protein Kinase in Mice Fed a High-Fat Diet. *Arch. Biochem. Biophys.* 518, 61–70. doi:10.1016/j.abb.2011.11.026

Puri, D., Prabhu, K. M., Dev, G., Agarwal, S., and Murthy, P. S. (2011). Mechanism of Antidiabetic Action of Compound GII Purified from Fenugreek (Trigonella Foenum Graecum) Seeds. *Indian J. Clin. Biochem.* 26, 335–346. doi:10.1007/s12291-011-0150-2

Raffaelli, F., Nanetti, L., Montecchiani, G., Borroni, F., Salvolini, E., Faloia, E., et al. (2015). *In Vitro* effects of Fermented Papaya (Carica Papaya, L.) on Platelets Obtained from Patients with Type 2 Diabetes. *Nutr. Metab. Cardiovasc. Dis.* 25, 224–229. doi:10.1016/j.numecd.2014.10.013

Raju, P., Mamidala, E., and Mamidala, E. (2015). Anti Diabetic Activity of Compound Isolated from Physalis Angulata Fruit Extracts in Alloxan Induced Diabetic Rats. *Ajsmr* 1, 40–43. doi:10.17812/ajsmr2015111

Randel, R. D., Chase, C. C., and Wyse, S. J. (1992). Effects of Gossypol and Cottonseed Products on Reproduction of Mammals. *J. Anim. Sci.* 70, 1628–1638. doi:10.2527/1992.7051628x

Rangari, V. D., Shukla, P., and Badole, S. L. (2015). "4-Hydroxyisoleucine: A Potential Antidiabetic Agent from Trigonella Foenum-Graecum," in *Glucose Intake and Utilization in Pre-diabetes and Diabetes* (Elsevier), 191–198. doi:10.1016/b978-0-12-800093-9.00015-6

Rashad, H., Metwally, F. M., Ezzat, S. M., Salama, M. M., Hasheesh, A., and Abdel Motaal, A. (2017). Randomized Double-Blinded Pilot Clinical Study of the Antidiabetic Activity of *Balanites Aegyptiaca* and UPLC-ESI-MS/MS Identification of its Metabolites. *Pharm. Biol.* 55, 1954–1961. doi:10.1080/13880209.2017.1354388

Ríos, J. L., Francini, F., and Schinella, G. R. (2015). Natural Products for the Treatment of Type 2 Diabetes Mellitus. *Planta Med.* 81, 975–994. doi:10.1055/s-0035-1546131

Salah El Dine, R., Ma, Q., Kandil, Z. A., and El-Halawany, A. M. (2014). Triterpenes as Uncompetitive Inhibitors of α-glucosidase from Flowers of *Punica granatum* L. *Nat. Product. Res.* 28, 2191–2194. doi:10.1080/14786419.2014.928292

Salehi, B., Albayrak, S., Antolak, H., Kręgiel, D., Pawlikowska, E., Sharifi-Rad, M., et al. (2018). Aloe Genus Plants: From Farm to Food Applications and Phytopharmacotherapy. *Int. J. Mol. Sci.* 19. doi:10.3390/ijms19092843

Sands, A. L., Leidy, H. J., Hamaker, B. R., Maguire, P., and Campbell, W. W. (2009). Consumption of the Slow-Digesting Waxy maize Starch Leads to Blunted Plasma Glucose and Insulin Response but Does Not Influence Energy Expenditure or Appetite in Humans. *Nutr. Res.* 29, 383–390. doi:10.1016/j.nutres.2009.05.009

Sanni, O., Erukainure, O. L., Chukwuma, C. I., Koorbanally, N. A., Ibeji, C. U., and Islam, M. S. (2019). *Azadirachta indica* Inhibits Key Enzyme Linked to Type 2 Diabetes *In Vitro*, Abates Oxidative Hepatic Injury and Enhances Muscle Glucose Uptake *Ex Vivo*. *Biomed. Pharmacother.* 109, 734–743. doi:10.1016/j.biopha.2018.10.171

Sato, H., Genet, C., Strehle, A., Thomas, C., Lobstein, A., Wagner, A., et al. (2007). Anti-hyperglycemic Activity of a TGR5 Agonist Isolated from *Olea Europaea*. *Biochem. Biophys. Res. Commun.* 362, 793–798. doi:10.1016/j.bbrc.2007.06.130

Sekiya, K., Ohtani, A., and Kusano, S. (2004). Enhancement of Insulin Sensitivity in Adipocytes by Ginger. *BioFactors* 22, 153–156. doi:10.1002/biof.5520220130

Sharma, A. K., Bharti, S., Kumar, R., Krishnamurthy, B., Bhatia, J., Kumari, S., et al. (2012). Syzygium Cumini Ameliorates Insulin Resistance and β-cell Dysfunction via Modulation of PPAR, Dyslipidemia, Oxidative Stress, and TNF-α in Type 2 Diabetic Rats. *J. Pharmacol. Sci.* 119, 205–213. doi:10.1254/jphs.11184FP

Shidfar, F., Rajab, A., Rahideh, T., Khandouzi, N., Hosseini, S., and Shidfar, S. (2015). The Effect of Ginger (*Zingiber Officinale*) on Glycemic Markers in Patients with Type 2 Diabetes. *J. Complement. Integr. Med.* 12, 165–170. doi:10.1515/jcim-2014-0021

Shinde, J., Taldone, T., Barletta, M., Kunaparaju, N., Hu, B., Kumar, S., et al. (2008). Alpha-glucosidase Inhibitory Activity of Syzygium Cumini (Linn.) Skeels Seed Kernel *In Vitro* and in Goto-Kakizaki (GK) Rats. *Carbohydr. Res.* 343, 1278–1281. doi:10.1016/j.carres.2008.03.003

Shivanagoudra, S. R., Perera, W. H., Perez, J. L., Athrey, G., Sun, Y., Jayaprakasha, G. K., et al. (2019). Cucurbitane-type Compounds from *Momordica Charantia*: Isolation, *In Vitro* Antidiabetic, Anti-inflammatory Activities and *In Silico* Modeling Approaches. *Bioorg. Chem.* 87, 31–42. doi:10.1016/j.bioorg.2019.02.040

Shyni, G. L., Kavitha, S., Indu, S., Arya, A. D., Anusree, S. S., Vineetha, V. P., et al. (2014). Chebulagic Acid from Terminalia Chebula Enhances Insulin Mediated Glucose Uptake in 3T3-L1 Adipocytes via PPARγ Signaling Pathway. *BioFactors* 40, 646–657. doi:10.1002/biof.1193

Silva, E. L., Almeida-Lafetá, R. C., Borges, R. M., and Staerk, D. (2017). Dual High-Resolution Inhibition Profiling and HPLC-HRMS-SPE-NMR Analysis for Identification of α-glucosidase and Radical Scavenging Inhibitors in Solanum Americanum Mill. *Fitoterapia* 118, 42–48. doi:10.1016/j.fitote.2017.02.002

Singh, A. B., Yadav, D. K., Maurya, R., and Srivastava, A. K. (2009). Antihyperglycaemic Activity of Alpha-Amyrin Acetate in Rats and Db/db Mice. *Nat. Prod. Res.* 23, 876–882. doi:10.1080/14786410802420416

Sugita, J., Yoneshiro, T., Hatano, T., Aita, S., Ikemoto, T., Uchiwa, H., et al. (2013). Grains of paradise (*Aframomum Melegueta*) Extract Activates Brown Adipose Tissue and Increases Whole-Body Energy Expenditure in Men. *Br. J. Nutr.* 110, 733–738. doi:10.1017/S0007114512005715

Sundaram Chinna Krishnan, S., Pillai Subramanian, I., and Pillai Subramanian, S. (2014). Isolation, Characterization of Syringin, Phenylpropanoid Glycoside from *Musa Paradisiaca* Tepal Extract and Evaluation of its Antidiabetic Effect in Streptozotocin-Induced Diabetic Rats. *Biomed. Prev. Nutr.* 4, 105–111. doi:10.1016/j.bionut.2013.12.009

Sundowo, A., Anita, Y., Maryani, F., Banjarnahor, S. D. S., and Widiyarti, G. (2018). Synthesis of Aniline Catalyzed by *Brassica Juncea* Peroxidase and its Antidiabetic Activities. *AIP Conf. Proc.* 2024. doi:10.1063/1.5064324

Suntar, I., Khan, H., Patel, S., Celano, R., and Rastrelli, L. (2018). An Overview onCitrus aurantiumL.: Its Functions as Food Ingredient and Therapeutic Agent. *Oxidative Med. Cell Longevity* 2018, 1–12. doi:10.1155/2018/7864269

Surya, S., Salam, A. D., Tomy, D. V., Carla, B., Kumar, R. A., and Sunil, C. (2014). Diabetes Mellitus and Medicinal Plants-A Review. *Asian Pac. J. Trop. Dis.* 4, 337–347. doi:10.1016/S2222-1808(14)60585-5

Sutedja, A. M., Yanase, E., Batubara, I., Fardiaz, D., and Lioe, H. N. (2020). Antidiabetic Components from the Hexane Extract of Red Kidney Beans (*Phaseolus vulgaris* L.): Isolation and Structure Determination. *Biosci. Biotechnol. Biochem.* 84, 598–605. doi:10.1080/09168451.2019.1691911

Tahora, S. B., Akhi, A. A., Karim, S. S., Hasan, R., Nabeela, S. A., and Ali, R. (2018). *In Silico* study of Some Isolated Compound Present in *Ageratum Conyzoides* against Diabetes. *J. Nutr. Diet. Prat.* 2, 1–4.

Tang, D., Liu, L., Ajiakber, D., Ye, J., Xu, J., Xin, X., et al. (2018). Anti-diabetic Effect of *Punica granatum* Flower Polyphenols Extract in Type 2 Diabetic Rats: Activation of Akt/GSK-3β and Inhibition of IRE1α-XBP1 Pathways. *Front. Endocrinol. (Lausanne)* 9, 586–611. doi:10.3389/fendo.2018.00586

Tang, G. Y., Li, X. J., and Zhang, H. Y. (2008). Antidiabetic Components Contained in Vegetables and Legumes. *Molecules* 13, 1189–1194. doi:10.3390/molecules13051189

Tardío, J., and Pardo-De-Santayana, M. (2008). Cultural Importance Indices: A Comparative Analysis Based on the Useful Wild Plants of Southern Cantabria (Northern Spain)1. *Econ. Bot.* 62, 24–39. doi:10.1007/s12231-007-9004-5

Tedong, L., Madiraju, P., Martineau, L. C., Vallerand, D., Arnason, J. T., Desire, D. D., et al. (2010). Hydro-ethanolic Extract of Cashew Tree (*Anacardium Occidentale*) Nut and its Principal Compound, Anacardic Acid, Stimulate Glucose Uptake in C2C12 Muscle Cells. *Mol. Nutr. Food Res.* 54, 1753–1762. doi:10.1002/mnfr.201000045

Teixeira Sousa Moura, L., Palomaris Mariano Souza, D., Mendonça, S., de Aquino Ribeiro, J. A., Fernandes Sousa, L., Tony Ramos, A., et al. (2017). Histopathological and Reproductive Evaluation in Male Rats Fed *Jatropha Curcas* Seed Cake with or without Alkaline Hydrolysis and Subjected to Heat Treatment. *Biomed. Res. Int.* 2017, 1–11. doi:10.1155/2017/6123408

Teodoro, T., Zhang, L., Alexander, T., Yue, J., Vranic, M., and Volchuk, A. (2008). Oleanolic Acid Enhances Insulin Secretion in Pancreatic Beta-Cells. *FEBS Lett.* 582, 1375–1380. doi:10.1016/j.febslet.2008.03.026

The World Medical Association (2001). Declaration of Helsinki. *Bull. World Health Organ.* 79, 373–374.

Tiong, S. H., Looi, C. Y., Hazni, H., Arya, A., Paydar, M., Wong, W. F., et al. (2013). Antidiabetic and Antioxidant Properties of Alkaloids from *Catharanthus Roseus* (L.) G. Don. *Molecules* 18, 9770–9784. doi:10.3390/molecules18089770

Tousch, D., Lajoix, A. D., Hosy, E., Azay-milhau, J., Ferrare, K., Jahannault, C., et al. (2008). Chicoric Acid, a New Compound Able to Enhance Insulin Release and Glucose Uptake. *Biochem. Biophys. Res. Commun.* 377, 131–135. doi:10.1016/j.bbrc.2008.09.088

Tuwisana Masunda, A., Inkoto, C. L., Bongo, G. N., Wa Oloko, J. D., Ngbolua, K.-T.-N., Tshibangu, D. S., et al. (2019). Ethnobotanical and Ecological Studies of Plants Used in the Treatment of Diabetes in Kwango, Kongo central and Kinshasa in the Democratic Republic of the Congo. *Ijde* 4, 18. doi:10.11648/j.ijde.20190401.14

Uwazie, J. N., Yakubu, M. T., Ashafa, A. O. T., and Ajiboye, T. O. (2020). Identification and Characterization of Anti-diabetic Principle in *Senna alata* (Linn.) Flower Using Alloxan-Induced Diabetic Male Wistar Rats. *J. Ethnopharmacol.* 261, 112997. doi:10.1016/j.jep.2020.112997

V, B. C., S, B. A., Philippe, O. N., J, K. B., Pierre, D., and Jb, L. S. (2020). Ethnobotanical Study of Plants Used as Antimalarial in Traditional Medicine in Bagira in Eastern RD Congo. *J. Pharmacogn. Phytochem.* 9, 01–14. doi:10.22271/phyto.2020.v9.i4a.11661

Vadivel, V., Nandety, A., and Biesalski, H. K. (2011). Antioxidant, Free Radical Scavenging and Type II Diabetes-Related Enzyme Inhibition Properties of Traditionally Processed Jequirity Bean (*Abrus precatorius* L.). *Int. J. Food Sci. Technol.* 46, 2505–2512. doi:10.1111/j.1365-2621.2011.02774.x

Vu, N. K., Kim, C. S., Ha, M. T., Ngo, Q. T., Park, S. E., Kwon, H., et al. (2020). Antioxidant and Antidiabetic Activities of Flavonoid Derivatives from the Outer Skins of *Allium cepa* L. *J. Agric. Food Chem.* 68, 8797–8811. doi:10.1021/acs.jafc.0c02122

Wang, F., Zhong, H. H., Chen, W. K., Liu, Q. P., Li, C. Y., Zheng, Y. F., et al. (2017a). Potential Hypoglycaemic Activity Phenolic Glycosides from *Moringa Oleifera* Seeds. *Nat. Prod. Res.* 31, 1869–1874. doi:10.1080/14786419.2016.1263846

Wang, H., Du, Y.-J., and Song, H.-C. (2010). α-Glucosidase and α-amylase Inhibitory Activities of Guava Leaves. *Food Chem.* 123, 6–13. doi:10.1016/j.foodchem.2010.03.088

Wang, J., Huang, M., Yang, J., Ma, X., Zheng, S., Deng, S., et al. (2017b). Anti-diabetic Activity of Stigmasterol from Soybean Oil by Targeting the GLUT4 Glucose Transporter. *Food Nutr. Res.* 61, 1364117. doi:10.1080/16546628.2017.1364117

Wang, Q., Wu, X., Shi, F., and Liu, Y. (2019). Comparison of Antidiabetic Effects of Saponins and Polysaccharides from *Momordica Charantia* L. In STZ-Induced Type 2 Diabetic Mice. *Biomed. Pharmacother.* 109, 744–750. doi:10.1016/j.biopha.2018.09.098

Wang-fischer, Y., and Garyantes, T. (2018). Improving the Reliability and Utility of Streptozotocin-Induced Rat Diabetic Model. *J. Diabetes Res.* 2018, 1–14. doi:10.1155/2018/8054073

Waterman, C., Rojas-silva, P., Tumer, T. B., Kuhn, P., Richard, A. J., Wicks, S., et al. (2016). Isothiocyanate-rich Moringa Oleifera Extract Reduces Weight Gain, Insulin Resistance, and Hepatic Gluconeogenesis in Mice. *Mol. Nutr. Food Res.* 59, 1013–1024. doi:10.1002/mnfr.201400679.Isothiocyanate-rich

WHO (2013). *WHO Traditional Medicine Strategy 2014-2023*. World Heal. Organ., 1–76.

Wikul, A., Damsud, T., Kataoka, K., and Phuwapraisirisan, P. (2012). (+)-Pinoresinol Is a Putative Hypoglycemic Agent in Defatted Sesame (Sesamum indicum) Seeds Though Inhibiting α-glucosidase. *Bioorg. Med. Chem. Lett.* 22, 5215–5217. doi:10.1016/j.bmcl.2012.06.068

Worawalai, W., Khongchai, P., Surachaitanawat, N., and Phuwapraisirisan, P. (2016). Synthesis of Furofuran Lignans as Antidiabetic Agents Simultaneously Achieved by Inhibiting α-glucosidase and Free Radical. *Arch. Pharm. Res.* 39, 1370–1381. doi:10.1007/s12272-016-0778-9

Xu, X., Shan, B., Liao, C. H., Xie, J. H., Wen, P. W., and Shi, J. Y. (2015). Anti-diabetic Properties of *Momordica Charantia* L. Polysaccharide in Alloxan-

Induced Diabetic Mice. *Int. J. Biol. Macromol.* 81, 538–543. doi:10.1016/j.ijbiomac.2015.08.049

Yagi, A., Hegazy, S., Kabbash, A., and Wahab, E. A. (2009). Possible Hypoglycemic Effect of *Aloe Vera* L. High Molecular Weight Fractions on Type 2 Diabetic Patients. *Saudi Pharm. J.* 17, 209–215. doi:10.1016/j.jsps.2009.08.007

Yang, D., Chen, X., Liu, X., Han, N., Liu, Z., Li, S., et al. (2020). Antioxidant and α-Glucosidase Inhibitory Activities Guided Isolation and Identification of Components from Mango Seed Kernel. *Oxidative Med. Cell Longevity* 2020, 1–15. doi:10.1155/2020/8858578

Yang, J., Wang, T., Yang, J., Rao, K., Zhan, Y., Chen, R. B., et al. (2013). S-allyl Cysteine Restores Erectile Function through Inhibition of Reactive Oxygen Species Generation in Diabetic Rats. *Andrology* 1, 487–494. doi:10.1111/j.2047-2927.2012.00060.x

Yonemoto, R., Shimada, M., Gunawan-Puteri, M. D., Kato, E., and Kawabata, J. (2014). α-Amylase Inhibitory Triterpene from *Abrus precatorius* Leaves. *J. Agric. Food Chem.* 62, 8411–8414. doi:10.1021/jf502667z

Yoon, K. D., Lee, J. Y., Kim, T. Y., Kang, H., Ha, K. S., Ham, T. H., et al. (2020). *In Vitro* and *In Vivo* Anti-hyperglycemic Activities of Taxifolin and its Derivatives Isolated from Pigmented Rice (Oryzae Sativa L. Cv. Superhongmi). *J. Agric. Food Chem.* 68, 742–750. doi:10.1021/acs.jafc.9b04962

Yue, J., Xu, J., Cao, J., Zhang, X., and Zhao, Y. (2017). Cucurbitane Triterpenoids from Momordica Charantia L. And Their Inhibitory Activity against α-glucosidase, α-amylase and Protein Tyrosine Phosphatase 1B (PTP1B). *J. Funct. Foods* 37, 624–631. doi:10.1016/j.jff.2017.07.041

Zameer, S., Najmi, A. K., Vohora, D., and Akhtar, M. (2017). A Review on Therapeutic Potentials of Trigonella Foenum Graecum (Fenugreek) and its Chemical Constituents in Neurological Disorders: Complementary Roles to its Hypolipidemic, Hypoglycemic, and Antioxidant Potential. *Nutr. Neurosci.* 21, 539–545. doi:10.1080/1028415X.2017.1327200

Zang, Y., Sato, H., and Igarashi, K. (2014). Anti-diabetic Effects of a Kaempferol Glycoside-Rich Fraction from Unripe Soybean (Edamame, Glycine max L. Merrill. 'Jindai') Leaves on KK-A(y) Mice. *Biosci. Biotechnol. Biochem.* 75, 1677–1684. doi:10.1271/bbb.110168

Zhang, C., Huang, M., Hong, R., and Chen, H. (2019). Preparation of a Momordica Charantia L. Polysaccharide-chromium (III) C-omplex and its A-nti-hyperglycemic A-ctivity in M-ice with S-treptozotocin-I-nduced D-iabetes. *Int. J. Biol. Macromol.* 122, 619–627. doi:10.1016/j.ijbiomac.2018.10.200

Zhang, H., Xu, J., Wang, M., Xia, X., Dai, R., and Zhao, Y. (2020). Steroidal Saponins and Sapogenins from Fenugreek and Their Inhibitory Activity against α-glucosidase. *Steroids* 161, 108690. doi:10.1016/j.steroids.2020.108690

Zhang, Z., Kong, F., Ni, H., Mo, Z., Wan, J. B., Hua, D., et al. (2016). Structural Characterization, α-glucosidase Inhibitory and DPPH Scavenging Activities of Polysaccharides from Guava. *Carbohydr. Polym.* 144, 106–114. doi:10.1016/j.carbpol.2016.02.030

Zhao, G., Li, X., Chen, W., Xi, Z., and Sun, L. (2012). Three New Sesquiterpenes from *Tithonia Diversifolia* and Their Anti-hyperglycemic Activity. *Fitoterapia* 83, 1590–1597. doi:10.1016/j.fitote.2012.09.007

Zhivov, A., Peschel, S., Schober, H. C., Stachs, O., Baltrusch, S., Bambi, M. T., et al. (2015). Diabetic Foot Syndrome and Corneal Subbasal Nerve Plexus Changes in Congolese Patients with Type 2 Diabetes. *PLoS One* 10, e0119842–12. doi:10.1371/journal.pone.0119842

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 27 October 2021
doi: 10.3389/fphar.2021.713855

Knowledge, Attitudes and Practices on the Use of Botanical Medicines in a Rural Caribbean Territory

*Tatijana Vujicic and Damian Cohall**

Faculty of Medical Sciences, The University of the West Indies, Cave Hill, St. Michael, Barbados

The worldwide use of medicinal plant products has been steadily increasing over the past few decades, whereas the traditional knowledge and practices of these botanical medicines appears to be diminishing. Considering the need to conserve and document these traditions, the objective of this study was to understand the knowledge, attitudes, and practices of people who are familiar with botanical medicines, as well as any factors that may influence the perceptions and behaviours associated with the use of medicinal plants. A previously validated survey instrument assessing knowledge, attitudes, and practices on the use medicinal plants was randomly administered to residents of three rural Barbadian communities. The data was analyzed using descriptive statistics and cross tabulations (Chi-Square Test, Fisher's Exact Test), with a confidence level of 95% and significance level of $p < 0.05$. One hundred and fifty-four participants completed the survey with a response rate of 96%. From participant responses we found that over 75% of the study population used botanical medicines. Key findings included a diverse repertoire of traditional knowledge on the use of medicinal plants, which included a total of 29 medicinal applications cited across 69 different plant species and 39 families. The most popular species among respondents (irrespective of use) were *Pimenta racemosa* (Mill.) J.W. Moore (FC = 30, RFC = 0.26), *Momordica charantia* L. (FC = 28, RFC = 0.24), *Zingiber officinale* Roscoe (FC = 22, RFC = 0.19) and *Annona muricata* L. (FC = 21, RFC = 0.18). The findings also show the persistence of medico-cultural concepts such as cleansing and cooling, and identified significant associations between the use of botanical medicines and related practices with demographic variables such as education ($p = 0.05$; Fisher's Exact Test) and health insurance, χ^2 (1, n = 152) = 4.645, $p = 0.003$. The findings of this study can be used in the identification and archiving of the medicinal plant practices in Barbados and the wider Caribbean, as well as for the larger purposes of biocultural exploration, preservation and further scientific assessment of botanical medicine practices.

Keywords: caribbean, drug-herb interaction, botanical, medicine, predictors, survey, medicinal plants

OPEN ACCESS

Edited by:
Michael Heinrich,
UCL School of Pharmacy,
United Kingdom

Reviewed by:
Fabien Schultz,
Technical University of Berlin,
Germany
Rebecca Lazarou,
Royal Botanic Gardens, Kew,
United Kingdom

****Correspondence:***
Damian Cohall
damian.cohall@cavehill.uwi.edu

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *24 May 2021*
Accepted: *06 October 2021*
Published: *27 October 2021*

Citation:
Vujicic T and Cohall D (2021) Knowledge, Attitudes and Practices on the Use of Botanical Medicines in a Rural Caribbean Territory. Front. Pharmacol. 12:713855. doi: 10.3389/fphar.2021.713855

INTRODUCTION

The Caribbean region is widely recognized for its rich plant diversity, ranking among the top 6 of 25 global biodiversity hotspots (Carrington, 2007; Cohall, 2014). These plants are utilized extensively throughout the Caribbean in the practice of folklore botanical medicine, which originated from the intense cultural convergence prompted by European colonialism, indigenous cultures of the Americas, the transatlantic slave trade, and immigration of indentured servants from Asia (Bayley, 1949; Feng 1956; Crawford-Daniel and Alexis, 2014). Many of the healing botanical medicines used in the Caribbean today are of West African origin, including the use of *Citrus aurantiifolia* [Christm.] Swingle (Lime), *Ricinus communis* L. (*Castor* Bean), and *Abrus precatorius* L. (Wild Licorice/Crab's Eye) (McCaskie, 2017). These were all important species used in West African healing traditions and are still commonly used in Caribbean countries such as Jamaica (Vandebroek and Picking, 2020), Cuba (Heredia-Diaz et al., 2018), the Virgin Islands (Soelberg et al., 2016), and others (Torres-Avilez et al., 2015). However, because of the cultural suppression and syncretism that occurred during the enslavement of West Africans in the Caribbean and after their emancipation, many of the islands differ in respect to their native pharmacopeias (Crawford-Daniel and Alexis, 2014; Sutherland et al., 2014).

Barbados is the easternmost Caribbean island and has a population of 287,000, 95.5% of which are descendants of the enslaved West African people (World Development Indicators, 2019; Jackson et al., 2021). Approximately 65% of the healthcare in Barbados is public and 35% private, however, the public system is universally accessible, free at delivery and, notably, includes access to drugs (Labonte et al., 2017). Despite this, the healthcare expenditure ranks high among other Caribbean or Central American countries, with an increase in disease burden on the island. One in ten Barbadian adults has a chronic, non-communicable disease and the associated mortality rate per 100,000 is increasing (Unwin et al., 2015; Global Burden of Disease Collaborative Network, 2020). Additionally, non-communicable diseases account for 9 of the top 10 causes of death in Barbados (Global Burden of Disease Collaborative Network, 2020).

Unlike its neighboring islands, Barbados was colonized primarily by the British and did not have the influx of indentured servants observed in other southern Caribbean islands (Lane, 1979). While a wealth of literature exists on traditional botanical medicine in larger Caribbean countries (Mahabir and Gulliford, 1997; Merritt-Charles, 2011; Clement et al., 2005), there is a need for more comprehensive studies to be done on this topic in smaller islands such as Barbados, as they were subject to a stronger degree of cultural suppression (Abrahams, 1967). For instance, smaller islands typically had smaller plantations, which meant fewer slaves and greater oversight from the plantation owners, as well as a greater adoption by slaves of the attitudes and expressions of the owners (Abrahams, 1967). This was especially the case amongst the liberated slaves who aspired for a better quality of life, which translated to attaining a British-based education, accessing westernised healthcare, and generally engaging in activities with the British colonial upper-class (Crawford-Daniel and Alexis, 2014). As a result, a gradient developed in the knowledge and use of traditional medicine between the uneducated vs educated, rural vs urban Barbadians (Crawford-Daniel and Alexis, 2014). Additionally, compared to the larger islands, more land mass had to be cultivated to ensure the plantation system was economically viable, which lead both to 1) the loss of hundreds of plant species across the island due to deforestation-many of which were potentially medicinal, and 2) newly freed slaves having few options but to remain on the plantations as labourers after the abolishment of slavery (Bayley, 1949; Watts, 1966; Abrahams, 1967). Currently, the island has approximately 650 species of flowering plants with only two species being endemic (Bayley, 1949; Carrington, 2007; Cohall, 2014). The persistence of many of these outcomes into present-day Barbados makes it a particularly compelling candidate for novel ethnopharmacological studies, as it not only provides information about local botanical medicine practices, but may also elucidate processes that underlie or drive the use of botanical medicine within the region, by allowing for comparisons with larger countries that did not experience the same degree of deculturation.

Even with the loss of some cultural practices and plant species throughout colonization, there are immense health and economic benefits associated with the cultivation, harvesting, extraction, research, and development of plant-based natural health products, as well as potential economic benefit in the marketing of Barbados and the broader Caribbean region as a health and wellness destination, addressing both spiritual and physical health. In fact, the medicinal uses of many of these plants have been investigated and confirmed mainly by *in vitro* and preclinical studies (Cohall, 2014). Therefore, substantial efforts should be made to document and preserve medicinal plant knowledge in the Caribbean and to identify barriers to use. Some such barriers may include the perceived superiority of western medical practices and the resultant attitudes of physicians towards botanical remedies, the possible effects of the unidentified chemical compounds in a plant, and the lack of public knowledge concerning drug-herb interactions.

Therefore, we conducted an observational study in College Lands, St. John, Barbados, to gain insight into the knowledge, attitudes, and practices on the use of botanical medicines in rural communities where the retention of these traditions has been reported to be higher (Crawford-Daniel and Alexis, 2014; Clement et al., 2015), and to determine whether there are factors that might predict or influence the use of these remedies in the surveyed communities. The knowledge gained from this research will be used to identify medicinal plants in the geographical area for further scientific investigation of pharmacological activity, cultivation, and the possible co-modification into effective and commercially viable products, which may help to mitigate some of these main barriers to the use and access of the benefits of botanical medicines.

MATERIALS AND METHODS

Study Setting, Participants, and Ethics Approval

Surveys were administered during April and May of 2018 in three rural communities situated in College Lands, a rural district in the parish of St. John located on the southeastern side of Barbados. These locations included the two communities adjacent to the Codrington College (Latitude/Longitude: 13° 10′ 31″ N/59° 28′ 32″ W) and College Savannah/Consett Bay (Latitude/Longitude: 13° 10′ 60.00″ N/-59° 27′ 59.99″ W). These communities were the sugarcane-producing estates, Society and Consett. They were established in the 17th century by Christopher Codrington, who, after his death, left the estates to be used for the spiritual and physical well-being of people by developing a religious college – known today as Codrington College – while the surrounding lands were left for the people's livelihoods (Holder, 1988). This bequest was intended to benefit the non-White population of Barbados, including the provision of an education for those who were enslaved; however, slave labour in the estates continued until it was abolished in 1833 (Holder, 1988). This resulted in a unique convergence of slavery and education in an area with a plant biodiversity reflective of ancestral practices and the tropical climate associated with Barbados' geographical location (Carrington, 2007; Crawford-Daniel and Alexis, 2014).

A map of College Lands was used with census data provided by the administration of the Codrington College and its Trust to assist in locating the community boundaries and the participants. Residents were recruited via written correspondences and flyers which were distributed in the area prior distributing the survey. The survey was randomly administered to persons meeting the eligibility criteria, which required them to be residents of College Lands, St. John, Barbados and at least 18 years of age. The number of surveys administered was based on a sample size of 160, which was calculated at a power of 80%, confidence level of 95%, confidence interval of 4%, and a population size of 216 (as determined from a census conducted in 2018). Surveys were applied and analyzed per person interviewed.

Study approval was obtained from The University of the West Indies (UWI)/Barbados Ministry of Health Ethics Committee/ Institutional Review Board (IRB No. 180303-A), and informed consent was obtained prior to administering the survey.

Survey Instrument

Semi-structured interviews were conducted using a questionnaire designed to probe information regarding the knowledge, attitudes, and practices surrounding the use of botanical medicines, as well as demographic data such as age, gender, education, and annual income. A total of 23 questions were asked, including single-response, multiple-response, and skip logic types. All questions were nominal in nature, excluding age and annual income. This instrument was validated and used in a prior ethnopharmacological study on the island (Cohall et al., 2012). Participants were asked to report information including, but not limited to, any chronic conditions they suffer from, their use of plant-based remedies, the specific plants they use and what they are used for, where they source the medicinal plants from, where they acquired their knowledge of botanical medicine, whether they discuss their use of plant-based remedies with their physician, as well as other non-botanical medicine-related prompts. The survey instrument can be found in **Supplementary Data Sheet 1**.

Plant Collection/Identification

Where possible, field officers collected samples of plants which were identified by a plant taxonomist, and vouchered samples were deposited at the herbarium at The University of the West Indies, Cave Hill Campus. In some instances, plants were easily identifiable as crop plants and some recalled as semi-processed products. Lastly, some plants with reported medicinal properties were identified with the use of high resolution pictures from peer-reviewed published sources by the interviewers. The scientific nomenclature of all the plant families and species were verified using the Kew Medicinal Plant Name Services (MPNS) and the final list of plants was reviewed by the plant taxonomist for accuracy.

Data Analysis

Measures

For the purposes of this study, botanical medicine use was determined through participants' response to the survey question asking "do you use herbs/bush to treat? If no, skip to question 19." Those citing one or more plants that they personally use in a healing capacity were considered users of botanical medicines and are referred to as "users" in following sections. Those who skipped questions 12–18 were considered "non-users" and referred to as such.

In relation to the primary research question, the knowledge, attitudes, and practices were ascertained through data provided by the users of botanical medicine on the following variables:

- Specific medicinal plants employed in treatment
- Uses/applications of the medicinal plants
- Sources of botanical medicine knowledge
- Sources of medicinal plants
- Communication with doctor about botanical medicine use
- Behaviours and knowledge surrounding drug-herb combination and associated risks
- Recommendation of botanical medicines to others

In relation to the secondary research question, potential predictors of botanical medicine use were identified through cross-tabulation and significance testing of the following variables with "Use of Botanical Medicine" (Y/N):

- Age
- Education Level
- Income
- Physician Relationship
- Parental Use of Botanical Medicine
- Health Insurance (Y/N)

A manual double data entry process was used to enter, verify, and validate the accuracy of data in Microsoft Excel (Microsoft

Corporation, Redmond, Washington, United States). All data analysis was performed using SPSS 27 software (SPSS Inc., Chicago, Illinois, United States). The response options for each question were numerically coded beginning at a value of 1. Missing values were assigned a code of "888", "999", or were left as "system missing". 888 codes corresponded to "not applicable" and were assigned if the missing value was from participants who did not identify with the subset of respondents that the question was intended for, and thus were correct in skipping it. 999 codes were labelled "no response" and were used if the missing value was from participants who incorrectly skipped a question that was intended for their specific demographic (e.g., only answers one of a two-part question about their chronic condition). When values were missing from stand-alone questions (e.g., what is your age), they were considered "missing at random" and were left as such.

Ethnobotanical data concerning reported plant species and medicinal uses among the study population were analyzed using the following indices:

Use Value

The use value determines the therapeutic versatility of locally known plant species and was calculated using the following formula (Napagoda et al., 2018; Andriamparany et al., 2014; Phillips and Gentry, 1993):

$$UV = \frac{\Sigma Ui}{N}$$

where Ui is the number of uses reported by each informant for a given species and N is the total number of informants. A high use value indicates that the cited species has a diversity of medicinal applications among the participants in the study.

Frequency of Citation and Relative Frequency of Citation

The frequency of citation is the number of informants reporting the use of a particular species, while the relative frequency of citation illustrates the local importance of each species among the study's participants and is given by the following formula (Amjad et al., 2017):

$$RFC = \frac{FC}{N}$$

where FC is the frequency of citation (number of informants reporting the use of a particular species), and N is the total number of informants. This index varies from 0, when nobody cited the use of the plant, to 1 in the case that all informants reported using this species.

All other data (e.g., demographic variables) were summarized using frequencies and percentages. To identify significant associations between variables, contingency tables were created, and Chi-squared analysis was performed. When more than 5 cells had an expected count less than 5, the variables were either recategorized into larger groups, or the Fisher's Exact Test was used instead of the Chi-squared analysis. If an association was found to have statistical significance ($p \leq 0.05$), a Z-test of column proportions with Bonferroni correction was carried out to highlight the specific relationships responsible for the significant result. Effect size was measured using Phi (for 2 × 2 tables) or Cramer's V (for RxC tables).

RESULTS

Characteristics of Sample

A total of 154 completed questionnaires were obtained from residents of College Lands, St. John, Barbados with a response rate of 96%. The median age group of respondents was 51–60 years old (n = 31, 20.1%), with 58.4% of the sample identifying as female and 41.6% identifying as male. Secondary school was the highest level of education attained by 45.4% of respondents, 30.9% had no higher than a primary level education, and 23.7% had attained an associate degree or higher. Among 136 total respondents, the most reported income range was less than $8,000 BDS per year ($n$ = 48, 35.3%) followed by those earning greater than $28,000 BDS per year ($n$ = 30, 22.6%). 62.1% of participants (n = 95) had at least one chronic condition, the most common of which were Hypertension (n = 57, 60.0%), Type II Diabetes Mellitus (n = 26, 27.4%), and Arthritis (n = 24, 25.3%). The treatment of chronic conditions with prescription medication was reported by 68 of the respondents, however, data was missing for 26 respondents. Participant characteristics are documented in **Table 1**, and data pertaining to the chronic conditions reported by participants are given in **Table 2**.

Knowledge, Attitudes, and Practices Surrounding the Use of Botanical Medicines

Results presented in *Characteristics of the Users of Botanical Medicines*, *Plants Used for Medicinal Purposes*, *Acquisition of Knowledge and Sources of Medicinal Plants*, *Discussion of Botanical Medicine Use with Doctor*, *Concomitant Use of Medicinal Plants and Prescription Medication*, and *Recommendation of Botanical Medicines to Others* only pertain to data collected from users of botanical medicines, or simply "users".

Characteristics of the Users of Botanical Medicines

Out of the 154 survey respondents, 116 (75.3%) were users of botanical medicines. The majority of the persons were over 50 years old (62.1%), female (56.9%), had "good" self-perceived health (51.7%), and reported at least one chronic condition (62.1%). Characteristics of the users are provided in **Table 1**.

Plants Used for Medicinal Purposes

As a group, the respondents cited a total of 29 different therapeutic applications across 69 different plants. The FC and RFC indices determined that *Pimenta racemosa* (Mill.) J.W. Moore (Bay Leaf), *Momordica charantia* L. (Cerasee), *Zingiber officinale* Roscoe (Ginger), *Annona muricata* L. (Soursop), *Moringa oleifera* Lam. (Moringa), *Cymbopogon citratus* (DC.) Stapf (Lemongrass), *Aloe vera* (L.) Burm.f. (Aloe), *Persea americana* Mill. (Pear), *Petroselinum crispum* (Mill.) Fuss (Parsley) and *Azadirachta indica* A. Juss. (Neem), were the 10 most cited ethnomedicinal plant species (**Table 3**) with reference to the respondents who cited the use of these plant species. In terms

TABLE 1 | Participant characteristics.

	Total N (%)	Proportion using botanical medicines	
		n/N	%
Age (Years)			
<20	5 (3.2)	2/5	40.0
21–30	13 (8.4)	8/13	61.5
31–40	20 (13.0)	17/20	85.0
41–50	23 (14.9)	17/23	73.9
51–60	31 (20.1)	24/31	77.4
61–70	29 (18.8)	24/29	82.8
71–80	30 (19.5)	21/30	70.0
81–90	3 (1.9)	3/3	100.0
Gender			
Male	64 (41.6)	50/64	78.1
Female	90 (58.4)	66/90	73.3
Education Level			
Primary or less	47 (30.9)	41/47	87.2
Secondary	69 (45.4)	52/69	75.4
Associate/Vocational	22 (14.5)	13/22	59.1
Undergraduate	6 (3.9)	4/6	66.7
Graduate	8 (5.3)	5/8	62.5
(Missing values)			
Missing at random	(2)	(1)	
Annual Income (BDS)[a]			
Less than $8,000	45 (33.8)	34/45	75.6
$8,000-$13,000	18 (13.5)	14/18	77.8
$13,000-$18,000	10 (7.5)	10/10	100.0
$18,000-$23,000	15 (11.3)	13/15	86.7
$23,000-$28,000	12 (9.0)	7/12	58.3
$28,000 +	30 (22.6)	19/30	63.3
Retired	3 (2.3)	2/3	66.7
(Missing values)			
Missing at random	(21)	(17)	
Relationship Status			
Married	44 (28.6)	33/44	75.0
Single	83 (53.9)	60/83	72.3
Separated	7 (4.5)	7/7	100.0
Divorced	6 (3.9)	6/6	100.0
Widowed	9 (5.8)	6/9	66.7
Common-Law	5 (3.2)	4/5	80.0
Country of Birth			
Barbados	147 (95.5)	111/145	76.6
Other Caribbean Country	7 (4.5)	5/7	71.4
Self-Perceived Health			
Excellent	9 (5.9)	8/9	88.9
Very Good	29 (19.0)	21/29	72.4
Good	81 (52.9)	60/81	74.1
Fair	29 (19.0)	24/29	82.8
Poor	5 (3.3)	3/5	60.0
(Missing values)			
Missing at random	(1)		
Chronic Condition			
Yes	95 (62.1)	72/95	75.8
No	58 (37.9)	43/58	74.1
(Missing values)			
Missing at random	(1)	(1)	

(Continued on following page)

TABLE 1 | (*Continued*) Participant characteristics.

	Total N (%)	Proportion using botanical medicines	
		n/N	%
Parental Use of Botanical Medicines			
Yes	70 (56.5)	58/70	82.9
No	54 (43.5)	37/54	68.5
(Missing values)			
Missing at random	(30)	(21)	
Health Insurance			
Yes	24 (15.8)	14/24	58.3
No	128 (84.2)	101/128	78.9
(Missing values)			
Missing at random	(2)	(1)	
Type of Healthcare			
Public	51 (35.9)	43/51	84.3
Private	71 (50.0)	47/71	66.2
Both	20 (14.1)	15/20	75.0
(Missing values)			
Missing at random	(12)		

[a]*1 BDS = 0.5 USD.*

of the medicinal uses reported for these plants, the UV index identified *A. indica* as the most versatile species (UV = 1.54), followed by *M. charantia* (UV = 1.32), *A. vera* (1.24), *P. racemosa* (1.17), *A. muricata* (1.14) and *Zingiber officinale Roscoe* (1.14), *P. americana* (1.13), *C. citratus* (1.11), *M. oleifera* (1.06) and *P. crispum* (1.06) among the reported citations. Irrespective of plant species, the most common therapeutic applications cited by respondents included "maintenance of health" (n = 49), "cooling" (n = 34), "hypertension" (n = 23), "cough" (n = 23), and "cold/flu" (n = 18). **Table 3** lists all of the plants cited by participants, along with their uses, methods of administration, and FC, RFC, and UV scores.

Acquisition of Knowledge and Sources of Medicinal Plants

For most of the respondents (n = 92), botanical medicine knowledge was acquired from family members. Friends were the second most frequent source of knowledge (n = 41), followed by the internet (n = 21), books (n = 11), and colleagues (n = 5). Pharmacists, the elderly, and a health and beauty company were cited by only one respondent each. None of the respondents reported acquiring any knowledge from doctors.

Most respondents (n = 79) retrieved medicinal plants from their backyard, although they were also commonly acquired from friends (n = 28), family (n = 20) and, to a lesser extent, natural product shops (n = 13) and the supermarket (n = 13). Only one respondent obtained medicinal plants from the pharmacy. Other sources cited by respondents included the community (n = 2), town (n = 2), gully (n = 2), market (n = 1), and neighbours (n = 1). **Figure 1** provides the specific plants acquired from each source.

Significant associations were found between the acquisition of knowledge and the source of medicinal plants. Respondents who acquired botanical medicine knowledge from family members were significantly more likely to source their medicinal plants from family than those who did not learn from their family members (22.5 vs 0.0%) (p = 0.02; Fisher's Exact Test) (**Table 4**). Similarly, respondents who acquired their knowledge from friends were significantly more likely to source medicinal plants from friends compared to those who learned from other sources (41.7 vs 18.1%), χ^2 (1, n = 108) = 6.967, p = 0.008) (**Table 5**). The respective effect sizes were 0.22 (Phi) and 0.25 (Phi).

The source of medicinal plants was also significantly associated with education, and with annual income. Participants with a primary level education were more likely to acquire medicinal plants from their backyard than were people with a secondary level education (89.5 vs 61.7%) (p = 0.02; Fisher's Exact Test) (**Table 6**), and participants earning over $18,000 per year were more likely to acquire the plants from their family members compared to those earning less than $18000 BDS per year (29.4 vs 10.7%), χ^2 (1, n = 90) = 5.060, p = 0.02) (**Table 7**). The effect sizes were 0.28 (Cramer's V) and −0.24 (Phi), respectively.

Discussion of Botanical Medicine Use With Doctor

Of the 103 users for which valid data were available, 80 (77.7%) indicated that they do not discuss the use of botanical medicines with their doctor. Most respondents who had discussed this topic with their doctor also reported them as having a positive attitude towards it (n = 15, 62.5%).

Concomitant Use of Medicinal Plants and Prescription Medication

Out of the 104 users with valid responses, 32 (30.8%) reported using medicinal plants alongside prescription drugs, while the remaining 72 (69.2%) did not combine the two. Reasons for abstaining were provided by 33 participants: 18 (54.5%) cited

TABLE 2 | Chronic Conditions and Drugs Prescribed for their Management Among Participants.

Chronic Conditions (*N* = 95)	*n* (%)	Proportion (%) using Botanical Medicines	Drugs Prescribed, *n*		Reported Costs (BDS)[a] *n*	
Type II Diabetes	26 (27.4)	22/26 (84.6)	Gliclazide	9	Free	6
			Metformin	14	Free	8
			Insulin	7	Free	2
					$27.5/m[b]	1
			Candesartan	1	Free	1
			Sitagliptin-Metformin	2	$104/m	2
Heart Disease	3 (3.2)	2/3 (66.7)	Bisoprolol	2	-	-
			Verapamil	1	Free	1
Hypertension	57 (60.0)	43/57 (75.4)	Valsartan	13	Free	6
			Amlodipine	7	Free	2
					$30/m	1
			Chlorthalidone	2	Free	1
			Telmisartan	3	$40/m	2
			Aspirin	2	Free	1
			Indapamide	4	Free	2
			Bezide (Gliclazide-Metformin)	3	Free	2
				5	Free	2
			Atenolol		$21/m	1
			Verapamil	1	Free	1
			Lisinopril	1	Free	1
			Indapamide-Amlodipine	4	Free	3
					Free	2
			Valsartan-Hydrochlorothiazide	6	< $20/m	1
High cholesterol	16 (16.8)	13/16 (81.3)	Atorvastatin	10	Free	7
			Amlodipine	1	Free	1
			Simvastatin	1	Free	1
			Rosuvastatin	1	Free	1
			Ezetimibe-Simvastatin	1	-	-
Back pain	11 (11.6)	8/11 (72.7)	Acetaminophen	3	Free	2
					≥ $5	1
Arthritis	24 (25.3)	19/24 (79.2)	Diclofenac	3	$6	1
			Acetaminophen	5	Free	2
			Carbamazepine	1	Free	1
			Enalapril	1	-	-
			Indapamide	1	-	-
			Tramadol	1	Free	1
			Glucosamine	1	Free	1
			Methotrexate	2	-	-
			L-ascorbic Acid	1	-	-
			Ferrous Sulphate	2	-	-
			Folic Acid	2	-	-
			Omega 3	1	Free	-
Osteoporosis	1 (1/1)	0	Strontium Ranelate	1	-	-
			Folic acid	1	-	-
			Calcium Carbonate	1	-	-
Sinus	6 (6.3)	6/6 (100.0)	Nasal spray	2	Free	2
Asthma	7 (7.4)	4/7 (57.1)	Albuterol	5	Free	3
			Betamethasone	1	$15	2
			Fluticosone Proprionate-Salmaterol Xinafoate	1	$20	1
			Beclometasone Diproprionate	1	Free	1
			Budesonide-Formoterol	1	Free	1
					Free	1
Neuro/Psychiatric	3 (3.2)	0	Citalopram	1	-	-
			Carbamazepine	1	-	-
			Zolpidem	1	-	-
Heart	2 (2.1)	2/2 (100.0)	Warfarin	1	-	-
Dyspnea	1 (1.1)	1/1 (100.0)	-	-	-	-
Blood/Circulatory	4 (4.2)	2/4 (50.0)	Diosmin	1	$5/m	1
			Ferrous sulphate	1	-	-
Eye	2 (2.1)	1/2 (50.0)	Bimatoprost	1	-	-
			Dorzolamide-timolol	1	$90	1
Cancer	2 (2.1)	1/2 (50.0)	Cyproterone	1	-	-

(Continued on following page)

TABLE 2 | (*Continued*) Chronic Conditions and Drugs Prescribed for their Management Among Participants.

Chronic Conditions (*N* = 95)	*n* (%)	Proportion (%) using Botanical Medicines	Drugs Prescribed, *n*		Reported Costs (BDS)[a] *n*	
Thyroid	7 (7.4)	4/7 (57.1)	Levothyroxine	7	Free	2
			Calcium carbonate	1	$12/m	1
					$5	1
					-	-
Autoimmune	1 (1.1)	1/1 (100.0)	-	-	-	-
Kidney	1 (1.1)	1/1 (100.0)	-	-	-	-
GERD	2 (2.1)	2/2 (100.0)	Omeprazole	1	$18	1

[a]*1 BDS = 0.5 USD.*
[b]*m is the abbreviation for month.*

interaction effects, 9 (27.3%) didn't take prescription medication at all, 3 (9.1%) believed botanical medicines to be the superior treatment, 2 (6.1%) wanted to ascertain the efficacy of botanical medicines alone, and 1 (3.0%) reported taking them each at different times. In terms of risk awareness, 40% of respondents ($n = 28$) reported being aware of the possible risks associated with herb-drug interactions.

Concomitant use of medicinal plants and prescription drugs was significantly less practiced among participants whose parents are users of botanical medicines (80.0 vs 60.6%) χ^2 (1, $n = 88$) = 3.911, $p = 0.05$, as well as among participants with no chronic conditions (88.2 vs 59.4%) χ^2 (1, $n = 103$) = 8.831, $p = 0.003$ (**Table 8**). The respective effect sizes were 0.21 (Phi) and 0.29 (Phi). Additionally, concomitant use was more frequent among respondents who did not discuss botanical medicines with their doctors ($n = 19$) than among those who did discuss with their doctors ($n = 11$), however, this result was marginally insignificant (26.8 vs 47.8%) χ^2 (1, $n = 94$) = 3.548, $p = 0.06$.

Recommendation of Botanical Medicines to Others

88.3% of respondents ($n = 88$) reported that they would recommend the use of botanical medicines to someone else.

Predictors of Medicinal Plant Use

A significant association was found between education and medicinal plant use. Respondents with a primary level education were significantly more likely to use medicinal plants compared to those with an associate/vocational degree (87.2 vs 59.1%) ($p = 0.05$; Fisher's Exact Test) (**Table 9**). The effect size was 0.2 (Cramer's V).

Health insurance was also significantly associated with medicinal plant use. Participants who did not have health insurance were more likely to use medicinal plants than those with insurance (58.3 vs 78.9%) χ^2 (1, $n = 152$) = 4.645, $p = 0.03$ (**Table 9**). The effect size was -0.2 (Phi).

Medicinal plant use was more frequently reported by respondents whose parents also use medicinal plants ($n = 58$), compared to those whose parents do not ($n = 37$); however, this result was marginally insignificant (82.9 vs 68.5%) χ^2 (1, $n = 124$) = 3.498, $p = 0.06$.

No significant relationships were found between the use of medicinal plants and age (55.6 vs 79.1% vs 80.0 vs 72.7%; $p = 0.19$, Fisher's Exact Test), annual income (75.0 vs 85.7% vs 74.1 vs 63.3%; χ^2 [1, $n = 133$] = 3.825, $p = 0.28$), nor physician relationship (74.6 vs 86.7%; $p = 0.52$, Fisher's Exact Test).

DISCUSSION

The primary purpose of this study was to determine the extent of use of botanical medicines by persons residing in three rural communities in College Lands, St. John, Barbados, and to gain insight into the knowledge, attitudes, and practices surrounding plant-based medicines in this locale. Secondary to this was the identification of potential factors that may influence the use of botanical medicines in that geographical location. The survey determined that over 75% of the study participants used plants for medicinal purposes, with the majority being female and over the age of 50. A diverse repertoire of medicinal plant knowledge was identified, both in terms of the specific plants used by respondents, as well as the multitude of conditions for which these plants were used. A total of 69 plant species belonging to 39 botanical families were reported, the most dominant being Lamiaceae (6 species) and Asteraceae (5 species). The high prevalence of botanical medicine use is expected considering the rural location of the study and thus these findings cannot be generalized to the whole population. In fact, other studies conducted in Barbados found that only ~30% of the whole Barbados population uses botanical medicines (Cohall et al., 2012; Peter, 2013). The low prevalence reported in these studies could partly be explained by the widespread adoption of Western medicine and practices, but could also be related to the deforestation that occurred in Barbados to make space for sugar cane plantations, which ultimately caused the number of plant species to diminish significantly and thus left few species to be used medicinally (Bayley, 1949). *P. racemosa* (Bay Leaf), *M. charantia* (Cerasee), *Zingiber officinale Roscoe* (Ginger), and *A. muricata* (Soursop) were the most frequently cited plants and were among those with the highest UV scores, indicating both the local importance and therapeutic versatility of these species within the study's population at the time of the survey. Although recent ethnobotanical studies are generally lacking in Barbados, a survey conducted in 2013 found similar results regarding the plant species used by participants and their medicinal applications (Peter, 2013). This study identified 93 plant species that were cited as useful, and approximately 41% of those species were common to those identified in this survey. Many of the same use reports for plant species were highlighted in both surveys, such as the use of *A. indica* (Neem) for diabetes and hypertension, or *Kalanchoe pinnata* [Lam.] Pers. (Wonder-of-the-World) for cough, colds/flu, and cleansing. However, our study highlighted novel use

TABLE 3 | List of medicinal plants, preparation methods and frequency of administration reported by users (*N* = 116).

Plant				FC (RFC)	UV	Applications		
Family	**Species**	**Voucher No.**	**Local Name(s)**			**Reported Uses**	**Preparation and Administration**	**Freq. of Treatment**
Acanthaceae	*Justicia secunda* Vahl		Bloodroot	2 (0.02)	1.0	Cleansing	Root (dried); decoction	Monthly
						Cold/Flu	Root (dried); decoction	As needed
Amaryllidaceae	*Allium sativum* L		Garlic	9 (0.08)	1.11	Blood/ Circulation	Decoction	Bi-weekly
						Joint pain	Leaves; decoction	Weekly
						Cuts	Ingested raw	As needed
						Hypertension	Pods; grinded and ingested with coconut water	Daily
						Immune booster	Infusion	As needed
						Sores	Ingested raw	As needed
Annonaceae	*Annona muricata* L		Soursop	21 (0.18)	1.14	Cooling	Leaves; decoction or infusion	Daily, weekly
						Health maintenance	Leaves; decoction or infusion	Daily, weekly
						Hypertension	Leaves; decoction or infusion	Daily
						Cancer prevention	Leaves; decoction	-
						Constipation	Infusion	Daily
						Diabetes	Leaves (dried); decoction	Daily
						Inflammatory conditions	Leaves; decoction	Weekly
	Annona squamosa L	DC012	Sugar Apple	4 (0.03)	1.25	Cooling	Leaves; decoction	Daily for 1 week then 2-weeks break
						Health maintenance	Infusion	Daily
						Diabetes	Leaves (dried); decoction	Daily
						Hypertension	Leaves; decoction	Daily
Apiaceae	*Petroselinum crispum* (Mill.) Fuss		Parsley	16 (0.14)	1.06	Hypertension	decoction or infusion	Daily, weekly
						Health maintenance	Decoction	Weekly
						Cooling	infusion	Weekly
						Headache	Infusion	Monthly
						Immune booster	Infusion	As needed
	Apium graveolens L		Celery	2 (0.02)	1.0	Hypertension	Decoction or infusion	Daily
						Health maintenance	-	Weekly
	Foeniculum vulgare Mill		Fennel	1 (0.01)	2.0	Fever	Infusion	Daily
						Cough	Infusion or inhaled	-
Arecaceae	*Cocos nucifera* L		Coconut	2 (0.02)	1.0	Cooling	Oil (+Epsom salts), applied topically	Every 3 months
						Back pain	Oil, applied topically	Weekly
Asphodelaceae	*Aloe vera* (L.) Burm.f	DC009	Aloe	17 (0.15)	1.24	Health maintenance	Gel; ingested raw, blended with juice, decoction, or infusion	Weekly, monthly
						Cleansing	Decoction or ingested raw	Weekly, monthly
						Cuts	Leaf; cut open, applied topically to wound	As needed
						Cold/Flu	Gel (blended); infusion	As needed
						Cough	Gel; ingested raw	As needed
						Diabetes	Ingested raw	Monthly
						Immune booster	Gel (blended); infusion	As needed
						Purgative	Ingested raw	Weekly
						Sores	Leaf; cut open, applied topically to sore	As needed

(Continued on following page)

TABLE 3 | (*Continued*) List of medicinal plants, preparation methods and frequency of administration reported by users (*N* = 116).

Plant				FC (RFC)	UV	Applications		
Family	**Species**	**Voucher No.**	**Local Name(s)**			**Reported Uses**	**Preparation and Administration**	**Freq. of Treatment**
Asteraceae	*Chromolaena odorata* (L.) R.M. King and H. Rob		Christmas Bush	4 (0.03)	1.0	Health maintenance	Decoction	Daily
						Cooling	Infusion	Daily
						Fever	Decoction (+Pambaram and Cerasee)	As needed
	Parthenium hysterophorus L		Whitehead Bush	2 (0.02)	1.0	Joint pain	Twigs; infusion	As needed
						Eczema	Soaked in water and left on skin until dry	As needed
	Pluchea carolinensis (Jacq.) G. Don		Cure-for-All	1 (0.01)	1.0	Cold/Flu	Infusion	As needed
	Lactuca virosa Habl		Wild Lettuce	1 (0.01)	2.0	Joint/Back pain	Infusion	Weekly
	Bidens pilosa L		Duppy needles/ Monkey Needles/ Spanish Needles	1 (0.01)	1.0	Hypertension	Decoction	-
Boraginaceae	*Cordia obliqua* Willd	DC006	Clammy Cherry	1 (0.01)	1.0	Cooling	Leaves; decoction	Daily
	Borago officinalis L		Starflower	1 (0.01)	1.0	Hormonal balance	Capsule; ingested orally	Daily
	Symphytum officinale L		Comfrey	1 (0.01)	1.0	Health maintenance	Decoction or infusion	Daily
Cannabaceae	*Cannabis sativus* L		Marijuana	1 (0.01)	1.0	Vomiting	Leaves; decoction	-
Caricaceae	*Carica papaya* L	DC011	Pawpaw	11 (0.1)	1.27	Hypertension	Decoction, infusion, or ingested raw	Daily, monthly, as needed
						Constipation	Infusion or eaten raw	Daily, as needed
						Cold/Flu	Leaves; decoction	As needed
Clusiaceae	*Clusia plukenetii* Urb		Rock Balsam	1 (0.01)	1.0	Cough	Leaves; chewed	As needed
Crassulaceae	*Kalanchoe pinnata* (Lam.) Pers	DC005	Wonder of the World	11 (0.1)	1.09	Cough	Leaves; decoction or ingested raw	As needed
						Cold/Flu	Dried, decoction	As needed
						Cleansing	Decoction or infusion	Annually
						Headache	Infusion	As needed
						Health maintenance	Decoction or infusion	Daily
						Sores	Applied topically to wound	As needed
Cucurbitaceae	*Momordica charantia* L	DC002	Cerasee	28 (0.24)	1.32	Health maintenance	Dried, decoction or infusion	Weekly, monthly
						Cold/Flu	Decoction	As needed
						Cleansing	Decoction or infusion	Monthly
						Cough	Decoction or infusion	As needed
						Fever	Leaves; decoction or chewed	As needed
						Hypertension	Infusion	Weekly, monthly
						Diabetes	Decoction	As needed, monthly
						Cancer prevention	Leaves; decoction	-
						Constipation	Leaves; infusion	Daily
						Cooling	Infusion	-
						Headache	Infusion	As needed
						Upset stomach	Decoction	As needed
	Cucumis sativus L		Cucumber	3 (0.03)	1.0	Sore eye	Placed on eyelids	As needed
						Hypertension	Infusion (+parsley)	Daily
	Citrullus lanatus (Thumb) Matsum. and Nakai		Melon	1 (0.01)	1.0	Cooling	Rinds; soaked in water and drunk	Daily
Dilleniaceae	*Doliocarpus dentatus* (Aubl.) Standl		Capadulla	1 (0.01)	1.0	Back pain	Bark; decoction	As needed
Ericaceae	*Vaccinium myrtillus L*		Bilberry	1 (0.01)	1.0	Sore eye	Leaves; infusion	As needed

(Continued on following page)

TABLE 3 | (*Continued*) List of medicinal plants, preparation methods and frequency of administration reported by users (*N* = 116).

Plant				FC (RFC)	UV	Applications		
Family	**Species**	**Voucher No.**	**Local Name(s)**			**Reported Uses**	**Preparation and Administration**	**Freq. of Treatment**
Euphorbiaceae	*Ricinus communis* L	DC004	*Castor* Bean, *Castor* Oil Plant	2 (0.02)	1.5	Sore eye	Oil; applied topically to eye	As needed
						Constipation	Oil; ingested orally	As needed
						Upset stomach	-	-
	Croton malabaricus Bedd		Pambaram	2 (0.02)	1.5	Cooling	decoction	Bi-weekly
						Health maintenance	decoction	Daily
						Fever	Decoction (+Christmas Bush and Cerasee)	Daily
Fabaceae	*Caesalpinia pulcherrima* (L.) Sw	DC014	Pride of Barbados, Flower Fence	3 (0.03)	0.67	Cooling	Leaves; decoction	-
	Abrus precatorius L		Crab Eye	2 (0.02)	1.0	Health maintenance	decoction	Daily
						Cough	infusion	As needed
	Senna bicapsularis (L.) Roxb		*Senna*, Money Bush, Monkey Tamarind	1 (0.01)	1.0	Cleansing	Decoction or infusion	Annually
	Trigonella foenum-graecum L		Fenugreek	1 (0.01)	1.0	Joint pain	Infusion	Weekly
Lamiaceae	*Mentha* × *piperita* L./ *Mentha* sp		Peppermint, Mint	9 (0.08)	1.0	Cooling	Infusion	Daily, weekly
						Fever	Leaves; decoction	As needed
						Cold/Flu	Infusion	As needed
						Hypertension	Infusion	Daily
						Health maintenance	Infusion	Weekly
	Ocimum basilicum L		Basil	3 (0.03)	1.0	Cooling	Infusion	Daily, as needed
	Thymus vulgaris L		Thyme	3 (0.03)	0.67	Headache	Infusion	Daily
						Health maintenance	Infusion	Daily
	Rosmarinus officinalis L		Rosemary	1 (0.01)	1.0	Cooling	Decoction (+Moringa)	Daily
	Marrubium vulgare L		Horehound	1 (0.01)	1.0	Cough	Infusion	As needed
Lauraceae	*Persea americana* Mill		Pear	16 (0.14)	1.13	Cooling	Leaf; decoction or infusion	Daily, weekly
						Hypertension	Leaf; decoction or infusion	Daily
						Health maintenance	Leaf; infusion	Weekly or monthly
						Cold/Flu	Leaf; decoction	As needed
						Diarrhea	Leaf; infusion	Once per week
						Headache	Leaf; infusion	Monthly
						Purgative	Leaf; decoction	Weekly
Linaceae	*Linum usitatissimum* L		Flax, Linseed	1 (0.01)	1.0	Joint pain	Ingested in powder form	Daily
Lythraceae	*Punica granatum* L	DC013	Pomegranate	2 (0.02)	1.0	Cooling	Decoction	-
						Health maintenance	Infusion	Monthly
Malvaceae	*Hibiscus rosa-sinensis* L	DC015	*Hibiscus* Flower	2 (0.02)	1.0	Health maintenance	-	-
Meliaceae	*Azadirachta indica* A. Juss		Neem	13 (0.11)	1.54	Diabetes	Leaf; decoction	Weekly or monthly
						Hypertension	Leaf; decoction	Monthly
						Health maintenance	Decoction	Weekly or monthly
						Cleansing	Leaf; infusion	Weekly
						Cooling	Leaf; infusion	Daily for 1 week then 2-weeks break
						Joint/Back pain	Decoction	Weekly
						Cough	Decoction	As needed
						Cuts	Pods; applied topically to wound	As needed
						Immune booster	Blended, infusion	As needed
						Sore eye	Leaf; decoction	As needed

(Continued on following page)

TABLE 3 | (*Continued*) List of medicinal plants, preparation methods and frequency of administration reported by users (*N* = 116).

Plant				FC (RFC)	UV	Applications		
Family	**Species**	**Voucher No.**	**Local Name(s)**			**Reported Uses**	**Preparation and Administration**	**Freq. of Treatment**
Moraceae	*Artocarpus altilis* (Parkinson) Fosberg		Breadfruit	3 (0.03)	1.0	Health maintenance	Infusion	Weekly
						Hypertension	Leaf; infusion	Weekly
Moringaceae	*Moringa oleifera* Lam		Moringa	18 (0.16)	1.06	Health maintenance	Leaf; decoction	Daily, weekly
							Nuts; ingested raw	
						Diabetes	Decoction or infusion	Weekly
						Hypertension	Decoction	Weekly
						Cleansing	Decoction (+sugar) or infusion	Weekly, monthly
						Cooling	Decoction	Daily
						Cough	Seeds; chewed	As needed
						Immune booster	Blended, infusion	As needed
Musaceae	*Musa acuminata* Colla	DC007	Banana	2 (0.02)	1.0	Iron supplement	-	-
						Toothache	Stub; decoction	As needed
Myristicaceae	*Myristica fragrans* Houtt		Nutmeg	2 (0.02)	1.0	Upset stomach	Grated, infusion	As needed
						Headache	Mixed with candle grease, applied to cloth, and placed on head	As needed
Myrtaceae	*Syzygium aromaticum* (L.) Merr. and L.M. Perry		Clove	2 (0.02)	1.0	Toothache	Cloves; decoction	As needed
						Health maintenance	-	-
	Pimenta racemosa (Mill.) J.W. Moore	DC018	Bay Leaf	30 (0.26)	1.17	Cooling	Leaves; decoction (+sugar) or infusion	Daily, weekly
						Health maintenance	Decoction or infusion	Daily, weekly
						Hypertension	Decoction or infusion	Daily
						Cleansing	Decoction (+sugar)	Monthly
						Cold/Flu	Decoction	As needed
						Headache	Infusion	As needed
						Sinus issues	Decoction	As needed
						Upset stomach	Infusion	Monthly
	Psidium guajava L		Guava	1 (0.01)	1.0	Sore throat	Ingested orally	As needed
Oleaceae	*Syringa vulgaris* L		Lilac	1 (0.01)	1.0	-	-	-
Onagraceae	*Oenothera biennis* L		Evening Primrose	1 (0.01)	1.0	Hormonal balance	Capsule, ingested orally	Daily
Papaveracae	*Argemome mexicana* L		Holly Hock	1 (0.01)	1.0	Nosebleed	-	-
Phytolaccaceae	*Petiveria alliacea* L		Gully Root	1 (0.01)	1.0	Cough	Decoction (+sugar and salt)	As needed
Poaceae	*Cymbopogon citratus* (DC.) Stapf	DC001	Lemongrass, Fever Grass	18 (0.16)	1.11	Cough	Decoction	As needed
						Cooling	Decoction	Daily
						Health maintenance	Decoction or infusion	Daily, monthly
						Cold/Flu	-	-
						Fever	Decoction	As needed
						Inflammatory conditions	-	-
						Joint pain	Infusion	Weekly
						Sinus issues	-	-
Ranunculaceae	*Hydrastis canadensis* L		Goldenseal	1 (0.01)	1.0	Health maintenance	-	-
	Nigella sativa L		Blackseed, Black Cumin	1 (0.01)	1.0	Hypertension	Oil extract	-
Rubiaceae	*Morinda citrifolia* L		Noni, Dog Dumpling	2 (0.02)	1.0	Joint pain	Leaf, fruit; decoction	As needed
						Health maintenance	Blended with juice	Monthly
	Psychotria tenuifolia Sw		Coffee Bush	2 (0.02)	1.0	Health maintenance	decoction	Daily
						Diabetes	Dried, decoction	Daily

(Continued on following page)

TABLE 3 | (*Continued*) List of medicinal plants, preparation methods and frequency of administration reported by users (*N* = 116).

Plant				FC (RFC)	UV	Applications		
Family	**Species**	**Voucher No.**	**Local Name(s)**			**Reported Uses**	**Preparation and Administration**	**Freq. of Treatment**
Rutaceae	*Citrus limon* (L.) Osbeck		Lemon	4 (0.03)	0.75	Health maintenance	Decoction	Daily
						Cooling	Infusion	-
						Diabetes	Infusion	As needed
	Dictamnus albus L		Moses Papa Bush	1 (0.01)	1.0	Cough	-	-
	Citrus sinensis (L.) Osbeck		Orange	1 (0.01)	1.0	Immune booster	Blended, infusion Capsule ingested orally	As needed
Salvadoraceae	*Salvadora persica* L		Mustard Bush	1 (0.01)	1.0	Nosebleed	-	-
Scrophulariaceae	*Bontia daphnoides* L		Wild Olive	2 (0.02)	1.0	Health maintenance	Infusion	Daily
						Cooling	Decoction	Daily
Solanaceae	*Solanum tuberosum* L		English Potato	1 (0.01)	1.0	Sore eye	Soak in water, mix with castor oil and use to wash eye	As needed
	Capsicum annum L		Cayenne pepper	1 (0.01)	1.0	Sinus issues	-	-
						Diverticulitis	-	-
Verbenaceae	*Lantana camara* L	DC019	Sage	1 (0.01)	0.5	Cooling	Infusion	Daily
						Diabetes	Infusion	Daily
	Stachytarpheta jamaicensis (L.) Vahl	DC003	Vervain	5 (0.04)	1.4	Cold/Flu	Ingested orally	As needed
						Cough	Infusion	Weekly
						Hypertension	Decoction or infusion	Weekly
						Joint pain	Infusion	-
						Headache	Infusion	Monthly
						Health maintenance	Infusion	Monthly
Zingiberaceae	*Zingiber officinale* Roscoe		Ginger	22 (0.19)	1.14	Health maintenance	Rhizome (grated or whole); decoction or infusion	Daily, weekly, monthly
						Joint/Back pain	Rhizome; decoction, infusion, or cooked in stew	Daily, weekly
						Blood/ Circulation	Rhizome; decoction	Bi-weekly
						Hypertension	Rhizome; decoction	Weekly
						Upset stomach	Rhizome; decoction or infusion	As needed
						Cooling	Infusion	Daily
						Diarrhea	Rhizome; decoction	As needed
						Dyspnea	Infusion	Daily
						Immune booster	Blended, infusion	As needed
						Inflammatory conditions	-	-
						Sinus issues	-	-
	Curcuma longa L		Turmeric	11 (0.1)	1.27	Health maintenance	Decoction, infusion or tablet ingested orally	Daily, weekly, monthly
						Hypertension	Decoction or infusion	Daily, weekly
						Joint/Back pain	Decoction, infusion, or cooked in stew	Daily, weekly
						Blood/ Circulation	Decoction	Bi-weekly
						Inflammatory conditions	Decoction	As needed

reports for some of the most reported species in both studies, including *M. charantia,* which our study identified as useful for cancer prevention, constipation, cooling, headache, and upset stomach, as well as *C. papaya* (Pawpaw), which was identified as useful in both colds/flu and constipation. Notably, there were no similarities in the use reports for *Stachytarpheta jamaicensis* (L.) Vahl (Vervain) identified by Peter (2013)-which were few and mainly neurological - and those identified in this study, which were more diverse and included cough, colds/flu, hypertension, joint pain, and health maintenance. This finding is noteworthy considering the much smaller and more focused rural setting of this study compared to that done by Peter, which surveyed 11 parishes across Barbados. In particular, this may provide support for the notion that traditional plant knowledge is retained and preserved to a greater extent in rural communities than in more urban localities.

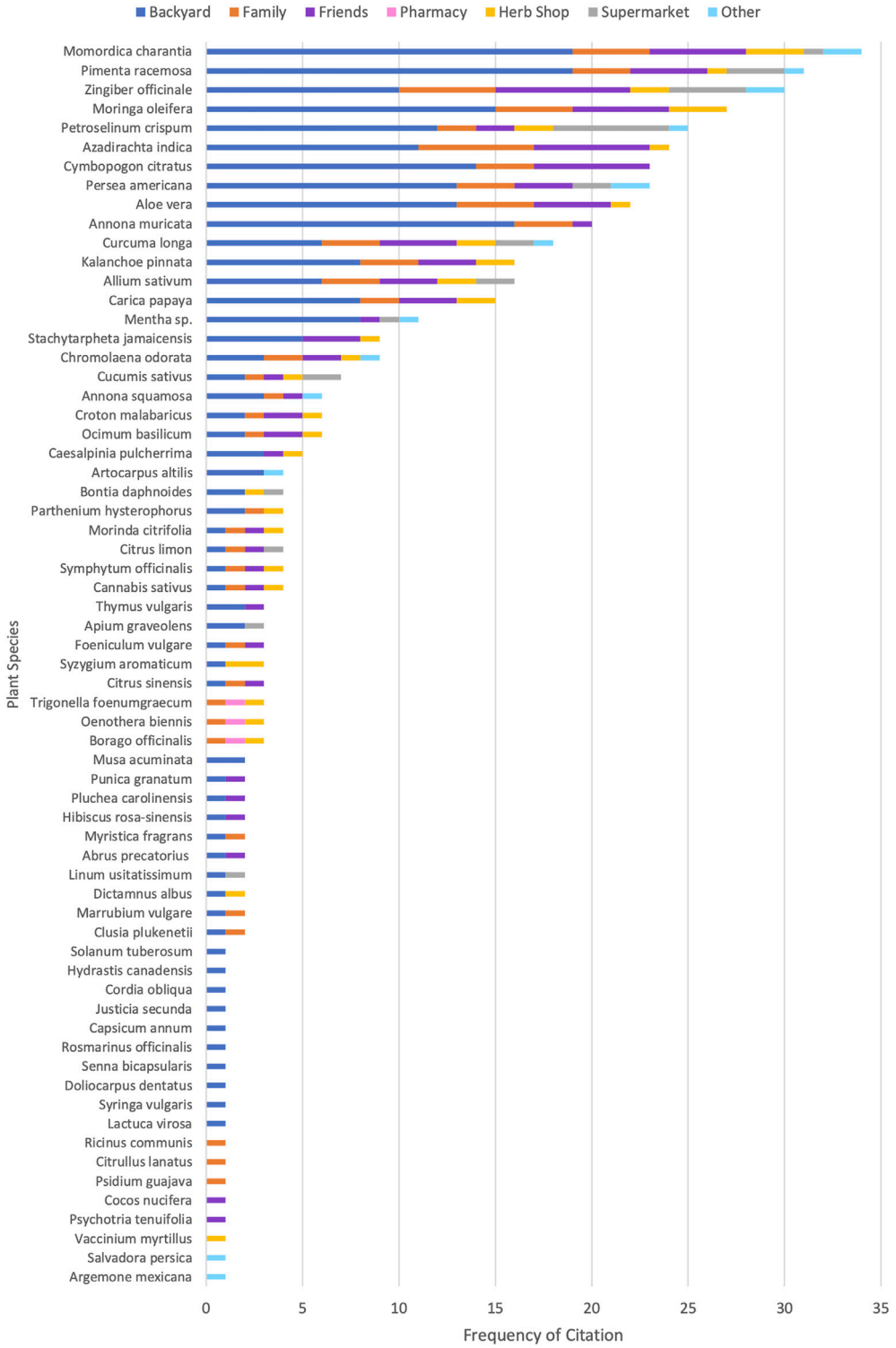


FIGURE 1 | Sources of acquisition of specific medicinal plants as cited by users. Frequency of citation reflects the number of respondents acquiring that species from the given source and includes cases in which respondents cited one species multiple times with different sources.

Hypertension, cough, and influenza proved to be the top three medical illnesses that participants in our study treated with botanical medicines, which is not surprising considering hypertension, heart disease and lower respiratory infections are among the top ten causes of morbidity and mortality in Barbados (Global Burden of Disease Collaborative Network,

TABLE 4 | Contingency Table and Fisher's Exact Test Results for Knowledge Acquired from Family x Medicinal Plants Sourced from Family (n = 108).

Medicinal Plants from Family	Knowledge from Family		*Row Total (%)*	
	Yes	No		*p*-value (ES)
Yes	20 (22.5)[a]	0 (0.0)[a]	20 (18.5)	
No	69 (77.5)[a]	19 (100.0)[a]	88 (81.5)	0.02 (0.22)
Column Total (%)	89 (100.0)	19 (100.0)	108 (100.0)	

[a]*Percent within* Knowledge from Family *(column %).*
Note: *ES = effect size.*

TABLE 5 | Contingency Table and Chi-Squared Results for Knowledge Acquired from Friends x Medicinal Plants Sourced from Friends (*n* = 108, df = 1).

Medicinal Plants from Friends	Knowledge from Friends		*Row Total (%)*	χ^2, *p*-value (ES)
	Yes	No		
Yes	15 (41.7)[a]	13 (18.1)[a]	28 (25.9)	
No	21 (58.3)[a]	59 (81.9)[a]	80 (74.1)	6.967, 0.008 (0.25)
Column Total (%)	36 (100.0)	72 (100.0)	108 (100.0)	

[a]*Percent within* Knowledge from Friends *(column %).*
Note: *ES = effect size.*

TABLE 6 | Contingency Table and Fisher's Exact Test Results for Education Level x Medicinal Plants Sourced from Backyard (*n* = 107).

Level of Education	Medicinal Plants from Backyard		*Row Total (%)*	
	Yes	No		*p*-value (ES)
Primary or less	34 (89.5)[a]	4 (10.5)[a]	38 (100.0)	
Secondary	29 (61.7)[a]	18 (38.3)[a]	46 (100.0)	
Associate/Vocational	9 (69.2)[a]	4 (30.8)[a]	13 (100.0)	0.02 (0.28)
Undergraduate +	6 (66.7)[a]	3 (33.3)[a]	9 (100.0)	
Column Total (%)	78 (72.9)	29 (27.1)	107 (100.0)	

[a]*Percent within* Level of Education *(row %).*
Note: *ES = effect size.*

TABLE 7 | Contingency Table and Chi-Squared Results for Annual Income x Medicinal Plants Sourced from Family (n = 90, df = 1).

Annual Income	Botanical Medicines from Family		*Row Total (%)*	χ^2, *p*-value (ES)
	Yes	No		
Less than $18,000	6 (10.7)[a]	50 (89.3)[a]	56 (100.0)	
More than $18,000	10 (29.4)[a]	24 (70.6)[a]	34 (100.0)	5.060, 0.02 (0.24)
Column Total (%)	16 (17.8)	74 (82.2)	90 (100.0)	

[a]*Percent within* Annual Income *(row %).*
Note: *ES = effect size.*

2020). Our study also showed that 62.1% of medicinal plant users suffered from at least one chronic non-communicable condition, with hypertension being the most prevalent. This is not alarming considering the median age group was 51–60 years, a popular age group for the onset of degenerative chronic diseases as well as a high prevalence of chronic conditions in Barbados (Unwin et al., 2015; Prasad et al., 2012). In fact, the 2015 Health of the Nation Survey found that roughly 45% of Barbadian adults are hypertensive, 25% have diabetes, and 21% are hypercholesterolemic; additionally, these figures were shown to increase with age (Unwin et al., 2015). Interestingly, botanical medicines were most frequently used for "health maintenance", cleansing (i.e., detoxifying), and cooling (i.e., eliminating heat or irritable behaviour), which are folkloric practices. It is likely that these practices persist in present-day Barbados as relics of the historical beliefs concerning health and disease that underpinned the traditions held by the enslaved West Africans and European colonialists. For example, two pillars of West African health and healing traditions include spiritual cleansing, as well as achieving and maintaining stability among the physical, mental, spiritual, and emotional domains – both of which involve the use of medicinal plants (White, 2015). The use of "cleansing" remedies in Caribbean society today echoes this spiritual cleansing practiced by slaves, although it has gradually become

TABLE 8 | Contingency Table and Chi-Squared Results for Parental Use of Botanical Medicines (n = 88, df = 1) and Chronic Condition (n = 103, df = 1) x Concomitant Use of Conventional and Botanical Medicines.

	Concomitant Use of Botanical and Conventional Medicines		*Row Total (%)*	χ^2, *p*-value (ES)
	Yes	**No**		
Parental Herb Use				
Yes	11 (20.0)[a]	44 (80.0)[a]	55 (100.0)	
No	13 (39.4)[a]	20 (60.6)[a]	33 (100.0)	3.911, 0.05 (0.21)
Column Total (%)	24 (27.3)	64 (72.7)	88 (100.0)	
Chronic Condition				
Yes	28 (40.6)[b]	41 (59.4)[b]	69 (100.0)	
No	4 (11.8)[b]	30 (88.2)[b]	34 (100.0)	8.831, 0.003 (0.29)
Column Total (%)	32 (31.1)	71 (68.9)	103 (100.0)	

[a]*Percent within* Parental Use of Botanical Medicines *(row %).*
[b]*Percent within* Chronic Condition *(row %).*
Note: *ES = effect size.*

TABLE 9 | Contingency Tables and Fisher's Exact Test/Chi-Squared Results for Level of Education (n = 152, df = 3) and Health Insurance (n = 152, df = 1) x Botanical Medicine Use.

	Use of Botanical Medicines		*Row Total (%)*	*p*-value (ES)
	Users	**Non-Users**		
Level of Education				
Primary or less	41 (87.2)[a]	6 (12.8)[a]	47 (100.0)	
Secondary	52 (75.4)[a]	17 (24.6)[a]	69 (100.0)	
Associate/Vocational	13 (59.1)	9 (40.9)	22 (100.0)	p = 0.05 (0.23)
Undergraduate +	9 (64.3)	5 (35.7)	14 (100.0)	
Column Total (%)	115 (75.7)	37 (24.3)	152 (100.0)	
Health Insurance				χ^2, *p*-value (ES)
Yes	14 (58.3)[b]	10 (41.7)[b]	24 (100.0)	
No	101 (78.9)[b]	27 (21.1)[b]	128 (100.0)	4.645, 0.003 (0.20)
Column Total (%)	115 (75.7)	37 (24.3)	152 (100.0)	

[a]*Percent within* Level of Education *(row %).*
[b]*Percent within* Health Insurance *(row %).*
Note: *ES = effect size.*

more focused on physical rather than extra-physical healing (Clement et al., 2015; White, 2015). Similarly, the botanical medicines used for "health maintenance" today are likely to reflect those used by West African slaves to achieve stability among the physical, mental, and spiritual domains. The use of cooling remedies, however, mirrors the European humoral theory of health and disease in which diseases were classified as hot or cold – the former being treated with cooling remedies and vice versa (Cook and Walker, 2013).

Other ethnobotanical studies conducted in the Caribbean basin have also documented the use of medicinal plants for cleansing and cooling (Clement et al., 2015; Picking et al., 2015; Tareau et al., 2017), supporting the notion that these medico-cultural concepts represent a lasting impact of the cultural diversity that began in the Caribbean during the colonial era. However, the most compelling support of this finding is provided by Handler and Jacoby (1993) in their examination of slave medicine and plant use, in which the medicinal plants used by slaves prior to 1834 are documented. The list cites several plants that are still used in Barbados today (as identified by this study and Peter (2013)), as well as the medico-cultural uses of these plants to "purify" or "cleanse" the body or protect against "poisons of a hot nature." While the former is likely a reflection of West African traditional remedies as discussed above, the latter implies the adoption of European healing beliefs by the slave population and the development of novel pharmacopeias in the region. From a clinicopathological viewpoint, cleansing and cooling remedies could have been used extensively during the period of enslavement due to prevalence of the tropical infectious conditions in the West Indies which affected the inhabitants of the islands. The coast of West Africa, the common site of the embarking of slaves to vessels en route to the Caribbean, was considered a breeding ground for infectious diseases which led to the spread of these conditions in the West Indies (Handler, 2006).

Many of the plant use reports highlighted in this study overlap with those reported in ethnobotanical studies conducted in West Africa and Europe. *M. oleifera* (Moringa), which informants in this study reported to be useful for hypertension, cough, and diabetes, has been documented for the same traditional uses in both Benin and Ghana (Oppong Bekoe et al., 2020; Agoyi et al., 2014). Additional parallels are seen with *A. indica* (Neem), which is used for diabetes and anti-bacterial purposes both in Nigeria

and in Barbados, as demonstrated by our findings (Lifongo et al., 2014). In Europe, on the other hand, similar use reports have been found for 1) *P. racemosa* (Bay Leaf), including headache, upset stomach, and colds, 2) *M. charantia* (Cerasee), including upset stomach, 3) *Mentha* x *piperita* L. (Peppermint), including colds/flu, and 4) *Aloe vera* Burm.f. (Aloe), including purgative, cuts and sores (Polat and Satil, 2012; Brussell, 2004; Gonzalez et al., 2010; Leonti et al., 2010). A study carried out in Trinidad and Tobago, a larger island in the south of the Caribbean, has also identified similar botanical families and plant species which can be found in Barbados and were reported to have medicinal properties related to ancestral practices. *Stachytarpheta jamaicensis* (L.). Vahl, *Senna alata* (L.) Roxb. and *Momordica charantia* L. were reported to be widely used for cooling/cleansing in that territory (Clement et al., 2015).

The preponderance of botanical medicine users acquiring knowledge from family members could be related to the rurality of the College Lands' communities. In the post-emancipation era, many liberated slaves migrated to urban centers in pursuit of a better education and achieving middle-class respectability, which meant conforming to British sociopolitical standards. As a result, traditional healing knowledge was largely confined to those who remained in the rural communities (Proctor, 1980). Therefore, the knowledge and use of medicinal plants by persons residing in these communities were likely passed down to them from older generations. This also supports our finding that people with a primary level education were more likely to source medicinal plants from their backyard as compared to those with a secondary level education. In fact, the adoption of western practices, including medicine, by the liberated slaves and their descendants who pursued higher education meant that traditional healing knowledge was essentially relegated the poorer and uneducated population (Proctor, 1980). Studies carried out in Jamaica and Trinidad have found interesting results concerning the relationship between education and the use of botanical medicines. In these studies, the respective prevalence of medicinal plant use among participants was inversely proportional to level of education attained; use was consistently lowest among participants with tertiary education (i.e., post-secondary), and the highest among participants with little to no education (Mahabir and Gulliford, 1997; Merritt-Charles, 2011; Picking et al., 2011). One study investigated the factors affecting the decision to use botanical medicines among asthmatic patients in Trinidad; interestingly, Garlic (*Allium sativum* L.) and Echinacea (*Echinacea purpurea* (L.) Moench) were the preferred plant species among users with higher levels of education – both of which have strong scientific evidence supporting their medicinal properties (Clement et al., 2005). In our study, significant differences in the use of botanical medicines were observed between participants with a primary level education and those with associates/vocational degrees. While this is similar to the general trends described above, no difference in use was observed between primary and tertiary (undergraduate/graduate degree) level participants. However, this could be the result of a small sample size or a lack of participants having a tertiary education level in our sample. Additionally, respondents who did not have health insurance were more likely to use botanical medicines than those with health insurance which further supports the socio-economic divide on the use of botanical medicines observed in the Caribbean territories. No significant associations were observed with annual income and the participants that used botanical medicines in this study. However, a modest number of persons identified as users earned an annual income greater than BDS $28,000.00 ($n = 19$). This supports a similar trend identified in Trinidad and Tobago where persons in the higher socio-economic class were exposed to the benefits of medicinal plants, but this may be limited to co-modified plant products with strong scientific evidence supporting their medical uses (Clement et al., 2005).

Our finding that combining medicinal plants and prescription medications was less common among people whose parents use medicinal plants could indicate that these participants were mirroring the practices they observed from their parents, whose traditional knowledge and experience may have elucidated some potential risks associated with those practices. A similar element of teaching/learning could be responsible for the observation that the concomitant use of botanical and conventional medicines was less frequent among people who discussed botanical medicines with their doctor. Although the association between age and the use of botanical medicine was not significant in our study, findings from other studies provide support for a general, cross-cultural trend of greater medicinal plant use and knowledge among older individuals and females, as observed in our study (Longuefosse and Nossin, 1996; Begossi et al., 2002; Quinlan and Quinlan, 2007). While the former could be interpreted as evidence of knowledge degradation through the younger generations, the latter may be explained by the notion that women are typically the principal caretakers and health custodians of the household, and in general seem to be more observant of their health than men. In fact, several studies have found that women are more likely to carry out their own forms of health management than are men (Liang et al., 1999; Turner and McClure, 2003; Stjernberg and Berglund, 2005). Interestingly, most participants reported earning an annual income, which may suggest that most of the sample may be employed. Thus, the relatively low gender disparity in botanical medicine use could be the result of more women being in the workforce rather than the sole traditional caretaking role in the household.

The 1969 Health Services Act of Barbados, Cap. 44 and the Drug Services Act 1980 provide the framework which ensures public access to quality drugs under specific categories, especially drugs for chronic non-communicable conditions (CNCDs), at affordable prices to citizens and permanent residents regardless of their socio-economic circumstances (Hennis et al., 2005). As observed in **Table 2**, persons who reported having one or more CNCDs ($n = 95$) had access to free or reasonable pharmaceutical products to treat their conditions and 75.8% of these persons were reported users of botanical medicines. This highlights an integration of traditional and westernized healing practices by these individuals as a result of cultural syncretism which may be irrespective of the cost of accessing health care. The lack of association found between the use of botanical medicines and the nature of doctor-patient relationships is also interesting due to the majority of medicinal plant users not discussing this practice with their physicians. It could be that these individuals have internalized the stigma

surrounding the use of botanical medicines and, in turn, do not discuss it with their physician for fear of being judged or ridiculed. While the majority of respondents would recommend the use of botanical medicines to their peers and family, only a minority were aware of risks associated with the use of these plants as medicines.

These findings support the need for more public education and awareness campaigns on the safe use of botanical medicines. It also highlights the need to archive information about the traditional uses of botanical medicines in a territory with low endemic plant species and diversity coupled with a relatively high burden of medical conditions. While the findings from this study are valuable, there are some limitations. The study data was collected by semi-structured interviews using a validated questionnaire and is likely to be affected by recall bias by the participants. This was evident due to differences in the accuracy and completeness of the recollections retrieved from the study participants on their knowledge and practices on the use of botanical medicines. Also, the use value (UV) and relative frequency of citation (RFC) are dynamic and will vary with changes in locations, the knowledge among the study participants and other factors unique to the study population and their practices. UV determines the extent to which a species can be used; thus, species with a high UV are more exploited in the study area to treat a particular ailment than those with a low UV (Amjad et al., 2017). Data have been analyzed and discussed to highlight comparisons within the dataset and other studies, but this may be limited because of variance and thus should be interpreted with caution. Further, this study was also done in a rural area of Barbados and while these findings may be generalizable to other rural Caribbean areas, they are not generalizable to the whole island of Barbados or urbanised areas of the Caribbean. Finally, field officers collected samples of the plants where possible and these were identified by plant taxonomist at the herbarium at the University of the West Indies, Cave Hill Campus. The identification of the other medicinal plants was by their recognition as crop plants and others were identified by the use of high-resolution photographs of the plants and by the informants' recall. To a lesser extent, some plants were reported as being sourced as semi-processed products, for example, *Lactuca virosa* Habl. (Wild Lettuce), *Oenothera biennis* L. (Evening Primrose), Linum *usitatissimum* L. (Linseed) and *Borago officinalis* L. (Starflower). Unfortunately, product details inclusive of batch numbers were not able to be retrieved by the interviewers.

CONCLUSION

The objectives of this study were achieved by the detailed documentation of the knowledge, attitudes, and practices of botanical medicines in rural Barbados, particularly in a district that has never been studied before. Demographic and socioeconomic variables which influence the use of botanical medicines in the surveyed communities were also identified. Additionally, our survey showed the persistence of medico-cultural concepts such as "cooling" and "cleansing" as well as the use of globally recognizable plants, some with West African origins. These findings demonstrate the permeation of ancestral healing practices to persons in these rural communities. Efforts must be mobilized to archive these practices for sensitizing the wider Barbadian and Caribbean population where the use of botanical medicines is much lower. The identification of practices and factors that may influence the use of botanical medicines can promote larger-scale studies to help determine how to overcome certain barriers to the use of medicinal plants, as well as to appropriately establish safety and efficacy studies to further evaluate the pharmacological effects of traditional plant-based remedies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of the West Indies/Barbados Ministry of Health Ethics Committee/Institutional Review Board (IRB No. 180303-A). Written informed consent by informants was provided prior to their participation. This includes written informed consent by the participants' legal guardian/next of kin as required.

AUTHOR CONTRIBUTIONS

DC designed and conducted the survey, reviewed and assisted with the writing and editing of the manuscript. TV carried out all data analyses and wrote the manuscript.

FUNDING

This study was funded by the UNOPS GEF Small Grants Programme (BAR/SGP/OP6/Y3/CORE/BD/17/02).

ACKNOWLEDGMENTS

To Michael Clarke, Principal of Codrington College, for support with the study. To Professor Sean Carrington, Taxonomist at The University of the West Indies, for his assistance with the identification and vouchering of the plant samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.713855/full#supplementary-material

REFERENCES

Abrahams, R. D. (1967). The Shaping of Folklore Traditions in the British West Indies. *J. Inter-am. Stud.* 9 (3), 456–480. doi:10.2307/164803

Agoyi, E. E., Assogbadjo, A. E., Gouwakinnou, G., Okou, F. A. Y., and Sinsin, B. (2014). Ethnobotanical Assessment of Moringa Oleifera Lam. In Southern Benin (West Africa). *Ethnobot. Res. App.* 12, 551–560. doi:10.17348/era.12.0.551-560

Amjad, M. S., Qaeem, M. F., Ahmad, I., Khan, S. U., Chaudhari, S. K., Zahid Malik, N., et al. (2017). Descriptive Study of Plant Resources in the Context of the Ethnomedicinal Relevance of Indigenous flora: A Case Study From Toli Peer National Park, Azad Jammu and Kashmir, Pakistan. *PLOS One* 12 (2), e0171896. doi:10.1371/journal.pone.0171896

Andriamparany, J. N., Brinkmann, K., Jeannoda, V., and Buerkert, A. (2014). Effects of Socio-Economic Household Characteristics on Traditional Knowledge and Usage of Wild Yams and Medicinal Plants in the Mahafaly Region of South-Western Madagascar. *J. Ethnobiol. Ethnomed.* 10 (82), 82. doi:10.1186/1746-4269-10-82

Bayley, I. (1949). The Bush Teas of Barbados. *J. Barbados Must Hist. Soc.* 16, 103–105.

Begossi, A., Hanazaki, N., and Tamashiro, J. Y. (2002). Medicinal Plants in the Atlantic Forest (Brazil): Knowledge, Use and Conservation. *Hum. Ecol.* 30 (3), 281–299. doi:10.1023/a:1016564217719

Carrington, S. (2007). *Wild Plants of Barbados*. 2nd ed. Oxford, England: Macmillan Caribbean.

Clement, Y. N., Baksh-Comeau, Y. S., and Seaforth, C. E. (2015). An Ethnobotanical Survey of Medicinal Plants in Trinidad. *J. Ethnobiol. Ethnomed.* 11, 67. doi:10.1186/s13002-015-0052-0

Clement, Y. N., Williams, A. F., Aranda, D., Chase, R., Watson, N., Mohammed, R., et al. (2005). Medicinal Herb Use Among Asthmatic Patients Attending a Specialty Care Facility in Trinidad. *BMC Complement. Altern. Med.* 5 (1), 3. doi:10.1186/1472-6882-5-3

Cohall, D. H. (2014). *Medicinal Plants of Barbados for the Treatment of Communicable and Non-Communicable Diseases*. Kingston, Jamaica: University of the West Indies Press.

Cohall, D. H., Scantlebury-Manning, T., Cadogan-McLean, C., Lallement, A., and Willis-O'Connor, S. (2012). The Impact of the Healthcare System in Barbados (Provision of Health Insurance and the Benefit Service Scheme) on the Use of Herbal Remedies by Christian Churchgoers. *West. Indian Med. J.* 61 (3), 258–263. doi:10.7727/wimj.2011.178

Cook, H. J., and Walker, T. D. (2013). Circulation of Medicine in the Early Modern Atlantic World. *Soc. Hist. Med.* 26 (3), 337–351. doi:10.1093/shm/hkt013

Crawford-Daniel, W. Y., and Alexis, J. M. (2014). in *Caribbean Healing Traditions: Implications for Health and Mental Health*. Editors P. Sutherland, R. Moodley, and B. Chevannes (New York (NY): Routledge), 29–40.

Eric Brussell, D. (2004). Medicinal Plants of MT. Pelion, Greece. *Econ. Bot.* 58 (S), S174–S202. doi:10.1663/0013-0001(2004)58[s174:mpompg]2.0.co;2

Feng, P. C. (1956). A Preliminary Survey of the Medicinal Plants of British Guiana. *West. Indian Med. J.* 5 (4), 265–270.

Global Burden of Disease Collaborative Network (2020). *Global Burden of Disease Study 2019 (GBD 2019)*. Seattle, United States of America: Institute for Health Metrics and Evaluation.

González, J. A., García-Barriuso, M., and Amich, F. (2010). Ethnobotanical Study of Medicinal Plants Traditionally Used in the Arribes del Duero, Western Spain. *J. Ethnopharmacol.* 131 (2), 343–355. doi:10.1016/j.jep.2010.07.022

Handler, J. S. (2006). Diseases and Medical Disabilities of Enslaved Barbadians, From the Seventeenth Century to Around 1838: Part 1. *Caribbean J. Hist.* 40, 1–38.

Handler, J. S., and Jacoby, J. A. (1993). Slave Medicine and Plant Use in Barbados. *J. Barbados Mus. Hist. Soc.* 41, 76–98.

Hennis, A., Hambleton, I., Crichlow, S., and Fraser, H. (2005). *Health, Welfare and Aging in Bridgetown, Barbados*. Washington, DC: SABE 2000.

Heredia-Diaz, Y., Garcia-Diaz, J., Lopez-Gonzalez, T., Chil-Nunez, I., Arias-Ramos, D., Escalona-Arranz, J. C., et al. (2018). An Ethnobotanical Survey of Medicinal Plants Used by Inhabitants of Holguin, Eastern Region, Cuba. *Bol Latinoam. Caribe Plantas Med. Aromat.* 15 (2), 160–196.

Holder, J. W. (1988). *Codrington College – A Brief History. Bridgetown*. Barbados: Codrington College.

Jackson, C. S., Phillips, A. D. V., and Marshall, W. K. (2021). Barbados. Encyclopedia Brittanica. Available at: https://www.britannica.com/place/Barbados (Accessed June 28, 2021).

Labonté, R., Runnels, V., Crooks, V. A., Johnston, R., and Snyder, J. (2017). What Does the Development of Medical Tourism in Barbados Hold for Health Equity? an Exploratory Qualitative Case Study. *Glob. Health Res. Pol.* 2, 5. doi:10.1186/s41256-017-0025-z

Lane, A. (1979). Race, Class and Development in Barbados. *Caribbean Q.* 25 (1/2), 45–51. doi:10.1080/00086495.1979.11671944

Leonti, M., Cabras, S., Weckerle, C. S., Solinas, M. N., and Casu, L. (2010). The Causal Dependence of Present Plant Knowledge on Herbals-Ccontemporary Medicinal Plant Use in Campania (Italy) Compared to Matthioli (1568). *J. Ethnopharmacol.* 130 (2), 379–391. doi:10.1016/j.jep.2010.05.021

Liang, W., Shediac-Rizkallah, M. C., Celentano, D. D., and Rohde, C. (1999). A Population-Based Study of Age and Gender Differences in Patterns of Health-Related Behaviors. *Am. J. Prev. Med.* 17, 8–17. doi:10.1016/s0749-3797(99)00040-9

Lifongo, L. L., Simoben, C. V., Ntie-Kang, F., Babiaka, S. B., and Judson, P. N. (2014). A Bioactivity versus Ethnobotanical Survey of Medicinal Plants from Nigeria, West Africa. *Nat. Prod. Bioprospect.* 4, 1–19. doi:10.1007/s13659-014-0005-7

Longuefosse, J. L., and Nossin, E. (1996). Medical Ethnobotany Survey in Martinique. *J. Ethnopharmacol.* 53 (3), 117–142. doi:10.1016/0378-8741(96)01425-0

Mahabir, D., and Gulliford, M. C. (1997). Use of Medicinal Plants for Diabetes in Trinidad and Tobago. *Rev. Panam Salud Publica.* 1 (3), 174–179. doi:10.1590/s1020-49891997000300002

McCaskie, T. C. (2017). "The Art or Mystery of Physick" - Asante Medicinal Plants and the Western Ordering of Botanical Knowledge. *Hist. Afr.* 44, 27–62. doi:10.1017/hia.2016.11

Merritt-Charles, L. (2011). The Prevalence of Herbal Medicine Use Among Surgical Patients in Trinidad. *Focus Altern. Complement. Ther.* 16 (4), 266–270. doi:10.1111/j.2042-7166.2011.01121.x

Napagoda, M. T., Sundarapperuma, T., Fonseka, D., Amarasiri, S., and Gunaratna, P. (2018). An Ethnobotanical Study of the Medicinal Plants Used as Anti-Inflammatory Remedies in Gampaha District, Western Province, Sri Lanka. *Scientifica (Cairo).* 2018, 9395052–9395058. doi:10.1155/2018/9395052

Oppong Bekoe, E., Agyare, C., Boakye, Y. D., Baiden, B. M., Asase, A., Sarkodie, J., et al. (2020). Ethnomedicinal Survey and Mutagenic Studies of Plants Used in Accra metropolis, Ghana. *J. Ethnopharmacol.* 248, 112309. doi:10.1016/j.jep.2019.112309

Peter, S. (2013). "Medicinal and Cooling Teas of Barbados," in *African Ethnobotany in the Americas*. Editors R. Voeks and J. Rashford (New York, NY: Springer). doi:10.1007/978-1-4614-0836-9_11

Phillips, O., and Gentry, A. H. (1993). The Useful Plants of Tambopata, Peru: I. Statistical Hypotheses Tests With a New Quantitative Technique. *Econ. Bot.* 47 (1), 15–32. doi:10.1007/bf02862203

Picking, D., Delgoda, R., Younger, N., Germosén-Robineau, L., Boulogne, I., and Mitchell, S. (2015). TRAMIL Ethnomedicinal Survey in Jamaica. *J. Ethnopharmacol.* 169, 314–327. doi:10.1016/j.jep.2015.04.027

Picking, D., Younger, N., Mitchell, S., and Delgoda, R. (2011). The Prevalence of Herbal Medicine Home Use and Concomitant Use With Pharmaceutical Medicines in Jamaica. *J. Ethnopharmacol.* 137 (1), 305–311. doi:10.1016/j.jep.2011.05.025

Polat, R., and Satıl, F. (2012). An Ethnobotanical Survey of Medicinal Plants in Edremit Gulf (Balıkesir-Turkey). *J. Ethnopharmacol.* 139 (2), 626–641. doi:10.1016/j.jep.2011.12.004

Prasad, S., Sung, B., and Aggarwal, B. B. (2012). Age-Associated Chronic Diseases Require Age-Old Medicine: Role of Chronic Inflammation. *Prev. Med.* 54, S29–S37. doi:10.1016/j.ypmed.2011.11.011

Proctor, R. (1980). Early Developments in Barbadian Education. *J. Negro Education* 49 (2), 184. doi:10.2307/2294967

Quinlan, M. B., and Quinlan, R. J. (2007). Modernization and Medicinal Plant Knowledge in a Caribbean Horticultural Village. *Med. Anthropol. Q.* 21 (2), 169–192. doi:10.1525/maq.2007.21.2.169

Soelberg, J., Davis, O., and Jäger, A. K. (2016). Historical versus Contemporary Medicinal Plant Uses in the US Virgin Islands. *J. Ethnopharmacol.* 192, 74–89. doi:10.1016/j.jep.2016.07.005

Stjernberg, L., and Berglund, J. (2005). Tick Prevention in a Population Living in a Highly Endemic Area. *Scand. J. Public Health.* 33, 432–438. doi:10.1080/14034940510005932

Sutherland, P., Moodley, R., and Chevannes, P. (2013). *Caribbean healing traditions: Implications for health and mental health*. 1st ed. New York: Routledge.

Tareau, M. A., Palisse, M., and Odonne, G. (2017). As Vivid as a Weed… Medicinal and Cosmetic Plant Uses Amongst the Urban Youth in French Guiana Medicinal and Cosmetic Plant Uses Among the Urban Youth in French Guiana. *J. Ethnopharmacol.* 203, 200–213. doi:10.1016/j.jep.2017.03.031

Torres-Avilez, W., Méndez-González, M., Durán-García, R., Boulogne, I., and Germosén-Robineau, L. (2015). Medicinal Plant Knowledge in Caribbean Basin: A Comparative Study of Afrocaribbean, Amerindian and Mestizo Communities. *J. Ethnobiol. Ethnomed.* 11 (18), 18. doi:10.1186/s13002-015-0008-4

Turner, C., and McClure, R. (2003). Age and Gender Differences in Risk-Taking Behaviour as an Explanation for High Incidence of Motor Vehicle Crashes as a Driver in Young Males. *Inj. Control. Saf. Promot.* 10, 123–130. doi:10.1076/icsp.10.3.123.14560

Unwin, N., Rose, A. M. C., George, K. S., Hambleton, I. R., and Howitt, C. (2015). *The Barbados Health of the Nation Survey: Core Findings*. St. Michael, Barbados: Barbados Ministry of Health and Chronic Disease Research Centre, The University of the West Indies. Available at: http://www.archive.healthycaribbean.org/newsletters/aug-2015/CDRC_HealthOfTheNationSurvey.pdf (Accessed May 27, 2021).

Vandebroek, I., and Picking, D. (2020). *Popular Medicinal Plants in Portland and Kingston, Jamaica*. New York (NY): Springer International Publishing.

Watts, D. H. (1966). *Man's Influence on the Vegetation of Barbados*. Yorkshire (England): University of Hull Publications, 1627–1800.

White, P. (2015). The Concept of Diseases and Health Care in African Traditional Religion in Ghana. *HTS Teol. Stud.* 71 (3), a2762. doi:10.4102/HTS.V71I3.2762

World Development Indicators (2019). The World Bank. Available at https://datacommons.org/place/country/BRB?utm_medium=explore&mprop=count&popt=Person&hl=en (Accessed June 28, 2021).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 02 November 2021
doi: 10.3389/fphar.2021.760242

Quantification of Chlorogenic Acid and Vanillin from Coffee Peel Extract and its Effect on α-Amylase Activity, Immunoregulation, Mitochondrial Oxidative Stress, and Tumor Suppressor Gene Expression Levels in H_2O_2-Induced Human Mesenchymal Stem Cells

Heba Khalil Alyahya[1], Pandurangan Subash-Babu[1], Ahmad Mohammad Salamatullah[1], Khizar Hayat[1], Nawal Albader[1], Mohammed Saeed Alkaltham[1], Mohammed Asif Ahmed[1], Shaista Arzoo[1] and Mohammed Bourhia[2]*

[1]Department of Food Science and Nutrition, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia, [2]Laboratory of Chemistry-Biochemistry, Environment, Nutrition and Health, Faculty of Medicine and Pharmacy, Hassan II University, Casablanca, Morocco

Background: Polyphenols and flavonoid-rich foods help in arresting reactive oxygen species development and protecting DNA from oxidative damage. Coffee peel (CP) preparations are consumed as beverages, and their total polyphenol or flavonoid content and their effect on oxidative stress–induced human mesenchymal stem cells (hMSCs) are poorly understood.

Method: We prepared hot water extracts of CP (CPE) and quantified the amount of total polyphenol and flavonoid using HPLC analysis. In addition, CPE have been studied for their α-amylase inhibitory effect and beneficial effects in oxidative stress–induced hMSCs.

Results: The obtained results show that the availability of chlorogenic acid, vanillin, and salicylic acid levels in CPE is more favorable for enhancing cell growth, nuclear integrity, and mitochondrial efficiency which is confirmed by propidium iodide staining and JC-1 staining. CPE treatment to hMSCs for 48 h reduced oxidative stress by decreasing mRNA expression levels of LPO and NOX-4 and in increasing antioxidant CYP1A, GSH, GSK-3β, and GPX mRNA expressions. Decreased pro-inflammatory (TNF-α, NF-κβ, IL-1β, TLR-4) and increased tumor suppressor genes (except Bcl-2) such as Cdkn2A, p53 expressions have been observed.

Conclusions: The availability of CGA in CPs effectively reduced mitochondrial oxidative stress, reduced pro-inflammatory cytokines, and increased tumor suppressor genes.

Keywords: coffee peel, polyphenol, flavonoid, chlorogenic acid, antioxidant, DNA integrity

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Ivana Tomac, University of Osijek, Croatia
Rehab Abdel-Megeed, National Research Centre, Egypt

****Correspondence:***
Ahmad Mohammad Salamatullah
asalamah@ksu.edu.sa

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *17 August 2021*
Accepted: *20 September 2021*
Published: *02 November 2021*

Citation:
Khalil Alyahya H, Subash-Babu P, Mohammad Salamatullah A, Hayat K, Albader N, Alkaltham MS, Ahmed MA, Arzoo S and Bourhia M (2021) Quantification of Chlorogenic Acid and Vanillin from Coffee Peel Extract and its Effect on α-Amylase Activity, Immunoregulation, Mitochondrial Oxidative Stress, and Tumor Suppressor Gene Expression Levels in H_2O_2-Induced Human Mesenchymal Stem Cells. Front. Pharmacol. 12:760242. doi: 10.3389/fphar.2021.760242

INTRODUCTION

Globally, coffee is the most frequently consumed drink, and the increasing interest in coffee consumption has led the manufacturers to produce more varieties of coffee and is still progressive (Song et al., 2019). In the year 2019, the global green coffee production reached 10.03 billion kilograms (FAOSTAT, 2019). The large-scale production of coffee powder leads to an enormous amount of coffee peel (CP) waste (Murthy and Naidu, 2012; Prihadi et al., 2020). CP contains high fiber, and it has been identified with antioxidant, antiallergic, antihypertensive, and antimicrobial potential (Prihadi et al., 2020). The phytochemicals present in the CP are caffeine, tannin, polyphenol, pectin, and monosaccharide and disaccharide compounds (Janissen and Huynh, 2018). In coffee, the major beneficial bioactive compounds are caffeine and various polyphenols such as chlorogenic acid (CGA), ferulic acid, sinapic acid, gallic acid, and quinic acid (Hečimović et al., 2011; Durak et al., 2014). The main component of the phenolic fraction of the green coffee bean is CGA (Farah and Donangelo, 2006). CGA possesses a variety of health benefits such as reducing the risk of diabetes, cancer, and liver disease and protecting against Parkinson's disease (Bhupathiraju et al., 2013; Cano-Marquina et al., 2013).

Antioxidant compounds are majorly obtained from coffee and they contribute to the total dietary antioxidant capacity (Fujioka and Shibamoto, 2006). CGA is the main antioxidant compound in coffee which provides an activity similar to ascorbic acid (Pari et al., 2010). In addition, CGA prevents oxidized LDL and H_2O_2-induced oxidative stress and effectively scavenges the free radicals (superoxide, hydroxyl, and peroxynitrite) and protects DNA from oxidative damage (Cha et al., 2014). Meantime, excessive intake of coffee leads to higher bioavailability of CGA, which inhibits endothelial cells and cancer cell growth and angiogenesis; inversely, inhibition of angiogenesis and neovascularization cause impaired cell growth, tissue ageing, and functional disability (Wang et al., 2021). The present study emphasizes on CP, which contains high fiber and limited CGA, to be used as an antioxidant, antiallergic, antihypertensive, and antimicrobial agent (Prihadi et al., 2020).

CPs containing biomolecules need to be considered as a major antioxidant agent to protect against reactive oxygen species (ROS)–induced oxidative stress in human mesenchymal stem cells (hMSCs) during tissue repair or tissue regeneration therapy. Human MSCs play a major role in regenerative and immunomodulatory properties due to their multipotent differentiation potential (Denu and Hematti, 2016). Human MSCs are more sensitive to oxidative stress, and excessive ROS or exogenous H_2O_2 can impair self-proliferation and multilineage capacity (Choo et al., 2014). They are implanted to injured tissues and contribute to tissue repair with suppressing inflammatory rejection (Di Nicola et al., 2002). The loss of transplanted MSCs at the ischemic site is a major problem due to the loss of chemokine receptors, after the generation of ROS at the graft site (Honczarenko et al., 2006). ROS are initially generated from the mitochondrial complex (I & III) and NOX4 during hMSCs differentiation (Devine et al., 2001). Excessive ROS react and damage the biomolecules, especially altering the integrity of genomic DNA, which is critical for cellular proliferation and functions (Kobayashi et al., 2012).

The present study aimed to quantify the concentration of CGA, vanillin, and salicylic acid (SA) in CP extract (CPE) using HPLC. Furthermore, the potential of CPE on α-amylase inhibitory effect, bioefficacy in hMSCs *via* analyzing the inhibitory effect of cell and nuclear damage, mitochondrial membrane polarization, oxidative damage, and immunomodulation-related gene expression levels in H_2O_2-induced oxidative stressed hMSCs have been explored.

MATERIALS AND METHODS

Raw Material

Coffea arabica was obtained from the Jazan region in Fifa Mountains (1800 m above sea level), Kingdom of Saudi Arabia. Fresh whole coffee fruits were selected, washed, dried in the sun, and saved in a dry place until their extraction. CP was removed (hulled) from the whole coffee fruit and ground by using a coffee grinder (SF Stardust, PCP-R400065, Japan). Finally, the ground CP was sieved with stainless steel (0.5 mm) wire mesh to ensure a consistent powder size and kept at room temperature (25°C) for further extractions.

Preparation of the Extracts

CP was weighed (0.5, 0.75, 1, and 1.25 g) and then mixed with 10 ml water (solvent). Then the samples were boiled and stirred using a heating magnetic stirrer at 100°C for 10 min. Furthermore, the samples were allowed to rest for 10 min at room temperature and then centrifuged at 3000 *g* for 10 min using a fixed rotor centrifuge (Thermo Fisher Scientific, MA, United States). Finally, the samples were filtered using a filter paper (Whatman 9.0 cm). The obtained CPE was labeled properly, such as 0.5 g as CPE-1, 0.75 g as CPE-2, 1 g as CPE-3, and 1.25 g as CPE-4, and were kept under refrigeration at 4°C until analysis.

Total Polyphenol Content

The total polyphenol content (TPC) of the CPE was determined according to the method described by Hayat et al. (2011). 25 µl of the CPEs (CPE-1, CPE-2, CPE-3, and CPE-4) was mixed with 1500 µl distilled water and 125 µl Folin–Ciocalteu reagent (0.2%) and allowed to stand for 1 min. After that, 375 µl of Na_2CO_3 (20% w/v) and 475 µl distilled water were added. Finally, the mixture was allowed to rest at room temperature for 30 min. The absorbance was measured at 760 nm using a spectrophotometer (JascoV-630 Spectrophotometer, United States). A blank was prepared without the extract. The TPC was expressed as a gallic acid equivalent per gram dry weight of the sample (mg GAE/g DW).

Total Flavonoid Content

The total flavonoid content (TFC) was determined as described by Hayat (2020). 250 µl of CPEs (CPE-1, CPE-2, CPE-3, and CPE-4) was mixed with 1000 µl of distilled water. Then, 75 µl of

each $NaNO_2$ (5%) and $AlCl_3$ (10%) was added and incubated at room temperature for 5 min. Then, 500 µl of NaOH (1 M) and 600 µl of distilled water were added. The absorbance was recorded with a spectrophotometer (JASCO V-630 Spectrophotometer, United States) at 510 nm. A blank was prepared without the extract, and each sample was repeatedly analyzed six times ($n = 6$) to get mean ± SD. The TFC was expressed as a catechin equivalent per gram dry weight of the sample (mg CE/g DW).

Quantification of Phenolic Compounds in Coffee Peel Extracts Using HPLC

The quantification of phenolic compounds (tannic acid, CGA, caffeic acid, resorcinol, vanillin, 1,2-dihydroxybenzene, salicylic acid, acetyl salicylic acid, 3,5-dinitrosalicylic acid, and quercetin) in CPE(s) was determined using HPLC according to the method of Santos et al. (2014). The CPEs (CPE-1, CPE-2, CPE-3, and CPE-4) were separated by using the Shimadzu HPLC system, prominence (Kyoto, Japan) equipped with a LC-20AB binary pump and a variable Shimadzu SPD-10A UV-Vis detector. The column used was Zorbax SB-C18 (250 × 4.6 mm, 5 µm) (Agilent, Santa Clara, CA, United States) and the mobile phase consisted of (0.1% formic acid, A) and MeOH (0.1% formic acid, B). The gradient program was the following: 0 min, 5% B; 4 min, 5% B; 20 min, 73% B; 50 min, 95% B; 57 min, 1% B; 58 min, 1% B; and 60 min, 5% B, with a low rate of 0.7 ml/min. The injection volume was 10 µl, and the detector was set at 280 nm. Compounds were identified by comparing their retention time with those of the standard. All samples were analyzed in duplicates.

Carbohydrate Hydrolysis Enzymatic α-Amylase Assay

The carbohydrate hydrolysis enzymatic α-amylase (α-A) assay was performed according to the procedure suggested previously by Subramanian et al. (2008) and Hasenah et al. (2006). The controlled sample contained 150 µl of α-A enzyme solution and 150 µl of distilled water. The sample mixture was prepared by adding 150 µl of α-A to 150 µl of extract (CPE-1, CPE-2, CPE-3, and CPE-4). Likewise, the positive control was prepared with 150 µl of 1 mM acarbose (dissolved in 1% DMSO (dimethylsulfoxide)) and 150 µl of α-A. The blank mixture was prepared by dissolving 150 µl of distilled water with 150 µl of 20 mM phosphate buffer (pH 6.9) without α-A and the extract. Each tube was gently mixed and incubated at 37°C for 10 min. Thereafter, 150 µl of 0.5% starch solution was added to initiate the reaction, and the tubes were incubated at 37°C for 30 min. Then, 300 µl of dinitrosalicylic acid was added to stop the reaction, and the tubes were placed in a water bath at 100°C for 10 min. Lastly, all tubes were cooled to room temperature and then diluted with 2 ml of distilled water. The absorbance was determined at 540 nm (JASCO V-630 Spectrophotometer, United States). The percentage of amylase inhibitory activity of each sample was calculated by using the following equations:

$$\%\ \text{Inhibition} = Ad\,justed\,control - \frac{Ad\,justed\,sample}{Ad\,justed\,control} X\,100. \quad (1)$$

In Vitro Cell Culture Method and Materials

Human mesenchymal stem cells (hMSCs) have been obtained from the American type culture collection (ATCC, Manassas, VA, United States). DMEM (Dulbecco's Modified Eagle medium), EDTA (ethylenediaminetetraacetic acid), and trypsin were purchased from Gibco (Paisley, United Kingdom). Cell culture materials such as FBS (fetal bovine serum) and penicillin–streptomycin were obtained from Hyclone Laboratories, United States. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], propidium iodide, JC-1 stain, and all other chemicals related to a molecular biology experiment have been purchased from Sigma-Aldrich (St. Louis, MO, United States). The cell to cDNA synthesis kits and SYBR green PCR master mix were purchased from QIAGEN (Hilden, Germany).

Human Mesenchymal Stem Cell Culture

Human mesenchymal stem cells (hMSCs) were cultured using DMEM added with 10% FBS and 100 U/ml penicillin–streptomycin at 37°C in a humidified 5% CO_2 incubator. According to the experimental design and need, hMSCs were seeded in 96-well (1×10^4 cells/well) or 24-well (2×10^4 cells/well) plates containing DMEM with 10% FBS at 37°C and 5% CO_2 in humidified air. After visual confirmation of 80% confluence under an inverted microscope, the culture was used for the experiments.

Cytotoxicity Analysis

Human mesenchymal stem cells (hMSCs) were cultured in a 96-well culture plate (1×10^4 cells/well) and allowed to adhere overnight to the growth medium. After discarding the medium, a culture medium containing an increasing concentration (1, 2, 4, 8, 16, 32, and 64 mg/ml) of CPEs, namely, CPE-1, CPE-2, CPE-3, and CPE-4, was added to each well, and the cells were incubated for 48 h; control cells were treated with vehicle alone. After completion of 48 h, the cells were added with 20 µl/well of MTT [3- (4, 5-dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide] at a concentration of 5 mg/ml in DMSO and incubated at 37°C for an additional 4 h. At the end of incubation, the supernatant solution was removed gently without disturbing the formed purple formazan crystals. The crystal was dissolved in 100 µl of 100% DMSO using a multiwell plate shaker. The absorbance of the solution was measured at 570 nm using a microplate reader (Thermo Scientific). Cell proliferation (%) was calculated by the following equation: (absorbance of the sample/mean absorbance of the control) × 100.

Experimental Design

Bioefficacy and oxidative stress inhibition capacity of CPE on normal and H_2O_2-induced oxidative stressed hMSCs were examined. Two sets of hMSCs were cultured in 24-well plates and treated with 4 mg/ml of CPEs, namely, CPE-1, CPE-2, CPE-3, and CPE-4, for 48 h, respectively. After incubation, one group was kept for normal observation, and the other group of hMSCs were treated with 10 µM of H_2O_2 for 30 min. CGA was used as a reference control in both the experimental sets. After incubation,

all the grouped cells were used for propidium iodide staining, JC-1 staining, and gene expression analysis.

Propidium Iodide Staining Assay for Nuclear Damage

Cellular morphology for characteristic nuclear damage, pyknosis, or apoptotic morphological changes after hMSCs were treated with CPE-1, CPE-2, CPE-3, and CPE-4 (with or without H_2O_2) was determined using propidium iodide (PI) staining analysis under inverted fluorescence microscopy as described by Leite et al. (1999).

Assay of Mitochondrial Membrane Potential ($\Delta\psi_m$) by JC-1 Dye Staining

The mitochondrial membrane potential ($\Delta\psi_m$) was determined using the JC-1 dye to assess mitochondrial efficiency in the vehicle control, and CPE-1–, CPE-2–, CPE-3–, and CPE-4–treated hMSCs (with and without H_2O_2). Briefly, the JC-1 staining solution (mixed with equal volumes of the culture medium) was added to experimental hMSCs and incubated for 20 min in the dark at 37°C. After incubation, the unbound JC-1 dye was gently removed by washing with 200 µl of JC-1 staining wash buffer at 4°C, repeatedly for two times. Then, the accumulation of J-aggregates against JC-1 staining was observed under a florescence microscope.

Quantitative Real-Time PCR Analysis

Vehicle control; CPE-1–, CPE-2–, CPE-3–, and CPE-4–treated hMSCs (with and without H_2O_2); total RNA; and cDNA were synthesized using a Fastlane® Cell cDNA kit using qPCR. mRNA expression levels of oxidative stress (LPO, NOS, and CYP1A), antioxidants (GSH, GSK-3β, and GPx), pro-inflammatory cytokines (TNF-α, NF-κb, IL-1β, and COX-2), and tumor suppressor (cdkn2a, p53, and BCL-2)–related genes, and the reference gene, β-actin, have been analyzed according to the method of Yuan et al. (2006). The amplification values (ΔCt) have been calculated by the difference between Ct (treated) and Ct (control). The gene expressions were plotted using the expression of $2^{-\Delta\Delta Ct}$ value.

Statistical Analysis

All the experiments were performed in triplicates, and the data were presented as mean values ± SD (standard deviation). SAS software (version 9.2, 2000–2008; SAS Institute Inc., Cary, NC, United States) was used to analyze the differences among the groups by one-way analysis of variance (ANOVA), and if significant differences were found, then the Duncan's multiple range test was conducted at a confidence interval of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Recently, there has been a surge of interest in phenolic compounds extracted from plant materials (Kähkönen et al., 1999; Tapiero et al., 2002; Murthy and Naidu, 2012). They are secondary metabolites that occur naturally in plants and are generally intricate in defense against oxidative stress or aggression by pathogens. Considerable populations in Europe and the Arab regions consume hot water–boiled dried CP powder as an energy drink or refreshing drink as a replacement for coffee (Liang and Kitts, 2016; Farah and de Paula, 2019). The polyphenol compound CGA is the major antioxidant present in the coffee seeds and peel (Ameca et al., 2018). Meanwhile, CGA is a more thermolabile compound, and depending upon the different hot preparation or extraction methods, a considerable amount of CGA is lost (Liang and Kitts, 2016). The processes to extract phenolic compounds vary from plant to plant. Thoo et al. (2010) demonstrated that the extraction efficacy is dependent on various factors such as the method of extraction, extraction time, solvent concentration, solvent type, temperature, and solid-to-solvent ratio. During extraction of phenolic compounds, the key effect of the solid-to-solvent ratio was applied to adjust the solubility and equilibrium constant, resulting in a maximum yield of bioactive compounds at the optimum solid-to-solvent ratio (Tan et al., 2011) and avoidance of saturation effect, as well as a reduction of the solvent waste disposal cost (Ho et al., 2008). The current research, on the other hand, demonstrated the effectiveness of aqueous extraction which is also known as a nonpolluting solvent for obtaining phenolic constituents. Many studies have been carried out in coffee, such as the optimum condition and extraction methods have been well explored, to attain maximum bioactive constituents with antioxidant potency from their raw materials (Boeira et al., 2018; Santos et al., 2014).

Total Polyphenol Content and Total Flavonoid Content in Coffee Peel

The TPC of the CPE has been shown in **Figure 1A**. A sample of CPE-1 (0.5 g CP) exhibited significantly ($p \leq 0.05$) the highest amount of polyphenol. An inverse relationship has been observed between the TPC and the concentration of CP. That is, the sample containing the least concentration of CP exhibited the highest TPC (CPE-1 > CPE-2 > CPE-3 > CPE-4). The TFC of the CPE has been shown in **Figure 1B**. A similar pattern of TPC has been observed in TFC also. Likewise, an inverse relationship has been observed between TFC and the concentration of CP. That is, the sample containing the least concentration of CP showed the highest TFC (CPE-1 > CPE-2 > CPE-3 > CPE-4). A significant ($p \leq 0.05$) difference in TFC has been observed between the samples except between CPE-3 and CPE-4. In this study, the TPC values ranged from 11.65 to 15.23 GAE mg/g CP and the TFC values ranged from 20.24 to 27.22 mg catechin equivalent. Our results are in line with those of Alkaltham et al. (2020). They reported the comparison of the TPC and TFC of coffee fruit beans, pulp, and parchment; the TPC was 0.392, 0.183, and 0.110 mg GAE/g DW, respectively, and the TFC was 30.30, 8.02, and 0.638 mg CE/g DW, respectively. In this context, Silva et al. (2021) have reported that comparison of the coffee husk extracted with different organic solvents and techniques with the conventional extraction method (water bath) exhibited the TPC and TFC that ranged from 31.35 to 97.89 mg CAE/g and 0.63–9.93 mg CE/g, respectively, and ultrasound-assisted extraction method exhibited TPC and TFC ranging from 16.54 to 90.95 and 0.21–15.69, respectively. Silva

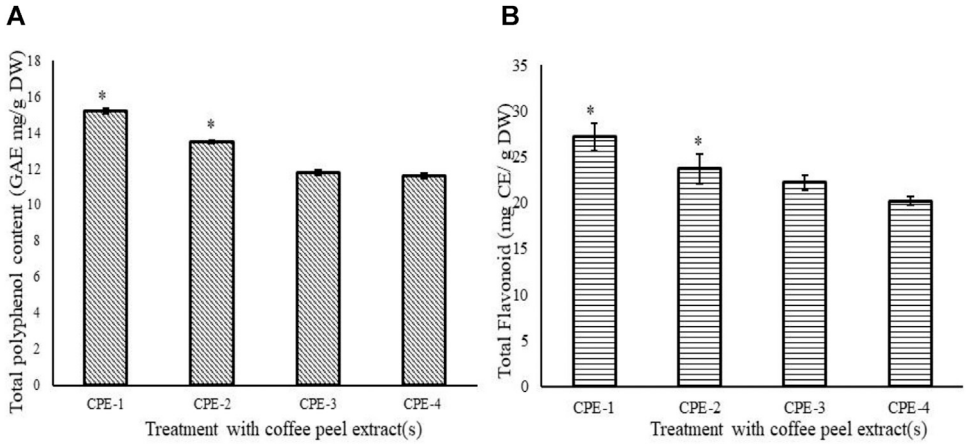


FIGURE 1 | TPC (GAE mg/g DW) **(A)** and flavonoid (mg CE/g DW) **(B)** in CPEs (CPE-1 = 0.5 g, CPE-2 = 0.75 g, CPE-3 = 1 g, and CPE-4 = 1.25 g). All the values are expressed as mean ± SD and are significantly different at $^*p \leq 0.05$.

TABLE 1 | Results of the phenolic compounds of CPEs (mg/100 g).

Sample	Chlorogenic acid (mg/100 g)	Vanillin (mg/100 g)	Salicylic acid (mg/100 g)
CPE-1	741.2	365.6	68.0
CPE-2	692.3	322.7	61.3
CPE-3	871.0	409.6	79.6
CPE-4	589.9	301.8	49.8

CPE-1, 0.5 g; CPE-2, 0.75 g; CPE-3, 1 g; and CPE-4, 1.25 g.

et al. (2021) confirmed that water: ethanol mixture was the extracting solution with a higher potential, and dehydration was a very significant factor to concentrate and provided higher levels of phenolic compounds. Andrade et al. (2012) reported that TPC ranged from 16.1 mg CAE/g to 423 mg CAE/g for coffee husk. The difference in results might be due to the differences in the variety and the extraction methods and extricating solvents used.

Quantification of Phenolic Compound in Coffee Peel Extracts Using HPLC

Individual polyphenols from coffee extracts (CPE-1, CPE-2, CPE-3, and CPE-4) were separated by HPLC and determined quantitatively. The major phenolic compounds which were found in CPE are presented in **Table 1**, and the results are expressed in mg/g.

Chromatograms of the standard phenolic compounds and samples have been presented in **Figures 2A,B**, respectively. It has been found that the amount of CGA present in 100 g of CPE ranges from 589.9 to 871.0 mg. The highest amounts of CGA present in CP are as follows: CPE-3 > CPE-1 > CPE-2 > CPE-4 (**Table 1**). The observed results confirmed that there was no significant correlation between the CGA quantity and the increasing amount of CP [CPE-1 (0.5 g), CPE-2 (0.75 g), CPE-3 (1 g), and CPE-4 (1.25 g)] taken for extraction. CPE-3 (1 g of CP) possessed a higher amount of phenolic compounds, such as CGA (871.0 mg/100 g), vanillin (409.6 mg/100 g), and salicylic acid (79.6 mg/100 g). As mentioned in **Table 1**, the average quantitative data showed that the CGA which is acknowledged for its antioxidant capacities ranged from 589.9 to 871.2 mg/100 g phenolic compound. The amount of vanillin ranged from 301.8 to 409.6 mg/100 g phenolic compound, and salicylic acid was the lowest (79.6–49.8 mg/100 g phenolic compound) among all. In this context, Jaiswal et al. (2012) have reported that the different roasting methods of coffee seeds in the laboratory consist of 40–209 mg/100 g in *Coffea arabica* and 40–509 mg/100 g in *Coffea canephora* seeds. In general, the level of CGA in coffee brews varies largely from 26 mg/100 ml to the extreme of 1141 mg/100 ml in different countries (Farah and de Paula, 2019). The amount of CGA differs due to various factors such as the species, the degree of maturation, the altitude, and the presence or absence of shade, as well as resistance to some diseases (Aerts and Baumann, 1994). According to our observation, the quantity of the CGA range in CP was found to be 589.9–871.0 mg/100 g in comparison with that in coffee seeds which varied from 200 to 301 mg/100 g. CGA is the main phenolic compound present in coffee beans that possesses antioxidant properties (Andrade et al., 2012; Clifford et al., 2017; Alkaltham et al., 2020). However, the CP contains a rich amount of polyphenols CGA along with total flavonoid, vanillin, and salicylic acid which favors effective inhibition of oxidative stress–associated cellular senescence and DNA damage (Xu et al., 2012; Cinkilic et al., 2013; Ameca et al., 2018).

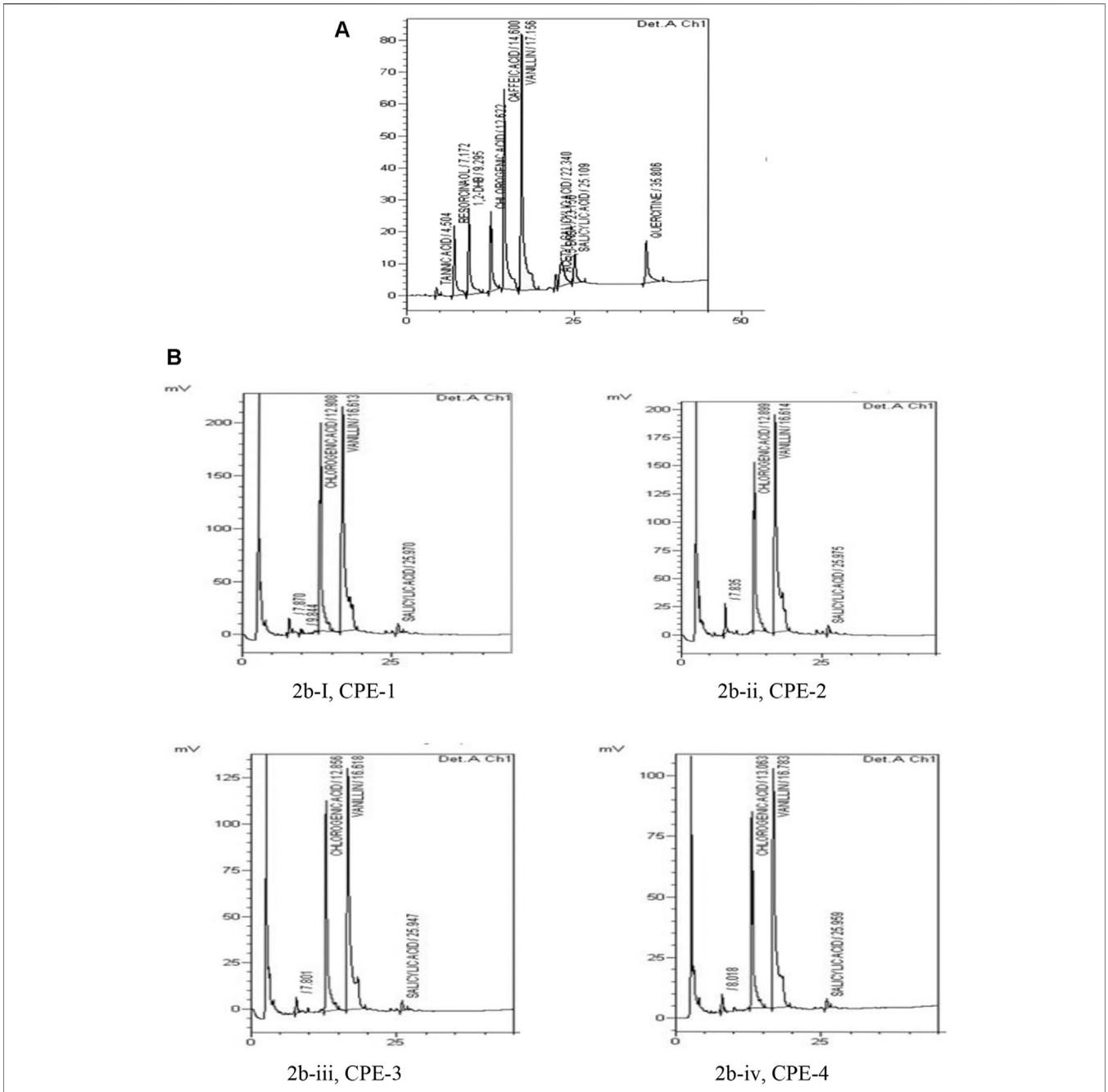


FIGURE 2 | Chromatogram of standard phenolic compounds **(A)** and chromatogram of samples **(B)**, such as CPE-1 = 0.5 g **(Bi)**, CPE-2 = 0.75 g **(Bii)**, CPE-3 = 1 g **(Biii)**, and CPE-4 = 1.25 g **(Biv)**.

Carbohydrate Hydrolysis Enzymatic α-Amylase Assay

Starch is one of the main sources of dietary energy which is mainly digested in the gastrointestinal tract by pancreatic α-amylase. Rate of starch digestion and absorption may help to control postprandial hyperglycemia, and so in diabetics, decelerating the digestion of starch may have a favorable effect on the glycemic index (Notkins, 2002). Hyperglycemia has been found to be linked with the threat of various diseases, such as obesity and cardiovascular- and kidney-related issues, which in turn increases the necessity for strict glycemic control (Blaak et al., 2012). In the intraluminal phase, α-A is the main enzyme accountable for starch digestion. So, an α-A inhibitor will slow down the carbohydrate digestion (Golay et al., 1991).

Four extracts from the CP (CPE-1, CPE-2, CPE-3, and CPE-4) were tested for porcine pancreatic α-A enzyme inhibition. All

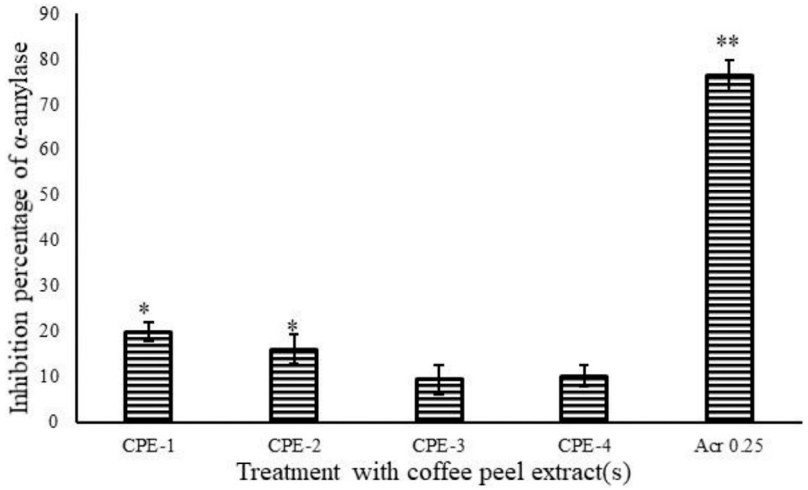


FIGURE 3 | Inhibition percentage of α-A by acarbose and CPEs (CPE-1 = 0.5 g, CPE-2 = 0.75 g, CPE-3 = 1 g, and CPE-4 = 1.25 g). All the values are expressed as mean ± SD and are significantly different at $*p \leq 0.05$ and $**p \leq 0.001$.

extracts weakly inhibited porcine pancreatic α-A. The extract CPE-1 (0.5 g) provided the highest inhibitory effect on α-A (19.88%/mg extract). It was able to inhibit porcine pancreatic α-A by 20%. We observed in CPE-2, CPE-3, and CPE-4 a lesser than 20% inhibition of porcine pancreatic α-A with an insignificant ($p \geq 0.05$) difference. IC_{50} can be defined as the lowermost concentration of a compound that is required to inhibit 50% of the enzyme activity (Oboh et al., 2015). Acarbose was able to inhibit the enzyme α-A at 70% as shown in **Figure 3**. For acarbose, an IC_{50} of 0.25 g/ml was determined (**Figure 3**). The inhibition by acarbose was higher when compared to CPE. α-A is one of the most significant digestive enzymes in humans, and it works as a catalyst in the reaction which implicates the hydrolysis of alpha-1,4 glycosidic linkages of large molecules like starch into smaller fragments of sugars, i.e., monosaccharides. The sugar level in the blood rises due to excess conversion of starch to sugar in response to which the insulin instructs cells to metabolize the surplus sugar and store them in the form of sugar. This cycle keeps on going interminably until in certain conditions where the activity of the amylase enzyme increases or a deficiency of insulin or insulin resistance occurs which in turn leads to an increase in blood sugar (Agarwal and Gupta, 2016). There are two main biochemical mechanisms related to raised blood glucose levels, and they are enzymatic (the polyol pathway) and nonenzymatic glycosylation (Creutzfeldt, 1999). Acarbose is used for treatment of diabetes, and it inhibits the activities of α-A (Jyothi et al., 2014). In this study, the observed inhibition of α-A even though it was weak might be due to the presence of CGA, which is inconsistent with earlier studies (Ranilla et al., 2010; Narita and Inouye, 2011). The inhibition of α-A by flavonoids is due to the formation of hydrogen bonds by hydroxyl groups with specific amino acids at the enzymes' active sites (De Sales et al., 2012). The extract CPE-1 (0.5 g) and CPE-2 of CP provided the highest inhibitory effect on α-A (19.88 and 16.07%/mg extract), which has been identified with the highest TPC and TF content compared to other extracts. Moreover, lower levels of TPC and TFC (μg/mg of extract) show lowest inhibition (statistically insignificant differences were observed between CP3 and CP4).

In Vitro Cell Culture Study Using hMSCs

Cells can normally protect themselves from ROS damage through their self-defense antioxidative mechanism. But, hMSCs have less antioxidant capacity and are more sensitive to oxidative stress, when compared to differentiated lineages such as adipocytes, chondrocytes, and osteoblasts (Yagi et al., 2013). In regenerative medicine, cellular stress produces excessive ROS or exogenous addition of H_2O_2 might impair the capacity of differentiation to multiline ages or self-renewal and proliferation were impaired (Zou et al., 2004). Excessive free radical damage to hMSCs might end with cell senescence and arrested cell divisions (Bajek et al., 2012). Orciani et al. (2010) reported that ROS inhibits hMSCs to osteogenesis differentiation and undifferentiated hMSCs, having a higher lactate production rate and glycolytic enzyme levels. Lesser osteogenic differentiation results in bone weakness and arthritis. In contrast, ROS increases adipocyte differentiation with upregulated antioxidant gene expressions, similar to osteogenic differentiation mitochondrial biogenesis of glycolysis, and the lactate level increased in adipogenesis (Denu and Hematti, 2016).

The quantified rich TPC and TFC of CPE favors the antioxidant and oxidative stress quenching capacity. It was confirmed by the present study that increasing the concentration of CPE treated to hMSCs resulted in increased cell proliferation and viability in CPE-2 (94%) when compared to the other extracts [CPE-3 (79%) or CPE-4 (74%)] (**Figure 4**). In addition, CPE-2 showed significantly ($p \leq 0.05$) increased cell viability (85%) against the increasing concentration of extract treatment. It may be due to the availability of rich amounts of phenolic and flavonoid components in CPE-2. The observed results were compared with the reference drug, CGA. In this context, Chen et al. (2008) have reported that the presence of antioxidant polyphenols, such as epigallocatechin and

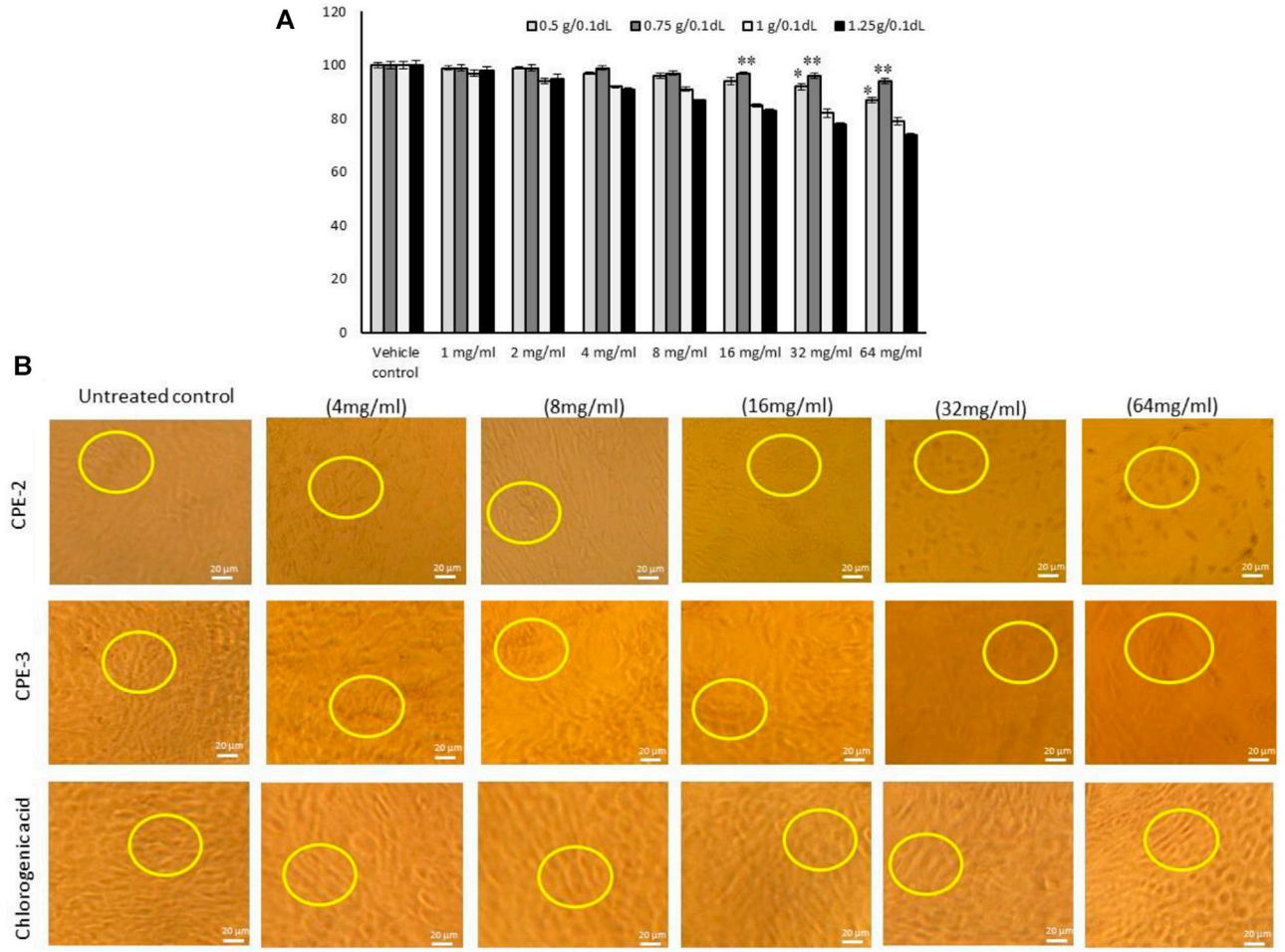


FIGURE 4 | In vitro cytotoxic effect **(A)** and light microscopy images for cell morphology **(B)** in hMSCs treated with increasing concentration of CPEs. All the images were captured with ×10 magnification. CPE-1 = 0.5 g, CPE-2 = 0.75 g, CPE-3 = 1 g, and CPE-4 = 1.25 g for 48 h. Each of the values are mean ± SD (n = 6). *$p \leq 0.05$ and **$p \leq 0.001$ by comparison with vehicle control.

tocopherol, protects the hMSCs from oxidative stress and increased proliferation capacity. Phenolic compounds and phenols are widely distributed from natural agents, so they have been considered as powerful agents for their antioxidative activities in hMSCs.

In propidium iodide (PI) staining analysis, CPE-2–treated cells were compared with CGA-treated cells; only 3% of the cells showed a nuclear morphology change in CPE-2 treatment, but CGA (80 µM) treatment showed 11% of different nuclear morphology (**Figure 5A**). H_2O_2-induced oxidative stressed hMSCs were treated with TPC- and TFC-rich CPE-2, with very less number (7%) of nuclear damaged cells, and H_2O_2 + CGA–treated cells showed 16% of morphologically different cells that were observed in PI nuclear staining. But, H_2O_2 alone treated cells showed 27% of pyknosis, and chromatin condensation was observed under an inverted microscope manually (**Figure 5B**). The observed results are in line with the previous reports that higher ROS levels cause cellular damage and dysfunction. The accumulation of ROS can damage cellular DNA, glycoproteins, and glycolipids. Human patients with atherosclerosis and diabetes have been identified with elevated oxidative stress and reduced capacity to inhibit T-cell proliferation (Mancini et al., 2015). Previously, Cha et al. (2014) have reported that CGA effectively protects oxidative stress induced by DNA damage in human keratinocytes.

Alterations in mitochondrial electrochemical gradient and transmembrane potential majorly affect the cellular ATP production progress. The mitochondria use oxidizable substrates majorly to produce an electrochemical proton gradient across the mitochondrial membrane (De Mello et al., 2018). Mitochondrial oxidative stress alters the inner membrane polarity and leads to a loss of mitochondrial transmembrane potential ($\Delta\psi_m$) further deregulating mitochondrial electronegative and transport capacity (Zorova et al., 2018). The internal electronegative organelle of the mitochondria promotes internal uptake of cations and outward transport of anions (ATP). During excessive ROS or free radicals, the mitochondrial permeability process decreases and a loss of the

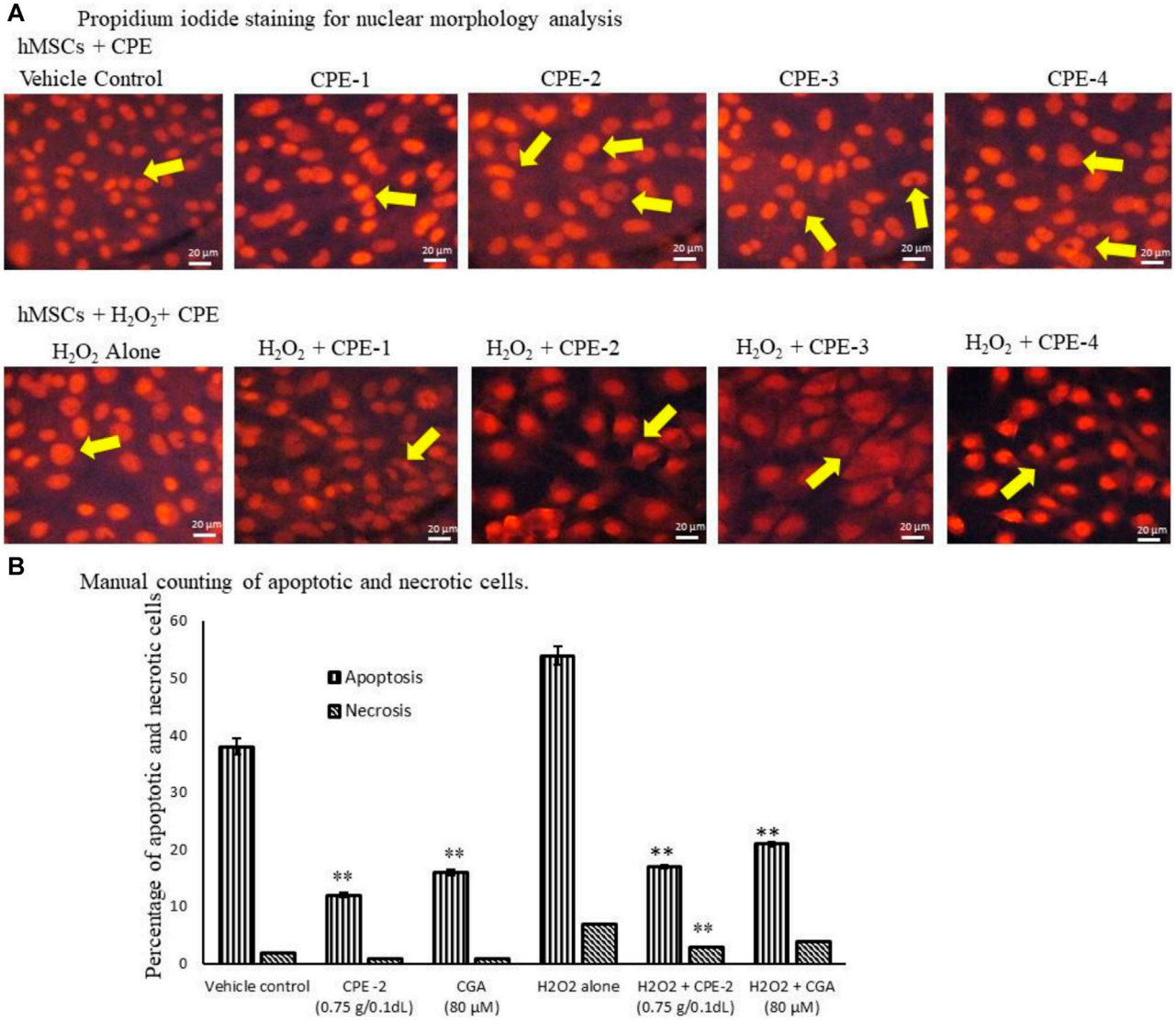


FIGURE 5 | Analysis of vehicle control and CPEs–treated hMSCs nuclear morphology by PI Staining for nuclear damage **(A)** and manual counting for apoptotic or necrosis cells **(B)** after 48 h. All the images were captured with ×20 magnification. CPE-1 = 0.5 g, CPE-2 = 0.75 g, CPE-3 = 1 g, and CPE-4 = 1.25 g. Each of the values are mean ± SD (n = 6). *$p \leq 0.05$ and **$p \leq 0.001$ were compared with vehicle control or H_2O_2 alone treated hMSCs.

electrochemical gradient which is the major indicator for mitochondrial function and cell health occurs (Sivandzade et al., 2019). Regulation of energy homeostasis has been majorly controlled by the mitochondria, and mitochondrial imbalance has been associated with the development of obesity (Picard et al., 2011). Polyphenols from plant sources have been described as antioxidants; they have the potential to eliminate ROS and free radicals, which normalize the mitochondrial membrane potential (Robb et al., 2017). In the present study, a rich amount of polyphenols and flavonoids in CPE-2 significantly enhanced the mitochondrial health when compared to the other extracts (**Figure 6**). It has been confirmed by the healthy electronegative mitochondria uptake of the cationic natural green florescent JC-1 dye, which was further converted internally into an irreversible red florescent J-aggregate. CPE-1 also showed moderate J-aggregate accumulation in oxidative stressed hMSCs; this effect was not observed in normal hMSCs. But, in CPE-3 and CPE-4, the conversion of J-aggregates from JC-1 was not observed both in normal as well as in oxidative stressed hMSCs, confirming the unhealthy mitochondria with a decreased mitochondrial membrane potential and membrane leakage.

In addition, changes in mRNA expression levels in oxidative stress, and immunomodulatory and tumor suppressive-related genes in normal and H_2O_2-induced oxidative stressed hMSCs after polyphenol rich CP treatment were observed. The results confirmed that the increased antioxidant and mitochondrial membrane potential capacity of CPE-2 significantly ($p \leq$

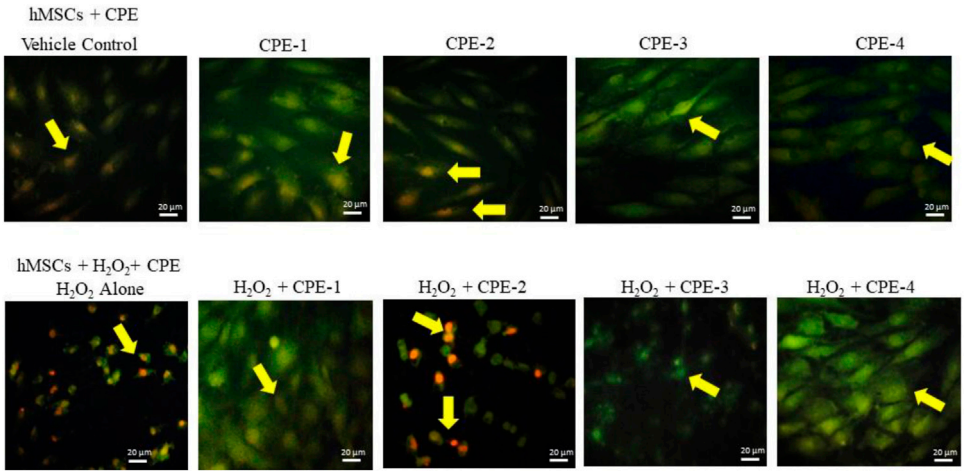


FIGURE 6 | Analysis of mitochondrial membrane potential using JC-1 staining for vehicle control and CPEs–treated hMSCs after 48 h. All the images were captured with ×20 magnification (CPE-1 = 0.5 g, CPE-2 = 0.75 g, CPE-3 = 1 g, and CPE-4 = 1.25 g).

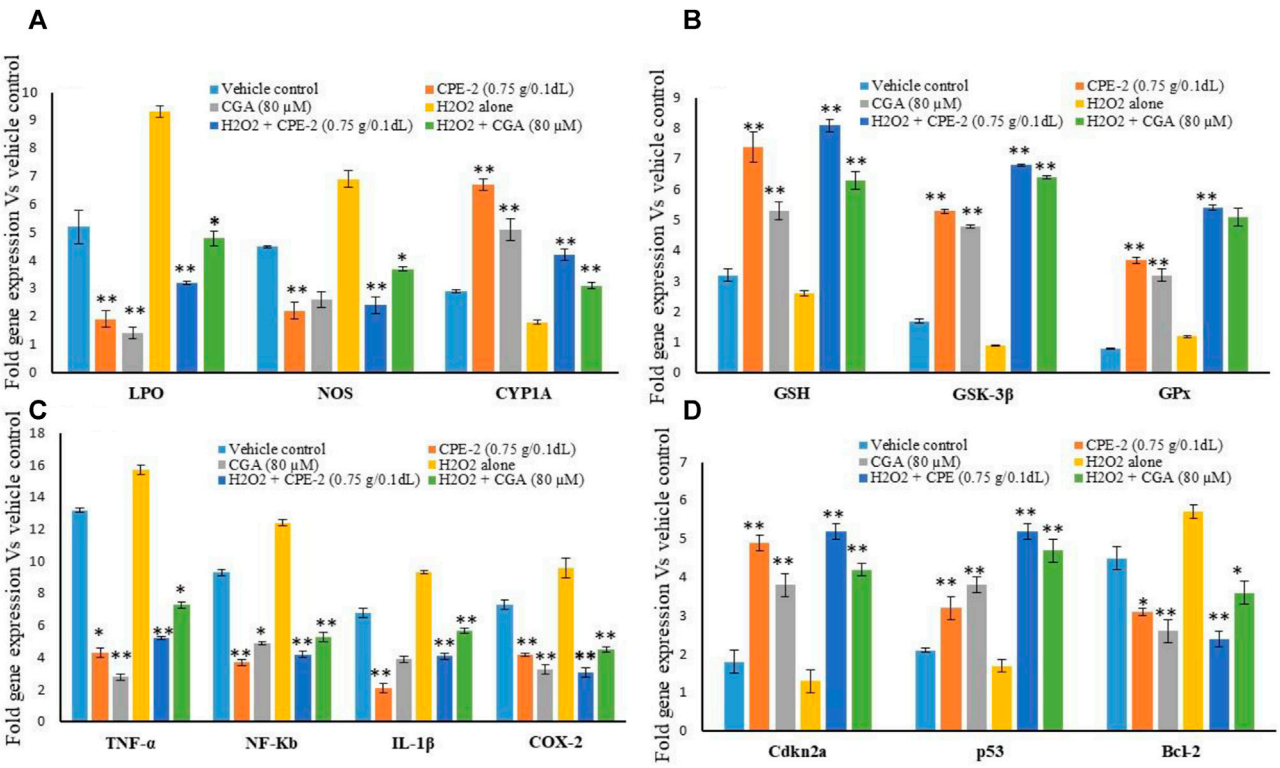


FIGURE 7 | Effect of vehicle control and CPEs (CPE-2; 0.75 g/0.1 dl) on oxidative stress **(A)**, antioxidant **(B)**, pro-inflammatory **(C),** and tumor suppression **(D)** related gene expression levels after 48 h. Each of the values are mean ± SD (n = 6). *$p \leq 0.05$ and **$p \leq 0.001$ were compared with vehicle control or H_2O_2 alone treated hMSCs.

0.001) decreased the oxidative stress markers of LPS and NOX-2 when compared to the untreated or H_2O_2 alone treated hMSCs (**Figure 7A**). The effect was observed in CGA-treated hMSCs with a significant level of $p \leq 0.05$. The expression level of CYP1A was significantly ($p \leq 0.001$) increased in CPE-2 treatment, and it was supported by the increased mitochondrial health (in JC-1 assay) after CPE treatment. The antioxidant potential of CPE-2 treatment was confirmed by the increased expression levels of GSH, GSK-3β, and GPx when compared to those of vehicle control or oxidative stress–induced hMSCs (**Figure 7B**). In

regenerative medicine, due to the multipotent differentiation potential of the hMSCs, these are implanted for tissue loss and contribute to tissue repair with suppressing inflammatory rejection (Di Nicola et al., 2002). After the generation of excessive ROS or exogenous H_2O_2 at the ischemic site, the transplanted MSCs might impair self-proliferation and multilineage capacity (Choo et al., 2014). The present finding confirmed the increased expression of antioxidant genes that was associated with quenching of oxidative stress aid to overcome the impairment of multiline age capacity of hMSCs at the graft site.

Pro-inflammatory cytokines and cellular metabolic inflammation–related gene expression levels also significantly ($p \leq 0.001$) decreased in CPE-2–treated cells. TNF-α and NF-κb expression levels were observed twofold higher in oxidative stressed hMSCs. IL-1β and COX-2 expressions were significantly increased to a fold in untreated and oxidative stressed cells when compared to the CPE-2–treated cells (**Figure 7C**). In addition, tumor suppressor-related genes cdkn2a and p53 expressions were significantly decreased twofold when compared to oxidative stressed hMSCs. BCL-2 expression was decreased in CPE-2–treated and CGA-treated hMSCs both in normal and H_2O_2-induced oxidative stressed hMSCs (**Figure 7D**). In response to diverse stresses such as DNA damage or hypoxia, the tumor suppressor p53 is accumulated and cellular proliferation arrested (Subash-Babu et al., 2017). Upon oxidative stress or hyperproliferation signaling conditions, mdm2 negatively regulates p53 which leads to transition from the resting phase (G1) to DNA synthesis phase (S), and subsequently, cancer cell progression continues. In unstressed cells, p53 is tightly regulated by murine double minute 2 (MDM2) by maintaining p53 at low levels. The cell-cycle gatekeeper gene p^{14ARF} neutralizing mdm2 function *via* cyclin-dependent protein kinase (Cdkn2A) leads to increased levels of active p53 (Chin et al., 1998).

External stimulus of oxidative stress or cellular stress causes DNA damage or cellular senescence. CP has the potential to overcome hypoxia and oxidative stress, therefore, it retains bioactive, secondary metabolites such as phenolic compounds, alkaloids, and flavonoid compounds. We identified CPE having a rich amount of polyphenol-CGA, vanillin, and salicylic acid. External stimulus of oxidative stress to hMSCs regulates stress-induced DNA damage, cell senescence, and impaired multilineage. But, CPE treatment to stressed hMSCs effectively downregulated the pro-oxidant LPO and NOX2 and enhanced the antioxidant mRNA. In addition, CP treated to stressed hMSCs potentially downregulated pro-inflammatory cytokines and enhanced tumor suppressor expressions, which is more beneficial for the cellular multilineage, such as osteogenic differentiation and cellular regeneration progress. In this context, several plant-derived compounds were found to fight against a wide range of cancer models, such as the colon, breast, liver, and prostate cancer suppression models (Wang, et al., 2012).

CONCLUSION

The present findings confirmed that the presence of total polyphenol-CGA, vanillin, and salicylic acid in CPE-2 (0.75g/10 ml) effectively inhibits pancreatic α-A inhibition and arrests H_2O_2-induced oxidative stress that may be due to the antioxidative nature of polyphenol and flavonoids. The antioxidant capacity of CP quenches the oxidative stress in hMSCs which decreases mitochondrial oxidative stress. Overall, the oxidative stress, and pro-inflammatory and tumor suppressor gene expression levels were normalized in CPE-treated oxidative stressed hMSCs. The present study confirmed that CP-derived polyphenol and flavonoid effectively quench the oxidative stress in hMSCs, which support to protect themselves from free radical or ROS damage through its self-defensive antioxidative mechanism during cellular multilineage and regenerative therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization: S-BP, AM, and NA; Data curation: S-BP, AM, and NA; Formal analysis: HK, S-BP, KH, MSA, and MAA; Funding acquisition: HK; Investigation: HK and AM; Methodology: HK, S-BP, KH, MSA, and MAA; Project administration: HK, AM, and NA; Resources: HK, AM, and MSA; Software: HK, S-BP, and KH; Supervision: AM and NA; Validation: S-BP and AM ; Visualization: KH and SA; Writing—original draft: HK, S-BP, SA, and MB; Writing—review and editing: AM, S-BP, and SA.

ACKNOWLEDGMENTS

The authors would like to thank the Deanship of Scientific Research in King Saud University for funding and supporting this research through the initiative of DSR Graduate Students Research Support (GSR).

REFERENCES

Aerts, R. J., and Baumann, T. W. (1994). Distribution and Utilization of Chlorogenic Acid inCoffeaseedlings, *J. Exp. Bot.* 45, 497–503. doi:10.1093/jxb/45.4.497

Agarwal, P., and Gupta, R. (2016). Alpha-amylase Inhibition Can Treat Diabetes Mellitus, *Res. Rev. J. Med. Health Sci.* 5, 1–9.

Alkaltham, M. S., Salamatullah, A., and Hayat, K. (2020), Determination of Coffee Fruit Antioxidants Cultivated in Saudi Arabia under Different Drying Conditions, *Food Measure* 14, 1306–1313. doi:10.1007/s11694-020-00378-4

Ameca, G. M., Cerrilla, M. E. O., Córdoba, P. Z., Cruz, A. D., Hernández, M. S., and Haro, J. H. (2018). Chemical Composition and Antioxidant Capacity of Coffee Pulp, *Ciênc. Agrotec.* 42, 307–313. doi:10.1590/1413-70542018423000818

Andrade, K. S., Gonçalvez, R. T., Maraschin, M., Ribeiro-Do-Valle, R. M., Martínez, J., and Ferreira, S. R. (2012). Supercritical Fluid Extraction from Spent Coffee Grounds and Coffee Husks: Antioxidant Activity and Effect of Operational Variables on Extract Composition, *Talanta* 88, 544–552. doi:10.1016/j.talanta.2011.11.031

Bajek, A., Czerwinski, M., Olkowska, J., Gurtowska, N., Kloskowski, T., and Drewa, T. (2012). Does Aging of Mesenchymal Stem Cells Limit Their Potential Application in Clinical Practice?, *Aging Clin. Exp. Res.* 24, 404–411. doi:10.3275/8424

Bhupathiraju, S. N., Pan, A., Malik, V. S., Manson, J. E., Willett, W. C., van Dam, R. M., et al. (2013). Caffeinated and Caffeine-free Beverages and Risk of Type 2 Diabetes, *Am. J. Clin. Nutr.* 97, 155–166. doi:10.3945/ajcn.112.048603

Blaak, E. E., Antoine, J. M., Benton, D., Björck, I., Bozzetto, L., Brouns, F., et al. (2012). Impact of Postprandial Glycaemia on Health and Prevention of Disease, *Obes. Rev.* 13, 923–984. doi:10.1111/j.1467-789X.2012.01011.x

Boeira, C. P., Piovesan, N., Soquetta, M. B., Flores, D. C. B., Lucas, B. N., Barin, J. S., et al. (2018). Ultrasonic Assisted Extraction to Obtain Bioactive, Antioxidant and Antimicrobial Compounds from Marcela, *Cienc. Rural* 48, 1–6. doi:10.1590/0103-8478cr20170772

Cano-Marquina, A., Tarín, J. J., and Cano, A. (2013). The Impact of Coffee on Health, *Maturitas* 75, 7–21. doi:10.1016/j.maturitas.2013.02.002

Cha, J. W., Piao, M. J., Kim, K. C., Yao, C. W., Zheng, J., Kim, S. M., et al. (2014). The Polyphenol Chlorogenic Acid Attenuates UVB-Mediated Oxidative Stress in Human HaCaT Keratinocytes, *Biomol. Ther. (Seoul)* 22, 136–142. doi:10.4062/biomolther.2014.006

Chen, C. T., Shih, Y. R., Kuo, T. K., Lee, O. K., and Wei, Y. H. (2008). Coordinated Changes of Mitochondrial Biogenesis and Antioxidant Enzymes during Osteogenic Differentiation of Human Mesenchymal Stem Cells, *Stem Cells* 26, 960–968. doi:10.1634/stemcells.2007-0509

Chin, L., Pomerantz, J., and DePinho, R. A. (1998). The INK4a/ARF Tumor Suppressor: One Gene-Ttwo Products-Ttwo Pathways, *Trends Biochem. Sci.* 23, 291–296. doi:10.1016/s0968-0004(98)01236-5

Choo, K. B., Tai, L., Hymavathee, K. S., Wong, C. Y., Nguyen, P. N., Huang, C. J., et al. (2014). Oxidative Stress-Induced Premature Senescence in Wharton's Jelly-Derived Mesenchymal Stem Cells, *Int. J. Med. Sci.* 11, 1201–1207. doi:10.7150/ijms.8356

Cinkilic, N., Cetintas, S. K., Zorlu, T., Vatan, O., Yilmaz, D., Cavas, T., et al. (2013). Radioprotection by Two Phenolic Compounds: Chlorogenic and Quinic Acid, on X-ray Induced DNA Damage in Human Blood Lymphocytes *In Vitro*, *Food Chem. Toxicol.* 53, 359–363. doi:10.1016/j.fct.2012.12.008

Clifford, M. N., Jaganath, I. B., Ludwig, I. A., and Crozier, A. (2017). Chlorogenic Acids and the Acyl-Quinic Acids: Discovery, Biosynthesis, Bioavailability and Bioactivity, *Nat. Prod. Rep.* 34, 1391–1421. doi:10.1039/C7NP00030H

Creutzfeldt, W. (1999). Effects of the Alpha-Glucosidase Inhibitor Acarbose on the Development of Long-Term Complications in Diabetic Animals: Pathophysiological and Therapeutic Implications, *Diabetes Metab. Res. Rev.* 15, 289–296. doi:10.1002/(sici)1520-7560(199907/08)15:4<289:aid-dmrr48>3.0.co;2-v

De Mello, A. H., Costa, A. B., Engel, J. D. G., and Rezin, G. T. (2018). Mitochondrial Dysfunction in Obesity, *Life Sci.* 192, 26–32. doi:10.1016/j.lfs.2017.11.019

Denu, R. A., and Hematti, P. (2016). Effects of Oxidative Stress on Mesenchymal Stem Cell Biology, *Oxid Med. Cel Longev* 2016, 1-9. doi:10.1155/2016/2989076

Devine, S. M., Bartholomew, A. M., Mahmud, N., Nelson, M., Patil, S., Hardy, W., et al. (2001). Mesenchymal Stem Cells Are Capable of Homing to the Bone Marrow of Non-human Primates Following Systemic Infusion, *Exp. Hematol.* 29, 244–255. doi:10.1016/S0301-472X(00)00635-4

Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P. D., Matteucci, P., et al. (2002). Human Bone Marrow Stromal Cells Suppress T-Lymphocyte Proliferation Induced by Cellular or Nonspecific Mitogenic Stimuli, *Blood* 99, 3838–3843. doi:10.1182/blood.v99.10.3838

Durak, A., Gawlik-Dziki, U., and Pecio, L. (2014). Coffee with Cinnamon - Impact of Phytochemicals Interactions on Antioxidant and Anti-inflammatory *In Vitro* Activity, *Food Chem.* 162, 81–88. doi:10.1016/j.foodchem.2014.03.132

Faostat Food and Agriculture Organization of the United Nations, Available online: http://www.fao.org/faostat/en/#data/QC (Accessed April 30, 2021).

Farah, A., and dePaula Lima, J. (2019). Consumption of Chlorogenic Acids through Coffee and Health Implications, *Beverages* 5, 11. doi:10.3390/beverages5010011

Farah, A., and Donangelo, C. M. (2006). Phenolic Compounds in Coffee, *Braz. J. Plant Physiol.* 18, 23–36. doi:10.1590/s1677-04202006000100003

Fujioka, K., and Shibamoto, T. (2006). Quantitation of Volatiles and Nonvolatile Acids in an Extract from Coffee Beverages: Correlation with Antioxidant Activity, *J. Agric. Food Chem.* 54, 6054–6058. doi:10.1021/jf060460x

Golay, A., Schneider, H., Temler, E., and Felber, J. P. (1991). Effect of Trestatin, an Amylase Inhibitor, Incorporated into Bread, on Glycemic Responses in normal and Diabetic Patients, *Am. J. Clin. Nutr.* 53, 61–65. doi:10.1093/ajcn/53.1.61

Hasenah, A., Houghton, P. J., and Soumyanath, A. (2006). α -Amylase Inhibitory Activity of Some Malaysian Plants Used to Treatdiabetes; with Particular Reference to Phyllanthus Amarus, *J. Ethno Pharma* 107, 449–455. doi:10.1016/j.jep.2006.04.004

Hayat, K., Abbas, S., Jia, C., Xia, S., and Zhang, X. (2011). Comparative Study on Phenolic Compounds and Antioxidant Activity of Feutrell's Early and Kinnow Peel Extracts. *J. Food Biochem.* 35, 454–471. doi:10.1111/j.1745-4514.2010.00395.x

Hayat, K. (2020). Impact of Drying Methods on the Functional Properties of Peppermint (Mentha Piperita L.) Leaves. *Sci. Lett. J.* 8, 36–42. doi:10.1111/j.1745-4514.2010.00395.x

Hečimović, I., Belščak-Cvitanović, A., Horžić, D., and Komes, D. (2011). Comparative Study of Polyphenols and Caffeine in Different Coffee Varieties Affected by the Degree of Roasting, *Food Chem.* 129, 991–1000. doi:10.1016/j.foodchem.2011.05.059

Ho, C. H. L., Cacace, J. E., and Mazza, G. (2008). Mass Transfer during Pressurized Low Polarity Water Extraction of Lignans from Flaxseed Meal, *J. Food Eng.* 89, 64–71. doi:10.1016/j.jfoodeng.2008.04.003

Honczarenko, M., Le, Y., Swierkowski, M., Ghiran, I., Glodek, A. M., and Silberstein, L. E. (2006). Human Bone Marrow Stromal Cells Express a Distinct Set of Biologically Functional Chemokine Receptors, *Stem cells* 24, 1030–1041. doi:10.1634/stemcells.2005-0319

Jaiswal, R., Matei, M. F., Golon, A., Witt, M., and Kuhnert, N. (2012). Understanding the Fate of Chlorogenic Acids in Coffee Roasting Using Mass Spectrometry Based Targeted and Non-targeted Analytical Strategies, *Food Funct.* 3, 976–984. doi:10.1039/C2FO10260A

Janissen, B., and Huynh, T. (2018). Chemical Composition and Value-Adding Applications of Coffee Industry By-Products: A Review, *Resour. Conservation Recycling* 128, 110–117. doi:10.1016/j.resconrec.2017.10.001

Jyothi, K. S. N., Shailaja, M., Viveni, J., and Suresh, C. (2014). Identification of a Proteinaceous Alpha Amylase Inhibitor from a Medicinal Herb Oxalis Corniculata L. (Oxalidaceae), *J. Homeop Ayurv Med.* 3, 165.

Kähkönen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S., et al. (1999). Antioxidant Activity of Plant Extracts Containing Phenolic Compounds, *J. Agric. Food Chem.* 47, 3954–3962. doi:10.1021/jf990146l

Kizilay Mancini, O., Shum-Tim, D., Stochaj, U., Correa, J. A., and Colmegna, I. (2015). Age, Atherosclerosis and Type 2 Diabetes Reduce Human Mesenchymal Stromal Cell-Mediated T-Cell Suppression, *Stem Cel Res Ther* 6, 140. doi:10.1186/s13287-015-0127-9

Kobayashi, C. I., and Suda, T. (2012). Regulation of Reactive Oxygen Species in Stem Cells and Cancer Stem Cells, *J. Cel Physiol* 227, 421–430. doi:10.1002/jcp.22764

Leite, M., Quinta-Costa, M., Leite, P. S., and Guimarães, J. E. (1999). Critical Evaluation of Techniques to Detect and Measure Cell Death-Sstudy in a Model of UV Radiation of the Leukaemic Cell Line HL60, *Anal Cel Pathol* 19, 139–151. doi:10.1155/1999/176515

Liang, N., and Kitts, D. D. (2016). Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions, *Nutrients* 8, 16. doi:10.3390/nu8010016

Murthy, P. S., and Madhava Naidu, M. (2012). Sustainable Management of Coffee Industry By-Products and Value Addition-A Review, *Resour. Conservation Recycling* 66, 45–58. doi:10.1016/j.resconrec.2012.06.005

Narita, Y., and Inouye, K. (2011). Inhibitory Effects of Chlorogenic Acids from green Coffee Beans and Cinnamate Derivatives on the Activity of Porcine Pancreas α-amylase Isozyme I, *Food Chem.* 127, 1532–1539. doi:10.1016/j.foodchem.2011.02.013

Notkins, A. L. (2002). Immunologic and Genetic Factors in Type 1 Diabetes, *J. Biol. Chem.* 277, 43545–43548. doi:10.1074/jbc.R200012200

Oboh, G., Agunloye, O. M., Adefegha, S. A., Akinyemi, A. J., and Ademiluyi, A. O. (2015). Caffeic and Chlorogenic Acids Inhibit Key Enzymes Linked to Type 2 Diabetes (*In Vitro*): A Comparative Study, *J. Basic Clin. Physiol. Pharmacol.* 26, 165–170. doi:10.1515/jbcpp-2013-0141

Orciani, M., Gorbi, S., Benedetti, M., Di Benedetto, G., Mattioli-Belmonte, M., Regoli, F., et al. (2010). Oxidative Stress Defense in Human-Skin-Derived Mesenchymal Stem Cells versus Human Keratinocytes: Different Mechanisms of protection and Cell Selection, *Free Radic. Biol. Med.* 49, 830–838. doi:10.1016/j.freeradbiomed.2010.06.007

Pari, L., Karthikesan, K., and Menon, V. P. (2010). Comparative and Combined Effect of Chlorogenic Acid and Tetrahydrocurcumin on Antioxidant Disparities in Chemical Induced Experimental Diabetes, *Mol. Cel Biochem* 341, 109–117. doi:10.1007/s11010-010-0442-5

Picard, M., Taivassalo, T., Gouspillou, G., and Hepple, R. T. (2011). Mitochondria: Isolation, Structure and Function, *J. Physiol.* 589, 4413–4421. doi:10.1113/jphysiol.2011.212712

Prihadi, A. R., Maimulyanti, A., Mellisani, B., and Nurhasanah, N. (2020). Antioxidant Activity, Tannin Content and Dietary Fiber from Coffee Husk Extract and Potential for Nutraceutical, *Rjc* 12, 955–959. doi:10.31788/rjc.2020.1325613

Ranilla, L. G., Kwon, Y. I., Apostolidis, E., and Shetty, K. (2010). Phenolic Compounds, Antioxidant Activity and *In Vitro* Inhibitory Potential against Key Enzymes Relevant for Hyperglycemia and Hypertension of Commonly Used Medicinal Plants, Herbs and Spices in Latin America, *Bioresour. Technol.* 101, 4676–4689. doi:10.1016/j.biortech.2010.01.093

Robb, E. L., Moradi, F., Maddalena, L. A., Valente, A. J. F., Fonseca, J., and Stuart, J. A. (2017). Resveratrol Stimulates Mitochondrial Fusion by a Mechanism Requiring Mitofusin-2, *Bioche Biophys. Res. Commun.* 485, 249–254. doi:10.1016/j.bbrc.2017.02.10

Sales, P. M., Souza, P. M., Simeoni, L. A., Silveira, D., and Silveira, D. (2012). α-Amylase Inhibitors: a Review of Raw Material and Isolated Compounds from Plant Source, *J. Pharm. Pharm. Sci.* 15, 141–183. doi:10.18433/j35s3k

Santos, J., Oliveira, M. B. P. P., Ibáñez, E., and Herrero, M. (2014). Phenolic Profile Evolution of Different Ready-To-Eat Baby-Leaf Vegetables during Storage, *J. Chromatogr. A.* 1327, 118–131. doi:10.1016/j.chroma.2013.12.085

Silva, M. D. O., Honfoga, J. N. B., Medeiros, L. L. D., Madruga, M. S., and Bezerra, T. K. A. (2021). Obtaining Bioactive Compounds from the Coffee Husk (Coffea Arabica L.) Using Different Extraction Methods, *Molecules* 26, 46. doi:10.3390/molecules26010046

Sivandzade, F., Bhalerao, A., and Cucullo, L. (2019). Analysis of the Mitochondrial Membrane Potential Using the Cationic JC-1 Dye as a Sensitive Fluorescent Probe, *Bio Protoc.* 9, e3128. doi:10.21769/BioProtoc.3128

Song, H. Y., Jang, H. W., Debnath, T., and Lee, K. (2019). Analytical Method to Detect Adulteration of Ground Roasted Coffee, *Int. J. Food Sci. Technol.* 54, 256–262. doi:10.1111/ijfs.13942

Subash-Babu, P., Alshammari, G. M., Ignacimuthu, S., and Alshatwi, A. (2017). Epoxy Clerodane Diterpene Inhibits MCF-7 Human Breast Cancer Cell Growth by Regulating the Expression of the Functional Apoptotic Genes Cdkn2A, Rb1, Mdm2 and P53, *Biomed. Pharmacother.* 87, 388–396. doi:10.1016/j.biopha.2016.12.091

Subramanian, R., Asmawi, M. Z., and Sadikun, A. (2008). *In Vitro* alpha-glucosidase and Alpha-Amylase Enzyme Inhibitory Effects of Andrographis Paniculata Extract and Andrographolide. *Acta Biochim. Pol.* 55, 391–398. doi:10.18388/abp.2008_3087

Tan, P. W., Tan, C. P., and Ho, C. W. (2011). Antioxidant Properties: Effect of Solid-To-Solvent Ratio on Antioxidant Compounds and Capacities of Pegaga (*Centella asiatica*), *Int. Food Res. J.* 18, 553–558.

Tapiero, H., Tew, K. D., Ba, G. N., and Mathe, G. (2002). Polyphenols: Do They Play a Role in the Prevention of Human Pathologies? *Biomed. Pharmacother.* 56, 200–207. doi:10.1016/S0753-3322(02)00178-6

Thoo, Y. Y., Ho, S. K., Liang, J. Y., Ho, C. W., and Tan, C. P. (2010). Effect of Binary Solvent Extraction System, Extraction Time and Extraction Temperature on Phenolic Antioxidants and Antioxidant Capacity from Mengkudu (*Morinda citrifolia*), *Food Chem.* 120, 2290–2295. doi:10.1016/j.foodchem.2009.09.064

Wang, H., Khor, T. O., Shu, L., Su, Z. Y., Fuentes, F., Lee, J. H., et al. (2012). Plants vs. Cancer: a Review on Natural Phytochemicals in Preventing and Treating Cancers and Their Drug Ability, *Anti-cancer Agents Med. Chem.* 12, 1281–1305. doi:10.2174/187152012803833026

Wang, T., Wu, Y., Wang, W., and Zhang, D. (2021). Association between Coffee Consumption and Functional Disability in Older US Adults, *Br. J. Nutr.* 125, 695–702. doi:10.1017/S0007114520003153

Xu, J. G., Hu, Q. P., and Liu, Y. (2012). Antioxidant and DNA-Protective Activities of Chlorogenic Acid Isomers, *J. Agric. Food Chem.* 60, 11625–11630. doi:10.1021/jf303771s

Yagi, H., Tan, J., and Tuan, R. S. (2013). Polyphenols Suppress Hydrogen Peroxide-Induced Oxidative Stress in Human Bone-Marrow Derived Mesenchymal Stem Cells, *J. Cel Biochem* 114, 1163–1173. doi:10.1002/jcb.24459

Yuan, J. S., Reed, A., Chen, F., and Stewart, C. N. (2006). Statistical Analysis of Real-Time PCR Data, *BMC bioinformatics* 7, 1–12. doi:10.1186/1471-2105-7-85

Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., et al. (2018). Mitochondrial Membrane Potential, *Anal. Biochem.* 552, 50–59. doi:10.1016/j.ab.2017.07.009

Zou, X., Li, H., Chen, L., Baatrup, A., Bunger, C., and Lind, M. (2004). Stimulation of Porcine Bone Marrow Stromal Cells by Hyaluronan, Dexamethasone and rhBMP-2, *Biomaterials* 25, 5375–5385. doi:10.1016/j.biomaterials.2003.12.041

Conflict of Interest: The authors declare that the research study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 05 November 2021
doi: 10.3389/fphar.2021.782962

The Quality Monitoring of Cistanches Herba (*Cistanche deserticola* Ma): A Value Chain Perspective

Linlin Jiang[1], Baochang Zhou[1], Xiaoqin Wang[1], Yaqiong Bi[2], Wenfang Guo[2], Jianhua Wang[1], Ruyu Yao[3]* and Minhui Li[1,2,4,5,6]**

[1]Department of Pharmacy, Inner Mongolia Medical University, Hohhot, China, [2]Inner Mongolia Hospital of Institute of Traditional Chinese Medicine, Hohhot, China, [3]Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [4]Inner Mongolia Key Laboratory of Characteristic Geoherbs Resources Protection and Utilization, Baotou, China, [5]Baotou Medical College, Baotou, China, [6]Inner Mongolia Hospital of Traditional Chinese Medicine, Hohhot, China

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Lifeng Han, Tianjin University of Traditional Chinese Medicine, China
Mihael Cristin Ichim, National Institute of Research and Development for Biological Sciences (NIRDBS), Romania

***Correspondence:**
Jianhua Wang
ny_wjh513@163.com
Ruyu Yao
yry0255@126.com
Minhui Li
prof_liminhui@yeah.net

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 25 September 2021
Accepted: 22 October 2021
Published: 05 November 2021

Citation:
Jiang L, Zhou B, Wang X, Bi Y, Guo W, Wang J, Yao R and Li M (2021) The Quality Monitoring of Cistanches Herba (Cistanche deserticola Ma): A Value Chain Perspective. Front. Pharmacol. 12:782962. doi: 10.3389/fphar.2021.782962

Cistanche deserticola Ma was used as a medicine food homology, which was mainly produced in the Alxa region of northwest China. In recent years, it has been widely used in various food items. The increasing demand for Cistanches Herba has led to problems such as overexploitation and quality deterioration. The quality and safety of herbal medicines are critical and have been shown to be affected by the value chain (VC). Using the VC framework, the study is embedded in a larger study aiming to investigate the effects of different VCs types on the quality and stakeholders of Cistanches Herba. In this study, 90 Cistanches Herba samples were collected during fieldwork. An additional 40 samples were obtained from the herbal markets and medicine purchasing stations. Semi-structured interviews and key informant interviews were performed to collect data on stakeholders in major production areas. These samples were analyzed using high performance liquid chromatography (HPLC) coupled with the *k*-means clustering method; a targeted quality assessment strategy based on chemical analysis was adopted to understand the quality of Cistanches Herba. Based on market research, the collected samples were divided into different grades through *k*-means clustering analysis. Moreover, quality differences of Cistanches Herba in Alxa region were explored through DNA barcoding and chemical analysis. Accordingly, 10 different types of VCs were determined in the production of Cistanches Herba. The results show that there is a close relationship between the quality of Cistanches Herba and stakeholder benefits. Vertical integration at different levels was found for independent farmer-based VCs, horizontal collaboration was found in the cooperative-based VCs. The vertical coordination has led to a more consistent traceability system and strict regulation of supply chains. At the same time, the Cistanches Herba were divided into three grades. Through DNA barcoding and chemical analysis, we found that the quality differences between Cistanches Herba in the Alxa area were not significant. It was found that geographical suitability and vertical integration could impact the quality and sustainable production of Cistanches Herba. At the same time, the well-developed VCs can provide products with reliable quality, and ensure adequate financial revenue for relevant stakeholders.

Keywords: cistanches herba, quality control, value chain, chemical analysis, specifications and grades, DNA barcoding

1 INTRODUCTION

Cistanche deserticola Ma (commonly known as *Rou Cong Rong* in Chinese or Cistanches Herba) is a parasitic plant. The dried fleshy stem of *C. deserticola* is a precious traditional Chinese medicine (TCM) that has been used for centuries (Fu et al., 2018). Several pharmacological studies have shown that Cistanches Herba displays a range of functions, including antioxidative, anti-nociceptive, anti-inflammatory, immunity, neuroprotective, and hepatoprotective functions (Li Z. et al., 2016; You et al., 2016). Cistanches Herba was used as a homology of medicine and food in 2018, which indicates that in addition to curing specific diseases, it can also be consumed daily for health care needs (Li et al., 2021). In recent years, it has been widely used in various food items, tea, drinks, and beverages. *Rou Cong Rong* tea is becoming increasingly popular as a health food supplement in Asian countries, such as China and Japan (Li et al., 2018; Hou et al., 2020; Lei et al., 2020). Cistanches Herba is known as desert ginseng due to its nutritional value (Wang et al., 2012). Consequently, Cistanches Herba is popularly used in TCM and health care practices.

With increasing interest in the clinical and health care functions of Cistanches Herba, more research is focused on its active ingredients. Phenylethanoid glycosides comprise a significant class of compounds that exhibit a wide range of pharmacological effects (Li Z. et al., 2016). Echinacoside and acteoside are the main active ingredients of Cistanches Herba. These are described as "marker components" in the Chinese Pharmacopeia 2020 edition (ChP., 2020) (Wang X. Y. et al., 2017). Galactitol is the main laxative constituent of Cistanches Herba. At the same time, it also performs a variety of biological activities, such as anti-aging, and regulation of immune function. Galactitol exhibits low toxicity and minimal side effects and has broad prospects in clinical applications and health food development (Gao et al., 2015). Thus, determination of galactitol confers a certain significance to the development and utilization of Cistanches Herba.

The demand for Cistanches Herba in the global market has grown rapidly, whereby the annual demand for Cistanches Herba is bordering on approximately 4,000 tons (Lu et al., 2019). However, the number of its hosts has been declining, as they are being cut down to be used as firewood by herdsmen. The populations of high-value plants that grow in the wild have rapidly declined due to the high market demand for medicinal plants, resulting in their scarcity in the market (Ndou et al., 2019). As a result, with the increase in demand year by year, the wild *C. deserticola* populations have been seriously affected, and the wild resources have been gradually exhausted. It has been listed as a "second grade" nationally protected plant in China, the International Union for Conservation of Nature Red List of Threatened Species and Convention on International Trade in Endangered Species of Wild Fauna and Flora (Appendix II) (Fan et al., 2020), resulting in restrictions being imposed on international trade. This has led to long-term stagnation of *C. deserticola*-related industries. Thus, artificial cultivation of *C. deserticola* is important. Li et al. (1989) have studied the artificial cultivation of *C. deserticola* since the 1980s. In the 1990s, researchers systematically elucidated the parasitic mechanism of *C. deserticola*, explored the key technologies associated with the artificial cultivation of parasitic plants, and established a sustainable *C. deserticola* cultivation system that produces high yields. At present, the artificial inoculation technology for *C. deserticola* cultivation is becoming increasingly advanced, and the cultivated area is expanding on a yearly basis. Therefore, artificial cultivation of *C. deserticola* may be considered to resolve issues arising from a scarcity of medicinal and protected wild resources (Bi et al., 2020). Moreover, the development of a series of health products based on Cistanches Herba has resulted in a substantial expansion in the international influence of Cistanches Herba.

C. deserticola has been cultivated in several plantations to meet the increasing demands of international and domestic herbal medicine markets (Hou et al., 2020). The quality of Cistanches Herba is affected by growing conditions, harvest seasons, and processing methods (Li et al., 2019). Due to natural conditions, ecology, and cultivation techniques, the Cistanches Herba in Alxa have larger and fleshier stems, are richer in pectin and tannin, and are of good quality. Therefore, Alxa is considered to be the ideal location for growing and producing top quality Cistanches Herba (*Daodi* herbs) (Li et al., 2019). *Daodi* herbs have self-adapting characteristics to adapt to their environments over long periods of time. Thus, they reportedly display advanced properties, such as superior active ingredients and better quality (Yin et al., 2019). Plants vary greatly in chemical composition; thus, any quality assessment must take the length, diversity, and uncertainty of value chains (VCs), from which these plants originate, into account (Heinrich et al., 2019). Recent studies have indicated that the VCs of medicinal plants have a significant impact on the quality and safety of herbal medicinal products, such as *Curcuma longa* L., *Ginkgo biloba* L., and *Lycium barbarum* L. (Booker et al., 2014; Booker et al., 2016; Yao et al., 2018). Improvements in medicinal quality and safety add value to the final product. Hence, maintaining supply of prime materials via VCs is considered vital for meeting the increasing demand for Cistanches Herba.

Starting from cultivation to processing and distribution, a medicinal plant product must pass through several levels of stakeholders before it reaches the final consumer (Székács et al., 2018). Besides production, VC research also focuses on other activities related to the supply chain, including distribution and marketing. VCs define the activities involved in different modes of production, while emphasizing the relationship between primary producers and other stakeholders in different production systems and their socio-economic impact (Bi et al., 2020). Although VC analyses have been widely used in various products, only a few studies have investigated the VCs of

medicinal plants and their derivatives. It was not until 2012 that people became increasingly aware of the broader role of VCs associated with medicinal plants in global markets (Booker et al., 2012; Booker et al., 2015).

Medicinal plants and their derived products appear to have varied VCs. Within a VC, there may be different stakeholders who contribute to different stages of supply. Understanding the relationship between production and supply helps identify the pressure points in different VCs. By comparing different VCs horizontally and vertically, a better understanding of the differences in the quality and price of herbal medicines in different markets may be acquired (Yao et al., 2018; Bi et al., 2020). In a short chain, such as one where the product is grown and used locally as an herbal medicine, the quality and safety of the product is relatively easy to control and manage. In contrast, using a TCM or food supplement in the domestic market or as an export product involves greater risks and challenges (Booker et al., 2015; Heinrich et al., 2019). It is worth noting that the maximum allowable levels of contaminants, authenticity, and purity are usually controlled at a very late stage. Only careful management along VCs allows improvement in the quality and safety of cultivated medicinal plants.

Alxa, being the main production area, accounts for more than 90% of the total output of Inner Mongolia and more than 70% of China's total output (Siqin et al., 2019). It is mainly cultivated via wild cultivation mode, without using chemical fertilizers or pesticides, and has great economic and ecological value. Due to its high medicinal value and nutritional effects, adulteration of Cistanches Herba often occurs under market conditions, which limits the safety and sustainable development of the products. There is a lack of information on the quality risks arising from these systems and risk management in production systems and VCs. In the current study, we aimed to investigate the quality differences in Cistanches Herba from the perspective of VCs. We investigated its production and circulation in the daodi area (Alxa) and analyzed specific issues affecting quality and yield. Differences in multiple stakeholders, capital flows, and information were clarified according to VCs. In addition, market research enabled us to develop a Cistanches Herba grading system; a targeted quality assessment strategy based on chemical analysis was adopted to understand the quality of Cistanches Herba, which provided a basis for ensuring and improving quality as well as the economic benefits of production and consumption.

2 MATERIALS AND METHODS

2.1 Fieldwork

Based on documentary research and on-site visits, starting August 2016, fieldwork was conducted during the cultivation and harvesting periods in Inner Mongolia, Gansu, Ningxia, and Xinjiang, covering the main production areas as well as several trading centers associated with Cistanches Herba. To maintain variability within an acceptable range, samples for content determination were collected only during the spring. We collected 90 samples, including 18 from Alxa Left Banner (105°42′E/38°50′N), 50 from Alxa Right Banner (101°68′E/39°20′N), and 22 from Ejina Banner (100°88′E/41°90′N). Moreover, we visited three national herbal markets, namely the Yulin Chinese herbal medicine market, Bozhou Chinese herbal medicine market, and An'guo Chinese herbal medicine market. The standard for grading Cistanches Herba was established by measuring the morphological characteristics of the samples. Participatory observations and semi-structured interviews were conducted with different stakeholders. Farmers, wholesalers, and retailers from core cultivation areas or trading centers of the visited areas were randomly selected to collect general information on VCs, current market conditions, and grade classification of Cistanches Herba.

2.2 Plant Material

90 Cistanches Herba samples were collected in Alxa during fieldwork. An additional 40 samples were offered either by the institutions we visited and bought in the above mentioned herbal markets and seven medicine purchasing stations in Inner Mongolia, Gansu, Ningxia, and Xinjiang. Whole Cistanches Herba plants were identified by Prof. Wang of Baotou Medical College. The voucher specimens were preserved at the Baotou Medical College, University of Inner Mongolia.

2.3 VCs Analysis

Analysis of VCs was carried out via the following steps: 1) identification of the main links in the production of Cistanches Herba; 2) identification of stakeholders in the production process and adding them to the corresponding process; 3) calculation of labor and non-labor costs of each transaction link based on collected data and information; and 4) building of a framework for the VCs to analyze VCs in relation to financial performance, production behavior and quality (Yao et al., 2018). In addition, the strengths and weaknesses of the different VCs were analyzed.

2.4 Reagents and Chemicals

A HPLC system was purchased from Thermo Fisher Scientific (Ultimate 3000, United States). A pulverizer (ST-08, 1800 W, China), laboratory water purifier (AMFI-5-P, Yiyang Enterprise, China), electronic analytical balance (AR153O, Mettler Toledo), Retsch-MIVMOO ball mill (model MM400, Retsch, Germany) and desk centrifuge (TGL-16G, Shanghai Anting) were also used. Methanol and acetonitrile were of HPLC grade, (Tianjin Kemiou Chemical Reagent Co., Ltd.) while all other reagents were analytical grade. The reference compounds echinacoside, acteoside, and galactitol were purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China).

2.5 Quantitative Analyses of Main Chemical Indexes in Cistanches Herba

Echinacoside and acteoside were detected under the following conditions: analysis was performed using a C18 column at a flow rate of 1.0 ml/min; the mobile phase was 35% methanol-0.1% acetic acid; the column temperature was set at 30°C; and the DAD detector wavelength was 334 nm. Conditions for the determination of galactitol were as follows: sample analysis was carried out on a Prevail Carbohydrate ES polymer gel column at a flow rate of 0.7 ml/min, the mobile phase was acetonitrile-water (77: 23), and

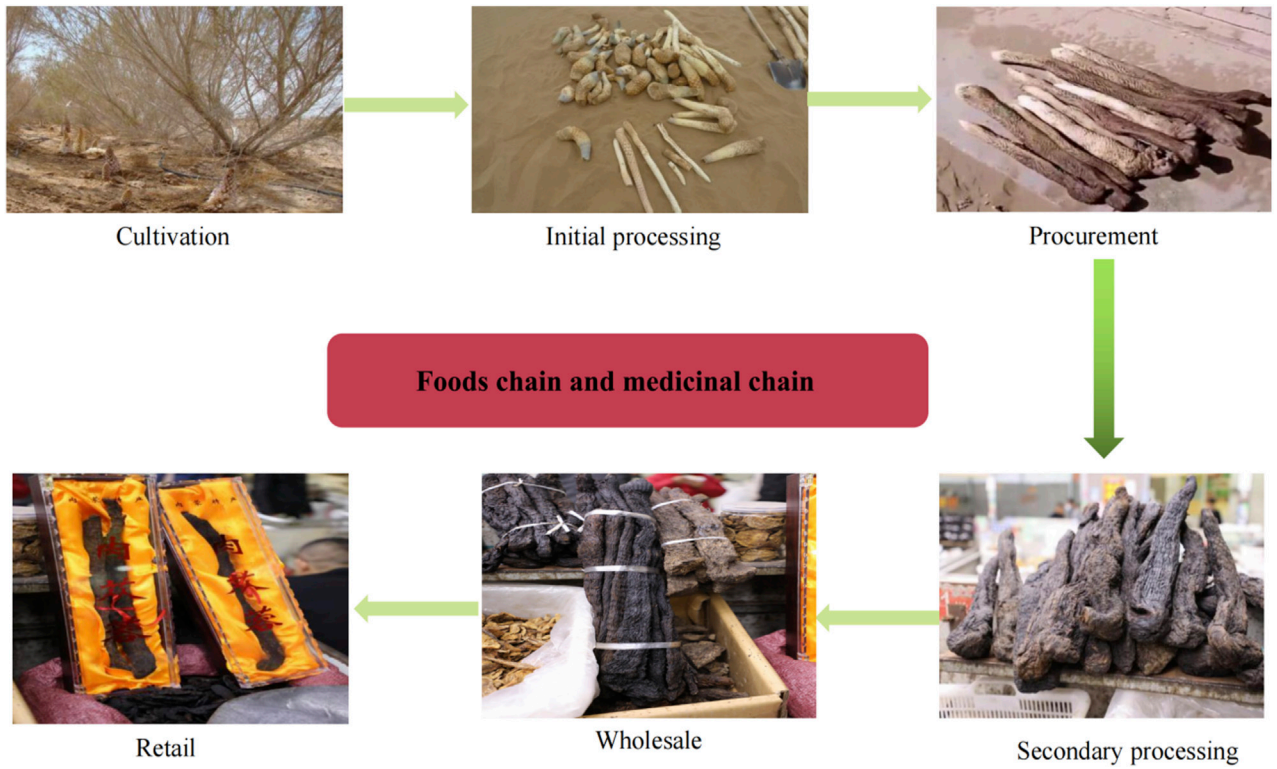


FIGURE 1 | Cistanches Herba goes through six production stages in food and medicinal chain.

the column temperature was set to 25°C. An Alltech ELSD 2000ES evaporation light scattering detector (Alltech Technology Ltd.) was used, the drift tube temperature was set to 40°C, and the gas pressure was set to 240 kPa.

2.6 Molecular Identification of Cistanches Herba

2.6.1 DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from silica gel-dried medicinal materials. Approximately 0.03 g of the molecular material was ground for 45 s at a frequency of 3,000 times/s in a Retsch-MIVMOO ball mill. DNA extraction was performed using an optimized cetyl trimethylammonium bromide method. Then, *rbc*L, *mat*K, *trn*H-*psb*A, and ITS regions were amplified by polymerase chain reaction (PCR) with primers. A 25 μL PCR amplification was performed. Purified PCR products were sequenced in the forward direction using the primers used for amplification on an ABI3730XL sequencer.

2.6.2 Phylogenetic Analysis

In this study, *Kopsiopsis hookeri* (Walp.) Govaerts, *Xylanche himalaica* (Hook. f. & Thomson) Beck, *Cistanche tinctoria* (Forssk.) Beck, and *Cistanche salsa* (C. A. Mey.) Beck were selected as the outgroup for *rbc*L, *mat*K, *trn*H-*psb*A, and ITS, respectively. A phylogenetic tree of four sequences of Cistanches Herba was constructed using the neighbor-joining (NJ) method with MEGA 6.0 software. The support rate of each branch was tested via 1,000 repetitive bootstrap tests, and 50% support was used as the threshold for successful identification.

2.7 Clustering Analysis

The *k*-means algorithm is capable of effectively analyzing large-scale data and is easy to understand. Automatic grouping was carried out according to the degree of natural affinity, which makes the structural characteristics of individuals within the group very similar, and the characteristics of the individuals between the groups different (Wang T. et al., 2017). *k*-means clustering was performed using SPSS and Rstudio to analyze market grades and the relationship between the contents of the active ingredients of the field sample and its place of origin.

3 RESULTS AND DISCUSSION

3.1 Industrial Structure and VCs

Cistanches Herba goes through six stages before it reaches the consumer as follows: 1) cultivation, including activities, such as plowing, inoculation, pruning, rodent control, and harvesting; 2) initial processing, including surface treatment, removing impurities, as well as drying; 3) procurement, which involves trading of unprocessed material produced in step 2; 4) secondary processing, including commercial grading, deep-processing and

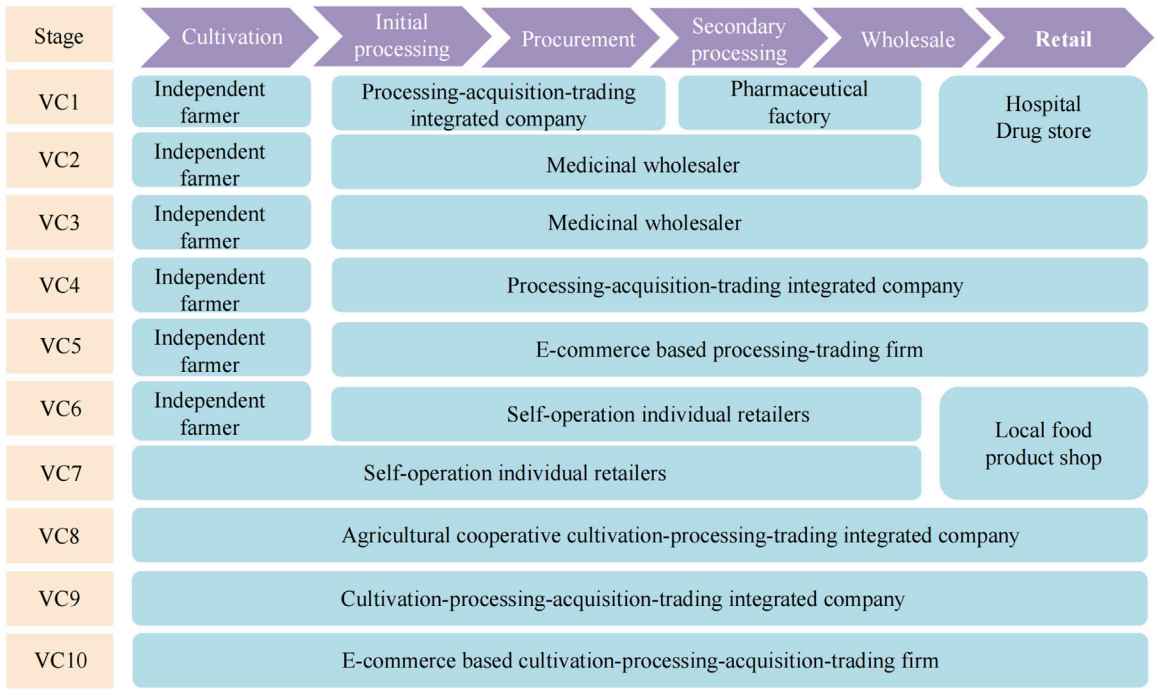


FIGURE 2 | Cistanches Herba production involves 10 mature VCs.

packaging; 5) wholesale, mainly to nonlocal markets; and 6) retail (**Figure 1**).

On account of its long production and supply lines, Cistanches Herba production involves 10 mature VCs, which may be distinguished by the various composite patterns of stakeholders. Different stakeholders play a role in each of these steps, resulting in various forms of VCs. The 10 primary VCs identified as being associated with Cistanches Herba as well as the stakeholders involved are shown in **Figure 2**.

VCs 1–6 are based on independent farmers with small Cistanches Herba fields offering harvested products to medicinal wholesalers, local individual retailers or other cultivation-processing-trading integrated companies. VC 7 is run by local individual retailers. Harvested products are often sold as local food products. VC 8 initiates with farmers involved in agricultural cooperatives (with relatively large fields). VCs 9 and 10 are characterized by cultivation-processing-acquisition-trading firms.

Independent farmers with relatively small fields are placed in VCs 1–6. These tend to be traditional, small-scale economies. In these VCs, independent farmers grow and harvest Cistanches Herba. Farmers near local individual retailers, medicinal wholesalers, or processing companies sell their products directly, without the involvement of middlemen, to increase profits earned from the cultivation of this plant. In VC 1, unprocessed Cistanches Herba is sold to processing firms; Cistanches Herba is processed into raw materials and sold to pharmaceutical factories, and then the products are sold to consumers through pharmacies and the like. Due to the particularities of the growing environment and strict storage requirements, many individual retailers, such as those in VCs 2, 3 and 6, are able to procure unprocessed Cistanches Herba directly from farmers, and reduce intermediate links, thus improving the quality. It is then further processed into herbal medicines or functional food and sold to consumers, hospitals, or pharmacies. At the same time, some are dried and processed as agricultural by-products for sale. However, farmers directly sell their products without processing, and the product quality is likely to be not stable due to insufficient drying during the transportation process. Additionally, the development of e-commerce makes it possible for a processing firm to expand downstream. VC 5, the trading firm plays a role that extends up to the retailer.

VC 7 is a one-stop VC conducted by self-operating individual retailers who hire local farmers or carry out their own production and processing. They usually sell their products through their own offline and online stores, which play an important part in a sale. These products are usually bought directly by consumers in the form of local agricultural products and functional food products.

VC 8 begins with agricultural cooperative-based VCs. Related governmental departments provide farmers with beneficial policies to plant Cistanches Herba. Thus, enterprises are encouraged to develop agricultural cooperatives. These farmers receive more technical support and equipment from cooperatives than independent farmers.

VC 9 and VC 10 are regulated by companies that control cultivation, processing and trading, and are based in large

production areas. They further process the products into functional food products, which are then directly sold to retailers or consumers, mainly through chain stores or online stores. Moreover, some products are sold to foreign wholesalers.

3.2 Trading along with VCs of Cistanches Herba

The value of a product is created by the production activities of the stakeholders and is generated during the transaction process. In different VCs, labor input and non-labor input have different effects on value (Yao et al., 2018).

Alxa has a vast land area and rich natural resources, which lays the foundation for its ecological construction and the development of the deserticulture industry. Herdsmen mostly contract pastures, where there are large areas of natural *Haloxylon ammodendron* (C. A. Mey.) Bunge ex Fenzl, as well as a large space suitable for planting *H. ammodendron*. Farmers usually plant *H. ammodendron* in their own fields or grasslands. After *H. ammodendron* survives, it is grafted onto *C. deserticola*, and finally the industrial model is based on selling *C. deserticola* raw materials. Therefore, the cultivation of Cistanches Herba is one of the income sources of herdsmen (Zhao and Zhang, 2014). However, the cultivation of Cistanches Herba is a long-term investment owing to the long planting cycle, large capital demand, and slow harvest.

During the planting period of *H. ammodendron*, a large amount of water is needed for irrigation. Owing to the serious shortage of groundwater in Alxa, the land can only be irrigated manually. At the same time, since *H. ammodendron* forests are more scattered and the area is larger, it is necessary to have the protection of fencing because the new young *H. ammodendron* seedlings planted every year cannot survive the grazing of animals. In the survey, many farmers and herdsmen generally reported serious issues with rats in *H. ammodendron* forests. Therefore, in the early stage of planting, the cost of watering, fencing, rodent control, machinery, and human labor in *H. ammodendron* forests are higher, resulting in higher costs for Cistanches Herba production. However, the wild cultivation model can save a portion of the non-labor costs of chemical fertilizers and pesticides while ensuring high quality. At the same time, the Alxa government pays a certain amount of compensation to the Cistanches Herba cultivators. However, Cistanches Herba cultivated by independent farmers has lower survival rates and yields than that cultivated by cooperative companies, which mainly leads to lower income for independent farmers.

In addition, some stakeholders, such as agricultural cooperatives and integrated cultivation-processing-trading companies, have well-established facilities that help reduce labor costs and lower inputs, thus guaranteeing high returns. By contrast, VCs 8–10 involve large-scale agricultural operations that are increasingly popular, including agricultural cooperatives and plantation companies. In addition to providing a traditional medicinal market, further processing into functional food adds to its benefits and efficiency.

Processing and wholesale are also labor-intensive stages (20–25 Chinese yuan (CNY)/kg). Before they move on to the second processing stage, processing companies spend a large amount of funds on production and testing equipment, production workshops and storage rooms. Storage of Cistanches Herba is an important factor affecting quality and price, and therefore a large amount of capital and labor is invested in storage. In addition, these factories must perform quality testing for each batch of TCM. At the same time, part of the product is processed into the food market. In addition, a large part of the input was made in the management of the factory.

Retail is the final stage of the VC. Here, the resources flow to high-quality hospitals, traditional herbal medicine markets, agricultural markets, and online markets. The trends seen in prices indicate that these are related to the dynamic balance between demand and supply. It is thus believed that as the demand for Cistanches Herba products grows, the price (and the economic benefits to farmers) will continue to rise in the short term. However, over the long term, progressively more Cistanches Herba will be harvested, causing prices (and farmers' financial gains) to decline.

The retail price of Cistanches Herba is generally higher than its wholesale price. The price of Cistanches Herba appreciates with grade. The market price of an entire plant is higher than that of its slices. An increase of 10 cm in the length of Cistanches Herba is equivalent to an increase of 100 CNY. In addition, slicing position and processing method causes a difference in the product phase, exerting a significant impact on the price. The size of Cistanches Herba shrinks sharply after drying in the shade or in the sun. The surface of the slices are obviously wrinkled, the color deeper, and the price is the lowest. After harvesting at the site of production, it is cleaned, peeled, and dried after being sliced. If the surface of the slice is smooth, then the price is intermediate. In pharmacies and hospitals, Cistanches Herba is further processed into decoction pieces or formula granules, and the retail price becomes generally twice the wholesale price.

3.3 Quality Evaluation of Cistanches Herba Along VCs

3.3.1 Grades of Cistanches Herba

In Alxa areas, the wild cultivation mode, without the use of chemical fertilizers or pesticides, helps sustainable development. Alxa has been considered as the *daodi* area of Cistanches Herba and has a reputation as "the hometown of Cistanches Herba in the world" (Siqin et al., 2019). Cultivation according to suitable ecology is a factor that guarantees the yield and quality of Cistanches Herba. However, the current planting of Cistanche Herba in Alxa has not been standardized. Therefore, the disordered classification of Cistanches Herba in the market leads to price differences. Currently, the size of obtainable medicinal materials is generally used as the main basis for grading (Li et al., 2019). We conducted an in-depth study of all samples collected. Based on requirements, the morphology, surface characteristics and size, and parameters, such as diameter and length, of Cistanches Herba were analyzed (Yan et al., 2019). More precisely, Cistanches Herba was divided into three grades (**Figure 3**). These results may be used to determine the specifications and grades of Cistanches Herba (**Table 1**). At

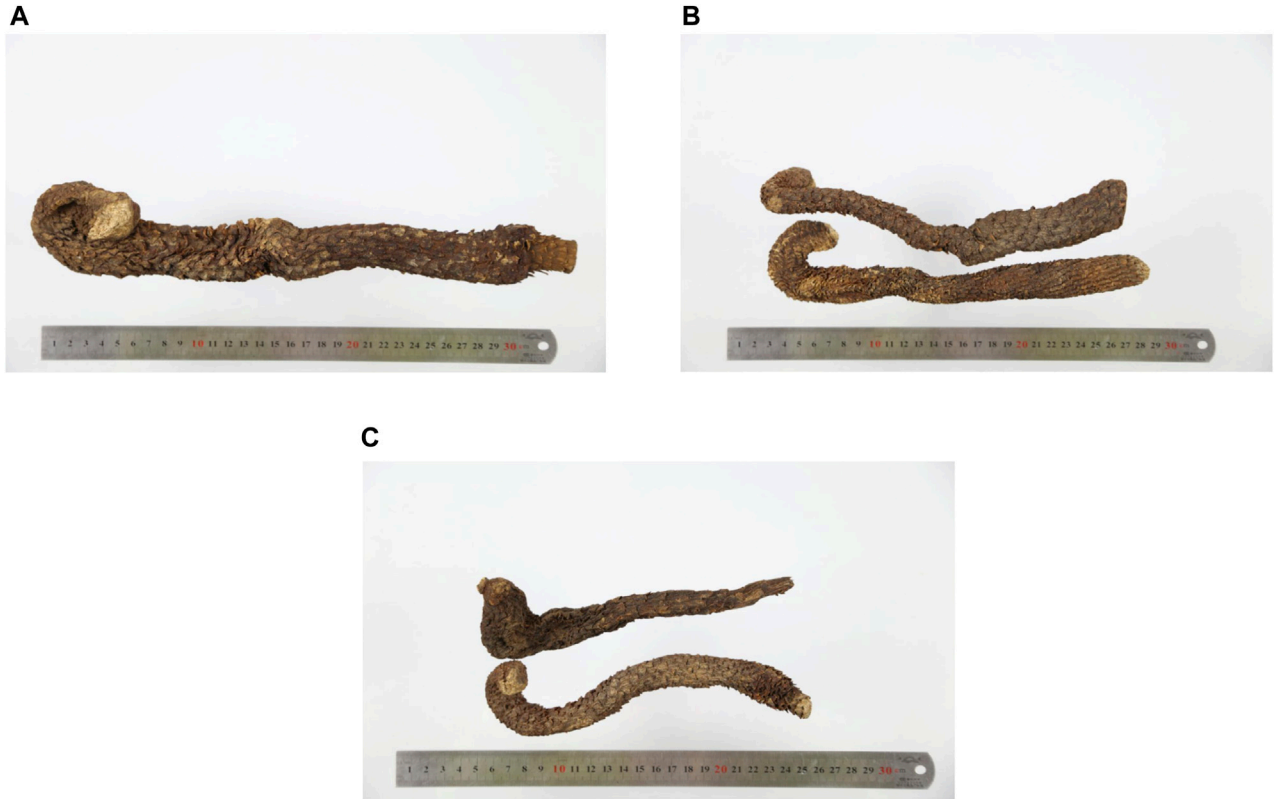


FIGURE 3 | Different grades of Cistanches Herba (**(A)**: first class, **(B)**: second class, **(C)**: third grade).

TABLE 1 | The specification and grade of Cistanches Herba.

Name	Grade	Shape	Surface character	Texture	Cross section	Taste	Size
Cistanches Herba	First grade	Flat cylindrical, slightly curved	The surface is brown or gray-brown, by most imbricate arrangement fleshy scaled leaf. Typically, the scales tip has been broken leaf	Hard, slightly flexible, not easy to break	The cross section is brown, with light brown punctate vascular bundles, arranged into undulate rings	Sweet, slightly bitter	length is greater than 25 cm, and the diameter is greater than 3.5 cm
	Second grade						length is 15–25 cm, and diameter is 2.5–3.5 cm
	Third grade						length is 3–15 cm, and the diameter is greater than 2 cm

the same time, the market survey found that the price of first-grade products was more than 200 CNY/kg; second-grade products cost about 180 CNY/kg; and third-grade products cost lower than 150 CNY/kg. By analyzing the quality differences between Cistanches Herba stocks in the market, a more reasonable standard for grading Cistanches Herba was established to guide a more standard production mode.

3.3.2 Quality Evaluation of Cistanches Herba

Daodi herbs has been widely recognized by the Chinese medicinal industry as a symbol of stable, high-quality TCM for a long time (Yin et al., 2019). Along the VCs, different criteria were considered as being indicative of good quality. For farmers, size is often a measure of quality; the bigger the fleshy stem, the better the quality. Retailers believe that habitat and size are important indices of the quality of Cistanches Herba. In addition, doctors believe that better quality would have a more effective clinical effect. At present, these measures can be used to evaluate the quality of Cistanches Herba that have been established in pharmacopeia. The quality of Cistanches Herba has been evaluated using methods, such as source, morphological, and physicochemical identification in the pharmacopeia (ChP., 2020). In addition, some planting companies and pharmaceutical factories impose their own regulations to ensure the quality of

TABLE 2 | Quality of the Cistanches Herba for the different VCs and likelihood of risks being made to its quality during its production.

VC	Traceability	Certify	Control	Heavy metal	Pesticide residue	Likelihood of hazard occurring		
						Cultivation	Processing	Wholesale
1	No	Maybe	Medium	No	No	Improbable	Probable	Probable
2	No	No	Weak	No	No	Improbable	Very probable	Very probable
3	No	No	Weak	No	No	Improbable	Very probable	Very probable
4	No	Maybe	Medium	No	No	Improbable	Probable	Probable
5	No	Maybe	Medium	No	No	Improbable	Probable	Very probable
6	No	No	Weak	No	No	Improbable	Probable	Probable
7	No	No	Weak	No	No	Improbable	Probable	Probable
8	Maybe	Maybe	Strong	No	No	Improbable	Improbable	Improbable
9	Yes	Maybe	Strong	No	No	Improbable	Improbable	Improbable
10	Yes	Maybe	Strong	No	No	Improbable	Improbable	Probable

TABLE 3 | One-way analysis results of variance between appearance of Cistanches Herba and echinacoside, acteoside, galactitol.

Compounds	Oiliness	Weight	Density of scales	Size	Texture
Echinacoside (%)	−1.336	1.152	0.179	0.287	0.644
Acteoside (%)	−2.445*	0.341	3.277*	0.880	1.947
Galactitol (%)	−2.254*	3.577*	0.626	0.464	0.551
The total contents (%)	−2.099*	0.744	1.317	0.444	1.455

*$p < 0.05$.

their herbal medicines. However, independent farmers are technologically not proactive enough to access them.

Along the VCs, different criteria were considered as being indicative of good quality. Therefore, a quality investigation of Cistanches Herba from different VCs was performed, and the results are shown in **Table 2**. The quality of Cistanches Herba is related to planting, storage, drying, and processing. In VCs 1–4, farmers directly sell their products without processing, and the product quality is not stable due to insufficient drying during the transportation process. In VCs 5 and 6, Cistanches Herba is usually sold as an agricultural product, making it difficult to trace or supervise effectively. In VCs 8–10, the integrated chain of cultivation, harvesting, and processing may reduce the loss of active ingredients. In addition, deep processing enterprises will have a stricter traceability and quality assurance system for products, thus guaranteeing quality.

Generally, the effective ingredient content of a plant is related to its growth period (Li Z. et al., 2016). Therefore, the evaluation of the main chemical components of Cistanche Herba can reflect its quality to a certain extent. Echinacoside and acteoside are the main active ingredients of Cistanches Herba (Li Y. et al., 2016). These are described as marker components in the Chinese Pharmacopeia 2020 edition (Wang X. Y. et al., 2017). Galactitol is the main laxative constituent of Cistanches Herba. Thus, determination of galactitol confers a certain significance to the development and utilization of Cistanches Herba (Gao et al., 2015). Moreover, a one-way analysis of variance of active ingredient content and characteristics (**Table 3**), indicated that the active ingredient content was correlated with the characteristics of Cistanches Herba. The higher the oiliness of the sample, the higher the content of its chemical components. At the same time, the study found that galactitol content is related to the weight of the sample, which further illustrates the significance of galactitol content determination.

We determined the content of all samples collected from the field. The results showed that the content of active ingredients in Alxa was high (**Supplementary Table S1**), clarifying the high quality of Alxa in the market and the high prices paid. *k*-means cluster analysis was used to analyze 90 samples collected from the field based on three variables: echinacoside, acteoside, and galactitol (**Figures 4A,B**). These results show that the samples can be divided into three different clusters, but the clusters representing Cistanches Herba from the three regions are relatively disorganized. Analysis of the chemical components of Cistanches Herba from different areas of Alxa revealed that there was no significant correlation between chemical composition and geographical distance. We believe that there is no substantial difference between in the quality of Cistanches Herba from different areas of Alxa.

Herbal products contain numerous ingredients, and phytochemical analyses used in the quality control of marker compounds are accurate but have limited applicability in the identification of biological constituents. The herbal products, sold worldwide as medicines or foods, are perceived as low risk because they are considered natural and thus safe (Ichim, 2019). The growing demand for herbal products due to their health benefits has resulted in a proportionally increase of their accidental contamination or intentional adulteration. To address this problem, DNA barcoding as powerful strategy has recently attracted considerable attention and, along with chemical methods, has started to enter the regulatory systems for quality control (Ichim et al., 2020). DNA barcoding is widely used for molecular identification for the purpose of solving a broad range of issues in taxonomy, molecular phylogenetics and population genetics, as well as for preventing illegal wildlife collection and product authenticity monitoring (Janjua et al., 2017; Raclariu et al., 2018). In this study, we randomly selected a few samples for DNA barcoding identification using four DNA barcode sequences (*rbc*L, *mat*K, *trn*H-*psb*A, and ITS) to identify Cistanches Herba from Alxa habitats. A *trn*H-*psb*A sequence analysis indicated a deletion in Alxa Left Banner at 298–337 bp, compared with Alxa Right Banner and Ejina. It remains to be

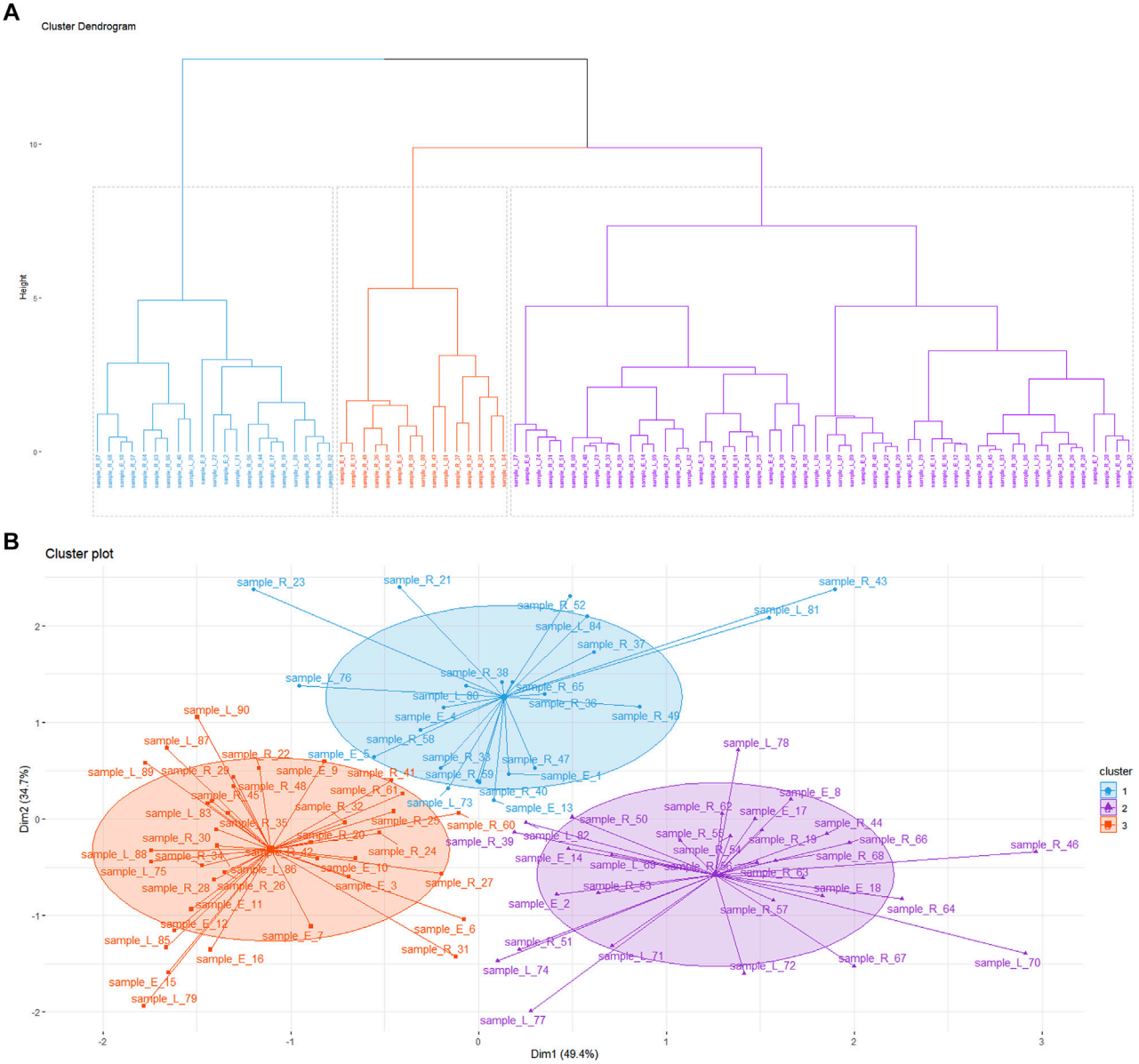


FIGURE 4 | (A,B) Results of the constituents of Cistanches Herba based on *k*-means cluster (sample_E: the sample of Ejin Bannerr; sample_R: the sample of Alxa Right Banner; sample_L: the sample of Alxa Left Banner).

verified whether this can be used as a specific identification site for Cistanches Herba. Based on the NJ tree established by the sequence, the sequence clustering of the four fragments of the sample cannot be distinguished (**Figures 5–8**). In addition, the genetic distance analysis results showed that there was no significant correlation between genetic distance and geographical distance (**Table 4–7**). A previous study of ours found that site-specific PCR may be used to screen site-specific primers for the purpose of identifying *C. deserticola*, *Cynomorium coccineum subsp. songaricum* (Rupr.) J. Léonard, and *Orobanche pycnostachya* Hance based on ITS sequences (Li et al., 2014). However, the identification of Cistanches Herba remains to be further studied because of the existence of other adulterants. By studying the DNA barcoding of Cistanches Herba, we found that the quality differences between Cistanches Herba populations in the Alxa area were not significant. We also quickly and accurately identified Cistanches Herba and its adulterants, which is of vital importance for guaranteeing food and medicine safety and regulating market circulation.

In conclusion, the genetic distance and chemical composition of Cistanches Herba were not correlated with geographical location in Alxa. However, this result may be limited by our representative sample and the availability of the sequences. Thus, these results require further verification.

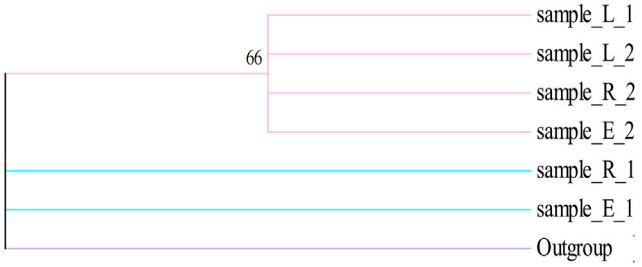


FIGURE 5 | Phylogenetic tree of Cistanches Herba based on the ITS sequences.

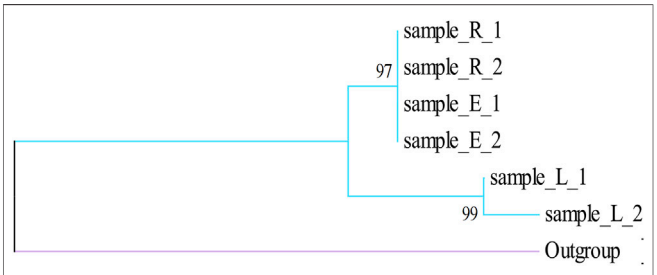


FIGURE 6 | Phylogenetic tree of Cistanches Herba based on the *trn*H-*psb*A sequences.

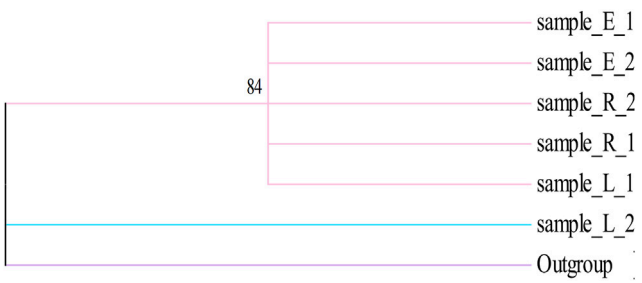


FIGURE 7 | Phylogenetic tree of Cistanches Herba based on the *rbc*L sequences.

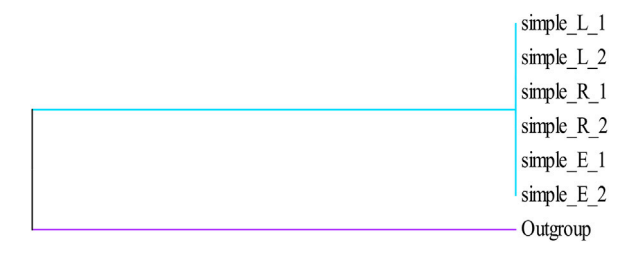


FIGURE 8 | Phylogenetic tree of Cistanches Herba based on the *mat*K sequences.

3.4 Relationship Between Behavior, Revenue, and Quality

Cistanches Herba was considered both a medicine and food (Li et al., 2021). The main retail channels of Cistanches Herba include hospital pharmacies, drug stores, medicine markets, agricultural by-product, markets, and online shops. Currently, due to its high medicinal value and nutritional function, a trend for developing Cistanches Herba is gradually forming in the form of traditional, food, and deep processing markets. Among these three major markets, due to the yield, geo-herbalism, and clinical function factors, the price of *C. deserticola* from Alxa is higher than that of *C. tinctoria* in the traditional market and *C. deserticola* rarely enters traditional markets. Usually, local people choose good quality Cistanches Herba as gifts from local retail outlets and pay a higher price.

The objectives of all stakeholders in these types of VCs are centered on profitability. However, in the traditional market, independent farmers generally adopt the sales mode of selling raw materials directly, which has low additional value. In the food products market, these mainly enter the market in the form of gifts, which are often higher in quality and price than those sold in the traditional market. In deep-processing-oriented chains, quality and additional value products are improved, thus increasing overall cost. Producers of deep-processed Cistanches Herba typically receive higher revenue from consumers, while stakeholders in traditional markets receive less. Therefore, the behavior and benefits of stakeholders are related to the quality of the product and the target market.

Regarding VC 1, some of the primary processing products are sold as raw materials to pharmaceutical factories by the primary processing companies, which are then further processed into other forms of drugs that bring greater profits. However, selling these products as drugs is often affected by higher regulations.

VCs 2 and 3 are typical examples of traditional markets, where medical wholesalers buy herbs directly from farmers. In these chains, it is difficult for farmers to expand the planting scale, due to the long planting cycle of Cistanches Herba, which requires high levels of early investment in time and labor. In addition, farmers resort to selling raw materials directly, resulting in less profits. Compared with farmers, medical wholesalers process and grade Cistanches Herba. However, storage of Cistanches Herba is usually an important factor that affects its quality, and also limits its circulation in the market, which is one reason for the high price. Moreover, to obtain higher profits, we found that medical wholesalers often retail *C. salsa* as a substitute for Cistanches Herba, which reduces product quality. Therefore, quality problems are common in these VCs, and the Cistanches Herba so produced can rarely enter traditional markets and therefore provides relatively low income for farmers.

VCs 6 and 7 mainly supply the food products market. They are mainly local individual retailers that sell online and offline. These individual retailers go through simple processing and sorting to select better products. This group deals mainly in gifts that enter

TABLE 4 | Genetic distance of Cistanches Herba based on ITS sequences.

	Sample_L_1	Sample_L_2	Sample_R_1	Sample_R_2	Sample_E_1	Sample_E_2
Sample_L_1						
Sample_L_2	0.000000					
Sample_R_1	0.002336	0.002336				
Sample_R_2	0.000000	0.000000	0.002336			
Sample_E_1	0.002336	0.002336	0.000000	0.002336		
Sample_E_2	0.000000	0.000000	0.002336	0.000000	0.002336	
Outgroup	0.004683	0.004683	0.002336	0.004683	0.002336	0.004683

(sample_E: the sample of Ejin Bannerr; sample_R: the sample of Alxa Right Banner; sample_L: the sample of Alxa Left Banner).

TABLE 5 | Genetic distance of Cistanches Herba based on *trn*H-*psb*A sequences.

	Sample_L_1	Sample_L_2	Sample_R_1	Sample_R_2	Sample_E_1	Sample_E_2
Sample_L_1						
Sample_L_2	0.017571					
Sample_R_1	0.000000	0.017571				
Sample_R_2	0.000000	0.017571	0.000000			
Sample_E_1	0.000000	0.017571	0.000000	0.000000		
Sample_E_2	0.000000	0.017571	0.000000	0.000000	0.000000	
Outgroup	0.421739	0.421739	0.421739	0.421739	0.421739	0.421739

TABLE 6 | Genetic distance of Cistanches Herba based on *rbc*L sequences.

	Sample_L_1	Sample_L_2	Sample_R_1	Sample_R_2	Sample_E_1	Sample_E_2
Sample_L_1						
Sample_L_2	0.019521					
Sample_R_1	0.000000	0.019521				
Sample_R_2	0.000000	0.019521	0.000000			
Sample_E_1	0.000000	0.019521	0.000000	0.000000		
Sample_E_2	0.000000	0.019521	0.000000	0.000000	0.000000	
Outgroup	0.047834	0.064703	0.047834	0.047834	0.047834	0.047834

TABLE 7 | Genetic distance of Cistanches Herba based on *mat*K sequences.

	Sample_L_1	Sample_L_2	Sample_R_1	Sample_R_2	Sample_E_1	Sample_E_2
Sample_L_1						
Sample_L_2	0.002391					
Sample_R_1	0.001194	0.001194				
Sample_R_2	0.000000	0.002391	0.001194			
Sample_E_1	0.000000	0.002391	0.001194	0.000000		
Sample_E_2	0.000000	0.002391	0.001194	0.000000	0.000000	
Outgroup	1.709952	1.709952	1.709952	1.709952	1.709952	1.709952

the market, which are generally good-quality Cistanches Herba. The prices of these are often higher than those in conventional markets due to being better quality products than those sold in traditional medicine markets. Furthermore, selling medicinal materials via e-commerce platforms greatly reduces store costs. However, there are still many gaps in the supervision process, leading to some irregular and speculative components, the origins of which remain untraceable.

With the continuous improvement in the value of Cistanches Herba products, it is gradually being recognized and accepted by consumers. Therefore, the demand for its deeply processed products in domestic and foreign markets increases yearly, bringing higher benefits and attracting a huge potential market. Furthermore, the government actively encourages local enterprises to carry out product research and development in order to enhance benefits for farmers as well as profits for enterprises, all of which increase tax revenue for the government. VCs 4 and 5 and VCs 8–10 are placed between the traditional and deep processing market models. Concerning VCs 4 and 5, processing companies purchase directly from farmers and further process the products in order to sell to consumers as TCM or health

care products. In these chains, farmers still earn lower benefits, but processing companies guarantee better quality than retailers. They engage more fully in various forms of self-regulation to improve their reputation. As a result, they increase their overall input costs while improving the safety and traceability of their products.

In VC 8, the agricultural cooperative mode of "company" + "farmer" further expands the planting area and drives farmer incomes. These companies provide effective technology and equipment that help improve planting efficiency and yield. At the same time, these companies conduct further processing research, thus improving the additional value of Cistanches Herba products and expanding the market for product demand, which in turn increases the sales of Cistanches Herba related products in multiple ways. These innovative deep processing technologies improve quality, enabling companies to obtain higher profits. The cooperative order mode guarantees the farmers a production income and reduces their market risk, which effectively stimulates the enthusiasm of local farmers to plant. In VCs 9 and 10, companies concentrate on deep processing to seek a bigger market and higher profits. These related companies employ a variety of processing methods, which allow flexibility for adjusting the variety and yield of production according to market demand and product revenues. In addition, resources are relatively adequate and the product quality is good, thereby enhancing market product space. Advanced industrial facilities and products are the key to the development of the company due to the need for better deep processing technology.

According to our survey, higher standards are being placed on the quality of materials, resulting in higher input costs. Therefore, the behavior and benefits of stakeholders, as well as the quality of the products and target markets, are all closely related. As a result, the price and quality of Cistanches Herba is higher in the gift (300–1200 CNY/kg) and deep processing markets than in traditional markets (150–800 CNY/kg). In these three major markets, the majority of sales are in the form of primary processed gifts. Compared with the price advantage of *C. tinctoria*, *C. deserticola* rarely enters the traditional medicine market, and its circulation is restricted. In conclusion, with the entry of the Cistanches Herba into the health food market, further processing and development of Cistanches Herba is expected to increase in the future.

4 CONCLUSION

C. deserticola is a parasitic plant growing in a unique environment, and Alxa appears to be the area that is most suitable for its growth. Regarding Cistanches Herba value chains, we have demonstrated the emergence and development of vertical integration, horizontal collaboration, and e-commerce at various levels. Vertical integration is induced by the expansion of stakeholders. VC 3 and VC 4 are the result of the vertical integration of wholesalers and processing companies via the development of downstream businesses. Among the 10 VCs, VCs 8–10 are fully vertically integrated. Farmers gain access to markets, as well as to technological and financial support by joining these vertically integrated VCs. Additionally, partial vertical integration is attributed to the reliable traceability of the products. Horizontal collaborations (e.g., agricultural cooperatives) demonstrate a higher level of communication and common goals, and bring higher incomes to relevant stakeholders. E-commerce offers a convenient and inexpensive approach for retailers to build direct access to consumers. However, quality assurance in such chains is poorly understood, and there is a need for certification of good practices in e-commerce.

On the one hand, due to the specific factors associated with Cistanches Herba cultivation, independent farmers did not have access to the latest technology and were not able to develop large-scale cultivations. Some key issues related to processing procedures as well as wholesale and retail activities, that were detected during the course of our interviews and quality analyses, indicated that earning higher profits via adulterated substitutes were hidden factors that reduced the value of products. These factors can affect the quality of herbal medicines, just as a value-added cooking process could improve the quality of foods (Chen et al., 2019). Thus, further regulation of the market may be necessary in order to eliminate adulteration by pharmaceutical wholesalers and to realize higher profits.

On the other hand, with the state supporting the development of the Cistanches Herba industry, it is predicted that planting Cistanches Herba may increase yearly in the future. However, if deep processing and terminal markets of Cistanches Herba do not develop sufficiently within the next few years, raw material obtained from Cistanches Herba will face a market environment of oversupply, causing the prices to drop. Large-scale stakeholders, such as processing-acquisition-trading integrated companies, are more capable of controlling the development of Cistanches Herba. They are supported by technology and management structures. Thus, they have an advantage in ensuring quality and stability due to being associated with deep processing and quality control. In addition, the development of agricultural cooperation promotes the standardization and scale of the Cistanches Herba planting industry, allowing the formation of a complete industrial chain. Local farmers and herdsmen have a remarkable effect on poverty alleviation and play an extremely important role in economic and social development.

Here, we have demonstrated that the quality of Cistanches Herba is affected by multiple stages of the production process, which suggests that deciding on an appropriate VC is the core of quality control. Furthermore, we demonstrated that product quality, stakeholder behavior, and revenue are interrelated. Therefore, coordination of relationships in VCs may be a strategy that may be utilized in the quality control of Cistanches Herba. Diverse VCs are induced by vertical integrations and horizontal collaborations, and this enhances both the quality of Cistanches Herba and the returns to stakeholders. Therefore, well-developed VCs may produce

products with good traceability as well as quality and ensure adequate financial revenues to relevant stakeholders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Conceptualization, ML, JW, RY, LJ, and XW; Writing–original draft preparation LJ, XW, and BZ; Writing–review and editing, WG, RY, ML, and YB; LJ, BZ, JW, YB, and WG generated the figures and tables; ML, JW, YB, RY, WG, BZ, and XW revising it critically for important intellectual content; All authors have read and agreed to the published version of the manuscript; All authors agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This work was supported by China Agriculture Research System (grant number CARS-21); 2019 Chinese medicine public health service subsidy special "the fourth survey on Chinese material medica resource" (grant number Finance Society (2019) 43); 2020 Inner Mongolia Autonomous Region Strategic Emerging Industry Development Fund Project (grant number 2020832).

ACKNOWLEDGMENTS

We would like to thank Li Yue and Li Zhenhua for their suggestions on revision and data sorting during this period. At the same time, We would like to thank the teachers of Inner Mongolia Institute of Traditional Chinese Medicine for helping with the field research and market research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.782962/full#supplementary-material

REFERENCES

Bi, Y., Bao, H., Zhang, C., Yao, R., and Li, M. (2020). Quality Control of Radix Astragali (The Root of *Astragalus Membranaceus* Var. Mongholicus) along its Value Chains. *Front. Pharmacol.* 11, 562376. doi:10.3389/fphar.2020.562376

Booker, A., Frommenwiler, D., Johnston, D., Umealajekwu, C., Reich, E., and Heinrich, M. (2014). Chemical Variability along the Value Chains of Turmeric (*Curcuma Longa*): a Comparison of Nuclear Magnetic Resonance Spectroscopy and High Performance Thin Layer Chromatography. *J. Ethnopharmacol.* 152, 292–301. doi:10.1016/j.jep.2013.12.042

Booker, A., Johnston, D., and Heinrich, M. (2012). Value Chains of Herbal Medicines-Rresearch Needs and Key Challenges in the Context of Ethnopharmacology. *J. Ethnopharmacol.* 140, 624–633. doi:10.1016/j.jep.2012.01.039

Booker, A., Frommenwiler, D., Reich, E., Horsfield, S., and Heinrich, M. (2016). Adulteration and Poor Quality of *Ginkgo Biloba* Supplements. *J. Herbal Med.* 6, 79–87. doi:10.1016/j.hermed.2016.04.003

Booker, A., Johnston, D., and Heinrich, M. (2015). Value Chains of Herbal Medicines-Ethnopharmacological and Analytical Challenges in a Globalizing World. *Evid-based Validation Herb. Med.*, 29–44. doi:10.1016/B978-0-12-800874-4.00002-7

Chen, L., Teng, H., and Xiao, J. (2019). A Value-Added Cooking Process to Improve the Quality of Soybean: Protecting its Isoflavones and Antioxidant Activity. *Food Sci. Hum. Wellness* 8, 195–201. doi:10.1016/j.fshw.2019.05.001

Fan, J. L., Li, B. W., Xu, X. W., and Li, S. Y. (2020). Technology Progress of Sand Industry Based on Desertification Control Engineering by Revegetation: Case Study on *Cistanche Deserticola* Ma Plantation Bulletin. *Chin. Acad. Sci.* 35, 717–723. doi:10.16418/j.issn.1000-3045.20200429002

Fu, Z., Fan, X., Wang, X., and Gao, X. (2018). Cistanches Herba: An Overview of its Chemistry, Pharmacology, and Pharmacokinetics Property. *J. Ethnopharmacol.* 219, 233–247. doi:10.1016/j.jep.2017.10.015

Gao, Y. J., Jiang, Y., Dai, F., Han, Z. L., Liu, H. Y., and Bao, Z. (2015). Studies on the Effective Substances of *Cistanche Deserticola* Ma to Relieve Constipation. *Mod. Chin. Med.* 17, 307–310. doi:10.13313/j.issn.1673-4890.2015.4.003

Heinrich, M., Scotti, F., Booker, A., Fitzgerald, M., Kum, K. Y., and Löbel, K. (2019). Unblocking High-Value Botanical Value Chains: Is There a Role for Blockchain Systems? *Front. Pharmacol.* 10, 396. doi:10.3389/fphar.2019.00396

Hou, L., Wang, B., Li, G. H., and Wang, X. J. (2020). Research Progress and Industrialization Status of *Cistanche Deserticola* Ma. *Shandong Agr. Sci.* 52, 133–140. doi:10.14083/j.issn.1001-4942.2020.12.025

Ichim, M. C. (2019). The DNA-Based Authentication of Commercial Herbal Products Reveals Their Globally Widespread Adulteration. *Front Pharmacol.* 10, 1227. doi:10.3389/fphar.2019.01227

Ichim, M. C., Häser, A., and Nick, P. (2020). Microscopic Authentication of Commercial Herbal Products in the Globalized Market: Potential and Limitations. *Front. Pharmacol.* 11, 876. doi:10.3389/fphar.2020.00876

Janjua, S., Fakhar-I-Abbas, W. K., William, K., Malik, I. U., and Mehr, J. (2017). DNA Mini-Barcoding for Wildlife Trade Control: A Case Study on Identification of Highly Processed Animal Materials. *Mitochondrial DNA A. DNA Mapp. Seq. Anal.* 28, 544–546. doi:10.3109/24701394.2016.1155051

Lei, H., Wang, X., Zhang, Y., Cheng, T., Mi, R., Xu, X., et al. (2020). Herba Cistanche (*Rou Cong Rong*): A Review of its Phytochemistry and Pharmacology. *Chem. Pharm. Bull. (Tokyo)* 68, 694–712. doi:10.1248/cpb.c20-00057

Li, D. Q., Xu, R., He, X., Feng, R., Xu, C. Q., Liu, T., et al. (2021). Market Investigation and Study of Standard Grade of Cistanches Herba. *Mod. Chin. Med.* 23, 401–408. doi:10.13313/j.issn.1673-4890.20200803006

Li, T. R., Xu, Y. Y., Ge, J. X., and Xu, G. Y. (1989). The Germination of *Cistanche Deserticola* Seeds and its Relationship with Host *Haloxylon Ammodendron* (C.A.Mey.) Bunge Ex Fenzl. *J. Inner Mongolia Univer.* 20, 107–112. CNKI:SUN:NMGX.0.1989-03-016.

Li, X., Qi, H. P., Wang, W. L., and Li, M. H. (2018). Arrangement and Analysis of Domestic Patents of *Cistanche Deserticola* Ma Health Products. *J. Food Saf. Qual.* 9, 4700–4705. doi:10.3969/j.issn.2095-0381.2018.17.038

Li, Y., Zhou, G., Peng, Y., Tu, P., and Li, X. (2016). Screening and Identification of Three Typical Phenylethanoid Glycosides Metabolites from Cistanches Herba by Human Intestinal Bacteria Using UPLC/Q-TOF-MS. *J. Pharm. Biomed. Anal.* 118, 167–176. doi:10.1016/j.jpba.2015.10.038

Li, Z., Lin, H., Gu, L., Gao, J., and Tzeng, C. M. (2016). Herba Cistanche (*Rou Cong-Rong*): One of the Best Pharmaceutical Gifts of Traditional Chinese Medicine. *Front. Pharmacol.* 7, 41. doi:10.3389/fphar.2016.00041

Li, Z., Zhang, C., Ren, G., Yang, M., Zhu, S., and Li, M. (2019). Ecological Modeling of Cistanche Deserticola Y.C. Ma in Alxa, China. *Sci. Rep.* 9, 13134. doi:10.1038/s41598-019-48397-6

Li, Z. H., Long, P., Zou, D. Z., Li, Y., Cui, Z. H., and Li, M. H. (2014). Study on Identification of Cistanche Hebra and its Adulterants by PCR Amplification of Specific Alleles Based on ITS Sequences. *Zhongguo Zhong Yao Za Zhi* 39, 3684–3688. doi:10.4268/cjcmm20141905

Lu, Y. H., Pi, W. M., Zhang, Y., Yilayimu, S., and Sun, Z. R. (2019). Comparative Study and Analysis of Market Status of *Cistanche Deserticola* Ma and *Cistanche Tinctoria* (Forssk.). *Beck. Mod. Chin. Med.* 21, 999–1005. doi:10.13313/j.issn.1673-4890.20190521009

Ndou, P., Taruvinga, B., Mofokeng, M. M., Kruger, F., Du Plooy, C. P., and Venter, S. L. (2019). Value Chain Analysis of Medicinal Plants in South Africa. *S-EM* 13, 226–236. doi:10.31901/24566772.2019/13.04.579

Raclariu, A. C., Heinrich, M., Ichim, M. C., and de Boer, H. (2018). Benefits and Limitations of DNA Barcoding and Metabarcoding in Herbal Product Authentication. *Phytochem. Anal.* 29, 123–128. doi:10.1002/pca.2732

Siqin, B., Liu, J., Xi Lin, T. Y., Qing, G. L., Lin, N., and Zhang, Z. Q. (2019). Clinical Value and Artificial Planting Situation of *Cistanche Deserticola* Ma. *World Latest Med. Inf.* 19, 260. doi:10.19613/j.cnki.1671-3141.2019.93.157

Székács, A., Wilkinson, M. G., Mader, A., and Appel, B. (2018). Environmental and Food Safety of Spices and Herbs along Global Food Chains. *Food Control* 83, 1–6. doi:10.1016/j.foodcont.2017.06.033

Wang, T., Zhang, X., and Xie, W. (2012). *Cistanche Deserticola* Y. C. Ma, "Desert Ginseng": a Review. *Am. J. Chin. Med.* 40, 1123–1141. doi:10.1142/S0192415X12500838

Wang, T., Xu, Y. X., Li, W. L., and Xu, J. F. (2017). The Application of Cluster Analysis in the Study of TCM Prescription. *Clin. J. Trad. Chin. Med.* 29, 2035–2037. doi:10.16448/j.cjtcm.2017.0672

Wang, X. Y., Xiao, B., Zhang, Z. F., He, Y. F., Cao, L. J., and Li, T. X. (2017). Study on Five Efficacy Components, Geoherbalism of *Cistanche Deserticola* Ma from Genuine Producing Area in Different Collecting Seasons. *Chin. Trad. Herbal Drug* 48, 3841–3846. doi:10.7501/j.issn.0253-2670.2017.18.027

Yan, W., Xu, J., Han, H., Feng, J. M., Cao, L. J., and Li, T. X. (2019). Establishment of Classification System for Commodity Specification and Character Characteristics of *Cistanche Deserticola* Ma. *Tianjin J. Trad. Chin. Med.* 36, 819–823. doi:10.11656/j.issn.1672-1519.2019.08.21

Yao, R., Heinrich, M., Wang, Z., and Weckerle, C. S. (2018). Quality Control of Goji (Fruits of *Lycium Barbarum* L. And *L. Chinense* Mill.): A Value Chain Analysis Perspective. *J. Ethnopharmacol.* 224, 349–358. doi:10.1016/j.jep.2018.06.010

Yin, M., Yang, M., Chu, S., Li, R., Zhao, Y., Peng, H., et al. (2019). Quality Analysis of Different Specification Grades of Astragalus Membranaceus Var. Mongholicus (Huangqi) from Hunyuan, Shanxi. *J. AOAC Int.* 102, 734–740. doi:10.5740/jaoacint.18-0308

You, S. P., Ma, L., Zhao, J., Zhang, S. L., and Liu, T. (2016). Phenylethanol Glycosides from Cistanche Tubulosa Suppress Hepatic Stellate Cell Activation and Block the Conduction of Signaling Pathways in TGF-B1/smad as Potential Anti-hepatic Fibrosis Agents. *Molecules* 21, 102. doi:10.3390/molecules21010102

Zhao, W. T., and Zhang, B. (2014). Feasibility of Artificial Cultivation of *Cistanche Deserticola* Ma and its Industrialization. *Inner Mongolia For. Invest. Design.* 37, 109–111. doi:10.3969/j.issn.1006-6993.2014.04.052

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 18 November 2021
doi: 10.3389/fphar.2021.738504

Valuable Hepatoprotective Plants - How Can We Optimize Waste Free Uses of Such Highly Versatile Resources?

*Lubov V. Krepkova[1], Aleksandra N. Babenko[1], Olga L. Saybel'[1], Irina A. Lupanova[1], Olga S. Kuzina[1], Kathleen M. Job[2], Catherine M. Sherwin[3†] and Elena Y. Enioutina[2,4†]**

[1]Center of Medicine, All-Russian Research Institute of Medicinal and Aromatic Plants (VILAR), Moscow, Russia, [2]Division of Clinical Pharmacology, Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, UT, United States, [3]Department of Pediatrics, Boonshoft School of Medicine, Dayton Children's Hospital, Wright State University, Dayton, OH, United States, [4]Department of Pharmaceutics & Pharmaceutical Chemistry, College of Pharmacy, University of Utah, Salt Lake City, UT, United States

OPEN ACCESS

Edited by:
Alessandra Durazzo,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Jadwiga Andrzejewska,
University of Technology and Life Sciences in Bydgoszcz, Poland
Bilal Haider Abbasi,
Quaid-i-Azam University, Pakistan
Kunjbihari Sulakhiya,
Indira Gandhi National Tribal University, India

***Correspondence:**
Elena Y. Enioutina
elena.enioutina@hsc.utah.edu

[†]These authors have contributed equally to this work and share senior authorship

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 08 July 2021
Accepted: 27 October 2021
Published: 18 November 2021

Citation:
Krepkova LV, Babenko AN, Saybel' OL, Lupanova IA, Kuzina OS, Job KM, Sherwin CM and Enioutina EY (2021) Valuable Hepatoprotective Plants - How Can We Optimize Waste Free Uses of Such Highly Versatile Resources? Front. Pharmacol. 12:738504. doi: 10.3389/fphar.2021.738504

Humans used plants for thousand of years as food, drugs, or fuel to keep homes warm. People commonly used fruits and roots, and other parts of the plant were often wasted. This review aims to discuss the potential of rational stem-to-stern use of three highly versatile and valuable plants with hepatoprotective properties. Milk thistle (*Silybum marianum* L. Gaertn.), artichoke (*Cynara cardunculus*), and chicory (*Cichorium intybus* L.) have well-characterized hepatoprotective properties. These plants have been chosen since liver diseases are significant diseases of concern worldwide, and all parts of plants can be potentially utilized. Artichoke and chicory are commonly used as food or dietary supplements and less often as phytodrugs. Various dietary supplements and phytodrugs prepared from milk thistle (MT) fruits/seeds are well-known to consumers as remedies supporting liver functions. However, using these plants as functional food, farm animal feed, is not well-described in the literature. We also discuss bioactive constituents present in various parts of these plants, their pharmacological properties. Distinct parts of MT, artichoke, and chicory can be used to prepare remedies and food for humans and animals. Unused plant parts are potentially wasted. To achieve waste-free use of these and many other plants, the scientific community needs to analyze the complex use of plants and propose strategies for waste-free technologies. The government must stimulate companies to utilize by-products. Another problem associated with plant use as a food or source of phytodrug is the overharvesting of wild plants. Consequently, there is a need to use more active cultivation techniques for plants.

Keywords: Milk Thistle, Chicory, Artichoke, rational use, food, phytodrug, functional food, animal feed

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; CCl4, carbon tetrachloride; DEC, dry extract obtained from wild chicory; DSHEA, dietary supplement health and education act; GGT, γ-glutamyl transferase; ERK1/2, extracellular signal-regulated kinase 1/2; HCV, hepatitis C virus; HgCl2, mercury chloride; IL1α, interleukin 1 alpha; IP6, inositol hexaphosphate; MT, Milk Thistle; NF-kB, nuclear factor kappa B; RNA, ribonucleic acid; STAT-1, signal transducer and activator of transcription 1; STAT-3, signal transducer and activator of transcription 3; TNFα, tumor necrosis factor-alpha; US FDA, the United States food and drug administration; VILAR, All-Russian research institute of medicinal and aromatic plants.

INTRODUCTION

Humans have been using plants as food and remedies for thousands of years. About 80% of the world's population uses medicinal plants and phytodrugs to treat various diseases (Ekor, 2014). Based on ethnopharmacological studies conducted by Sun et al., plants have a critical role in people's diets, with roots and fruits most commonly used. Currently, wild and cultivated plants are used by the food, pharmaceutical, and cosmetic industries (Schmidt, 2012; Nofal et al., 2019; Rahman et al., 2019). The demand for wild plants, specially harvested in ecologically clean areas, is growing. Therefore, overharvesting of wild plants can lead to their endangerment (United Plant Savers, 2021). Often distinct parts of the plant are used as food or medicine. This raises the question of what happens to by-products and whether it is possible to develop waste-free processes of plant handling. Parts of the plants that may be wasted can be used as a source of food, functional food, or feed for farm animals. Therefore, researchers urge companies to introduce ethically reasonable use of plants.

According to the encyclopedia Britannica, food is a "substance consisting essentially of protein, carbohydrate, fat, and other nutrients used in the body of an organism to sustain growth and vital processes and to furnish energy" (Britannica Encyclopaedia., 2020). The Japanese government first introduced the term "functional food" in the early 1980s (Martirosyan and Singh, 2015). Later functional food was introduced to the European and American markets (Martirosyan and Singh, 2015). A function food "may provide a health benefit beyond the traditional nutrients it contains" (Thomas and Earl, 1994). In 1994, a new category of natural products, a dietary supplement, was introduced to consumers (1994). The Dietary Supplement Health and Education Act (DSHEA) describes dietary supplements as preparations intended to supplement the diet and may contain plants, vitamins, minerals, amino acids, tissue from organs, enzymes, and probiotics (1994). Dietary supplements are usually minimally regulated by medical authorities. According to the United States Food and Drug Administration (US FDA), about 50% of United States adults regularly use medicinal plants/herbs (Gottlieb, 2019). Phytodrugs, another category of natural products, are purified extracts from various parts of the plant or single-molecule phytochemicals isolated from natural products. These products are tightly regulated by regulatory authorities worldwide (Enioutina et al., 2020). The US FDA considers the highly purified extracts prepared from medicinal plants as botanical drugs and similarly regulates them as conventional synthetic drugs (Enioutina et al., 2020).

The primary purpose of this article is to discuss the potential of rational "stem-to-stern" use of plants. To prove the potential of rational use, we have chosen three plants with confirmed hepatoprotective properties. These plants are milk thistle (MT, *Silybum marianum* L. Gaertn.), artichoke (*Cynara cardunculus*), and Chicory (*Cichorium intybus* L.) (Baginskaya et al., 2000; Abenavoli et al., 2010; Li et al., 2014; Ben Salem et al., 2019; Achufusi and Patel, 2020; Mukhtar et al., 2021; Perovic et al., 2021; Song et al., 2021). The term "hepatoprotective" is often used to describe the ability of a drug or plant to prevent liver damage, whereas "antihepatotoxic" drugs can prevent or treat liver damage done by hepatotoxic substances. In general, these terms are often used interchangeably. In this article, we have chosen to use the term "hepatoprotective" plants.

The chief reason for selecting these plants is that liver disorders, including cirrhosis, viral hepatitis, hepatocellular carcinoma, and drug-, heavy metal-, and alcohol-induced liver injuries, are significant diseases of concern worldwide (Rehm et al., 2013; Asrani et al., 2019; Bashir et al., 2021). One-third to nearly 90% of patients with liver diseases or cancer diagnoses used herbal products with hepatoprotective properties (Fenclova et al., 2019).

Another reason is that the consumers widely use these plants as food, food substitute, phytodrugs, or dietary supplements. Artichoke flowers are primarily used as food or dietary supplements, while wasted aerial parts have the potential to be used as phytodrug, animal feed, or biofuel (Fernández et al., 2006; Gostin and Waisundara, 2019; Barbosa et al., 2020). The artichoke is often added to the hepatoprotective proprietary blends along with MT. Chicory is also best known as food (e.g., coffee substitute) (Wu and Cadwallader, 2019) or dietary supplement (inulin, a prebiotic driven from chicory root) (Shoaib et al., 2016) and less known as a phytodrug. The chicory wasted aerial part is occasionally a part of the phytodrug and can be used as animal feed (Niness, 1999; Githiori et al., 2006; Kandeler and Ullrich, 2009; Himalaya Wellness Company., 2021b). Chicory is a part of a multi-component phytodrug developed by the Himalaya Drug Company (Himalaya Wellness Company., 2021a). Unlike the other two plants, milk thistle fruits/seeds are mainly used as phytodrugs or dietary supplements, but the remaining plant parts can also be used as food additives for animals and biofuel (Chabaev et al., 2011; Andrzejewska et al., 2015; Ataei Nukabadi et al., 2021). Several MT-based phytodrugs were marketed [e.g., Carsil (SoPharma, Bulgaria); and Legalon (Flordis, Australia)]. Currently, MT-based dietary supplements are in the top-40 best-selling herbal supplements (Smith et al., 2017).

Distinct parts of these plants have different compositions of nutrients and phytochemicals. For example, the bioactive constituents responsible for the hepatoprotective properties of MT are present in the highest concentration in plant fruits and seeds (Baginskaya et al., 2000; Vargas-Mendoza et al., 2014). The hepatoprotective phytochemicals are present in roots and aerial part of chicory (Thabrew et al., 1982; Huseini et al., 2005; Abenavoli et al., 2010; Cha et al., 2010; Abd El-Mageed, 2011).

The demand for phytodrugs and dietary supplements with hepatoprotective properties will grow. This may increase the exhaustion of wild-grown resources and waste parts of plants not used for drug/supplement preparation. This article will show the potential use of distinct parts of MT, artichoke, and chicory as food, phytodrugs, dietary supplements, functional food, and animal feed.

METHODS

This extensive review (Munn et al., 2018) of existing literature and evaluation of the existing literature found numerous gaps in

TABLE 1 | The areas of usage of distinct parts of the plants.

Plant name	Part of the plant	Areas of usage
Milk thistle	Fruits (oilseed cake, oil, and seeds)	✓Phytodrugs Brailski et al. (1986); Baginskaya et al. (2000) ✓Dietary supplements Smith et al. (2017); Fenclova et al. (2019). ✓Human functional food & bakery products Semenkina (2010); Nekrasova and Popov (2020) ✓Animal functional foodKolesnyk et al. (2009); Chabaev et al. (2011) ✓Cosmetics Nofal et al. (2019) ✓Biodiesel (fruit oil) Ullah et al. (2015)
	Aerial part	✓Animal feed Andrzejewska et al. (2015) ✓Phytodrug Himalaya Wellness Company (2021b)
Artichoke	Flowers	✓Food Gostin and Waisundara (2019) ✓Dietary supplements GistGear (2021) ✓Cosmetics D'Antuono et al. (2018)
	Aerial part	✓Dietary supplements Zeaiter et al. (2019) ✓Animal feed Barbosa et al. (2020) ✓Biofuel Barbosa et al. (2020)
Chicory	Aerial part	✓Phytodrugs Himalaya Wellness Company. (2021a) ✓Dietary supplements Perovic et al. (2021) ✓Functional food Perovic et al. (2021) ✓Animal feed Nwafor et al. (2017)
	Roots	✓Dietary supplements Shoaib et al. (2016) ✓Biofuel Hughes et al. (2017)

our knowledge related to the use of distinct parts of MT, chicory, and artichoke in food and pharmaceutical industries animal husbandry. The authors conducted a comprehensive search in six electronic databases (PubMed, Embase, Scopus, ScienceDirect, Web Science, and elibrary.ru), the All-Russian Institute of Aromatic and Medicinal Plants (VILAR) medical library, and other relevant medicinal herbs websites for literature published between January 1976 and May 2021. Searches were conducted using the keywords: artichoke, chicory, *Silybum marianum*, *Cichorium intybus*, phytodrugs, drugs, dietary supplements, food, functional food, and animal feed.

MILK THISTLE

Milk thistle (MT, *Silybum marianum* L. Gaertn.,), a member of the Asteraceae family, is native to Southern Europe and Asia and widely naturalized in Europe, North America, South America, Australia, and New Zealand (Missouri Botanical Garden., 2019). It is not used actively in agriculture and is considered to be an invasive weed in North America, Australia, New Zealand, and South Africa (King County, 2018).

MT is best known for its hepatoprotective and antioxidant properties (Siegel and Stebbing, 2013) (**Table 1**). Bioactive compounds isolated from MT, specifically a bioactive flavonolignan, silymarin, have antioxidant properties, protect against damage by the free radicals generated from the metabolism of ethanol, acetaminophen, and carbon tetrachloride ingested in excessive amounts (Krepkova et al., 2008; Vargas-Mendoza et al., 2014). In addition, MT has anti-inflammatory, anti-cancer, antiviral, and immunomodulatory properties (Deak et al., 1990; Krepkova et al., 2008; Vargas-Mendoza et al., 2014) (**Table 2**). Carsil, Legalon, and Silimar are popular phytodrugs prepared from the fruit of MT (Brailski et al., 1986; Feher et al., 1989; Soleimani et al., 2019; Gillessen and Schmidt, 2020). Now, MT and plant extracts are immensely popular as dietary supplements (**Table 1**).

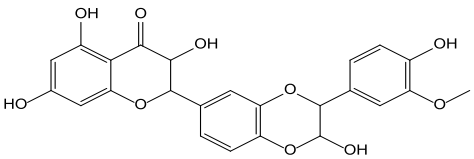
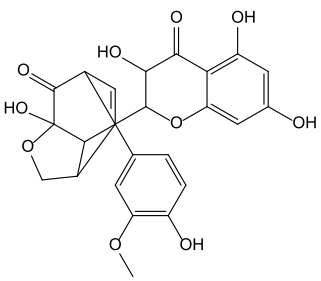
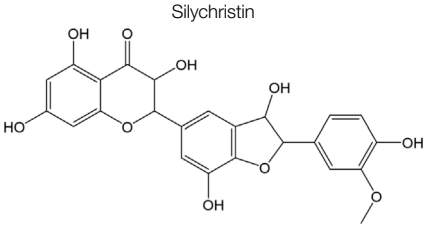
However, the use of MT as food, functional food for humans and animals is not well studied. Several publications describe the usefulness of bread or pastry fortification with MT fruits and oil and the supplementation to animal feed with bioactive compounds from the plant. It appears that plant stems and foliage are not used following phytodrugs and dietary supplements preparations and have the potential to be wasted.

Milk Thistle Bioactive Constituents and Their Biological Activity

Silymarin is the main bioactive constituent found in the MT fruit oilseed cake (Vargas-Mendoza et al., 2014) (**Table 2**). It is a complex flavonolignan responsible for the plant's hepatoprotective properties. Silymarin is a complex flavonolignan comprising silybin A and B, isosilybin A and B, silydianin, silychristin, isosilychristin, and taxifolin, a flavonoid precursor (Pradhan and Girish, 2006; Vargas-Mendoza et al., 2014). The silibinin, a mixture of silybins A and B, is also named silibinin. The ratio of flavonolignan isomers may differ depending on the region where MT was grown (Lee et al., 2007; Poppe and Petersen, 2016). Flavonolignan's concentrations can also be influenced by oil extraction technology from MT fruits. The cold-pressed oil extraction preserves a unique combination and activity of bioactive constituents present in the plant fruits (Hou et al., 2010; Lee et al., 2013). The MT oilseed cake or seeds are the primary raw material used by the pharmaceutical and dietary supplement companies to prepare phytodrugs and dietary supplements.

The hepatoprotective properties of silibinin include blocking stabilizing liver cell membranes and blocking phosphodiesterase (Romanova and Krasavtsev, 2007). In addition, silibinin can bind radicals due to its phenolic structure, resulting in a significant

TABLE 2 | Phytochemical composition and pharmacological properties of milk thistle.

Plant name/part of the plant commonly used for medicinal purposes	Bioactive constituents	Pharmacological properties
Milk thistle *Silybum marianum* L. Gaertn./fruits and seeds 	Silibinin  Silidianin  Silychristin 	✓Hepatoprotective Flora et al. (1998); Baginskaya et al. (2000); Sokol'skaya (2000); Krepkova and Sokol'skaya (2007); Romanova and Krasavtsev (2007); Krepkova et al. (2008); Abenavoli et al. (2010); Schmidt (2012); Vargas-Mendoza et al. (2014) ✓Antioxidant Siegel and Stebbing (2013) ✓Anti-inflammatory Krepkova and Sokol'skaya (2007); Lee et al. (2007); Krepkova et al. (2008) ✓Anti-cancer Lee et al. (2013) ✓Immunomodulatory Deak et al. (1990)

increase in the reduced glutathione content in the liver. This reduction in glutathione increases the organ's protection from oxidative stress, maintaining its normal detoxification function.

Silymarin reduces inflammation via downregulation of the expression of transcription factors [nuclear factor-kappa B (NF-kB) and signal transducer and activator of transcription 1 (STAT-1)] and inflammation-associated proteins. The downregulation of transcription factors led to a significant reduction of interleukin 1 alpha (IL1α) and tumor necrosis factor-alpha (TNFα) production (Lee et al., 2013) (**Table 2**). It has also been reported that silibinin may suppress melanoma cell growth by reducing the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2. The reduction of ERK-1/2 resulted in downregulation of the MEK1/2 and reduced activity of NF-κB and STAT-3, followed by the cell-cycle arrest in the G1 (Lee et al., 2013). Interestingly, silymarin, but not silibinin, could attenuate ischemia-reperfusion-induced brain injury (Hou et al., 2010). Silibinin also reduced chemotherapeutic drug resistance in human bladder cancer cell lines via NF-kB dependent and independent pathways.

Milk Thistle Hepatoprotective Properties

Bioactive flavonoids have been used to treat hepatobiliary system diseases (Flora et al., 1998; Pradhan and Girish, 2006; Abenavoli et al., 2010) (**Table 1**). Flavonolignans isolated from the fruit of MT prevail in this group of drugs. Studies have shown that the hepatoprotective effect of these phytodrugs are due to the presence of three flavonolignans: silibinin, silidianin, and silicristin (Abenavoli et al., 2010). Numerous preclinical studies were published demonstrating hepatoprotective properties of MT or MT extracts in animal models of liver injury. Only the PubMed database holds 311 articles, including review articles, published between 1989 and 2021. Several animal models of hepatotoxicity were used by investigators, including acetaminophen, alcohol, carbon tetrachloride, phenylhydrazine, and thioacetamide (Abenavoli et al., 2010; Kantah et al., 2011; Ahmed et al., 2020).

It has been reported that liver fibrosis in rats induced by the 8-weeks intragastric administration of carbon tetrachloride (CCl4) was successfully treated with a standardized extract of the MT (Tsai et al., 2008). The extract was administered at a 200 mg/kg dose four times per week for 3 weeks. The treatment has significantly decreased aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in the serum of experimental animals.

A study confirming the hepatoprotective activity of Silimar, an original phytodrug developed by the VILAR, was conducted on a model of experimental hepatitis in rats induced by a single subcutaneous administration of oil-based CCl_4 (Baginskaya et al., 2000; Sokol'skaya, 2000). Silimar at a dose of 100 mg/kg was administered 5 days before CCl_4 administration and

continued 21 days after liver damage. The administration of CCl_4 resulted in a significant elevation of the γ-glutamyl transferase (GGT), ALP, ALT, and AST compared with healthy controls (Baginskaya et al., 2000). Following chronic administration of Silimar resulted in significantly reduced levels of GGT, ALT, AST. In an *in vitro* experiment, Silimar suppressed induced lipid peroxidation, evidenced by reducing the amount of malondialdehyde by 15–41% compared to the control (Baginskaya et al., 2000).

It appears that MT extracts have low bioavailability (El-Gazayerly et al., 2014; Mukhtar et al., 2021). The treatment of rats receiving CCl_4 with silymarin incorporated into phytosomes significantly decreased superoxide dismutase activities and glutamic pyruvic transaminase compared to the plant extract free of phytosomes (El-Gazayerly et al., 2014). Additionally, the treatment with encapsulated silymarin inhibited liver fibrosis induced in rats by administering 2 mg/kg acetaminophen for 2 weeks (Mukhtar et al., 2021). The treatment significantly decreased levels of ALT, AST, and ALP.

Several investigators have studied the pharmacokinetics of MT in healthy volunteers and patients with various liver diseases. The study published by Calani et al. investigated the bioavailability and metabolism of flavonolignans after a single administration of the water-soluble MT extract (Calani et al., 2012). After overnight fasting, the healthy volunteers consumed 8 g of the extract dissolved in the water in this study. The study showed that MT flavonolignans have low bioavailability (0.45%). Urinary excretion was studied following 48 h. Thirty-one metabolites of flavonolignans have been found in the urine. The most common metabolites were monoglucuronides followed by sulfate–glucuronides and diglucuronides. Zhu et al. investigated the pharmacokinetics of individual flavonolignans in humans following single or chronic administration of MT extract (Zhu et al., 2013). The authors determined that all investigated flavonolignans were rapidly absorbed and eliminated by the organism. Flavonolignan exposure was dose-dependent. The most prevalent flavonolignans detected in volunteers were silybin A and silybin B, followed by isosilybin B and isosilybin A. The pharmacokinetics of MT flavonolignans was significantly altered in patients infected with Hepatitis C virus and patients with non-alcoholic fatty liver disease (Abenavoli et al., 2018). High exposure to bioactive flavonolignans was observed in patients with live cirrhosis.

Several MT phytodrugs are marketed under different brand names (e.g., Silymarin, Legalon, Carsil, and Silibor). Legalon is a standardized phytodrug containing 70–140 mg silymarin (calculated as silibinin) in 86–186 mg of dry extract of the fruit (European OTC medicines and devices., 2020). The phytodrug is intended to treat acute and chronic hepatitis and cirrhosis. Legalon at a 25 mg/kg dose reduced lipid peroxidation and increased superoxide dismutase activity in hepatic tissues (Batakov, 2001). It has been reported that workers chronically exposed to toluene and/or xylene vapors receiving oral Legalon for 30 days had significant improvements in liver function and platelet counts compared with workers that did not receive this treatment (Szilard et al., 1988). Legalon-SIL® has been used for intravenous treatment of patients infected with hepatitis C virus (HCV) and not responding to pegylated interferon/ribavirin treatment (Rutter et al., 2011). Most of the patients (85%) had undetectable HCV ribonucleic acid (RNA) after a 14-days course of Legalon-SIL®.

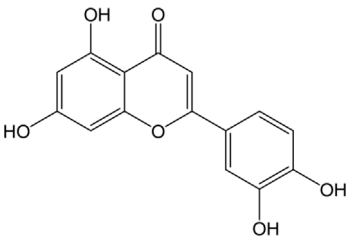
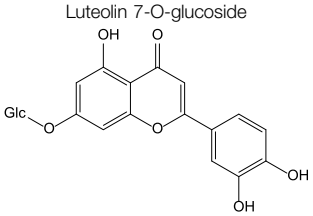
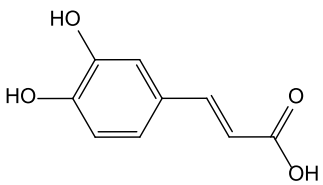
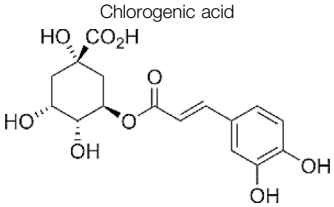
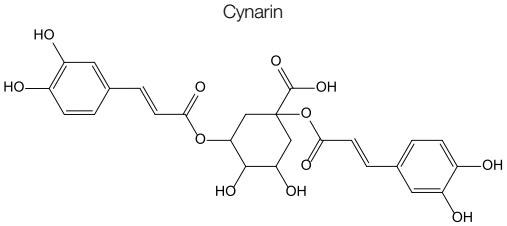
Many clinical trials were conducted to find the efficacy of MT extracts in humans with various liver diseases. The first trial that we were able to find was conducted in 1980 and investigated the effectiveness of the MT derivatives for the treatment of chronic hepatopathies (De Martiis et al., 1980). The first randomized placebo-controlled trial was performed in 1989 and investigated the efficacy of silymarin in patients with liver cirrhosis (Ferenci et al., 1989). The patients in this trial received 140 mg of silymarin three times a day for an extended period. It has been determined that the 4-years survival rate in the treatment group was significantly higher compared with patients receiving placebo. Another double-blind placebo-controlled clinical trial proved the efficacy of milk thistle for treating patients with acute hepatitis (El-Kamary et al., 2009). The therapy reduced symptoms of biliary retention such as jaundice and scleral icterus. Interestingly, unlike in animal experiments, the reduction of ALT and AST in human subjects was not significant (El-Kamary et al., 2009). The evaluation of the efficacy of silymarin for treatment of non-alcoholic steatohepatitis has shown that patients receiving 700 mg of silymarin for 48-weeks had comparable to control non-alcoholic fatty liver disease activity scores; however, silymarin-treated patients had significantly lower liver fibrosis (Wah Kheong et al., 2017).

A more detailed analysis of available clinical data confirming hepatoprotective properties of MT is available in the recently published articles by Abenavoli et al. (Abenavoli et al., 2018) and Marmouzi et al. (Marmouzi et al., 2021). The literature analysis led us to conclude that MT standardized extracts can improve liver function following several hepatic disorders, including chronic exposure to toxic chemicals, viral hepatitis, liver cirrhosis, and non-alcoholic liver steatosis. However, this treatment cannot cure the disease. In our opinion, this treatment can be combined with the standard of care therapies, especially when there is no improvement following conventional treatment.

Milk Thistle as Food and MT-Based Functional Food

MT oil, oilseed cake, or seeds could fortify bakery products (Semenkina, 2010; Ataei Nukabadi et al., 2021) (**Table 1**). MT fruits contain a complex of biologically active substances (vitamins, minerals, flavonoids, a significant amount of dietary fiber, and amino acids) (Semenkina, 2010). The MT seed oil is enriched with omega-6 and omega-3 fatty acids, tocopherols, and carotenoids. The addition of MT in bakery products may increase the nutritional value by supplying extra proteins, linoleic acid, vitamin E, and calcium. The presence of flavonolignans in the MT oilseed cake could supply additional support for liver function. MT flavonolignans may help increase bone calcium absorption when added to bakery products combined with fat-free milk (Semenkina, 2010; Nekrasova and Popov, 2020). The addition of nettle leaf and MT seed powder to sponge cakes could reduce

TABLE 3 | Phytochemical composition and pharmacological properties of artichoke.

Plant name/part of the plant commonly used for medicinal purposes	Bioactive constituents	Pharmacological properties
Artichoke (*Cynara cardunculus* L.)/ immature flower 	Luteolin  Luteolin 7-O-glucoside  Caffeic acid  Chlorogenic acid  Cynarin 	✓Hepatoprotective Ben Salem et al. (2015) ✓Anti-oxidant Ben Salem et al. (2015) ✓Anti-inflammatory Ben Salem et al. (2015) ✓Anti-cancer Abdel-Moneim et al. (2021); Villarini et al. (2021) ✓Anti-viral Elsebai et al. (2016) ✓Immunomodulatory ✓Hypolipidemic Bundy et al. (2008); Ben Salem et al. (2015)

blood sugar levels (Ataei Nukabadi et al., 2021) and improve liver functions.

Andrzejewska with colleague suggested that whole MT plant can be used as food and cosmetics (Andrzejewska et al., 2015). According to the Edible Wild Food resource, "the young stalks, leaves, roots and flowers can be eaten" (Edible Wild Food., 2021). Mediterranean communities have been using MT young stems as food for centuries (Andrzejewska et al., 2015). Spanish natives have been adding aerial parts to salads or eating them cooked (Andrzejewska et al., 2015). Unfortunately, the reports on the use of MT as food or functional food for humans are limited and primarily focused on the use of plant fruit, not aerial parts, potentially due to the presence of spikes on the leaves. MT may be an active ingredient of the cream intended to treat melasma (Nofal et al., 2019).

Milk Thistle as Animal Feed

The MT can also be used as a part of functional food for farm animals (**Table 1**). MT oilseed cake is enriched in crude proteins, dietary fiber, and fat (Stastnik et al., 2020). The seed cake contains ~4% of flavonolignans (Stastnik et al., 2020). It has been reported that the addition of MT oilseed cake in the diet of lactating dairy cows in the amount of 25% of the required digestible protein increased the protein and fat content in the milk, improved its amino acid composition, and augmented the average daily milk yield (Chabaev et al., 2011). The administration of MT oilseed cake at a dose of 44 mg/kg/day to Ayrshire heifers during the first twenty-first months of their lives reduced the number of diseases caused by impaired liver function and metabolic processes by ~35% and prevented mortality of the livestock from the first month of cattle life to the end first lactation (Kravainis et al., 2014). The supplementation of the sow diet with MT at the dose 100 mg/kg/day starting from 88 days of gestation to farrowing positively affected the fetal formation and the weight of newborn piglets (Kolesnyk and Bankovska, 2008). The addition of MT to the sow fodders prevented suckling pig's mortality and increased the average daily gain by ~19% (Kolesnyk et al., 2009). Chickens receiving MT extract at doses of 0.1; 1.0; 1.5 and 2.0 mg/kg had the average daily body weight gain during the rearing period by 1.1–5.3%, the European productivity index increased 2.4–10.2%, and feed consumption per 1 kg of the weight gain increased 1.6–4.8% compared to the control group (Bagno et al., 2020). Feeding MT oilseed cake in combination with ascorbic acid to broiler chickens reduced the concentration of lead and cadmium in meat by 2.72 and 2.08 times and increased its biological value (Mildzikhov et al., 2013). Adding oilseed cake to the hens' feed increased egg production and egg size (Stastnik et al., 2020). Some studies reported no effect on chicken wellbeing (Stastnik et al., 2020). As shown above, the fortification of farm animals' fodder with the MT oilseed cake has significantly improved animals' survival.

It has been hypothesized that the aerial part of the plant can also be used to fortify farm animal feed (Andrzejewska et al.,

2015). It has been reported that MT can be used as supplemental food in cattle and chicken (Bagno et al., 2020; Shemuranova and Garifullina, 2020). Unfortunately, the nutritional value of the MT areal part is lower than barley (Stastnik et al., 2020).

ARTICHOKE

Cynara cardunculus L. is a member of the Asteraceae family. There are three taxonomic variants of the *Cynara cardunculus* species: *Cynara cardunculus* L., var. *scolymus* (L.) Fiori (globe artichoke), *Cynara cardunculus* L., var. *altilis* DC. (cultivating cardoon) and *Cynara cardunculus* L., var. *sylvestris* (Lamk), Fiori (wild cardoon) (Gostin and Waisundara, 2019). The plant is native to the Mediterranean area, and nowadays, it is cultivated in other countries (Gostin and Waisundara, 2019). Artichoke prefers hot and dry climates and can live in an adverse environment. The artichoke immature flower heads have a bitter-sweet flavor and are part of the diet in many countries.

Artichoke bioactive constituents have antibacterial, anti-malarian, antiviral (Hep C virus), anti-inflammatory, antioxidative, hepatoprotective, and metabolic effects (Ben Salem et al., 2015; Elsebai et al., 2016; Villarini et al., 2021) (**Table 3**). Constituents isolated from artichoke (e.g., cynaropicrin) are involved in the regulation of the NF-κB pathway and downregulate inflammatory cytokines (Elsebai et al., 2016). An extract of the artichoke leaves has anti-hypercholesterolemic and antioxidative properties in an *in vivo* model of rats with high-fat diet-induced obesity (Ben Salem et al., 2019). Prophylactic use of artichoke leaf and flower head extracts may also protect against cancer development (Abdel-Moneim et al., 2021). The preparations from artichoke can be added to cosmetics intended for prophylaxis of skin photoaging (D'Antuono et al., 2018).

Numerous companies market artichoke to improve liver function dietary supplement (Zeaiter et al., 2019; GistGear, 2021) (**Table 1**). The artichoke is often used in combination with MT and dandelion (GistGear, 2021). The immature flower is the most used part of the plant in the food industry. As a result, >80% of biomass can be wasted (Barbosa et al., 2020). The use of wasted artichoke biomass could produce phytodrugs, dietary supplements, biofuel, biodiesel, cellulose, and animal feed.

Artichoke Bioactive Constituents and Their Biological Activity

The artichoke plant has significant levels of polyphenols, sesquiterpene lactones, terpenoids, carotenoids, and chlorophylls (Turkiewicz et al., 2019; Zeaiter et al., 2019; Rocchetti et al., 2020) (**Table 3**). The artichoke is an excellent source of inulin, minerals (e.g., potassium and phosphorus), and vitamins (vitamins group B and vitamin C) (Bundy et al., 2008; Zeaiter et al., 2019). Levels of phytochemicals can vary significantly depending on the taxonomic variant, cultivar, or hybrid plant (Turkiewicz et al., 2019). The artichoke bracts have elevated levels of inulin and polyphenols (Turkiewicz et al., 2019). The analysis of phytochemicals in the seed-propagated artichoke hybrids revealed that leaves of artichoke contain >90 polyphenols and >120 sesquiterpene lactones (Rocchetti et al., 2020). Seven cultivars were investigated by Rocchetti and others (Rocchetti et al., 2020). The authors reported significant differences in the composition of bioactive compounds present in these cultivars. As proved by Turkiewicz and others, the health-promoting benefits of artichoke depended on the levels of bioactive phytochemicals present in cultivars and hybrids (Turkiewicz et al., 2019).

The preclinical studies investigating pharmacological properties of the artichoke leaf compounds have found that the hepatoprotective properties of the phenolic compounds (e.g., luteolin, chlorogenic and caffeic acids, cynarin and luteolin 7-O-glucoside) are associated with their ability to reduce plasma levels of malondialdehyde induced by t-BHP and as a result decrease of free radicals and hepatocyte damage (Ben Salem et al., 2015) (**Table 3**). The phenolic leaf compounds also prevent lipid peroxidation. Additionally, phenolic compounds may exhibit hypocholesterolemic, anti-cholestatic, and antimicrobial effects (Ben Salem et al., 2015). The treatment of volunteers with total plasma cholesterol levels 6.0–8.0 mmol/l with artichoke leaf extract (ALE) significantly decreased total cholesterol levels, but not low-density lipoprotein and high-density lipoprotein (Bundy et al., 2008). An *in vitro* study demonstrated that treatment of HT-29 and RKO tumor cells with cynaropicrin, caffeoylquinic acids, and chlorogenic acid-induced cell apoptosis suggests that ALEs could be potentially used as an effective tool chemotherapeutic agent (Villarini et al., 2021) (**Table 3**).

Artichoke Hepatoprotective Properties

The hepatoprotective properties of the artichoke were actively studied in animal models of acute and chronic liver injury (**Tables 1**, **3**). In 1987, Adzed and others showed that cynarine and caffeic acid possess cytoprotective properties *in vitro* experiments (Adzet et al., 1987). The ethanolic ALE was given to experimental rats for 60 days, and a high-fat diet was significantly reduced by high-fat diet levels of ALT, AST, and ALP (Ben Salem et al., 2019). Another study reported that the ALE treatment of rats with diazinon-induced liver injury reduced levels of ALT, AST, ALT, and TNFα gene expression in hepatocytes (Ahmadi et al., 2019). The treatment of rats with ALE for 2 weeks improved ALT and AST levels after CCl_4 hepatic injury and decreased DNA fragmentation and caspase 3 levels in liver tissues of CCl_4-exposed rats (Colak et al., 2016). While most of the studies evaluating the hepatoprotective properties of the artichoke were done using leaf extracts. Sumer and others investigated the hepatoprotective properties of artichoke stems and bracts in an animal model of acetaminophen-induced liver injury in rats (Sumer et al., 2020). They found that stem and bract extract also effectively reduced ALT and AST levels but not ALP. It appears that the intensity of hepatoprotective effects of artichoke extracts is associated with the levels of phenolic compounds present in the extract (Speroni et al., 2003).

Artichoke extracts are marketed as phytodrugs for the treatment of liver disorders (Lattanzio et al., 2009). A double-blind clinical trial in patients with non-alcoholic liver injury confirmed the results of preclinical studies. Patients receiving ALE 600 mg daily for 2 months had an increased hepatic vein flow, reduced portal vein diameter, and improved lipid and

hepatic enzyme profiles (Panahi et al., 2018). Another trial investigated the efficacy of a standardized ALE in patients with chronic Hep C infection (Huber et al., 2009). Unfortunately, the treatment of patients for 12 weeks with ALE 3,200 mg/day did not improve hepatic enzymes levels while reducing the patient's fatigue and joint problems.

The reviewed literature confirmed that the artichoke extracts from leaves and other parts of the plant exhibit strong hepatoprotective properties (**Table 1**). More clinical trials are needed to verify the hepatoprotective properties of artichoke extracts in humans.

Artichoke Use as Food or Functional Food

The artichoke was used as food from ancient times in Egypt, Greece, and Rome. Immature flowers and stems were used to prepare dishes, while mature flowers coagulate milk (Gostin and Waisundara, 2019) (**Table 1**). Modern-day artichoke flowers are exceedingly popular worldwide as a part of the everyday diet or delicatessen food. Immature flowers can be used fresh and canned or frozen for future use (Gostin and Waisundara, 2019). The artichoke was traditionally used in Mediterranean cuisine to prepare salads, dips, or soups (Barbosa et al., 2020). It can also be roasted with garlic and olive oil. The artichoke flowers and stigmas are used as a vegetable alternative to animal-driven rennet (Barbosa et al., 2020). The ability to clot milk is attributed to two enzymes mature artichoke flowers, cardosins A and B. The artichoke seed oil is enriched by linoleic, oleic, palmitic, and stearic fatty acids (Barbosa et al., 2020). The oil obtained from artichoke seeds is similar in composition to sunflower oil and can be used for human consumption and as a dietary supplement.

Artichoke is a rich source of non-digestible inulin, a heterogenous fructose polymer (Niness, 1999; Nwafor et al., 2017) and oligofructose, a shorter-chain oligomer, belonging to the inulin subgroup, which can be used as a part of dietary supplement blends or can be added to food to enhance its health benefits (Lattanzio et al., 2009). Inulin is well-known prebiotic that can support grows of a healthy intestinal microbiome. Antioxidant properties of phenolic compounds can be used in a functional food to support immune, endocrine, and cardiovascular systems (**Table 3**).

The biomass that is not used by the food industry could be a source of animal feed or feed supplement for farm animals, especially in areas of extensive artichoke cultivation. Silage artichoke (e.g., artichoke bracts and plants) has been investigated as an animal feed with generally positive results for animals and animal products (Meneses et al., 2007; Monllor et al., 2020) (**Table 1**). The analysis of artichoke foliage revealed that it is safe to use as animal feed.

Artichoke By-Products Use

A significant part of the artichoke is wasted after the food industry uses artichoke flowers. The alternative use of the artichoke aerial part is presented in **Figure 1**. It has been proposed to use remaining lignocellulosic biomass as solid biofuel for heating houses or energy generation (Fernández et al., 2006; Barbosa et al., 2020). Several studies evaluated the heating value and combustion of artichoke biomass (Fernández et al., 2006; Oliveira et al., 2012; Gominho et al., 2018). Artichoke biomass and oil can be used for biodiesel production (Fernández et al., 2006). A bioplastic prepared from a plant's leaves can potentially replace slowly degrading plastic (Mirpoor et al., 2022). Gominho et al. suggested using artichoke stalks as a source for paper production (Gominho et al., 2001). It appears that the artichoke has good potential to be used from stem-to-stern in food, pharmaceutical, and bioenergetic industries. The use of waste by food industry by-products could have a significant impact on the environment.

CHICORY

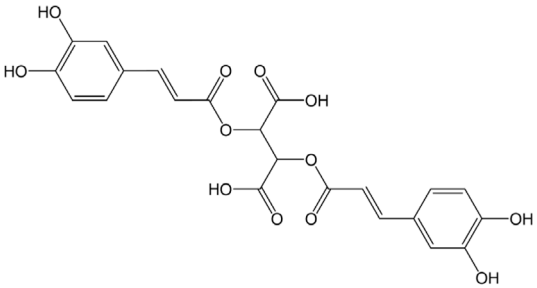
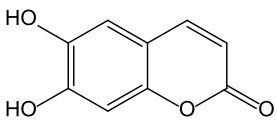
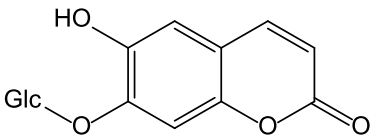
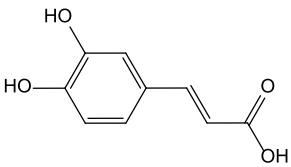
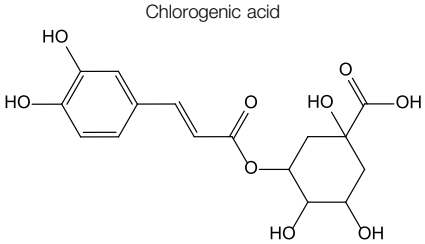
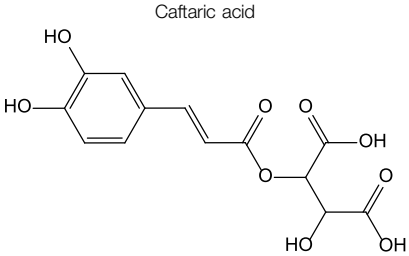
Chicory, *Cichorium intybus* L., a member of the Asteraceae family, is a well-known plant cultivated in Europe, Asia, Canada, the eastern part of the United States, northern Africa, and Australia (Kandeler and Ullrich, 2009). Practically, all plant parts are used as a source of food, phytodrugs, and dietary supplements (Perovic et al., 2021) (**Table 1**). In the past, fresh plant roots and young leaves were used as vegetables (Kandeler and Ullrich, 2009; Al-Snafi, 2016; Perovic et al., 2021). The plant was used to treat pulmonary and reproductive systems diseases, the biliary tract, hepatic disorders, diarrhea, and cancer (Al-Snafi, 2016). The hepatoprotective activities of chicory have been established in animals and humans (Sama et al., 1976; Saxena and Garg, 1979; Thabrew et al., 1982; Cimen et al., 2020). Inulin, a chicory root constituent, is extremely popular among consumers as a prebiotic and source of dietary fiber (Shoaib et al., 2016). It is sold as a dietary supplement worldwide. Additionally, chicory root and root bioactive constituent, inulin, are often used in the food industry as a coffee substitute or substitute for fat and sugar in pastry and ice cream (Kandeler and Ullrich, 2009; Shoaib et al., 2016; Wu and Cadwallader, 2019).

Chicory Bioactive Constituents and Their Biological Activity

The chicory plant contains numerous biologically active constituents, including inulin, sesquiterpene lactones (e.g., chicoroisides B and C, sonchuside C), flavonoids, alkaloids, caffeic acid derivatives (e.g., chicoric acid, chlorogenic acid), vitamins E, β-carotene, and minerals (calcium, phosphorus, magnesium, and potassium) (Al-Snafi, 2016; Perovic et al., 2021) (**Table 4**). The distinct parts of the plant may contain different amounts of bioactive constituents. Plant roots are enriched in inulin and tannins but contain low amounts of phenolic acids (Al-Snafi, 2016). Inulin comprises ~70% of constituents present in the fresh chicory root (Nwafor et al., 2017). The leaves and seeds contain high levels of phenolic compounds and flavonoids and low levels of inulin (Al-Snafi, 2016). Both wild and cultivated chicory is used in the food and dietary supplement industries. The aerial part of the plant may be used as a raw material for hepatoprotective phytodrugs, while the food and dietary supplement industries use roots.

Chicory is reported to have several pharmacological properties, including antimicrobial, anthelmintic, antimalarial, anti-inflammatory, antioxidant, gastroprotective, and

TABLE 4 | Phytochemical composition and pharmacological properties of chicory.

Plant name/part of the plant commonly used for medicinal purposes	Bioactive constituents	Pharmacological properties
Chicory (*Cichorium intybus* L.) Aerial part and roots  	Chicoric acid  Esculetin  Cichoriin  Caffeic acid  Chlorogenic acid  Caftaric acid 	✓Hepatoprotective Ferenci et al. (1989); Huseini et al. (2005); Kolesnyk et al. (2009); Bortnikova et al. (2021) ✓Antibacterial/anthelmintic Perovic et al. (2021) Street et al. (2013) ✓Antiviral Janda et al. (2021) ✓Anti-inflammatory Huseini et al. (2005); Street et al. (2013) ✓Antioxidant Huseini et al. (2005);Street et al. (2013) ✓Gastroprotective Street et al. (2013) ✓Hypolipidemic Janda et al. (2021) ✓Immunomodulatory Huseini et al. (2005) ✓Anticancer Janda et al. (2021)

hepatoprotective (Street et al., 2013; Al-Snafi, 2016; Janda et al., 2021; Perovic et al., 2021) (**Table 1**). In addition, chicoric acid has hypoglycemic activity and antiviral effects (Zhang et al., 2014; Ferrare et al., 2018). Aqueous extracts from cultivated chicory possess antioxidant properties that were confirmed in animal models (Saybel', 2020). Hepatoprotective properties were reported for flavonoids isolated from *Cichorium glandulosum* Boiss. et Huet (Tong et al., 2015) (**Table 4**). Phenolic flavonoids extracted from leaves of chicory and artichoke also demonstrated hepatoprotective properties (Mohafrash and Mossa, 2020). The constituents present in the chicory extract are oxycoumarins: esculetin and cichoriin, hydroxycinnamic acids: chicory, chlorogenic, caftaric acids (Petropoulos et al., 2017; Saybel', 2020) (**Table 4**). These constituents have antioxidant and

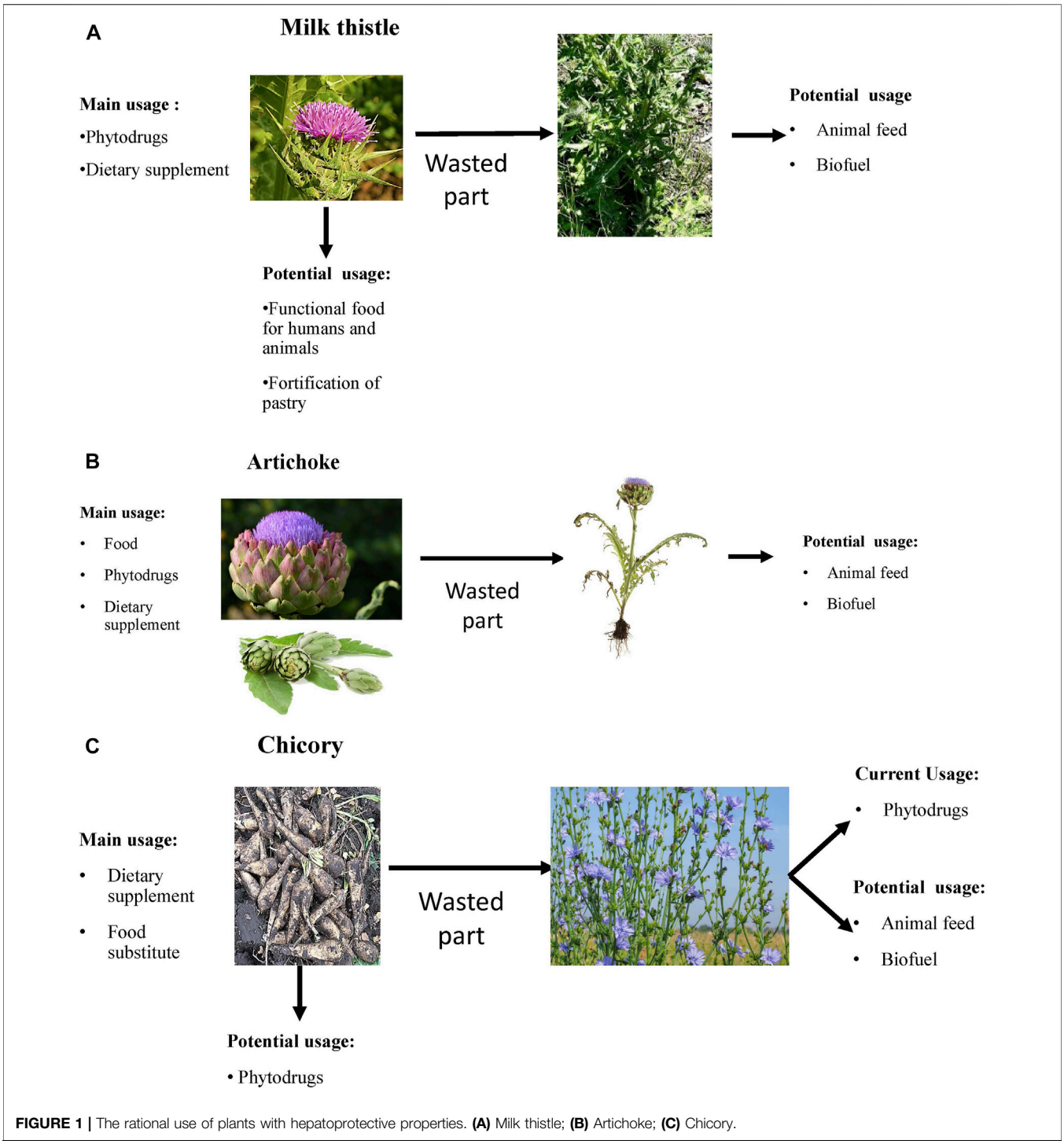


FIGURE 1 | The rational use of plants with hepatoprotective properties. **(A)** Milk thistle; **(B)** Artichoke; **(C)** Chicory.

hepatoprotective properties. It is suggested that the complex biologically active constituents such as polyfructose, hydroxycinnamic acids, coumarins, and flavonoids, responsible for the plant's hepatoprotective properties (Domitrovic and Potocnjak, 2016).

A double-blind, randomized controlled trial conducted in patients with chronic periodontitis has determined that treatment with 1 g of the chicory leaf extract decreases serum levels of total cholesterol, low-density lipoprotein cholesterol, and triglycerides, while increased serum levels of high-density lipoprotein cholesterol, uric acid, and total antioxidant capacity (Babaei et al., 2018). Additionally, the authors reported that the treatment decreased periodontal pocket depth.

Several clinical trials were conducted to evaluate inulin or chicory coffee's effects on various human organ and system functions (e.g., lipid profile, bowel movement, and platelet

aggregation Schumacher et al., 2011; Grela et al., 2014; Buddington et al., 2017). One of these studies evaluated whether chicory coffee has any beneficial effects on the cardiovascular system (Schumacher et al., 2011). The consumption of chicory coffee for 1 week resulted in a significant decrease in whole blood, red blood cell deformability, plasma viscosity, and macrophage migration inhibitory factors. A more recent randomized crossover trial has determined that supplementation of diets with chicory inulin contacting snack bars of people with low fiber intake led to an increase of beneficial members of the human intestinal microbiome, *Bifidobacterium* genus (Reimer et al., 2020).

Chicory Hepatoprotective Properties

Cichorium intybus is a part of a phytodrug (Liv-52) introduced to the pharmaceutical market by the Himalaya Drug Company in 1955 (Himalaya Wellness Company., 2021b). Liv-52 includes *Capparis spinosa, Cichorium intybus, Solanum nigrum, Cassia occidentalis, Terminalia arjuna, Achillea millefolium,* and *Tamarix gallica* (Huseini et al., 2005; Himalaya Wellness Company., 2021b). Currently, Liv-52 and its modifications are widely used as dietary supplements in the United States and European countries. Liv-52 prevents the loss of functional integrity of the cell membrane, maintains cytochrome P-450 activity, and promotes hepatocellular regeneration (Himalaya Wellness Company., 2021b). The high hepatoprotective properties of this phytodrug were confirmed in patients with viral hepatitis, alcoholic and fatty liver, cirrhosis, anorexia (Sama et al., 1976; Huseini et al., 2005). Patients receiving Liv-52 for six consecutive months in a double-blind placebo-controlled trial had decreased ascites and significantly lower ALT and AST levels (Huseini et al., 2005). Huseini et al. attributed the hepatoprotective properties of the phytodrug to its antioxidant, anti-inflammatory, and immunomodulatory properties (Huseini et al., 2005). Recently, it has been proven that Liv-52 could treat ischemia reperfusion-induced liver damage in rats (Cimen et al., 2020).

Another clinical trial reported that supplementation of the diets of patients with non-alcoholic fatty liver disease for 12 weeks with a mixture of turmeric and chicory seeds resulted in a significant decrease of participants' BMI and waist circumference and levels of serum alkaline phosphatase (Ghaffari et al., 2019).

We recently presented data demonstrating hepatoprotective properties of standardized dry extract obtained from the aerial part of wild chicory (*Cichorium intybus* L., DEC) (Bortnikova et al., 2021). DEC was developed at the VILAR. The analysis of the chemical composition revealed that the primary constituents present in DEC are phenol carboxylic acids [e.g., esters of caffeic, ferulic, coumaric acids with organic acids (quinic and tartaric)], flavonoids (isoquercetin, astragalin, rutin, luteolin, and kaempferol), and oxycoumarins (esculetin, cichoriin) (Saybel', 2020). The DEC contains 9.20 ± 0.43% of phenolic constituents calculated as chicoric acid.

DEC has significant hepatoprotective properties confirmed in a model of acute toxic hepatitis induced by a single subcutaneous injection of mercuric chloride ($HgCl_2$). Mercuric chloride is a potent thiol poison leading to protein structure damage and inhibition of thiol-containing antioxidants and liver damage caused by the development of oxidative stress (Deng et al., 2012).

Mercuric chloride intoxication led to increased relative liver weight in the $HgCl_2$ treated group (Bortnikova et al., 2021). Rats treated with $HgCl_2$ had a statistically significant increase in total protein, glucose, total cholesterol, total bilirubin, triglycerides, and the GGT, ALP, AST, and AST activities. The histological liver analysis showed the presence of hepatocyte dystrophy, characterized by hyaline droplet dystrophy. Treatment of rats with DEC in doses of 100 and 500 mg/kg for 3 weeks prevented a sharp decrease in the animals' body weight and physical activity. The relative liver weight was lower than in animals treated with $HgCl_2$ (Bortnikova et al., 2021). The administration of the DEC resulted in a decrease in the activity of several liver enzymes characterizing the functional state of the liver and contributed to experimental rats' fast recovery (Bortnikova et al., 2021). These changes were more noticeable in animals receiving the maximal DEC dose. Lipid, protein, glucose, and bilirubin levels decreased significantly in the DEC groups compared to the $HgCl_2$ group. The dystrophic changes in hepatocytes of rats treated with DEC 500 mg/kg were not visible. The comparison of hepatoprotective activity of DEC and Silimar indicates that the hepatoprotective effect of DEC at the dose 500 mg/kg was comparable with the effects observed in the group receiving 100 mg/kg of Silimar (Bortnikova et al., 2021). Preclinical data demonstrating DEC hepatoprotective properties in rats need to be confirmed in clinical studies.

Additionally, we have reported that DEC has immunomodulatory properties (Saybel', 2020). Oral administration of DEC to immunosuppressed mice at a 50 mg/kg dose for five consecutive days resulted in upregulation of innate, humoral, and cell-mediated immune responses. Similar upregulations were not observed in animals with normal immune responsiveness.

Chicory-Based Functional Food and Supplements

The food and dietary supplement industries actively use chicory roots (e.g., inulin and oligofructose) as a food substitute, dietary, functional, or food supplements (Wu and Cadwallader, 2019) (**Table 1**). Inulin is widely used as a prebiotic supporting the intestinal microbiome, specifically stimulating the development of the bifidobacteria in the colon (Niness, 1999). Additionally, inulin acts as dietary fiber (Niness, 1999). Therefore, inulin consumption may increase intestinal peristalsis and may help people experiencing constipation.

Overconsumption of fatty and sugary food has contributed to the disproportional increase in the number of people who are obese or have diabetes. Therefore, reducing fat and sugar in food products could help reduce this trend. Moreover, many people are interested in a healthy lifestyle and healthy eating habits. When dissolved in the water or milk, inulin forms a creamy texture and gives the food a fatty feel (Niness, 1999). Oligofructose has a sweet taste and can be used as a sugar substitute (Niness, 1999). Replacement of high-fat milk and

sugar with inulin and oligofructose in milk products (e.g., ice cream) may significantly reduce fat and sugar consumption. At the same time, it will not compromise product taste. Significantly, inulin and oligofructose do not influence glucose levels and insulin secretion (Niness, 1999).

Animal Feed

Lastly, the aerial part and root of chicory can be used as fodder and complementary treatment of livestock (Nwafor et al., 2017) (**Table 1**). It appears that chicory extracts or phytochemicals like inulin and volatile oils can eliminate or suppress the growth of intestinal worms and other parasites found in animals (Githiori et al., 2006). Therefore, fodder fortified by chicory foliage can promote overall livestock health and prevent intestinal parasite development. Adding 0.1% of chicory powder to the broiler's diet increased body weight but significantly lower abdominal fat compared to control birds (Khoobani et al., 2020). The same study reported that adding probiotics or chicory to the diet improved the broiler's ileal microbiome. When chicory is given in excessive amounts to farm animals, it can negatively affect the growth and performance of the livestock.

DISCUSSION

This review article has focused on the complex use of distinct parts of MT, artichoke, and chicory for medicinal purposes, food for humans and animals, and potentially biofuel. As presented in the preceding sections, all parts of these plants can potentially be used as phytodrugs, dietary supplements, components of functional food, and food for humans and animals. It is important to note that usually, only one part of these plants is actively used by the food or drug/dietary supplement industry, and as a result, dozens of articles focus on the biological properties of this part of the plant and constituents responsible for its activity. There are very few articles analyzing real-world aspects of by-product use. Review articles summating available literature on by-product use cite 1–2 articles or state that based on the known biological activity of by-product, it can be used as a dietary supplement, animal feed, or biofuel.

The actively used part of MT is plant fruit (e.g., whole fruit, seeds, oilseed cake, or oil) (**Figure 1**). It is actively used for medicinal purposes to produce phytodrugs and dietary supplements but can also be added to bakery products. The fortification of the bread with MT oil and oilseed cake improves the bread quality and may help support proper liver function and provide an additional source of amino acids, minerals, and vitamins. Several reports demonstrate the benefits of oilseed cake as a supplement in improving the wellbeing and survival of farm animals (see Section 3.4). The question then becomes what happens to the remaining parts of the plant. Are they wasted? MT leaves are occasionally used to prepare salads. The addition of the aerial part of MT to animal feed may have nutritional benefits, helping to improve the weight gain and survival of farm animals (Andrzejewska et al., 2015; Bagno et al., 2020). It is doubtful that the aerial part of the plant will be used as the main feed for animals due to its low nutritional value (Stastnik et al., 2020). The aerial part represents 70–80% of the whole plant and can be potentially used as a biofuel source.

The food industry uses flowers of artichoke (**Figure 1**). It appears that 80% of the plant is wasted. Artichoke leaves demonstrate many biological activities, including hepatoprotective. Therefore, they can be used for medicinal purposes as a phytodrug or dietary supplement. It has been proposed that the aerial part of artichoke can be used as animal feed and biofuel.

The most used part of chicory is the root (**Figure 1**). Roots from cultivated chicory are inulin's core sources, actively used as dietary supplements and substitutes for fat and sugar. The aerial part of the plant is usually wasted. Bioactive constituents present in the aerial part of the plant possess hepatoprotective properties. The aerial part of the cultivated plant can serve as a raw material for the pharmaceutical or dietary supplement industry, and potentially, as biofuel.

As another example of a plant with the predominant use of roots is licorice (*Glycyrrhiza glabra*). It is a cough remedy (Kamei et al., 2003; Nosalova et al., 2013; Ruetzler et al., 2013; Kuang et al., 2018) and a gum flavor for candies and food (Kwon et al., 2020). It can treat digestive problems, atopic dermatitis, bacterial and viral infections (Kwon et al., 2020). The licorice roots also possess hepatoprotective properties (Li et al., 2019). It has been reported that licorice ethanolic extract prepared in the leaves possesses antimicrobial activity against Gram-positive bacteria, which was higher than in extract prepared from roots (Irani et al., 2010). Compounds isolated from the leaves of *G. uralensis* demonstrated anti-inflammatory properties *in vitro* (Wang et al., 2019).

One of the best examples is the stem-to-stern use of pumpkin (*Cucurbita pepo*) and corn (*Zea mays*). Pumpkin is a well-known source of food for humans and farm animals. It is highly nutritional (Klyuchnikova et al., 2011; Brennan, 2020). Pumpkin is rich in proteins, carbohydrates, dietary fiber, vitamin A, vitamin C, magnesium, and potassium (Brennan, 2020). All parts of the pumpkin are edible, including seeds, seed oil, and even leaves. Pumpkins can be given to domestic animals to support their digestive system (Lans, 2019). Elevated levels of α-carotene, β-carotene and β-cryptoxanthin, well-known antioxidants, and vitamin C could help to improve immune system functions and protect against infections, reduce risk of cancer development (Ben-Amotz and Fishier, 1998; Zhou et al., 2016).

Corn is high in dietary fiber, regulates bowel movement, prevents constipation, and decreases cholesterol and glucose levels (Cooper et al., 2017). Various parts of corn are used as a human food source (ear of corn, corn flour, cornmeal, and high-fructose corn syrup), food for farm animal food (plant), a source for fuel, ethanol, and plastic (Fowler, 2012; Gwirtz and Garcia-Casal, 2014). Corn constituents are also used as dietary supplements. For example, corn contains inositol hexaphosphate (IP6), a phosphorylated carbohydrate that has anti-tumor properties and modulatory effects on macrophages (Wee et al., 2021).

The potential of the whole plant use for MT, artichoke, chicory, and other plants is evident. Unfortunately, different

industries use distinct parts of the plant (**Figure 1**). Other parts of the plant are likely wasted. Considering the growing human population, responsible management of plant resources could provide sufficient medicine and food supplies for humans and animals.

The whole plant use should be a priority for businesses and the government. For example, when chicory plant is collected at the field, roots can be sold to the food industry and aerial part to pharmaceutical/dietary supplement companies. Alternatively, dietary supplement companies producing inulin from roots can use the aerial part of the plant to produce supplements supporting liver function.

We feel that the research community needs to analyze and summarize the potential use of distinct parts of plants. This includes estimating the economic judiciousness and proposing a complex and rational use plan for plants to pharmaceutical/dietary supplements, food industries, and country government. The joint efforts with the government must stimulate companies to utilize by-products coming from their technological processes by providing, for example, tax credits.

Many plants used by pharmaceutical and dietary supplement companies are wild plants. About 20 North American medicinal plants are at risk of endangerment due to overharvesting and decreased natural habitats (United Plant Savers, 2021). The development of new plant cultivation techniques could help overcome the overharvesting of precious plants.

CONCLUSION

Milk thistle, artichoke, and chicory are highly versatile and valuable plants that can be potentially used as phytodrugs, dietary supplements, functional food, food for humans and animals, or biofuel. Distinct parts of these plants are used by pharmaceutical/dietary supplement and food industries. Other parts of plants are possibly wasted. These are only three examples of the potential use of the whole plant. There are many other plants, some parts of which are used, and others are wasted. A plan for the rational stem-to-stern use of the whole plant needs to be developed for most, if not all, plants actively used by food or drug/supplement industries. This should include measures to prevent wild plant overharvesting and the active introduction of cultivation techniques utilizing all plant parts.

AUTHOR CONTRIBUTIONS

LK, EE, and CS contributed to the conception, design, and preparation of the manuscript. EE, LK, AB, OS, IL, OK, KJ, and CS contributed to the literature search and interpretation of published data. EE, LK, AB, OS, IL, OK, KJ, and CS made substantial contributions to draft the manuscript and revise it critically for valuable intellectual content. All authors have read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank the Senior Research Scientist of the laboratory of natural resources, Fadeev N., for supplying the photographs of the milk thistle and chicory. We want to acknowledge leopictures for the photo of an artichoke flower, Tom Viggars for the photo of the whole Artichoke plant, Iryna Denysova for the photo of the fresh artichoke with a stalk and leaves, Purple artichoke Elisa Galceran Garcia, LifeCollectionPhotography for the photo of a field of blue Chicory. Licenses for these images have been purchased from https://www.shutterstock.com/.

REFERENCES

Abd El-Mageed, N. M. (2011). Hepatoprotective Effect of Feeding Celery Leaves Mixed with Chicory Leaves and Barley Grains to Hypercholesterolemic Rats. *Pharmacogn Mag.* 7 (26), 151–156. doi:10.4103/0973-1296.80675

Abdel-Moneim, A., Ahmed, O. M., Abd El-Twab, S. M., Zaky, M. Y., and Bakry, L. N. (2021). Prophylactic Effects of Cynara Scolymus L. Leaf and Flower Hydroethanolic Extracts against Diethylnitrosamine/acetylaminoflourene-Induced Lung Cancer in Wistar Rats. *Environ. Sci. Pollut. Res. Int.* 28 (32), 43515–43527. doi:10.1007/s11356-021-13391-x

Abenavoli, L., Capasso, R., Milic, N., and Capasso, F. (2010). Milk Thistle in Liver Diseases: Past, Present, Future. *Phytother Res.* 24 (10), 1423–1432. doi:10.1002/ptr.3207

Abenavoli, L., Izzo, A. A., Milić, N., Cicala, C., Santini, A., and Capasso, R. (2018). Milk Thistle (Silybum marianum): A Concise Overview on its Chemistry, Pharmacological, and Nutraceutical Uses in Liver Diseases. *Phytother Res.* 32 (11), 2202–2213. doi:10.1002/ptr.6171

Achufusi, T. G. O., and Patel, R. K. (2020). *Milk Thistle*. in *StatPearls [Internet]*. Treasure Island (FL: StatPearls Publishing.

Adzet, T., Camarasa, J., and Laguna, J. C. (1987). Hepatoprotective Activity of Polyphenolic Compounds from Cynara Scolymus against CCl4 Toxicity in Isolated Rat Hepatocytes. *J. Nat. Prod.* 50 (4), 612–617. doi:10.1021/np50052a004

Ahmadi, A., Heidarian, E., and Ghatreh-Samani, K. (2019). Modulatory Effects of Artichoke (Cynara Scolymus L.) Leaf Extract against Oxidative Stress and Hepatic TNF-α Gene Expression in Acute Diazinon-Induced Liver Injury in Rats. *J. Basic Clin. Physiol. Pharmacol.* 30 (5). doi:10.1515/jbcpp-2018-0180

Ahmed, H. S., Mohamed, W. R., Moawad, A. S., Owis, A. I., Ahmed, R. R., and AbouZid, S. F. (2020). Cytotoxic, Hepatoprotective and Antioxidant Activities of Silybum marianum Variety Albiflorum Growing in Egypt. *Nat. Prod. Res.* 34 (24), 3540–3544. doi:10.1080/14786419.2019.1582039

Al-Snafi, A. E. (2016). Medical Importance of Cichorium Intybus – A Review. *J. Pharm. Pharmacol.* 6 (3), 41–56.

Andrzejewska, J., Martinelli, T., and Sadowska, K. (2015). Silybum marianum: Non-medical Exploitation of the Species. *Ann. Appl. Biol.* 167 (3), 285–297. doi:10.1111/aab.12232

Asrani, S. K., Devarbhavi, H., Eaton, J., and Kamath, P. S. (2019). Burden of Liver Diseases in the World. *J. Hepatol.* 70 (1), 151–171. doi:10.1016/j.jhep.2018.09.014

Ataei Nukabadi, F., Hojjatoleslamy, M., and Abbasi, H. (2021). Optimization of Fortified Sponge Cake by Nettle Leaves and Milk Thistle Seed Powder Using Mixture Design Approach. *Food Sci. Nutr.* 9 (2), 757–771. doi:10.1002/fsn3.2041

Babaei, H., Forouzandeh, F., Maghsoumi-Norouzabad, L., Yousefimanesh, H. A., Ravanbakhsh, M., and Zare Javid, A. (2018). Effects of Chicory Leaf Extract on Serum Oxidative Stress Markers, Lipid Profile and Periodontal Status in Patients with Chronic Periodontitis. *J. Am. Coll. Nutr.* 37 (6), 479–486. doi:10.1080/07315724.2018.1437371

Baginskaya, A. I., Kolchir, V. K., Shkarenkov, A. A., Gorodniuk, T. I., Glazova, N. G., Leskova, T. E., et al. (2000). MIlk Thistle Based Medicines. *Ann. scientific works VILAR: Chem. Technol. Med.*, 282–291.

Bagno, O. A., Shevchenko, S. A., Fedorov, Y. N., Shevchenko, A. I., Prokhorov, O. N., and Shentseva, A. V. (2020). The Influence of Milk Thistle Extract on Productive Qualities and Survivial of Broiler Chickens. *Vestnik Altai State. Agric. Univ.* 5 (187), 84–91.

Barbosa, C. H., Andrade, M. A., Vilarinho, F., Castanheira, I., Fernando, A. L., Loizzo, M. R., et al. (2020). A New Insight on Cardoon: Exploring New Uses besides Cheese Making with a View to Zero Waste. *Foods* 9 (5), 564. doi:10.3390/foods9050564

Bashir, A., Hoilat, G. J., Sarwal, P., and Mehta, D. (2021). *Liver Toxicity*. in *StatPearls*. Treasure Island FL: StatPearls Publishing.

Batakov, E. A. (2001). Effect of Silybum marianum Oil and Legalon on Lipid Peroxidation and Liver Antioxidant Systems in Rats Intoxicated with Carbon Tetrachloride. *Eksp Klin Farmakol* 64 (4), 53–55.

Ben Salem, M., Affes, H., Ksouda, K., Dhouibi, R., Sahnoun, Z., Hammami, S., et al. (2015). Pharmacological Studies of Artichoke Leaf Extract and Their Health Benefits. *Plant Foods Hum. Nutr.* 70 (4), 441–453. doi:10.1007/s11130-015-0503-8

Ben Salem, M., Ksouda, K., Dhouibi, R., Charfi, S., Turki, M., Hammami, S., et al. (2019). LC-MS/MS Analysis and Hepatoprotective Activity of Artichoke (Cynara Scolymus L.) Leaves Extract against High Fat Diet-Induced Obesity in Rats. *Biomed. Res. Int.* 2019, 4851279. doi:10.1155/2019/4851279

Ben-Amotz, A., and Fishier, R. (1998). Analysis of Carotenoids with Emphasis on 9-cis β-carotene in Vegetables and Fruits Commonly Consumed in Israel. *Food Chem.* 62 (4), 515–520. doi:10.1016/S0308-8146(97)00196-9

Bortnikova, V. V., Krepkova, L. V., Babenko, A. N., Saybel, O. L., Borovkova, M. V., Kuzina, O. S., et al. (2021). "A Perspective Botanical Drug: Hepatoprotective Activity of Dry Extract Prepared from the Aerial Part of Chicory Plant (*Cichorium Intybus* L.)," in American College Of Clinical Pharmacology (ACCP)), 100.

Brailski, Kh., Chernev, K., Etŭrska, M., Donov, M., and Velev, G. (1986). Effect of Carsil and Legalon in Treating Chronic Liver Diseases. *Vutr Boles* 25 (3), 43–49.

Brennan, D. (2020). *Health Benefits Of Pumpkin*. WebMD Medical Reference. Available: https://www.webmd.com/diet/health-benefits-pumpkin#2 (Accessed May 12, 2021).

Britannica Encyclopaedia (2020). Food. Available: https://www.britannica.com/topic/food (Accessed May 12, 2021).

Buddington, R. K., Kapadia, C., Neumer, F., and Theis, S. (2017). Oligofructose Provides Laxation for Irregularity Associated with Low Fiber Intake. *Nutrients* 9 (12). doi:10.3390/nu9121372

Bundy, R., Walker, A. F., Middleton, R. W., Wallis, C., and Simpson, H. C. (2008). Artichoke Leaf Extract (Cynara Scolymus) Reduces Plasma Cholesterol in Otherwise Healthy Hypercholesterolemic Adults: a Randomized, Double Blind Placebo Controlled Trial. *Phytomedicine* 15 (9), 668–675. doi:10.1016/j.phymed.2008.03.001

Calani, L., Brighenti, F., Bruni, R., and Del Rio, D. (2012). Absorption and Metabolism of Milk Thistle Flavanolignans in Humans. *Phytomedicine* 20 (1), 40–46. doi:10.1016/j.phymed.2012.09.004

Cha, J.-Y., Park, C.-K., and Cho, Y.-S. (2010). Hepatoprotective Effect of Chicory (Chicorium Intybus) Root Extract against Orotic Acid-Induced Fatty Liver in Rats. *Food Sci. Biotechnol.* 19 (4), 865–871. doi:10.1007/s10068-010-0123-x

Chabaev, M. G., Ryijkov, I. V., Nikolaichenko, N. V., and Khabibulina, V. A. (2011). Productivity and Metabolism in Lactating Cows when by Feeding Milk Thistle Meal. *Zootechnika* (6), 8–10.

Cimen, O., Eken, H., Keskin Cimen, F., Cekic, A. B., Kurt, N., Ozbek Bilgin, A., et al. (2020). The Effect of Liv-52 on Liver Ischemia Reperfusion Damage in Rats. *BMC Pharmacol. Toxicol.* 21 (1), 2. doi:10.1186/s40360-019-0380-0

Colak, E., Ustuner, M. C., Tekin, N., Colak, E., Burukoglu, D., Degirmenci, I., et al. (2016). The Hepatocurative Effects of Cynara Scolymus L. Leaf Extract on Carbon Tetrachloride-Induced Oxidative Stress and Hepatic Injury in Rats. *Springerplus* 5, 216. doi:10.1186/s40064-016-1894-1

Cooper, D. N., Kable, M. E., Marco, M. L., De Leon, A., Rust, B., Baker, J. E., et al. (2017). The Effects of Moderate Whole Grain Consumption on Fasting Glucose and Lipids, Gastrointestinal Symptoms, and Microbiota. *Nutrients* 9 (2). doi:10.3390/nu9020173

D'Antuono, I., Carola, A., Sena, L. M., Linsalata, V., Cardinali, A., Logrieco, A. F., et al. (2018). Artichoke Polyphenols Produce Skin Anti-age Effects by Improving Endothelial Cell Integrity and Functionality. *Molecules* 23 (11). doi:10.3390/molecules23112729

De Martiis, M., Fontana, M., Assogna, G., D'Ottavi, R., and D'Ottavi, O. (1980). Milk Thistle (Silybum marianum) Derivatives in the Therapy of Chronic Hepatopathies. *Clin. Ter* 94 (3), 283–315.

Deák, G., Müzes, G., Láng, I., Niederland, V., Nékám, K., Gonzalez-Cabello, R., et al. (1990). Immunomodulator Effect of Silymarin Therapy in Chronic Alcoholic Liver Diseases. *Orv Hetil* 131 (24), 12911295–12961296.

Deng, Y., Xu, Z., Liu, W., Yang, H., Xu, B., and Wei, Y. (2012). Effects of Lycopene and Proanthocyanidins on Hepatotoxicity Induced by Mercuric Chloride in Rats. *Biol. Trace Elem. Res.* 146 (2), 213–223. doi:10.1007/s12011-011-9242-3

Domitrović, R., and Potočnjak, I. (2016). A Comprehensive Overview of Hepatoprotective Natural Compounds: Mechanism of Action and Clinical Perspectives. *Arch. Toxicol.* 90 (1), 39–79. doi:10.1007/s00204-015-1580-z

Edible Wild Food (2021). *Milk Thistle*. Available: https://www.ediblewildfood.com/milk-thistle.aspx (Accessed May 12, 2021).

Ekor, M. (2014). The Growing Use of Herbal Medicines: Issues Relating to Adverse Reactions and Challenges in Monitoring Safety. *Front. Pharmacol.* 4, 177. doi:10.3389/fphar.2013.00177

El-Gazayerly, O. N., Makhlouf, A. I., Soelm, A. M., and Mohmoud, M. A. (2014). Antioxidant and Hepatoprotective Effects of Silymarin Phytosomes Compared to Milk Thistle Extract in CCl4 Induced Hepatotoxicity in Rats. *J. Microencapsul* 31 (1), 23–30. doi:10.3109/02652048.2013.805836

El-Kamary, S. S., Shardell, M. D., Abdel-Hamid, M., Ismail, S., El-Ateek, M., Metwally, M., et al. (2009). A Randomized Controlled Trial to Assess the Safety and Efficacy of Silymarin on Symptoms, Signs and Biomarkers of Acute Hepatitis. *Phytomedicine* 16 (5), 391–400. doi:10.1016/j.phymed.2009.02.002

Elsebai, M. F., Mocan, A., and Atanasov, A. G. (2016). Cynaropicrin: A Comprehensive Research Review and Therapeutic Potential as an Anti-hepatitis C Virus Agent. *Front. Pharmacol.* 7, 472. doi:10.3389/fphar.2016.00472

Enioutina, E. Y., Job, K. M., Krepkova, L. V., Reed, M. D., and Sherwin, C. M. (2020). How Can We Improve the Safe Use of Herbal Medicine and Other Natural Products? A Clinical Pharmacologist mission. *Expert Rev. Clin. Pharmacol.* 13 (9), 935–944. doi:10.1080/17512433.2020.1803739

European OTC medicines and devices (2020). Carsil 22,5 Mg Silymarinum N80 Tabs. Stabilizes Liver Cell Membrane. Available: https://bbeautybureau.com/carsil-225mg-silymarinum-n80-liver-cell-membrane/(Accessed May 12, 2021).

Fehér, J., Deák, G., Müzes, G., Láng, I., Niederland, V., Nékám, K., et al. (1989). Liver-protective Action of Silymarin Therapy in Chronic Alcoholic Liver Diseases. *Orv Hetil* 130 (51), 2723–2727.

Fenclova, M., Novakova, A., Viktorova, J., Jonatova, P., Dzuman, Z., Ruml, T., et al. (2019). Poor Chemical and Microbiological Quality of the Commercial Milk Thistle-Based Dietary Supplements May Account for Their Reported Unsatisfactory and Non-reproducible Clinical Outcomes. *Sci. Rep.* 9 (1), 11118. doi:10.1038/s41598-019-47250-0

Ferenci, P., Dragosics, B., Dittrich, H., Frank, H., Benda, L., Lochs, H., et al. (1989). Randomized Controlled Trial of Silymarin Treatment in Patients with Cirrhosis of the Liver. *J. Hepatol.* 9 (1), 105–113. doi:10.1016/0168-8278(89)90083-4

Fernández, J., Curt, M. D., and Aguado, P. L. (2006). Industrial Applications of Cynara Cardunculus L. For Energy and Other Uses. *Ind. Crops Prod.* 24 (3), 222–229. doi:10.1016/j.indcrop.2006.06.010

Ferrare, K., Bidel, L. P. R., Awwad, A., Poucheret, P., Cazals, G., Lazennec, F., et al. (2018). Increase in Insulin Sensitivity by the Association of Chicoric Acid and Chlorogenic Acid Contained in a Natural Chicoric Acid Extract (NCRAE) of Chicory (Cichorium Intybus L.) for an Antidiabetic Effect. *J. Ethnopharmacol* 215, 241–248. doi:10.1016/j.jep.2017.12.035

Flora, K., Hahn, M., Rosen, H., and Benner, K. (1998). Milk Thistle (Silybum marianum) for the Therapy of Liver Disease. *Am. J. Gastroenterol.* 93 (2), 139–143. doi:10.1111/j.1572-0241.1998.00139.x

Fowler, J. (2012). *FarmProgress. 13 Ways Corn Is Used in Our Everyday Lives*. Available: https://www.farmprogress.com/vegetables/13-ways-corn-used-our-everyday-lives (Accessed May 12, 2021).

Ghaffari, A., Rafraf, M., Navekar, R., Sepehri, B., Asghari-Jafarabadi, M., and Ghavami, S. M. (2019). Turmeric and Chicory Seed Have Beneficial Effects on Obesity Markers and Lipid Profile in Non-alcoholic Fatty Liver Disease (NAFLD). *Int. J. Vitam Nutr. Res.* 89 (5-6), 293–302. doi:10.1024/0300-9831/a000568

Gillessen, A., and Schmidt, H. H. (2020). Silymarin as Supportive Treatment in Liver Diseases: A Narrative Review. *Adv. Ther.* 37 (4), 1279–1301. doi:10.1007/s12325-020-01251-y

GistGear (2021). Best Artichoke Herbal Supplements - Buying Guide. Available: https://gistgear.com/health-personal-care/nutrition-dietary/artichoke-herbal-supplements (Accessed August 31, 2021).

Githiori, J. B., Athanasiadou, S., and Thamsborg, S. M. (2006). Use of Plants in Novel Approaches for Control of Gastrointestinal Helminths in Livestock with Emphasis on Small Ruminants. *Vet. Parasitol.* 139 (4), 308–320. doi:10.1016/j.vetpar.2006.04.021

Gominho, J., Curt, M. D., Lourenço, A., Fernández, J., and Pereira, H. (2018). Cynara Cardunculus L. As a Biomass and Multi-Purpose Crop: A Review of 30 Years of Research. *Biomass and Bioenergy* 109, 257–275. doi:10.1016/j.biombioe.2018.01.001

Gominho, J., Fernandez, J., and Pereira, H. (2001). Cynara Cardunculus L. - a New Fibre Crop for Pulp and Paper Production. *Ind. Crops Prod.* 13 (1), 1–10. doi:10.1016/S0926-6690(00)00044-3

Gostin, A.-I., and Waisundara, V. Y. (2019). Edible Flowers as Functional Food: A Review on Artichoke (Cynara Cardunculus L.). *Trends Food Sci. Technology* 86, 381–391. doi:10.1016/j.tifs.2019.02.015

Gottlieb, S. (2019). "Statement from FDA Commissioner on the agency's New Efforts to Strengthen Regulation of Dietary Supplements by Modernizing and Reforming FDA's Oversight," in *The U.S. Food and Drug Administration* (Washington, D.C.: U.S. Department of Health and Human Services.).

Grela, E. R., Sobolewska, S., and Roziński, T. (2014). Effect of Inulin Extracts or Inulin-Containing Plant Supplement on Blood Lipid Indices and Fatty Acid Profile in Fattener Tissues. *Pol. J. Vet. Sci.* 17 (1), 93–98. doi:10.2478/pjvs-2014-0012

Gwirtz, J. A., and Garcia-Casal, M. N. (2014). Processing maize Flour and Corn Meal Food Products. *Ann. N. Y Acad. Sci.* 1312, 66–75. doi:10.1111/nyas.12299

Himalaya Wellness Company (2021a). *Himalaya, Our story* [Online]. Makali, Bengaluru: Himalaya Wellness Company. Available: https://himalayawellness.in/pages/our-story (Accessed September 11, 2021).

Himalaya Wellness Company (2021b). Liv.52. Description. Available: https://himalayawellness.in/products/liv-52 (Accessed June 1, 2021).

Hou, Y. C., Liou, K. T., Chern, C. M., Wang, Y. H., Liao, J. F., Chang, S., et al. (2010). Preventive Effect of Silymarin in Cerebral Ischemia-Reperfusion-Induced Brain Injury in Rats Possibly through Impairing NF-Kb and STAT-1 Activation. *Phytomedicine* 17 (12), 963–973. doi:10.1016/j.phymed.2010.03.012

Huber, R., Müller, M., Naumann, J., Schenk, T., and Lüdtke, R. (2009). Artichoke Leave Extract for Chronic Hepatitis C - a Pilot Study. *Phytomedicine* 16 (9), 801–804. doi:10.1016/j.phymed.2009.03.007

Hughes, S. R., Qureshi, N., López-Núñez, J. C., Jones, M. A., Jarodsky, J. M., Galindo-Leva, L. Á., et al. (2017). Utilization of Inulin-Containing Waste in Industrial Fermentations to Produce Biofuels and Bio-Based Chemicals. *World J. Microbiol. Biotechnol.* 33 (4), 78. doi:10.1007/s11274-017-2241-6

Huseini, H. F., Alavian, S. M., Heshmat, R., Heydari, M. R., and Abolmaali, K. (2005). The Efficacy of Liv-52 on Liver Cirrhotic Patients: a Randomized, Double-Blind, Placebo-Controlled First Approach. *Phytomedicine* 12 (9), 619–624. doi:10.1016/j.phymed.2004.10.003

Irani, M., Sarmadi, M., Bernard, F., Ebrahimi Pour, G. H., and Shaker Bazarnov, H. (2010). Leaves Antimicrobial Activity of Glycyrrhiza Glabra L. *Iran J. Pharm. Res.* 9 (4), 425–428.

Janda, K., Gutowska, I., Geszke-Moritz, M., and Jakubczyk, K. (2021). The Common Cichory (Cichorium Intybus L.) as a Source of Extracts with Health-Promoting Properties-A Review. *Molecules* 26 (6), 1814. doi:10.3390/molecules26061814

Kamei, J., Nakamura, R., Ichiki, H., and Kubo, M. (2003). Antitussive Principles of Glycyrrhizae Radix, a Main Component of the Kampo Preparations Bakumondo-To (Mai-Men-Dong-Tang). *Eur. J. Pharmacol.* 469 (1-3), 159–163. doi:10.1016/s0014-2999(03)01728-x

Kandeler, R., and Ullrich, W. R. (2009). Symbolism of Plants: Examples from European-Mediterranean Culture Presented with Biology and History of Art: NOVEMBER: Chicory. *J. Exp. Bot.* 60 (14), 3973–3974. doi:10.1093/jxb/erp248

Kantah, M. K., Kobayashi, R., Sollano, J., Naito, Y., Solimene, U., Jains, S., et al. (2011). Hepatoprotective Activity of a Phytotherapeutic Formula on Thioacetamide-Iinduced Liver Fibrosis Model. *Acta Biomed.* 82 (1), 82–89.

Khoobani, M., Hasheminezhad, S. H., Javandel, F., Nosrati, M., Seidavi, A., Kadim, I. T., et al. (2020). Effects of Dietary Chicory (Chicorium Intybus L.) and Probiotic Blend as Natural Feed Additives on Performance Traits, Blood Biochemistry, and Gut Microbiota of Broiler Chickens. *Antibiotics (Basel)* 9 (1), 5. doi:10.3390/antibiotics9010005

King County, W. (2018). *King County, Washington (2018). Milk Thistle – May 2018 Weed of the Month*. King County, Washington. Available at: https://kingcountyweeds.com/2018/05/14/milk-thistle-may-2018-weed-of-the-month/#:~:text=Left%20unchecked%2C%20they%20produce%20purple (Accessed May 12, 2021).

Klyuchnikova, O. V., Skogoreva, E. A., Kozhevnikova, N. P., and Slobodyanik, V. S. (2011). "Vegetable Raw Materials in the Creation of Functional Meat Products," in *Successes in Modern Natural Sciences*.

Kolesnyk, M. D., and Bankovska, I. (2008). Application of Milk Thistle to Spotted Piglets. *Livestock of Ukraine* (2), 32–34.

Kolesnyk, M. D., Bankovska, I. B., and Kostenko, O. I. (2009). Components of the Effectiveness of the Use Milk Thistle. *Vestnyl Poltava State. Adgicultural Acad.* 1, 76–77.

Kravainis, Y. Y., Tanifa, V. V., and Kravaine, R. S. (2014). Application of Milk Thistle Cake in Cattle Breading. *Vestnik Russ. Acad. Argicultural Sceince* (4), 44–46.

Krepkova, L. V., Shkarenkov, A. A., and Sokol'skaya, T. A. (2008). Experimental and Clinical Study of Herbal Remedies from Milk Thistle. *Probl. Biol. Med. Pharm. Chem.* (4), 3–6.

Krepkova, L. V., and Sokol'skaya, T. A. (2007). Preclinical Investigation of the Safety of the Hepatoprotective Drug Silimar. *Khimiko-Farmatsevticheskii Zhurnal* 41 (9), 32–34. doi:10.1007/s11094-007-0108-0

Kuang, Y., Li, B., Fan, J., Qiao, X., and Ye, M. (2018). Antitussive and Expectorant Activities of Licorice and its Major Compounds. *Bioorg. Med. Chem.* 26 (1), 278–284. doi:10.1016/j.bmc.2017.11.046

Kwon, Y. J., Son, D. H., Chung, T. H., and Lee, Y. J. (2020). A Review of the Pharmacological Efficacy and Safety of Licorice Root from Corroborative Clinical Trial Findings. *J. Med. Food* 23 (1), 12–20. doi:10.1089/jmf.2019.4459

Lans, C. (2019). Do recent Research Studies Validate the Medicinal Plants Used in British Columbia, Canada for Pet Diseases and Wild Animals Taken into Temporary Care? *J. Ethnopharmacol* 236, 366–392. doi:10.1016/j.jep.2019.02.030

Lattanzio, V., Kroon, P. A., Linsalata, V., and Cardinali, A. (2009). Globe Artichoke: A Functional Food and Source of Nutraceutical Ingredients. *J. Funct. Foods* 1 (2), 131–144. doi:10.1016/j.jff.2009.01.002

Lee, J. I., Narayan, M., and Barrett, J. S. (2007). Analysis and Comparison of Active Constituents in Commercial Standardized Silymarin Extracts by Liquid Chromatography-Electrospray Ionization Mass Spectrometry. *J. Chromatogr. B Analyt Technol. Biomed. Life Sci.* 845 (1), 95–103. doi:10.1016/j.jchromb.2006.07.063

Lee, M. H., Huang, Z., Kim, D. J., Kim, S. H., Kim, M. O., Lee, S. Y., et al. (2013). Direct Targeting of MEK1/2 and RSK2 by Silybin Induces Cell-Cycle Arrest and Inhibits Melanoma Cell Growth. *Cancer Prev. Res. (Phila)* 6 (5), 455–465. doi:10.1158/1940-6207.CAPR-12-0425

Li, G. Y., Gao, H. Y., Huang, J., Lu, J., Gu, J. K., and Wang, J. H. (2014). Hepatoprotective Effect of Cichorium Intybus L., a Traditional Uighur Medicine, against Carbon Tetrachloride-Induced Hepatic Fibrosis in Rats. *World J. Gastroenterol.* 20 (16), 4753–4760. doi:10.3748/wjg.v20.i16.4753

Li, X., Sun, R., and Liu, R. (2019). Natural Products in Licorice for the Therapy of Liver Diseases: Progress and Future Opportunities. *Pharmacol. Res.* 144, 210–226. doi:10.1016/j.phrs.2019.04.025

Marmouzi, I., Bouyahya, A., Ezzat, S. M., El Jemli, M., and Kharbach, M. (2021). The Food Plant Silybum marianum (L.) Gaertn.: Phytochemistry, Ethnopharmacology and Clinical Evidence. *J. Ethnopharmacol* 265, 113303. doi:10.1016/j.jep.2020.113303

Martirosyan, D. M., and Singh, J. (2015). A New Definition of Functional Food by FFC: what Makes a New Definition Unique? *Ffhd* 5 (66), 209209–223223. doi:10.31989/ffhd.v5i6.183

Meneses, M., Megías, M. D., Madrid, J., Martínez-Teruel, A., Hernández, F., and Oliva, J. (2007). Evaluation of the Phytosanitary, Fermentative and Nutritive Characteristics of the Silage Made from Crude Artichoke (Cynara Scolymus L.) By-Product Feeding for Ruminants. *Small Ruminant Res.* 70 (2-3), 292–296. doi:10.1016/j.smallrumres.2006.05.008

Mildzikhov, T. Z., Temiraev, R. B., Khadikova, M. A., and Malieva, E. V. (2013). Quality of Broiler Meat in Heavy Metal Detoxication. *Meat Industry* (12), 55–57.

Mirpoor, S. F., Varriale, S., Porta, R., Naviglio, D., Spennato, M., Gardossi, L., et al. (2022). A Biorefinery Approach for the Conversion of Cynara Cardunculus Biomass to Active Films. *Food Hydrocolloids* 122, 107099. doi:10.1016/j.foodhyd.2021.107099

Missouri Botanical Garden (2019). *Plant Finder Database: Silybum Marianum*. St. Louis, MO. Available: https://www.missouribotanicalgarden.org/PlantFinder/PlantFinderDetails.aspx?taxonid=277472&isprofile=0& (Accessed May 12, 2021).

Mohafrash, S. M. M., and Mossa, A. H. (2020). Herbal Syrup from Chicory and Artichoke Leaves Ameliorate Liver Damage Induced by Deltamethrin in Weanling Male Rats. *Environ. Sci. Pollut. Res. Int.* 27 (7), 7672–7682. doi:10.1007/s11356-019-07434-7

Monllor, P., Romero, G., Sendra, E., Atzori, A. S., and Díaz, J. R. (2020). Short-Term Effect of the Inclusion of Silage Artichoke By-Products in Diets of Dairy Goats on Milk Quality. *Animals (Basel)* 10 (2), 339. doi:10.3390/ani10020339

Mukhtar, S., Xiaoxiong, Z., Qamer, S., Saad, M., Mubarik, M. S., Mahmoud, A. H., et al. (2021). Hepatoprotective Activity of Silymarin Encapsulation against Hepatic Damage in Albino Rats. *Saudi J. Biol. Sci.* 28 (1), 717–723. doi:10.1016/j.sjbs.2020.10.063

Munn, Z., Peters, M. D. J., Stern, C., Tufanaru, C., McArthur, A., and Aromataris, E. (2018). Systematic Review or Scoping Review? Guidance for Authors when Choosing between a Systematic or Scoping Review Approach. *BMC Med. Res. Methodol.* 18 (1), 143. doi:10.1186/s12874-018-0611-x

Nekrasova, K. L., and Popov, V. G. (2020). Scientific Approach to the Production of Functional Food Ingredients Based on Unconventional Plant Materials. *Vestn. Voronež. Gos. Univ. Inž. Tehnol.* 82 (2), 77–82. doi:10.20914/2310-1202-2020-2-77-82

Niness, K. R. (1999). Inulin and Oligofructose: what Are They? *J. Nutr.* 129, 1402S–6S. doi:10.1093/jn/129.7.1402S

Nofal, A., Ibrahim, A. M., Nofal, E., Gamal, N., and Osman, S. (2019). Topical Silymarin versus Hydroquinone in the Treatment of Melasma: A Comparative Study. *J. Cosmet. Dermatol.* 18 (1), 263–270. doi:10.1111/jocd.12769

Nosalova, G., Fleskova, D., Jurecek, L., Sadlonova, V., and Ray, B. (2013). Herbal Polysaccharides and Cough Reflex. *Respir. Physiol. Neurobiol.* 187 (1), 47–51. doi:10.1016/j.resp.2013.03.015

Nwafor, I. C., Shale, K., and Achilonu, M. C. (2017). Chemical Composition and Nutritive Benefits of Chicory (Cichorium Intybus) as an Ideal Complementary And/or Alternative Livestock Feed Supplement. *ScientificWorldJournal* 2017, 7343928. doi:10.1155/2017/7343928

Oliveira, I., Gominho, J., Diberardino, S., and Duarte, E. (2012). Characterization of Cynara Cardunculus L. Stalks and Their Suitability for Biogas Production. *Ind. Crops Prod.* 40, 318–323. doi:10.1016/j.indcrop.2012.03.029

Panahi, Y., Kianpour, P., Mohtashami, R., Atkin, S. L., Butler, A. E., Jafari, R., et al. (2018). Efficacy of Artichoke Leaf Extract in Non-alcoholic Fatty Liver Disease: A Pilot Double-Blind Randomized Controlled Trial. *Phytother Res.* 32 (7), 1382–1387. doi:10.1002/ptr.6073

Perović, J., Tumbas Šaponjac, V., Kojić, J., Krulj, J., Moreno, D. A., García-Viguera, C., et al. (2021). Chicory (Cichorium Intybus L.) as a Food Ingredient - Nutritional Composition, Bioactivity, Safety, and Health Claims: A Review. *Food Chem.* 336, 127676. doi:10.1016/j.foodchem.2020.127676

Petropoulos, S. A., Levizou, E., Ntatsi, G., Fernandes, Â., Petrotos, K., Akoumianakis, K., et al. (2017). Salinity Effect on Nutritional Value, Chemical Composition and Bioactive Compounds Content of Cichorium Spinosum L. *Food Chem.* 214, 129–136. doi:10.1016/j.foodchem.2016.07.080

Poppe, L., and Petersen, M. (2016). Variation in the Flavonolignan Composition of Fruits from Different Silybum marianum Chemotypes and Suspension Cultures Derived Therefrom. *Phytochemistry* 131, 68–75. doi:10.1016/j.phytochem.2016.09.003

Pradhan, S. C., and Girish, C. (2006). Hepatoprotective Herbal Drug, Silymarin from Experimental Pharmacology to Clinical Medicine. *Indian J. Med. Res.* 124 (5), 491–504.

Rahman, I. U., Afzal, A., Iqbal, Z., Ijaz, F., Ali, N., Shah, M., et al. (2019). Historical Perspectives of Ethnobotany. *Clin. Dermatol.* 37 (4), 382–388. doi:10.1016/j.clindermatol.2018.03.018

Rehm, J., Samokhvalov, A. V., and Shield, K. D. (2013). Global burden of Alcoholic Liver Diseases. *J. Hepatol.* 59 (1), 160–168. doi:10.1016/j.jhep.2013.03.007

Reimer, R. A., Soto-Vaca, A., Nicolucci, A. C., Mayengbam, S., Park, H., Madsen, K. L., et al. (2020). Effect of Chicory Inulin-type Fructan-Containing Snack Bars on the Human Gut Microbiota in Low Dietary Fiber Consumers in a Randomized Crossover Trial. *Am. J. Clin. Nutr.* 111 (6), 1286–1296. doi:10.1093/ajcn/nqaa074

Rocchetti, G., Lucini, L., Corrado, G., Colla, G., Cardarelli, M., Pascale, S., et al. (2020). Phytochemical Profile, Mineral Content, and Bioactive Compounds in Leaves of Seed-Propagated Artichoke Hybrid Cultivars. *Molecules* 25 (17), 3795. doi:10.3390/molecules25173795

Romanova, E. I., and Krasavtsev, E. L. (2007). Modern Aspects of Hepatoprotective Therapy. *Health Ecol. Issues* 12 (12), 42–50.

Ruetzler, K., Fleck, M., Nabecker, S., Pinter, K., Landskron, G., Lassnigg, A., et al. (2013). A Randomized, Double-Blind Comparison of Licorice versus Sugar-Water Gargle for Prevention of Postoperative Sore Throat and Postextubation Coughing. *Anesth. Analg* 117 (3), 614–621. doi:10.1213/ANE.0b013e318299a650

Rutter, K., Scherzer, T. M., Beinhardt, S., Kerschner, H., Stättermayer, A. F., Hofer, H., et al. (2011). Intravenous Silibinin as 'rescue Treatment' for On-Treatment Non-responders to Pegylated Interferon/ribavirin Combination Therapy. *Antivir. Ther.* 16 (8), 1327–1333. doi:10.3851/IMP1942

Sama, S. K., Krishnamurthy, L., Ramachandran, K., and Lal, K. (1976). Efficacy of an Indigenous Compound Preparation (Liv-52) in Acute Viral Hepatitis-A Double Blind Study. *Indian J. Med. Res.* 64 (5), 738–742.

Saxena, A., and Garg, N. K. (1979). Effect of Liv-52 on Hepatic Enzymes. *Indian J. Exp. Biol.* 17 (7), 662–664.

Saybel, O. L., Radimich, A. I., Adamov, G. V., and Dargaeva, T. D. (2020). Comparative Phytochemical Study of the Aerial Parts of the Wild-Growing and Cultivat-Ed Chicory Ordinary (Cichorium Intybus L.). *Jcprm* (3), 187–195. doi:10.14258/jcprm.2020037386

Schmidt, B. M. (2012). Responsible Use of Medicinal Plants for Cosmetics. *horts* 47 (8), 985–991. doi:10.21273/hortsci.47.8.985

Schumacher, E., Vigh, E., Molnár, V., Kenyeres, P., Fehér, G., Késmárky, G., et al. (2011). Thrombosis Preventive Potential of Chicory Coffee Consumption: a Clinical Study. *Phytother Res.* 25 (5), 744–748. doi:10.1002/ptr.3481

Semenkina, N. G. (2010). Using Milk Thistle as a Source of Functional Ingredients. *Food Industry* (5), 40–41.

Shemuranova, N. A., and Garifullina, N. A. (2020). Plants as the Basis for the Development of Environmentally Friendly Highly Functional Bioadditives for Animals (Review). АҐрарная наука Евро-Северо-Востока 21 (5), 483–502. doi:10.30766/2072-9081.2020.21.5.483-502

Shoaib, M., Shehzad, A., Omar, M., Rakha, A., Raza, H., Sharif, H. R., et al. (2016). Inulin: Properties, Health Benefits and Food Applications. *Carbohydr. Polym.* 147, 444–454. doi:10.1016/j.carbpol.2016.04.020

Siegel, A. B., and Stebbing, J. (2013). Milk Thistle: Early Seeds of Potential. *Lancet Oncol.* 14 (10), 929–930. doi:10.1016/S1470-2045(13)70414-5

Smith, T., Kawa, K., Eckl, V., Morton, C., and Stredney, R. (2017). Herbal Supplement Sales in US Increase 7.7% in 2016 Consumer Preferences Shifting toward Ingredients with General Wellness Benefits, Driving Growth of Adaptogens and Digestive Health Products. *HerbalGram* 115, 56–65.

Sokol'skaya, T. A. (2000). Complex Processing of Milk Thistle Fruits and Production of the Drug "Silimar" on its Basis. *Khimiko-Farmatsevticheskii Zhurnal* 34 (9), 227–230.

Soleimani, V., Delghandi, P. S., Moallem, S. A., and Karimi, G. (2019). Safety and Toxicity of Silymarin, the Major Constituent of Milk Thistle Extract: An Updated Review. *Phytother Res.* 33 (6), 1627–1638. doi:10.1002/ptr.6361

Song, X. Y., Li, R. H., Liu, W. W., Hayashi, T., Mizuno, K., Hattori, S., et al. (2021). Effect of Silibinin on Ethanol- or Acetaldehyde-Induced Damge of Mouse Primary Hepatocytes *In Vitro*. *Toxicol. Vitro* 70, 105047. doi:10.1016/j.tiv.2020.105047

Speroni, E., Cervellati, R., Govoni, P., Guizzardi, S., Renzulli, C., and Guerra, M. C. (2003). Efficacy of Different Cynara Scolymus Preparations on Liver Complaints. *J. Ethnopharmacol* 86 (2-3), 203–211. doi:10.1016/s0378-8741(03)00076-x

Stastnik, O., Pavlata, L., and Mrkvicova, E. (2020). The Milk Thistle Seed Cakes and Hempseed Cakes Are Potential Feed for Poultry. *Animals (Basel)* 10 (8), 1384. doi:10.3390/ani10081384

Street, R. A., Sidana, J., and Prinsloo, G. (2013). Cichorium Intybus: Traditional Uses, Phytochemistry, Pharmacology, and Toxicology. *Evid. Based Complement. Alternat Med.* 2013, 579319. doi:10.1155/2013/579319

Sümer, E., Senturk, G. E., Demirel, Ö. U., and Yesilada, E. (2020). Comparative Biochemical and Histopathological Evaluations Proved that Receptacle Is the Most Effective Part of Cynara Scolymus against Liver and Kidney Damages. *J. Ethnopharmacol* 249, 112458. doi:10.1016/j.jep.2019.112458

Szilárd, S., Szentgyörgyi, D., and Demeter, I. (1988). Protective Effect of Legalon in Workers Exposed to Organic Solvents. *Acta Med. Hung* 45 (2), 249–256.

Thabrew, M. I., Emerole, G. O., and Subbarao, V. V. (1982). Effect of Liv-52 on Carbon Tetrachloride-Induced Changes in Hepatic Microsomal Drug-Metabolizing Enzymes of the Rat. *Toxicol. Lett.* 14 (3-4), 183–188. doi:10.1016/0378-4274(82)90049-2

Thomas, P. R., and Earl, R. (1994). Institute of Medicine; Committee On Opportunities In the Nutrition And Food Sciences: Research Challenges And the Next Generation Of Investigators *[Online]*. Washington, DC: The National Academies Press. doi:10.17226/2133

Tong, J., Yao, X., Zeng, H., Zhou, G., Chen, Y., Ma, B., et al. (2015). Hepatoprotective Activity of Flavonoids from Cichorium Glandulosum Seeds *In Vitro* and *In Vivo* Carbon Tetrachloride-Induced Hepatotoxicity. *J. Ethnopharmacol* 174, 355–363. doi:10.1016/j.jep.2015.08.045

Tsai, J. H., Liu, J. Y., Wu, T. T., Ho, P. C., Huang, C. Y., Shyu, J. C., et al. (2008). Effects of Silymarin on the Resolution of Liver Fibrosis Induced by Carbon Tetrachloride in Rats. *J. Viral Hepat.* 15 (7), 508–514. doi:10.1111/j.1365-2893.2008.00971.x

Turkiewicz, I. P., Wojdyło, A., Tkacz, K., Nowicka, P., and Hernández, F. (2019). Antidiabetic, Anticholinesterase and Antioxidant Activity vs. Terpenoids and Phenolic Compounds in Selected New Cultivars and Hybrids of Artichoke Cynara Scolymus L. *Molecules* 24 (7), 1222. doi:10.3390/molecules24071222

Ullah, K., Ahmad, M., Sofia, and Qiu, F. (2015). Assessing the Experimental Investigation of Milk Thistle Oil for Biodiesel Production Using Base Catalyzed Transesterification. *Energy* 89, 887–895. doi:10.1016/j.energy.2015.06.028

United Plant Savers (2021). Species at Risk. Available at: https://unitedplantsavers.org/species-at-risk/(Accessed Sep, 2021).

Vargas-Mendoza, N., Madrigal-Santillán, E., Morales-González, A., Esquivel-Soto, J., Esquivel-Chirino, C., García-Luna Y González-Rubio, M., et al. (2014). Hepatoprotective Effect of Silymarin. *World J. Hepatol.* 6 (3), 144–149. doi:10.4254/wjh.v6.i3.144

Villarini, M., Acito, M., di Vito, R., Vannini, S., Dominici, L., Fatigoni, C., et al. (2021). Pro-Apoptotic Activity of Artichoke Leaf Extracts in Human HT-29 and RKO Colon Cancer Cells. *Int. J. Environ. Res. Public Health* 18 (8). doi:10.3390/ijerph18084166

Wah Kheong, C., Nik Mustapha, N. R., and Mahadeva, S. (2017). A Randomized Trial of Silymarin for the Treatment of Nonalcoholic Steatohepatitis. *Clin. Gastroenterol. Hepatol.* 15 (12), 1940–e8. doi:10.1016/j.cgh.2017.04.016

Wang, L., Zhang, K., Han, S., Zhang, L., Bai, H., Bao, F., et al. (2019). Constituents Isolated from the Leaves of Glycyrrhiza Uralansis and Their Anti-inflammatory Activities on LPS-Induced RAW264.7 Cells. *Molecules* 24 (10). doi:10.3390/molecules24101923

Wee, Y., Yang, C. H., Chen, S. K., Yen, Y. C., and Wang, C. S. (2021). Inositol Hexaphosphate Modulates the Behavior of Macrophages through Alteration of Gene Expression Involved in Pathways of Pro- and Anti-inflammatory Responses, and Resolution of Inflammation Pathways. *Food Sci. Nutr.* 9 (6), 3240–3249. doi:10.1002/fsn3.2286

Wu, T., and Cadwallader, K. R. (2019). Identification of Characterizing Aroma Components of Roasted Chicory "Coffee" Brews. *J. Agric. Food Chem.* 67 (50), 13848–13859. doi:10.1021/acs.jafc.9b00776

Zeaiter, Z., Regonesi, M. E., Cavini, S., Labra, M., Sello, G., and Di Gennaro, P. (2019). Extraction and Characterization of Inulin-type Fructans from Artichoke Wastes and Their Effect on the Growth of Intestinal Bacteria Associated with Health. *Biomed. Res. Int.* 2019, 1083952. doi:10.1155/2019/1083952

Zhang, H. L., Dai, L. H., Wu, Y. H., Yu, X. P., Zhang, Y. Y., Guan, R. F., et al. (2014). Evaluation of Hepatocyteprotective and Anti-hepatitis B Virus Properties of Cichoric Acid from Cichorium Intybus Leaves in Cell Culture. *Biol. Pharm. Bull.* 37 (7), 1214–1220. doi:10.1248/bpb.b14-00137

Zhou, Y., Wang, T., Meng, Q., and Zhai, S. (2016). Association of Carotenoids with Risk of Gastric Cancer: A Meta-Analysis. *Clin. Nutr.* 35 (1), 109–116. doi:10.1016/j.clnu.2015.02.003

Zhu, H. J., Brinda, B. J., Chavin, K. D., Bernstein, H. J., Patrick, K. S., and Markowitz, J. S. (2013). An Assessment of Pharmacokinetics and Antioxidant Activity of Free Silymarin Flavonolignans in Healthy Volunteers: a Dose Escalation Study. *Drug Metab. Dispos* 41 (9), 1679–1685. doi:10.1124/dmd.113.052423

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

SYSTEMATIC REVIEW
published: 26 November 2021
doi: 10.3389/fphar.2021.777561

Effectiveness of Medicinal Plants for Glycaemic Control in Type 2 Diabetes: An Overview of Meta-Analyses of Clinical Trials

Merlin L. Willcox[1]*, *Christina Elugbaju*[1], *Marwah Al-Anbaki*[2], *Mark Lown*[1] and *Bertrand Graz*[2]

[1]Primary Care Research Centre, Aldermoor Health Centre, University of Southampton, Southampton, United Kingdom, [2]Medicines Unit, Antenna Foundation, Geneva, Switzerland

Aims: To rank the effectiveness of medicinal plants for glycaemic control in Type 2 Diabetes (T2DM).

Methods: MEDLINE, EMBASE, CINAHL and Cochrane Central were searched in October 2020. We included meta-analyses of randomised controlled clinical trials measuring the effectiveness of medicinal plants on HbA1c and/or Fasting Plasma Glucose (FPG) in patients with T2DM.

Results: Twenty five meta-analyses reported the effects of 18 plant-based remedies. Aloe vera leaf gel, Psyllium fibre and Fenugreek seeds had the largest effects on HbA1c: mean difference −0.99% [95% CI−1.75, −0.23], −0.97% [95% CI −1.94, −0.01] and −0.85% [95% CI −1.49, −0.22] respectively. Four other remedies reduced HbA1c by at least 0.5%: *Nigella sativa*, *Astragalus membranaceus,* and the traditional Chinese formulae Jinqi Jiangtang and Gegen Qinlian. No serious adverse effects were reported. Several other herbal medicines significantly reduced FPG. Tea and tea extracts (*Camellia sinensis*) were ineffective. However, in some trials duration of follow-up was insufficient to measure the full effect on HbA1c (<8 weeks). Many herbal remedies had not been evaluated in a meta-analysis.

Conclusion: Several medicinal plants appear to be as effective as conventional antidiabetic treatments for reducing HbA1c. Rigorous trials with at least 3 months' follow-up are needed to ascertain the effects of promising plant-based preparations on diabetes.

Keywords: type 2 diabetes mellitus, phytomedicines, medicinal plants, herbal preparations, metaanalysis, randomised controlled clinical trials, glycaemic control, HbA1c

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=220291, PROSPERO.

HIGHLIGHTS

- Aloe vera, Psyllium fibre and Fenugreek seeds had the largest effects on HbA1c: −0.99, −0.97, and −0.85% respectively.

OPEN ACCESS

Edited by:
Massimo Lucarini, Council for Agricultural Research and Economics, Italy

Reviewed by:
Adolfo Andrade-Cetto, National Autonomous University of Mexico, Mexico
Rachael Frost, University College London, United Kingdom

***Correspondence:**
Merlin L. Willcox
M.L.Willcox@soton.ac.uk

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *15 September 2021*
Accepted: *09 November 2021*
Published: *26 November 2021*

Citation:
Willcox ML, Elugbaju C, Al-Anbaki M, Lown M and Graz B (2021) Effectiveness of Medicinal Plants for Glycaemic Control in Type 2 Diabetes: An Overview of Meta-Analyses of Clinical Trials. Front. Pharmacol. 12:777561. doi: 10.3389/fphar.2021.777561

- Four other remedies reduced HbA1c by >0.5%, including *Nigella sativa* and *Astragalus membranaceus*.
- Tea (*Camellia sinensis*) and tea extracts were ineffective.
- No serious adverse effects were reported.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a major, growing health problem. It is estimated that 9.3% of the world's population (463 million people) were living with diabetes in 2019 and this is projected to increase to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2,045 (Saeedi et al., 2019). Over 90% of these have T2DM and over 1 million deaths per year are attributable to diabetes (Khan et al., 2020). The costs are huge: the USA alone spends $294 billion per year on management of diabetes in the population aged 20–79 (International Diabetes Federation, 2019).

Initial treatment of diabetes involves lifestyle modifications including changes to the diet and increasing physical activity, but dietary advice does not usually extend to herbs and phytomedicines. On average, compared to normal diets, low carbohydrate diets reduce HbA1c by only 0.09% (1 mmol/mol) (Korsmo-Haugen et al., 2019). Individualised dietary advice is recommended alongside a personalised management plan that aims to reduce and maintain HbA1c to below 6.5% (National Institute for Health and Care Excellence, 2020). Pharmacotherapy is initiated if patients fail to maintain HbA1c levels below this threshold.

Among adults with T2DM, 45% had not achieved adequate glycaemic control, in a national cross-sectional survey in the USA (Wong et al., 2013); poor adherence to medications is a major reason (Polonsky and Henry, 2016). Less than 50% of patients prescribed metformin were adherent and a third discontinued within 12 months, in a retrospective study in the UK Clinical Practice Research Datalink database (CPRD) (Tang et al., 2020). Side-effects of medication are the commonest reason for non-adherence (Grant et al., 2003). As many as 62% of patients taking metformin complain of diarrhoea (Florez et al., 2010).

Diabetes mellitus has been recognised for thousands of years and treated by traditional systems of medicine in Egypt, China, India, and Africa (Simmonds et al., 2006). Many patients with diabetes still use complementary therapies, ranging from 17% in the UK to 72% in the USA (Chang et al., 2007). Herbal medicines are among the most popular: they are used by 68% of diabetic patients in Saudi Arabia (Alqathama et al., 2020), 62% in Mexico (Chang et al., 2007), 62% in Ethiopia (Mekuria et al., 2018) and 58% in Sudan (Ali and Mahfouz, 2014). In India, 67% of diabetic patients use naturopathy or Ayurveda (Chang et al., 2007). However, the majority do not inform their doctors about their use of herbal medicine (Mekuria et al., 2018; Alqathama et al., 2020). In a qualitative study of members of the Pakistani community in Bradford (UK), two-thirds preferred using herbal medicine compared to conventional medicine and many believed that the vegetable "Karela" (*Momordica charantia*) could cure diabetes (Pieroni et al., 2008). Worldwide, about 1,200 plant species are reportedly used for the treatment of diabetes (Simmonds et al., 2006).

Although there has been a wealth of laboratory and clinical research on herbal medicines for diabetes, this has not been translated into user-friendly evidence-based information to guide patients or clinicians. Most patients base their choice of herbal medicines on advice from family and friends (Ali and Mahfouz, 2014; Mekuria et al., 2018). Although there have been several systematic reviews about herbal medicines for diabetes (Yeh et al., 2003; Wang et al., 2013; Gupta et al., 2017; Governa et al., 2018), none has yet provided a ranking of remedies for their effectiveness on glycaemic control in patients with T2DM. We aim to determine the relative effectiveness of common herbal remedies for treatment of type 2 diabetes through a systematic overview of meta-analyses of controlled clinical trials.

METHODS

The protocol for this study was registered on PROSPERO prior to starting data extraction: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=220291.

The protocol included the research question, search strategy, inclusion criteria and quality appraisal.

Data Sources and Searches

We searched the following databases on October 7, 2020 for systematic reviews of randomised controlled clinical trials: EMBASE via OVID (from 1947), MEDLINE via OVID (from 1946), CINAHL (Cumulated Index to Nursing and Allied Health Literature from 1977) and the Cochrane Library including the Cochrane Central Register of Controlled Trials (CENTRAL). Each search strategy was adapted to take into account differences in controlled vocabulary and syntax rules. An example search strategy is given in **Supplementary Material**. We also contacted experts in the field to identify any relevant studies which had not been found by the search.

Study Selection

Two reviewers independently screened titles and abstracts to select articles for full-text screening. Two reviewers then independently screened the full-text articles. We selected articles which met the following inclusion criteria:

- Study type: Systematic reviews of randomised controlled clinical trials with a reported systematic search strategy and with the intention to perform a meta-analysis.
- Participants: human subjects diagnosed with Type 2 diabetes, both diet-controlled and those on oral hypoglycaemic medications.
- Interventions: one specific herb or standardised herbal remedy
- Comparison: An inactive treatment (placebo) or standard care (oral hypoglycaemic medications, conventional diets)
- Outcomes: quantified change in HbA1c and/or fasting plasma glucose (FPG), reported as a numerical effect size.

We excluded reviews which only presented results in a narrative format and did not attempt to meta-analyse the

outcomes. We did however include systematic reviews which found only a single relevant trial and presented its results in the correct format–where a meta-analysis had been intended but included only a single trial. Some reviews included trials both on T2DM and also on prediabetes. If results for T2DM were presented separately, and/or if trials in T2DM were the majority of included trials, we included these. We excluded reviews where the majority of included trials were not on patients with T2DM and where it was not possible to separate out the results for T2DM patients. We also excluded reviews where results for type 1 diabetes (T1DM) were not presented separately. We excluded reviews of multiple different herbal remedies and of pure compounds extracted from herbs, because none of these presented meta-analyses of individual medicinal plants. We did not apply any language restrictions.

Data Extraction and Quality Assessment

Two reviewers independently extracted relevant data using a data extraction form created on Microsoft Excel, and any discrepancies were checked by a third reviewer. Where a review reported several patient groups and/or outcomes, we extracted the number of trials and participants which matched our inclusion criteria (type 2 diabetes) and which reported each relevant outcome (HbA1c and FPG). When results were presented separately for different types of control, we preferentially chose the comparison against placebo (rather than comparison against standard treatment), in order to gauge the effect size of the medicinal plant itself. Where HbA1c results were reported in mmol/mol, they were multiplied by the conversion factor 0.09148 to give the equivalent as a percentage (National Glycohemoglobin Standardization Program, 2010). Where FPG results were presented as mg/dL, they were divided by 18 to convert to mmol/L. For each review we extracted the number of trials which had reported on adverse effects, and the number of these which reported any specific adverse effects.

Two reviewers independently appraised the quality of the studies using the AMSTAR-2 tool (Shea et al., 2017) and discrepancies were resolved by discussion with a third reviewer.

Data Synthesis and Analysis

Results from meta-analyses of HbA1c and FPG were ranked in order of effect size and presented on a Forest plot. A clinically significant reduction in HbA1c has been defined by clinicians as a reduction of at least ≥0.5% (Lenters-Westra et al., 2014); we defined a clinically significant reduction in FPG as a change of 0.5 mmol/l or more. We conducted a narrative synthesis of the other results. We calculated the Spearman's rank correlation coefficient for the correlation between rank of effect on HbA1c and FPG. In this analysis we only included remedies for which both measures were reported. Where a remedy had differing results from several reviews, we took the rank of the best result for each of HbA1c and FPG.

RESULTS

Included Studies

Our initial search identified 2,363 articles after removing duplicates (**Figure 1**). Forty-nine full texts were screened and of these, 25 met all our inclusion criteria (Davis and Yokoyama, 2011; Kim et al., 2011; Leach and Kumar, 2012; Ooi et al., 2012; Allen et al., 2013; Ooi and Loke, 2013; Neelakantan et al., 2014; Gibb et al., 2015; Gui et al., 2016; Li et al., 2016; Shin et al., 2016; Suksomboon et al., 2016; Tian et al., 2016; Zhang et al., 2016; Daryabeygi-Khotbehsara et al., 2017; Poolsup et al., 2017; Schwingshackl et al., 2017; Gu et al., 2018; Deyno et al., 2019; Gao et al., 2019; Huang et al., 2019; Namazi et al., 2019; Peter et al., 2019; Yang et al., 2019; Ziaei et al., 2020). The commonest reason for exclusion was that the review did not attempt a quantitative meta-analysis of randomised controlled trials. One of the meta-analyses was excluded because it had incorrectly reported underlying data from included studies and its results were inaccurate (Gong et al., 2016).

There were reviews on 18 different medicinal plants (**Table 1**). Some herbal remedies had more than one review: cinnamon (Davis and Yokoyama, 2011; Leach and Kumar, 2012; Allen et al., 2013; Deyno et al., 2019; Namazi et al., 2019), ginseng (Kim et al., 2011; Gui et al., 2016), *Aloe vera* (Suksomboon et al., 2016; Zhang et al., 2016) and karela (*Momordica charantia*) (Ooi et al., 2012; Peter et al., 2019). Three reviews evaluated the effect of a standard traditional Chinese herbal formula which contained a mixture of several herbs. Gegen formulae contained *Pueraria lobata* root as their main constituent alongside other ingredients such as *Salvia miltiorrhiza* root, liquorice root and *Dioscorea opposita* rhizome (Yang et al., 2019). Jinqi Jiangtang contains *Astragalus membranaceus* root, *Coptis* spp rhizome and *lonicera japonica* (Gao et al., 2019). Tianmai Xiaoke contains *Trichosanthes* root, *Ophiopogon japonicus* root, *Schisandra chinensis* fruit and chromium picolinate (Gu et al., 2018). Some reviews studied the effect of specific plant products which are also used as foods: olive oil (Schwingshackl et al., 2017), sweet potato (Ooi and Loke, 2013), dragon fruit (Poolsup et al., 2017), and fenugreek powder incorporated into chapatis (Neelakantan et al., 2014).

All the reviews included mainly clinical trials in patients with T2DM (see **Table 1**) but four also included a few trials in patients with pre-diabetes. One included a single trial in patients with T1DM, but its results were presented separately and excluded from this review. Five reviews included only trials of patients with diet-controlled diabetes, not taking any conventional antidiabetic medications. Fourteen reviews included trials in which both intervention and control groups received concomitant conventional treatment with oral hypoglycaemic agents (OHA). Five reviews did not specify whether concomitant treatment was given. In 19 reviews, the control groups received a placebo, in four reviews they received only the conventional care (diet and/or medications) and in one, some control groups received a fish oil supplement. In three reviews, some studies gave a conventional OHA to the control group only, not to the treatment group (Ooi et al., 2012; Shin et al., 2016; Peter

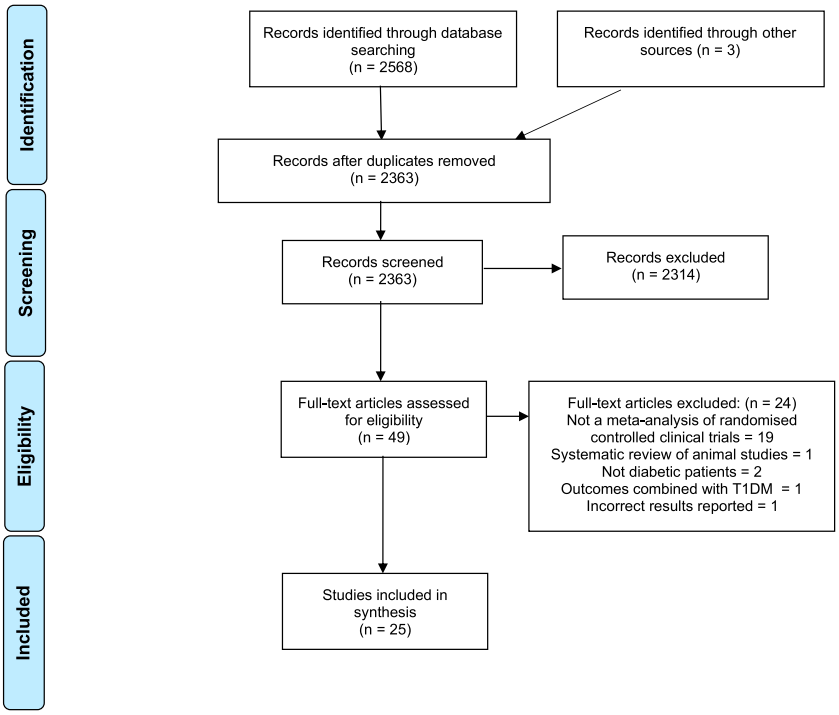


FIGURE 1 | PRISMA flowchart.

et al., 2019) but for this review we only extracted the outcomes from the studies using a placebo control.

Duration of follow-up was most often 4–12 weeks, but there was a wide range with a few included studies following up for as little as 1 week or for as long as 4 years. All the reviews included randomised controlled trials but two also included a few non-randomised controlled trials. The reviews included a median of eight trials and 390 participants but the smallest included only a single trial and the largest review included 25 studies (1724 participants).

Quality Assessment

The AMSTAR-2 scores for each study are shown in **Supplementary Material**. Only three reviews scored "yes" on all the criteria–all of them Cochrane reviews (Leach and Kumar, 2012; Ooi et al., 2012; Ooi and Loke, 2013). Several quality issues were identified with the other reviews. Most did not report that there was a pre-established published protocol. Most did not have a fully comprehensive search strategy including the grey literature. Most did not list all excluded studies and most did not report on the sources of funding for the studies included in the review. Seven did not adequately investigate publication bias. Six did not report conflicts of interest, including the review on Psyllium which was led by a company marketing a Psyllium product (Gibb et al., 2015)—this review is at high risk of bias.

Effect Size on HbA1c

Twenty-one studies on 16 remedies attempted to conduct a meta-analysis quantifying the reduction in HbA1c (**Figure 2**). The most effective remedy appeared to be Aloe vera (freshly extracted juice) (Suksomboon et al., 2016). Psyllium fibre (Gibb et al., 2015) and Fenugreek seeds (Neelakantan et al., 2014) also led to similar reductions in HbA1c although with wider confidence intervals. *Nigella sativa* seeds (Daryabeygi-Khotbehsara et al., 2017), *Astragalus membranaceus* root (Tian et al., 2016), and two complex traditional Chinese formulae (Gegen Qinlian (Yang et al., 2019) and Jinqi Jiantang (Gao et al., 2019)) also led to clinically and statistically significant reductions in HbA1c. Nettle (*Urtica dioica*) appeared to lead to a clinically significant reduction but this was not statistically significant because of very wide confidence intervals (Ziaei et al., 2020).

Several remedies produced a statistically significant reduction in HbA1c but the standard mean difference fell below the pre-determined threshold of 0.5%. These were the patent traditional Chinese formula Tianmai Xiaoke (Gu et al., 2018), ginger (Huang et al., 2019), sweet potato tablets (Ooi and Loke, 2013), olive oil (Schwingshackl et al., 2017), karela (*Momordica charantia*) (Peter et al., 2019) and cinnamon (Namazi et al., 2019). *Momordica charantia* was studied by two reviews which came to differing conclusions; an early Cochrane review found only a single small RCT with 40 participants, which concluded that Karela dried powder in capsules appeared to be ineffective (Ooi et al., 2012). However, a more recent and comprehensive review including five RCTs (243 participants) found that there was a statistically significant reduction in HbA1c by 0.26% (Peter et al., 2019). Similarly, the four reviews on cinnamon which reported HbA1c came to slightly different conclusions; only one

TABLE 1 | Characteristics of included studies.

Herbal remedy	References	Plant species	Plant part	Preparation	Daily dose (mg)	Control	Concomitant treatment (both groups)	Patient type	Follow-up duration for measuring outcomes (weeks)	Study types included	Number of trials	Total number of participants
Aloe vera	Suksomboon et al., (2016)	*Aloe vera* (L) Burm. F. (Xanthorrhoeaceae)	leaf	raw/juice/gel powder	600–30,000	Placebo/ no treatment	OHA/insulin	T2DM	8–12	RCTs	5	235
Aloe vera	Zhang et al., (2016)	*Aloe vera* (L) Burm. F. (Xanthorrhoeaceae)	leaf	juice/powder	200–2,800	Placebo	None	T2DM + prediabetes	6–12	RCTs	5	415
Astragalus	Tian et al., (2016)	*Astragalus membranaceus* (Fisch) Bunge (Fabaceae)	root	aqueous decoction/ injection	1,200–320,000	No treatment	OHA	T2DM	2–16	RCTs	13	1,054
Cinnamon	Allen et al., (2013)	*Cinnamomum cassia* (L.) J.Presl (Lauraceae)	Bark	aqueous extract/raw powder	120–6,000	Placebo	OHA	T2DM	4–18	RCTs	10	464
Cinnamon	Davis and Yokoyama, (2011)	*Cinnamomum cassia* (L.) J.Presl (Lauraceae)	Bark	powder/ aqeuous extract	250–6,000	Placebo	OHA/none	T2DM + prediabetes	4–16	RCTs	8	369
Cinnamon	Deyno et al., (2019)	*Cinnamomum cassia* (L.) J.Presl and *Cinnamomum verum* J.Presl (Lauraceae)	NS	capsules	1,000–14,400	Placebo	OHA/none	T2DM + prediabetes	4–16	RCTs	16	1,098
Cinnamon	Leach and Kumar, (2012)	*Cinnamomum cassia* (L.) J.Presl and *Cinnamomum burmanni* (Nees & T.Nees) Blume (Lauraceae)	NS	tablet/ capsule	500–6,000	Placebo	OHA/insulin	T1+T2DM	4–16	RCTs	10	304
Cinnamon	Namazi et al., (2019)	*Cinnamomum cassia* (L.) J.Presl (Lauraceae)	NS	Powder/ extract	120–6,000	Placebo	None	T2DM	6–17	RCTs	18	1,100
Dragon Fruit	Poolsup et al., (2017)	*Hylocereus polyrhizus* (F.A.C.Weber) Britton & Rose; *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose (Cactaceae)	Fruit	Fresh fruit	100,000–600,000	No treatment	NS	T2DM	2–4	RCTs	2	58
Fenugreek	Neelakantan et al., (2014)	*Trigonella foenum-graecum* L (Fabaceae)	seed	powder/ extract in capsules/ chapati	1,000–100,000	Placebo	OHA	T2DM	1.5–12	9 RCTs and 1 controlled trial	10	278

(Continued on following page)

TABLE 1 | (*Continued*) Characteristics of included studies.

Herbal remedy	References	Plant species	Plant part	Preparation	Daily dose (mg)	Control	Concomitant treatment (both groups)	Patient type	Follow-up duration for measuring outcomes (weeks)	Study types included	Number of trials	Total number of participants
Gegen formulae	Yang et al., (2019)	*Pueraria lobata* (Willd.) Ohwi (Fabaceae)	Root	mixture	NS	Placebo	OHA/insulin	T2DM	2–24	RCTs	13	1,440
Ginger	Huang et al., (2019)	*Zingiber officinale* Roscoe (Zingiberaceae)	NS	NS	1,600–4,000	NS	NS	T2DM	8–12	RCTs	8	454
Ginseng	Gui et al., (2016)	*Panax quinquefolius* L and *Panax ginseng* C.A.Mey (Araliaceae)	NS	Raw herb/ hydrolysed extract in capsules	960–13,500	Placebo	Nil	Untreated early diabetes + prediabetes	4–20	RCTs	8	390
Ginseng	Kim et al., (2011)	*Panax ginseng* C.A.Mey (Araliaceae)	Root	Red Ginseng powder/ fermented powder	780–3,000	Placebo	OHA	T2DM	12–24	RCTs	3	76
Jinqi Jiangtang	Gao et al., (2019)	*Astragalus membranaceus* (Fisch) Bunge (Fabaceae); *Coptis chinensis* Franch. (Ranunculaceae); *Lonicera japonica* Thunb. (Caprifoliaceae)	Root (*Astragalus* and *Coptis*), Flower (*Lonicera*)	Tablets	2,520–16,800	No treatment	OHA	T2DM	3–26	RCTs	17	1,365
Momordica charantia	Ooi et al., (2012)	*Momordica charantia* L (Cucurbitaceae)	fruit	dried powder in capsules	3,000	Placebo, OHA	Diet only	T2DM	12	RCTs	1	40
Momordica charantia	Peter et al., (2019)	*Momordica charantia* L (Cucurbitaceae)	Fruit	dried pulp/ juice	1,200–6,000	Placebo, OHA	OHA/none	T2DM	4–16	RCTs	6	243
Mulberry	Shin et al., (2016)	*Morus alba* L. (Moraceae)	leaf	Extract in capsules	1,000	Placebo, OHA	None	T2DM	12	RCTs	1	23
Nettle	Ziaei et al., (2020)	*Urtica dioica* L (Urticaceae)	NS	NS	1,500–10,000	placebo	None	T2DM	8–12	RCTs	8	266
Nigella sativa	Daryabeygi-Khotbehsara et al., (2017)	*Nigella sativa* L (Ranunculaceae)	seed	powder/oil	500–2000mg; 1–5 ml	Placebo	OHA/none	T2DM	8–52	4 RCTs and 3 non-randomised controlled trials	7	505
Olive Oil	Schwingshackl et al., (2017)	*Olea europaea* L. (Oleaceae)	fruit	oil	10,000–75,000	Low-fat diet/fish oils/PUFA	NS	T2DM	2–208	RCTs	25	1724

(Continued on following page)

TABLE 1 | (*Continued*) Characteristics of included studies.

Herbal remedy	References	Plant species	Plant part	Preparation	Daily dose (mg)	Control	Concomitant treatment (both groups)	Patient type	Follow-up duration for measuring outcomes (weeks)	Study types included	Number of trials	Total number of participants
Psyllium Fiber	Gibb et al., (2015)	*Plantago psyllium* L.; *Plantago ovata* Forssk. (Plantaginaceae)	Seed	husk	6,800–15,000	Placebo/ no treatment	None	T2DM	6–12	RCTs	4	245
Sweet Potato	Ooi and Loke, (2013)	*Ipomoea batatas* (L.) Lam. (Convolvulaceae)	Rhizome	Dry powder in tablets	4,000	Placebo	None	T2DM	6–20	RCTs	2	122
Tea	Li et al., (2016)	*Camellia sinensis* (L.) Kuntze (Theaceae)	Leaf	green/black/ oolong tea/ capsules	150–1,500	Placebo/ water	NS	T2DM	4–16	RCTs	12	658
Tianmai Xiaoke	Gu et al., (2018)	*Trichosanthes kirilowii* Maxim. (Cucurbitaceae); *Ophiopogon japonicus* (Thunb.) Ker Gawl. (Asparagaceae); *Schisandra chinensis* (Turcz.) Baill. (Schisandraceae)	Root (*Trichosanthes*, Ophiopogon); Fruit (Schisandra)	Tablets, also containing chromium picolinate (1.6 mg per tablet)	480	No treatment	OHA/insulin	T2DM	8–16	RCTs	7	717

Abbreviations: OHA, oral hypoglycaemic agent; T1DM, Type 1 Diabetes Mellitus; T2DM, type 2 diabetes mellitus; NS, Not Specified; RCTs, Randomised Controlled Trials.

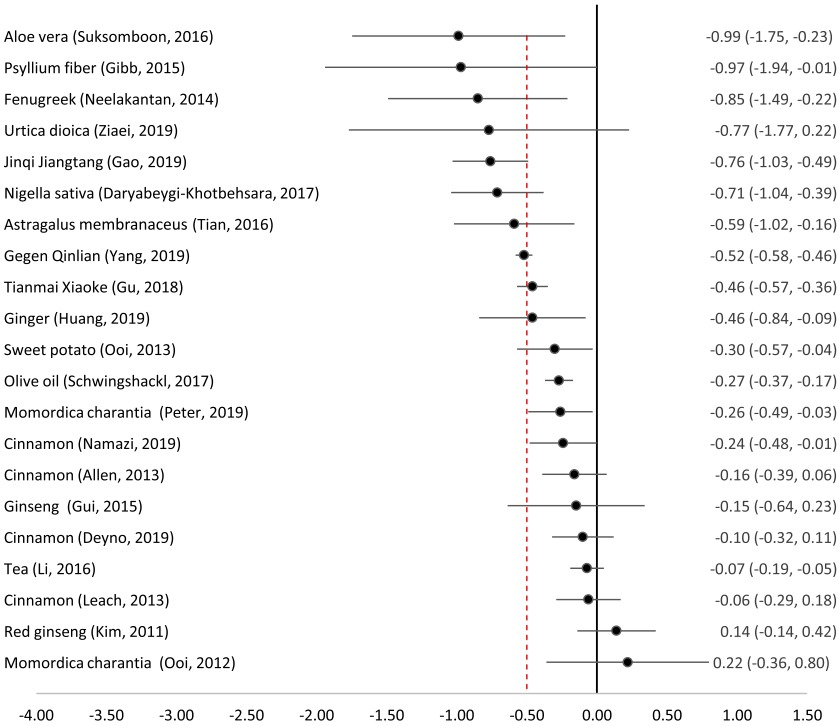


FIGURE 2 | Effect of medicinal plants on HbA1c (%). The red dotted line indicates the threshold for a clinically significant effect (reduction by 0.5%). Point indicates the effect size, and the line (and figures to the right) indicate the 95% confidence interval.

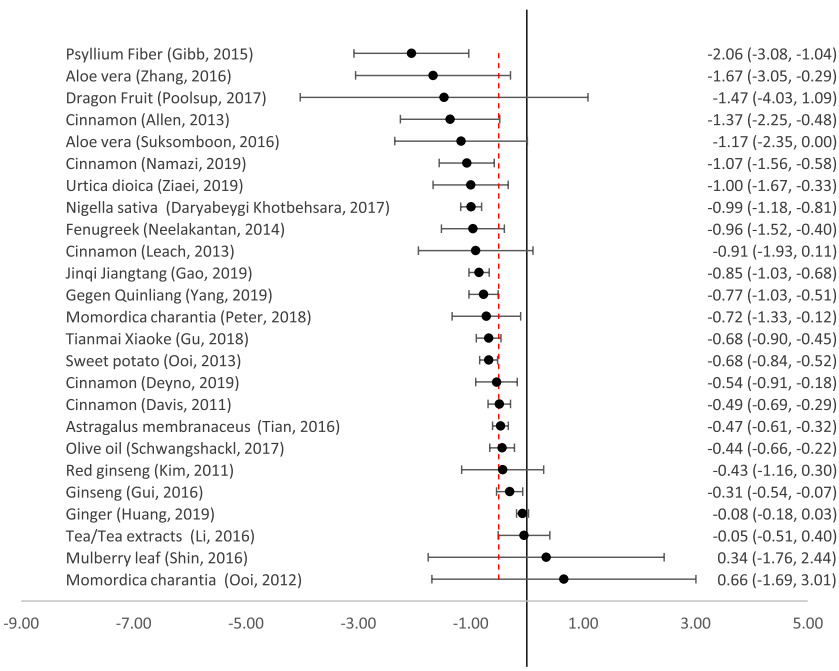


FIGURE 3 | Effect of medicinal plants on Fasting Plasma Glucose (mmol/l). The red dotted line indicates the threshold for a clinically significant effect (reduction by 0.5mmol/l). The point indicates the effect size, and the line (and figures to the right) indicate the 95% confidence interval.

TABLE 2 | Reported adverse effects.

Herbal remedy	References	Number of trials	Adverse effects (N of trials reporting)
Aloe vera	Suksomboon et al., (2016)	5	None (4); diarrhoea/vomiting (1)
Aloe vera	Zhang et al., (2016)	5	Only one trial reported one adverse event (not specified)
Astragalus	Tian et al., (2016)	13	None (3)
Cinnamon	Allen et al., (2013)	10	No significant adverse effects (10)
Cinnamon	Davis and Yokoyama, (2011)	8	Not reported
Cinnamon	Deyno et al., (2019)	16	"Well tolerated"
Cinnamon	Leach and Kumar, (2012)	10	No difference in incidence between treatment and control (3). One case of hives, and one of hypoglycaemic seizure (in a trial in T1DM patients on insulin).
Cinnamon	Namazi et al., (2019)	18	One case of "skin allergy" after taking the remedy for 90 days.
Dragon Fruit	Poolsup et al., (2017)	2	None (2)
Fenugreek	Neelakantan et al., (2014)	10	Mild gastrointestinal symptoms (3)
Gegen Qinlian	Yang et al., (2019)	13	None reported (15); fewer than in control group (12); mild g-i effects (2)
Ginger	Huang et al., (2019)	8	Not reported
Ginseng	Gui et al., (2016)	8	No serious adverse events (8)
Ginseng	Kim et al., (2011)	3	No difference in incidence compared to control; reports of tachycardia, headache, blurry vision, insomnia, irritability and hypoglycemia (1)
Ginseng	Shishtar et al., (2014)	16	No difference in incidence compared to control (4)
Jinqi Jiangtang	Gao et al., (2019)	17	No difference in incidence compared to control (8)
Momordica charantia	Ooi et al., (2012)	1	No serious adverse events (3); gastrointestinal symptoms (2)
Momordica charantia	Peter et al., (2019)	6	Gastrointestinal symptoms (5), headache/dizziness (2), rash (1), sore throat (1), hypotension (1)
Mulberry	Shin et al., (2016)	1	Not reported
Nettle	Ziaei et al., (2020)	8	No significant adverse events (7), itching (1)
Nigella sativa	Daryabeygi-Khotbehsara et al., (2017)	7	None (6); mild g-i side effects (1)
Olive Oil	Schwingshackl et al., (2017)	25	Not reported
Psyllium Fiber	Gibb et al., (2015)	4	Not reported
Sweet Potato	Ooi and Loke, (2013)	2	No difference in incidence compared to control (2)
Tea	Li et al., (2016)	12	Not reported
Tianmai Xiaoke	Gu et al., (2018)	7	Gastrointestinal symptoms, nervous system symptoms, and hypoglycemia (7)

found a statistically significant reduction and none of them reported a clinically significant reduction.

Two meta-analyses of ginseng (Kim et al., 2011; Gui et al., 2016) and one of tea (*Camellia sinensis*) and tea extracts (Li et al., 2016) all showed that these remedies had no clinically or statistically significant effect on HbA1c.

Effect Size on Fasting Plasma Glucose (FPG)

Twenty-five reviews meta-analysed the reduction in FPG (**Figure 3**). All the remedies which produced clinically significant reductions in HbA1c also produced clinically and statistically significant reductions in FPG, with the exception of *Astragalus membranaceus*, which reduced FPG slightly less than the predetermined clinically significant threshold of 0.5 mmol/l. Nettle (*Urtica dioica*), *Momordica charantia* and sweet potato also all produced clinically significant reductions in FPG.

There were varying results in the five meta-analyses of cinnamon, but the largest and most recent (which only included patients with T2DM) showed a clinically significant reduction in FPG of −1.07 mmol/l (95% CI-1.56 to −0.58) (Namazi et al., 2019). Other reviews also included patients with T1DM (Leach and Kumar, 2012) or pre-diabetes (Davis and Yokoyama, 2011; Deyno et al., 2019).

For several remedies, there was a wide degree of uncertainty regarding their effectiveness in reducing FPG. Dragon fruit appeared to have a large effect but this was not statistically significant as there were very wide confidence intervals (Poolsup et al., 2017). There was also a large degree of uncertainty about the effect of Mulberry leaf–there was a wide confidence interval, and a second trial (not included in the meta-analysis) reported that it was more effective than glibenclamide (Shin et al., 2016). Of the two reviews on Ginseng, that by Kim et al. (2011) was the only one to focus purely on T2DM; it showed a non-significant reduction in FPG. Another meta-analysis did report a significant reduction in FPG but also included pre-diabetic patients (Gui et al., 2016).

It can be stated with some certainty that ginger and tea (*Camellia sinensis*) extracts were ineffective for reducing FPG. Neither had a significant effect, and confidence intervals were tight.

Correlation Between Effect on HbA1c and FPG

Spearman's rank correlation coefficient was 0.70, indicating that there was a moderate correlation between effect on HbA1c and FPG.

Adverse Effects

None of the included reviews reported any serious adverse events. In most cases there was no significant difference in the incidence of adverse events between the treatment and control groups (**Table 2**). Mild gastrointestinal symptoms such as diarrhoea, vomiting and abdominal discomfort were reported in a few cases for certain herbal remedies, in particular *Momordica charantia* (three participants) and Fenugreek seeds (three participants). There was no specific mention of drug interactions although 14 of the reviews included trials in which the herbal medicine was given in addition to conventional oral hypoglycaemic agents. Only three of these reviews mentioned cases of hypoglycaemia, including only one reported case of a hypoglycaemic seizure in a clinical trial of cinnamon given to adolescent T1DM patients on insulin (Leach and Kumar, 2012).

DISCUSSION

Summary of Main Findings

There have been many RCTs on different phytomedicines and herbal medicines for T2DM, and 25 published meta-analyses on 18 different medicinal plants. Of these, seven have a clinically and statistically significant effect on HbA1c and 12 on FPG (**Figures 2**, **3**). The most effective on both measures appear to be *Aloe vera*, Psyllium fibre, Fenugreek seeds, *Nigella sativa* seeds, and the complex traditional Chinese formula Jinqi Jiangtang. Tea and tea extracts were ineffective. The 12 other remedies showed some degree of effectiveness on either HbA1c or FPG, but in some cases with a wide degree of uncertainty. All of the medicinal plants evaluated in this review appeared to be safe, with no serious adverse effects reported. However, some were associated with minor side-effects, in particular gastrointestinal disturbances.

Strengths and Limitations

This the first study to provide a systematic, evidence-based overview of meta-analyses of the effectiveness of medicinal plants for glycaemic control in type 2 diabetes. Our systematic approach with broad search terms ensured that we probably found most relevant articles. One limitation is that we did not have the time to search the grey literature or databases in foreign languages such as Chinese. Another limitation is that we were not able to include medicinal plants for which there had been no systematic review with a meta-analysis. For example there was a systematic review of the Ayurvedic remedy *Gymnema sylvestre* (Leach, 2007) but this found no clinical trials which met its inclusion criteria. It is also likely that there are other potentially effective medicinal plants which have been evaluated in RCTs but not reviewed in a meta-analysis, and others which have not been evaluated in an RCT although lower-level evidence suggests they could be effective (Sissoko et al., 2020).

Our results are also limited by the quality of the trials included in the meta-analyses. Although most only included RCTs, in some cases the preparation or dosage of the phytomedicine may have been suboptimal; in some reviews both herbal remedies and standardised phytomedicines were included. The clinical condition of the patients may have been different between trials where patients were taking concomitant oral antidiabetics and those who were purely diet controlled. In some trials, the duration of follow-up was insufficient to measure the effect on HbA1c, which should be measured at least 3 months after the start of treatment to reveal its full effect. Follow-up duration was generally short: only three reviews included studies with follow-up of 1 year or more, so there is little information on long-term adherence to herbal remedies.

Comparison With the Existing Literature

The effect of the most promising medicinal plants was similar to that of standard oral hypoglycaemic agents. In a meta-analysis, metformin monotherapy lowered HbA1c by 1.12% (95% CI 0.92–1.32) versus placebo. Metformin added to oral therapy lowered HbA1c by 0.95% (0.77–1.13) versus placebo added to oral therapy (Hirst et al., 2012). In another meta-analysis, metformin reduced FPG by −2.0 mmol/l (95% CI: −2.4, −1.7) (Johansen, 1999). Other conventional hypoglycaemic medications have a smaller effect, for example sitagliptin lowers HbA1c by −0.94% and FPG by 1.2 mmol/l (Aschner et al., 2006).

Several mechanisms of action explain the effect of medicinal plants. Firstly, many plant products contain gel-forming fibres which delay gastric emptying and interfere with glucose absorption from the intestines–for example *Aloe vera* (Suksomboon et al., 2016), Fenugreek (Madar and Shomer, 1990) and Psyllium (Gibb et al., 2015). Secondly, some medicinal plants contain substances which inhibit enzymes involved in digestion of carbohydrates (eg α-amylase, α-glucosidase), such as nettle (Ziaei et al., 2020) and the Chinese formula Jinqui Jiangtan (Gao et al., 2019). Third, others stimulate release of insulin; these include Fenugreek seeds (Neelakantan et al., 2014) and *Nigella sativa* seeds (Daryabeygi-Khotbehsara et al., 2017). Fourth, some medicinal plants inhibit gluconeogenesis, including *Nigella sativa* (Daryabeygi-Khotbehsara et al., 2017). Fifth, some, such as nettle (Ziaei et al., 2020), mimic the effect of insulin by increasing peripheral uptake of glucose, while others such as *Nigella sativa* induce insulin sensitivity (Daryabeygi-Khotbehsara et al., 2017).

Implications for Policy and Practice

Dietary and lifestyle advice for patients with diabetes rarely includes information on natural remedies, herbs and spices that can help with glycaemic control. The results presented here can guide patients who wish to try herbal supplements and foods as part of their self-care and diet, and clinicians who wish to advise them. Several of the remedies tested are effective and safe. Many of these herbs and spices with clinically assessed hypoglycemic properties are common food products, and as such generally considered very safe. Some can easily be incorporated into the diet–for example in some studies fenugreek seed powder was mixed with flour for baking chapatis, to reach a total daily dose of 100 g (Neelakantan et al., 2014); but the most effective preparation appeared to be a standardised extract of Fenugreek seed total saponins given in six capsules three times daily after meals (Lu et al., 2008). Other herbs can easily be purchased

without a prescription (for example *Aloe vera*, Psyllium fibre, and *Nigella* seeds). However, it would be necessary to ensure that an adequate dosage is taken of the most effective preparations. The most effective preparation of *Aloe vera* appeared to be freshly extracted juice, followed by powdered gel in capsules (Suksomboon et al., 2016). In the case of Psyllium, the most effective preparation appeared to be the seed husk of *Plantago ovata* Forssk (Ziai et al., 2005). For *Nigella sativa*, the seed powder (at a dose of 2 g daily) was more effective than the oil (Daryabeygi-Khotbehsara et al., 2017). It is equally important to inform patients and clinicians about remedies which appear to be ineffective–such as tea extracts–and those for which there is insufficient evidence of effectiveness–for example cinnamon and ginseng.

Priorities for Future Research

Firstly, some of the meta-analyses were performed more than 5 years ago and need to be updated to include the most recent trials. Some reviews were not performed to the highest standards and could be improved. In particular we recommend that the meta-analysis on Fenugreek should be updated because this appears to be one of the most effective remedies but the systematic review was done in 2014 (Neelakantan et al., 2014). A later systematic review suggested an even greater effect but incorrectly reported some of the underlying data (Gong et al., 2016). It would also be useful to perform a network meta-analysis to estimate the relative effects between the different herbal interventions.

Secondly, it would be interesting to evaluate the impact on glycaemic control of including information on effective medicinal plants and herbal remedies within dietary and lifestyle advice for patients with type 2 diabetes. These may have an additional benefit, and for some patients may be more acceptable, so may be a useful addition to the "menu" of options. This information would need to include clear instructions on the most effective preparations and dosages, and to warn patients about potential side-effects.

Thirdly, this review found a large number of potentially effective medicinal plants for which there is insufficient evidence of effectiveness. For example, Nettle (*Urtica dioica*) appears to have a significant effect on HbA1c and FPG (Ziaei et al., 2020) but the confidence intervals are very wide. Larger trials are needed to provide a more precise estimate of efficacy. Although it appears effective, the results on Psyllium were at high risk of bias because the review was undertaken by a company selling it–a higher quality review, with low risk of bias, would be helpful. In some studies, cinnamon appears to significantly reduce FPG, but not HbA1c. However, there is a wide variety of cinnamon species, preparations and doses–it is likely that some are more effective than others. Further research is needed to identify the most effective preparations and dosages, and to conduct high-quality clinical trials of these.

Fourth, for the majority of the 1,200 remedies which have been traditionally used in the treatment of diabetes (Simmonds et al., 2006), no meta-analyses and/or no RCTs have been conducted. Some of these have preliminary evidence of effectiveness, for example on post-prandial glucose; these include the Ayurvedic remedy *Gymnema sylvestre* (Leach, 2007) and the West African tree *Moringa oleifera* (Sissoko et al., 2020). It is important to conduct high-quality clinical trials of these (at low risk of bias, using a standardised, replicable dosage and preparation, and measuring HbA1c after at least 12 weeks).

CONCLUSION

Several medicinal plants have the potential to lower HbA1c and could be effective as an adjunct to other lifestyle measures and current treatment, in particular Aloe vera, Psyllium fibre, Fenugreek seeds, Nigella sativa seeds and the Chinese formula Jinqi Jiangtang. It is also clear that tea and tea extracts are ineffective. Rigorous trials with at least 3 months follow-up are needed to ascertain the safety and effectiveness of promising plant-based preparations on diabetes. Practical information on safe plant-based preparations with hypoglycaemic effects should be made widely available to clinicians and patients with diabetes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MW, ML, and BG conceived and designed the study. CE and MA-A conducted the literature searches, screening, quality appraisal and data extraction. MW checked quality appraisal and data extraction and wrote the first draft of the manuscript. All authors contributed to revising the manuscript and approved the final version.

FUNDING

MLW's and ML's salaries were funded by the National Institute of Health Research (grants CL-2016-26-005, NIHR301108). MA-A's and BG's salaries were funded by the Antenna Foundation (Geneva, Switzerland).

ACKNOWLEDGMENTS

The authors would like to thank Paula Sands for her assistance in developing and refining the search strategy and her guidance throughout the search process.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.777561/full#supplementary-material

REFERENCES

Ali, B., and Mahfouz, M. (2014). Herbal Medicine Use Among Patients with Type 2 Diabetes in North Sudan. *Annual Research and Review in Biology* 4, 1827–1838. doi:10.9734/arrb/2014/8015

Allen, R. W., Schwartzman, E., Baker, W. L., Coleman, C. I., and Phung, O. J. (2013). Cinnamon Use in Type 2 Diabetes: an Updated Systematic Review and Meta-Analysis. *Ann. Fam. Med.* 11, 452–459. doi:10.1370/afm.1517

Alqathama, A., Alluhiabi, G., Baghdadi, H., Aljahani, L., Khan, O., Jabal, S., et al. (2020). Herbal Medicine from the Perspective of Type II Diabetic Patients and Physicians: what Is the Relationship? *BMC Complement. Med. Ther.* 20, 65. doi:10.1186/s12906-020-2854-4

Aschner, P., Kipnes, M. S., Lunceford, J. K., Sanchez, M., Mickel, C., and Williams-Herman, D. E. (2006). Effect of the Dipeptidyl Peptidase-4 Inhibitor Sitagliptin as Monotherapy on Glycemic Control in Patients with Type 2 Diabetes. *Diabetes Care* 29, 2632–2637. doi:10.2337/dc06-0703

Chang, H. Y., Wallis, M., and Tiralongo, E. (2007). Use of Complementary and Alternative Medicine Among People Living with Diabetes: Literature Review. *J. Adv. Nurs.* 58, 307–319. doi:10.1111/j.1365-2648.2007.04291.x

Daryabeygi-Khotbehsara, R., Golzarand, M., Ghaffari, M. P., and Djafarian, K. (2017). Nigella Sativa Improves Glucose Homeostasis and Serum Lipids in Type 2 Diabetes: A Systematic Review and Meta-Analysis. *Complement. Ther. Med.* 35, 6–13. doi:10.1016/j.ctim.2017.08.016

Davis, P. A., and Yokoyama, W. (2011). Cinnamon Intake Lowers Fasting Blood Glucose: Meta-Analysis. *J. Med. Food* 14, 884–889. doi:10.1089/jmf.2010.0180

Deyno, S., Eneyew, K., Seyfe, S., Tuyiringire, N., Peter, E. L., Muluye, R. A., et al. (2019). Efficacy and Safety of Cinnamon in Type 2 Diabetes Mellitus and Pre-diabetes Patients: A Meta-Analysis and Meta-Regression. *Diabetes Res. Clin. Pract.* 156, 107815. doi:10.1016/j.diabres.2019.107815

Florez, H., Luo, J., Castillo-Florez, S., Mitsi, G., Hanna, J., Tamariz, L., et al. (2010). Impact of Metformin-Induced Gastrointestinal Symptoms on Quality of Life and Adherence in Patients with Type 2 Diabetes. *Postgrad. Med.* 122, 112–120. doi:10.3810/pgm.2010.03.2128

Gao, H., Yang, Y., Deng, J., Liang, J., Zhang, W., and Feng, X. (2019). A Systematic Review and Meta-Analysis on the Efficacy and Safety of Traditional Chinese Patent Medicine Jinqi Jiangtang Tablet in the Treatment of Type 2 Diabetes. *Complement. Ther. Med.* 47, 102021. doi:10.1016/j.ctim.2019.01.016

Gibb, R. D., McRorie, J. W., Jr, Russell, D. A., Hasselblad, V., and D'Alessio, D. A. (2015). Psyllium Fiber Improves Glycemic Control Proportional to Loss of Glycemic Control: a Meta-Analysis of Data in Euglycemic Subjects, Patients at Risk of Type 2 Diabetes Mellitus, and Patients Being Treated for Type 2 Diabetes Mellitus. *Am. J. Clin. Nutr.* 102, 1604–1614. doi:10.3945/ajcn.115.106989

Gong, J., Fang, K., Dong, H., Wang, D., Hu, M., and Lu, F. (2016). Effect of Fenugreek on Hyperglycaemia and Hyperlipidemia in Diabetes and Prediabetes: A Meta-Analysis. *J. Ethnopharmacol* 194, 260–268. doi:10.1016/j.jep.2016.08.003

Governa, P., Baini, G., Borgonetti, V., Cettolin, G., Giachetti, D., Magnano, A. R., et al. (2018). Phytotherapy in the Management of Diabetes: A Review. *Molecules* 23, 105. doi:10.3390/molecules23010105

Grant, R. W., Devita, N. G., Singer, D. E., and Meigs, J. B. (2003). Polypharmacy and Medication Adherence in Patients with Type 2 Diabetes. *Diabetes Care* 26, 1408–1412. doi:10.2337/diacare.26.5.1408

Gu, Y., Xu, X., Wang, Z., Xu, Y., Liu, X., Cao, L., et al. (2018). Chromium-Containing Traditional Chinese Medicine, Tianmai Xiaoke Tablet, for Newly Diagnosed Type 2 Diabetes Mellitus: A Meta-Analysis and Systematic Review of Randomized Clinical Trials. *Evid. Based Complement. Alternat Med.* 2018, 3708637. doi:10.1155/2018/3708637

Gui, Q. F., Xu, Z. R., Xu, K. Y., and Yang, Y. M. (2016). The Efficacy of Ginseng-Related Therapies in Type 2 Diabetes Mellitus: An Updated Systematic Review and Meta-Analysis. *Medicine (Baltimore)* 95, e2584. doi:10.1097/MD.0000000000002584

Gupta, R. C., Chang, D., Nammi, S., Bensoussan, A., Bilinski, K., and Roufogalis, B. D. (2017). Interactions between Antidiabetic Drugs and Herbs: an Overview of Mechanisms of Action and Clinical Implications. *Diabetol. Metab. Syndr.* 9, 59. doi:10.1186/s13098-017-0254-9

Hirst, J. A., Farmer, A. J., Ali, R., Roberts, N. W., and Stevens, R. J. (2012). Quantifying the Effect of Metformin Treatment and Dose on Glycemic Control. *Diabetes Care* 35, 446–454. doi:10.2337/dc11-1465

Huang, F. Y., Deng, T., Meng, L. X., and Ma, X. L. (2019). Dietary Ginger as a Traditional Therapy for Blood Sugar Control in Patients with Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis. *Medicine (Baltimore)* 98, e15054. doi:10.1097/MD.0000000000015054

International Diabetes Federation (2019). *IDF Diabetes Atlas*. 9th edn ed. Brussels, Belgium: International Diabetes Federation.

Johansen, K. (1999). Efficacy of Metformin in the Treatment of NIDDM. Meta-Analysis. *Diabetes Care* 22, 33–37. doi:10.2337/diacare.22.1.33

Khan, M. A. B., Hashim, M. J., King, J. K., Govender, R. D., Mustafa, H., and Al Kaabi, J. (2020). Epidemiology of Type 2 Diabetes - Global Burden of Disease and Forecasted Trends. *J. Epidemiol. Glob. Health* 10, 107–111. doi:10.2991/jegh.k.191028.001

Kim, S., Shin, B. C., Lee, M. S., Lee, H., and Ernst, E. (2011). Red Ginseng for Type 2 Diabetes Mellitus: A Systematic Review of Randomized Controlled Trials. *Chin. J. Integr. Med.* 17, 937–944. doi:10.1007/s11655-011-0937-2

Korsmo-Haugen, H. K., Brurberg, K. G., Mann, J., and Aas, A. M. (2019). Carbohydrate Quantity in the Dietary Management of Type 2 Diabetes: A Systematic Review and Meta-Analysis. *Diabetes Obes. Metab.* 21, 15–27. doi:10.1111/dom.13499

Leach, M. J. (2007). Gymnema Sylvestre for Diabetes Mellitus: a Systematic Review. *J. Altern. Complement. Med.* 13, 977–983. doi:10.1089/acm.2006.6387

Leach, M. J., and Kumar, S. (2012). Cinnamon for Diabetes Mellitus. *Cochrane Database Syst. Rev.* 2012, CD007170. doi:10.1002/14651858.cd007170.pub2

Lenters-Westra, E., Schindhelm, R. K., Bilo, H. J., Groenier, K. H., and Slingerland, R. J. (2014). Differences in Interpretation of Haemoglobin A1c Values Among Diabetes Care Professionals. *Neth. J. Med.* 72, 462–466.

Li, Y., Wang, C., Huai, Q., Guo, F., Liu, L., Feng, R., et al. (2016). Effects of tea or tea Extract on Metabolic Profiles in Patients with Type 2 Diabetes Mellitus: a Meta-Analysis of Ten Randomized Controlled Trials. *Diabetes Metab. Res. Rev.* 32, 2–10. doi:10.1002/dmrr.2641

Lu, F. R., Shen, L., Qin, Y., Gao, L., Li, H., and Dai, Y. (2008). Clinical Observation on Trigonella Foenum-Graecum L. Total Saponins in Combination with Sulfonylureas in the Treatment of Type 2 Diabetes Mellitus. *Chin. J. Integr. Med.* 14, 56–60. doi:10.1007/s11655-007-9005-3

Madar, Z., and Shomer, I. (1990). Polysaccharide Composition of a Gel Fraction Derived from Fenugreek and its Effect on Starch Digestion and Bile Acid Absorption in Rats. *J. Agric. Food Chem.* 38, 1535–1539. doi:10.1021/jf00097a023

Mekuria, A. B., Belachew, S. A., Tegegn, H. G., Ali, D. S., Netere, A. K., Lemlemu, E., et al. (2018). Prevalence and Correlates of Herbal Medicine Use Among Type 2 Diabetic Patients in Teaching Hospital in Ethiopia: a Cross-Sectional Study. *BMC Complement. Altern. Med.* 18, 85. doi:10.1186/s12906-018-2147-3

Namazi, N., Khodamoradi, K., Khamechi, S. P., Heshmati, J., Ayati, M. H., and Larijani, B. (2019). The Impact of Cinnamon on Anthropometric Indices and Glycemic Status in Patients with Type 2 Diabetes: A Systematic Review and Meta-Analysis of Clinical Trials. *Complement. Ther. Med.* 43, 92–101. doi:10.1016/j.ctim.2019.01.002

National Glycohemoglobin Standardization Program (2010). *IFCC Standardization of HbA1c*. Available at http://www.ngsp.org/ifccngsp.asp.

National Institute for Health and Care Excellence (2020). *Type 2 Diabetes in Adults: Management*. London: NICE.

Neelakantan, N., Narayanan, M., De Souza, R. J., and Van Dam, R. M. (2014). Effect of Fenugreek (Trigonella Foenum-Graecum L.) Intake on Glycemia: A Meta-Analysis of Clinical Trials. *Nutr. J.* 13, 7. doi:10.1186/1475-2891-13-7

Ooi, C. P., and Loke, S. C. (2013). Sweet Potato for Type 2 Diabetes Mellitus. *Cochrane Database Syst. Rev.* 2017, CD009128. doi:10.1002/14651858.CD009128.pub3

Ooi, C. P., Yassin, Z., and Hamid, T. A. (2012). Momordica Charantia for Type 2 Diabetes Mellitus. *Cochrane Database Syst. Rev.* 2012, CD007845. doi:10.1002/14651858.CD007845.pub2

Peter, E. L., Kasali, F. M., Deyno, S., Mtewa, A., Nagendrappa, P. B., Tolo, C. U., et al. (2019). Momordica Charantia L. Lowers Elevated Glycaemia in Type 2 Diabetes Mellitus Patients: Systematic Review and Meta-Analysis. *J. Ethnopharmacol* 231, 311–324. doi:10.1016/j.jep.2018.10.033

Pieroni, A., Sheikh, Q. Z., Ali, W., and Torry, B. (2008). Traditional Medicines Used by Pakistani Migrants from Mirpur Living in Bradford, Northern England. *Complement. Ther. Med.* 16, 81–86. doi:10.1016/j.ctim.2007.03.005

Polonsky, W. H., and Henry, R. R. (2016). Poor Medication Adherence in Type 2 Diabetes: Recognizing the Scope of the Problem and its Key Contributors. *Patient Prefer Adherence* 10, 1299–1307. doi:10.2147/PPA.S106821

Poolsup, N., Suksomboon, N., and Paw, N. J. (2017). Effect of Dragon Fruit on Glycemic Control in Prediabetes and Type 2 Diabetes: A Systematic Review and Meta-Analysis. *PLoS ONE* 12, e0184577. doi:10.1371/journal.pone.0184577

Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., et al. (2019). Global and Regional Diabetes Prevalence Estimates for 2019 and Projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th Edition. *Diabetes Res. Clin. Pract.* 157, 107843. doi:10.1016/j.diabres.2019.107843

Schwingshackl, L., Lampousi, A. M., Portillo, M. P., Romaguera, D., Hoffmann, G., and Boeing, H. (2017). Olive Oil in the Prevention and Management of Type 2 Diabetes Mellitus: a Systematic Review and Meta-Analysis of Cohort Studies and Intervention Trials. *Nutr. Diabetes* 7, e262. doi:10.1038/nutd.2017.12

Shea, B. J., Reeves, B. C., Wells, G., Thuku, M., Hamel, C., Moran, J., et al. (2017). AMSTAR 2: a Critical Appraisal Tool for Systematic Reviews that Include Randomised or Non-randomised Studies of Healthcare Interventions, or Both. *BMJ* 358, j4008. doi:10.1136/bmj.j4008

Shin, S.-O., Seo, H.-J., Park, H., and Song, H. J. (2016). Effects of mulberry Leaf Extract on Blood Glucose and Serum Lipid Profiles in Patients with Type 2 Diabetes Mellitus: A Systematic Review. *Eur. J. Integr. Med.* 8, 602–608. doi:10.1016/j.eujim.2016.06.008

Simmonds, M. S. J., and Howes, M-J. R. (2006). "Plants Used in the Treatment of Diabetes," in *Antidiabetic Plants*. Editor A. Soumyanath (Boca Raton: CRC Press).

Sissoko, L., Diarra, N., Nientao, I., Stuart, B., Togola, A., Diallo, D., et al. (2020). *Moringa Oleifera* Leaf Powder for Type 2 Diabetes: a Pilot Clinical Trial. *Afr. J. Traditional, Complement. Altern. Medicines* 17, 29–36. doi:10.21010/ajtcam.v17i2.3

Suksomboon, N., Poolsup, N., and Punthanitisarn, S. (2016). Effect of Aloe Vera on Glycaemic Control in Prediabetes and Type 2 Diabetes: a Systematic Review and Meta-Analysis. *J. Clin. Pharm. Ther.* 41, 180–188. doi:10.1111/jcpt.12382

Tang, Y., Weiss, T., Liu, J., Rajpathak, S., and Khunti, K. (2020). Metformin Adherence and Discontinuation Among Patients with Type 2 Diabetes: A Retrospective Cohort Study. *J. Clin. Transl Endocrinol.* 20, 100225. doi:10.1016/j.jcte.2020.100225

Tian, H., Lu, J., He, H., Zhang, L., Dong, Y., Yao, H., et al. (2016). The Effect of Astragalus as an Adjuvant Treatment in Type 2 Diabetes Mellitus: A (Preliminary) Meta-Analysis. *J. Ethnopharmacol* 191, 206–215. doi:10.1016/j.jep.2016.05.062

Wang, Z., Wang, J., and Chan, P. (2013). Treating Type 2 Diabetes Mellitus with Traditional Chinese and Indian Medicinal Herbs. *Evid. Based Complement. Alternat Med.* 2013, 343594. doi:10.1155/2013/343594

Wong, N. D., Patao, C., Wong, K., Malik, S., Franklin, S. S., and Iloeje, U. (2013). Trends in Control of Cardiovascular Risk Factors Among US Adults with Type 2 Diabetes from 1999 to 2010: Comparison by Prevalent Cardiovascular Disease Status. *Diab Vasc. Dis. Res.* 10, 505–513. doi:10.1177/1479164113496828

Yang, L., Chen, J., Lu, H., Lai, J., He, Y., Liu, S., et al. (2019). Pueraria Lobata for Diabetes Mellitus: Past, Present and Future. *Am. J. Chin. Med.* 47, 1419–1444. doi:10.1142/S0192415X19500733

Yeh, G. Y., Eisenberg, D. M., Kaptchuk, T. J., Phillips, R. S., Yeh, G. Y., Eisenberg, D. M., et al. (2003). Systematic Review of Herbs and Dietary Supplements for Glycemic Control in Diabetes. *Diabetes Care* 26, 1277–1294. doi:10.2337/diacare.26.4.1277

Zhang, Y., Liu, W., Liu, D., Zhao, T., and Tian, H. (2016). Efficacy of Aloe Vera Supplementation on Prediabetes and Early Non-treated Diabetic Patients: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Nutrients* 8, 388. doi:10.3390/nu8070388

Ziaei, R., Foshati, S., Hadi, A., Kermani, M. A. H., Ghavami, A., Clark, C. C. T., et al. (2020). The Effect of Nettle (Urtica Dioica) Supplementation on the Glycemic Control of Patients with Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis. *Phytother Res.* 34, 282–294. doi:10.1002/ptr.6535

Ziai, S. A., Larijani, B., Akhoondzadeh, S., Fakhrzadeh, H., Dastpak, A., Bandarian, F., et al. (2005). Psyllium Decreased Serum Glucose and Glycosylated Hemoglobin Significantly in Diabetic Outpatients. *J. Ethnopharmacol* 102, 202–207. doi:10.1016/j.jep.2005.06.042

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 26 November 2021
doi: 10.3389/fphar.2021.746808

In Vitro Selective Antibacterial and Antiproliferative Effects of Ethanolic Extracts from Cambodian and Philippine Plants Used in Folk Medicine for Diarrhea Treatment

*Tomas Kudera[1], Barbora Fiserova[1], Marie Korytakova[1], Ivo Doskocil[2], Hana Salmonova[2], Edgardo E. Tulin[3], Samnang Nguon[4], Marlito M. Bande[5] and Ladislav Kokoska[1]**

[1]Laboratory of Ethnobotany and Ethnopharmacology, Department of Crop Sciences and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, Prague, Czechia, [2]Faculty of Agrobiology, Food and Natural Resources, Department of Microbiology, Nutrition and Dietetics, Czech University of Life Sciences Prague, Prague, Czechia, [3]PhilRootcrops, Visayas State University, Baybay, Philippines, [4]Graduate School, Royal University of Agriculture, Phnom Penh, Cambodia, [5]Institute of Tropical Ecology and Environmental Management, Visayas State University, Baybay, Philippines

OPEN ACCESS

Edited by:
Alessandra Durazzo,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Abdul Majeed,
Bahauddin Zakariya University, Pakistan
Jamuna Aswathanarayan,
JSS Academy of Higher Education and Research, India
Folorunso Oludayo Fasina,
University of Pretoria, South Africa
Hasanga Rathnayake,
University of Ruhuna, Sri Lanka

***Correspondence:**
Ladislav Kokoska
kokoska@ftz.czu.cz

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 24 July 2021
Accepted: 08 October 2021
Published: 26 November 2021

Citation:
Kudera T, Fiserova B, Korytakova M, Doskocil I, Salmonova H, Tulin EE, Nguon S, Bande MM and Kokoska L (2021) In Vitro *Selective Antibacterial and Antiproliferative Effects of Ethanolic Extracts from Cambodian and Philippine Plants Used in Folk Medicine for Diarrhea Treatment. Front. Pharmacol. 12:746808. doi: 10.3389/fphar.2021.746808*

Bacterial diarrhea remains a global health problem, especially in developing tropical countries. Moreover, dysbiosis caused by diarrheagenic bacteria and inappropriate antimicrobial treatment has been associated with intestinal carcinogenesis. Despite the rich tradition of the use of herbs for the treatment of gastrointestinal disorders in Cambodian and Philippine folk medicine, many of them have not yet been systematically studied for their *in vitro* selective inhibitory effects on intestinal bacteria and cells. In the present study, *in vitro* inhibitory activities of 35 ethanolic extracts derived from 32 Cambodian and Philippine medicinal plants were determined by broth microdilution method against 12 pathogenic bacteria. Furthermore, cytotoxicity against intestinal cancer cells (Caco-2 and HT-29) using thiazolyl blue tetrazolium bromide cytotoxicity assay and safety to six beneficial intestinal bacteria (bifidobacteria and lactobacilli) and intestinal normal cells (FHs 74 Int) were determined for the antimicrobially active extracts. Selectivity indices (SIs) were calculated among the averages of minimum inhibitory concentrations (MICs), half-maximal inhibitory concentrations (IC_{50}), and 80% inhibitory concentrations of proliferation (IC_{80}) for each type of the tested agents. The extracts of *Artocarpus blancoi* (Elmer) Merr. (Moraceae), *Ancistrocladus tectorius* (Lour.) Merr. (Ancistrocladaceae), and *Pentacme siamensis* (Miq.) Kurz (Dipterocarpaceae) produced significant growth-inhibitory effects (MICs = 32–512 µg/ml) against intestinal pathogenic bacteria at the concentrations nontoxic to normal intestinal cells (IC_{80} values >512 µg/ml; SIs = 0.11–0.2). Moreover, the extract of *P. siamensis* (Miq.) Kurz was relatively safe to beneficial bacteria (MICs ≥512 µg/ml; SI = 0.1), and together with *A. blancoi* (Elmer) Merr., they selectively inhibited intestinal cancer cells (IC_{50} values ≥51.98 ± 19.79 µg/ml; SIs = 0.3 and 0.6). Finally, a strong selective antiproliferative effect on cancer cells (IC_{50} values 37.89 ± 2.68 to 130.89 ± 13.99 µg/ml; SIs = 0.5) was exerted by *Ehretia microphylla* Lam. (Boraginaceae), *Lagerstroemia cochinchinensis* Pierre ex Gagnep. (Lythraceae), and *Melastoma saigonense* (Kuntze)

Merr. (Melastomataceae) (leaves with flower buds). The results suggest that the above-mentioned species are promising materials for the development of new selective antibacterial and antiproliferative agents for the treatment of infectious diarrhea and associated intestinal cancer diseases. However, further research is needed regarding the isolation and identification of their active constituents.

Keywords: diarrhea, Cambodia, Philippines, medicinal plant, antibacterial, anticancer

INTRODUCTION

According to the latest data of the World Health Organization, infectious diarrhea is still the third leading cause of death among all communicable diseases worldwide, especially affecting under-five children in developing countries (World Health Organization, 2020a). Moreover, common risk factors associated with these gastrointestinal infections, such as inappropriate changes in the host-gut microbiome (Sun et al., 2018), have been considered as a crucial precondition for several noncommunicable intestinal diseases, including colorectal cancer, which is the third leading cause of cancer death globally (Taddese et al., 2020). The developed countries are at the highest risk, but the incidence of gastrointestinal cancers in developing nations is steadily increasing (Rawla et al., 2019). Infectious and toxigenic strains of *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium difficile*, *Clostridium perfringens*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus,* and *Yersinia enterocolitica* are the major causes of bacterial diarrhea (Casburn-Jones, 2004). Among these, *Enterococcus* spp., *Escherichia* spp., and *Shigella* spp. were previously observed to be richer in the fecal microbiota of patients with colorectal cancer (Xu and Jiang, 2017).

Despite the advantages of treatment utilizing antibiotic agents, disruption of the gut microbiota is usually considered as one of the negative consequences of their use in infectious diarrhea (Francino, 2016). Moreover, the antimicrobial resistance rate among diarrheagenic bacteria recovered from human patients has significantly increased, especially in developing countries (Meng et al., 2011; World Health Organization, 2020b). Therefore, it is important to seek new sources of efficient antimicrobial agents against which the infectious bacteria are less prone to develop resistance (Sibanda and Okoh, 2007). Additionally, agents that are highly selective, thus less disruptive for human microbial ecology, should be preferred (Garrett, 2019). Therefore, an investigation on the *in vitro* antimicrobial effects of promising candidates is recommended to involve both representatives of diarrheagenic and probiotic bacteria. The most common bacteria recognized to date as probiotics are *Lactobacillus* spp. and *Bifidobacterium* spp., belonging to the dominant bacterial phyla that can be found in human intestines (Behnsen et al., 2013). Although it is a simplified representation of beneficial gut microbiota, the counterscreen of *in vitro* inhibitory activities on gut commensals appears to be an effective way to avoid unnecessarily promiscuous agents (Gavrish et al., 2014).

Additionally, assessment of interactions with intestinal epithelial cells and toxicity profiles is another important factor for the evaluation of antibacterial agents, which target the intestinal site (Maher and McClean, 2006). To lower the potential toxic responses of intestinal epithelial cells, the use of antibacterial agents with mechanisms enabling toxicity to be prokaryotic but not eukaryotic cells, such as in the case of the antibiotic ceftriaxone, should be prioritized (Neftel and Hübscher, 1987). However, the antiproliferative activity can be acknowledged in cases where the immune responses caused by ongoing intestinal infection and dysbiosis have already promoted the carcinogenesis of epithelial cells. Initially, colorectal cancer usually has an oligosymptomatic characteristic; thus, many cases are diagnosed only at advanced stages, at which stage the therapeutic outcomes are poor (Rogowski and Sulzyc-Bielicka, 2016). Antibacterial agents able to eliminate diarrheagenic pathogens and having the additional selective antiproliferative properties could potentially help to prevent progression of yet not diagnosed intestinal cancers. The use of antibiotics—such as quinolones and tetracyclines, which are both utilized in diarrheal infections—as anticancer drugs has been previously suggested (Onoda et al., 2005; Batalha et al., 2016).

Plant-derived products provide novel chemical scaffolds for anti-infective drugs and leads that have chemically been modified and developed as antimicrobial agents. Several over-the-counter pharmaceuticals, dietary supplements, and herbal medicines recommended for the support and maintenance of gastrointestinal health, containing antibacterially active plant extracts and derivatives of their constituents, are already available at the international market. The benzylisoquinoline alkaloid berberine (e.g., *Hydrastis canadensis* L. [Ranunculaceae]); simple phenol bismuth subsalicylate, the analog of salicylic acid derived from salicin (*Salix alba* L. [Salicaceae]) (Kokoska et al., 2019); and picrosides, an iridoid glycoside of *Picrorhiza kurroa* Royle ex Benth. (Plantaginaceae), are some examples (Rathee et al., 2016). The *in vitro* selective antibacterial effects of plant-derived products have also been reported. For example, Chan et al. (2018) reported that the phenolic-rich extracts from various dietary spices and medicinal herbs (*Cinnamomum burmannii* Nees and T.Nees] Blume [Lauraceae], *Cinnamomum cassia* [L.] J.Presl [Lauraceae], *Origanum vulgare* L. [Lamiaceae], *Punica granatum* L. [Lythraceae], *Reynoutria japonica* Houtt. [Polygonaceae], and *Syzygium aromaticum* [L.]. Merr. and L.M.Perry [Myrtaceae]) exerted *in vitro* growth-inhibitory effects against selected foodborne pathogenic bacteria but not against lactic-acid bacteria. Selective *in vitro* antibacterial activity was also

described in the study by Novakova et al. (2013), where the anticlostridial effect of 8-hydroxyquinoline (*Microstachys corniculata* [Vahl] Griseb. [Euphorbiaceae]) was higher than the activities revealed against different strains of bifidobacteria. In our previous study, we also reported that 8-hydroxyquinoline exerts selective *in vitro* antiproliferative activity against some intestinal cancer cell lines with a comparably lower effect on normal cells (Kudera et al., 2020). Quinoline alkaloids, such as camptothecin extracted from the bark of *Camptotheca acuminata* Decne. (Cornaceae), are already being used in chemotherapy for the treatment of colon cancer (Zeng et al., 2013). Anticancer activities of other antibacterially active phytochemicals (e.g., berberine) utilized against infectious diarrhea are currently studied (Lin et al., 2008). Based on these studies, it is evident that plant-derived products have great potential for the development of antibacterial and anticancer preparations for the treatment of infectious diarrhea and associated intestinal cancer diseases. Although many of such products work based on antidiarrheal activity (e.g., antisecretory and astringent effects) (Palombo, 2006), their antimicrobial effect is not an uncommon feature.

The Southeast Asian region is one of the world's major sources of useful plant resources and has long been recognized as a center of plant biodiversity (Duriyaprapan et al., 2005). Situated in the humid tropics with areas of high rainfall, Southeast Asia has one of the largest numbers of vascular plants species globally. For centuries, people living in this region have relied on traditional medicine using available plants for daily healthcare. Cambodia and the Philippines are two geographically distinct Southeast Asian countries, each having numerous plant biodiversity hotspots and a long tradition of herbalism (de Padua et al., 1999). While the former is situated in the mainland, having rich ecosystems, especially around the Mekong River (Chassagne et al., 2016), the latter is a huge archipelago consisting of approximately 7,107 islands, many of which are the center of endemicity and biodiversity (Guzman et al., 2016).

Diarrhea has been a significant issue in both Cambodia and the Philippines (Our World in Data, 2011). Therefore, plant resources in these countries have extensively been utilized medicinally to treat this ailment. The Philippines also has the highest estimated number of cases of colorectal cancer in Southeast Asia and the tenth highest number of deaths in the world (Rawla et al., 2019). In certain provinces of Cambodia, treatment of digestive disorders, such as abdominal pain (chhu poh), diarrhea (reak ach), and dysentery (reak muol), has particularly been based on herbal medicine. Alcohol maceration is a common method of preparation of antidiarrheal medicines, whereas a majority of the preparations are administered orally: drunk, eaten, or chewed (Kham, 2004). Grilling the plant part over a fire and then boiling it into a form of decoction is also common. As an example, Bunong people in Mondulkiri province treat diarrhea using a "step-by-step" process using a sole ingredient from one plant that is substituted by a different species if the condition becomes persistent. In the Philippines, conditions such as diarrhea (pagtatae) and dysentery (pagdidisenyo) have similarly been treated by orally administered herbal preparations that are processed by alcohol maceration, decoction, or infusion or eaten and chewed raw. According to the Philippine traditional medicinal system, the disease is usually conceptualized as a disruption (dys-krasia) of the balance of forces (whether germs or evil spirits), both external and internal to humans. Therefore, it can be assumed that the use of herbal preparations is intended to also defend the immunological mechanisms, helping the body to overcome the disease itself (Tan, 1980). Despite the existence of several reports on the antibacterial and antiproliferative effects of Cambodian and Philippine medicinal plants used for the treatment of diarrhea (Beloy et al., 1976; Chea et al., 2007), there are several species in both regions that have not yet been appropriately studied using modern scientific techniques. In this study, we, therefore, examine the *in vitro* selective antibacterial and antiproliferative effects of ethanolic extracts from various parts of plant species that have been used in Cambodian and Philippine traditional herbal systems the treatment of gastrointestinal disorders and determine which bioactive properties have not been properly tested in such form and degree before.

MATERIALS AND METHODS

Plant Materials

The criteria for selection of promising plant species included their uses for the treatment of diarrhea, dysentery, abdominal pain, and other gastrointestinal complaints in traditional herbal systems of Southeast Asia, particularly Cambodia and the Philippines. Therefore, the appropriate literature on ethnobotany and ethnomedicine of this region was primarily used (Chassagne et al., 2016; de Padua et al., 1999; van Duong, 1993; Kham, 2004; Langenberger et al., 2008; Lemmens and Bunyapraphatsara, 2003; Lim, 2012; Stuart, 2017; Tan, 1980; van Valkenburg and Bunyapraphatsara, 2001). Additionally, several species were identified through meetings with local herbalists in Cambodia (2) and the Philippines (1), assembled by local experts Dr. Nguon and Dr. Bande, respectively. Overall, more than 100 plant species were selected, referring to a limited number of previous studies testing their bioactivity *in vitro*. A total of 35 samples from different parts (bark, fruit, leaves, or roots, one per plant except three of the species) of 13 Cambodian and 19 Philippine medicinal plant species were collected from various locations in the Republic of the Philippines in April–May 2017 and 2018 and in the Kingdom of Cambodia in March–April 2019 (**Table 1**). The collected fresh samples were subsequently air-dried for several days and sent to the Czechia for further processing and bioactivity testing. Ethnobotany expert Prof. Kokoska and local experts Dr. Bande and Dr. Nguon authenticated the species. Their voucher specimens have been deposited in the herbarium of the Department of Botany and Plant Physiology of the Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences Prague (Prague, Czechia). The scientific names of the collected species were reviewed using (The Plant List, 2013), and their local names were verified with data from literature and local herbalists (Tan, 1980; van Duong, 1993; de Padua et al., 1999; van Valkenburg and

TABLE 1 | Ethnobotanical data on Cambodian and Philippine medicinal plants.

Latin name (family)	GPS coordinates (country)	Local name	Voucher specimen	Tested part(s)	Extract yield (%)	Ethnomedicinal use
Aganonerion polymorphum Spire (Apocynaceae)	12.3966000N, 107.1934975E (C)	Vor Thneung	02559KBFRC	Whole plant	19.7	Diarrhea (Chassagne et al. (2016))
Acalypha grandis Benth. (Euphorbiaceae)	10.7623297N, 124.8062889E (P)	Unknown	02537KBFR8	Leaves	23.6	Diarrhea and dysentery; sapped/crushed into water/food (van Valkenburg and Bunyapraphatsara (2001))
Acanthus ebracteatus Vahl (Acanthaceae)	10.6888289N, 124.7956483E (P)	Diluario	02505KBFR3	Whole plant	17.9	Abdominal pain; decoction of 30–60 gDW (Stuart. (2017))
Ancistrocladus tectorius (Lour.) Merr. (Ancistrocladaceae)	13.7333775N, 107.0151108E (C)	Khan Maa	02560KBFR4	Leaves	16	Dysentery; decoction (Lemmens and Bunyapraphatsara (2003); interviewed herbalist)
Aporosa villosa (Lindl.) Baill. (Phyllanthaceae)	12.3965292N, 107.1938597E (C)	Krong	02561KBFR5	Leaves	6.1	Diarrhea and abdominal pain; decoction (Chassagne et al. (2016))
Artocarpus blancoi (Elmer) Merr. (Moraceae)	10.7435833N, 124.8020564E (P)	Antipolo	02538KBFR9	Fruit	25.7	Diarrhea; cooked (Tan. (1980); Stuart. (2017))
Artocarpus camansi Blanco (Moraceae)	10.6819242N, 124.8001064E (P)	Kamansi	02512KBFR1	Bark	13.3	Diarrhea; cooked (Tan. (1980); Stuart. (2017))
Artocarpus elasticus Reinw. ex Blume (Moraceae)	10.7435939N, 124.8019275E (P)	Terap	02539KBFRA	Bark	11.8	Dysentery (Lim, 2012; interviewed herbalist)
Artocarpus odoratissimus Blanco (Moraceae)	10.7436072N, 124.8017989E (P)	Marang	02540KBFR2	Fruit	22.9	Diarrhea (Lim. (2012); interviewed herbalist)
Bauhinia malabarica Roxb. (Leguminosae)	12.4428908N, 107.1592217E (C)	Choeung Koo	02562KBFR6	Bark and leaves	14.7 and 11.2	Diarrhea and abdominal pain; alcohol maceration or decoction (Chassagne et al. (2016))
Breynia cernua (Poir.) Müll.Arg. (Phyllantaceae)	9.8153556N, 124.3597258E (P)	Mutang-Ulang	02541KBFR3	Bark	10.6	Dysentery; infusion (van Valkenburg and Bunyapraphatsara (2001))
Breynia vitis-idaea (Burm.f.) C.E.C.Fisch. (Phyllanthaceae)	11.5627122N, 104.9167906E (C)	Phnek Preab	02563KBFR7	Wood with bark	9.9	Dysentery; infusion (Kham, 2004)
Commelina communis L. (Commelinaceae)	10.6159294N, 124.9272431E (P)	Alibangon	02542KBFR4	Whole plant	13.3	Diarrhea (van Valkenburg and Bunyapraphatsara (2001))
Cyathula prostrata (L.) Blume (Amaranthaceae)	10.7433806N, 124.8001225E (P)	Dayang	02543KBFR5	Whole plant	12.8	Dysentery and cholera; decoction or infusion (van Valkenburg and Bunyapraphatsara (2001); Stuart. (2017))
Diplazium esculentum (Retz.) Sw. (Athyriaceae)	10.7577433N, 124.7975153E (P)	Paco	02545KBFR7	Rhizome	5.4	Diarrhea and dysentery; pulverization and cold water maceration (Stuart. (2017); interviewed herbalist)
Ehretia microphylla Lam. (Boraginaceae)	10.7442369N, 124.7897825E (P)	Tsaang-Gubat	02489KBFRE	Leaves	15.3	Diarrhea, dysentery, and abdominal pain; decoction or infusion (8 tbsp of chopped leaves in 2 glasses) (de Padua et al. (1999); Stuart. (2017))
Emilia sonchifolia (L.) DC. ex DC. (Compositae)	10.7407072N, 124.8002914E (P)	Tagulinaw	02520KBFR0	Whole plant	20.9	Diarrhea, dysentery, and enteritis; decoction (6–15 gDW) (Tan. (1980); Stuart. (2017))
Helicteres angustifolia L. (Malvaceae)	12.3963028N, 107.1938622E (C)	Sambok Cheas	02564KBFR8	Root	9.2	Diarrhea, dysentery, and abdominal pain; decoction (Chassagne et al. (2016))
Hyptis capitata Jacq. (Lamiaceae)	10.7590292N, 124.8020589E (P)	Botonesan	02546KBFR8	Whole plant	10.1	Gastrointestinal problems; decoction (Lemmens and Bunyapraphatsara (2003))
Ixora nigricans R.Br. ex Wight and Arn. (Rubiaceae)	13.7291931N, 107.0113667E (C)	Phka Mochul Pich	02565KBFR9	Leaves	10.8	Dysentery and abdominal pain (Kham. (2004))
Kyllinga brevifolia Rottb. (Cyperaceae)	11.0610592N, 124.7009597E (P)	Pugo-Pugo	02544KBFR6	Whole plant	11.4	Diarrhea (de Padua et al. (1999); Stuart. (2017))
Lagerstroemia cochinchinensis Pierre ex Gagnep. (Lythraceae)	13.4692872N, 105.8909203E (C)	Sralao	02566KBFRA	Bark	2.8	Diarrhea; decoction (Chassagne et al. (2016))
Leea indica (Burm. f.) Merr. (Vitaceae)	11.5627122N, 104.9167906E (C)	Kdaing Baay	02567KBFRB	Root	8.3	Diarrhea, dysentery, digestive and intestinal complaints; decoction or infusion (Kham. (2004))
Melastoma dodecandrum Lour. (Melastomataceae)	12.4089644N, 107.3133011E (C)	Unknown	02568KBFRC	Bark and leaves with flower buds	12.7 and 9.9	Diarrhea (van Duong. (1993))
Melastoma saigonense (Kuntze) Merr. (Melastomataceae)	11.5627122N, 104.9167906E (C)	Baay Nhenh	02569KBFRD	Wooden stem and leaves with flower buds	7.3 and 17.3	Diarrhea (Chassagne et al. (2016))

(Continued on following page)

TABLE 1 | (*Continued*) Ethnobotanical data on Cambodian and Philippine medicinal plants.

Latin name (family)	GPS coordinates (country)	Local name	Voucher specimen	Tested part(s)	Extract yield (%)	Ethnomedicinal use
Parkia javanica (Lam.) Merr. (Leguminosae)	10.7448892N, 124.8059375E (P)	Kupang	02547KBFR9	Bark	25.7	Diarrhea and dysentery; decoction (Tan. (1980); Stuart. (2017))
Pentacme siamensis (Miq.) Kurz (Dipterocarpaceae)	13.4474300N, 105.8756317E (C)	Raing Phnom	02571KBFR6	Bark	5.8	Diarrhea (Chassagne et al. (2016)
Picrasma javanica Blume (Simaroubaceae)	10.7438825N, 124.8039956E (P)	Manunggal	02548KBFRA	Bark	6.3	Digestive and abdominal pain; decoction (Langenberger et al. (2008))
Pseudelephantopus spicatus (Juss. ex Aubl.) Rohr (Compositae)	9.8110686N, 124.3551231E (P)	Kokunbanog	02553KBFR6	Whole plant	12.5	Diarrhea; decoction (Langenberger et al. (2008))
Rourea minor (Gaertn.) Alston (Connaraceae)	12.3965372N, 107.1933392E (C)	Unknown	02570KBFR5	Leaves	11.4	Diarrhea (Chassagne et al. (2016))
Tabernaemontana pandacaqui Lam. (Apocynaceae)	14.1667808N, 121.2143336E (P)	Pandakaking-Puti	02503KBFR1	Bark	10.1	Gastroenteritis (Tan. (1980))
Triumfetta bartramia L. (Malvaceae)	10.7467864N, 124.8152500E (P)	Kulutkulutan	02554KBFR7	Root	14.9	Diarrhea and intestinal ulcers (van Valkenburg and Bunyapraphatsara (2001); Stuart. (2017))

C, Cambodia; P, Philippines.

Bunyapraphatsara, 2001; Lemmens and Bunyapraphatsara, 2003; Kham, 2004; Langenberger et al., 2008; Lim, 2012; Chassagne et al., 2016; Stuart, 2017). For all assayed species, the scientific names, families, local names, voucher specimen codes, GPS coordinates, collected parts (plant samples), and their uses in folk medicine are presented in **Table 1**.

Preparation of Plant Extracts

Although the most common procedures of processing antidiarrheal plants in Cambodia and the Philippines are decoction and infusion (**Table 1**), ethanol was selected for the extraction of plant samples since it is an efficient solvent for herbal drugs with a well-established tradition in herbal medicine (Kelber et al., 2016). With the aim of preventing possible loss or changes of active constituents due to storage of plant samples, the extraction was performed immediately after their arrival in the Czechia. Each dried sample was homogenized into powder using Grindomix mill (Retsch, Haan, Germany), and 15 g of dry matter was extracted in 450 ml 80% ethanol (Penta, Prague, Czechia) for 24 h at room temperature using a laboratory shaker (GFL3005, GFL, Burgwedel, Germany). Therefore, the drug extract ratio was 1:30. Extracts were subsequently filtered and concentrated using a rotary vacuum evaporator (R-200, Buchi Labortechnik, Flawil, Switzerland) *in vacuo* at 40°C. According to the recommendations of Cos et al. (2006), the dried residue was finally diluted in 100% dimethylsulfoxide (DMSO) (Penta, Prague, Czechia) to obtain stock solutions with a final concentration of 51.2 mg/ml and stored at −20°C until their use. Some of the extracts were not completely soluble in other solvents, such as distilled water. Yields (%) of the dried residues are shown in **Table 1**.

Bacterial Strains and Media

The intestinal bacterial type strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, United States), Czech Collection of Microorganisms (CCM, Brno, Czechia), German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and National Collection of Type Cultures (NCTC, London, United Kingdom).

In accordance with the diversity of diarrheagenic gram-positive and gram-negative bacteria responsible for globally distributed foodborne, waterborne, and nosocomial infections (Diniz-Santos et al., 2006; Rajkovic et al., 2020), the following 12 strains were used in this study: *B. cereus* (ATCC 14579), *C. difficile* (DSMZ 12056), *C. perfringens* (DSMZ 11778), *E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *E. coli* 0175:H7 (NCTC 12900), *L. monocytogenes* (ATCC 7644), *Shigella flexneri* (ATCC 12022), *Salmonella enterica* ssp. *enterica* serovar Enteritidis (ATCC 13076), *S. enterica* ssp. *enterica* serovar Typhimurium (ATCC 14028), *V. parahaemolyticus* (ATCC 17802), and *Y. enterocolitica* (ATCC 9610). The above-mentioned strains were considered as obligate or facultative pathogens. The following six bacterial strains, which belong to the dominant bacterial phyla in the human gut and exhibit probiotic functions (Behnsen et al., 2013), were used in this study: *Bifidobacterium adolescentis* (DSMZ 20087), *Bifidobacterium animalis* spp. *lactis* (DSMZ 10140), *Bifidobacterium breve* (ATCC 15700), *Lactobacillus casei* (DSMZ 20011), *Lactobacillus reuteri* (CCM 3625), and *Lactobacillus rhamnosus* (CCM 7091). All these strains were considered beneficial gut bacteria.

As the maintenance and growth medium, Mueller-Hinton Broth (Oxoid, Basingstoke, United Kingdom) was used for the majority of bacteria that grow aerobically (*E. faecalis* supp. 1% glucose, *V. parahaemolyticus* supp. 3% NaCl). *Y. enterocolitica* was stored and cultured in Brain Heart Infusion Broth (Oxoid, Basingstoke, United Kingdom). Bifidobacteria and lactobacilli were maintained and cultured in Wilkins-Chalgren Broth (Oxoid, Basingstoke, United Kingdom) supplemented with 5 g/L soya peptone and 0.5 g/L cysteine. Although the same growth medium was used for clostridia, they were stored in cooked meat medium (both from Oxoid, Basingstoke, United Kingdom) at room temperature. The standard safety guidelines for handling

microorganisms were followed. Therefore, all items, such as culture tubes, syringes, and gloves, were discarded in the biohazard autoclave bag after every use.

Cell Cultures

One representative of normal intestinal cell line (FHs 74 Int [ATCC CCL 241]) and two of cancer intestinal cell lines (Caco-2 [ATCC HTB 37]) and HT-29 [ATCC HTB 38]) were purchased from ATCC (Rockville, MD, United States). Normal cells were cultured in Hybri-Care medium supplemented with 10% fetal bovine serum, 1% sodium bicarbonate, 1% nonessential amino acids, 30 ng/ml of epidermal growth factor, and 1% penicillin-streptomycin solution (10,000 units/ml and 100 mg/ml, respectively). The cancer cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 1% sodium pyruvate, 10% fetal bovine serum, 1% sodium bicarbonate, 1% nonessential amino acids, and 1% penicillin-streptomycin solution (10,000 units/ml and 100 mg/ml, respectively) (all purchased from Biowest, Nuaille, France). The cultures were incubated at 37°C and 5% CO_2. The culture medium was replaced every 2–3 days, and cells were passaged every 7 days.

Antibacterial Assay

Initially, all 35 extracts (**Table 1**) were evaluated for their antibacterial activities against the pathogenic strains. Those showing any inhibitory action were subsequently tested against the probiotic strains. The growth-inhibitory activities against aerobic and anaerobic bacteria were evaluated using the broth microdilution method using 96-well microtiter plates, following the protocols of (Clinical and Laboratory Standards Institute, 2021) and Hecht (1999), respectively. For the effective assessment of the anti-infective potential of natural products, slight modifications were implemented as described by Cos et al. (2006).

Prior to testing, the strains that grow aerobically were subcultured in the appropriate media at 37°C (*Y. enterocolitica* at 30°C) for 24 h. Bifidobacteria, clostridia, and lactobacilli were cultured at 37°C for 48 h using Whitley A35 Anaerobic Workstation (Don Whitley Scientific, Bingley, United Kingdom). The anaerobic conditions were created by supplying a standard anaerobic gas mixture of 10% H_2, 10% CO_2, and 80% N_2 (Linde Gas, Prague, Czechia).

The extracts were diluted twofold in appropriate growth media (initial concentration of 512 μg/ml) using the Freedom EVO 100 automated pipetting platform (Tecan, Männedorf, Switzerland) and multichannel pipette (Eppendorf, Hamburg, Germany) in case of aerobic and anaerobic bacteria, respectively. After the optimalization of bacterial cultures to inoculum density of 1.5×10^8 CFU/ml by 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, Czechia), the cultures were inoculated in 96-well plates (5 μl/well). Bacterial cultures in microplates were incubated by employing the same protocols as used for their cultivation prior to the test. The optical density of the cultures was measured at 405 nm ($OD_{450\ nm}$) using a Cytation 3 Imaging Reader (BioTek, Winooski, VT, United States) before and after the growth period.

The lowest concentration (μg/ml) of the extracts that inhibited the bacterial growth by ≥80% was defined as the minimum inhibitory concentration (MIC). Ciprofloxacin (Sigma-Aldrich, Prague, Czechia), an antibiotic commonly recommended for the treatment of infectious diarrhea (Casburn-Jones, 2004), was dissolved in distilled water and used as a positive control drug. All tests were performed as three independent experiments, each conducted in triplicate. The mode and median were used for the final MIC value calculation when the triplicate endpoints were within the two- and three-dilution ranges, respectively. The antibacterial activities were classified as strong (MICs ≤64 μg/ml), moderate (MICs = 128–256 μg/ml), and weak (MIC = 512 μg/ml) (Kokoska et al., 2019). As a result of experiments performed without dissolved extracts and ciprofloxacin (Sigma-Aldrich, Prague, Czechia), their respective solvents, namely, DMSO (Sigma-Aldrich, Prague, Czechia) and distilled water, did not inhibit bacterial growth of any strain at the tested concentrations (≤1%).

Cytotoxicity Assay

The antiproliferative activities of the extracts that showed some inhibitory action against the tested bacteria were further assessed using the modified thiazolyl blue tetrazolium bromide (MTT) cytotoxicity assay developed by Mosmann (1983). Cancer (2.5×10^3) and normal intestinal (2.5×10^5) cells were seeded in a 96-well microtiter plate for 24 h. Cells were incubated with twofold serially diluted plant extracts (0.25–512 μg/ml) for 72 h. Next, the cells were incubated with MTT reagent (1 mg/ml) (Sigma-Aldrich, Prague, Czechia) in EMEM or Hybri-Care medium for an additional 2 h at 37°C and 5% CO_2. The medium with MTT was removed, and the intracellular formazan product was dissolved in 100 μl DMSO. The absorbance was measured at 555 nm using a Tecan Infinite M200 spectrometer (Tecan, Männedorf, Switzerland), and the percentage of viability was calculated when compared to an untreated control.

The antiproliferative activity of the tested plant extracts was represented as half-maximal inhibitory concentration (IC_{50}; μg/ml). The colon cancer chemotherapeutic drug 5-fluorouracil (Sigma-Aldrich, Prague, Czechia) was used as a positive control (Fuente et al., 2020). Three independent experiments (two replicates each) were performed for every test. Data are presented as mean ± standard deviation. The antiproliferative activity was evaluated as follows: cytotoxic (IC_{50} values ≤100 μg/ml), moderately cytotoxic (IC_{50} values = 100–400 μg/ml), and weakly cytotoxic (IC_{50} values = 401–512 μg/ml) (Srisawat et al., 2013). The solvents did not affect the viability of normal and cancer intestinal cell lines at the tested concentration (≤1%).

Calculations

For comparison of microbiological and toxicological data, 80% bacterial growth inhibition (IC_{80}) was calculated as equivalent to the MIC endpoint (Houdkova et al., 2018). Subsequently, $\bar{x}$-MIC, $\bar{x}$-IC_{50}, and $\bar{x}$-IC_{80} values (±standard deviations) were calculated to quantify the inhibitory activity of the tested plant extracts against pathogenic/beneficial bacteria and intestinal cancer/normal cells. Subsequently, the selectivity index (SI) was calculated between normal intestinal cells and pathogenic

TABLE 2 | *In vitro* selective inhibitory activities of ethanolic extracts of Cambodian and Philippine plants against intestinal bacteria and cells.

Cultures tested			Plant species with their parts and positive antibiotic and anticancer control																
			AP(w)	*AG(l)*	*AT(l)*	*AB (f)*	*AC(b)*	*BM(b)*	*BV(wb)*	*DE(r)*	*EM(l)*	*IN(l)*	*LC(b)*	*MD(b)*	*MD(lf)*	*MS(lf)*	*PJ(b)*	*PS(b)*	CIP	5-FU
Bacterial strain/		BC	-[a]	512	64	64	256	512	-	-	512	-	-	-	-	512	-	256	1	nd
MIC		CD	512	512	512	32	128	-	-	-	512	512	-	-	512	512	-	-	16	nd
(μg/ml)		CP	-	512	512	32	256	-	-	-	-	-	-	-	-	-	-	-	1	nd
		EF	-	-	-	128	-	-	-	-	-	-	-	-	-	-	-	-	2	nd
		EC	512	-	-	-	-	256	256	512	-	-	-	256	-	-	256	256	0.062	nd
		ECS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.016	nd
		LM	-	-	128	256	-	-	-	-	-	-	-	-	-	512	-	512	4	nd
		SF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.016	nd
		SE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	256	0.031	nd
		ST	-	-	-	-	-	-	256	-	-	-	-	-	512	-	-	-	0.031	nd
		VP	-	256	-	-	-	512	-	-	512	-	512	512	512	512	-	-	0.062	nd
		YE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.125	nd
		$\bar{x}$-PB ± SD	938.7 ± 191	832 ± 279	784 ± 390	640 ± 458	821.3 ± 352	874.7 ± 266	896 ± 286	981.3 ± 142	896 ± 222	981.3 ± 142	981.3 142±	917.3 ± 244	896 ± 222	853.3 ± 241	960 ± 212	789.3 ± 338	2 ± 6	nd
		BA	64	512	256	16	-	-	-	-	512	512	-	-	256	512	-	-	8	nd
		BB	-	64	64	16	512	-	-	-	128	128	-	-	-	256	128	-	64	nd
		BLC	-	-	256	16	-	-	-	-	256	512	-	-	-	512	-	-	32	nd
		LC	512	256	128	16	256	-	-	-	128	128	-	-	-	512	512	512	32	nd
		LR	-	-	256	16	-	-	-	-	-	-	-	-	-	-	-	-	32	nd
		LRM	-	-	128	16	512	-	-	-	512	-	-	-	-	-	-	-	4	nd
		$\bar{x}$-BB ± SD	778.7 ± 370	650.7 ± 395	181.3 ± 78	16 ± 0	725.3 ± 311	1,024 ± 0	1,024 ± 0	1,024 ± 0	426.7 ± 311	554.7 ± 367	1,024 ± 0	1,024 ± 0	896 ± 286	640 ± 286	789.3 ± 350	938.7 ± 191	29 ± 20	nd
Cell line (μg/ml)	IC_{50} ± SD	HT-29	130.52 ± 2.57	96.53 ± 12.41	82.19 ± 17.22	53.70 ± 16.02	84.77 ± 4.20	35.195 ± 5.32	81.79 ± 20.79	-	130.89 ± 13.99	125.55 ± 13.92	37.89 ± 2.68	248.56 ± 23.13	210.85 ± 16.83	49.75 ± 3.53	155.87 ± 41.79	51.98 ± 19.79	88.81 ± 13.44	6.35 ± 2.07
		Caco-2	148.96 ± 17.14	78.42 ± 23.18	33.82 ± 10.57	79.41 ± 6.90	48.40 ± 0.93	-	-	-	52.49 ± 8.81	135.60 ± 3.11	122.86 ± 13.16	193.73 ± 1.61	77.57 ± 10.22	87.40 ± 19.18	121.26 ± 15.34	-	90.87 ± 17.98	181.79 ± 151.51
		$\bar{x}$-CC ± SD	139.7 ± 9.2	87.5 ± 9	58 ± 24.2	66.5 ± 12.8	66.5 ± 18	529.6 ± 494	552.9 ± 471	-	91.7 ± 39.2	130.6 ± 5	80.4 ± 42.5	221.1 ± 27.4	144.2 ± 66.6	68.575 ± 18.8	138.6 ± 17.3	538 ± 486	89.84 ± 1	94 ± 88
		FHs 74 Int	297.39 ± 22.54	118.76 ± 36.04	45.50 ± 7.29	273.32 ± 7.50	68.23 ± 12.39	158.36 ± 23.75	68.75 ± 8.89	-	303.41 ± 18.00	243.50 ± 21.92	282.05 ± 0.57	-	368.07 ± 30.00	195.19 ± 8.94	342.62 ± 3.54	-	58.90 ± 4.27	492.43 ± 22.92
	IC_{80} ± SD	HT-29	130.50 ± 12.06	-	498.17 ± 16.74	-	356.20 ± 19.04	-	-	-	-	-	-	351.26 ± 43.24	378.50 ± 34.93	143.70 ± 16.74	-	-	181.53 ± 15.05	367.26 ± 0.57
		Caco-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		$\bar{x}$-CC ± SD	577.25 ± 446.75	-	761.1 ± 263	-	690.1 ± 333.9	-	-	-	-	-	-	687.63 ± 336.37	701.25 ± 322.75	583.85 ± 440.15	-	-	602.77 ± 421.23	695.63 ± 328.37
		FHs 74 Int	-	-	-	-	264.64 ± 6.82	-	-	-	-	-	-	-	-	-	-	-	83.37 ± 8.60	-
SI		(a)	0.04	0.09	0.12	0.2	–0.49	0.07	0.06	0.02	0.06	0.02	0.02	0.05	0.06	0.08	0.03	0.11	1.62	nd
		(b)	–0.1	–0.1	–0.6	–1.6	–0.1	0.1	0.1	0.02	–0.3	–0.2	0.02	0.05	0	–0.1	–0.04	0.1	1.2	nd
		(c)	0.3	0.1	–0.1	0.6	0.01	–0.5	–0.9	0	0.5	0.3	0.5	0.7	0.4	0.5	0.4	0.3	–0.2	0.4
		(d)	0.13	–0.2	–0.62	–1.81	0.02	0	0	0	–0.38	–0.27	0	0.17	0.11	0.04	–0.11	–0.04	–1.32	nd

MIC, minimum inhibitory concentration; IC_{50}, half-maximal inhibitory concentration; IC_{80}, 80% inhibitory concentration of proliferation; SD, standard deviation; [a]Not active (MIC/$IC_{50/80}$ >512 μg/ml, the value 1,024 μg/ml was used for average calculation); nd: no data. AP(w), Aganonerion polymorphum Spire (whole plant); AG(l), Acalypha grandis Benth. (leaves); AT(l), Ancistrocladus tectorius (Lour.) Merr. (leaves); AB (f), Artocarpus blancoi (Elmer) Merr. (fruit); AC(b), Artocarpus camansi Blanco (bark); BM(b), Bauhinia malabarica Roxb. (bark); BV(wb), Breynia vitis-idaea (Burm.f.) C.E.C.Fisch. (wood with bark); DE(r), Diplazium esculentum (Retz.) S roots); EM(l), Ehretia microphylla Lam. (leaves); IN(l), Ixora nigricans R.Br. ex Wight and Arn. (leaves); LC(b), Lagerstroemia cochinchinensis Pierre ex Gagnep. (bark); MD(b), Melastoma dodecandrum Lour. (bark); MD(lf), Melastoma dodecandrum Lour. (leaves with flower buds); MS(lf), Melastoma saigonense (Kuntze) Merr. (leaves with flower buds); PJ(b), Picrasma javanica Blume (bark); PS(b), Pentacme siamensis (Miq.) Kurz; CIP, ciprofloxacin; 5-FU, 5-fluorouracil. BC, Bacillus cereus; CD, Clostridium difficile; CP, Clostridium perfringens; EF, Enterococcus faecalis; EC, Escherichia coli; ECS, E. coli 0,175:H7; LM, Listeria monocytogenes; SF, Shigella flexneri; SE, Salmonella Enteritidis; ST, Salmonella Typhimurium; VP, Vibrio parahaemolyticus; YE, Yersinia enterocolitica; BA, Bifidobacterium adolescentis; BB, Bifidobacterium breve; BLC, Bifidobacterium animalis spp. lactis, LC, Lactobacillus casei; LR, Lactobacillus reuteri; LRM, Lactobacillus rhamnosus; $\bar{x}$-PB, mean MIC for pathogenic bacteria; $\bar{x}$-BB, mean MIC for beneficial bacteria; $\bar{x}$-CC, mean $IC_{50/80}$ for intestinal cancer cells; FHs 74 Int (intestinal normal cells); SI (selectivity index): (a) normal cells/diarrheagenic bacteria, (b) beneficial bacteria/diarrheagenic bacteria, (c) normal cells/cancer cells, and (d) beneficial bacteria/cancer cells.

strains (SIa), beneficial and pathogenic strains (SIb), normal and cancer intestinal cells (SIc), and beneficial strains and cancer intestinal cells (SId) using the following formulas where X1 = IC_{80} against normal intestinal cells; X2 = $\bar{x}$-MIC against beneficial strains; X3 = IC_{50} against normal intestinal cells; Y1 = $\bar{x}$-MIC against pathogenic strains; Y2 = $\bar{x}$-IC_{50} against cancer intestinal cells; and Y3 = IC_{80} against cancer intestinal cells:

$$SIa = \log (X1/Y1), \quad (1)$$

$$SIb = \log (X2/Y1), \quad (2)$$

$$SIc = \log (X3/Y2), \quad (3)$$

$$SId = \log (X2/Y3). \quad (4)$$

The SI values >0 and <0 indicate selective toxicity against pathogenic strains/cancer cell lines and beneficial strains/normal cell lines, respectively.

RESULTS

Antibacterial Activity

Diarrheagenic Bacterial Pathogens

Considering the antibacterial activity against the pathogens, 16 of 35 tested extracts revealed a growth-inhibitory effect on at least one of these bacterial strains. While *B. cereus*, *C. difficile*, *E. coli,* and *V. parahaemolyticus* were the most susceptible bacteria inhibited by the highest number of extracts, none of the extracts exerted activity against *E. coli* O157:H7 and *S. flexneri*. The MICs (32–512 μg/ml) of all 16 plant extracts for the diarrheagenic bacterial pathogens are presented in **Table 2**.

There were four extracts showing promising antibacterial actions against multiple pathogenic bacteria, especially the gram-positive strains. Namely, the fruit extract of *Artocarpus blancoi* (Elmer) Merr. (Moraceae) inhibited *B. cereus* and both clostridia at MICs 64 and 32 μg/ml, respectively. This plant was also moderately active against *E. faecalis* (MIC = 128 μg/ml) and *L. monocytogenes* (MIC = 256 μg/ml). Similarly, the leaf extract of *Ancistrocladus tectorius* (Lour.) Merr. (Ancistrocladaceae) revealed a strong inhibitory effect on *B. cereus* (MIC = 64 μg/ml) and moderate activity against *L. monocytogenes* (MIC = 128 μg/ml). However, it produced only weak inhibitory action against both clostridia (MICs = 512 μg/ml). Next, bark extract of *Artocarpus camansi* Blanco (Moraceae) inhibited *B. cereus* and both clostridia at MICs ranging from 128 to 256 μg/ml. Although the antibacterial activities of bark extract of *Pentacme siamensis* (Miq.) Kurz (Dipterocarpaceae) were rather moderate, it exerted inhibitory action against several gram-positive as well as gram-negative pathogenic strains. Namely, it inhibited *B. cereus*, *E. coli,* and *S.* Enteritidis at MICs of 256 μg/ml and *L. monocytogenes* at MIC of 512 μg/ml.

Additionally, there were five more plant extracts exerting moderate activity (MIC = 256 μg/ml) against a single gram-negative strain (**Table 2**). Namely, *Bauhinia malabarica* Roxb. (Leguminosae) (bark), *Breynia vitis-idaea* (Burm.f.) C.E.C.Fisch. (Phyllanthaceae), *Melastoma dodecandrum* Lour. (Melastomataceae) (bark), and *Picrasma javanica* Blume (Simaroubaceae) inhibited *E. coli*; *B. vitis-idaea* (Burm.f.) C.E.C.Fisch. inhibited *S.* Typhimurium; and *Acalypha grandis* Benth. (Euphorbiaceae) inhibited *V. parahaemolyticus*. Finally, *Aganonerion polymorphum* Spire (Apocynaceae), *Diplazium esculentum* (Retz.) Sw. (Athyriaceae), *Ehretia microphylla* Lam. (Boraginaceae), *Ixora nigricans* R.Br. ex Wight and Arn. (Rubiaceae), *Lagerstroemia cochinchinensis* Pierre ex Gagnep. (Lythraceae), *Melastoma dodecandrum* Lour. (Melastomataceae) (leaves with flower buds), and *Melastoma saigonense* (Kuntze) Merr. (Melastomataceae) (leaves with flower buds) produced only weak inhibitory actions at MICs of 512 μg/ml (**Table 2**).

The remaining 19 extracts of *Acanthus ebracteatus* Vahl (Acanthaceae), *Aporosa villosa* (Lindl.) Baill. (Phyllanthaceae), *Artocarpus elasticus* Reinw. ex Blume (Moraceae), *Artocarpus odoratissimus* Blanco (Moraceae), *B. malabarica* Roxb. (leaves), *Breynia cernua* (Poir.) Müll.Arg. (Phyllantaceae), *Commelina communis* L. (Commelinaceae), *Cyathula prostrata* (L.) Blume (Amaranthaceae), *Emilia sonchifolia* (L.) DC. ex DC. (Compositae), *Helicteres angustifolia* L. (Malvaceae), *Hyptis capitata* Jacq. (Lamiaceae), *Kyllinga brevifolia* Rottb. (Cyperaceae), *Leea indica* (Burm. f.) Merr. (Vitaceae), *M. saigonense* (Kuntze) Merr. (wooden stem and leaves), *Parkia javanica* (Lam.) Merr. (Leguminosae), *Pseudelephantopus spicatus* (Juss. ex Aubl.) Rohr (Compositae), *Rourea minor* (Gaertn.) Alston (Connaraceae), *Tabernaemontana pandacaqui* Lam. (Apocynaceae), and *Triumfetta bartramia* L. (Malvaceae) did not show any inhibitory action; thus, they have not been further discussed.

Beneficial Gut Bacteria

Subsequently, 16 extracts that exerted growth-inhibitory effect against diarrheagenic pathogens were verified for their safety to beneficial bacteria. The final MICs are presented in **Table 2**. Five extracts, namely, *B. malabarica* Roxb., *B. vitis-idaea* (Burm.f.) C.E.C.Fisch, *D. esculentum* (Retz.) Sw., *L. cochinchinensis* Pierre ex Gagnep., and *M. dodecandrum* Lour. (bark), did not have any inhibition of these strains (MICs >512 μg/ml), suggesting their harmless effect on gut commensals.

The remaining 11 extracts affected to some degree the growth of beneficial gut bacteria, particularly of bifidobacteria and *L. casei* (**Table 2**). The single strain was inhibited by *P. siamensis* (Miq.) Kurz (*L. casei*) and leaf with flower bud of *M. dodecandrum* Lour. (*B. adolescentis*) at MICs of 256 and 512 μg/ml, respectively. Moreover, *P. javanica* Blume inhibited *B. breve* (MIC = 128 μg/ml) and *L. casei* (MIC = 512 μg/ml). Although *A. polymorphum* Spire significantly affected the growth of *B. adolescentis* (MIC = 64 μg/ml), the remaining probiotic strains were rather resistant toward this extract (MICs ≥512 μg/ml). Three and four probiotic bacteria were inhibited (MICs = 256–512 μg/ml) by *A. camansi* Blanco and *M. saigonense* (Kuntze) Merr., respectively. At MICs ranging from 128 to 512 μg/ml (**Table 2**), *E. microphylla* Lam. and *I. nigricans* R.Br. ex Wight and Arn. affected the growth of the majority of beneficial strains. Although half of the bacteria were not inhibited by *A. grandis* Benth., this extract inhibited *B. breve* at low MIC (64 μg/ml). Finally, all six strains were inhibited by *A. blancoi* (Elmer) Merr. and *A. tectorius* (Lour.) Merr. Whereas the

former uniformly affected the growth at very low MICs (16 μg/ml), the latter inhibited *B. breve* (MIC = 64 μg/ml) only.

Cytotoxic Effect

The outcomes of the MTT assay for all 16 antibacterially active plant extracts against normal and cancer intestinal cells are presented in **Table 2**. With the exception of *D. esculentum* (Retz.) Sw. (IC_{50} values > 512 μg/ml), all the 16 extracts produced a certain antiproliferative effect on at least one of the tested cell lines (IC_{50} values = 33.82 ± 10.57–368.07 ± 30.00 μg/ml).

Normal Intestinal Cells

Considering the toxicity to normal intestinal cells (FHs 74 Int), *M. dodecandrum* Lour. (bark) and *P. siamensis* (Miq.) Kurz did not show inhibitory action at the concentrations tested (IC_{50} > 512 μg/ml) (**Table 2**). Moderate toxicity was shown by *A. polymorphum* Spire, *A. grandis* Benth., *A. blancoi* (Elmer) Merr., *B. malabarica* Roxb., *E. microphylla* Lam., *I. nigricans* R.Br. ex Wight and Arn., *L. cochinchinensis* Pierre ex Gagnep., *M. dodecandrum* Lour. (leaves with flower buds), *M. saigonense* (Kuntze) Merr., and *P. javanica* Blume at IC_{50} values ranging from 118.76 ± 36.04 to 368.07 ± 30.00 μg/ml. Finally, the extracts of *A. tectorius* (Lour.) Merr., *A. camansi* Blanco, and *B. vitis-idaea* (Burm.f.) C.E.C.Fisch. were shown to be cytotoxic (IC_{50} values = 45.50 ± 7.29, 68.23 ± 12.39 and 68.75 ± 8.89 μg/ml, respectively).

Cancer Intestinal Cells

Regarding the antiproliferative activities against cancer intestinal cells (**Table 2**), the plants producing strong effects on Caco-2 (IC_{50} values = 33.82 ± 10.57–87.40 ± 19.18 μg/ml) have been ordered as follows: *A. tectorius* (Lour.) Merr., *A. camansi* Blanco, *E. microphylla* Lam., *M. dodecandrum* Lour. (leaves with flower buds), *A. grandis* Benth., *A. blancoi* (Elmer) Merr., and *M. saigonense* (Kuntze) Merr. With the exception of moderately cytotoxic *E. microphylla* Lam. and *M. dodecandrum* Lour. (leaves with flower buds), the same plant extracts with the addition of *B. malabarica* Roxb., *B. vitis-idaea* (Burm.f.) C.E.C.Fisch., *L. cochinchinensis* Pierre ex Gagnep., and *P. siamensis* (Miq.) Kurz also produced strong antiproliferative effect on HT-29 (IC_{50} values = 35.195 ± 5.32–96.53 ± 12.41 μg/ml) (**Table 2**). A moderate cytotoxic effect on both these cancer cell lines was then shown by *A. polymorphum* Spire, *I. nigricans* R.Br. ex Wight and Arn., *M. dodecandrum* Lour. (bark), and *P. javanica* Blume (IC_{50} values = 121.26 ± 15.34–248.56 ± 23.13 μg/ml). The majority of extracts revealed higher activities against Caco-2 than that of 5-fluorouracil (IC_{50} = 181.79 ± 151.51 μg/ml).

Selective Toxicity

The calculated mean values for pathogenic/beneficial bacteria, cancer cells ($\bar{x}$-MIC, $\bar{x}$-IC_{50}, and $\bar{x}$-IC_{80}), and derived SIs are presented in **Table 2**. Comparing the concentrations inhibiting 80% of growth for pathogenic bacteria and normal intestinal cells, the antibacterially active extracts were shown to be relatively safe (SIa values = 0.02–0.2; IC_{80} values >512 μg/ml) except *A. camansi* Blanco (SIa = −0.49; IC_{80} = 264.64 ± 6.82 μg/ml). Selective antibacterial effect (SIb values = 0.1) with relative safety for beneficial strains was shown by *B. malabarica* Roxb., *B. vitis-idaea* (Burm.f.) C.E.C.Fisch., and *P. siamensis* (Miq.) Kurz (**Table 2**). However, none of the selective effects were as significant as in the case of ciprofloxacin (SIb = 1.2). Other extracts did not show any noticeable selectivity or were comparably more harmful to beneficial bacteria, especially *A. blancoi* (Elmer) Merr., and *A. tectorius* (Lour.) Merr. (SIb values = −1.6 and −0.6, respectively). Regarding the selective antiproliferative effects against cancer intestinal cells, *A. blancoi* (Elmer) Merr., *E. microphylla* Lam., *L. cochinchinensis* Pierre ex Gagnep., *M. dodecandrum* Lour. (bark), and *M. saigonense* (Kuntze) Merr. revealed higher selectivity (SIc values = 0.5–0.7) than that of 5-fluorouracil (SIc = 0.4) (**Table 2**). Other extracts produced either the same or lower degree of selective effects than that of this cytotoxic drug, whereas *A. tectorius* (Lour.) Merr., *B. malabarica* Roxb., and *B. vitis-idaea* (Burm.f.) C.E.C.Fisch. were relatively more toxic to normal intestinal cells (SIc values = −0.9 to −0.1). The probiotic strains were not affected by the antiproliferative concentrations of *A. polymorphum* Spire (SId = 0.13), mainly because of moderate inhibition of HT-29 (IC_{80} = 130.50 ± 12.06 μg/ml) (**Table 2**). Interestingly, the extract of *P. siamensis* (Miq.) Kurz produced noticeable selective actions combining antibacterial and antiproliferative effects on pathogenic bacteria and intestinal cancer cells without affecting beneficial bacteria and normal intestinal cells.

DISCUSSION

In the present study, 16 of 35 tested extracts revealed *in vitro* growth-inhibitory effect on the diarrheagenic bacterial pathogens, especially *A. blancoi* (Elmer) Merr., *A. camansi* Blanco, *A. tectorius* (Lour.) Merr., and *P. siamensis* (Miq.). Except *A. camansi* Blanco, the antibacterially active concentrations of the three were nontoxic to normal intestinal cells. Among the 16 extracts, *A. blancoi* (Elmer) Merr., *E. microphylla* Lam., *L. cochinchinensis* Pierre ex Gagnep., *M. saigonense* (Kuntze) Merr., and *P. siamensis* (Miq.) Kurz also revealed a strong selective antiproliferative effect against intestinal cancer lines. The extract of *P. siamensis* (Miq.) Kurz exhibited activities combining selective inhibition of pathogenic bacteria and intestinal cancer cells without affecting beneficial bacteria and normal intestinal cells. To the best of our knowledge, this is the first study on antibacterial and antiproliferative activities of *A. polymorphum* Spire, *B. vitis-idaea* (Burm.f.) C.E.C.Fisch., *I. nigricans* R.Br. ex Wight and Arn., *L. cochinchinensis* Pierre ex Gagnep., *P. siamensis* (Miq.) Kurz, and *M. saigonense* (Kuntze) Merr. Moreover, there are no previous studies on the cytotoxic effects of *A. blancoi* (Elmer) Merr. Although the cytotoxic effect of products isolated from *B. malabarica* Roxb. was described previously (Kittakoop et al., 2000), its antibacterial activity is herein reported for the first time. Our results correspond with those of previous studies on antibacterial and antiproliferative activities of *A. grandis* Benth. (Bradacs et al., 2009), *D. esculentum* (Retz.) Sw. (Mackeen et al., 1997; Rahmat et al., 2003), and *P. javanica* Blume (Khan et al.,

2001; Win et al., 2015). The above highlighted seven plant extracts with promising activities have mainly been discussed.

Two of the four tested species of the genus *Artocarpus* (Moraceae), namely, *A. blancoi* (Elmer) Merr. and *A. camansi* Blanco, exhibited strong antibacterial and antiproliferative activities in our study. *Artocarpus* spp. are rich in phenolic compounds, such as flavonoids, stilbenoids, and arylbenzofurans, which are known to possess a wide range of biological activities, including antibacterial and anticancer effects (Hafid et al., 2017). Our study is the first to report on anticlostridial activities of *Artocarpus* spp. As flavonoids have been reported to have potent *in vitro* inhibitory effect on some clostridia (Wu et al., 2013), these compounds might be responsible for significant antibacterial activities revealed by *A. blancoi* (Elmer) Merr. and *A. camansi* Blanco against *C. difficile* and *C. perfringens*. Beloy et al. (1976) isolated the flavonoid 5,7,4′-trihydroxyflavanone-3-O-α-L-rhamnopyranoside from the bark extract of *A. blancoi* (Elmer) Merr., showing antibacterial activity against *Mycobacterium tuberculosis*. Ante et al. (2016) showed that bark essential oil of *A. camansi* Blanco produced antibacterial activity against some diarrheagenic bacteria. Our results show that both of these plants inhibited gram-positive bacteria only. Beside their anticlostridial effect, this selectivity probably also contributed to their relative toxicity to beneficial bacteria. *In vitro* inhibitory effect against lactobacilli was previously reported for *Artocarpus lacucha* Buch.-Ham. (Teanpaisan et al., 2014). An example of a compound isolated from the plant of this genus and showing similar activities is artocarpin. In the study by Sato et al. (1996), this flavonoid exhibited strong inhibition of all gram-positive bacteria, including *L. casei*, whereas in another study, it produced higher MICs against *E. coli* and *Pseudomonas aeruginosa* (Septama and Panichayupakaranant, 2015). The absence of antibacterial action of *A. odoratissimus* Blanco found herein will correlate with rather low levels of phenolic content detected in its fruit methanolic extract (Abu Bakar et al., 2015), compared to antibacterially active species (Jalal et al., 2015). Although hexane bark extract of *A. elasticus* Reinw. ex Blume exhibited activity against *B. cereus* and *E. coli* in the study by Ramli et al. (2016), its lack of activity in the present study could be influenced by the use of different extraction procedures. According to our results, *A. blancoi* (Elmer) Merr. and *A. camansi* Blanco had a selective cytotoxic effect on intestinal cancer cells, whereas the former did not show cytotoxicity to normal cells at the inhibitory concentrations against several pathogens. Various terpenoids and phytosterols were previously isolated from methanolic and dichloromethane extract of stem and leaves of *A. camansi* Blanco, respectively. Among them, friedelinol, cycloartenol, and cycloartenol acetate inhibited the growth of HT-29 cells; squalene has profound chemopreventive activity against colon carcinogenesis; and ß-sitosterol has been shown to induce apoptosis in human colon tumors (Tsai et al., 2013). Regarding cytotoxic compounds isolated from other *Artocarpus* spp., the prenylated flavone artelastin revealed strong *in vitro* activity against five colon cancer cell lines (COLO 205, HCT 116, HCT 15, HT-29, and SW 620) in the study by Pedro et al. (2005).

Similar to *Artocarpus* spp., the leaf extract of *A. tectorius* (Lour.) Merr. exhibited growth-inhibitory effects only against gram-positive bacteria. This corresponds with the study by Wiart et al. (2004), where its methanolic leaf extract produced antibacterial activity against *B. cereus* but not *E. coli*. We also found that the overall cytotoxic effect of this plant was strong. Although the antiproliferative effect on cancer cells was not selective, the extract concentrations inhibiting the pathogens were generally nontoxic to normal intestinal cells. Previous phytochemical analysis of leaf ethanolic/methanolic extracts of this plant showed the presence of various naphthylisoquinoline alkaloids, such as 7-epiancistrobrevine D, ancistrocladinine, ancistrotectoquinone A-B, ancistrotectoriline A–C, and hamatinine (Anh et al., 1997; Tang et al., 2000; Tang et al., 2010; Bringmann et al., 2016). Since these isoquinoline alkaloids are known to possess various biological activities, including antimicrobial and cytotoxic effects, we suspect them to be responsible for the growth-inhibitory effects revealed by *A. tectorius* (Lour.) Merr. in the present study. For example, in the study by Mihalyi et al. (2014), michellamine B isolated from *Ancistrocladus korupensis* D.W.Thomas and Gereau inhibited *B. subtilis*. Jiang et al. (2013) showed that naphthylisoquinolines isolated from *A. tectorius* (Lour.) Merr. exhibited cytotoxic effect against three leukemia cells *in vitro*. In another study, 7-epiancistrobrevine and ancistrotectoriline exhibited activity against pancreatic cancer cells (Shang et al., 2020). The present study is the first to report on *in vitro* selective antiproliferative activity of *A. tectorius* (Lour.) Merr. against intestinal cells.

Regarding *P. siamensis* (Miq.) Kurz, there are no comparable studies dealing with species of the same genus. However, our results showing a noticeable combination of selective antibacterial and cytotoxic effects of its bark extract can be compared to the data available for closely related genus *Shorea* (Dipterocarpaceae). For example, Marandi et al. (2016) showed that bark ethanolic extract from Indian antidiarrheal and antidysenteric medicinal plant *Shorea robusta* Gaertn. exhibited inhibitory action against *B. cereus*, *B. subtilis*, *E. faecalis*, *E. coli*, *S.* Typhi, and *V. cholerae*. Stilbene derivatives isolated from barks of *Shorea* spp. previously showed strong antibacterial effects against some of these strains (Nitta et al., 2002; Sudto et al., 2019). Some polyphenols, such as stilbenes, can inhibit several nonbeneficial bacteria from the human microbiota, with no noticeable effects on the growth of probiotic bacteria (Requena et al., 2010). Therefore, we suggest that some of these agents could also contribute to the selective antibacterial activities of *P. siamensis* (Miq.) Kurz shown in the present study. Regarding cytotoxic effect, oligostilbenoids were usually the constituents derived from *Shorea* spp., with reported antiproliferative action against various cancer cell lines (Rohaiza, 2011; Zawawi et al., 2012; Moriyama et al., 2016). Among them, ampelopsin E exhibited obvious antiproliferative properties on COLO205 and HT-29 cells (Tian et al., 2019), whereas α-viniferin showed selective inhibition of colon cancer cells (HCT-116, HT-29, and Caco-2) with twofold lower IC_{50} compared to normal colon cells (CCD-18Co) (Gonzalez-Sarrias et al., 2011). To identify phytochemicals responsible for the *in vitro* selective

inhibitory actions shown by *P. siamensis* (Miq.) Kurz in the present study, an accurate chemical analysis of this plant is needed.

Finally, *E. microphylla* Lam., *L. cochinchinensis* Pierre ex Gagnep., and *M. saigonense* (Kuntze) Merr. revealed a strong selective antiproliferative effect against intestinal cancer lines. It has been reported that triterpenes urs-12-en-24-oic acid, 3-oxo-, methyl ester, and *ß*-amyrin are involved in anticancer activities of products derived from leaves of *E. microphylla* Lam. (Rajkumar et al., 2019). Our study corresponds with other studies dealing with these chemicals. For example, in the study by Kuete et al. (2018), *ß*-amyrin produced a selective cytotoxic effect against Caco-2 compared to that on normal cell line HEK293. In another study, extract of *Alstonia macrophylla* Wall. ex G.Don containing *ß*-amyrin produced a selective cytotoxic effect against HT-29 compared to that on normal cell line HDFn (Tan et al., 2019). The present study is the first to report the antiproliferative activities of *E. microphylla* Lam. against intestinal cell lines. Compounds such as triterpenes, tannins, ellagic acids, glycosides, and flavones were previously attributed to bioactive properties of *Lagerstroemia* spp. (Chan et al., 2014). In previous studies, triterpenes isolated from species of this genus produced significant *in vitro* activity against colon cancer cells, for instance, betulinic acid and 3*β*-acetoxyolean-12-en-28-acid against HCT15 (Woo et al., 2016) and corosolic acid against HCT116 (Sung et al., 2014). Regarding *M. saigonense* (Kuntze) Merr., there are various previously published studies on related species showing corresponding results. For example, the methanolic leaf extract of *Melastoma malabathricum* L. produced an antiproliferative effect on HT-29 in the study by Kamsani et al. (2019). Asiatic acid, caffeic acid, *p*-coumaric acid, kaempferol, quercetin, rutin, and ursolic acid were isolated compounds with previously profound antiproliferative action to this cell line. In the study by Karakurt et al. (2020), *p*-coumaric acid exhibited selective inhibition of Caco-2 and HT-29 cells compared to that of healthy colon epithelial cells (CCD-18Co). Since the decoction from the leaves of *M. malabathricum* L. is also traditionally consumed to treat diarrhea, we suggest a similar composition of bioactive compounds to be present in *M. saigonense* (Kuntze) Merr. (Ong and Nordiana, 1999). Regarding the moderate selective antiproliferative activities of bark and leaf with flower bud extracts of *M. dodecandrum* Lour., three pentacyclic triterpenoids (ursolic acid, asiatic acid, and terminolic acid) and one tannin (casuarinin) were previously isolated from this plant and found to significantly decrease interleukin-8 production in HT-29 (Yang et al., 2014).

In summary, *A. blancoi* (Elmer) Merr., *A. tectorius* (Lour.) Merr., and *P. siamensis* (Miq.) Kurz produced significant growth-inhibitory effects against diarrheagenic bacterial pathogens at concentrations nontoxic to normal intestinal cells. Except the strong anticlostridial actions of *A. blancoi* (Elmer) Merr., the MICs determined for these plant extracts in the present study reflect rather moderate antibacterial activities. However, the discrimination of specific cell toxicity indicates that higher amounts of these products necessary to acquire the appropriate efficiency may still be safe to use (Cos et al., 2006). A long tradition of their use in folk medicinal systems supports this assumption. Moreover, it has been reported that microorganisms are less likely to develop resistance to phytochemicals with anti-infective potential, mainly because of their high diversity in plants. Some were even considered as antibiotic resistance modifying compounds (Sibanda and Okoh, 2007). Additionally, our study showed that the extract of *P. siamensis* (Miq.) Kurz was relatively safe for probiotic bacteria, and together with *A. blancoi* (Elmer) Merr., they exerted selective anticancer activities *in vitro*. Similar to the cytotoxic activities revealed by *E. microphylla* Lam., *L. cochinchinensis* Pierre ex Gagnep., and *M. saigonense* (Kuntze) Merr., the inhibitory effect of *A. blancoi* (Elmer) Merr. on cancer cell line Caco-2 and the selectivity of its overall antiproliferative actions were generally higher than those of anticancer drug 5-fluorouracil.

These results suggest that extracts from the above-mentioned Cambodian and Philippine plant species are promising materials for further research focused on the development of new plant-derived selective antibacterial and antiproliferative agents used in the treatment of infectious diarrhea and associated intestinal cancer diseases. For instance, the combination of strong anticlostridial and anticancer actions of *A. blancoi* (Elmer) Merr. may in the future be utilized in the treatment of digestive cancers associated with *C. difficile* infections (Han et al., 2013). However, further phytochemical and pharmacological research is needed for the isolation and proper identification of their bioactive constituents. Referring to studies dealing with taxonomically related plants to estimate the presence of their bioactive principles is a very limited approach as their composition can vary greatly. On the other hand, our results could serve as an indicator of bioactive potentials of products derived from species of the same taxa. This is mainly the case of *P. siamensis* (Miq.) Kurz that exhibited selective inhibition of pathogenic bacteria and intestinal cancer cells without affecting beneficial bacteria and normal intestinal cells. Future research combining the ethnomedicinal and chemotaxonomic approaches might help to identify more plants with promising bioactivities (Hao and Xiao, 2020).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TK collected the plant materials, coordinated antibacterial activity testing and its related data analysis, and prepared the article. BF and MK processed the voucher specimens and participated in the collection of plant materials and testing of antibacterial activity. ID conducted the cytotoxicity assays and their related data analysis. HS was responsible for maintenance and culturing of anaerobic bacteria tested. ET and MB participated in the collection of plant materials and

verification of the data on the ethnobotanical use of plants in the Philippines. SN participated in the collection of plant materials and verification of the data on the ethnobotanical use of plants in Cambodia. LK conceptualized and coordinated the whole study and provided the botanical identification of plant samples. All authors have read and agreed to the published version of the article.

FUNDING

This work was supported by the Internal Grant Agency of the Faculty of Tropical AgriSciences (IGA 20213109), the METROFOOD-CZ Research Infrastructure Project (MEYS LM2018100), and the Strategic Setting of Human Resources Development at CZU (CZ.02.2.69/0.0/0.0/18_054/0014642).

REFERENCES

Abu Bakar, M. F., Abdul Karim, F., and Perisamy, E. (2015). Comparison of Phytochemicals and Antioxidant Properties of Different Fruit Parts of Selected *Artocarpus* Species from Sabah, Malaysia. *Sains Malays* 44 (3), 355–363. doi:10.17576/jsm-2015-4403-06

Anh, N. H., Porzel, A., Ripperger, H., Bringmann, G., Schäffer, M., God, R., et al. (1997). Naphthylisoquinoline Alkaloids from *Ancistrocladus cochinchinensis*. *Phytochemistry* 45 (6), 1287–1291. doi:10.1016/s0031-9422(97)00110-6

Ante, I., Aboaba, S., Siddiqui, H., and Choudhary, M. I. (2016). Essential Oils of the Leaf, Stem-Bark, and Nut of *Artocarpus camansi*: Gas Chromatography-Mass Spectrometry Analysis and Activities against Multidrug-Resistant Bacteria. *J. Herbs, Spices Med. Plants* 22 (3), 203–210. doi:10.1080/10496475.2016.1159638

Batalha, P. N., Vieira de Souza, M. C., Peña-Cabrera, E., Cruz, D. C., and da Costa Santos Boechat, F. (2016). Quinolones in the Search for New Anticancer Agents. *Curr. Pharm. Des.* 22 (39), 6009–6020. doi:10.2174/1381612822666160715115025

Behnsen, J., Deriu, E., Sassone-Corsi, M., and Raffatellu, M. (2013). Probiotics: Properties, Examples, and Specific Applications. *Cold Spring Harb. Perspect. Med.* 3 (3), a010074. doi:10.1101/cshperspect.a010074

Beloy, F. B., Masilungan, V. A., Cruzde, la. R. M., and Ramos, E. V. (1976). Investigation of Some Philippine Plants for Antimicrobial Substances. *Philipp. J. Sci.* 105, 205–213.

Bradacs, G., Maes, L., and Heilmann, J. (2009). *In Vitro* Cytotoxic, Antiprotozoal and Antimicrobial Activities of Medicinal Plants from Vanuatu. *Phytother. Res.* 24 (6), 800–809. doi:10.1002/ptr.2981

Bringmann, G., Xu, M., Seupel, R., Feineis, D., and Wu, J. (2016). Ancistrotectoquinones A and B, the First Quinoid Naphthylisoquinoline Alkaloids, from the Chinese Liana *Ancistrocladus tectorius*. *Nat. Prod. Commun.* 11 (7), 971–976. doi:10.1177/1934578x1601100725

Casburn-Jones, A. C., and Farthing, M. J. (2004). Management of Infectious Diarrhoea. *Gut* 53 (2), 296–305. doi:10.1136/gut.2003.022103

Chan, C.-L., Gan, R.-Y., Shah, N. P., and Corke, H. (2018). Polyphenols from Selected Dietary Spices and Medicinal Herbs Differentially Affect Common Food-Borne Pathogenic Bacteria and Lactic Acid Bacteria. *Food Control* 92, 437–443. doi:10.1016/j.foodcont.2018.05.032

Chan, E. W. C., Tan, L. N., and Wong, S. K. (2014). Phytochemistry and Pharmacology of *Lagerstroemia Speciosa*: a Natural Remedy for Diabetes. *Int. J. Herb. Med.* 2 (1), 81–87.

Chassagne, F., Hul, S., Deharo, E., and Bourdy, G. (2016). Natural Remedies Used by Bunong People in Mondulkiri Province (Northeast Cambodia) with Special Reference to the Treatment of 11 Most Common Ailments. *J. Ethnopharmacol.* 191, 41–70. doi:10.1016/j.jep.2016.06.003

Chea, A., Jonville, M. C., Bun, S. S., Laget, M., Elias, R., Duménil, G., et al. (2007). *In Vitro* antimicrobial Activity of Plants Used in Cambodian Traditional Medicine. *Am. J. Chin. Med.* 35 (05), 867–873. doi:10.1142/s0192415x07005338

Clinical and Laboratory Standards Institute (2021). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard-2021 Ed. CLSI Document M07-M100. Wayne: CLSI.

Cos, P., Vlietinck, A. J., Berghe, D. V., and Maes, L. (2006). Anti-infective Potential of Natural Products: How to Develop a Stronger *In Vitro* 'proof-Of-Concept'. *J. Ethnopharmacol.* 106, 290–302. doi:10.1016/j.jep.2006.04.003

de Guzman, G. Q., Dacanay, A. T., Andaya, B. A., and Alejandro, G. J. (2016). Ethnopharmacological Studies on the Uses of *Euphorbia hirta* in the Treatment of Dengue in Selected Indigenous Communities in Pangasinan (Philippines). *J. Intercult. Ethnopharmacol.* 5 (3), 239–243. doi:10.5455/jice.20160330124637

de Padua, L. S., Bunyapraphatsara, N., and Lemmens, R. H. M. J. (1999). *Plant Resources of South-East Asia (PROSEA) No 12 (1), Medicinal and Poisonous Plants 1*. (Leiden: Backhuys Publishers).

Diniz-Santos, D. R., Silva, L. R., and Silva, N. (2006). Antibiotics for the Empirical Treatment of Acute Infectious Diarrhea in Children. *Braz. J. Infect. Dis.* 10 (3), 217–227. doi:10.1590/s1413-86702006000300011

Duriyaprapan, S., Tanpanich, S., and Khuankhamnuan, C. (2005). The Plant Resources of South-East Asia (PROSEA). *Acta Hortic.* 675, 15–21. doi:10.17660/actahortic.2005.675.1

Francino, M. P. (2015). Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Front. Microbiol.* 6, 1543. doi:10.3389/fmicb.2015.01543

Fuente, B., López-García, G., Máñez, V., Alegría, A., Barberá, R., and Cilla, A. (2020). Antiproliferative Effect of Bioaccessible Fractions of Four Brassicaceae Microgreens on Human Colon Cancer Cells Linked to Their Phytochemical Composition. *Antioxidants (Basel)* 9 (5), 368. doi:10.3390/antiox9050368

Garrett, W. S. (2019). The Gut Microbiota and Colon Cancer. *Science* 364 (6446), 1133–1135. doi:10.1126/science.aaw2367

Gavrish, E., Shrestha, B., Chen, C., Lister, I., North, E. J., Yang, L., et al. (2014). *In Vitro* and *In Vivo* Activities of HPi1, a Selective Antimicrobial against *Helicobacter pylori*. *Antimicrob. Agents Chemother.* 58 (6), 3255–3260. doi:10.1128/aac.02573-13

González-Sarrías, A., Gromek, S., Niesen, D., Seeram, N. P., and Henry, G. E. (2011). Resveratrol Oligomers Isolated from *Carex* Species Inhibit Growth of Human Colon Tumorigenic Cells Mediated by Cell Cycle Arrest. *J. Agric. Food Chem.* 59 (16), 8632–8638. doi:10.1021/jf201561e

Hafid, A. F., Aoki-Utsubo, C., Permanasari, A. A., Adianti, M., Tumewu, L., Widyawaruyanti, A., et al. (2017). Antiviral Activity of the Dichloromethane Extracts from *Artocarpus heterophyllus* Leaves against Hepatitis C Virus. *Asian Pac. J. Trop. Biomed.* 7 (7), 633–639. doi:10.1016/j.apjtb.2017.06.003

Han, X. H., Du, C. X., Zhang, C. L., Zheng, C. L., Wang, L., Li, D., et al. (2013). *Clostridium difficile* Infection in Hospitalized Cancer Patients in Beijing, China Is Facilitated by Receipt of Cancer Chemotherapy. *Anaerobe* 24, 82–84. doi:10.1016/j.anaerobe.2013.05.004

Hao, D.-c., and Xiao, P.-g. (2020). Pharmaceutical Resource Discovery from Traditional Medicinal Plants: Pharmacophylogeny and Pharmacophylogenomics. *Chin. Herbal Medicines* 12, 104–117. doi:10.1016/j.chmed.2020.03.002

Hecht, D. W. (1999). "Antimicrobial Agents and Susceptibility Testing: Susceptibility Testing of Anaerobic Bacteria," in *Manual of Clinical Microbiology*. Editors P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken. 7th ed (Washington DC: American Society for Microbiology ASM), 1555–1563.

Houdková, M., Urbanová, K., Doskočil, I., Rondevaldová, J., Nový, P., Nguon, S., et al. (2018). *In Vitro* Growth-Inhibitory Effect of Cambodian Essential Oils against Pneumonia Causing Bacteria in Liquid and Vapour Phase and Their Toxicity to Lung Fibroblasts. *South Afr. J. Bot.* 118, 85–97. doi:10.1016/j.sajb.2018.06.005

Jalal, T. K., Ahmed, I. A., Mikail, M., Momand, L., Draman, S., Isa, M. L., et al. (2015). Evaluation of Antioxidant, Total Phenol and Flavonoid Content and Antimicrobial Activities of *Artocarpus altilis* (Breadfruit) of Underutilized Tropical Fruit Extracts. *Appl. Biochem. Biotechnol.* 175 (7), 3231–3243. doi:10.1007/s12010-015-1499-0

Jiang, C., Li, Z. L., Gong, P., Kang, S. L., Liu, M. S., Pei, Y. H., et al. (2013). Five Novel Naphthylisoquinoline Alkaloids with Growth Inhibitory Activities against Human Leukemia Cells HL-60, K562 and U937 from Stems and Leaves of *Ancistrocladus tectorius*. *Fitoterapia* 91, 305–312. doi:10.1016/j.fitote.2013.09.010

Kamsani, N. E., Zakaria, Z. A., Md Nasir, N. L., Mohtarrudin, N., and Mohamad Alitheen, N. B. (2019). Safety Assessment of Methanol Extract of *Melastoma malabathricum* L. Leaves Following the Subacute and Subchronic Oral Consumptions in Rats and its Cytotoxic Effect against the HT29 Cancer Cell Line. *Evid. Based Complement. Alternat Med.* 2019, 5207958. doi:10.1155/2019/5207958

Karakurt, S., Abuşoğlu, G., and Arituluk, Z. C. (2020). Comparison of Anticarcinogenic Properties of *Viburnum opulus* and its Active Compound P-Coumaric Acid on Human Colorectal Carcinoma. *Turk. J. Biol.* 44 (5), 252–263. doi:10.3906/biy-2002-30

Kelber, O., Steinhoff, B., Nauert, C., Biller, A., Adler, M., Abdel-Aziz, H., et al. (2016). Ethanol in Herbal Medicinal Products for Children : Data from Pediatric Studies and Pharmacovigilance Programs. *Wien Med. Wochenschr.* 167 (7–8), 183–188. doi:10.1007/s10354-016-0474-x

Kham, L. (2004). Medicinal Plants of Cambodia: Habitat, Chemical Constituents and Ethnobotanical Uses. Golden Square: Bendigo Scientific Press.

Khan, M. R., Kihara, M., and Omoloso, A. D. (2001). Antibacterial Activity of *Picrasma javanica*. *Fitoterapia* 72 (4), 406–408. doi:10.1016/s0367-326x(00)00274-4

Kittakoop, P., Kirtikara, K., Tanticharoen, M., and Thebtaranonth, Y. (2000). Antimalarial Preracemosols A and B, Possible Biogenetic Precursors of Racemosol from *Bauhinia malabarica* Roxb. *Phytochemistry* 55 (4), 349–352. doi:10.1016/s0031-9422(00)00318-6

Kokoška, L., Klouček, P., Leuner, O., and Nový, P. (2019). Plant-Derived Products as Antibacterial and Antifungal Agents in Human Health Care. *Curr. Med. Chem.* 26 (29), 5501–5541. doi:10.2174/0929867325666180831144344

Kudera, T., Doskočil, I., Salmonová, H., Petrtýl, M., Skřivanová, E., and Kokoška, L. (2020). *In Vitro* Selective Growth-Inhibitory Activities of Phytochemicals, Synthetic Phytochemical Analogs, and Antibiotics against Diarrheagenic/Probiotic Bacteria and Cancer/Normal Intestinal Cells. *Pharmaceuticals (Basel)* 13 (9), 233. doi:10.3390/ph13090233

Kuete, V., Ngnintedo, D., Fotso, G. W., Karaosmanoğlu, O., Ngadjui, B. T., Keumedjio, F., et al. (2018). Cytotoxicity of Seputhecarpan D, Thonningiol and 12 Other Phytochemicals from African Flora towards Human Carcinoma Cells. *BMC Complement. Altern. Med.* 18 (1), 36. doi:10.1186/s12906-018-2109-9

Langenberger, G., Prigge, V., Martin, K., Belonias, B., and Sauerborn, J. (2008). Ethnobotanical Knowledge of Philippine Lowland Farmers and its Application in Agroforestry. *Agrofor. Syst.* 76 (1), 173–194. doi:10.1007/s10457-008-9189-3

Lemmens, R. H. M. J. L., and Bunyapraphatsara, N. (2003). *Plant Resources of South-East Asia (PROSEA) No 12 (3), Medicinal and Poisonous Plants 3*. Leiden: Backhuys Publishers.

Lim, T. K. (2012). *Edible Medicinal and Non Medicinal Plants*. Fruits. New York: Springer.

Lin, J. P., Yang, J. S., Wu, C. C., Lin, S. S., Hsieh, W. T., Lin, M. L., et al. (2008). Berberine Induced Down-Regulation of Matrix Metalloproteinase-1, -2 and -9 in Human Gastric Cancer Cells (SNU-5). *In Vivo* 22 (2), 223–230.

Mackeen, M. M., Ali, A. M., El-Sharkawy, S. H., Manap, M. Y., Salleh, K. M., Lajis, N. H., et al. (1997). Antimicrobial and Cytotoxic Properties of Some Malaysian Traditional Vegetables (Ulam). *Int. J. Pharmacognosy* 35 (3), 174–178. doi:10.1076/phbi.35.3.174.13294

Maher, S., and McClean, S. (2006). Investigation of the Cytotoxicity of Eukaryotic and Prokaryotic Antimicrobial Peptides in Intestinal Epithelial Cells *In Vitro*. *Biochem. Pharmacol.* 71 (9), 1289–1298. doi:10.1016/j.bcp.2006.01.012

Marandi, R. R., Britto, S. J., and Soreng, P. K. (2016). Phytochemical Profiling, Antibacterial Screening and Antioxidant Properties of the Sacred Tree (*Shorea robusta* Gaertn.) of Jharkhand. *Int. J. Pharm. Sci. Res.* 7 (7), 2874–2888. doi:10.13040/IJPSR.0975-8232.7(7).2874-88

Meng, C. Y., Smith, B. L., Bodhidatta, L., Richard, S. A., Vansith, K., Thy, B., et al. (2011). Etiology of Diarrhea in Young Children and Patterns of Antibiotic Resistance in Cambodia. *Pediatr. Infect. Dis. J.* 30 (4), 331–335. doi:10.1097/inf.0b013e3181fb6f82

Mihalyi, A., Jamshidi, S., Slikas, J., and Bugg, T. D. (2014). Identification of Novel Inhibitors of Phospho-MurNAc-Pentapeptide Translocase MraY from Library Screening: Isoquinoline Alkaloid Michellamine B and Xanthene Dye Phloxine B. *Bioorg. Med. Chem.* 22 (17), 4566–4571. doi:10.1016/j.bmc.2014.07.035

Moriyama, H., Moriyama, M., Ninomiya, K., Morikawa, T., and Hayakawa, T. (2016). Inhibitory Effects of Oligostilbenoids from the Bark of *Shorea roxburghii* on Malignant Melanoma Cell Growth: Implications for Novel Topical Anticancer Candidates. *Biol. Pharm. Bull.* 39 (10), 1675–1682. doi:10.1248/bpb.b16-00420

Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* 65 (1–2), 55–63. doi:10.1016/0022-1759(83)90303-4

Neftel, K. A., and Hübscher, U. (1987). Effects of Beta-Lactam Antibiotics on Proliferating Eucaryotic Cells. *Antimicrob. Agents Chemother.* 31 (11), 1657–1661. doi:10.1128/aac.31.11.1657

Nitta, T., Arai, T., Takamatsu, H., Inatomi, Y., Murata, H., Iinuma, M., et al. (2002). Antibacterial Activity of Extracts Prepared from Tropical and Subtropical Plants on Methicillin-Resistant *Staphylococcus aureus*. *J. Health Sci.* 48 (3), 273–276. doi:10.1248/jhs.48.273

Novaková, J., Vlková, E., Bonušova, B., Rada, V., and Kokoška, L. (2013). *In Vitro* Selective Inhibitory Effect of 8-Hydroxyquinoline against Bifidobacteria and Clostridia. *Anaerobe* 22, 134–136. doi:10.1016/j.anaerobe.2013.05.008

Ong, H. C., and Nordiana, M. (1999). Malay Ethno-Medico Botany in Machang, Kelantan, Malaysia. *Fitoterapia* 70 (5), 502–513. doi:10.1016/s0367-326x(99)00077-5

Onoda, T., Ono, T., Dhar, D. K., Yamanoi, A., and Nagasue, N. (2005). Tetracycline Analogues (Doxycycline and COL-3) Induce Caspase-dependent and -independent Apoptosis in Human Colon Cancer Cells. *Int. J. Cancer* 118 (5), 1309–1315. doi:10.1002/ijc.21447

Our World in Data (2011). Diarrheal Diseases. Available at: https://ourworldindata.org/diarrheal-diseases (Accessed October 19, 2020).

Palombo, E. A. (2006). Phytochemicals from Traditional Medicinal Plants Used in the Treatment of Diarrhoea: Modes of Action and Effects on Intestinal Function. *Phytother Res.* 20 (9), 717–724. doi:10.1002/ptr.1907

Pedro, M., Ferreira, M. M., Cidade, H., Kijjoa, A., Bronze-da-Rocha, E., and Nascimento, M. S. (2005). Artelastin Is a Cytotoxic Prenylated Flavone that Disturbs Microtubules and Interferes with DNA Replication in MCF-7 Human Breast Cancer Cells. *Life Sci.* 77 (3), 293–311. doi:10.1016/j.lfs.2004.09.049

Rahmat, A., Kumar, V., Fong, L. M., Endrini, S., and Sani, H. A. (2003). Determination of Total Antioxidant Activity in Three Types of Local Vegetables Shoots and the Cytotoxic Effect of Their Ethanolic Extracts against Different Cancer Cell Lines. *Asia Pac. J. Clin. Nutr.* 12 (3), 292–295.

Rajković, A., Jovanović, J., Monteiro, S., Decleer, M., Andjelković, M., Foubert, A., et al. (2020). Detection of Toxins Involved in Foodborne Diseases Caused by Gram-Positive Bacteria. *Compr. Rev. Food Sci. Food Saf.* 19 (4), 1605–1657. doi:10.1111/1541-4337.12571

Rajkumar, R., Shivakumar, M. S., Senthil Nathan, S., and Selvam, K. (2019). Preparation and Characterization of Chitosan Nanocomposites Material Using Silver Nanoparticle Synthesized *Carmona retusa* (Vahl) Masam Leaf Extract for Antioxidant, Anti-Cancerous and Insecticidal Application. *J. Clust. Sci.* 30 (4), 1145–1155. doi:10.1007/s10876-019-01578-9

Ramli, F., Rahmani, M., Ismail, I. S., Sukari, M. A., Abd Rahman, M., Zajmi, A., et al. (2016). A New Bioactive Secondary Metabolite from *Artocarpus elasticus*. *Nat. Prod. Commun.* 11 (8), 1103–1106. doi:10.1177/1934578x1601100818

Rathee, D., Rathee, P., Rathee, S., and Rathee, D. (2016). Phytochemical Screening and Antimicrobial Activity of *Picrorrhiza kurroa*, an Indian Traditional Plant Used to Treat Chronic Diarrhea. *Arabian J. Chem.* 9, S1307–S1313. doi:10.1016/j.arabjc.2012.02.009

Rawla, P., Sunkara, T., and Barsouk, A. (2019). Epidemiology of Colorectal Cancer: Incidence, Mortality, Survival, and Risk Factors. *Prz. Gastroenterol.* 14 (2), 89–103. doi:10.5114/pg.2018.81072

Requena, T., Monagas, M., Pozo-Bayón, M. A., Martín-Álvarez, P. J., Bartolomé, B., del Campo, R., et al. (2010). Perspectives of the Potential Implications of Wine Polyphenols on Human Oral and Gut Microbiota. *Trends Food Sci. Tech.* 21 (7), 332–344. doi:10.1016/j.tifs.2010.04.004

Rogowski, W., and Sulżyc-Bielicka, V. (2016). Optimal Duration of a First-Line Palliative Chemotherapy in Disseminated Colorectal Cancer - a Review of the Literature from a Developing Country Perspective. *Contemp. Oncol. (Pozn)* 20, 210–214. doi:10.5114/wo.2016.61561

Rohaiza, S. (2011). Cytotoxic Oligostilbenes from *Shorea hopeifolia*. *Afr. J. Pharm. Pharmacol.* 5 (10), 1272–1277. doi:10.5897/ajpp10.386

Sato, M., Fujiwara, S., Tsuchiya, H., Fujii, T., Iinuma, M., Tosa, H., et al. (1996). Flavones with Antibacterial Activity against Cariogenic Bacteria. *J. Ethnopharmacol.* 54 (2–3), 171–176. doi:10.1016/s0378-8741(96)01464-x

Septama, A. W., and Panichayupakaranant, P. (2015). Synergistic Effect of Artocarpin on Antibacterial Activity of Some Antibiotics against Methicillin-Resistant *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*. *Pharm. Biol.* 54 (4), 686–691. doi:10.3109/13880209.2015.1072566

Shang, X. F., Yang, C. J., Morris-Natschke, S. L., Li, J. C., Yin, X. D., Liu, Y. Q., et al. (2020). Biologically Active Isoquinoline Alkaloids Covering 2014-2018. *Med. Res. Rev.* 40 (6), 2212–2289. doi:10.1002/med.21703

Sibanda, T., and Okoh, A. I. (2007). The Challenges of Overcoming Antibiotic Resistance: Plant Extracts as Potential Sources of Antimicrobial and Resistance Modifying Agents. *Afr. J. Biotechnol.* 6 (25), 2886–2896.

Srisawat, T., Chumkaew, P., Heed-Chim, W., Sukpondma, Y., and Kanokwiroon, K. (2013). Phytochemical Screening and Cytotoxicity of Crude Extracts of *Vatica diospyroides* Symington Type I.S. *Trop. J. Pharm. Res.* 12 (1), 71–76. doi:10.4314/tjpr.v12i1.12

Stuart, G. U., Jr. (2017). List of Philippine Herbal Medicinal Plants. Philippine medicinal plants. StuartXchange-SX. Available at: http://www.stuartxchange.org/CompleteList.html (Accessed February 20, 2017).

Sudto, K., Saparpakorn, P., Tancharoen, C., Phromyothin, D., Techasakul, S., Khunnawutmanotham, N., et al. (2019). Diptoindonesin D, a Potent Antibacterial Activity against Gram-Positive Bacteria, an Inhibitor of Penicillin-Binding Protein 2a from the Stem Bark of *Shorea roxburghii* G.Don. *Chiang Mai J. Sci.* 46 (6), 1161–1175.

Sun, X., Gao, Y., Wang, X., Hu, G., Wang, Y., Feng, B., et al. (2018). *Escherichia coli* O101-Induced Diarrhea Develops Gut Microbial Dysbiosis in Rats. *Exp. Ther. Med.* 17 (1), 824–834. doi:10.3892/etm.2018.6997

Sung, B., Kang, Y. J., Kim, D. H., Hwang, S. Y., Lee, Y., Kim, M., et al. (2014). Corosolic Acid Induces Apoptotic Cell Death in HCT116 Human Colon Cancer Cells through a Caspase-dependent Pathway. *Int. J. Mol. Med.* 33 (4), 943–949. doi:10.3892/ijmm.2014.1639

Taddese, R., Garza, D. R., Ruiter, L. N., de Jonge, M. I., Belzer, C., Aalvink, S., et al. (2020). Growth Rate Alterations of Human Colorectal Cancer Cells by 157 Gut Bacteria. *Gut Microbes* 12 (1), 1–20. doi:10.1080/19490976.2020.1799733

Tan, M. C. S., Carranza, M. S. S., Linis, V. C., Malabed, R. S., and Oyong, G. G. (2019). Antioxidant, Cytotoxicity, and Antiophidian Potential of *Alstonia macrophylla* Bark. *ACS Omega* 4 (5), 9488–9496. doi:10.1021/acsomega.9b00082

Tan, M. L. (1980). *Philippine Medicinal Plants in Common Use*. (Quezon City: AKAP).

Tang, C. P., Xin, Z. Q., Li, X. Q., and Ye, Y. (2010). Two New Naphthylisoquinoline Alkaloids from Stems and Leaves of *Ancistrocladus tectorius*. *Nat. Prod. Res.* 24 (11), 989–994. doi:10.1080/14786410902836727

Tang, C. P., Yang, Y. P., Zhong, Y., Zhong, Q. X., Wu, H. M., and Ye, Y. (2000). Four New Naphthylisoquinoline Alkaloids from *Ancistrocladus tectorius*. *J. Nat. Prod.* 63 (10), 1384–1387. doi:10.1021/np000091d

Teanpaisan, R., Senapong, S., and Puripattanavong, J. (2014). *In Vitro* Antimicrobial and Antibiofilm Activity of *Artocarpus lakoocha* (Moraceae) Extract against Some Oral Pathogens. *Trop. J. Pharm. Res.* 13 (7), 1149. doi:10.4314/tjpr.v13i7.20

The Plant List (2013). Royal Botanic Gardens and Missouri Botanical Garden. Kew and St. Louis. Version 1.1. Available at: http://www.theplantlist.org/(Accessed March 11, 2017).

Tian, X., Guo, S., Zhang, S., Li, P., Wang, T., Ho, C. T., et al. (2019). Chemical Characterization of Main Bioactive Constituents in *Paeonia ostii* Seed Meal and GC-MS Analysis of Seed Oil. *J. Food Biochem.* 44 (1), e13088. doi:10.1111/jfbc.13088

Tsai, P.-W., De Castro-Cruz, K. A., Shen, C.-C., Chiou, C.-T., and Ragasa, C. Y. (2013). Chemical Constituents of *Artocarpus camansi*. *Pharmacognosy J.* 5 (2), 80–82. doi:10.1016/j.phcgj.2012.06.001

van Duong, N. (1993). *Medicinal Plants of Vietnam*. (Cambodia and Laos: Nguyen Van Duong).

van Valkenburg, J. L. C. H., and Bunyapraphatsara, N. (2001). *Plant Resources of South-East Asia (PROSEA) No 12 (2), Medicinal and Poisonous Plants 2*. Leiden: Backhuys Publishers.

Wiart, C., Mogana, S., Khalifah, S., Mahan, M., Ismail, S., Buckle, M., et al. (2004). Antimicrobial Screening of Plants Used for Traditional Medicine in the State of Perak, Peninsular Malaysia. *Fitoterapia* 75 (1), 68–73. doi:10.1016/j.fitote.2003.07.013

Win, N. N., Ito, T., IsmailKodama, T., Kodama, Y. Y., Tanaka, M., et al. (2015). Picrajavanicins A-G, Quassinoids from *Picrasma javanica* Collected in Myanmar. *J. Nat. Prod.* 78 (12), 3024–3030. doi:10.1021/acs.jnatprod.5b00824

Woo, K. W., Cha, J. M., Choi, S. U., and Lee, K. R. (2016). A New Triterpene Glycoside from the Stems of *Lagerstroemia indica*. *Arch. Pharm. Res.* 39 (5), 631–635. doi:10.1007/s12272-016-0746-4

World Health Organization (2020b). Antimicrobial Resistance. Available at: https://www.who.int/en/news-room/fact-sheets/detail/antimicrobial-resistance (Accessed September 13, 2021).

World Health Organization (2020a). The Top 10 Causes of Death. Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (Accessed September 13, 2021).

Wu, X., Alam, M. Z., Feng, L., Tsutsumi, L. S., Sun, D., and Hurdle, J. G. (2013). Prospects for Flavonoid and Related Phytochemicals as Nature-Inspired Treatments for *Clostridium difficile* Infection. *J. Appl. Microbiol.* 116 (1), 23–31. doi:10.1111/jam.12344

Xu, K., and Jiang, B. (2017). Analysis of Mucosa-Associated Microbiota in Colorectal Cancer. *Med. Sci. Monit.* 23, 4422–4430. doi:10.12659/msm.904220

Yang, G. X., Zhang, R. Z., Lou, B., Cheng, K. J., Xiong, J., and Hu, J. F. (2014). Chemical Constituents from *Melastoma dodecandrum* and Their Inhibitory Activity on Interleukin-8 Production in HT-29 Cells. *Nat. Prod. Res.* 28 (17), 1383–1387. doi:10.1080/14786419.2014.903480

Zawawi, N. K., Ahmat, N., Mazatulikhma, M. Z., Shafiq, R. M., Wahid, N. H., and Sufian, A. S. (2012). Bioactive Oligostilbenoids from *Shorea maxwelliana* King and Their Chemotaxonomic Significance. *Nat. Prod. Res.* 27 (17), 1589–1593. doi:10.1080/14786419.2012.730047

Zeng, X. H., Li, Y. H., Wu, S. S., Hao, R. L., Li, H., Ni, H., et al. (2013). New and Highly Efficient Column Chromatographic Extraction and Simple Purification of Camptothecin from *Camptotheca acuminata* and *Nothapodytes pittosporoides*. *Phytochem. Anal.* 24 (6), 623–630. doi:10.1002/pca.2441

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 26 November 2021
doi: 10.3389/fphar.2021.779352

Ginger from Farmyard to Town: Nutritional and Pharmacological Applications

Jeremiah Oshiomame Unuofin[1]*, *Nelisiwe Prenate Masuku*[1], *Oluwatomiwa Kehinde Paimo*[2] *and Sogolo Lucky Lebelo*[1]

[1]Department of Life and Consumer Sciences, University of South Africa, Florida, South Africa, [2]Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Ibadan, Nigeria

Ginger (*Zingiber officinale*) is one of the most widely used natural products consumed as a spice and medicine for treating diabetes, flatulent intestinal colic, indigestion, infertility, inflammation, insomnia, a memory booster, nausea, rheumatism, stomach ache, and urinary tract infections. To date, over 400 bioactive components, such as diarylheptanoids, gingerol analogues, phenylalkanoids, sulfonates, monoterpenoid glycosides, steroids, and terpene compounds have been derived from ginger. Increasing evidence has revealed that ginger possesses a broad range of biological activities, especially protective effects against male infertility, nausea and vomiting, analgesic, anti-diabetic, anti-inflammatory, anti-obesity, and other effects. The pharmacological activities of ginger were mainly attributed to its active phytoconstituents such as 6-gingerol, gingerdiol, gingerol, gingerdione, paradols, shogaols, sesquiterpenes, zingerone, besides other phenolics and flavonoids. In recent years, *in silico* molecular docking studies revealed that gingerol (6-gingerol, 8-gingerol, and 10-gingerol) and Shogaol (6-shogaol, 8-shogaol, 10-shogaol) had the best binding affinities to the receptor protein in disease conditions such as diabetes, inflammation, obesity, and SARS-CoV-2. Furthermore, some clinical trials have indicated that ginger can be consumed for alleviation of nausea and vomiting induced by surgery, pain, diabetes, obesity, inflammation, male infertility. This review provides an updated understanding of the scientific evidence on the development of ginger and its active compounds as health beneficial agents in future clinical trials.

Keywords: ginger, gingerols and shogaols, nutritional composition, *in silico* molecular docking studies, clinical trials, *Zingiber officinale*

OPEN ACCESS

Edited by:
Alessandra Durazzo, Research Centre for Food and Nutrition, Italy

Reviewed by:
Syaripah Za'imah Syed Jaapar, Polytechnic Tun Syed Nasir Syed Ismail, Malaysia
Vijendra Kumar Nanjundaswamy, Central Food Technological Research Institute (CSIR), India
Ali El Halawany, Cairo University, Egypt

***Correspondence:**
Jeremiah Oshiomame Unuofin
unuofinjeremiah@gmail.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *18 September 2021*
Accepted: *08 October 2021*
Published: *26 November 2021*

Citation:
Unuofin JO, Masuku NP, Paimo OK and Lebelo SL (2021) Ginger from Farmyard to Town: Nutritional and Pharmacological Applications. Front. Pharmacol. 12:779352. doi: 10.3389/fphar.2021.779352

INTRODUCTION

Herbs and spices have been the most sort after throughout history because of the roles they play as food preservatives, flavor, coloring, and therapeutic agents. Although herbs and spices are relatively low-cost commodities, they have been valued as precious jewels by ancient Egypt, India, and China for many centuries (El-Sayed and Youssef, 2019). In recent years, their popularity has increased in developed countries owing to the belief that they are more effective and possess mild adverse effects than synthetic pharmaceuticals for preventing or treating various ailments (Unuofin et al., 2018; Masuku et al., 2020; Unuofin and Lebelo, 2020, 2021). In addition, herbs and spices have gained significant recognition in the food industry due to their incorporation in foodstuffs and their ability to improve health. It has been reported that 70–80% of the world population rely on the use of

complementary and alternative traditional medicine (herbal medicine) for major primary healthcare concerns (Chan, 2003; Martín-Domingo et al., 2017; El-Sayed and Youssef, 2019).

According to Herman (2015), herbs are obtained from the leaves of the plant while spices are collected from different parts of the plant such as arils, barks, flowers, fruit berries, pods, rhizomes, roots, and seeds. *Zingiber officinale* (Roscoe) also known as ginger (rhizomes) belongs to the family Zingiberaceae. It is native to East and Southern Asia and consists of 53 genera and 1300 species worldwide, with 80–90 of which are Zingiber (Shahrajabian et al., 2019).

Ginger (*Z. officinale*) is a popular flowering plant whose rhizome, ginger root, or ginger, is widely used as a culinary and folk medicine (Nour et al., 2017). The underground stem (rhizome) is used for the preparation of ginger and can be obtained in colors varying from white to brown, depending on whether the exterior is scraped off and how it is initially treated (Singletary, 2010). The economic importance of ginger has been restricted by challenges such as biotic stress, climatic fluctuations (drought or floods), other external shocks, prominent instability in food price have caused a decline in returns when compared with high production costs (Mmasa and Kizito Mhagama, 2017). According to Mahat et al. (2019), ginger thrives best in a temperature range of 19–28°C, pH (6.0–6.5), and relative humidity (70–90%).

Sowley and Kankam (2019) cited the Transparency Market Research report on ginger, which showed that ginger is among the herbal commodities with great economic value. The top ten ginger-producing countries according to Dhanik and colleagues are India, China, Nepal, Indonesia, Nigeria, Thailand, Bangladesh, Japan, Cameroon, and the Philippines (Dhanik et al., 2017). The global production of ginger in 2017 was estimated to be 3.3 million tonnes. India accounted for 34% of the production while Nigeria, China, and Indonesia produced a substantial amount. Subsequently, China the second-largest producer produced about 0.58 million tons of ginger with a huge amount being exported to Japan, Korea, and Vietnam. In the same year, ginger production witnessed a boost of 6.5% per year in market value and it was projected that by 2022, the consumption rate of ginger would be 7.5% with a valuation as high as US$ 4.18 billion (Dhanik et al., 2017; Sowley and Kankam, 2019; Zhang et al., 2021).

The beneficial effects of ginger to mankind have been dated as far back as the 13th century (Singletary, 2010). The rhizome has been processed into powder, syrup, volatile oil, and oleoresin. The findings of (Ali and Hassan Gilani, 2007) revealed that both the fresh and dried forms of ginger are renowned globally for their culinary and medicinal properties. Literature is replete with information regarding its aromatic, penetrating, spicy, hot, and biting attributes. These attributes, however, are either diminished or lost upon exposure to light and air (Ghayur and Gilani, 2005; Ali and Hassan Gilani, 2007). The use of ginger in Asian and Ayurvedic medicine for the treatment of diabetes, flatulent intestinal colic, indigestion, infertility, inflammation, insomnia, as memory booster, nausea, rheumatism, stomach ache, and urinary tract infections had also been reported (Ali and Hassan Gilani, 2007; Rehman et al., 2010; Kumar Gupta and

TABLE 1 | Comparative data on the nutritional composition of ginger rhizome on a dry weight basis from different geographical locations.

Composition (%)	Dhaka, Bangladesh	Hisar, India	Mysore, India	Enugu, Nigeria	Imo, Nigeria	Kaduna, Nigeria	Kano, Nigeria	Kwara, Nigeria	Port hacourt, Nigeria	Faisalabad, Pakistan
Moisture	7.16 ± 0.04	3.70 ± 0.08	15.02 ± 0.04	6.45 ± 0.00	6.32 ± 0.35	4.61 ± 0.40	4.74 ± 0.30	6.37 ± 0.01	6.67 ± 0.01	8.60 ± 0.23
Ash	3.31 ± 0.12	3.50 ± 0.04	3.85 ± 0.61	6.63 ± 0.00	6.57 ± 0.18	5.25 ± 0.20	5.05 ± 0.10	6.30 ± 0.13	6.40 ± 0.15	1.74 ± 0.04
Crude fiber	4.80 ± 0.12	5.4 ± 0.08	ND	0.92 ± 0.18	10.36 ± 0.67	0.60 ± 0.02	0.20 ± 0.05	3.25 ± 0.13	ND	ND
Crude fat	1.39 ± 0.25	0.90 ± 0.02	3.72 ± 0.03	5.71 ± 0.00	6.48 ± 0.38	7.30 ± 0.50	11.15 ± 0. 00	5.35 ± 0.13	5.53 ± 0.15	5.03 ± 0.43
Crude protein	6.32 ± 0.03	5.80 ± 0.09	5.087 ± 0.09	8.83 ± 0.00	5.45 ± 0.46	4.38 ± 0.30	4.92 ± 0.10	8.58 ± 0.01	8.58 ± 0.01	7.88 ± 0.01
Carbohydrate	77.21 ± 0.22	80.3 ± 0.40	38.35 ± 0.1	71.46 ± 0.00	64.40 ± 0.42	77.86 ± 0.3	73.94 ± 0.20	68.15 ± 0.01	ND	76.4 ± 1.30
Reference	Sarker et al. (2021)	Sangwan et al. (2014)	Shirin Adel and Jamuna, (2010)	Ugwoke and Nzekwe, (2010)	Ogbuewu et al. (2014)	Abubakar et al. (2019)	Abubakar et al. (2019)	Otunola et al. (2010)	Nwinuka et al. (2005)	Shahid and Hussain, (2012)

Sharma, 2014; Mele, 2019). Furthermore, ginger possesses life-improving potential and other pharmacological activities such as anticancer, anti-diabetes antioxidant, antimicrobial, anti-neuroinflammation, chemotherapy-induced nausea, and vomiting (Nile and Park, 2015; Zhu et al., 2018; Crichton et al., 2018; Mao et al., 2019).

Recently the demand and use of ginger and its products (gingerbread, ginger cake, ginger coffee, ginger drink, ginger oil, ginger spice, ginger syrup, and ginger wine) in households, pharmaceutical, brewery, food, and other related industries have skyrocketed (Bag, 2018; Sowley and Kankam, 2019).

Results of recent studies summarized in this review have shown that ginger's richness in phytochemicals, nutritional potential, economic benefits of cultivation, and export. These studies uncovered the ameliorative effect of ginger against diabetes, obesity, male infertility, and inflammation. Furthermore, the preclinical studies depicting the mechanisms by which ginger elicits its effects are complemented by recent clinical trials that support the traditional view that ginger has analgesic, anti-diabetic, anti-obesity, pain, nausea and vomiting, male infertility, and anti-inflammatory properties. In addition, *in silico* studies (anti-diabetic, anti-obesity, anti-inflammatory, and SARS-CoV-2) have also been reported. This review comprises scientific data on ginger compiled over the past 10 years. These findings strongly support and affirm the widespread belief that ginger's nutritional and therapeutic properties cannot be downplayed.

NUTRITIONAL AND MINERAL COMPOSITION OF GINGER RHIZOME FROM DIFFERENT GEOGRAPHICAL LOCATION

As is the case with many other spices, ginger is rich in proximate composition and mineral elements that are beneficial to the body. The nutritional compositions of ginger grown in Bangladesh, India, Nigeria, and Pakistan are shown in **Table 1**. with high fluctuations found in the different geographical locations. Indeed, moisture contents varied between 3.7 ± 0.08 and 15.02 ± 0.04%, ash content between 1.74 ± 0.04 and 6.57 ± 0.18%, crude fibers from 0.20 ± 0.05 to 10.36 ± 0.67%, protein contents varied from 4.38 ± 0.3 to 8.58 ± 0.01%, crude fat content ranged from 0.90 ± 0.02 to 11.15 ± 0.00%, and carbohydrates from 38.35 ± 0.1 to 80.3 ± 0.40%.

We observed that ginger grown in Hisar, India exhibited the lowest moisture content (3.7 ± 0.08%) when compared with other studies from different locations(Sangwan et al., 2014). This low moisture level in any food sample is an indicator of its longer shelf life (Unuofin et al., 2017b; Ohikhena et al., 2017). Different reports have shown that low moisture levels of food and agricultural products aid in eliminating the risks of microbial growth and preventing deterioration that may occur during storage (Unuofin et al., 2017b; Ohikhena et al., 2017; Alp and Bulantekin, 2021).

The ash content of ginger gotten from Enugu, Nigeria (Ugwoke and Nzekwe, 2010) had the highest ash content

TABLE 2 | Comparative data on mineral and metal composition of ginger of different geographical location.

	Mineral and metals contents in ginger rhizome of different geographical location. Average concentration (mean ± SD, n = 3, µg/g dry weight basis)													
Parameter	**Dhaka, Bangladesh**	**China**	**Tepi, Ethiopia**	**Bombae, Ethiopia**	**Hadaro, Ethiopia**	**Illubabur, Ethiopia**	**Mysore, India**	**Imo, Nigeria**	**Kwara, Nigeria**	**Kano, Nigeria**	**Kaduna, Nigeria**	**Faisalabad, Pakistan**	**Karachi, Pakistan**	**Multan, Pakistan**
Ca	2080 ± 0.00	2810 ± 0.00	2000 ± 47.00	2540 ± 93.00	2190 ± 24.00	2490 ± 41.00	8840 ± 97.00	34.55 ± 1.39	2800 ± .00	7287.86 ± 0.76	1641.95 ± 0.03	4.9 ± 1.90	ND	ND
Mg	2476 ± 0.00	2762 ± 00.00	2990 ± 9.00	2700 ± 57.00	2760 ± 11.00	4090 ± 105.00	ND	ND	ND	2030.80 ± 1.78	1258.06 ± 0.02	12 ± 14.3	ND	ND
Na	440.19 ± 0.8	474.13 ± 0.6	ND	ND	ND	ND	ND	38.96 ± 3.58	ND	625.00 ± 0.17	333.35 ± 0.76	ND	ND	ND
K	27860 ± 12600	16970 ± 8600	ND	ND	ND	ND	ND	36.34 ± 1.93	ND	429.00 ± 0.76	666.50 ± 0.43	ND	ND	ND
P	ND	ND	ND	ND	ND	ND	17400 ± 120.0	26.70 ± 1.59	8068.0 ± 0.00	3.68 ± 0.02	2.64 ± 0.01	ND	ND	ND
Cu	ND	ND	4.78 ± 0.34	1.86 ± 0.18	2.53 ± 0.19	1.10 ± 0.05	5.45 ± 0.02	0.86 ± 0.01	88.0 ± 0.00	ND	ND	125.0 ± 2.90	1.7 ± 0.01	49.2 ± 2.70
Zn	ND	ND	55.2 ± 3.90	39.6 ± 0.50	38.5 ± 0.50	54.0 ± 2.70	92 ± 0.00	4.19 ± 0.06	640.0 ± 0.00	4.86 ± 0.02	0.99 ± 0.01	122.3 ± 1.60	3.4 ± 0.02	19.7 ± 1.90
Mn	ND	ND	385.00 ± 9.00	285.00 ± 4.30	184.00 ± 3.60	401.00 ± 12.00	913.00 ± 1.00	18.90 ± 1.60	59.0 ± 0.00	20.97 ± 0.07	45.78 ± 0.06	73.30 ± 2.2	1.6 ± 0.01	1014 ± 52.00
Ni	0.558 ± 0.00	ND	5.61 ± 0.44	5.46 ± 0.48	6.78 ± 0.53	8.40 ± 0.32	ND	ND	ND	ND	ND	ND	ND	ND
Fe	195.55 ± 0.00	215.04 ± 0.00	44.2 ± 3.30	55.4 ± 5.00	41.8 ± 2.8	89.0 ± 6.10	800.00 ± 20.0	1.59 ± 0.08	279.74 ± 0.00	39.13 ± 0.05	7.61 ± 0.03	800.00 ± 28.90	13.30 ± 0.01	2475.00 ± 1110.00
Co	0.023 ± 0.00	0.043 ± 0.00	7.58 ± 0.46	5.68 ± 0.40	2.04 ± 0.14	2.18 ± 0.18	ND	ND	ND	ND	ND	ND	ND	ND
Cr	ND	ND	9.28 ± 0.61	6.02 ± 0.14	9.17 ± 0.62	10.80 ± 0.20	7.00 ± 0.00	ND	ND	ND	ND	ND	0.50 ± 0.01	ND
Cd	0.014 ± 0.00	0.042 ± 0.00	0.97 ± 0.08	0.38 ± 0.02	0.38 ± 0.02	0.70 ± 0.07	ND	ND	ND	ND	ND	ND	ND	ND
Reference	Nandi et al. (2013)	Nandi et al. (2013)	Wagesho and Chandravanshi, (2015)	Wagesho and Chandravanshi, (2015)	Wagesho and Chandravanshi, (2015)	Wagesho and Chandravanshi, (2015)	Shirin Adel and Jamuna, (2010)	Ogbuewu et al. (2014)	Latona et al. (2012)	Abubakar et al. (2019)	Abubakar et al. (2019)	Shahid and Hussain, (2012)	Hashmi et al. (2007)	Anita et al. (2004)

(6.63 ± 0.00%) when compared to other locations as shown in **Table 1**. It has been revealed that plant materials with high ash content also possess rich dietary fibres content which provides shelter for digestive organisms in the alimentary tract (Schroeder and Bäckhed, 2016; Jimoh et al., 2020). Furthermore, a high level of ash in food and plant material is an indicator of its richness in mineral nutrients (Unuofin et al., 2017a; Ohikhena et al., 2017).

Literature is replete with information regarding the essentiality of fibre in food substances (Unuofin et al., 2017b; Adegbaju et al., 2019; Idris et al., 2019; Abifarin et al., 2021). Our findings revealed that ginger grown in Imo state, Nigeria had the highest crude fibre content (10.36 ± 0.67%) when compared with other geographical locations studied as shown in **Table 1**. It has been nutritionally established that relatively high dietary fibre composition in food samples is pivotal for proper peristaltic action and aids the absorption of trace elements in the gut and reduces cholesterol absorption (Adebowale et al., 2013; Unuofin et al., 2017a; Ohikhena et al., 2017).

The value of the crude fat of ginger rhizome powder from the different geographical locations ranged from 0.90 ± 0.02 to 11.15 ± 0.00%. It has been suggested that 1–2% of caloric energy from fat is best for healthy living (Antia et al., 2006; Unuofin et al., 2017b). In this regard, the low crude fat content of ginger grown in Hispar, India and Dhaka, Bangladesh (0.90 ± 0.02 and 1.39 ± 0.25%) respectively are within this set caloric energy range when compared with other locations. The onset of several cardiovascular diseases, cancer, and aging have been attributed to excess intake of dietary fat.

On the other hand, the protein contents of ginger grown in Kwara and River States, Nigeria had the highest contents (8.58 ± 0.01%) when compared with other geographical locations as shown in **Table 1**. Dietary proteins are essential in the human body because they aid in the manufacturing and safeguarding of certain organic materials necessary for their smooth functioning (Hayat et al., 2014).

Carbohydrate had the highest nutritional composition in the ginger derived from all the different locations. However, ginger obtained from Hisar, India (80.3 ± 0.40%) has the highest composition when compared with other geographical locations as revealed in **Table 1**. The high carbohydrate content makes ginger a rich source of energy and this could be used to enrich the energy content of diets. Ginger is rich in phenolic compounds, i.e., gingerol and shogaol, which have been shown to have molecular structures capable of influencing the thermic effect of food (TEF) (Iwasaki et al., 2006; Fagundes et al., 2021). However, the thermogenic potential of ginger is controversial (Mansour et al., 2012; Gregersen et al., 2013; Fagundes et al., 2021).

Minerals are essential components of the human diet. They support life in the provision of vital nutrients needed for the psychophysical well-being of the body (Unuofin et al., 2017b; Jiménez-Aguilar and Grusak, 2017; Jimoh et al., 2020).

Eleven essential minerals and two heavy metals derived from powdered rhizome samples procured from different geographical locations are compared with reported data (**Table 2**). The ginger rhizome from the different understudied locations contained beneficial nutrients viz. calcium, potassium, phosphorous, and magnesium in higher concentrations with appreciable amounts of copper, cobalt, iron, nickel, manganese, sodium, and zinc. Calcium, potassium, phosphorous, and magnesium play a crucial role in the bone skeleton, biochemical reactions, and energy metabolism (Haddy et al., 2006; Unuofin et al., 2017b; Srivastava et al., 2019). Furthermore, the data gathered from the different studies revealed that ginger rhizome possesses therapeutic action against growth disorders and anemia due to the availability of iron, manganese, and other mineral antioxidants, e.g., zinc, cobalt, and nickel. The variability in the mineral content of ginger rhizome between different countries, e.g., Bangladesh, China, Ethiopia, India, Nigeria, and Pakistan also within the same country can be observed (**Table 2**). The variation observed in the nutritional and mineral contents in ginger rhizome could be attributed to the variety used for the study and environmental factors such as climatic conditions, geographical location, genetic and environmental (G×E) interactions like any other plant, soil type, Sun exposure, grazing stress, seasonal changes etc (Hussain et al., 2009; Liu et al., 2016; Srivastava et al., 2019; Unuofin and Lebelo, 2021).

The ginger rhizome for the different geographical origins was safe with no critical load of harmful heavy metals. The concentration of metal in plants is largely dependent on its geochemical environment. We observed that there were sufficient essential micronutrients such as Co, Cu, Fe, Mg, Mn, Ni, and Zn (**Table 2**).

According to Zeiner and Cindrić (2017), it is important to monitor the levels of the heavy metal in medicinal plants and spices used as a nutraceutical/functional food for health-promoting benefits. The levels of heavy metals in medicinal food plants could be increased during postharvest and storage conditions (Srivastava et al., 2019).

THE PHYTOCHEMISTRY OF GINGER

Over 400 bioactive components have been found in ginger (Mele, 2019). These chemical constituents have been grouped into different active chemical constituents such as diarylheptanoids, gingerol analogues, phenylalkanoids, sulfonates, monoterpenoid glycosides, steroids, and terpene compounds (Prasad and Tyagi, 2015; Zhang et al., 2021).

Diarylheptanoids

Recently, diarylheptanoids with a class term of 1,7-diarylheptane skeleton have gathered an increasing interest (Zhang et al., 2021). Literature is replete with a total of 41 diarylheptanoids compounds derived from ginger e.g. Bisgingerdiones B, Dihydrocurcumin, Hexahydrocurcumin, 6-gingeroldiacetate, and 8-isodehydrogingerdion (**Figure 1**) (Ma et al., 2004; Jiang et al., 2007; Feng et al., 2011; Hong and Oh, 2012; Olennikov and Kashchenko, 2015). These metabolites possess chemopreventive, anti-hepatotoxic, anti-inflammatory, antioxidant, and anti-tumor (Zhang et al., 2021).

Gingerol Analogues

Several gingerol analogues such as gingerols, paradols, shogaols, and zingerone possess biting and hot sensations

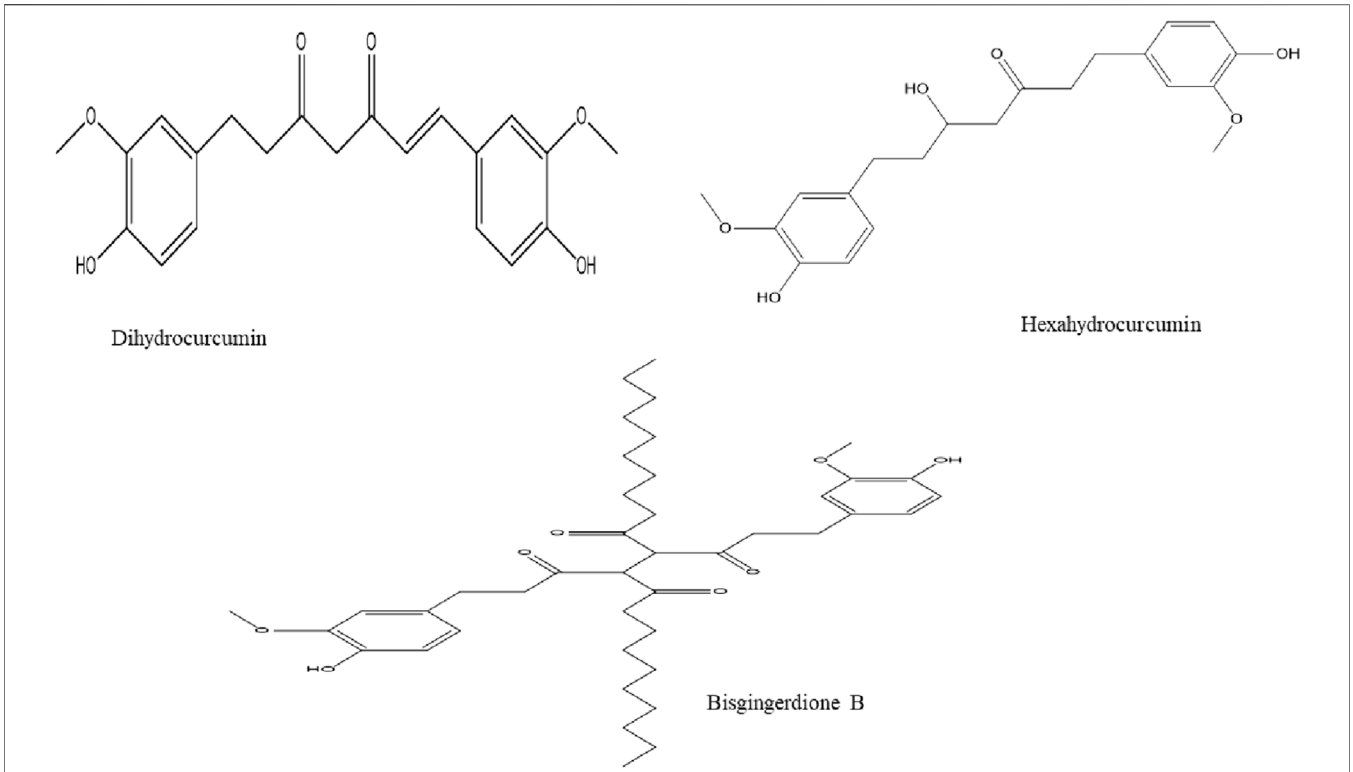


FIGURE 1 | Chemical structures of diarylheptanoids compound isolated from ginger.

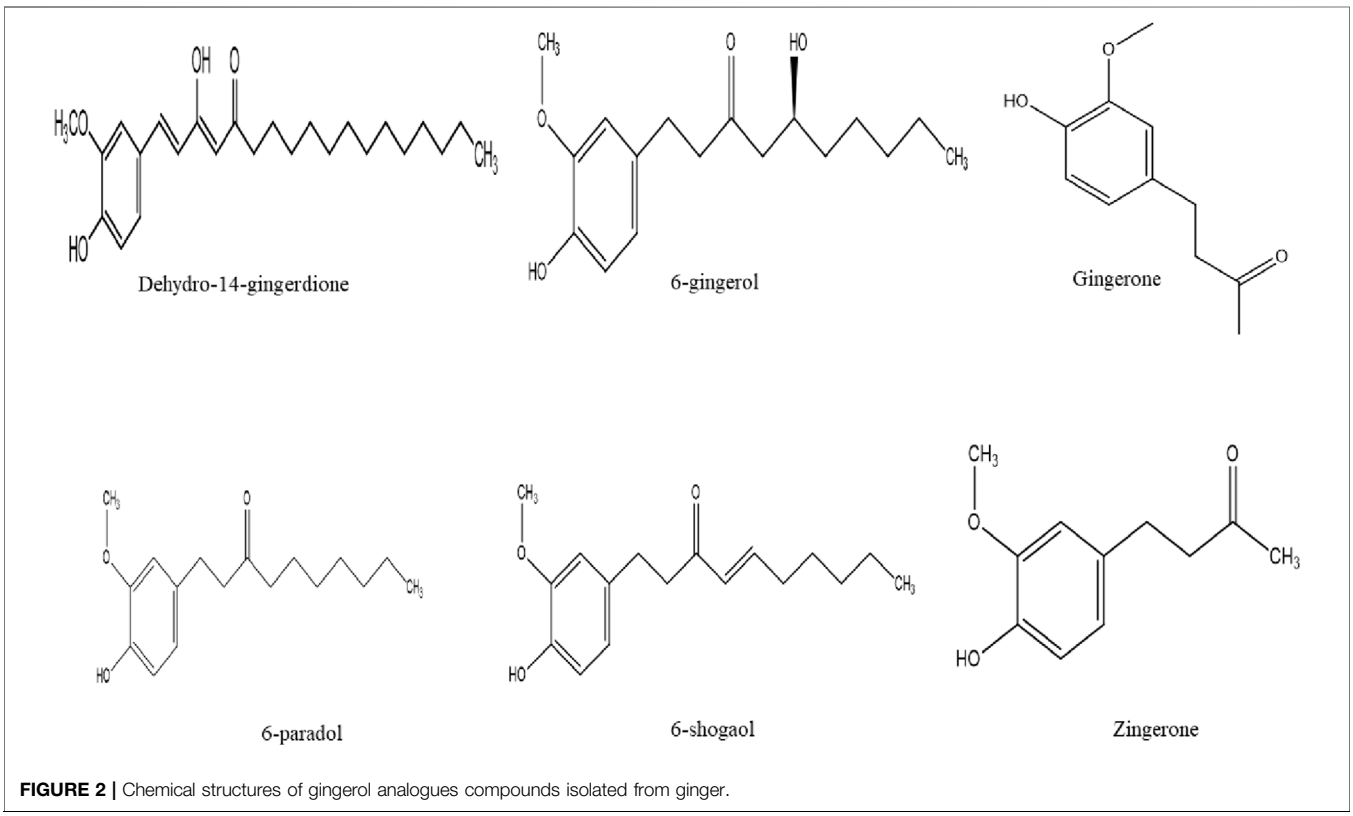


FIGURE 2 | Chemical structures of gingerol analogues compounds isolated from ginger.

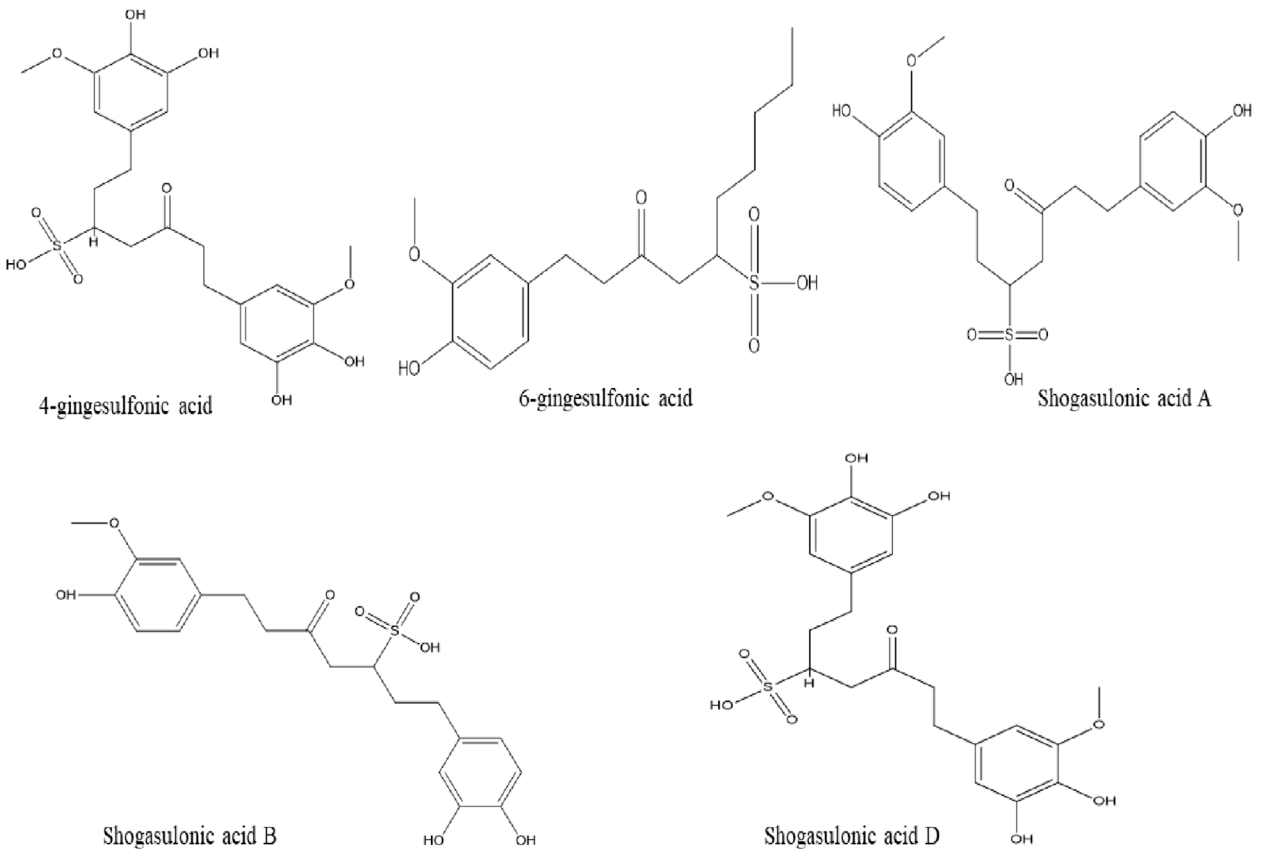


FIGURE 3 | Chemical structures of phenylalkanoids and sulfonates compound isolated from ginger.

in the mouth and they also exhibit pharmacological effects (**Figure 2**) (Kubra and Rao, 2012). Gingerols (6-gingerol, 8-gingerol, and 10-gingerol) are the most abundant polyphenol in fresh ginger. Upon dehydration or long-time storage, it can be converted into shogaols which is twice hotter than gingerols. The hydrogenation of shogaols produces paradols (Stoner, 2013). According to Zhang et al. (2021) fresh ginger is not as pungent as dried ginger. In recent years, seventy gingerols analogues compounds have been isolated from ginger such as (5S)-6-gingerol, (5S)-methyl-8-gingerol, (5S)-5-methoxy-6-gingerol, (3R,5S)-6-gingerdiol, dehydro-14-gingerdione, just to mention a few (Jiang et al., 2005; Feng et al., 2011; Lee et al., 2011; Pan et al., 2019).

Phenylalkanoids and Sulfonates

Six phenylalkanoids compounds (3-hydroxy-1-(4′-hydroxy-3′-methoxy-phenyl)-hexan-5-one, 3-hydroxy-1-(3′,5′-dimethoxy-4′-hydroxyphenyl)-hexan-5-one, 5-hydroxy-1-(4′,5′-dihydroxy-3′-methoxyphenyl) dodecan-3-one, 1-(4′,5′-dihydroxy-3′-methoxyphenyl)dodec-4-en-3-one, (*E*)-3-hydroxy-1-(4′-dihydroxy-3′,5′-dimethoxy-phenyl)-dodecan-6-en-5-one and (E)-3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-tetradecan-6-en-5-one) have been reported to present in ginger rhizome (Chen et al., 2011; Chen and Yeh, 2011; Kuo et al., 2012; Li et al., 2013; Wang et al., 2018).

Additionally, six sulfonates compounds have been isolated from ginger, they are 4-gingesulfonic acid, 6-gingesulfonic acid, and shogasulfonic acids A-D (**Figure 3**) (Hori et al., 2003).

Steroids and Monoterpenoid Glycosides

A total of six steroids compounds including β-sitosterol, daucosterol, stigmast-4-en-3,6-dione, 6β-hydroxystigmast-4-en-3-one, stigmast-4-en-3-one, and stigmasterol have been reported in ginger (Feng et al., 2011). Furthermore, Guo et al. (2018) isolated six monoterpenoid glycosides (Trans-3-hydroxy-1,8-cineole 3,6-dihydroxy 3-O-β-D-glucopyranoside, Trans-3-hydroxy-1,8-cineole3-O-β-D-glucopyranoside,5,9-dihydroxy borneol2-O-β-D-glucopyranoside, Angelicoidenol 2-O-β-D-glucopyranoside, Vicodiol 2-O-β-D-glucopyranoside, and Zingiberoside C) from the fresh rhizome of tongling white ginger (**Figure 4**).

Terpenes

Several components of terpenes such as monoterpenes and sesquiterpenes are known to be volatile fractions (Butt and Sultan, 2011). The savory component of ginger is attributed to the presence of sesquiterpenes, while monoterpenes are the most abundant terpenes in fresh ginger oil (Dhanik et al., 2017; Balogun et al., 2019). Furthermore, diverse components present in ginger essential oils are responsible for

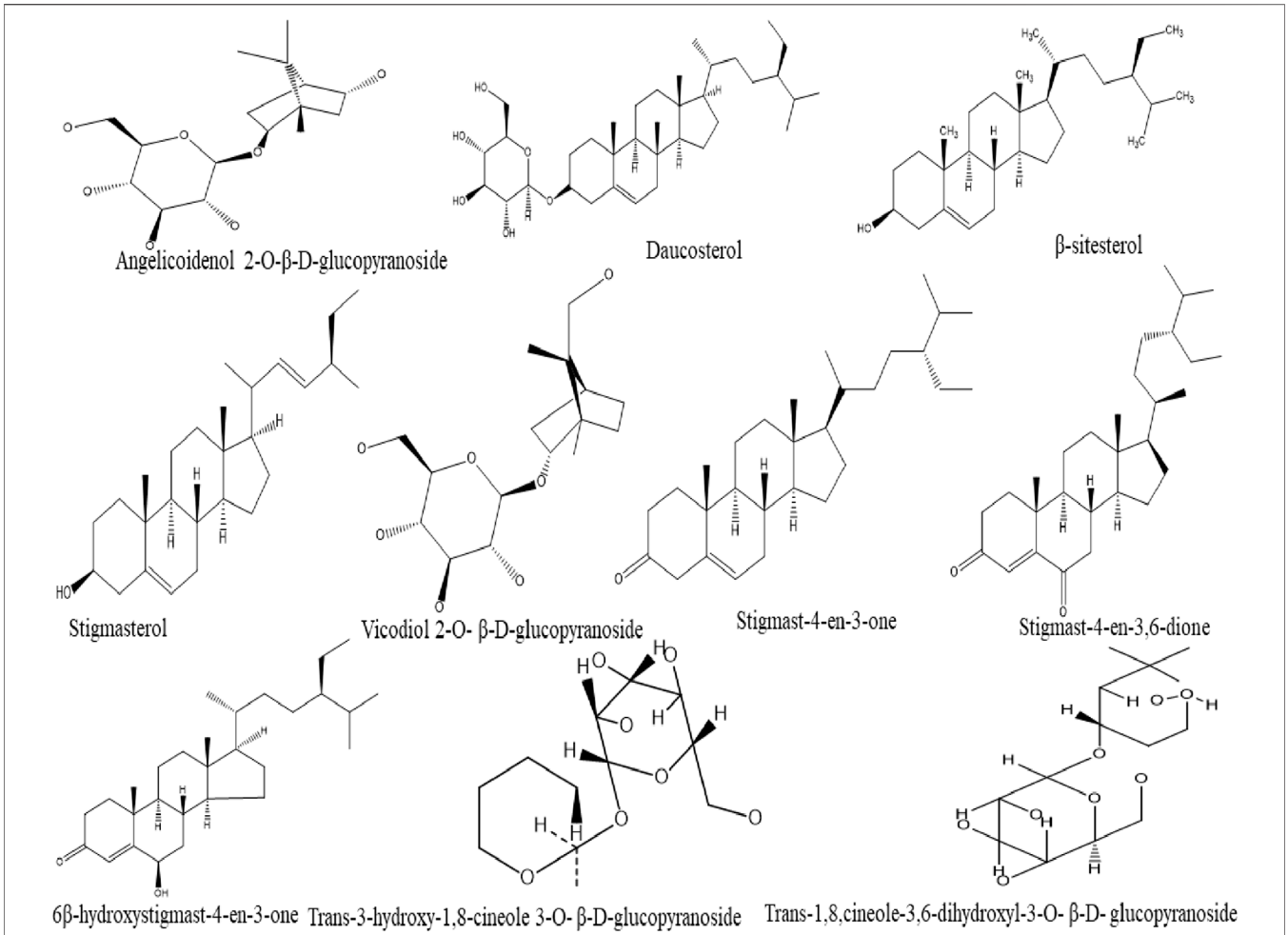


FIGURE 4 | Chemical structures of steroids and monoterpenoid glycosides compounds isolated from ginger.

its aromatic scent, these are such as β-bisabolene, α-curcumene, α-farnesene, β-sesquiphellandrene, and zingiberene (**Figure 5**) (Prasad and Tyagi, 2015).

PHARMACOLOGICAL POTENTIAL OF GINGER AND ITS ANALOGUES

A large number of bioactive constituents in ginger possess pharmacological activities and they have been comprehensively investigated. These include protective effects against male infertility, nausea and vomiting, analgesic, anti-diabetic, anti-inflammatory, anti-obesity, and other effects. The pharmacological effects of ginger analogous *in vitro* and *in vivo* studies on cells and experimental animals are summarized in **Table 3**.

ANTI-DIABETES ACTIVITIES

Ginger has been reported to possess anti-diabetic properties. The aqueous extracts of ginger rhizomes (5, 10, 20, and 40 g/L) were examined on protein glycation and the diffusion of glucose. The results showed that the extract can mitigate diabetes via inhibition of glucose diffusion and by causing a reduction in glycation (Sattar et al., 2012).

A recent study by Ademosun et al. (2021) evaluated the antioxidant properties, glycemic indices, and carbohydrate hydrolysing enzymes activities of ginger-based fruit drinks prepared by mixing ginger (G), pineapple (P), and apple (A) (G50:P40:A10, G50:P30:A20, G50:P20:A30, G50:P10:A40, and G100). The *in vitro* antioxidant activities were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) radicals, as well as ferric reducing antioxidant power (FRAP) assays. Results showed that G50:P10:A40 had stronger antioxidant properties against DPPH and ABTS radicals and FRAP compared to other ginger-based drinks and commercial ginger drinks. Also, G50:P10:A40 blend displayed the highest phenolic content and strongest inhibition effect on carbohydrate hydrolyzing enzymes. However, all drinks had low glycemic indices. The study has been suggested that consumption of G50:P10:A40

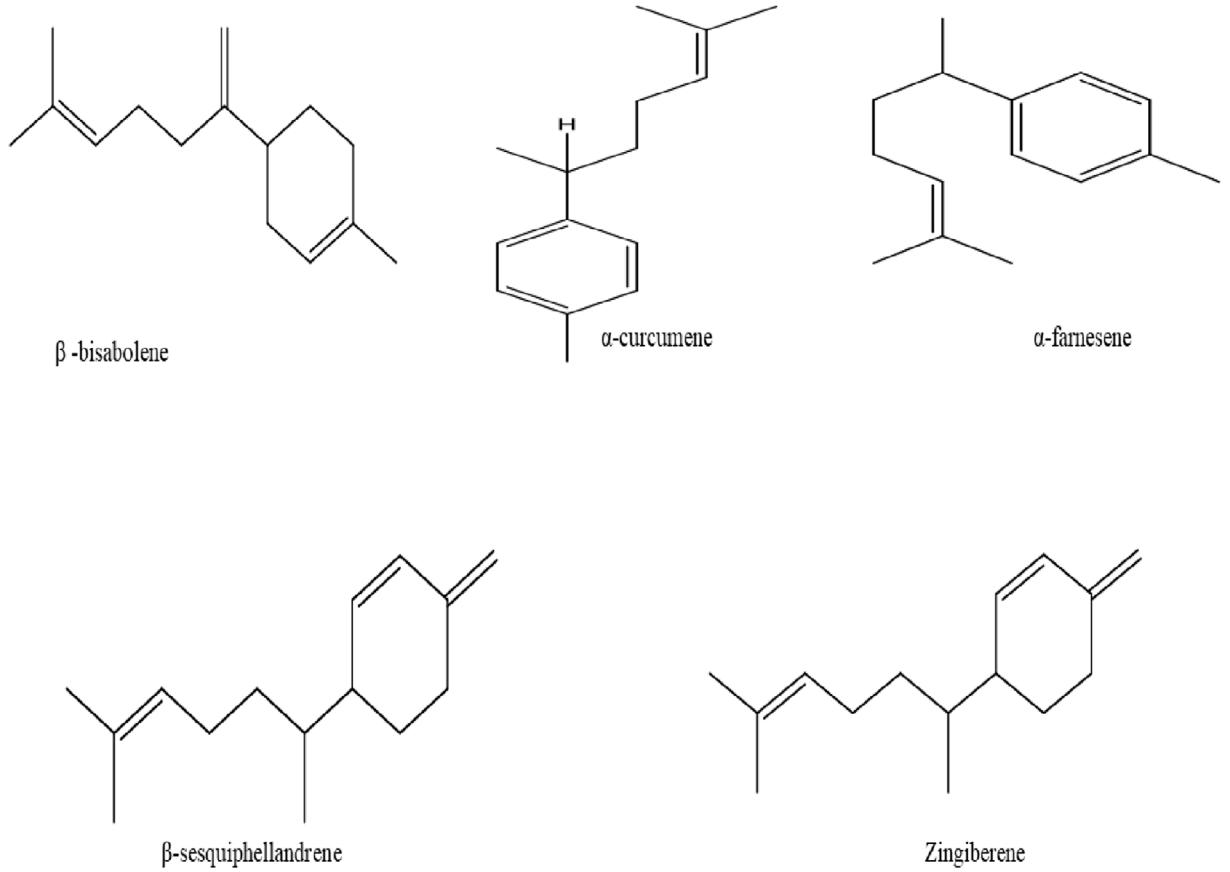


FIGURE 5 | Chemical structure of β-bisabolene, α-curcumene, α-farnesene, β-sesquiphellandrene, and zingiberene isolated from ginger.

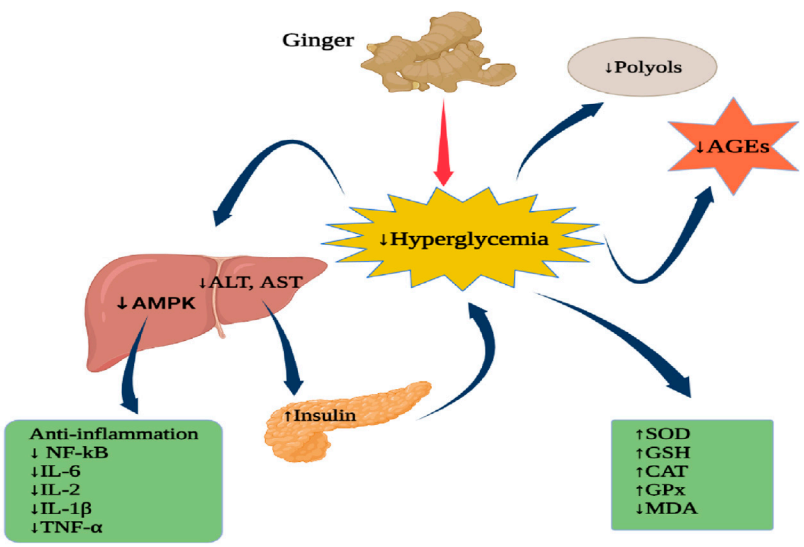


FIGURE 6 | Anti-diabetic actions of ginger. AMPK, Mitogen-activated protein kinase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; AGEs, Advanced glycation end-products; NF-kB, nuclear factor kappa B; IL-6, Interleukin-6; IL-2, Interleukin-2; IL-1β, Interleukin-1 beta; TNF-α, Tumour necrosis factor-alpha; SOD, Superoxide dismutase; GSH, glutathione; CAT, Catalase; GPx, Glutathione peroxidase, MDA, Malondialdehyde. Figure was created using BioRender.com by the authors.

TABLE 3 | Pharmacological effects of ginger analogues.

Activity	Ginger phytocompounds	Dosage used	Mode of action	Experimental Models	References
Anti-emetic	Gingerol	50, 100, and 200 mg/kg, i.g	Inhibits vomiting by attenuating 5-hydroxytryptamine (5-HT) and dopamine (DA) levels in the area postrema and ileum Suppresses Substance P (SP) in the mucosa and submucosa of ileum, and neurons of area postrema	Cisplatin-induced emesis in adult castrated male minks	Qian et al. (2009)
Anti-emetic	Gingerol	50, 100, 200 mg/kg, i.g	Stops vomiting through the inhibition of NK1 receptor in the muscular and submucosa of ileum and the neurons of area postrema, and Substance P receptor in the mucosa and submucosa of the ileum and neurons of the area postrema	Cisplatin-induced emesis in adult castrated male minks	Qian et al. (2010)
Chemotherapy-induced nausea and vomiting (CINV)	Gingerol	20, and 200 mg/kg body weight	Ameliorates gastric emptying through the inhibition of dopamine D2 receptor (D2R) and tyrosine hydroxylase (TH) expression levels and increasing dopamine transporter (DAT)	Cisplatin-induced nausea and vomiting in male Wistar rats	Qian et al. (2016)
Chemotherapy-induced nausea and vomiting (CINV)	Gingerol	10 mg/kg, 20, and 40 mg/kg, i.g in rats 50, 100, 200 mg/kg, i.g in minks	Alleviates chemotherapy-induced nausea and vomiting by reducing the levels of 5-TH, 5-HT3 receptor, TPH, SP, NK1 receptor, PPT, DA, D2R, TH, and boosts accumulation of SERT, NEP, and DAT in the area postrema and ileum	Cisplatin-induced acute and delayed emesis in rats and minks	Tian et al. (2020)
Anti-emetic	6-Gingerol	50 and 100 mg/kg	Alleviates vomiting by attenuating 5-hydroxytryptamine (serotonin, 5-HT) concentration through the modulation of tryptophan hydroxylase (TPH), monoamine oxidase A (MAO-A), serotonin reuptake transporter (SERT), and 5-HT3 receptor	Chemotherapy-induced emesis in rats	Cheng et al. (2020)
Anti-obesity	6-Gingerol	50 μmol/L	Suppresses adipogenesis by decreasing the expression of PPARγ, C/EBP, mRNA and adipocyte-specific fatty acid binding protein 4 and fatty acid synthase	Rosiglitazone (RGZ)-induced adipogenesis in 3T3-L1 cells	Tzeng et al. (2014)
Anti-obesity	6-Gingerol	6.25, 12.5, and 25 μM	1) Attenuates adipogenesis by suppressing the markers PPARγ, C/EBPα, and adipocyte protein 2, and triglyceride synthesis enzymes, such as sterol regulatory element- binding protein-1, fatty acid synthase, lysophosphatidic acid acyltransferase, and acyl-coA: diacylglycerol acyltransferase 2) Reduction in the expression of the proinflammatory cytokines such as TNFα, IL-1β, and IL-6, elevation of cytokine interleukin-10, and inhibition of c-JUN N-terminal kinase (JNK) and I kappa B kinase (IKK) 3) Inhibits the induction on nitric oxide synthase (NOS)	3T3-L1 cells with RAW 264.7 macrophages	Choi et al. (2017)
	Gingerol	25, 50 and 75 mg/kg	Cutting down the levels of blood glucose, leptin, insulin, amylase, lipase, and lipids; improve body weight when compared with control group	HFD-induced obese rats	Saravanan et al. (2014)
Anti-obesity	6-Gingerol and 6-Shogaol	0–80 μM	1) Inhibits adipogenesis, induces up-regulation of the brown fat-specific genes expression such as UCP1, PGC1α, PRDM16, Fgf21, Tmem26 and Cidea	Pre-adipocyte cell line (3T3-L1) and Inguinal fat-derived stromal vascular fraction (SVF) cells	Sampath et al. (2021)

(Continued on following page)

TABLE 3 | (*Continued*) Pharmacological effects of ginger analogues.

Activity	Ginger phytocompounds	Dosage used	Mode of action	Experimental Models	References
			2) Upregulation of mitochondrial biogenesis 3) Better binding affinity to the β3-adrenergic receptor membrane protein (β3-AR)		
Anti-obesity	6-shogaol	40 μM	Anti-adipogenic activity by lowering the expression of the PPARγ, C/EBPα, and fatty acid synthase (FAS)	MID-induced adipogenesis in 3T3-L1 Preadipocytes	Suk et al. (2016)
Asthma	Zingerone	5, 20, and 50 μM	1) Increases SOD activity and reduces the MDA levels 2) Suppresses NF-κB activation, reduces mRNA expression of TNF-α and IL-1β 3) Inhibit the expression of p65 (nucleus) and p-IκB 4) Upregulation of p-AMPK, Nrf2, and HO-1 expression	MLE12 cells stimulated with hydrogen peroxide (H_2O_2)	Zhu et al. (2021)
Anti-diabetes	6-Gingerol	25 μM	Reduction in level of glucose; enhances cell viability; and inhibition of polyol pathway via decreasing aldose reductase enzyme activity	High glucose-induced human retinal pigment epithelial (HRPE) cells toxicity	Sampath et al. (2016)
Anti-diabetes	6-Gingerol	75 mg/kg	Reduction in the levels of plasma glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), advanced glycation end-products (AGEs), and insulin levels Decreasing levels of AGEs and N(ε)-(carboxymethyl)lysine (CML) levels through the Nrf2 pathway, increasing GSH/GSSG ratio, heme oxygenase-1 and glyoxalase 1 in liver tissue	High-fat diet (HFD)-induced high blood glucose in C57BL/6 mice	Sampath et al. (2017)
Diabetic nephropathy	Gingerol	12.5, 25, 50, and 100 mg/kg (*in vivo*) 1–50 μM (*in vitro*)	Cutting down the levels of blood glucose, creatinine, and blood urea nitrogen (BUN) Upsurge in the levels of SOD, GSH, GSH/GSSG ratio, GPx, and CAT. Suppresses activation of NF-κB, renal p38 mitogen-activated protein kinase (p38MAPK) and transforming growth factor-beta (TGF-β); and down-regulation of IL-6, TNF-α, and IL-1β release	Hf D/STDZ-induced type 2 diabetes in a rat and Normal renal proximal tubular epithelial (NRK 52E) cells treated with high glucose	Song et al. (2019)
Anti-diabetes	Zingerone	50 and 100 mg/kg body weight	Increases the levels of GSH, SOD, CAT, GPX, and reduces lipid peroxidation Also, decreases the level of NF-kB levels, and down-regulated inflammatory cytokines such as IL1-β, IL-2, IL-6, and TNF-α	Alloxan-induced diabetic rats	Ahmad et al. (2018)
Anti-inflammatory	6-shogaol	20, 30 μM	Inhibition of NF-κB activation, and COX-2 expression by TLR4 pathway Inhibition of NF-κB activation by MyD88 or IKKβ pathway, and degradation of IRAK-1	RAW 264.7 cells (a murine monocytic cell line) and 293T human embryonic kidney cells treated lipopolysaccharide (LPS)	Ahn et al. (2009)
Anti-inflammatory	6-gingerol	50 mg/kg body weight	1) Elevation of hepatic glutathione (GSH), superoxide dismutase (SOD), and glutathione-S-transferase (GST) enzymes, reduction in MDA levels 2) Restores serum AST, ALT, and ALP, and markedly increases serum total proteins	Diethylenetriamine-induced liver injury in rats	Alsahli et al. (2021)

(Continued on following page)

TABLE 3 | (*Continued*) Pharmacological effects of ginger analogues.

Activity	Ginger phytocompounds	Dosage used	Mode of action	Experimental Models	References
			3) Diminishes the expression of inflammatory indicators TNF-α, IL-6, ICAM1, and CRP		
Anti-inflammatory	6-gingerol	0–128 μM	Ameliorates sepsis through the inhibition of pyroptosis and caspase-1p20 release, HMGB1, mature IL-1β, IL-18 by suppressing AMPK activation	ATP and LPS treated RAW264.7 cell line and bone marrow-derived macrophages (BMDMs)	Zhang et al. (2020)
Anti-inflammatory	6-gingerol	2.5, 50, and 100 μM	Suppresses T lymphocyte proliferation through inhibition of DNA synthesis and interferon-γ synthesis, expression of CD25 and CD69 activation markers, cytokine synthesis, and interleukin (IL)-2 receptor signaling	IL-2-dependent mouse CTLL-2 $CD8^+$ T lymphocytes	Bernard et al. (2015)
	1-dehydro-10-gingerdione	30 μM	Suppresses NF-kB activation through the inhibition of IkBα phosphorylation by IKKβ	LPS-activated RAW 264.7 macrophages	Lee et al. (2012)
	1-dehydro-10-gingerdione	1–30 μM	1) Attenuates TLR4-mediated expression of NF-kB 2) Down-regulation of activator protein 1 (AP-1) target genes, TNF-a and IL-1β, interferon (IFN) regulatory factor 3 (IRF3) target IFN-β gene and IFN-γ inducible protein 10 (IP-10)	LPS-stimulated RAW 264.7 macrophages	Park et al. (2012)
	1-dehydro-10-gingerdione	50, 100, 150, and 200 ng/m	Hinders the production of NO, IL-6, and PGE_2 via modulating iNOS and COX-2 mRNA expression	LPS-stimulated Raw 264.7 cells	(Han et al., 2013)(Han et al., 2013)
	10-Dehydogingerdione	10 mg/kg	Reduces LDL cholesterol and elevates HDL-cholesterol by suppressing cholesteryl ester transfer protein (CETP) Also, decreases cardiovascular risks such as high sensitivity C-reactive protein (hsCRP), oxidized LDL (Ox-LDL), matrix metalloproteinase 9 (MMP9), homocysteine, lipoprotein a (Lp(a))	New Zealand male rabbits fed an atherogenic or high cholesterol diet	Elseweidy et al. (2013)
	10-Dehydogingerdione	10 mg/kg	Improvement of nuclear factor kappa (NF-kB), insulin-like growth factor I (IGF-I), fibroblast growth factor-23 (FGF-23) of the kidney Reduces MDA and increases GSH of the kidney	Cisplatin-induced nephrotoxicity and renal fibrosis in male Wistar albino rats	Elseweidy et al. (2016)
Anti-neuroinflammatory	12-Dehydogingerdione	2.5, 5, 10 μM	Reduces the production of NO and PGE_2, and the expression of iNOS, COX-2, and mRNA expression of IL-6 Ameliorates neuro-inflammation by suppressing the Akt/IKK/NF-κB pathway Promotes the production of NO and TNF-α through the activation of NF-E2-related factor (Nrf)-2 and heme oxygenase (Nrf-2/HO-1) pathway	LPS-activated microglial cells and BV-2 cells	Zhao et al. (2019)
Anti-inflammatory	Zingerone	25 and 50 mg/kg body weight	1) Attenuates levels of TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), COX-2, p53, cysteine aspartate specific protease-3 (caspase-3), cysteine aspartate specific protease-8 (caspase-8), cytochrome *c*, Bcl-2 associated X protein (Bax), and B-cell lymphoma-2 (Bcl-2) 2) Boosting activities of SOD, GPX, and CAT.	Vancomycin-induced hepatotoxicity in rats	Kucukler et al. (2020)

(Continued on following page)

TABLE 3 | (*Continued*) Pharmacological effects of ginger analogues.

Activity	Ginger phytocompounds	Dosage used	Mode of action	Experimental Models	References
			3) Alleviation of hepatic aspartate aminotransferase, alkaline phosphatase, and alanine aminotransferase		
	Zingerone	50 and 100 mg/kg, p.o	Attenuates accumulation of collagen bundles, TNF-α, and IL-1β levels, MDA level, TGF-β1, and iNOS expression and enhances SOD and GPx activities	Bleomycin-induced pulmonary fibrosis in Wistar-albino rats	Gungor et al. (2020)
	Zingerone	10, 20, and 40 mg/kg	Increases the levels of SOD, GPx, and GSH, and decreases MDA, NO, COX-2, PGE2, TNF-α, and IL-1β	Carrageenan-induced Inflammation in rats	Mehrzadi et al. (2021)
	Zingerone	Orally 25 mg/kg body weight	Improve activities of SOD, catalase and GPx, in the hepatic and joint tissues Also, reducing levels of NF-κB, TGF-β, TNF-α, IL-1β, IL-6, and Hs-CRP, and induces a significant increase in IL-10 levels	Freund's adjuvant (FCA) immunized arthritic Wistar rats	Bashir et al. (2021)
Nephroprotective	Zingerone	An oral dose of 25 mg/kg body weight	Reduction in levels of malondialdehyde (MDA), nitric oxide (NO) and 8-hydroxy-2-deoxyguanosine in the renal Elevation of nuclear factor erythroid 2-related factor 2 mRNA expressions, CAT, SOD, and GSH levels Reduction in the renal levels of NF-κB, TNF-α, IL-1β, and myeloperoxidase activity, thus bringing about anti-inflammation	Adriamycin (doxorubicin)-mediated nephrotoxicity in Swiss albino male mice	Elshopakey et al. (2021)
Anti-inflammatory	Zingerone	50 mg/kg body weight	1) Reduces the level of MDA, and increases the levels of GSH and CAT, SOD activities 2) Decreases the level of IL-4, IL-5, IL-13, and increases IFN-γ 3) Suppresses the expression of the p-IκB and p65 4) Activates the expression of AMPK, Nrf2 (nucleus), and HO-1	Ovalbumin-induced asthmatic mice	Zhu et al. (2021)
Neuroprotective	6-shogaol and 6-paradol	Oral 5 mg/kg/day, p.o	Diminishing the expression level of TNFα Reducing cell accumulation in the white matter of the spinal cord; and also inhibits astrogliosis and microglial activation in the central nervous system	Experimental autoimmune encephalomyelitis (EAE) C57BL/6 mice	Sapkota et al. (2019)
Gastroprotective	Zingerone	50, 100, and 200 mg/kg, oral	Lowers the level of MDA and restores the NO level	Ethanol-induced gastric ulcers in rat	Karampour et al. (2019)
Anti-inflamma- tory	Zingerone	10, 50, and 100 nM	Stimulates the expression of markers α smooth muscle actin (α-SMA) and smooth muscle 22α (SM22α)), upregulation of AMPK phosphorylation and TIMP4 expression, and reduces the expression of core-binding factor α-1 (CBFA1)	Pi-induced vascular calcification	Lim et al. (2021)
Anti-melanogenesis	8-gingerol	5–100 μM	Suppresses melanogenesis via down-regulation of mitogen-activated protein kinases (MAPK) and protein kinase A (PKA) signaling pathways Also, reduces microphthalmia-associated transcription factor (MITF) expression and inhibits tyrosinase activity	B16F10 cells and B16F1 cells (melatonin cells)	Huang et al. (2013)

drinks could be useful in the mitigation of high blood glucose and also the prevention of diabetes mellitus (Ademosun et al., 2021).

Fajrin et al. (2020a) explored the mode of action of ginger extract and 6- shogaol on pancreatic islets and expressions of transient receptor potential vanilloid-1 (TRPV1) and N-methyl-D-aspartate receptor subunit 2B (NMDAR2B) in the spinal cord of streptozotocin (STZ)-induced mice model of Painful Diabetic Neuropathy (PDN). In this study, oral administration of ginger extracts (400 mg/kg body weight), 6-shogaol (15 mg/kg body weight), or gabapentin once daily for 49 days in mice induced a marked decrease of TRPV1 and NMDAR2B expressions in the spinal cord as compared to controls. Also, results revealed that there were no significant differences in the total volume of pancreatic islets and insulin expression between PDN groups. Therefore, the study concluded that ginger extracts and its 6-shogaol compound alleviated pain in PDN by downregulation of TRPV1 and NMDAR2B expressions in the spinal cord, with minor changes on pancreatic islets (Fajrin et al., 2020a).

Clinical Studies

A double-blind clinical trial study by Javid et al. (2019) evaluated the effects of ginger supplementation on inflammatory, antioxidant, and periodontal parameters in 46 patients with type 2 diabetes mellitus (T2DM) and chronic periodontitis (CP). Treatment with 4 tablets of 500 mg (2 g) of ginger twice per day for 8 weeks with non-surgical periodontal therapy (NSPT) significantly decreased mean levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), pocket depth (PD), hs-C-reactive protein (hs-CRP), clinical attachment loss (CAL) as compared to controls. Also, treatment induced a significant increase in mean serum levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx). It has been suggested that ginger supplementation with NSPT could be recommended for type 2 diabetic patients with CP (Javid et al., 2019).

In another randomized double-blind clinical trial was conducted by (Mohammadzadeh Honarvar and colleagues (2019), 48 diabetic patients were grouped for ginger (2 g) or placebo treatment for 10 weeks. The results demonstrated decreased nuclear factor kappa B (NF-κB) concentration after ginger consumption, but statistically not important. Also, the results showed no significant effect on the anthropometric parameters (hip and waist circumference, and body mass index (BMI) compared to placebo. Owing to insignificant findings, the study suggested that further studies are needed (Mohammadzadeh Honarvar et al., 2019).

In a randomized controlled clinical trial was conducted by Rahimlou et al. (2019), 37 patients with metabolic syndrome (MetS) were randomly allocated to receive ginger powder (2 g) or a placebo for 12 weeks. The application of ginger significantly improved the levels of triglyceride (TG), fasting blood glucose, and insulin resistance as compared to the placebo group. The authors also observed improvements in patients' body weight, waist circumference, total cholesterol level (LDL and HDL), blood pressure, as well as energy intake (Rahimlou et al., 2019).

Recently, a randomized double-blind placebo-controlled clinical trial performed by Hajimoosayi et al. (2020), the determined effect of ginger on the blood glucose level of 70 pregnant women with gestational diabetes mellitus (GDM). They were randomly separated into the ginger group obtained 126 tablets, and the placebo group had 126 tablets for 6 weeks. A significant decrease in fasting blood glucose, fasting insulin, Homeostasis Model Assessment (HOMA) index was observed in the ginger group compared to the placebo group. However, there was no significant reduction in mean blood sugar 2 h post-prandial in both groups (Hajimoosayi et al., 2020).

Another randomized, controlled, and triple-blind clinical trial by Badooei et al. (2021) compared the effects of ginger and aloe vera mouthwashes on xerostomia in type 2 diabetic (TD2) patients. Ginger mouthwash, aloe vera mouthwash, or placebo were used by one-hundred and five (105) patients for 20 ccs three times a day for 14 consecutive days. The study revealed a considerable decreased in all symptoms and severity of xerostomia using ginger and aloe vera mouthwashes. In diabetic patients, a 6.12 ± 2.04 cm decrease of xerostomia was recorded in the ginger group, 4.08 ± 2.09 cm in the aloe vera group when comparing with placebo. The results concluded that ginger mouthwash can effectively ameliorate xerostomia, hence could be prescribed for dry mouth in T2D patients (Badooei et al., 2021).

In Silico Molecular Docking Studies

Research by Fajrin et al. (2018) examined and predicted the binding ability of two ginger constituents to the Transient Receptor potential Vanilloid 1 (TRPV1) using *in silico* molecular docking method. Shogaol (6-shogaol, 8-shogaol, 10-shogaol) and gingerol (6-gingerol, 8-gingerol, and 10-gingerol) as well as were Capsaicin (reference), were docked against TRPV1. The study revealed that 10-gingerol had many hydrogen bonds (six H-bonds), 8-gingerol (5 H-bonds), 8-shogaol, 10-shogaol, 6-gingerol, and 10-gingerol had (four H-bonds), and 6-shogaol (3 H-bonds) bonded to Phe 49 and Ile 293 residues. However, capsaicin had three H-bonds bonded to Phe 54 and Ile 265 of TRPV1. Among the compounds, 6-shogaol also showed potent binding affinity (-7.10 kcal/mol) for TRPV and capsaicin (-7.36 kcal/mol). Because there was no important difference in kcal/mol between 6-shogaol and the drug; therefore, it was noted that 6-shogaol could be developed as TRPV1 for the treatment of Painful Diabetic Neuropathy (PDN) (Fajrin et al., 2018; Fajrin et al., 2020b).

Another study by (Fajrin et al., 2020b) determined the potential activity of 6-paradol and its derivatives to Transient Receptor potential Vanilloid 1 (TRPV1), a target receptor in Painful Diabetic Neuropathy (PDN). In this study, 2-paradol, 4-paradol, 6-paradol, 8-paradol, and 10-paradol were used as potential inhibitors of TRPV1. Capsaicin used in the treatment of PDN was utilized as a reference. The findings demonstrated that 2-paradol, 4-paradol, 6-paradol,

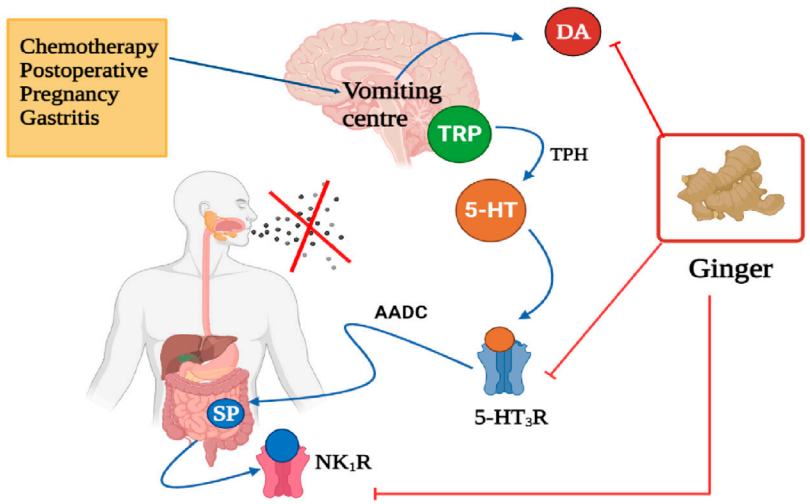


FIGURE 7 | Anti-emetic effect of ginger, targets, and mode of action. TRP, Tryptophan; 5-HT, 5-Hydroxytrptamine (serotonin); 5-HT_3R, 5-Hydroxytryptamine 3 receptor; AADC, Aromatic L-amino acid decarboxylase; SP, Substance P; NK_1R, Neurokinin 1 receptor; DA, Dopamine. X represent ceased vomiting. Figure was created using BioRender.com by the authors.

8-paradol, and 10-paradol had a strong binding affinity to the TRPV1. 2-paradol, 4-paradol, and 8-paradol had hydrogen bond interaction with Leu 32 and Thr 28 as capsaicin. 6-paradol had hydrogen bond interaction with Gln 135 and 143 Glu 140. However, 10-paradol had steric interaction with TRV1. It was concluded that 6-paradol and the derivatives potentially inhibited the TRPV1, hence could be used as a drug for PDN therapy (Fajrin et al., 2020a).

Another research examined the potential inhibition activity of ginger compound 6-gingerol on the insulin receptor kinase (3EKK), pancreatic lipase-colipase complex (1N8S), and human alpha-ketoglutarate- dependent dioxygenase FTO (protein 4CXW) for the treatment diabesity. The docking results showed that 6-Gingerol had different binding energy with proteins, 3EKK (−69.79 kcal/mol), IN8S (−53.47 kcal/mol), and 4CXW (−79.33). 6-Gingerol had van der Waal's interactions with amino residues of 3EKK such as Leu 1002A, Gly 1003A, Gln 1004A, Val 1010A, Ala 1028A, Lys 1030A, Val 1060A, Met 1076A, Met 1139A, Gly 1149A, and Asp 1150A. This compound had also formed hydrophobic interactions with Gly 1003A, Gln 1004A, Gly 1005A, Val 1010A, Ala 1028A, Lys 1030A, Met 1076A, and Met 1139A. On the human pancreatic lipase protein (IN8S), 6-Gingerol formed hydrogen bond interactions with Gln368A andTyr403A amino acid residues. On the human alpha-ketoglutarate-dependent dioxygenase FTO (4CXW) active sites, 6-Gingerol had van der Waal's interactions with Ile 85A, Pro 93A, Arg 96A, Tyr 108A, Leu 109A, Met 226A, Ala 227A, Val 228A, Ser 229A, His 231A, His 232A, Asp 233A, Glu 234A, and Arg 322A; hydrophobic interactions with Ile 85A, Pro 93A, Leu 109A, Leu 203A, Leu 215A, Val 228A, Ser 229A, and His 231A; aromatic interactions with Tyr 108A and His 231A; hydrogen bonding with Tyr 106A, Glu 234A, and Arg 322A (De et al., 2020). **Figure 6** exemplifies the potential anti-diabetic actions of ginger.

ANTI-EMETIC PROPERTIES

Clinical Studies

Several studies investigated the utilization of ginger for the alleviation of nausea and vomiting induced by surgery. Kamali et al. (2020) conducted a study that determined the effectiveness of ginger to prevent nausea and vomiting after abdominal hysterectomy as compared to dexmedmoidine (**Figure 7**). The study involved 92 patients who underwent an abdominal hysterectomy, were randomized to receive orally ginger (1 g) and injection dexmedmoidine (25 mg) before and after the anesthesia. The results demonstrated that ginger was most effective than dexmedetomidine. This study also found that ginger treatment significantly reduced vomiting scores, 2 h after the operation as compared to dexmedetomidine; however, 4 h later both treatments completely stopped vomiting. Also, ginger reduced the number or frequency of nausea than dexmedetomidine (Kamali et al., 2020).

In a double-blind study conducted by Naemi et al. (2020) in 88 patients, age ranges from 30–70 years old (both genders) who were randomly allocated to take various treatments such as ginger (4 capsules), Haloperidol, Metoclopramide, and Dexmedetomidine after laparoscopy found no significant effects on vomiting after the operation (**Figure 7**). Also, there was no significant difference observed in nausea between treatments. Due to the findings, the study suggested that ginger and these drugs can be further used in the management of vomiting and nausea; however, when there are no serious adverse effects (Naemi et al., 2020). Therefore, patients should be advised to cease using therapy when experiencing side effects.

A triple-blind clinical trial study by Sedighmaroufi and co-workers (2020) assessed the effectiveness of ginger in reducing the frequency and severity of both nausea and vomiting in

patients who had eye operations (**Figure 7**). One hundred and forty-eight (148) patients (all genders) were randomized to obtain treatment with ginger, ondansetron (reference), or placebo. It was found that oral administration of ginger capsules (1000 mg) and ondansetron significantly reduced vomiting as compared to placebo, but no significant difference was observed in the number of vomiting and severity of nausea. They also reported that ginger was more effective, safe, and cheaper as compared to ondansetron, hence be used as an alternative therapy for nausea and vomiting (Sedighmaroufi et al., 2020).

A study carried out by Uthaipaisanwong et al. (2020) examined the efficacy of ginger in 47 gynaecological cancer patients who take combined carboplatin-paclitaxel chemotherapy (**Figure 7**). As compared to the placebos, dried ginger capsules (500 mg) were showed to be effective in reducing the acute nausea score, but no significant difference was noticed in delayed nausea. The study also observed no significant difference in reducing both acute and delayed vomiting. Besides, heartburn, diarrhoea, and constipation were common adverse effects (Uthaipaisanwong et al., 2020).

A systematic review involved three studies, a pilot, randomized, open-label clinical trial; a randomized controlled trial; and a randomized, double-blind clinical trial was performed to examine the efficacy of ginger on nausea and vomiting associated with chemotherapy (**Figure 7**). The study found that oral administration of ginger (1–1.5 g) was effective in reducing nausea in patients who had breast cancer chemotherapy, and did not reduce the number of vomiting (Fitriyanti and Sulung, 2020).

Furthermore, the health benefits of ginger in controlling nausea and vomiting during pregnancy have been reported. A study conducted by Anita et al. (2020) evaluated the efficacy of ginger candy in 51 first-trimester pregnant women who experience vomiting three to five times per day (**Figure 7**). They were grouped (17 each) and recommended to take ginger candy (1/day), vitamin B6 (3 times/day), or a placebo for 7 days. Treatment with ginger candy was effective in reducing the frequency of vomiting (76.5%) as compared to vitamin B6 (5.9%) and placebo (no changes). The study concluded that ginger candy can be used to ameliorate emesis gravidarum or morning sickness (Anita et al., 2020).

A meta-analysis by Hu et al. (2020) conducted on 30 studies or 1174 patients reported that the application of ginger appears to be effective in reducing nausea and vomiting symptoms in pregnant women and nausea as compared to control, but no important effect was detected on vomiting (**Figure 7**). Besides, intake of ginger showed to be effective than vitamin B6 in the alleviation of nausea and vomiting during gestation (Hu et al., 2020).

ANTI-INFLAMMATORY ACTIVITIES

The innate immunity, the defense system that protects against pathogens plays a key role in the onset of inflammation *via* germline-encoded pattern-recognition receptors (PRRs). The PRRs such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic-acid-inducible gene-I (RIG-I)-like receptors (RLRs), and receptor for advanced glycation end products (RAGE). The binding of foreign pathogens to PRRs is linked to several signalling pathways such as nuclear factor kappa B (NF-kB), mitogen-activated protein kinase (MAPK), TANK-binding kinase 1 (TBK1)-interferon regulator factor 3 (IRF-3), and the inflammasome, their activation results in the production of proinflammatory factors (Horiguchi et al., 2018; Hu et al., 2018; Li and Wu, 2021).

The increase in levels of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6, play an essential role in rheumatoid arthritis (Bode and Dong, 2011; Aryaeian et al., 2019). It has been reported that 6- gingerol and 6- shogaol attenuate the production of pro-inflammatory molecules such as prostaglandins by suppressing the enzymes cyclooxygenase (COX)-1 and COX-2 (Grzanna et al., 2005; Saptarini et al., 2013; Mutthuraj et al., 2020).

Several studies demonstrated the anti-inflammatory actions of ginger and its phytocompounds. Zhang et al. (2013) found that 6-gingerol and 6-shogaol, 6-Dehydroshogaol (6-DHSG) (2.5, 5, and 10 μM) were the most powerful inhibitors of inflammation in lipopolysaccharide (LPS)-induced nitric oxide and prostaglandin E_2 (PGE_2) production in RAW 264.7 cells. 6-DHSG was powerful in reducing NO and PGE_2 generation as compared to 6-shogaol and 6-gingerol (Zhang et al., 2013; Mao et al., 2019).

In the study by Tripathi et al. (2008), alcoholic ginger extract (1 μl/ml) caused reduced production of pro-inflammatory cytokines (IL-12, TNF-α, IL-1β) and chemokines (RANTES, MCP-1) on macrophages treated with LPS. The ginger extract decreased the expression of B7.1, B7.2, and MHC class II molecules on macrophages. The study further evaluated the effect of ginger against an antigen and found that ginger extract induced a marked reduction in T cell proliferation as well as production of IFN-γ and IL-2 by T cells (Tripathi et al., 2008).

Luettig et al. (2016) revealed the preventative effect of 6-shogaol pro-inflammatory cytokine tumor necrosis factor α (TNF-α)-induced intestinal barrier inflammation using HT-29/B6 and Caco-2 cells. 6-shaogol prevented upregulation in protein expression of claudin-2 by suppressing phosphatidylinositol-3-kinase/Akt signaling, and dissemble of claudin-1 by inhibition of phosphorylation of nuclear factor kappa light chain enhancer of activated B cells (NF-kB) (Luettig et al., 2016; Mao et al., 2019).

In a recent study, aqueous extract of ginger has been found to possess an anti-inflammatory effect on the paw edema induced by carrageenan 1% of (CAR) injection. Rats treated with an aqueous extract of ginger (100 mg/kg BW) for 1 week before CAR injection showed a marked decrease in edema thickness, size, and percentage of inflammation as compared to indomethacin. The use of ginger extract was more effective, exhibited a considerable anti-inflammatory effect by >80%, at the 5 h after CAR injection. Also, ginger extract normalized the inflammatory markers, fibrinogen, and C-reactive protein (CRP). Besides, this finding was further confirmed by determining the antioxidant activity against inflammation. The SOD, CAT, and GPx activities

were significantly higher than the control group (Zammel et al., 2021).

Clinical Studies

Recently, the study by (Bauer Faria et al., 2021), examined the anti-inflammatory and antimicrobial activity of mouthwash containing 0.5% of ginger essential oil in 31 adult males and females with orthodontic appliances compared with chlorhexidine (0.12%) mouthwash and placebo. The patients were randomized to receive mouthwashes with CLX, ginger, and flavored sterile water (placebo) for 7 days with 15-days intervals between each treatment. Saliva and bleeding were used to assess the efficacy of the mouthwashes. Both ginger and CLX mouthwashes exhibited antimicrobial activity against *Streptococcus mutans*, but different substantivity. Also, ginger mouthwash showed an anti-inflammatory effect, markedly reduced the bleeding as compared to placebo; however, the taste was unpleasant. The study suggested that ginger taste should be improved (Bauer Faria et al., 2021).

A randomized, double-blind, controlled clinical trial conducted by Heidari-Beni et al. (2020) evaluated the efficacy of turmeric extract, black pepper, and ginger formulation on the prostaglandin E_2 (PGE_2) in 60 patients with Grade 2 and chronic knee osteoarthritis. The patients were grouped to receive herbal formulation or Naproxen capsule twice a day for 4 weeks. Both oral administration of herbal formulation and Naproxen significantly reduced PGE_2 levels. The anti-inflammatory effect is associated with gingerol and piperine, some of the known active constituents from ginger and black pepper. It has been concluded that oral intakes of turmeric extract, black pepper, and ginger formulation can ameliorate the PGE_2 levels in patients with chronic knee osteoarthritis. Therefore, future research examining the anti-inflammatory effect on biomarkers such as ILs and TNF-α is of paramount importance (Heidari-Beni et al., 2020).

Another interesting study by Mutthuraj and co-workers (2020) found that topical application of ginger essential oil on patients suffering from arthritis for 30 days inhibited the pro-inflammatory molecules by reducing the serum levels of rheumatoid arthritis (RA) factor, C reactive protein (CPR), and erythrocyte sedimentation rate (ESR). These results suggest that ginger has pungent anti-inflammatory potential, which can be used in the treatment of joint pain and swelling (Mutthuraj et al., 2020).

A systematic review and meta-analysis by Jalali et al. (2020) assessed the efficacy of ginger on the biomarkers of inflammatory and oxidative stress. The study included clinical studies that determined the effects of ginger on serum CRP (C- reactive protein), TNF-α (tumour necrosis factor-alpha), IL-6 (interleukin-6), PGE2 (prostaglandin E2), TAC (total antioxidant capacity), and MDA (malondialdehyde) until 2019. The effects of ginger on serum CRP, TNF-α, IL-6, TAC, MDA, PGE2 levels were statistically significant. It was concluded that ginger may be used in the treatment or mitigation of inflammation and oxidative stress. Therefore, large-scale randomized clinical trials should be conducted to confirm its safety (Jalali et al., 2020).

A randomized double-blind placebo-controlled clinical trial conducted by Aryaeian et al. (2019) evaluated the effects of ginger on the expression of some immune factors and inflammatory genes in 70 patients with rheumatoid arthritis (RA). The patients were randomized to receive 1500 mg of ginger powder or placebo daily for 12 weeks. The treatment with ginger improved RA through increasing genes expression such as forkhead box P3 (FoxP3), peroxisome proliferator-activated receptor-gamma (PPAR-γ), and GATA binding protein 3(GATA- 3) genes expressions. And downregulation of T-box transcription factor TBX (T-bet), and RAR-related orphan receptor γt (RORγt) genes expression (Aryaeian et al., 2019).

In Silico Molecular Docking Studies

The current study by Zammel et al. (2021) investigated the potential ability of ginger bioactive constituents to bind to the crystal structure of Toll-like receptor 6 (TRL6 4OM7) protein using *in silico* molecular docking. The results showed several ginger phytocompounds were bonded to the TRL6 with different binding affinities ranging between −5.4 and −10.8 kcal/mol. 6-Gingerol had four hydrogen bonds bonded to His651 residue. 8-gingerol showed three hydrogen bonds bonded to Glu 710. 10-Gingerol showed three hydrogen bonds interacting with Tyr 648. 6-Shogaol had five hydrogen bonds linked to Ser 728. Caffeic acid exhibited three hydrogen bonds interacting with Lys 769. Rosmarinic acid and syringic acid formed seven and five hydrogen bonds bonded to His 674 and Ser 728, respectively. Also, amentoflavone and ferulic acid showed five and three hydrogen bonds connected to His 674 and Gln 757 residues. The compounds were further evaluated into human TLR6 and indomethacin, the anti-inflammatory drug was used as a reference. The study found that 6-shogaol was bonded to Ile 684, Asn 687, Glu 675, His 674, Asn 705, Glu 710, and Tyr 648 residues in the pocket region of TLR6. And Rosmarinic acid was interacting with residues Ala 780, Ile 732, Leu 733, Leu 731, Thr 759, His 725, Ser 728, and Gly 727; as compared to indomethacin was bonded to Glu 675, His 651, Glu 650, Glu 710, and Ile 684 (Zammel et al., 2021).

Saptarini et al. (2013), investigated the potential inhibitory effect of gingerol, 6-shogaol, and 6-paradol for anti-inflammation. Molecular docking was studied on COX-1 and COX-2 enzymes. The interaction energy of the compounds toward COX-1 and 2 was ranging from −2.40 to −7.40 kcal/mol, and −7.80 to −11.13 kcal/mol, respectively. The selective index value was further calculated, revealed that all these compounds could induce anti-inflammation vis COX-2. The results of the research suggested that gingerol, 6-shogaol, and 6-paradol should be developed as COX-2 inhibitors for the treatment of inflammation (Saptarini et al., 2013).

Murugesan et al. (2020) examined the potential molecular interactions of nine bioactive constituents of ginger selected from gas chromatography-mass spectrometry (GC-MS) analysis with novel rheumatoid arthritis (RA) target proteins (COX-2, IL-1b,

MCSF, MMP-9, and TNF-alpha) for the treatment of rheumatoid arthritis. Ginger methanol extract active compounds included 2,5 dibutylfuran, 6-gingerol, 8-gingingerol, benzoic acid, dihydrocapsaicin, dihydropseudoionone, ferulic acid ethyl ester, geranylacetone, and zingerone. The results indicated different binding affinities toward the proteins, COX-2 (−4.4 to −7.8 kcal/mol), TNF-a (−3.3 to 5.6 kcal/mol, MCSF (−3.6 to 5.7 kcal/mol), IL-1b (−3.2 to 5.7 kcal/mol), and MMP-9 (−4.9 to −7.4 kcal/mol). Amongst nine phytoconstituents, 6-gingerol displayed the best binding affinity with COX-2 and IL-1b (−7.8 and −5.7 kcal/mol, respectively). 8-Gingerol showed a good binding affinity with MCSF (−5.7 kcal/mol) and TNF alpha (−5.6 kcal/mol). Zingerone had a higher binding affinity with MMP-9 protein (−7.4 kcal/mol). 8-Gingerol, 6-gingerol, and zingerone formed the strongest interactions with RA target proteins residues. Moreover, the pharmacokinetic and bioactivity analysis results showed that 6- and 8-Gingerol can act as enzyme inhibitors of Rheumatoid arthritis (RA) proteins (Murugesan et al., 2020).

PROTECTIVE EFFECTS AGAINST MALE INFERTILITY

Infertility is defined as incompetence to attain pregnancy after a year or more of copulation without contraception. Approximately 50% of men suffer from infertility, 40–90% is attributable to oligospermia and 152 million are associated with erectile dysfunction (Abdillahi and Van Staden, 2012; Masuku et al., 2020, 2021). Several factors that can interfere with fertility include exposure to various chemical compounds, drugs, chronic diseases, and lifestyle factors (Masuku et al., 2020; Kasonga et al., 2021). Numerous studies have investigated the protective and ameliorative properties of ginger male fertility.

Mustafa et al. (2016) investigated the protective effect of ginger on the testicular tissue and testosterone hormone in rats exposed to monosodium glutamate (MSG). The concomitant administration of MSG and ginger aqueous extract (100 mg/kg body weight) for 14 days prevented changes on the stratified epithelium of the seminiferous tubules, spermatogenic cells were normal and well-organized, and normal interstitial space and Leydig cells. The results also showed a significant increase in serum testosterone levels (Mustafa et al., 2016).

Soleimanzadeh et al. (2018) examined the ameliorative effect of ginger against formaldehyde (FA, CH2O)-induced reproductive toxicity in mice. Treatment with FA has been reported to decrease spermatozoa, levels of sex hormones, and antioxidant enzymes activities, and alters the expression of Bcl-2 and Bax genes in the testes. The concomitant administration of FA (10 mg/kg i.p) and ginger ethanolic extract (500, 1000, and 2000 mg/kg/day) in adult male NMRI mice for 35 days demonstrated improvement in spermatozoa parameters, sexual hormones, and antioxidant enzymes. Also, treatment with ginger suppressed the upregulation of Bc1-2 expression and downregulation of Bax gene expression in mice testes. The researchers concluded that the ameliorative effect of ginger was attributed to its androgen, antioxidant, and anti-apoptotic properties. Also, the use of ginger could be useful in patients exposed to FA (Soleimanzadeh et al., 2018).

In another study, oral administration of 70% ethanolic extract of ginger (200 mg/kg/day) for 21 days in aluminum-treated rats induced reduction in activities of liver enzymes (such as AST, ALT, and ALP) and malondialdehyde (MDA). Ginger was found to upsurge the levels of the antioxidants enzymes such as glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). Also, it was found to enhance the levels of the Follicle-stimulating hormone (FSH), luteinising hormone (LH), and testosterone. Also, the consumption of ginger rejuvenated spermatogenesis. However, coadministration of ginger and taurine was most effective than ginger alone (Kuoti Al-Rekabi, 2019).

Recently, Olusanya et al. (2018) evaluated the protective effect of coadministration of ginger and garlic against *Hibiscus sabdariffa* L. (Roselle)-induced testicular damage in rats. Treatment with a concoction of ginger and garlic aqueous extracts (250 mg/kg) for 28 days displayed an improvement in plasma levels of testosterone, estradiol, prolactin, LH, and FSH. In this study, coadministration ginger plus garlic also preserved the seminiferous tubule structural integrity and prevented the morphological changes of testes. The beneficial effects of ginger are proposed to be associated with its antioxidant properties (Olusanya et al., 2018).

Another recent study by Al-Muswie et al. (2021) examined the effect of ginger aqueous extract on the histological changes of testis and kidneys of male rats treated subjected to hydrogen peroxide (H_2O_2). Oral administration of the ginger aqueous extract (0.5 ml of 200 mg/kg) for 30 days attenuated the kidney damage and preserved the testicular damage as manifested by the normal structure of the seminiferous tubules, number, and distribution of spermatogenic cells as well as Leydig cells (Al-Muswie et al., 2021).

Sulfite metabisulfite (SMB) are added to food as preservatives and pharmaceutical agents. SMB impairs spermatogenesis, epidermal morphometry, and spermatozoa parameters in treated rats. Also, SMB decreased levels of enzymatic activities of glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT), and increased malondialdehyde (MDA) of SMB and ginger (500 mg/kg/day) for 28 days preserved histological features of the testes and epididymis and improved levels of enzymatic antioxidants (GPx, CAT, and GR) as well as MDA. They concluded that the prophylactic effects of ginger are related to its androgenic properties and free radicals scavenging capacity (Afkhami Fathabad et al., 2018).

Yaghubi Beklar and co-workers (2019) examined the effect of oral administration of ginger extract (100 mg/kg) on diazinon-induced testicular toxicity in mice. Treatment with ginger for 30 consecutive days caused a significant increase in testosterone levels, inhibitory effect on caspase-3 immunoexpression, and improved the spermatozoa parameters as manifested by a higher percentage of sperm motility and lower sperm abnormality. Also, testicular histopathological examination displayed an improvement. The authors concluded that the protective effects of ginger may be attributed to its anti-apoptotic, antioxidant, and free-radical scavenging activities (Yaghubi Beklar et al., 2019).

The study by Rafiee et al. (2019) assessed the effect of ginger on the zinc oxide nanoparticles (ZNP)-induced spermatogenesis defects in mice. The effects were assessed on the epididymal sperm parameters, testicular histology oxidative stress, serum testosterone level, viability of mouse Leydig (TM3) and mouse Sertoli (TM4) cell lines. Treatment with ginger (10, 20, and 40 mg/kg) before ZNP improved testosterone levels, sperm quality, morphometric parameters, enhanced superoxide dismutase (SOD) and catalase (CAT) activities, and decreased malondialdehyde (MDA). Also, the treatment increased the viability of TM3 and TM4 cells (Rafiee et al., 2019).

Odo et al. (2020) evaluated the preventive effect of ginger extract against lead acetate on sperm quality and haematology in male rats. In this study, the simultaneous ingestion of lead acetate with aqueous ginger extract (300 mg/kg) for 6 weeks induced a significant increase in the spermatozoa parameters (viability and motility), and the production of red blood cells. Also, the level of white blood cells was significantly reduced. The ameliorative impact of ginger on spermatozoa parameters may be associated with its antioxidant properties, and the presence of vitamin C (Odo et al., 2020).

In a study conducted by Mohammed et al. (2019), male rabbit bucks consumed a diet supplemented with fresh ginger (700 g/100 kg) and 6% of *Moringa oleifera* significantly enhanced sexual desire, had higher spermatozoa (count, motility, and morphology) (Mohammed et al., 2019).

A similar study by Adeyemi et al. (2020), assessed the antioxidant properties of ginger on rabbit semen. Consumption of diet ginger (5, 10, and 15 g/kg diet) for 7 weeks by rabbits induced higher semen volume, spermatozoa motility, and seminal total antioxidant capacity especially those fed 15 g of ginger (Adeyemi et al., 2020).

Another study by Kandeil et al. (2019) showed that oral intake of water added ginger extract (100 mg/kg body weight) caused a significant increase in semen volume, and spermatozoa parameters (count, motility, viability), testicular size, production of testosterone, and body weight in V-line male rabbits (Kandeil et al., 2019).

El-Naggar et al. (2020) found that oral intake of ginger extract (400 mg/tablet) before exposure to chronic stress is more effective than after posttreatment. Pre-treatment with ginger improved the gonadosomatic index, testosterone level, and prevented testicular degeneration in male rats (El-Naggar et al., 2020).

The study by Donkor et al. (2018) evaluated the efficacy of ethanolic ginger extract on the semen parameters. Treatment with ginger extract (100 mg/kg, 300 mg/kg, and 500 mg/kg) for 30 days enhanced sperm count, sperm morphology, sperm viability, and sperm motility. The study concluded that could be used in the treatment of male infertility (Donkor et al., 2018).

Muhammad et al. (2019) assessed the effect of ginger (100 mg/kg/day) on reproductive dysfunction in STZ diabetes-induced rats. Treatment with ginger mitigated the inflammation, damage of testicular morphology and also improved spermatozoa quality. The positive effect of ginger on male fertility might be due to its free radical scavenging capacity and anti-inflammatory activity (Muhammad et al., 2019).

The study of Al-Shathly et al. (2020) evaluated the efficacy of ginger in maintaining the structural integrity of testis in streptozotocin (STZ)-induced diabetic rats compared to the efficacy of metformin. Oral administration of aqueous ginger root extracts (500 mg/kg body weight) and metformin for 6 weeks in STZ-induced diabetic male rats resulted in a significant reduction in fasting blood significantly, significantly increased in total antioxidant capacity compared to untreated diabetic rats. Also, treatment with ginger and metformin significantly improved the testicular damage, causes inhibition of caspase-3 immuno-expression, and a significant increase in immune expression of androgen receptors and proliferating cell nuclear antigen. The study concluded that ginger can be given as adjuvant therapy in the treatment of diabetes (Al-Shathly et al., 2020).

Another research evaluated the effects of hydro-alcoholic extract of ginger on HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase level in the testis of streptozotocin (STZ)-induced diabetic rats. Oral treatment with hydroalcoholic extract (200 and 400 mg kg/kg) for 56 consecutive days resulted in a significantly upsurged in serum insulin levels, reduction in serum glucose concentration, and HMG-COA reductase level in the rat's testis compared to the diabetic group. Also, treatment with ginger improved body weight in STZ-induced diabetic rats. The study concluded that the results support the use of ginger in the alleviation of diabetes (Moradi-Podeh et al., 2018).

Clinical Studies

A few clinical studies have been published that evaluate the use of ginger or phytochemicals for the prevention, alleviation, and or treatment of male infertility.

In 2012, Mares and Najam evaluated the efficacy of taking ginger supplements in 75 infertile men patients aged between 19 and 40 years. In these patients, administration of ginger supplements resulted in a significant increase in spermatozoa parameters (count, motility, viability, and normal morphology), and semen volume compared to before therapy. Also, the use of ginger induced a significant increase in serum glutathione, FSH, LH, and decreased MDA (Mares and Najam, 2012; Gholamnezhad et al., 2018).

A double-blind randomized clinical study by Hosseini et al. (2016) examined the efficacy of ginger on spermatozoa DNA fragmentation in 100 infertile men patients. Patients were randomized to receive 250 mg of ginger powder per capsule and placebo, two times a day for 3 months. Semen samples, before and after treatment were used to assess the spermatozoa count, motility, and DNA fragmentation. Treatment with ginger significantly reduced spermatozoa DNA fragmentation compared to placebo. However, there were no statistically important differences for spermatozoa parameters (count and motility) between the study groups. Besides, no side effects were reported by patients (Hosseini et al., 2016; Gholamnezhad et al., 2018; Banihani, 2019).

ANTI-OBESITY POTENTIAL

In a mouse model of diet-induced obesity (DIO), consumption of a high-fat diet (HFD, 60% fat w/w) containing ethanolic extract of

steamed ginger (SGE) (40 mg/kg and 80 mg/kg) by male C57BL/6J mice for 12 weeks prevented lipid accumulations by suppressing adipogenesis and lipogenesis genes, PPARγ, and C/EBPα expression in 3T3-L1 cells and epididymal adipose tissue of DIO mice. PPARγ and C/EBPα modulate the expressions of aP2, GLUT4, fatty acids synthase (FAS), acetyl-CoA carboxylase (ACC), and adiponectin (ApN). Oral administration of SGE showed a significant reduction in obesity via marked inhibition of aP2, GLUT4, FAS, ACC, and ApN expression. Besides, SGE HFD fed mice exhibited a drastic reduction in total cholesterol triglyceride levels compared to control (Kim et al., 2021).

Concurrently administration of ginger and garlic aqueous extracts (1000 mg/k body weight) in male Wistar rats fed high fat diet (HFD) showed a reduction of body weight and a dose-dependent reduction in the total cholesterol, triacylglycerol (TAG), and low-density lipoprotein (LDL) level (Adegbola et al., 2021).

The study examining the effect of ginger water on body weight and energy expenditure revealed that oral administration of drinking water supplemented ginger water (25 and 50%) in male Wistar rats for a month lowered the total cholesterol and serum triglycerides and significantly reduced the body weight. Also, ginger water attenuated the expressions of mRNA of Sterol regulatory element-binding protein 1 (SREBP-1c) in the liver and leptin in adipose tissues, and increase the expressions of adiponectin, hepatic carnitine palmitoyltransferase1 (CPT-1), acyl-CoA oxidase (ACO), Glucose transporter 2 (GLUT-2), and pyruvate kinase (PK) (Sayed et al., 2020).

In another study, consumption of a high-fat diet supplemented with ginger powder (5%) caused improvements in body weight and gain, hepatic lipid levels, hyperglycemia, hypercholesterolemia, and lipogenic levels in C57BL/6 mice. Also, ginger enhanced levels of the fatty-acid oxidation gene, carnitine palmitoyltransferase 1 (CPT1), and downregulated the adipocyte inflammatory gene expression (Seo et al., 2021).

Clinical Studies

In a randomized, double-blind, placebo-controlled clinical trial, 80 healthy obese participants were assigned randomly to steam ginger ethanolic extract (SGE) capsules (100 mg plus 5.89–8.83 mg/g of 6-Shogaol) and placebo for 12 weeks. A significant decreased in mean body, body mass index, and body fat level was observed in SGE subjects as compared to placebo. Common cold and dyspepsia were observed in participants after SGE consumption. Nevertheless, the study suggested that SGE and lifestyle modifications together may be useful for maintaining body weight and fat mass (Park et al., 2020).

In another study, Farhadi et al. (2020) assessed the health benefits of aerobic exercise and consumption of ginger extract on lipid profiles, body composition, and liver enzymes in obese menopause women (53–58 years old). Fourth-eighth (48) women were recruited and assigned randomly to the ginger extract (500 mg capsules, 3 times/day) for 24 weeks, aerobic training (3 times/week), and aerobic training -ginger extract, and control. The study found that 12- and 24-weeks of training ginger supplementation, and a combination of ginger and training significantly reduced aspartate aminotransferase (AST) and alanine aminotransferase (ALT) liver enzymes. Also, the combination of ginger and training (12- and 24-weeks) improved lipid profiles and body composition in obese women, and changes were most after 6 months. The results of this study suggested that the combination of aerobic training and ginger extract consumption might be an effective way to improved obesity (Farhadi et al., 2020).

El Gayar et al. (2019) conducted a study on the effect of ginger powder supplementation on glycemic status, lipid profile, and beta-cell function in 80 obese patients with newly diagnosed type 2 diabetes mellitus. The patients were randomized to receive a daily dose of 1.8 g (~600 mg powered/capsule, 3 times/day) of ginger plus one metformin tablet (850 mg, 2 times/day) with meals for 8 weeks or placebo. The results showed that supplementation with ginger powder resulted in a significant reduction of body mass index, fasting plasma glucose, 2-h postprandial blood glucose, glycated hemoglobin, total cholesterol levels, low-density lipoprotein cholesterol, triglycerides, fasting insulin levels, and homeostasis model assessment-insulin resistance index (HOMA2-IR). The study also found a significant increase in beta-cell function index (HOMA2-%β), insulin sensitivity index (HOMA2-%S), as well as high-density lipoprotein cholesterol levels in the ginger group compared to placebo. The authors concluded that ginger supplements could be used as an adjuvant therapy to improve the efficacy of type 2 diabetes mellitus treatment (El Gayar et al., 2019).

In the study by (Ebrahimzadeh Attari et al., 2016), eighty (80) obese women (18–45 years old) were randomized to receive ginger powered (2 g/day) and placebo for 12 weeks. As compared to the placebo group, oral administration of ginger significantly reduced body mass index, serum insulin, and HOMA-IR index and correlated with increasing quantitative insulin sensitivity check index (QUICKI). Ginger supplementation also significantly reduced serum leptin, resistin, and glucose with both groups, but there was no significant difference. Moreover, there were no significant changes were observed in the body composition and serum levels of adiponectin in both groups. It was concluded that consumption of 2 g of ginger for 12 weeks was not effective enough for weight loss and reducing some of the metabolic features associated with obesity (Ebrahimzadeh Attari et al., 2016; Anh et al., 2020).

A systematic review with meta-analysis was conducted on 14 randomized controlled trials/473 participants to evaluate the effects of ginger on bodyweight loss, glycemic control, and lipid profiles in overweight and obese individuals. The study revealed that ginger supplementation significantly reduced body weight, waist-to-hip ratio, hip ratio, fasting blood glucose, and insulin resistance index (HOMA-IR), and significantly improved HDL-cholesterol levels. But had no significant effect was found on body mass index (BMI), insulin, triglycerides, and low-density lipoprotein (LDL) (Maharlouei et al., 2019; Venkatakrishnan et al., 2019). The molecular mechanisms for the anti-obesity effects of ginger phytochemicals are illustrated in **Figure 8**.

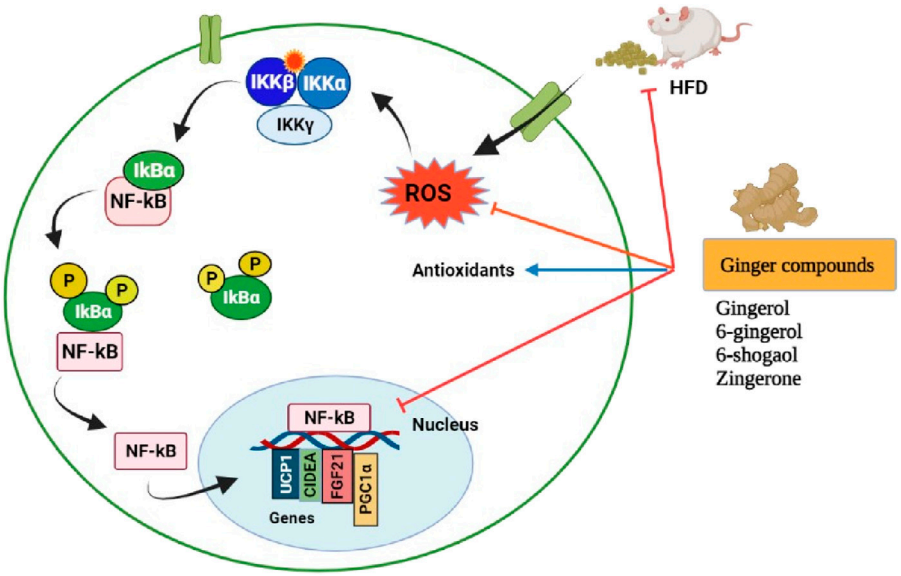


FIGURE 8 | Pharmacological actions of ginger phytoconstituents on obesity. NF-kB, nuclear factor kappa B; IKK (βαγ); I kappa B kinase complex; IkBα, I kappa-B-alpha; UCP1, uncoupling protein 1; CIDEA, Cell death-inducing DFFA like effector A; FGF21, Fibroblast growth factor 21; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator; ROS, Reactive oxygen species; P, Phosphorylation; HDF, High fat diet. Figure was created using BioRender.com by the authors.

In Silico Molecular Docking Studies

The β3-AR has become a promising therapeutic target for the treatment of obesity and other metabolic disorders. This is owing to its presence in the adipose tissue and its ability to activate brown adipocytes which promote energy expenditure, weight loss, improved glucose, and lipid metabolism (Arch and Ainsworth, 1983; Hao et al., 2019). A study conducted by Sampath and co-worker (2021) determined the interactions of 6-gingerol and 6-shogaol as potential ligands for β3-adrenergic receptor membrane protein (β3-AR). They also compared their binding affinity with β3-AR protein by *in silico* molecular docking studies. The study revealed that both 6-gingerol and 6-shogaol had the best binding affinities to the receptor, but 6-gingerol indicated a better −82.364 kcal/mol docking score as compared to 6-shogaol (−79.987 kcal/mol). The study also found that 6-gingerol has powerful hydrogen bond interaction with β3-AR than 6-shogaol. 6-gingerol showed hydrogen bond interaction with serine amino acid residue, and 6-shogaol was bonded to the serine and tryptophan residues. Moreover, the results showed that 6-gingerol enhances the browning effects in white adipocytes via activation of the β3-AR signaling pathway. Therefore, 6-gingerol is a potential treatment for obesity (Sampath et al., 2021).

PAIN-RELIEVING POTENTIAL

Clinical Studies

A systemic review and meta-analysis of Negi et al. (2021) compared the efficacy of ginger, non-steroidal anti-inflammatory (NSAID), and placebo for primary dysmenorrhea. The application of ginger was found to be effective in the alleviation of menstrual pain as compared to placebo, but no significant difference was observed in the duration of pain. Nevertheless, the use of ginger and non-steroidal anti-inflammatory (NSAID) was useful to treat pain severity (Negi et al., 2021).

A randomized clinical trial by Munir et al. (2021) determined the effectiveness and safety of ginger capsules and naproxen tablets for the treatment of knee osteoarthritis. The study involved 60 male and female patients (>50 years of age), were randomized to receive twice-daily doses of 550 mg of ginger capsules (550 mg) plus 550 mg of naproxen tablets and placebo for 6 weeks. The concomitant administration of ginger with naproxen was more effective than naproxen alone, alleviated pain and stiffness in knee osteoarthritis. Minimal adverse effects were reported. Mild diarrhea was common in patients. In addition, the authors suggested long-term multicenter studies (Munir et al., 2021).

In Silico Study on SARS-Cov-2

Coronaviruses disease 2019 (COVID-19) is a pandemic virus caused by severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2). The virus was first discovered or diagnosed in Wuhan, China by the Chinese Center for Disease Control and Prevention (Guan et al., 2020; Zhu et al., 2020). SARS-Cov-2 is a single-stranded genome (positive sense) made of ribonucleic acid (RNA). The structure of SARS-Cov-2 consists of four main proteins including the membrane (M) glycoprotein, the envelope (E) protein, nucleoprotein (N), and the spike (S) protein (Boheemen et al., 2012; Lu et al., 2020; Tai et al.,

2020). The main target of the virus is the angiotensin-converting enzyme 2 (ACE2), an enzyme expressed in several body tissues such as the heart, lungs, testis, and kidneys (Santos et al., 2018; Ni et al., 2020; Fan et al., 2021). The attachment of virus spike protein to the ACE2 receptor on the host cells is the critical step for SARS-Cov-2 entry into cells. After binding to the receptor, the protease enzyme (TMPRSS2) activates the spike protein, and the virus enters the human cells. Once in the cells, the viral replication process takes place (Baughn et al., 2020; Hoffmann et al., 2020; Ren et al., 2020). Fever, dry cough, and feeling tiredness have been reported to be the most common symptoms of COVID-19. Besides, some patients suffer from diarrhea, headache, nasal congestion or runny nose, sore throat, body aches and pains, lymphopenia, and dyspnea (Guan et al., 2020; Orisakwe et al., 2020; Silveira et al., 2020; Wang et al., 2020). The inhibition of SARS-Cov-2 spike protein binding ACE2 using natural herbs or phytochemicals is the promising approach in the prevention and treatment of Covid-19 infection (Abinaya et al., 2020; Mehrotra, 2020; Orisakwe et al., 2020; Silveira et al., 2020; Haridas et al., 2021).

Owing to the urgent need for effective treatment of COVID-19 and fewer treatment options (Ibrahim et al., 2020), ginger extract is prepared by patients as a medication for the management, and melioration of SARS-Cov-2 (Abinaya et al., 2020; Orisakwe et al., 2020). A homemade ginger remedy has been reported. Ginger is prepared alone or in combination with other natural herbs. Ginger, garlic cloves (20), and lime are blended. One tablespoon of paste, Lipton tea and crushed paracetamol tablets (1000 mg) is added to boiling water. One cup of concoction is drunk once after 4 h. The remission of signs and symptoms of COVID-19 has been observed to occur within 3 days. Ginger is cooked with turmeric powder, garlic, and lemon for steam inhalation. The patient covers their head with a towel and deep breath through the nose. The patient recovers from the illness within 3 days. Also, ginger is cooked with garlic, paw leaf, Neem (Dogoyaro) leaf, artemisia, lime, and oranges. Steam is inhaled for 30 min. This is most effective therapy to clear lungs or chest congestion. It takes one to 2 weeks to recuperate (Orisakwe et al., 2020). At the moment, there are no scientific studies and or clinical trials support the efficacy and safety of these medications. Therefore, further studies are needed to prevent their chronic health effects.

In silico and molecular docking studies have been conducted to screen the potential inhibition activity of ginger phytochemicals against SARS-Cov-2 and to assess its binding affinities to block SARS-Cov-2. Research by Rajagopal et al. (2020) explored the potential anti-COVID-19 (PD: id-5R82) effectiveness of ginger phytocompounds. The docking results revealed that 8-Gingerol and 10-Gingerol had significant inhibition towards Covid-19. The compounds exhibited higher Glidescore (−5.88 and −5.72 kcal/mol, respectively), high binding affinity to the receptor when compared to that of Hydroxychloroquine drug (−5.47 kcal/mol), and other ginger constituents. Multiple active site residues such Ser 46, Met 49, Hie 41, Gln 189, Arg 189, Asp 187, Met 165, Hie 164, Thr 24, Thr 25, Leu 27, Asn 142, and Gly 143 were involved in the antiviral (5R82) potential of ginger phytochemicals (Rajagopal et al., 2020).

In another study, Haridas et al. (2021) examined 16 compounds isolated from ginger as inhibitors of SARS-Cov-2 spike protein and ACE-2. This study was targeted against COVID-19 protein called SARS-CoV-2 main protease receptor cocrystallized with 6-(ethylamino) pyridine-3-carbonitrile (PDB ID:5R82) using *in silico* molecular docking model. Molecular docking results at the active sites of spike protein two possible inhibitors, adenine and 6-Gingerol showed higher binding affinity (−24.18 and −36.60 kcal/mol, respectively). Adenine formed two hydrogen bonds by interacting with residues Tyr 495, and Gly 496. 6-Gingerol formed four hydrogen bonds with residues Tyr 453, Ser 494, Gly 496, and Tyr 505. Results of the ACE-2 revealed that 6 compounds (10-paradol, 8-paradol, scopoletin, 10-shogaol, 8-gingerol, and 10-gingerol) could be powerful inhibitors of ACE-2 against Covid-19. Docking scores was ranging from −33.72 to −51.27 kcal/mol. 10-Paradol formed two bonds with Arg 273 and Lys 363 residues. 8-paradol formed three bonds with Asn 149 and Lys 363. Scopoletin formed three hydrogen bonds, interacting with residues Asn 149, Asn 368, and Lys 363. 10-shogaol formed three hydrogen bonds, interacting with Asn 277, Lys 363, and Asp 367 residues. 8-gingerol formed three bonds with residues Asp 367 and Lys 363. Also, 10-gingerol formed two hydrogen bonds, interacting with residues Glu 406 and Arg 518 (Haridas et al., 2021).

Moreover, comparative research by Al-Sanea et al. (2021) evaluated the potential inhibition activity of methanolic extract of ginger, ginger silver nanoparticles (AgNPs), strawberry methanolic extract, and strawberry AgNPs to treat SARS-Cov-2. The MTT assay was used to assess the anti-SARS-CoV-2 activity. Ginger AgNPs and strawberry methanolic extract displayed antivirus potentials against Covid-19 with IC_{50} values of 0.034 μg/ml and 0.0062 μg/ml, respectively. Strawberry AgNPs with IC_{50} value of 0.0989 μg/ml and ginger methanolic extract with IC_{50} value of 206.4 mg/ml. To further evaluate the antiviral activity, *in silico* molecular docking study was conducted to examine the potential phytochemicals that might bind to and inhibit SARS-CoV-2 proteins. Among 30 compounds, neohesperidin showed better binding affinity to SARS-CoV-2 NSP16 protein (Al-Sanea et al., 2021).

CONCLUSION

Numerous studies have shown that ginger possesses nutritional components required for wellness and its cultivation could boost the economy of many countries especially the developing ones. The presence of superabundant pungent constituents (>400), for instance, gingerols, shogaols, zingerone, and many others provide therapeutic benefits of this plant. In addition, ginger contains high amounts of antioxidants and nutrients which are important in many physiological and biochemical processes in the body. The pharmacological activities of ginger and its chemical compounds are promising in the mitigation, treatment, prevention of

diabetes, male infertility, obesity, nausea, emesis, as well as inflammation. Presently, the potential inhibition activity of ginger phytochemicals against SARS-Cov-2 and to assess its binding affinities to block SARS-Cov-2 have been reported. The application of *in silic*o molecular docking tool for the identification of inhibitors or drugs can revolutionize the treatments. The findings of *in silico* molecular studies of ginger compounds interacting with various receptors or proteins (e.g. β3-AR, TRPV1) are promising for the design of novel drugs/inhibitors in combating obesity, diabetes, inflammation, nausea, vomiting, and SARS-CoV-2. However, further studies are needed to investigate their mechanism of action and safety. Moreover, few clinical trials have validated the efficacy and safety of ginger and its constituents especially for the treatment of male fertility. Therefore, future well-designed large-scale randomized clinical investigations of long-term effects are warranted to further evaluate the effectiveness and safety of ginger or phytocompounds for the development of sufficient medications. In addition, owing to the myriad pharmacological properties of ginger, consuming a little bit of ginger in our diet may boost the immune system to fight against various ailments.

AUTHOR CONTRIBUTIONS

Conceptualization, JOU, NPM, OKP, and LSL; Methodology, JOU, NPM, and OKP, Investigation, JOU, NPM, and OKP, Writing–Original Draft, JOU, NPM, and OKP, Writing–Review & Editing, JOU, NPM, OKP, and LSL Funding Acquisition, LSL; Resources, JOU, NPM, and OKP, Supervision, JOU and LSL.

ACKNOWLEDGMENTS

The authors would like to thank the Department of Life and Consumer Sciences and the College of Agriculture and Environmental Sciences (CAES) through the University of South Africa (UNISA) for support.

REFERENCES

Abdillahi, H. S., and Van Staden, J. (2012). South African Plants and Male Reproductive Healthcare: Conception and Contraception. *J. Ethnopharmacol.* 143, 475–480. doi:10.1016/j.jep.2012.06.047

Abifarin, T. O., Otunola, G. A., and Afolayan, A. J. (2021). Nutritional Composition and Antinutrient Content of Heteromorpha Arborescens (Spreng.) Cham. & Schltdl. Leaves: An Underutilized Wild Vegetable. *Food Sci. Nutr.* 9, 172–179. doi:10.1002/FSN3.1978

Abinaya, S., Gayatri Devi, R., and Lakshmanan, G. (2020). Knowledge and Awareness about Ginger and Turmeric as a Herbal Cure for COVID-19. *Ijpr* 12, 768–777. doi:10.31838/ijpr/2020.SP2.093

Abubakar, U. S., Tajo, S. M., Masha Joseph, K. M., Tijjani, A., Adamu, J. G., and Fatima, S. S. (2019). *Comparative Study of the Proximate, mineral and Phytochemical Composition of Zingiber Officinale (Zingiberaceae) Cultivated in Kano and Kaduna States*, 4, 1-6. Available at: https://sciarena.com/storage/models/article/yihaZ4f7isb9kieePYKsxOfJm6uk4zgZEiVxqLVPpq0VeVSNOksmtQAA1VFu/comparative-study-of-the-proximate-mineral-and-phytochemical-composition-of-zingiber-officinale-zi.pdf.

Adebowale, K. O., Nwokocha, L. M., and Agbaje, W. B. (2013). Composition of Cissus Populnea Stem. *J. Food Compost. Anal.* 30, 41–46. doi:10.1016/J.JFCA.2013.01.001

Adegbaju, O. D., Otunola, G. A., and Afolayan, A. J. (2019). Proximate, mineral, Vitamin and Anti-nutrient Content of Celosia Argentea at Three Stages of Maturity. *South Afr. J. Bot.* 124, 372–379. doi:10.1016/J.SAJB.2019.05.036

Adegbola, P. I., Fadahunsi, O. S., Ajilore, B. S., Akintola, A. O., and Olorunnisola, O. S. (2021). Combined Ginger and Garlic Extract Improves Serum Lipid Profile, Oxidative Stress Markers and Reduced IL-6 in Diet Induced Obese Rats. *Obes. Med.* 23, 100336. doi:10.1016/j.obmed.2021.100336

Ademosun, M. T., Omoba, O. S., and Olagunju, A. I. (2021). Antioxidant Properties, Glycemic Indices, and Carbohydrate Hydrolyzing Enzymes Activities of Formulated Ginger-Based Fruit Drinks. *J. Food Biochem.* 45, e13324–10. doi:10.1111/jfbc.13324

Adeyemi, A. A., Yekini, S. D., and Oloyede, O. J. (2020). Antioxidant Impact of Zingiber Officinale and Allium Sativum on Rabbit Semen. *J. Vet. Androl.* 5, 18–22.

Afkhami Fathabad, A., Shekarforoush, S., Hoseini, M., and Ebrahimi, Z. (2018). Attenuation of Sulfite-Induced Testicular Injury in Rats by Zingiber Officinale Roscoe. *J. Diet. Suppl.* 15, 398–409. doi:10.1080/19390211.2017.1349233

Ahmad, B., Rehman, M. U., Amin, I., Mir, M. U. R., Ahmad, S. B., Farooq, A., et al. (2018). Zingerone (4-(4-Hydroxy-3-Methylphenyl) Butan-2-One) Protects against Alloxan-Induced Diabetes via Alleviation of Oxidative Stress and Inflammation: Probable Role of NF-kB Activation. *Saudi Pharm. J.* 26, 1137–1145. doi:10.1016/j.jsps.2018.07.001

Ahn, S. I., Lee, J. K., and Youn, H. S. (2009). Inhibition of Homodimerization of Toll-like Receptor 4 by 6-shogaol. *Mol. Cell* 27, 211–215. doi:10.1007/s10059-009-0026-y

Al-Muswie, R. T., Majid, A., Al-Rekabi, E. A., and Al-Fartosi, K. G. (2021). Prophylactic Role of Curcuma Longa and Zingiber Officinale on Histological Changes of Testis and Kidney of Male Rats Treated with Hydrogen Peroxide. *Ann. Rom. Soc. Cel Biol.* 25, 3883–3891. Available at: http://annalsofrscb.ro.

Al-Sanea, M. M., Abelyan, N., Abdelgawad, M. A., Musa, A., Ghoneim, M. M., Al-Warhi, T., et al. (2021). Strawberry and Ginger Silver Nanoparticles as Potential Inhibitors for Sars-Cov-2 Assisted by In Silico Modeling and Metabolic Profiling. *Antibiotics (Basel)* 10. doi:10.3390/antibiotics10070824

Al-Shathly, M. R., Ali, S. S., and Ayuob, N. N. (2020). Zingiber Officinale Preserves Testicular Structure and the Expression of Androgen Receptors and Proliferating Cell Nuclear Antigen in Diabetic Rats. *Andrologia* 52, e13528–8. doi:10.1111/and.13528

Ali, A., and Gilani, A. H. (2007). Medicinal Value of Ginger with Focus on its Use in Nausea and Vomiting of Pregnancy. *Int. J. Food Properties* 10, 269–278. doi:10.1080/10942910601045297

Alp, D., and Bulantekin, Ö. (2021). The Microbiological Quality of Various Foods Dried by Applying Different Drying Methods: a Review. *Eur. Food Res. Technol.* 247, 1333–1343. doi:10.1007/S00217-021-03731-Z

Alsahli, M. A., Almatroodi, S. A., Almatroudi, A., Khan, A. A., Anwar, S., Almutary, A. G., et al. (2021). 6-Gingerol, a Major Ingredient of Ginger Attenuates Diethylnitrosamine-Induced Liver Injury in Rats through the Modulation of Oxidative Stress and Anti-inflammatory Activity. *Mediators Inflamm.* 2021, 1–17. doi:10.1155/2021/6661937

Anh, N. H., Kim, S. J., Long, N. P., Min, J. E., Yoon, Y. C., Lee, E. G., et al. (2020). Ginger on Human Health: A Comprehensive Systematic Review of 109 Randomized Controlled Trials. *Nutrients* 12, 1–28. doi:10.3390/nu12010157

Anita, N., Sartiniand Alam, G. (2020). Ginger Candy (Zingiber Officinale) Reduces the Frequency of Vomiting of First-Trimester Pregnant Women with Emesis Gravidarum. *Enferm. Clin.* 30, 536–538. doi:10.1016/j.enfcli.2020.03.014

Antia, B. S., .Akpan, E. J., Okon, P. A., and Umoren, I. U. (2006). Nutritive and Anti-nutritive Evaluation of Sweet Potatoes (Ipomoea Batatas) Leaves. *Pakistan J. Nutr.* 5, 166–168. doi:10.3923/PJN.2006.166.168

Antia, T. M., Ikram, N., Najam-ul-Haq, M., Fayyaz, I., Fayyaz, Q., and Ghafoor, I. (2004). Essential Trace Metal (Zinc, Manganese, Copper and Iron) Levels in Plants of Medicinal Importance. *J. Biol. Sci.* 4, 95–99. doi:10.3923/jbs.2004.95.99

Arch, J., and Ainsworth, A. (1983). Thermogenic and Antiobesity Activity of a Novel Beta-Adrenoceptor Agonist (BRL 26830A) in Mice and Rats. *Am. J. Clin. Nutr.* 38, 549–558. doi:10.1093/AJCN/38.4.549

Aryaeian, N., Shahram, F., Mahmoudi, M., Tavakoli, H., Yousefi, B., Arablou, T., et al. (2019). The Effect of Ginger Supplementation on Some Immunity and Inflammation Intermediate Genes Expression in Patients with Active Rheumatoid Arthritis. *Gene* 698, 179–185. doi:10.1016/j.gene.2019.01.048

Badooei, F., Imani, E., Hosseini-Teshnizi, S., Banar, M., and Memarzade, M. (2021). Comparison of the Effect of Ginger and Aloe Vera Mouthwashes on Xerostomia in Patients with Type 2 Diabetes: A Clinical Trial, Triple-Blind. *Med. Oral* 26, e408–e413. doi:10.4317/medoral.23998

Bag, B. (2018). Ginger Processing in India (Zingiber Officinale): A Review. *Int.J.Curr.Microbiol.App.Sci* 7, 1639–1651. doi:10.20546/ijcmas.2018.704.185

Balogun, F. O., Tayo AdeyeOluwa, E., and Omotayo Tom Ashafa, A. (2019). Pharmacological Potentials of Ginger. *Ginger Cultiv. Its Antimicrob. Pharmacol. Potentials*. doi:10.5772/intechopen.88848

Banihani, S. A. (2019). Effect of Ginger (Zingiber Officinale) on Semen Quality. *Andrologia* 51, e13296–7. doi:10.1111/and.13296

Bashir, N., Ahmad, S. B., Rehman, M. U., Muzamil, S., Bhat, R. R., Mir, M. U. R., et al. (2021). Zingerone (4-(four-Hydroxy-3-Methylphenyl) Butane-Two-1) Modulates Adjuvant-Induced Rheumatoid Arthritis by Regulating Inflammatory Cytokines and Antioxidants. *Redox Rep.* 26, 62–70. doi:10.1080/13510002.2021.1907518

Bauer Faria, T. R., Furletti-Goes, V. F., Franzini, C. M., de Aro, A. A., de Andrade, T. A. M., Sartoratto, A., et al. (2021). Anti-inflammatory and Antimicrobial Effects of Zingiber Officinale Mouthwash on Patients with Fixed Orthodontic Appliances. *Am. J. Orthod. Dentofacial Orthopedics* 159, 21–29. doi:10.1016/j.ajodo.2019.10.025

Baughn, L. B., Sharma, N., Elhaik, E., Sekulic, A., Bryce, A. H., and Fonseca, R. (2020). Targeting TMPRSS2 in SARS-CoV-2 Infection. *Mayo Clin. Proc.* 95, 1989–1999. doi:10.1016/j.mayocp.2020.06.018

Bernard, M., Furlong, S. J., Power Coombs, M. R., and Hoskin, D. W. (2015). Differential Inhibition of T Lymphocyte Proliferation and Cytokine Synthesis by [6]-Gingerol, [8]-Gingerol, and [10]-Gingerol. *Phytother Res.* 29, 1707–1713. doi:10.1002/ptr.5414

Bode, A., and Dong, Z. (2011). The Amazing and Mighty Ginger. *Herb. Med. Biomol. Clin. Asp*, 131–156. doi:10.1201/b10787-8

Boheemen, S. V., Graaf, M. D., Lauber, C., Bestebroer, T. M., Raj, V. S., Zaki, A. M., et al. (2012). Genomic Characterization of a Newly Discovered Coronavirus. *MBio* 3, 1–9. doi:10.1128/mBio.00473-12

Butt, M. S., and Sultan, M. T. (2011). Ginger and Its Health Claims: Molecular Aspects. *Crit. Rev. Food Sci. Nutr.* 51, 383–393. doi:10.1080/10408391003624848

Chan, K. (2003). Some Aspects of Toxic Contaminants in Herbal Medicines. *Chemosphere* 52, 1361–1371. doi:10.1016/S0045-6535(03)00471-5

Chen, C.-Y., and Yeh, Y.-T. (2011). Two New Phenylalkanoids from the Rhizomes of Zingiber Officinale. *Nat. Prod. Res.* 25, 62–67. doi:10.1080/14786419.2010.490917

Chen, C. Y., Yeh, Y. T., and Yang, W. L. (2011). New Phenylalkanoids from Zingiber Officinale. *Nat. Prod. Commun.* 6, 855–856. doi:10.1177/1934578x1100600624

Cheng, Q., Feng, X., Meng, Q., Li, Y., Chen, S., Wang, G., et al. (2020). [6]-Gingerol Ameliorates Cisplatin-Induced Pica by Regulating the TPH/MAO-A/SERT/5-HT/5-HT3 Receptor System in Rats. *Drug Des. Devel Ther.* 14, 4085–4099. doi:10.2147/DDDT.S270185

Choi, J., Kim, K. J., Kim, B. H., Koh, E. J., Seo, M. J., and Lee, B. Y. (2017). 6-Gingerol Suppresses Adipocyte-Derived Mediators of Inflammation *In Vitro* and in High-Fat Diet-Induced Obese Zebra Fish. *Planta Med.* 83, 245–253. doi:10.1055/s-0042-112371

Crichton, M., Marshall, S., Marx, W. M., McCarthy, A. L., and Isenring, E. (2019). Efficacy of Ginger (Zingiber Officinale) in Ameliorating Chemotherapy-Induced Nausea and Vomiting and Chemotherapy-Related Outcomes: A Systematic Review Update and Meta-Analysis. *J. Acad. Nutr. Diet.* 119, 2055–2068. doi:10.1016/J.JAND.2019.06.009

De, B., Bhandari, K., Singla, R. K., Saha, G., and Goswami, T. K. (2020). In Silico molecular GRIP Docking of Some Secondary Metabolites Combating Diabesity. *Bull. Natl. Res. Cent.* 44. doi:10.1186/s42269-020-00327-7

Dhanik, J., Arya, N., and Nand, V. (2017). A Review on Zingiber Officinale. *J. Pharmacogn. Phytochem.* 6, 174–184. Available at: https://www.phytojournal.com/archives/2017.v6.i3.1207/a-review-on-zingiber-officinale (Accessed August 26, 2021).

Donkor, Y. O., Abaidoo, C. S., Abaidoo, C. S., Tetteh, J., Darko, N. D., Atuahene, O. O.-D., et al. (2018). The Effect of Zingiber Officinale (Ginger) Root Ethanolic Extract on the Semen Characteristics of Adult Male Wistar Rats. *Ijar* 6, 5481–5487. doi:10.16965/ijar.2018.245

Ebrahimzadeh Attari, V., Ostadrahimi, A., Asghari Jafarabadi, M., Mehralizadeh, S., and Mahluji, S. (2016). Changes of Serum Adipocytokines and Body Weight Following Zingiber Officinale Supplementation in Obese Women: a RCT. *Eur. J. Nutr.* 55, 2129–2136. doi:10.1007/s00394-015-1027-6

El Gayar, M. H., Aboromia, M. M. M., Ibrahim, N. A., and Abdel Hafiz, M. H. (2019). Effects of Ginger Powder Supplementation on Glycemic Status and Lipid Profile in Newly Diagnosed Obese Patients with Type 2 Diabetes Mellitus. *Obes. Med.* 14, 100094. doi:10.1016/j.obmed.2019.100094

El-Naggar, H. A. E.-M., El-Safty, F. E.-N. A., El-mehi, A. E., and Reafie, A. A. (2020). Effect of Chronic Stress on the Testis of the Adult Male Albino Rat and the Role of Ginger. *Egypt. J. Hosp. Med.* 81, 2184–2194. doi:10.21608/ejhm.2020.127968

El-Sayed, S. M., and Youssef, A. M. (2019). Potential Application of Herbs and Spices and Their Effects in Functional Dairy Products. *Heliyon* 5, e01989. doi:10.1016/J.HELIYON.2019.E01989

Elseweidy, M. M., Abdallah, F. R., Younis, N. N., Aldohmy, S., and Kassem, H. M. (2013). 10-Dehydrogingerdione Raises HDL-Cholesterol through a CETP Inhibition and Wards off Oxidation and Inflammation in Dyslipidemic Rabbits. *Atherosclerosis* 231, 334–340. doi:10.1016/j.atherosclerosis.2013.09.024

Elseweidy, M. M., Zaghloul, M. S., and Younis, N. N. (2016). 10-DHGD Ameliorates Cisplatin-Induced Nephrotoxicity in Rats. *Biomed. Pharmacother.* 83, 241–246. doi:10.1016/j.biopha.2016.06.032

Elshopakey, G. E., Almeer, R., Alfaraj, S., Albasher, G., Abdelgawad, M. E., Abdel Moneim, A. E., et al. (2021). Zingerone Mitigates Inflammation, Apoptosis and Oxidative Injuries Associated with Renal Impairment in Adriamycin-Intoxicated Mice. *Toxin Rev.* 0, 1–12. doi:10.1080/15569543.2021.1923528

Fagundes, G. B. P., Rodrigues, A. M. d. S., Martins, L. B., Monteze, N. M., Correia, M. I. T. D., Teixeira, A. L., et al. (2021). Acute Effects of Dry Extract of Ginger on Energy Expenditure in Eutrophic Women: A Randomized Clinical Trial. *Clin. Nutr. ESPEN* 41, 168–174. doi:10.1016/j.clnesp.2020.10.001

Fajrin, F. A., Nugroho, A. E., Nurrochmad, A., and Susilowati, R. (2020a). Ginger Extract and its Compound, 6-shogaol, Attenuates Painful Diabetic Neuropathy in Mice via Reducing TRPV1 and NMDAR2B Expressions in the Spinal Cord. *J. Ethnopharmacol.* 249, 112396. doi:10.1016/j.jep.2019.112396

Fajrin, F. A., Nugroho, A. E., Susilowati, R., and Nurrochmad, A. (2018). Molecular Docking Analysis of Ginger Active Compound on Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1). *Indones. J. Chem.* 18, 179–185. doi:10.22146/ijc.28172

Fajrin, F. A., Rahmayanti, F., and Pratoko, D. K. (2020b). The Binding Prediction of 6-Paradol and its Derivatives on TRPV1 Agonist as a New Compound for Treating Painful Diabetic Neuropathy. *J. Dasar* 21, 133. doi:10.19184/jid.v21i2.15501

Fan, C., Lu, W., Li, K., Ding, Y., and Wang, J. (2021). ACE2 Expression in Kidney and Testis May Cause Kidney and Testis Infection in COVID-19 Patients. *Front. Med.* 7, 1–9. doi:10.3389/fmed.2020.563893

Farhadi, M., Homaee, H., and Farzanegi Arkhazlou, P. (2020). The Effect of Aerobic Training and Ginger Extract on Lipid Profiles, Body Composition and Liver Enzymes in Obese Menopausal Women. *Ijdoj. Diabetes Obes.* 12. doi:10.18502/ijdo.v12i3.4458

Feng, T., Su, J., Ding, Z. H., Zheng, Y. T., Li, Y., Leng, Y., et al. (2011). Chemical Constituents and Their Bioactivities of "Tongling White Ginger" (Zingiber Officinale). *J. Agric. Food Chem.* 59, 11690–11695. doi:10.1021/JF202544W

Fitriyanti, D., and Sulung, R. (2020). Effectiveness of Ginger to Overcome Nausea and Vomiting Caused by Chemotherapy in Breast Cancer Patients. *Can. Oncol. Nurs. J.* 30, 3–5. doi:10.5737/2368807630135

Ghayur, M. N., and Gilani, A. H. (2005). Pharmacological Basis for the Medicinal Use of Ginger in Gastrointestinal Disorders. *Dig. Dis. Sci.* 50, 1889–1897. doi:10.1007/s10620-005-2957-2

Gholamnezhad, Z., Ghorani, V., Saadat, S., Shakeri, F., and Boskabady, M. H. (2018). The Effects of Medicinal Plants on Muscarinic Receptors in Various Types of Smooth Muscle. *Phytother Res.* 32, 2340–2363. doi:10.1002/ptr.6179

Gregersen, N. T., Belza, A., Jensen, M. G., Ritz, C., Bitz, C., Hels, O., et al. (2013). Acute Effects of Mustard, Horseradish, Black Pepper and Ginger on Energy Expenditure, Appetite, Ad Libitum Energy Intake and Energy Balance in Human Subjects. *Br. J. Nutr.* 109, 556–563. doi:10.1017/S0007114512001201

Grzanna, R., Lindmark, L., and Frondoza, C. G. (2005). Ginger--an Herbal Medicinal Product with Broad Anti-inflammatory Actions. *J. Med. Food* 8, 125–132. doi:10.1089/jmf.2005.8.125

Guan, W. J., Ni, Z. Y., Hu, Y., Liang, W. H., Ou, C. Q., He, J. X., et al. (2020). Clinical Characteristics of Coronavirus Disease 2019 in China. *N. Engl. J. Med.* 382, 1708–1720. doi:10.1056/nejmoa2002032

Gungor, H., Ekici, M., Onder Karayigit, M., Turgut, N. H., Kara, H., and Arslanbas, E. (2020). Zingerone Ameliorates Oxidative Stress and Inflammation in Bleomycin-Induced Pulmonary Fibrosis: Modulation of the Expression of TGF-B1 and iNOS. *Naunyn. Schmiedebergs. Arch. Pharmacol.* 393, 1659–1670. doi:10.1007/s00210-020-01881-7

Guo, T., Tan, S. B., Wang, Y., and Chang, J. (2018). Two new monoterpenoid glycosides from the fresh rhizome of Tongling White Ginger (Zingiber officinale). *Nat. Prod. Res.* 32, 71–76. doi:10.1080/14786419.2017.1333994

Haddy, F. J., Vanhoutte, P. M., and Feletou, M. (2006). Role of Potassium in Regulating Blood Flow and Blood Pressure. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290, 546–552. doi:10.1152/ajpregu.00491.2005

Hajimoosayi, F., Jahanian Sadatmahalleh, S., Kazemnejad, A., and Pirjani, R. (2020). Effect of Ginger on the Blood Glucose Level of Women with Gestational Diabetes Mellitus (GDM) with Impaired Glucose Tolerance Test (GTT): a Randomized Double-Blind Placebo-Controlled Trial. *BMC Complement. Med. Ther.* 20, 116–117. doi:10.1186/s12906-020-02908-5

Han, Y. A., Song, C. W., Koh, W. S., Yon, G. H., Kim, Y. S., Ryu, S. Y., et al. (2013). Anti-inflammatory Effects of the Zingiber Officinale roscoe Constituent 12-dehydrogingerdione in Lipopolysaccharide-Stimulated Raw 264.7 Cells. *Phytother Res.* 27, 1200–1205. doi:10.1002/ptr.4847

Hao, L., Scott, S., Abbasi, M., Zu, Y., Khan, M. S. H., Yang, Y., et al. (2019). Beneficial Metabolic Effects of Mirabegron In Vitro and In High-Fat Diet-Induced Obese Mice. *J. Pharmacol. Exp. Ther.* 369, 419–427. doi:10.1124/jpet.118.255778

Haridas, M., Sasidhar, V., Nath, P., Abhithaj, J., Sabu, A., and Rammanohar, P. (2021). Compounds of Citrus Medica and Zingiber Officinale for COVID-19 Inhibition: In Silico Evidence for Cues from Ayurveda. *Futur. J. Pharm. Sci.* 7. doi:10.1186/s43094-020-00171-6

Hashmi, D. R., Ismail, S., and Shaikh, G. H. (2007). Assessment of the Level of Trace Metals in Commonly Edible Vegetables Locally Available in the Markets of Karachi City. *Pakistan J. Bot.* 39, 747–751.

Hayat, I., Ahmad, A., Ahmed, A., Khalil, S., Gulfraz, M., and Kashmir, A. (2014). Exploring the Potential of Red Kidney Beans (Phaseolus Vulgaris l.) to Develop Protein Based Product for Food Applications. *J. Anim. Plant Sci.* 24, 860–868.

Heidari-Beni, M., Moravejolahkami, A. R., Gorgian, P., Askari, G., Tarrahi, M. J., and Bahreini-Esfahani, N. (2020). Herbal Formulation "turmeric Extract, Black Pepper, and Ginger" versus Naproxen for Chronic Knee Osteoarthritis: A Randomized, Double-Blind, Controlled Clinical Trial. *Phytother Res.* 34, 2067–2073. doi:10.1002/ptr.6671

Herman, L. (2015). Herb & Spice Companion: The Complete Guide to over 100 Herbs & Spices - Lindsay Herman - Google Books. Available at: https://books.google.co.za/books?hl=en&lr=&id=B6WSCgAAQBAJ&oi=fnd&pg=PP1&ots=L_r-p5iTU&sig=qzO6tx0iU-MwNh0hq5punJPaRH0&redir_esc=y#v=onepage&q&f=false (Accessed September 16, 2021).

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181, 271–e8. doi:10.1016/j.cell.2020.02.052

Hong, S. S., and Oh, J. S. (2012). Phenylpropanoid Ester from Zingiber Officinale and Their Inhibitory Effects on the Production of Nitric Oxide. *Arch. Pharm. Res.* 35, 315–320. doi:10.1007/S12272-012-0211-Y

Hori, Y., Miura, T., Hirai, Y., Fukumura, M., Nemoto, Y., Toriizuka, K., et al. (2003). Pharmacognostic Studies on Ginger and Related Drugs-Ppart 1: Five Sulfonated Compounds from Zingiberis Rhizome (Shokyo). *Phytochemistry* 62, 613–617. doi:10.1016/S0031-9422(02)00618-0

Horiguchi, H., Loftus, T. J., Hawkins, R. B., Raymond, S. L., Stortz, J. A., Hollen, M. K., et al. (2018). Innate Immunity in the Persistent Inflammation, Immunosuppression, and Catabolism Syndrome and its Implications for Therapy. *Front. Immunol.* 9, 1–20. doi:10.3389/fimmu.2018.00595

Hosseini, J., Mardi Mamaghani, A., Hosseinifar, H., Sadighi Gilani, M. A., Dadkhah, F., and Sepidarkish, M. (2016). The Influence of Ginger (Zingiber Officinale) on Human Sperm Quality and DNA Fragmentation: A Double-Blind Randomized Clinical Trial. *Int. J. Reprod. Biomed.* 14, 533–540. doi:10.29252/ijrm.14.8.533

Hu, Y., Amoah, A. N., Zhang, H., Fu, R., Qiu, Y., Cao, Y., et al. (2020). Effect of Ginger in the Treatment of Nausea and Vomiting Compared with Vitamin B6 and Placebo during Pregnancy: a Meta-Analysis. *J. Matern. Fetal Neonatal. Med.* 0, 1–10. doi:10.1080/14767058.2020.1712714

Hu, Y. W., Zhang, J., Wu, X. M., Cao, L., Nie, P., and Chang, M. X. (2018). TANK-binding Kinase 1 (TBK1) Isoforms Negatively Regulate Type I Interferon Induction by Inhibiting TBK1-IRF3 Interaction and IRF3 Phosphorylation. *Front. Immunol.* 9, 84–17. doi:10.3389/fimmu.2018.00084

Huang, H. C., Chou, Y. C., Wu, C. Y., and Chang, T. M. (2013). [8]-Gingerol Inhibits Melanogenesis in Murine Melanoma Cells through Down-Regulation of the MAPK and PKA Signal Pathways. *Biochem. Biophys. Res. Commun.* 438, 375–381. doi:10.1016/j.bbrc.2013.07.079

Hussain, J., Latif Khan, A., ur Rehman, N., ., Z., Khan, F., Tasleem Hu, S., et al. (2009). Proximate and Nutrient Investigations of Selected Medicinal Plants Species of Pakistan. *Pakistan J. Nutr.* 8, 620–624. doi:10.3923/PJN.2009.620.624

Ibrahim, M. A. A., Abdelrahman, A. H. M., Hussien, T. A., Badr, E. A. A., Mohamed, T. A., El-Seedi, H. R., et al. (2020). In Silico drug Discovery of Major Metabolites from Spices as SARS-CoV-2 Main Protease Inhibitors. *Comput. Biol. Med.* 126, 104046. doi:10.1016/j.compbiomed.2020.104046

Idris, O. A., Wintola, O. A., and Afolayan, A. J. (2019). Comparison of the Proximate Composition, Vitamins (Ascorbic Acid, α-Tocopherol and Retinol), Anti-nutrients (Phytate and Oxalate) and the GC-MS Analysis of the Essential Oil of the Root and Leaf of Rumex Crispus L. *Plants* 8. doi:10.3390/plants8030051

Iwasaki, Y., Morita, A., Iwasawa, T., Kobata, K., Sekiwa, Y., Morimitsu, Y., et al. (2006). A Nonpungent Component of Steamed Ginger--[10]-shogaol--increases adrenaline secretion via the activation of TRPV1. *Nutr. Neurosci.* 9, 169–178. doi:10.1080/110284150600955164

Jalali, M., Mahmoodi, M., Moosavian, S. P., Jalali, R., Ferns, G., Mosallanezhad, A., et al. (2020). The effects of ginger supplementation on markers of inflammatory and oxidative stress: A systematic review and meta-analysis of clinical trials. *Phytother Res.* 34, 1723–1733. doi:10.1002/ptr.6638

Javid, A. Z., Bazyar, H., Gholinezhad, H., Rahimlou, M., Rashidi, H., Salehi, P., et al. (2019). The effects of ginger supplementation on inflammatory, antioxidant, and periodontal parameters in type 2 diabetes mellitus patients with chronic periodontitis under non-surgical periodontal therapy. A double-blind, placebo-controlled trial. *Diabetes Metab. Syndr. Obes.* 12, 1751–1761. doi:10.2147/DMSO.S214333

Jiang, H., Sólyom, A. M., Timmermann, B. N., and Gang, D. R. (2005). Characterization of gingerol-related compounds in ginger rhizome (Zingiber officinale Rosc.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass. Spectrom.* 19, 2957–2964. doi:10.1002/RCM.2140

Jiang, H., Timmermann, B. N., and Gang, D. R. (2007). Characterization and identification of diarylheptanoids in ginger (Zingiber officinale Rosc.) using high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass. Spectrom.* 21, 509–518. doi:10.1002/RCM.2858

Jiménez-Aguilar, D. M., and Grusak, M. A. (2017). Minerals, vitamin C, phenolics, flavonoids and antioxidant activity of Amaranthus leafy vegetables. *J. Food Compost. Anal.* 58, 33–39. doi:10.1016/J.JFCA.2017.01.005

Jimoh, M. O., Afolayan, A. J., and Lewu, F. B. (2020). Nutrients and antinutrient constituents of Amaranthus caudatus L. Cultivated on different soils. *Saudi J. Biol. Sci.* 27, 3570–3580. doi:10.1016/J.SJBS.2020.07.029

Kamali, A., Beigi, S., Shokrpour, M., and Pazuki, S. (2020). The efficacy of ginger and doxedetomidine in reducing postoperative nausea and vomiting in patients undergoing abdominal hysterectomy. *Altern. Ther. Health Med.* 26, 28–33.

Kandeil, M. A., Mohameddeen, A., Abdel Gabbar, M., Ahmed, R. R., and Ali, S. M. (2019). Ameliorative effects of oral ginger and/or thyme aqueous extracts on productive and reproductive performance of V-line male rabbits. *J. Anim. Physiol. Anim. Nutr. (Berl)* 103, 1437–1446. doi:10.1111/jpn.13147

Karampour, N. S., Arzi, A., Rezaie, A., Pashmforoosh, M., and Kordi, F. (2019). Gastroprotective effect of zingerone on ethanol-induced gastric ulcers in rats. *Medicina (Kaunas)* 55, 1–9. doi:10.3390/medicina55030064

Kasonga, T. K., Coetzee, M. A. A., Kamika, I., Ngole-Jeme, V. M., and Benteke Momba, M. N. (2021). Endocrine-disruptive chemicals as contaminants of emerging concern in wastewater and surface water: A review. *J. Environ. Manage.* 277, 111485. doi:10.1016/j.jenvman.2020.111485

Kim, B., Kim, H. J., and Cha, Y. S. (2021). The protective effects of steamed ginger on adipogenesis in 3t3-l1 cells and adiposity in diet-induced obese mice. *Nutr. Res. Pract.* 15, 279–293. doi:10.4162/nrp.2021.15.3.279

Kubra, I. R., and Rao, L. J. (2012). An Impression on Current Developments in the Technology, Chemistry, and Biological Activities of Ginger (Zingiber officinale Roscoe). *Crit. Rev. Food Sci. Nutr.* 52, 651–688. doi:10.1080/10408398.2010.505689

Kucukler, S., Darendelioğlu, E., Caglayan, C., Ayna, A., Yıldırım, S., and Kandemir, F. M. (2020). Zingerone attenuates vancomycin-induced hepatotoxicity in rats through regulation of oxidative stress, inflammation and apoptosis. *Life Sci.* 259, 118382. doi:10.1016/j.lfs.2020.118382

Kumar Gupta, S., and Sharma, A. (2014). Medicinal properties of Zingiber officinale Roscoe-A Review. *IOSR J. Pharm. Biol. Sci.* 9, 124–129. doi:10.9790/3008-0955124129

Kuo, S. Y., Lin, R. J., Yeh, Y. T., Lee, J. Y., and Chen, C. Y. (2012). A new phenylalkanoid from the rhizomes of Zingiber officinale. *Nat. Prod. Res.* 26, 1318–1322. doi:10.1080/14786419.2011.576396

Kuoti Al-Rekabi, B. K. (2019). The protective role of taurine and Zingiber officinale on aluminum chloride induced testicular injury and impaired spermatogenesis in adults male rabbits: Biochemical, hormonal and histopathological study. *Plant Arch.* 19, 1755–1767.

Latona, D. F., Oyeleke, O. G., and Olayiwola, O. A. (2012). Chemical Analysis of Ginger Root. *IOSR J. Appl. Chem.* 1, 47–49. doi:10.9790/5736-0114749

Lee, H. Y., Park, S. H., Lee, M., Kim, H. J., Ryu, S. Y., Kim, N. D., et al. (2012). 1-Dehydro-[10]-gingerdione from ginger inhibits IKKβ activity for NF-κB activation and suppresses NF-κB-regulated expression of inflammatory genes. *Br. J. Pharmacol.* 167, 128–140. doi:10.1111/j.1476-5381.2012.01980.x

Lee, S. W., Lim, J.-H., Kim, M. S., Jeong, J.-H., Song, G.-Y., Lee, W. S., et al. (2011). Phenolic compounds isolated from Zingiber officinale roots inhibit cell adhesion. *Food Chem.* 128, 778–782. doi:10.1016/J.FOODCHEM.2011.03.095

Li, D., and Wu, M. (2021). Pattern recognition receptors in health and diseases. *Sig Transduct Target. Ther.* 6, 1–24. doi:10.1038/s41392-021-00687-0

Li, W.-J., Lin, R.-J., Yeh, Y.-T., Chen, H.-L., and Chen, C.-Y. (2013). Phenylalkanoids from Zingiber officinale. *Chem. Nat. Compd.* 49, 440–442. doi:10.1007/S10600-013-0633-X

Lim, Y. J., Min, H. Y., and Jang, W. G. (2021). Zingerone attenuates pi-induced vascular calcification via ampk-mediated timp4 expression. *J. Lipid Atheroscler.* 10, 62–73. doi:10.12997/jla.2021.10.1.62

Liu, W., Yin, D., Li, N., Hou, X., Wang, D., Li, D., et al. (2016). Influence of Environmental Factors on the Active Substance Production and Antioxidant Activity in Potentilla fruticosa L. and Its Quality Assessment. *Sci. Rep.* 6, 1–18. doi:10.1038/srep28591

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395, 565–574. doi:10.1016/S0140-6736(20)30251-8

Luettig, J., Rosenthal, R., Lee, I. M., Krug, S. M., and Schulzke, J. D. (2016). The ginger component 6-shogaol prevents TNF-α-induced barrier loss via inhibition of PI3K/Akt and NF-κB signaling. *Mol. Nutr. Food Res.* 60, 2576–2586. doi:10.1002/mnfr.201600274

Ma, J., Jin, X., Yang, L., and Liu, Z. L. (2004). Diarylheptanoids from the rhizomes of Zingiber officinale. *Phytochemistry* 65, 1137–1143. doi:10.1016/J.PHYTOCHEM.2004.03.007

Maharlouei, N., Tabrizi, R., Lankarani, K. B., Rezaianzadeh, A., Akbari, M., Kolahdooz, F., et al. (2019). The effects of ginger intake on weight loss and metabolic profiles among overweight and obese subjects: A systematic review and meta-analysis of randomized controlled trials. *Crit. Rev. Food Sci. Nutr.* 59, 1753–1766. doi:10.1080/10408398.2018.1427044

Mahat, S., Sapkota, S., Sapkota, S., and Katuwal, K. (2019). Factors Affecting Ginger Production in Surkhet District, Nepal. *Int. J. Appl. Sci. Biotechnol.* 7, 269–273. doi:10.3126/ijasbt.v7i2.24650

Mansour, M. S., Ni, Y. M., Roberts, A. L., Kelleman, M., Roychoudhury, A., and St-Onge, M. P. (2012). Ginger consumption enhances the thermic effect of food and promotes feelings of satiety without affecting metabolic and hormonal parameters in overweight men: A pilot study. *Metabolism* 61, 1347–1352. doi:10.1016/J.METABOL.2012.03.016

Mao, Q. Q., Xu, X. Y., Cao, S. Y., Gan, R. Y., Corke, H., Beta, T., et al. (2019). Bioactive compounds and bioactivities of ginger (zingiber officinale roscoe). *Foods* 8, 1–21. doi:10.3390/foods8060185

Mares, W. A. A., and Najam, W. S. (2012). The effect of Ginger on semen parameters and serum FSH, LH & testosterone of infertile men. *Tikrit Med. J.* 18, 322–329.

Martín-Domingo, M. C., Pla, A., Hernández, A. F., Olmedo, P., Navas-Acien, A., Lozano-Paniagua, D., et al. (2017). Determination of metalloid, metallic and mineral elements in herbal teas. Risk assessment for the consumers. *J. Food Compost. Anal.* 60, 81–89. doi:10.1016/J.JFCA.2017.03.009

Masuku, N. P., Unuofin, J. O., and Lebelo, S. L. (2021). Advances in Nanoparticle Delivery System for Erectile Dysfunction: An Updated Review. *Sex. Med.* 9, 100420. doi:10.1016/j.esxm.2021.100420

Masuku, N. P., Unuofin, J. O., and Lebelo, S. L. (2020). Promising role of medicinal plants in the regulation and management of male erectile dysfunction. *Biomed. Pharmacother.* 130, 110555. doi:10.1016/j.biopha.2020.110555

Mehrotra, N. (2020). Medicinal plants, aromatic herbs and spices as potent immunity defenders: Antiviral (COVID-19) perspectives. *Ap* 9, 30–49. doi:10.21276/ap.2020.9.2.4

Mehrzadi, S., Khalili, H., Fatemi, I., Malayeri, A., Siahpoosh, A., and Goudarzi, M. (2021). Zingerone Mitigates Carrageenan-Induced Inflammation Through Antioxidant and Anti-inflammatory Activities. *Inflammation* 44, 186–193. doi:10.1007/s10753-020-01320-y

Mele, M. A. (2019). Bioactive compounds and biological activity of ginger. *J. Multidiscip. Sci.* 1, 1–7. doi:10.33888/jms.2019.111

Mmasa, J. J., and Kizito Mhagama, J. (2017). *Social-Economic Factors Influencing Ginger (Zingiber Officinale) Productivity Among Smallholders Growers in Tanzania-Case of Same District the Impact of Fiscal Reforms on Poverty and Income Distribution in Tanzania: Macro-Micro Simulation Modelling View*, 12–29. Available at: https://www.researchgate.net/publication/316683733.

Mohammadzadeh Honarvar, N., Zarezadeh, M., Khorshidi, M., Makhdoomi Arzati, M., Yekaninejad, M. S., Abdollahi, M., et al. (2019). The effect of an oral ginger supplementation on NF-κB concentration in peripheral blood mononuclear cells and anthropomorphic data of patients with type 2 diabetes: A randomized double-blind, placebo-controlled clinical trial. *Complement. Ther. Med.* 42, 7–11. doi:10.1016/j.ctim.2018.10.019

Mohammed, A. A., Iyeghe-erakpotobor, G., Zahraddeen, D., Samuel, F. U., and Barje, P. P. (2019). Growth and Reproductive Performance of Rabbit Does fed Moringa Oleifera Leaf Meal Based Diets Supplemented With Garlic , Ginger or. *J. Anim. Prod. Amd Reseaarch* 31, 74–87.

Moradi-Podeh, B., Kheirollah, A., Ahmmadpour, F., Lamuchi-Deli, N., Payami, S.-A., and Mohammadzadeh, G. (2018). Effects of Zingiber officinale hydro-alcoholic extract on HMG-COA reductase level in the testis of streptozotocin-induced diabetic rats. *J. Herbmed Pharmacol.* 7, 94–99. doi:10.15171/jhp.2018.16

Muhammad, S., Sangi, A., Bawadekji, A., Alotaibi, N. M., and Aljalaud, N. A. (2019). Preventive and Curative Effects of Metformin, Nigella sativa, Punica granatum and Zingiber officinale on Male Reproductive Dysfunction in Diabetic Rats. *Allied Sci.* 8, 48–57. Available at: www.ijpras.com.

Munir, M., Memon, K. M., Jaffri, S. A., and Ali, H. (2021). Effect of Ginger-Naproxen on Knee Osteoarthritis: A Clinical Study. *J. Bahria Univ. Med. Dent. Coll.* 11, 17–21. doi:10.51985/fagq7533

Murugesan, S., Venkateswaran, M. R., Jayabal, S., and Periyasamy, S. (2020). Evaluation of the antioxidant and anti-arthritic potential of Zingiber officinale Rosc. by *In Vitro* and In Silico analysis. *South Afr. J. Bot.* 130, 45–53. doi:10.1016/j.sajb.2019.12.019

Mustafa, S. J., Qader, G. I., Qader, G. I., and Mahmood, S. A. F. (2016). Protective Role of Ginger on Testicular Tissue and Testosterone Hormone of Male Rats Exposed To Mono-Sodium Glutamate. *Jsmc* 6, 99–105. doi:10.17656/jsmc.10093

Mutthuraj, D., Vinutha, T., Gopenath, T., Kaginelli, B., Karthikeyan, M., Ashok, G., et al. (2020). Inhibition of Pro-Inflammatory Molecules by Ginger (Zingiber officinale Roscoe) and its Anti-Inflammatory Effects on Arthritis Patients. *J. Drug Deliv. Ther.* 10, 125–139. doi:10.22270/jddt.v10i2-s.3963

Naemi, A. R., Kashanitabar, V., Kamali, A., and Shiva, A. (2020). Comparison of the Effects of Haloperidol, Metoclopramide, Dexmedetomidine and Ginger on Postoperative Nausea and Vomiting After Laparoscopic Cholecystectomy. *J. Med. Life* 13, 206–210. doi:10.25122/jml-2019-0070

Nandi, S., Saleh-e-In, M., Rahim, M., Bhuiyan, M., Sultana, N., Ahsan, M., et al. (2013). Quality Composition and Biological Significance of the Bangladeshi and China Ginger (Zingiber Officinale Rosc.). *J. Microbiol. Biotechnol. Food Sci.* 2, 2283–2290.

Negi, R., Sharma, D. S., Gaur, D. R., Bahadur, A., and Jelly, P. (2021). Efficacy of Ginger in the Treatment of Primary Dysmenorrhea: A Systematic Review and Meta-analysis. *Cureus* 13. doi:10.7759/cureus.13743

Ni, W., Yang, X., Yang, D., Bao, J., Li, R., Xiao, Y., et al. (2020). Role of angiotensin-converting enzyme 2 (ACE2) in COVID-19. *Crit. Care* 24, 1–10. doi:10.1186/s13054-020-03120-0

Nile, S. H., and Park, S. W. (2015). Chromatographic analysis, antioxidant, anti-inflammatory, and xanthine oxidase inhibitory activities of ginger extracts and its reference compounds. *Ind. Crops Prod.* 70, 238–244. doi:10.1016/J.INDCROP.2015.03.033

Nour, A. H., Sook, S., and Yap, A. H. N. (2017). Extraction And Chemical Compositions of Ginger (Zingiber Officinale Roscoe) Essential Oils As Cockroaches Repellent. *Aust. J. Basic Appl. Sci.* 11, 1–8.

Nwinuka, N. M., Ibeh, G. O., and Ekeke, G. I. (2005). Proximate Composition And Levels Of Some Toxicants Four Commonly Consumed Spices. *J. Appl. Sci. Environ. Manag.* 9, 150–155. Available at: www.bioline.org.br/ja (Accessed August 30, 2021).

Odo, R. I., Mbegbu, E. C., and Anyanwu, L. N. (2020). Effect of aqueous ginger (Zingiber officinale) extract on sperm quality and haematology in lead acetate-treated male albino rats. *Trop. J. Pharm. Res.* 19, 1481–1485. doi:10.4314/tjpr.v19i7.21

Ogbuewu, I., Jiwuba, P., Ezeokeke, C., Uchegbu, M., Okoli, I., and Iloeje, M. (2014). Evaluation of Phytochemical and Nutritional Composition of. *Int. J. Agric. Rural Dev.* 17, 1663–1670.

Ohikhena, F. U., Wintola, O. A., and Afolayan, A. J. (2017). Proximate Composition and Mineral Analysis of Phragmanthera capitata (Sprengel) Balle, a Mistletoe Growing on Rubber Tree. *Res. J. Bot.* 12, 23–31. doi:10.3923/RJB.2017.23.31

Olennikov, D. N., and Kashchenko, N. I. (2015). 1-Dehydro-[14]-Gingerdione, a New Constituent from Zingiber officinale. *Chem. Nat. Compd.* 51, 877–881. doi:10.1007/s10600-015-1438-x

Olusanya, V., Rasaq, N., and Rasaq, O. (2018). Ameliorative effects of aqueous extracts of ginger and garlic on *Hibiscus sabdariffa*-induced testicular damage in male Wistar rats. *Egypt. Pharmaceut J.* 17, 21. doi:10.4103/EPJ.EPJ_29_17

Orisakwe, O. E., Orish, C. N., and Nwanaforo, E. O. (2020). Coronavirus disease (COVID-19) and Africa: Acclaimed home remedies. *Sci. Afr.* 10, e00620. doi:10.1016/j.sciaf.2020.e00620

Otunola, G. A., Oloyede, O. B., Oladiji, A. T., and Afolayan, A. J. (2010). Comparative Analysis of the Chemical Composition of Three Spices - Allium Sativum L. Zingiber Officinale Rosc. And Capsicum Frutescens L. Commonly Consumed in Nigeria. *Afr. J. Biotechnol.* 9, 6927–6931. doi:10.5897/ajb10.183

Pan, D., Zeng, C., Zhang, W., Li, T., Qin, Z., Yao, X., et al. (2019). Non-volatile pungent compounds isolated from Zingiber officinale and their mechanisms of action. *Food Funct.* 10, 1203–1211. doi:10.1039/c8fo02019a

Park, S. H., Jung, S. J., Choi, E. K., Ha, K. C., Baek, H. I., Park, Y. K., et al. (2020). The effects of steamed ginger ethanolic extract on weight and body fat loss: a randomized, double-blind, placebo-controlled clinical trial. *Food Sci. Biotechnol.* 29, 265–273. doi:10.1007/s10068-019-00649-x

Park, S. H., Kyeong, M. S., Hwang, Y., Ryu, S. Y., Han, S. B., and Kim, Y. (2012). Inhibition of LPS binding to MD-2 co-receptor for suppressing TLR4-mediated expression of inflammatory cytokine by 1-dehydro-10-gingerdione from dietary ginger. *Biochem. Biophys. Res. Commun.* 419, 735–740. doi:10.1016/j.bbrc.2012.02.091

Prasad, S., and Tyagi, A. K. (2015). Ginger and its constituents: Role in prevention and treatment of gastrointestinal cancer. *Gastroenterol. Res. Pract.* 2015, 1–11. doi:10.1155/2015/142979

Pourebrahim, M., Delirezh, N., Kian, M., and Soleimanzadeh, A. (2018). Ginger ameliorates reproductive toxicity of formaldehyde in male mice: Evidences for bcl-2 and bax. *J. Herbmed Pharmacol.* 7, 259–266. doi:10.15171/jhp.2018.39

Qian, Q. H., Yue, W., Chen, W. H., Yang, Z. H., Liu, Z. T., and Wang, Y. X. (2010). Effect of gingerol on substance P and NK1 receptor expression in a vomiting model of mink. *Chin. Med. J. (Engl)* 123, 478–484. doi:10.3760/cma.j.issn.0366-6999.2010.04.017

Qian, Q. H., Yue, W., Wang, Y. X., Yang, Z. H., Liu, Z. T., and Chen, W. H. (2009). Gingerol inhibits cisplatin-induced vomiting by down regulating 5-hydroxytryptamine, dopamine and substance P expression in minks. *Arch. Pharm. Res.* 32, 565–573. doi:10.1007/s12272-009-1413-9

Qian, W., Cai, X., Wang, Y., Zhang, X., Zhao, H., Qian, Q., et al. (2016). Effect of gingerol on cisplatin-induced pica analogous to emesis via modulating expressions of dopamine 2 receptor, dopamine transporter and tyrosine hydroxylase in the vomiting model of rats. *Yonago Acta Med.* 59, 100–110.

Rafiee, Z., Khorsandi, L., and Nejad-Dehbashi, F. (2019). Protective effect of Zingerone against mouse testicular damage induced by zinc oxide nanoparticles. *Environ. Sci. Pollut. Res. Int.* 26, 25814–25824. doi:10.1007/s11356-019-05818-3

Rahimlou, M., Yari, Z., Rayyani, E., Keshavarz, S. A., Hosseini, S., Morshedzadeh, N., et al. (2019). Effects of ginger supplementation on anthropometric, glycemic and metabolic parameters in subjects with metabolic syndrome: A randomized, double-blind, placebo-controlled study. *J. Diabetes Metab. Disord.* 18, 119–125. doi:10.1007/s40200-019-00397-z

Rajagopal, K., Byran, G., Jupudi, S., and Vadivalan, R. (2020). Activity of phytochemical constituents of black pepper, ginger, and garlic against coronavirus (COVID-19): An In Silico approach. *Int. J. Heal. Allied Sci.* doi:10.4103/ijhas.IJHAS

Rehman, R., Akram, M., Akhtar, N., Jabeen, Q., and Shah, S. M. A. (2010). Zingiber officinale Roscoe (pharmacological activity). *J. Med. Plants Res.* 5, 344–348.

Ren, X., Wang, S., Chen, X., Wei, X., Li, G., Ren, S., et al. (2020). Multiple expression assessments of ace2 and tmprss2 sars-cov-2 entry molecules in the urinary tract and their associations with clinical manifestations of covid-19. *Infect. Drug Resist.* 13, 3977–3990. doi:10.2147/IDR.S270543

Sampath, C., Rashid, M. R., Sang, S., and Ahmedna, M. (2017). Specific bioactive compounds in ginger and apple alleviate hyperglycemia in mice with high fat diet-induced obesity via Nrf2 mediated pathway. *Food Chem.* 226, 79–88. doi:10.1016/j.foodchem.2017.01.056

Sampath, C., Sang, S., and Ahmedna, M. (2016). *In Vitro* and *In Vivo* inhibition of aldose reductase and advanced glycation end products by phloretin, epigallocatechin 3-gallate and [6]-gingerol. *Biomed. Pharmacother.* 84, 502–513. doi:10.1016/j.biopha.2016.09.073

Sampath, S. J. P., Birineni, S., Perugu, S., Kotikalapudi, N., and Venkatesan, V. (2021). Therapeutic efficacy of 6-Gingerol and 6-Shogaol in promoting browning of white adipocytes vis-à-vis enhanced thermogenesis portrayed in high fat milieu. *Food Biosci.* 42, 101211. doi:10.1016/j.fbio.2021.101211

Sangwan, A., Kawatra, A., and Sehgal, S. (2014). Nutritional composition of ginger powder prepared using various drying methods. *J. Food Sci. Technol.* 51, 2260–2262. doi:10.1007/S13197-012-0703-2

Santos, R. A. S., Sampaio, W. O., Alzamora, A. C., Motta-Santos, D., Alenina, N., Bader, M., et al. (2018). The ACE2/Angiotensin-(1-7)/Mas axis of the renin-angiotensin system: Focus on Angiotensin-(1-7). *Physiol. Rev.* 98, 505–553. doi:10.1152/physrev.00023.2016

Sapkota, A., Park, S. J., and Choi, J. W. (2019). Neuroprotective effects of 6-shogaol and its metabolite, 6-paradol, in a mouse model of multiple sclerosis. *Biomol. Ther. (Seoul)* 27, 152–159. doi:10.4062/biomolther.2018.089

Saptarini, N. M., Sitorus, E. Y., and Levita, J. (2013). Structure-Based In Silico Study of 6-Gingerol, 6-Ghogaol, and 6-Paradol, Active Compounds of Ginger (Zingiber officinale) as COX-2 Inhibitors. *Ijc* 5, 12–18. doi:10.5539/ijc.v5n3p12

Saravanan, G., Ponmurugan, P., Deepa, M. A., and Senthilkumar, B. (2014). Anti-obesity action of gingerol: Effect on lipid profile, insulin, leptin, amylase and

lipase in male obese rats induced by a high-fat diet. *J. Sci. Food Agric.* 94, 2972–2977. doi:10.1002/jsfa.6642

Sarker, A., Rashid, M., Roy, D., Musarrat, M., and Bithi, U. (2021). Ginger (Zingiber officinale) powder from low temperature drying technique. *Bangladesh J. Sci. Ind. Res.* 56, 133–140. doi:10.3329/bjsir.v56i2.54320

Sattar, N. A., Hussain, F., Iqbal, T., and Sheikh, M. A. (2012). Determination of *In Vitro* antidiabetic effects of Zingiber officinale Roscoe. *Braz. J. Pharm. Sci.* 48, 601–607. doi:10.1590/S1984-82502012000400003

Sayed, S., Ahmed, M., El-Shehawi, A., Alkafafy, M., Al-Otaibi, S., El-Sawy, H., et al. (2020). Ginger water reduces body weight gain and improves energy expenditure in rats. *Foods* 9, 2–14. doi:10.3390/foods9010038

Schroeder, B. O., and Bäckhed, F. (2016). Signals from the gut microbiota to distant organs in physiology and disease. *Nat. Med.* 22, 1079–1089. doi:10.1038/NM.4185

Sedighmaroufi, S., Abbaskhani Davanloo, A., Moradimajd, P., Samaee, H., and Lavaie, M. (2020). The Effect of Ginger and Ondansetron on Post-Operative Nausea and Vomiting in Patients Undergoing Eye Surgery: A Triple-Blind Clinical Trial. *Shiraz E-med J.* 22. doi:10.5812/semj.102883

Seo, S. H., Fang, F., and Kang, I. (2021). Ginger (Zingiber officinale) attenuates obesity and adipose tissue remodeling in high-fat diet-fed c57bl/6 mice. *Int. J. Environ. Res. Public Health* 18, 1–13. doi:10.3390/ijerph18020631

Shahid, M., and Hussain, F. (2012). Chemical Composition and Mineral Contents of Zingiber officinale and Alpinia allughas (Zingiberaceae) Rhizomes. *IJCBS 2*, 101–104. Available at: www.iscientific.org/Journal.html (Accessed August 30, 2021).

Shahrajabian, M. H., Sun, W., and Cheng, Q. (2019). Clinical aspects and health benefits of ginger (Zingiber officinale) in Both traditional Chinese medicine and modern industry. *Soil Plant Sci.* 69, 546–556. doi:10.1080/09064710.2019.1606930

Shirin Adel, P. R., and Jamuna, P. (2010). Chemical composition and antioxidant properties of ginger root (Zingiber officinale). *J. Med. Plants Res.* 4, 2674–2679. doi:10.5897/JMPR09.464

Silveira, D., Prieto-Garcia, J. M., Boylan, F., Estrada, O., Fonseca-Bazzo, Y. M., Jamal, C. M., et al. (2020). COVID-19: Is There Evidence for the Use of Herbal Medicines as Adjuvant Symptomatic Therapy? *Front. Pharmacol.* 11, 581840–581844. doi:10.3389/fphar.2020.581840

Singletary, K. (2010). Ginger. *Nutr. Today* 45, 171–183. doi:10.1097/NT.0b013e3181ed3543

Song, S., Dang, M., and Kumar, M. (2019). Anti-inflammatory and renal protective effect of gingerol in high-fat diet/streptozotocin-induced diabetic rats via inflammatory mechanism. *Inflammopharmacology* 27, 1243–1254. doi:10.1007/s10787-019-00569-6

Sowley, E., and Kankam, F. (2019). Harnessing the Therapeutic Properties of Ginger (Zingiber officinale Roscoe) for the Management of Plant Diseases. *Ginger Cultiv. Its Antimicrob. Pharmacol. Potentials*. doi:10.5772/INTECHOPEN.90464

Srivastava, N., Ranjana, S., Singh, A. C., Shanker, K., Bawankule, D. U., and Luqman, S. (2019). Aromatic ginger (Kaempferia galanga L.) extracts with ameliorative and protective potential as a functional food, beyond its flavor and nutritional benefits. *Toxicol. Rep.* 6, 521–528. doi:10.1016/J.TOXREP.2019.05.014

Stoner, G. D. (2013). Ginger: is it ready for prime time? *Cancer Prev. Res.* 6, 257–262. doi:10.1158/1940-6207.CAPR-13-0055

Suk, S., Seo, S. G., Yu, J. G., Yang, H., Jeong, E., Jang, Y. J., et al. (2016). A Bioactive Constituent of Ginger, 6-Shogaol, Prevents Adipogenesis and Stimulates Lipolysis in 3T3-L1 Adipocytes. *J. Food Biochem.* 40, 84–90. doi:10.1111/jfbc.12191

Tai, W., He, L., Zhang, X., Pu, J., Voronin, D., Jiang, S., et al. (2020). Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell. Mol. Immunol.* 17, 613–620. doi:10.1038/s41423-020-0400-4

Tian, L., Qian, W., Qian, Q., Zhang, W., and Cai, X. (2020). Gingerol inhibits cisplatin-induced acute and delayed emesis in rats and minks by regulating the central and peripheral 5-HT, SP, and DA systems. *J. Nat. Med.* 74, 353–370. doi:10.1007/s11418-019-01372-x

Tripathi, S., Bruch, D., and Kittur, D. S. (2008). Ginger extract inhibits LPS induced macrophage activation and function. *BMC Complement. Altern. Med.* 8, 1–7. doi:10.1186/1472-6882-8-1

Tzeng, T. F., Chang, C. J., and Liu, I. M. (2014). 6-Gingerol Inhibits Rosiglitazone-Induced Adipogenesis in 3T3-L1 Adipocytes. *Phytother Res.* 28, 187–192. doi:10.1002/ptr.4976

Ugwoke, C., and Nzekwe, U. (2010). *Phytocvhemistry and Proximate Composition of*, 1–7.

Unuofin, J. O., and Lebelo, S. L. (2020). Antioxidant Effects and Mechanisms of Medicinal Plants and Their Bioactive Compounds for the Prevention and Treatment of Type 2 Diabetes: An Updated Review. *Oxidative Med. Cell Longevity* 2020, 1–36. doi:10.1155/2020/1356893

Unuofin, J. O., and Lebelo, S. L. (2021). UHPLC-QToF-MS characterization of bioactive metabolites from Quercus robur L. grown in South Africa for antioxidant and antidiabetic properties. *Arabian J. Chem.* 14, 102970. doi:10.1016/j.arabjc.2020.102970

Unuofin, J. O., Otunola, G. A., and Afolayan, A. J. (2017a). Essential Oil Composition, Nutrient and Anti-nutrient Analysis of Vernonia mespilifolia Less. *Res. J. Bot.* 12, 38–45. doi:10.3923/rjb.2017.38.45

Unuofin, J. O., Otunola, G. A., and Afolayan, A. J. (2018). *In Vitro* α-amylase, α-glucosidase, lipase inhibitory and cytotoxic activities of tuber extracts of Kedrostis africana (L.) Cogn. *Heliyon* 4, e00810. doi:10.1016/j.heliyon.2018.e00810

Unuofin, J. O., Otunola, G. A., and Afolayan, A. J. (2017b). Nutritional evaluation of Kedrostis africana (L.) Cogn: An edible wild plant of South Africa. *Asian Pac. J. Trop. Biomed.* 7, 443–449. doi:10.1016/j.apjtb.2017.01.016

Uthaipaisanwong, A., Oranratanaphan, S., and Musigavong, N. (2020). Effects of ginger adjunct to the standard prophylaxis on reducing carboplatin and paclitaxel-induced nausea vomiting: a randomized controlled study. *Support. Care Cancer* 28, 3831–3838. doi:10.1007/s00520-019-05201-5

Venkatakrishnan, K., Chiu, H. F., and Wang, C. K. (2019). Extensive review of popular functional foods and nutraceuticals against obesity and its related complications with a special focus on randomized clinical trials. *Food Funct.* 10, 2313–2329. doi:10.1039/c9fo00293f

Wagesho, Y., and Chandravanshi, B. S. (2015). Levels of essential and non-essential metals in ginger (Zingiber officinale) cultivated in Ethiopia. *SpringerPlus* 4, 107–113. doi:10.1186/S40064-015-0899-5

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., et al. (2020). Clinical Characteristics of 138 Hospitalized Patients with 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 323, 1061–1069. doi:10.1001/jama.2020.1585

Wang, H. M., Kao, C. L., Li, W. J., Li, H. T., and Chen, C. Y. (2018). Two New Phenylalkanoids from the Rhizomes of Zingiber officinale. *Chem. Nat. Compd.* 54, 7–9. doi:10.1007/S10600-018-2246-X

Yaghubi Beklar, S., Hamzeh, M., Karimpour, A., and Talebpour Amiri, F. (2019). Zingiber Officinale Mitigates Diazinon-Induced Testicular Toxicity via Suppression of Oxidative Stress and Apoptosis in Mice Model. *J. Res. Pharm.* 23, 224–234. doi:10.12991/jrp.2019.128

Zammel, N., Saeed, M., Bouali, N., Elkahoui, S., Alam, J. M., Rebai, T., et al. (2021). Antioxidant and Anti-Inflammatory Effects of Zingiber officinale roscoe and Allium subhirsutum: In Silico, Biochemical and Histological Study. *Foods* 10, 1383. doi:10.3390/foods10061383

Zeiner, M., and Juranović Cindrić, I. (2017). Review - trace determination of potentially toxic elements in (medicinal) plant materials. *Anal. Methods* 9, 1550–1574. doi:10.1039/C7AY00016B

Zhang, F. L., Zhou, B. W., Yan, Z. Z., Zhao, J., Zhao, B. C., Liu, W. F., et al. (2020). 6-Gingerol attenuates macrophages pyroptosis via the inhibition of MAPK signaling pathways and predicts a good prognosis in sepsis. *Cytokine* 125, 154854. doi:10.1016/j.cyto.2019.154854

Zhang, G., Nitteranon, V., Chan, L. Y., and Parkin, K. L. (2013). Glutathione conjugation attenuates biological activities of 6-dehydroshogaol from ginger. *Food Chem.* 140, 1–8. doi:10.1016/j.foodchem.2013.02.073

Zhang, M., Zhao, R., Wang, D., Wang, L., Zhang, Q., Wei, S., et al. (2021). Ginger (Zingiber officinale Rosc.) and its bioactive components are potential resources for health beneficial agents. *Phytother Res.* 35, 711–742. doi:10.1002/ptr.6858

Zhao, D., Gu, M. Y., Xu, J. L., Zhang, L. J., Ryu, S. Y., and Yang, H. O. (2019). Anti-neuroinflammatory Effects of 12-Dehydrogingerdione in LPS-Activated Microglia through Inhibiting Akt/IKK/NF-κB Pathway and Activating Nrf-2/HO-1 Pathway. *Biomol. Ther. (Seoul)* 27, 92–100. doi:10.4062/biomolther.2018.104

Zhu, J., Chen, H., Song, Z., Wang, X., and Sun, Z. (2018). Effects of Ginger (Zingiber officinale Roscoe) on Type 2 Diabetes Mellitus and Components of the Metabolic Syndrome: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Evidence-Based Complement. Altern. Med.* 2018, 1–11. doi:10.1155/2018/5692962

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi:10.1056/nejmoa2001017

Zhu, Y., Wang, C., Luo, J., Hua, S., Li, D., Peng, L., et al. (2021). The protective role of Zingerone in a murine asthma model via activation of the AMPK/Nrf2/HO-1 pathway. *Food Funct.* 12, 3120–3131. doi:10.1039/d0fo01583k

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 November 2021
doi: 10.3389/fphar.2021.760492

Medicinal Plants Used by Traditional Healers in Algeria: A Multiregional Ethnobotanical Study

*Khadidja Belhouala and Bachir Benarba**

Laboratory Research on Biological Systems and Geomatics, Faculty of Nature and Life Sciences, University of Mascara, Mascara, Algeria

Traditional medicine is the cornerstone that boosts scientific research to explore new therapeutic approaches. The study aimed to assess the traditional knowledge and use of medicinal plants to treat various ailments by Algerian traditional healers. Forty traditional healers were face-to-face interviewed in three different Algerian areas (West, Kabylia, and Sahara). The data collected were analyzed using quantitative indices such as fidelity level (FL) and informant consensus factor (F_{IC}). A total of 167 species belonging to 70 families were recorded. Lamiaceae (13%), Asteraceae (13%), Apiaceae (7%), and Rosaceae and Fabaceae (5% each) were the most cited families. The survey revealed that leaves were the most used parts of the plants (29%). Furthermore, decoction (35%), raw (24%), and infusion (19%) were the common modes for the remedies' preparation. Here, 15% of the total species were newly reported as medicinal plants. Besides, it was reported for the first time a total of 47 new therapeutic uses for 20 known plant species. Of 17 ailments categories, cancer was presented by 44 species, showing the highest F_{IC} of 0.46. *Marrubium vulgare* L., *Artemisia herba-alba* Asso., *Zingiber officinale* Roscoe., and *Juniperus phoenicea* L. recorded the maximum fidelity value of 100%. Therefore, our study reveals strong ethnomedicinal knowledge shared by local populations living in the three regions studied. The medicinal species with a high FL could be promising candidates for identifying new bioactive molecules.

Keywords: Algeria, medicinal plants (herbal drugs), traditional healers, phytotherapy, ethnobotany

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Gizem Emre, Marmara University, Turkey
Emin Ugurlu, Bursa Technical University, Turkey
Cecilia Cordero, St. Dominic College of Asia, Philippines

****Correspondence:***
Bachir Benarba
bachirsb@yahoo.fr

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *18 August 2021*
Accepted: *25 October 2021*
Published: *29 November 2021*

Citation:
Belhouala K and Benarba B (2021) Medicinal Plants Used by Traditional Healers in Algeria: A Multiregional Ethnobotanical Study. Front. Pharmacol. 12:760492. doi: 10.3389/fphar.2021.760492

INTRODUCTION

Medicinal plants are still considered important and promising sources of drugs to treat various diseases. Their therapeutic uses, vernacular names, modes of preparation, and routes of administration were orally transmitted to constitute a local ancestral knowledge characterizing each population or ethnic group living in a specified area. Actually, from the identification of morphine in opium in the 19th century, drug discovery is based on ethnobotanical investigations and local ethnomedicinal knowledge (Ojah, 2020). Moreover, almost 35% of drugs and about 80% of anticancer drugs used in clinical practice are plants- or natural products-derived (Calixto, 2019).

Algeria is the largest country in the Mediterranean basin, Africa, and the Arab region with a total area of almost 2.4 million km^2 and 1,600 of coastline. In addition to a diversified climate, Algeria is characterized by a rich flora consisting of 4,000 taxa, 917 genera, and 131 families. Moreover, owing to its ancient history as one of the first cradles of *Homo sapiens* and civilization in the world, Algeria possesses an important and rich cultural diversity. Although several studies have been undertaken to

FIGURE 1 | Study areas.

document the local knowledge regarding the use of medicinal plants to treat different diseases (Benarba, 2015; Benarba et al., 2016; Chelghoum et al., 2021; Mechaala et al., 2021), the Algerian ancestral ethnomedicinal knowledge deserves more ethnobotanical investigations. On the other hand, almost all of these ethnobotanical studies covered one region and therefore the same culture and traditions. The present study was carried out in three important regions of Algeria: North-West, Kabylia (Center), and Sahara (South) to 1) record the medicinal species used for medicinal purposes and the local therapeutic practices of traditional healers and 2) document the species newly reported as medicinal plants and new uses.

MATERIAL AND METHODS

Description of the Study Area

The multiregional study was carried out in three regions in Algeria: North-West, Kabylia (Center), and Sahara (South) (**Figure 1**). The ethnobotanical investigations in the North-West were performed in five departments: Mascara (area = 5,139 Km^2), Oran (area = 2,114 Km^2), Mostaganem (area = 2,269 Km^2), Sid Bel Abbas (area = 9,150 Km^2), and Tiaret (area = 20,673 Km^2) and their surrounding villages located from the Mediterranean Sea to the Moroccan borders. Although no data is available regarding the flora of each department, that of the region of Oran showed the presence of 92 taxa; out of them, 72 remain endemic (Miara et al., 2018). The ethnobotanical study carried out in Center Algeria covered one city named Tizi Ouzou and its surrounding villages covering an area of 3,568 Km^2, located 100 km east of the capital (Algiers) and 30 km south of the Mediterranean Sea. Owing to its favorable climate, this region is characterized by an important vegetal diversity, including 659 species, 95 subspecies, 2 varieties, and 1 forma from 381 genera and 88 botanical families (Meddour and Sahar, 2021). The south areas included in the present study covered three of the main cities of the Algerian large desert: Ghardaïa (area = 32,256 Km^2), Bechar (area = 161,400 Km^2), and El Bayad (area = 71,686 Km^2), characterized by important cultural, ecological, climatic, and botanical diversity (Taïbi et al., 2020; Taïbi et al., 2021). This desert wide region is characterized by sparse vegetation, grasses appearing during a short period of the year, and rare trees. According to its adaptation mode to the drought, Saharan flora can be divided into ephemeral plants, called "achebs" with a short vegetative cycle of one to four months, and perennial plants with morphological and anatomical adaptations based on an enhanced absorbent system and reduced evaporating surface. The local flora comprises 130 species belonging to 40 families (Chehma and Djebbar, 2008).

Data Collection

The ethnobotanical investigations were carried out from December 2019 to June 2020. During this period, we visited

TABLE 1 | Demographic characteristics of the traditional healers.

Gender	n	100%
F	23	57.5%
M	17	42.5%
Areas		
West	26	65.0%
Kabylia	6	15.0%
Sahara (desert)	8	20.0%
Age		
34–49	4	10.0%
50–65	11	27.5%
66–81	15	37.5%
82–98	10	25.0%
Education		
Illiterate	27	67.5%
Literate	13	32.5%
Inherited	28	70.0%
Acquired	7	17.5%
Unknown	5	12.5%

13 cities and 19 villages in the study areas, searching for traditional healers. The data had been gathered from 40 informants; 87.5% of them were professionals, acquiring the therapeutic knowledge by the transition from generation to generation, and 12.5% were herbalists. The traditional healers were interviewed by a face-to-face interview in their homes or workplaces to fill out a questionnaire and collect the data. The responses included the demographic characteristics of healers (**Table 1**) and other information related to the uses of medicinal plants, such as the vernacular name, ailments treated, parts used, preparation, and administration modes. The species were given in their local names in Arabic or Amazigh.

Botanical Identification

The medicinal species mentioned by the traditional healers were collected, coded, and dried. Voucher specimens were deposited at the Herbarium of the Laboratory of Research on Biological Systems and Geomatics (LRSBG), University of Mascara, Algeria.

The taxonomic identification was performed by Professor Bachir Benarba using the standard literature (Baba Aissa, 1999; Kunkele and Lohmeyer, 2007; Trabut, 2015).

Ailment Categories

Table 2 shows more than 100 diseases recorded from the ethnobotanical investigations. All the ailments were classified into 17 categories based on the vital system/organ affected or type of damage.

Data Analysis

Ethnobotanical indices, fidelity level (FL) and informant consensus factor (F_{IC}), were calculated to analyze the data obtained. Consensus indicators FL and F_{IC} were used to quantify the relevance and importance of a species for a given ailment category and the agreement of its use among healers, respectively (Hoffman and Gallaher, 2007; Khan et al., 2014). FL and F_{IC} were calculated using the following formulas (Morvin Yabesh et al., 2014):

Fidelity level: FL (%) = (Np/N)*100

Np is the number of use reports for a given species reported for a particular ailment category, and *N* is the total number of use reports cited for any given species.

Informant Consensus Factor: F_{IC} = (Nur-Nt)/(Nur-1)

Nur is the number of use citations in each category, and Nt is the number of species reported in each category.

TABLE 2 | Ailments categories.

Category	Ailments/disorders	Abbreviation
Kidney diseases	Kidney failure, kidney problems, and urolithiasis	KD
Gastrointestinal system diseases	Irritable bowel syndrome (IBS), ulcers, heartburn, hemorrhoids, stomach ache, diarrhea, constipation, colitis, flatulence, gastrointestinal diseases, gallstones, liver diseases, and jaundice/icterus	GISD
Skin diseases	Limb swelling, itchy skin, tinea capitis, scalp ringworm, heel fissures, skin diseases and ulcer, urticaria, lichen, chalazion, albinism, dermatitis or eczema, boils, head ulcers, skin ulcers, leprosy, festering wounds, and burns	SD
Cancer	Cancer, blood cancer, gum tumors, tumors, skin pimples, uterine cysts/tumors, breast cysts, breast tumors lung tumors, liver cancer, breast cancer, legs cancer, skin cancer, early stage cancer, and stomach cancer	Can
Endocrine system diseases	Goiter and diabetes	ESD
Respiratory tract diseases	Sinusitis, bronchitis, nasal-lung inflammation, pneumonia, lung filtering/smoker, chest and lung diseases, cough, pulmonary-breathing problem, asthma, allergy, cold, and chest pain	RTD
Skeletomuscular system disorder	Osteoarthritis, bones pain, acute arthritis, gout, back pain, arthritis, arthrosis, fracture, osteoporosis, and moving difficulty	SMSD
Cardiovascular system diseases	Cardiovascular diseases, hypertension, clogged arteries, and hypercholesterolemia	CVSD
General health	Earache and deafness, hoarseness, sore throat, fever, mouth ulcer, halitosis, gingivitis, anxiety disorders, and hypochondria, tonsillitis, and incurable diseases	GH
Haircare	Baldness, alopecia areata, and hair loss	HC
Nervous system	Migraine, headache, dizziness, head problems, psychosis, insomnia, epilepsy, and sciatica	NS
Sexual-reproductive problems	Uterine problems, uterine microbe, infections, infertility, breast milk outage, and prostatitis	SRP
Infectious diseases	Laryngitis	ID
Poisoning	Scorpion sting and poisoning	P
Hematological system diseases	Anemia, spleen diseases, and blood purification	HSD
Urology system diseases	Bladder disease, urinary tract infection/inflammation, and cystolithiasis	USD

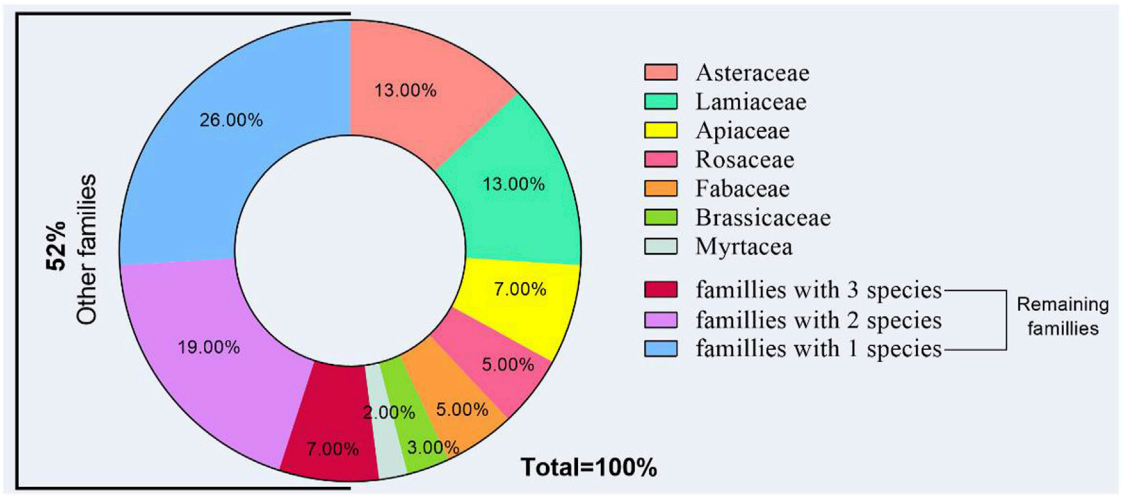


FIGURE 2 | Distribution of reported species among the botanical families.

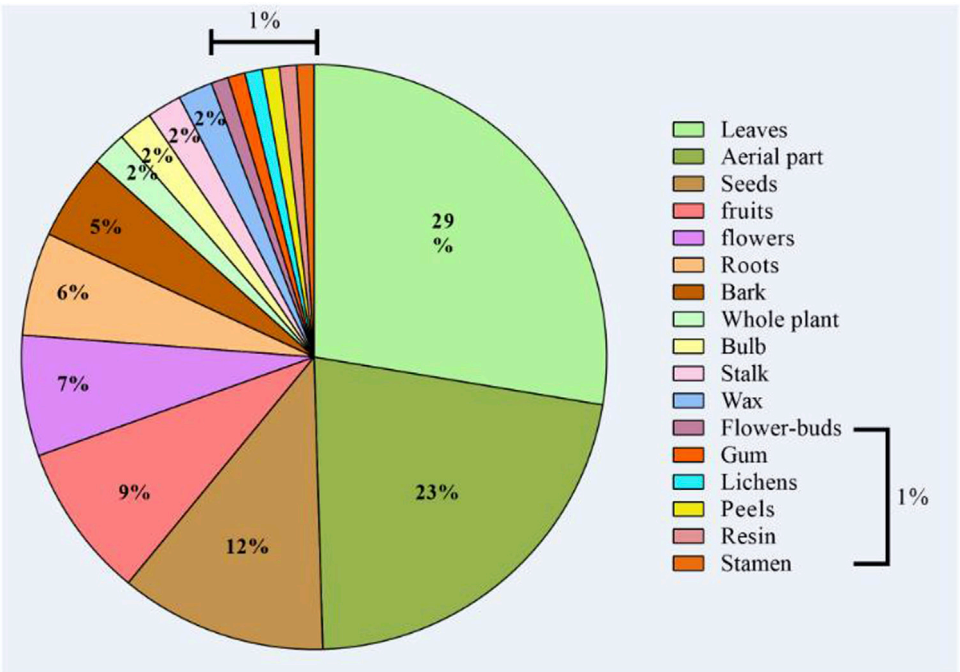


FIGURE 3 | Plants parts used by traditional healers.

RESULTS

Botanical Diversity, Parts Used, Modes of Preparation, and Administration

This study revealed 167 species of medicinal species used for therapeutic purposes, belonging to 70 families. Lamiaceae (13%), Asteraceae (13%), Apiaceae (7%), Rosaceae (5%), and Fabaceae (5%) were the most cited families, while the 66 remaining families (57%) had between 1 and 5 species in each (**Figure 2**). As shown in **Figure 3**, the plant parts most frequently used were leaves (29%), followed by aerial part (23%), seeds (12%), fruits (9%), and flowers (7%). Some used parts were lower than those, such as roots (6%), bark (5%), and whole plant, bulb, wax, and stalk (2% each). Besides, peels, flower buds, stamen, and gum were slightly used (1%).

Regarding the preparation methods (**Figure 4**), decoction (35%), raw (24%), infusion (19%), paste (10%), and maceration (8%) were the dominant methods for remedies preparation. Surprisingly, the current study recorded burning (2%) as an uncommon/novel mode used by traditional healers. In addition,

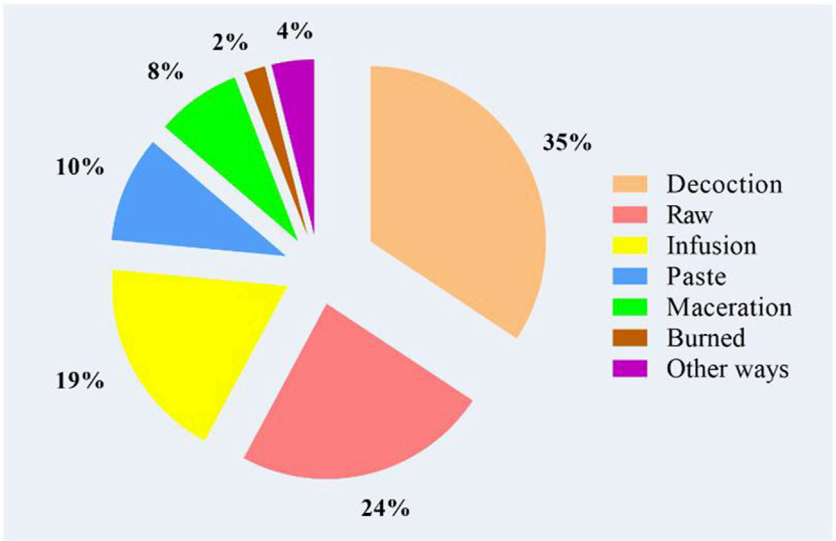


FIGURE 4 | Modes of preparation used by traditional healers.

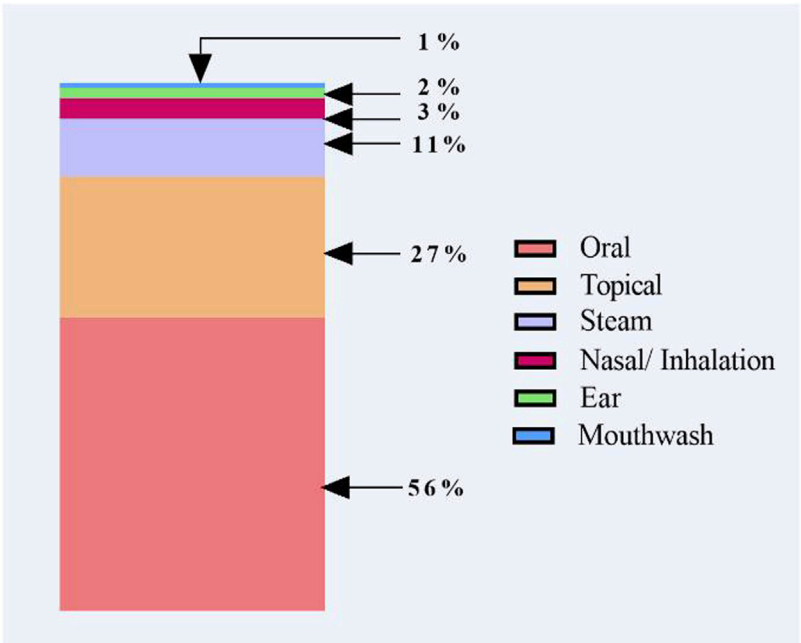


FIGURE 5 | Modes of administration.

the common administration route was the oral ingestion (56%) followed by external application as an ointment on the skin and compress (27%), steam (11%), or internally tract as nasal inhalation (3%), intraear (2%), and the mouthwash (1%) (**Figure 5**). Of the remedy's prescription, 64% of medicinal plants were mixed with other ingredients, and 36% were taken without addition. Indeed, there were 32 species combined with one plant, 21 plants with two plants, 19 plants with three or four plants, and 14 plants with more than four plants. Furthermore, some herbal mixtures (43%, n = 74 species) were prepared by adding different adjuvants (**Figure 6**). These adjuvants include honey (25 use reports) followed by olive oil (22), fat (8), vinegar (7), plant oil, and sulfur and tar (6 times each).

New Reports and New Uses

By comparing the data from this study with other ethnobotanical researches carried out in Algeria and neighboring countries (Morocco, Tunisia, Mauritania, Nigeria, and Mali), we found that 11% of total species have not been previously reported as medicinal plants. Of them, 11 species were documented in

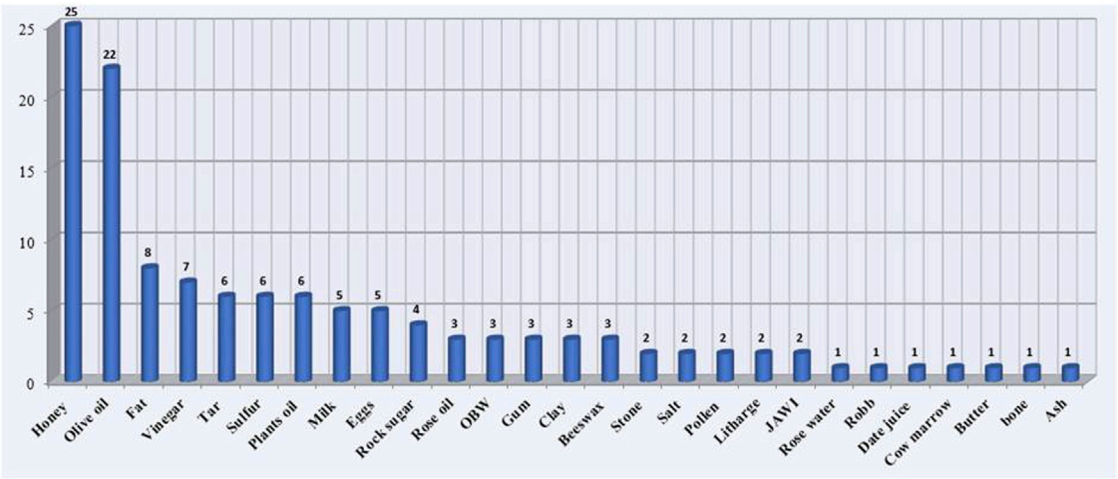


FIGURE 6 | Adjuvants added.

TABLE 3 | New recorded medicinal plants used by traditional healers in Algeria (West-Kabylia-Sahara).

Scientific name	Local name	Ailments	Number of informants citing plants	Number of citations
Inula helenium L.	مطهر	Can: 2* breast cancer and legs cancer	1	2
Centaurea acaulis L.	سنتوريا او القنطريون	Can: 2* breast cancer and legs cancer	1	2
Melilotus officinalis (L.) Pall.	الهندقوق إكليل الملك	RTD: 1* chest and lung diseases GISD: 1* IBS	1	2
Lupinus micranthus Guss.	الترمز المر الدقيق	ESD: 1* diabetes	1	1
Boswellia ameero Balf.f.	اللبان	RTD: 1* chest and lung diseases	1	1
Carduus nutans L.	شوك المحني	HC: 1* alopecia areata	1	1
Quercus faginea Lam.	العفص	SRP: 1* uterine microbe	1	1
Gentiana acaulis L.	كف الذئب او الجنطيانا	Can: 1* breast cancer and legs cancer	1	1
Digitalis purpurea L.	القمعية او الديجيتال	CVSD: 1* cardiovascular diseases	1	1
Cistanche tubulosa (Schenk) Wight	ذنون	GISD: 1* colitis	1	1
Hypecoum procumbens L.	الخشاش) زهرة)	Can: 1* skin pimples and tumors	1	1
Phyllanthus niruri L.	الأملج	Can: 1* cancer RTD: 1* cough	1	2
Verbascum sinuatum L.	مصلحة الأنظار أو البوصير أو تاراو	SMSD: 1* osteoarthritis	1	1
Lycium shawii Roem. and Schult.	العوسج	SD: 2* skin ulcers and leprosy RTD: 1* pneumonia	2	3
Tamarix aphylla (L.) H.Karst.	طرفاء	NS: 1* headache	1	1
Ulmus rubra Muhl.	الدردار	ID: 1* laryngitis SMSD: 1* moving difficulty	2	2
Telephium imperati L.	تنيغرمست	GH: 1* mouth ulcer HSD: 1* anemia	1	2
Humulus lupulus L.	لجنج	HC: 1* alopecia areata and baldness GISD: 2* hemorrhoids	2	3
Cirsium creticum (Lam.) d'Urv.	شوك الرمح	GISD: 1* hemorrhoids	1	1

Sahara, 5 in Kabylia, and 3 in West Algeria. These species are used as remedies to treat both common ailments and incurable diseases. The new reports are listed in **Table 3** with their vernacular names, parts used, therapeutic uses, and modes of administration.

Surprisingly, 4 out of the 19 new species (*Lycium shawii* Roem. and Schult, *Humulus lupulus* L., *Crataegus azarolus* L., *Centaurea acaulis* L., and *Verbascum sinuatum* L.) were highly cited by the informants. *V. sinuatum* is used to treat gastrointestinal and respiratory tract diseases such as pneumonia, using the

TABLE 4 | List of new therapeutic uses recorded in Algeria (West-Kabylia-Sahara).

Botanical name	Part used	New uses	Preparation methods	Previously reported uses	References
Silybum marianum (L.) Gaertn.	Leaves	Can: 2 breast cancer and legs cancer	Raw	Biliary, liver disorders, and degenerative necrosis Jaundice and enlarged spleen	Lahlah et al. (2012)
Prunus persica (L.) Batsch.	Leaves	Can: 2* cancer Sd: 1* limb swelling	Raw Infusion	Cough, constipation, and menstruation absent	Lin et al. (2021) Al-Fatimi. (2019)
Inula helenium L.	Capitulum	Can: 2* breast cancer and legs cancer	Raw	Hematomas, relief of bruises, joint pains, rheumatism, and gastrointestinal, otolaryngological, and respiratory diseases	Teixidor-Toneu et al. (2016) Obón et al. (2012)
Calendula arvensis M. Bieb.	Capitulum	Rtd: 1* pneumonia	Decoction	Burns, varicose veins, eczema, fungus, warts, and wounds	Lievre et al. (1992) Lavagna et al. (2001)
Artemisia campestris L.	Leaves	P: 1* scorpion sting	Raw	Digestive troubles, gastric ulcer, and menstrual pains	Baba Aissa (1991)
Cichorium intybus L.	Aerial part/ roots	Usd: 2* cystolithiasis and bladder disease	Decoction Decoction and raw	Urinary tract infections and urolithiasis, digestive problems, kidney diseases, diabetes, and nervous disorders	Sekkoum et al. (2011) Miara et al. (2013)
		Gisd: 2* hemorrhoids and liver diseases	Raw		El-Hilaly et al. (2003)
			Decoction		Daoudi et al. (2016) Benarba et al. (2015)
		Can: 4* breast cancer and legs cancer	Decoction		
		Hsd: 1* spleen diseases	Decoction		
Carlina gummifera (L.) Less.	Capitulum/ leaves/roots	Srp: 2* infertility and uterine problems	Decoction Decoction	Epilepsy, psoriasis, ulcers, and hemorrhage	Bellakhdar (1997) Ahid et al. (2012)
		Usd: 2* urinary tract infection and bladder disease	Decoction Decoction		Hammiche et al. (2013)
		Smsd: 1* osteoarthritis	Decoction		
Echinops spinosissimus Turra.	Aerial part	Can: 1* skin pimples and tumors	Decoction	Hypotensive, diuretic, hypoglycemic, for stomachic effects, liver disorders, and postpartum care	Bouzabata (2013)
Clinopodium nepeta (L.) Kuntze.	Aerial part	Gisd: 1* IBS	Decoction	Colon ailments, abdominal pain, influenza, heart problems, bee, and insect stings	Mattalia et al. (2020)
		Esd: 1* diabetes	Decoction		Çelik et al. (2021)
		Cvsd: 1* cholesterol	Decoction		
		Kd: 1* kidney failure	Decoction		
		Usd: 1* bladder disease	Decoction		
Mentha rotundifolia (L.) Huds.	Aerial part	Gisd: 1* IBS	Decoction	Mental illnesses, colds, respiratory problems and to protect removal of “curses” and “evil spirits”	Arnold and Gulumian (1984) Pooley (2005)
Potentilla erecta (L.) Raeusch.	Roots	Srp: 1* breast milk outage	Maceration	Wounds, certain forms of cancer, infections, diarrhea, and diabetes mellitus	Synowiec et al. (2014)
		Rtd: 1* chest and lung diseases	Raw		
		Gisd: 2* stomach ache and ulcers	Maceration Decoction		
Amaranthus spinosus L.	Aerial part	Srp: 1* infertility	Decoction	Internal bleeding, diarrhea, excessive menstruation, and snake bites. Ulcerated mouths, nosebleeds, and wounds Menorrhagia, gonorrhea, eczema and colic, fevers, and urinary troubles	Saravanan (2016)
Mahonia aquifolium (Pursh) Nutt.	Whole plant	Can: 2* breast cancer and legs cancer	Raw	Skin diseases, psoriasis, and diabetes	Galle et al. (1994) Missoun et al. (2018)
Boswellia ameero Balf.f.	Gum	Rtd: 1* chest and lung diseases	Maceration	Antitumor activity	Shao et al. (1998)
Commiphora myrrha (Nees) Engl.	Wax	Can: 2* breast cancer and legs cancer	Raw	Mouth ulcers, gingivitis, sinusitis, glandular fever, brucellosis, and antiparasitic agent	Abdel-Hay et al. (2002)
			Raw	Autoimmune diseases, rheumatic pains, amenorrhea, fever, stomach complaints, gall bladder, nephrosis syndrome, chest ailments, snake and scorpion bites, mouth ulcer, and skin infections	Abdul-Ghani et al. (2009) Boual et al. (2020) El Ashry et al. (2003) Massoud et al. (2001)
Cymbopogon schoenanthus (L.) Spreng.	Leaves	Can: 1* skin pimples and tumors	Decoction	Termites and bruchid, digestive diseases, aerophagia, flatulence and urinary decrease, analeptic, bad breath, gumboils, and urinary incontinence	Koba et al. (2007) Hammiche and Maiza (2006)

(Continued on following page)

TABLE 4 | (*Continued*) List of new therapeutic uses recorded in Algeria (West-Kabylia-Sahara).

Botanical name	Part used	New uses	Preparation methods	Previously reported uses	References
Daphne gnidium L.	Leaves	Hc: 1* hair loss Rtd: 1* sinusitis	Raw Steaming	Constipation and toothache, wounds, hair lice or ticks in animals hair washing and as hair tonic	Allal et al. (2019)
Cistanche tubulosa (Schenk) Wight	Whole plant	Gisd: 1* colitis	Raw	For blood circulation and impotence, female infertility, lumbago, body weakness, and tonic substance	Namba (1994) Kobayashi et al. (1987)
Phyllanthus niruri L.	Leaves	Can: 1* cancer Rtd: 1* cough	Raw Decoction	Hepatoprotective functions	Bhattacharjee & Sil (2007)
Tetraena alba (L.f.) Beier and Thulin.	Leaves/seeds	Esd: 1* diabetes	Decoction	Diabetes, intoxication (toukal), gastrointestinal disorders, hypertension, and arteriosclerosis	Benali et al. (2017) Mnafghi et al. (2016)

decoction method with oral and topical application, respectively. The plant is termed locally "Moslih el-Andar" meaning in the local dialect "tract's fixer" relating to its effect that repairs the continuous elongated anatomical structure in the body. Similarly, the decoction of *L. shawii* is used to treat two ailments categories: skin diseases (skin ulcers and leprosy) and skeletomuscular system disorder (osteoarthritis). Nevertheless, these ethnomedicinal uses and their pharmacological properties have not been documented in previous studies.

Furthermore, some species were previously reported to be used for culinary purposes such as *Telephium imperati* L. called in local dialect as tassarghit/sarghina. The stems of the plant are usually consumed as soup' spice for postpartum women in Algeria (Sahara and Kabylia region). As reported here, for the first time, it is newly mentioned to be used for medicinal purposes by the local healers treating mouth ulcers and anemia. Moreover, we found that decoction of *Quercus faginea* Lam. seeds, a popular tree in West Algeria (Alcaraz, 1989), is used to treat sexual-reproductive problems besides the fruits (raw) of *Lupinus micranthus* Guss., a species widely distributed in Algeria and the Mediterranean countries (Msaddak et al., 2017). On the other hand, our results showed that species such as *Phyllanthus niruri* L., *Hypecoum procumbens* L., and *Gentiana acaulis* L. are used to treat skin diseases and cancer *via* topical application. These species have not been previously reported to be used as medicinal species in the Mediterranean region.

In the present study, we found that 89% of total species have already been mentioned as medicinal plants. In fact, more than 100 species cited in this survey were reported in previous studies from different regions of Algeria. Besides, we found that, despite having similar therapeutic uses, the species had different vernacular names from a region to another. Interestingly, we report here 47 new therapeutic uses for 20 known plant species. **Table 4** shows these new uses compared to those previously reported in the world.

Informant Consensus Factor and FL

Table 5 shows the 16 ailments categories arranged in descending order based on the F_{IC} values. Cancer had the highest F_{IC} value of 0.49 with 44 species used, such as *C. colocynthis, Panax ginseng* C.A. Mey., *E. alata, Aquilaria malaccensis, Aristolochia longa* L., and *Taraxacum officinale.* On the other hand, we found that sexual-reproductive problems (F_{IC} = 0.46), gastrointestinal system diseases (F_{IC} = 0.44), and skeletomuscular system disorders (F_{IC} = 0.39) were recorded to have the second, third, and fourth highest F_{IC} values, respectively. Respiratory tract diseases were ranked to be the fifth ailment group with an F_{IC} value of 0.36.

TABLE 5 | Informant consensus factor for commonly used medicinal.

Ailment category	Nur	Nt	F_{IC}
Cancer	86	44	0.49
Sexual-reproductive problems	70	38	0.46
Gastrointestinal system diseases	100	56	0.44
Skeletomuscular system disorder	32	20	0.39
Respiratory tract diseases	51	33	0.36
Skin diseases	42	29	0.32
Urology system diseases	26	21	0.20
General health	39	32	0.18
Nervous system	36	30	0.17
Kidneys diseases	9	8	0.13
Hair care	11	8	0.30
Endocrine system diseases	12	11	0.09
Hematological system diseases	17	16	0.06
Cardiovascular system diseases	18	17	0.06
Poisoning	2	2	0.00
Infectious diseases	2	2	0.00

According to their knowledge and experience, the local healers preferred some species to treat particular diseases. The highest FL values of the commonly used plants are listed in **Table 6**. Our results indicated that *M. vulgare, A. herba-alba, Z. officinale,* and *J. phoenicia* had the absolute FL value of 100% in several ailment categories (SRD, cancer, respiratory diseases, and GISD).

DISCUSSION

Botanical Diversity, Parts Used, Modes of Preparation, and Administration

In the present study, we recorded 167 species belonging to 70 families with a dominance of Lamiaceae, Asteraceae, Apiaceae, Rosaceae, and Fabaceae. Our findings agreed with those we previously reported. Indeed, in Mascara (North-West Algeria), most of the medicinal species used by local healers belonged to these five families (Benarba, 2015). Similar findings were reported in Algeria (Miara et al., 2018; Taibi et al., 2020), Morocco (Barkaoui et al., 2017; Skalli et al., 2019), and Italy (Tuttolomondo et al., 2014). It has been suggested that plants belonging to these families are mainly used by local populations

TABLE 6 | FL of commonly used medicinal plants.

Ailment category	Species	FL (100 (%)
KD	*Cichorium alatum* Hochst. and Steud.	100
	Artemisia herba-alba Asso.	50
	Parietaria officinalis L.	100
GISD	*Marrubium vulgare* L.	100
	Zingiber officinale Roscoe	100
	Juniperus Phoenicea L.	100
	Artemisia herba-alba Asso.	100
	Matricaria chamomilla L.	80
	Punica granatum L.	67
	Rhamnus alaternus L.	67
	Curcuma longa L.	67
SD	*Thymus vulgaris* L.	100
	Origanum majorana L.	50
	Eruca sativa L.	50
Can	*Carum carvi* L.	50
	Thapsia garganica L.	33
	Marrubium vulgare L.	100
	Zingiber officinale Roscoe	100
	Juniperus phoenicea L.	100
	Artemisia herba-alba Asso.	100
	Matricaria chamomilla L.	40
	Ziziphus spina-christi (L.) Desf.	50
ESD	*Pimpinella anisum* L.	17
	Saccocalyx satureioides Coss. and Durieu.	100
RTD	*Marrubium vulgare* L.	100
	Zingiber officinale Roscoe	100
	Glycyrrhiza glabra L.	67
	Juniperus phoenicea L.	100
	Artemisia herba-alba Asso.	100
	Pinus maritima L.	50
	Calendula arvensis M.Bieb.	50
SMSD	*Echinops spinosissimus* Turra.	67
	Tussilago farfara L.	100
	Echinops ritro L.	100
CVSD	*Myrtus nivellei* Batt. and Trab.	51
	Crataegus azarolus L.	50
GH	*Nicotiana tabacum* L.	50
	Pistacia lentiscus L.	53
	Carthamus tinctorius L.	50
HC	*Carduus nutans* L.	100
	Daphne gnidium L.	69
NS	*Crocus sativus* L.	53
	Eriobotrya japonica (Thunb.) Lindl.	67
SRP	*Asarum europaeum* L.	100
	Hyacinthus orientalis L.	80
	Marrubium vulgare L.	100
	Zingiber officinale Roscoe	100
	Juniperus phoenicea L.	100
	Artemisia herba-alba Asso.	100
ID	*Ulmus rubra* Muhl.	50
P	*Artemisia campestris* L.	100
HSD	*Cichorium alatum* Hochst. and Steud.	100
	Salvia hispanica L.	100
	Vitis vinifera L.	50
	Rubia tinctorum L.	33
USD	*Urtica dioica* L.	34
	Nitraria retusa (Forssk.) Asch.	50

in Africa owing to their pharmacological effects offering a cheap therapeutic alternative (Sawadogo et al., 2012). Furthermore, leaves, aerial parts, and seeds were the most frequently used parts by local healers. Our results confirm the dominance of leaves as the most common used important plants' part used in local phytotherapy as has been demonstrated in Algeria (Benarba, 2015; Benarba, 2016; Bouasla and Bouasla 2017; Miara et al., 2018; Taibi et al., 2020) and neighboring countries such as Mauritania (Yebouk et al., 2020), Morocco (Barkaoui et al., 2017; Skalli et al., 2019), or Italy (Leto et al., 2013). The dominance of leaves in most of the ethnobotanical studies could be explained by their ease collecting and abundance besides the fact that they are considered the site of photosynthesis and therefore of bioactive molecules.

Our results showed that the traditional healers used different preparation methods, including decoction, infusion, paste, or maceration. Decoction was found to be the preferred method. Similar results were found in previous ethnobotanical studies (Benarba, 2015; Merrouni and Elachouri, 2020). In fact, decoction and infusion were found to be the most used in the recent ethnobotanical studies in Algeria (Benarba et al., 2015; Benarba, 2016; Mechaala et al., 2021; Zatout et al., 2021) and neighboring countries such as Tunisia, Egypt, Spain, and Italy in Africa and in Europe (Giday et al., 2009; Benitez et al., 2010; Amri and Kisangau, 2012; Menale et al., 2016; Savić et al., 2019). The dominance of decoction or infusion could be explained by the disinfection potential of heating besides its extraction enhancing effects (Benarba, 2015).

We also found that oral ingestion was the most frequently used mode of administration, followed by external application, steam, and nasal inhalation. Our findings are consistent with those we previously reported in South-West Algeria (Benarba, 2016), North-West Algeria (Benarba, 2015), and Extreme-West Algeria (Tlemcen) (Zatout et al., 2021). Likewise, oral and topical applications were found to be the most frequently used administration methods used by local healers or populations in other regions in Algeria (Hammiche and Maiza, 2006; Boudjelal et al., 2013; Sarri et al., 2014; Miara et al., 2018) and neighboring countries (Mrabti et al., 2019; Fakchich and Elachouri, 2014; Benitez et al., 2010). In this same line, oral and topical administrations are frequently used in traditional medicine. The choice of administration routes is based on the pharmacological effect of each species, the therapy target, duration, and the limitation of treatment to a precise area (Sargin et al., 2015; Benarba, 2016).

The traditional healers in the study areas reported that 64% of medicinal species were mixed with other medicinal plants, whereas 43% of herbal mixtures were prepared by adding different adjuvants with a dominance of honey, olive oil, animal fat, or vinegar. In concordance with our findings, several ethnobotanical investigations carried out in Algeria demonstrated that honey was the adjuvant most frequently added to prepare medicinal herbal mixtures (Benarba, 2016; Ouelbani et al., 2016; Zatout et al., 2021). Our findings are also in perfect consistency with those reported in other regions around the world (Yabesh et al., 2014; Amri and Kisangau, 2012; Pranskuniene et al., 2016). These ingredients could enhance the plant effect, maintain the blend texture, and facilitate the treatment administration. To the best of our knowledge, some adjuvants were not previously mentioned, such as tar and litharge.

TABLE 7 | List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
Amaranthaceae	*Amaranthus spinosus* L. (LRSBG/AB/20/067)	القطيفة سالف العروس	Aerial part	SRP: 1* infertility	Decoction	Oral/topical
	Haloxylon salicornicum (Moq.) Bunge ex Boiss. (LRSBG/AB/20/068)	رمث الاحمر او تاساي	Leaves	Can: 1* cancer	Raw	Oral
				P: 1* poisoning	Decoction	Oral
	Atriplex halimus L. (LRSBG/AB/20/069)	القطف او السرمق الملوخ،	Leaves	Can: 3* uterine cysts and tumors	Decoction	Oral
				Breast cysts and tumors	Decoction	Oral
				Cancer	Maceration	Oral
Amaryllidaceae	*Allium sativum* L. (LRSBG/AB/20/072)	الثوم	Bulb	RTD: 5* asthma	Decoction	Topical
				Chest and lung diseases	Decoction	Topical
				Cough	Raw	Topical
				Nasal-lung inflammation	Maceration/decoction	Topical/oral Oral
				SRP: 2* infertility	Decoction	Inhalation
				GH: 2* tonsillitis	Decoction	Topical
				Earache and deafness	Decoction	Topical
				GISD: 2* jaundice/icterus	Raw	Topical
				Liver diseases	Raw	Topical
				HC: 1* alopecia areata	Frying	Topical
				SD: 1* boils	Frying	Topical
				Can: 1* skin pimples and tumors	Decoction	Oral
	Allium cepa L. (LRSBG/AB/20/073)	بصل	Bulb	SRP: 3* infertility + uterine problems	Decoction	Topical
						Topical
					Decoction	Topical
				SD: 2* boils and head ulcers	Frying	Topical
					Frying	Topical
				Can: 1* skin pimples and tumors	Raw	Topical
				GISD: 1* jaundice/icterus	Raw	Topical
Anacardiaceae	*Pistacia lentiscus* L. (LRSBG/AB/20/123)	المصطكى أو المسكة او الضرو	Leaves/wax	RTD: 1* pulmonary-breathing problem	Raw	Oral
				GISD: 4* heartburn	Decoction	Oral
				stomach ache	Decoction	Oral
				Diarrhea	Decoction/raw	Oral
				GH: 2* mouth ulcer	Decoction	Oral
				Earache and deafness	Decoction	Topical
Apiaceae	*Ferula assa-foetida* L. (LRSBG/AB/20/145)	الكلخ او الحلتيت	Whole plant	GH: 2* tonsillitis	Infusion/raw	Topical
					Raw/decoction	Oral/topical
	Cuminum cyminum L. (LRSBG/AB/20/001)	الكمون	Seeds	SMSD: 1* arthritis	Infusion	Topical/oral
				SRP: 2* infertility	Raw/decoction	Topical
				GISD: 2* stomach ache	Maceration	Oral
				Jaundice/icterus	Decoction	Oral
	Ammoides pusilla (Brot.) Breistr. (LRSBG/AB/20/002)	النوخة أو النانخة	Aerial part	GH: 1* fever	Decoction	Oral/topical
				HSD: 1* jaundice/icterus	Decoction	Oral
	Pimpinella anisum L. (LRSBG/AB/20/003)	حبة الحلاوة أو اليانسون	Fruits	ESD: 1* diabetes	Decoction	Oral
				CVSD: 1* cholesterol	Decoction	Oral
				KD: 1* kidney failure	Decoction	Oral
				USD: 1* bladder disease	Decoction	Oral
				RTD: 1* asthma	Decoction	Topical
				GISD: 1* stomach ache	Maceration	Oral
				NS: 1* insomnia	Decoction	Oral
				SD: 1* skin disease	Infusion	Topical
				SMSD: 2* arthritis	Infusion	Oral/topical
				SRP: 1* infertility	Decoction	Topical
	Coriandrum sativum L. (LRSBG/AB/20/004)	القزبر أو الكزبرة	Aerial part	SD: 2* limb swelling	Infusion	Topical
				RTD: 1* chest and lung diseases	Infusion	Topical
	Foeniculum vulgare Mill. (LRSBG/AB/20/005)	البسباس	Seeds	GISD: 3* IBS	Infusion	Oral
				stomach ache	Maceration	Oral
				Flatulence	Decoction	Oral
				NS: 1* headache	Infusion	Oral
		تالغودة او آ ألثار	Roots/seeds	ESD: 1* goiter	Raw	Oral

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
	Bunium mauritanicum L. (LRSBG/AB/20/006)					
	Carum carvi L. (LRSBG/AB/20/007)	كرويا	Seeds	Can: 1* early stage cancer GISD: 1* stomach ache GH: 1* anxiety disorders and hypochondria SMSD: 1* bones pain	Decoction Maceration Raw Raw	Raw Oral Topical Oral
	Apium graveolens L. (LRSBG/AB/20/008)	الكرفس	Leaves	SMSD: 2* osteoarthritis	Decoction	Oral/topical
	Thapsia garganica L. (LRSBG/AB/20/009)	درياس أو بونافع	Aerial part	RTD: 2* chest and lung diseases Can: 1* lung tumors	Maceration/frying Raw	Topical/oral Topical
	Petroselinum crispum (Mill.) Fuss. (LRSBG/AB/20/010)	البقدونس أو المعدنوس	Aerial part	KD: 1* urolithiasis GH: 1* mouth ulcer	Decoction Decoction	Oral Oral
Apocynaceae	*Nerium oleander* L. (LRSBG/AB/20/109)	الدفلة	Leaves	GH: 1* mouth ulcer Can: 1* skin pimples and tumors SD: 3* chalazion Tinea capitis and scalp ringworm Urticaria	Decoction Burned Decoction Raw Maceration	Topical Topical Topical Topical Topical
Araliaceae	*Panax ginseng* C.A.Mey. (LRSBG/AB/20/124)	الجنسنغ أو الجنسة	Aerial part	Can: 1* stomach cancer SRP: 1* infertility GISD: 1* liver diseases HSD: 1* spleen diseases	Maceration Raw Decoction Decoction	Oral Oral Oral Oral
Aristolochiaceae	*Asarum europaeum* L. (LRSBG/AB/20/096)	أسارون	Leaves	SRP: 1* uterine microbe and infections	Decoction	Oral
	Aristolochia longa L. (LRSBG/AB/20/097)	برسطم—برزطم	Stalk	HC: 1* baldness Can: 3* breast cancer Legs cancer Cancer	Raw Raw Raw Raw	Topical Topical Topical Oral
Asparagaceae	*Hyacinthus orientalis* L. (LRSBG/AB/20/098)	الخزامى	Flowers	SRP: 4* infertility Uterine problems USD: 2* urinary tract infection/inflammation Bladder disease GH: 1* fever	Raw/decoction Decoction Decoction Maceration Decoction	Topical Topical Oral Oral Oral/topical
	Drimia maritima (L.) Stearn. (LRSBG/AB/20/099)	البصل البري أو بصل الحلوف	Bulb	SRP: 2* infertility Uterine problems HC: 1* alopecia areata	Decoction Decoction Decoction	Topical Topical Topical
Asteraceae	*Cynara scolymus* L. (LRSBG/AB/20/156)	العسلوج او ساق الخرشوف	Stalk	GISD: 1* hemorrhoids	Decoction	Oral
	Arctium atlanticum (Pomel) H.Lindb. (LRSBG/AB/20/119)	الأرقطيون	Leaves/capitulum	SD: 1* boils Can: 1* skin pimples and tumors	Frying Frying	Topical Topical
	Cirsium creticum (Lam.) d'Urv. (LRSBG/AB/20/160)			GISD: 1* hemorrhoids	Decoction	Topical/oral
	Carthamus tinctorius L. (LRSBG/AB/20/011)	العصفر أو الجرجوم	Capitulum	GH: 1* anxiety disorders and hypochondria	Raw	Oral/topical
	Dittrichia viscosa (L.) Greuter (LRSBG/AB/20/012)	مقرمان	Aerial part	SD: 2* festering wounds Skin diseases	Maceration/raw Maceration	Oral/topical Topical
	Tussilago farfara L. (LRSBG/AB/20/013)	حشيشة السعال أو تافيرا	Aerial part	SMSD: 2* osteoarthritis	Decoction	Oral/topical
	Echinops ritro L. (LRSBG/AB/20/014)	تاسكرا أو الشوك الأزرق ، و أبونقار	Aerial part	SMSD: 2* osteoarthritis	Decoction	Oral/topical
	Saussurea costus (Falc.) Lipsch. (LRSBG/AB/20/015)	القسط الهندي	Roots	SRP: 1* infertility	Infusion	Topical
	Silybum marianum (L.) Gaertn. (LRSBG/AB/20/016)	الخرفيش	Leaves	Can: 2* breast cancer Legs cancer	Raw	Topical
	Centaurea acaulis L. (LRSBG/AB/20/017)	سنتوريا او القنطريون	Aerial part	Can: 4* breast cancer Legs cancer Can: 4* tumors and skin pimples	Raw	Topical
		المطهر	Capitulum	Can: 2* breast cancer	Raw	Topical

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
	Inula helenium L. (LRSBG/AB/20/018)			Legs cancer		
	Calendula arvensis M.Bieb. (LRSBG/AB/20/019)	عين البقر	Capitulum	RTD: 1* pneumonia	Decoction	Oral
	Artemisia campestris L. (LRSBG/AB/20/020)	القوگتف	Leaves	P: 1* scorpion sting	Raw	Topical
	Anacyclus valentinus L. (LRSBG/AB/20/021)	القرطوفة	Aerial part	HSD: 1* anemia	Decoction	Oral
	Taraxacum officinale (L.) Weber ex F.H.Wigg. (LRSBG/AB/20/022)	هندباء البر، العيصيب	Aerial part	Can: 2* breast cancer Legs cancer	Raw	Topical
	Anacyclus pyrethrum (L.) Lag. (LRSBG/AB/20/023)	أوعاقر تيسيطنقت حرقا	Leaves	RTD: 1* pulmonary-breathing problem	Raw	Topical
				SRP: 1 infertility	Raw	Oral
				SMSD: 2* arthritis	Maceration	Topical and oral
	Cichorium alatum Hochst. and Steud. (LRSBG/AB/20/024)	العلت. تمرزوق	Aerial part/ roots	USD: 2* cystolithiasis	Decoction	Oral
				Bladder disease	Decoction	Oral
				GISD: 2* hemorrhoids liver diseases	Raw	Topical
					Raw	Topical
				Can: 4* breast cancer	Decoction	Oral
				Legs cancer	Decoction	Oral
				KD: 1* urolithiasis	Decoction	Oral
				HSD: 1* spleen diseases	Decoction	Oral
	Matricaria chamomilla L. (LRSBG/AB/20/025)	البابونج	Capitulum	GISD: 4* liver diseases	Decoction	Oral
				IBS stomach ache	Decoction	Oral
				Heartburn	Raw	Topical
					Raw	Topical
				HSD: 1* spleen diseases	Raw	Topical
				SD: 1* skin ulcers	Decoction	Oral
				Can: 2* breast cancer	Decoction	Oral
				Legs cancer	Decoction	Oral
				USD: 2* urinary tract infection/inflammation	Decoction	Oral
				Bladder diseases	Infusion	Oral
				NS: 1* insomnia	Infusion	Oral
	Carlina gummifera (L.) Less. (LRSBG/AB/20/026)	الأداد	Capitulum leaves/roots	SRP: 2* infertility	Decoction	Topical
				Uterine problems	Decoction	Topical
				USD: 2* urinary tract infection and bladder disease	Decoction	Topical
					Decoction	Topical
				SMSD: 1* osteoarthritis	Decoction	Oral/topical
	Echinops spinosissimus Turra (LRSBG/AB/20/027)	شوك الجمل	Aerial part	Can: 1* skin pimples and tumors	Decoction	Oral
	Artemisia herba-alba Asso. (LRSBG/AB/20/028)	الشيح	Aerial part	GH: 1* tonsillitis	Infusion	Topical
				Can: 2* skin cancer	Raw	Topical
				Breast cancer	Maceration	Oral
				ESD: 2* diabetes	Decoction	Oral
				CVSD: 1* cholesterol	Decoction	Oral
				KD: 1* kidney failure	Decoction	Oral
				USD: 1* bladder disease	Decoction	Topical
				RTD: * asthma	Decoction	Topical/oral
				GISD: 2* IBS and liver diseases	Decoction	Topical
				SRP: 2* infertility and uterine problems	Decoction	Topical
	Carduus nutans L. (LRSBG/AB/20/104)	شوك المحني	Capitulum	HC: 1* alopecia areata	Raw	Topical
Berberidaceae	*Berberis vulgaris* L. (LRSBG/AB/20/070)	عود الريح	Roots/bark	SRP: 1* infertility	Decoction	Topical
	Mahonia aquifolium (Pursh) Nutt. (LRSBG/AB/20/071)	اريغون	Whole plant	Can: 2* breast cancer and legs cancer	Raw	Topical
Betulaceae	*Betula pendula Roth* (LRSBG/AB/20/149)	عصير الشجر (البات ولية)	Bark	GISD: 1* ulcers	Infusion	Oral
Boraginaceae		عشبة الثور	Aerial part	SRP: 1* infertility	Decoction	Oral

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
	Borago officinalis L. (LRSBG/AB/20/165)					
Brassicaceae	*Armoracia rusticana* P.Gaertn., B.Mey. and Scherb. (LRSBG/AB/20/085)	فجل العود او الخيل	Aerial part	GH: 2* mouth ulcer Halitosis	Decoction Decoction	Topical Topical
	Sinapis arvensis L. (LRSBG/AB/20/086)	الخردل	Seeds	RTD: 1* chest and lung diseases	Decoction	Inhalation
	Eruca sativa Mill. (LRSBG/AB/20/087)	الكثأ أو الجرجير	Aerial part	SD: 1* boils Can: 1* skin pimples and tumors	Frying Frying	Topical Topical
	Lepidium sativum L. (LRSBG/AB/20/088)	حب الرشاد أو الحبة الحمراء الحرف	Seeds	SRP: 1* breast milk outage RTD: 1* chest and lung diseases Can: 1* cancer GISD: 2* colitis Flatulence	Maceration Raw Raw Raw Raw	Oral Oral Oral Oral Oral
	Anastatica hierochuntica L. (LRSBG/AB/20/154)	عشبة مريم	Leaves	GISD: 1* gastrointestinal diseases	Decoction	Oral
Burseraceae	*Boswellia ameero* Balf.f. (LRSBG/AB/20/074)	اللبان	Resin	RTD: 1* chest and lung diseases	Maceration	Topical
	Commiphora myrrha (Nees) Engl. (LRSBG/AB/20/075)	المر	Wax	Can: 2* breast cancer Legs cancer	Raw Raw	Topical Topical
Cactaceae	*Opuntia ficus-indica* (L.) Mill. (LRSBG/AB/20/126)	التين الشوكي الكرموس	Leaves	GISD: 1* liver diseases NS: 2* headache and dizziness	Maceration Decoction	Oral Oral
Cannabaceae	*Humulus lupulus* L. (LRSBG/AB/20/153)	حنجل	Leaves	HC: 3* alopecia areata Baldness NS: 1* headache GISD: 2* hemorrhoids ID: 2* mouth and ears infections	Raw Raw Raw Raw	Topical Topical Topical Topical
Cucurbitaceae	*Cucurbita maxima* Duchesne (LRSBG/AB/20/100)	القرع البلدي	Seeds	NS: 1* migraine	Decoction	Inhalation
	Citrullus colocynthis (L.) Schrad (LRSBG/AB/20/101)	الحنظل	Fruits	SD: 1* skin ulcers and leprosy GISD: 1* constipation Can: skin cancer	Decoction Decoction Maceration	Oral Oral Oral/topical
Cupressaceae	*Juniperus foetidissima* Willd. (LRSBG/AB/20/089)	العرعر	Aerial part	GISD: 5* IBS and stomach ache Heartburn RTD: 2* chest and lung diseases Can: 5* breast cancer Legs cancer SD: 1* urticaria SRP: 5* infertility	Decoction Decoction Decoction Raw Raw Raw Maceration Decoction	Oral Oral Oral Oral Topical Topical Topical Topical
	Cupressus sempervirens L. (LRSBG/AB/20/090)	السرو	Leaves	USD: 1* bladder disease SMSD: 1* arthritis	Decoction Infusion	Oral Topical
Cyperaceae	*Cyperus esculentus* L. (LRSBG/AB/20/111)	حب عزيز	Seeds	HSD: 1* anemia	Infusion	Oral
Ephedraceae	*Ephedra alata Decne.* (LRSBG/AB/20/127)	العلندى	Aerial part	Can: 1* breast cysts and breast tumors	Raw	Topical
Equisetaceae	*Equisetum arvense* L. (LRSBG/AB/20/128)	ذيل الحصان ، وذنب الخيل	Aerial part	SMSD: 1* arthritis	Decoction	Oral
Fabaceae	*Ceratonia siliqua* L. (LRSBG/AB/20/162)	الخروب	Seeds	GISD: 1* gastrointestinal diseases	Raw	Oral
	Glycyrrhiza glabra L. (LRSBG/AB/20/029)	العرقسوس	Roots	RTD: 2* cough Lung filtering/smoker HSD: 1* spleen diseases NS: 1* head problems Psychosis	Decoction Infusion Decoction Decoction Raw	Oral Oral Oral Oral Topical
	Senna alexandrina Mill. (LRSBG/AB/20/030)	السنامكي	Leaves	SD: 1* skin diseases GISD: 4* colitis Flatulence	Maceration Infusion/ decoction	Topical Oral

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
				IBS		
				Constipation		
				NS: 1* head problems	Decoction	Topical
				Psychosis		Oral
	Acacia senegal (L.) Willd. (LRSBG/AB/20/031)	الصمغ العربي	Gum	SD: 1* lichen	Infusion	Topical
	Acacia gummifera Willd. (LRSBG/AB/20/032)	أم غيلان	Leaves	Can: 3* cancer, stomach cancer, and liver cancer	Decoction	Oral
				GH: 1* incurable diseases	Decoction	Oral
	Trigonella foenum-graecum L. (LRSBG/AB/20/033)	الحلبة	Seeds	HSD: 1* anemia	Maceration	Oral
				Can: 5* breast cancer	Raw	Topical
				Legs cancer	Raw	Topical
				Cancer	Raw	Topical
				Skin pimples	Maceration	Oral
				Tumors	Raw	Oral
				SMSD: 2* fracture back pain	Raw	Topical
				GISD: 1* stomach ache	Raw	Oral
				RTD: 1* chest and lung diseases	Decoction/raw	Oral/topical
				SRP: 2* infertility	Infusion	Topical
				GH: 1* anxiety disorders and hypochondria	Raw	Topical
	Melilotus officinalis (L.) Pall. (LRSBG/AB/20/034)	الهندقوق إكليل الملك	Aerial part	RTD: 1* chest and lung diseases	Infusion	Topical
				GISD: 1* IBS	Decoction	Oral
	Lupinus micranthus Guss. (LRSBG/AB/20/035)	الترمز المر الدقيق	Fruits	SRP: 1* infertility	Raw	Oral
Fagaceae	*Quercus faginea* Lam. (LRSBG/AB/20/110)	العفص	Seeds	SRP: 1* uterine microbe	Decoction	Topical/oral
Gentianaceae	*Gentiana acaulis* L. (LRSBG/AB/20/112)	كف الذئب او الجنطيانا	Leaves/ flowers	Can: 1* breast cancer	Raw	Topical
				Legs cancer		
Iridaceae	*Crocus sativus* L. (LRSBG/AB/20/113)	الزعفران	Stamen	SD: 1* albinism	Raw	Topical
				NS: 1* headache	Raw	Topical
Juglandaceae	*Juglans regia* L. (LRSBG/AB/20/159)	الدير م	Aerial part/ park	GISD: 1* gallstones	Decoction	Oral
Lamiaceae	*Lavandula angustifolia* Mill. (LRSBG/AB/20/163)	ضرم الحار	Aerial parts	GISD: 1* hemorrhoids	Raw	Topical
	Mentha pulegium L. (LRSBG/AB/20/036)	النعناع الأوروبي او الفليو	Aerial part	RTD: 1* chest and lung diseases	Decoction	Oral
				USD: 1* urinary tract infection/inflammation	Decoction	Oral
				SRP: 1* infertility	Decoction	Oral/topical
				NS: 1* insomnia	Decoction	Oral
	Saccocalyx satureioides Coss. and Durieu (LRSBG/AB/20/037)	يزير البل	Leaves	GISD: 1* stomach ache	Decoction	Oral
				ESD: 2* diabetes	Infusion	Oral
	Thymus capitatus (L.) Hoffmanns. and Link. (LRSBG/AB/20/038)	صعتر أو الزعتر	Aerial part	SD: 1* burns	Frying	Topical
	Mentha arvensis L. (LRSBG/AB/20/039)	النعناع	Aerial part	CVSD: 1* cardiovascular diseases	Raw	Oral
				SRP: 1* infertility	Raw	Topical
				RTD: 1* chest and lung diseases	Raw	Oral
				GISD: 1* IBS	Decoction	Oral
				GH: 1* anxiety disorders and hypochondria	Raw	Oral
				NS: 2* head problems	Raw	Oral
				Psychosis insomnia	Decoction	Oral
	Ocimum basilicum L. (LRSBG/AB/20/040)	الريحان	Leaves	SRP: 4* infertility	Raw/decoction	Topical
				Uterine problems	Decoction	Topical
				GISD: 1* IBS	Decoction	Oral
				USD: 2* urinary tract infection/inflammation	Decoction	Oral
				Bladder disease	Infusion	Oral
				GH: 2* fever	Decoction	Oral/topical
				NS: 1* dizziness	Maceration	Topical

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
				CVSD: 1* hypertension	Infusion	Topical
	Melissa officinalis L. (LRSBG/AB/20/041)	مليسا	Leaves	NS: 1* insomnia	Decoction	Oral
	Rosmarinus officinalis L. (LRSBG/AB/20/042)	إكليل الجبل	Aerial part	CVSD: 1* cholesterol	Decoction	Oral
				GISD: 2* IBS jaundice/ icterus	Decoction	Oral
					Infusion	Oral
	Origanum majorana L. (LRSBG/AB/20/043)	المردقوش	Aerial part	SD: 1* limb swelling	Maceration	Topical
	Clinopodium nepeta (L.) Kuntze. (LRSBG/AB/20/044)	النباطة أو الفوذنج الجبلي	Aerial part	GISD: 1* IBS	Decoction	Oral
				ESD: 1* diabetes	Decoction	Oral
				CVSD: 1* cholesterol	Decoction	Oral
				KD: 1* kidney failure	Decoction	Oral
				USD: 1* bladder disease	Decoction	Oral
	Lavandula stoechas L. (LRSBG/AB/20/045)	الحلحال أو أسنان داود	Aerial part	ESD: 1* diabetes	Decoction	Oral
				CVSD: 1* cholesterol	Decoction	Oral
				KD: 1* kidney failure	Decoction	Oral
				USD: 1* bladder diseases	Decoction	Oral
				HSD: 1* blood purify	Decoction	Oral
	Thymus vulgaris L. (LRSBG/AB/20/046)	الزعتر البري	Aerial part	GISD: 1* IBS jaundice/ icterus	Decoction	Oral
					Decoction/raw	Topical
				SRP: 2* infertility	Raw	Topical
				HC: 1* baldness	Raw	Topical
				Can: 1* breast cancer legs cancer	Raw	Topical
				USD: 1* urinary tract infection	Decoction	Oral
				GH: 1* fever	Decoction	Oral/topical
				SD: 4* skin diseases and ulcer	Decoction	Topical
					Infusion	Oral
	Salvia officinalis L. (LRSBG/AB/20/047)	المريمية او القصعين المخزني	Leaves	CVSD: 1* cholesterol	Decoction	Oral
	Salvia hispanica L. (LRSBG/AB/20/048)	بذور شيا	Seeds	HSD: 1* anemia	Raw	Oral
	Teucrium spinosum L. (LRSBG/AB/20/049)	الجعدة	Aerial part	HSD: 1* blood purify	Maceration	Oral
				GISD: 1* ulcers	Raw	Topical
				CVSD: 1* diabetes	Decoction	Oral
				RTD: 1* chest and lung diseases	Raw	Topical
	Mentha aquatica L. (LRSBG/AB/20/050)	حبق الماء	Aerial part	GH: 1* anxiety disorders and hypochondria	Raw	Oral
	Marrubium vulgare L. (LRSBG/AB/20/051)	المرية	Aerial part	Can: 3* skin pimples and tumors	Raw	Topical
				Skin cancer	Raw	Topical
				Breast cancer	Decoction/raw	Topical/oral
				SRP: 4* infertility	Decoction	Topical
				Uterine problems	Decoction	Topical
				RTD: 1* pulmonary-Breathing problem	Frying/ decoction	inhalation/ topical
	Vitex agnus-castus L. (LRSBG/AB/20/052)	كف مريم	Leaves	Can: 3* breast tumor	Decoction	Oral
				Uterus tumor	Decoction	Oral
				Gum tumor	Decoction	Oral
				NS: 1* sciatica	Raw	Oral
	Ajuga iva (L.) Schreb. (LRSBG/AB/20/053)	الشندقورة	Leaves	CVSD: 1* cholesterol	Infusion	Oral
	Teucrium polium L. (LRSBG/AB/20/054)	خياطة الجراح	Aerial part	GISD: 1* ulcers	Raw	Oral
	Mentha rotundifolia (L.) Huds (LRSBG/AB/20/055)	تيمرصاد	Aerial part	GISD: 1* IBS	Decoction	Oral
Lauraceae	*Cinnamomum camphora* (L.) J.Presl. (LRSBG/AB/20/076)	الكافور	Wax	NS: 1* migraine	Infusion	Topical
	Cinnamomum verum J.Presl (LRSBG/AB/20/077)	قرفة	Bark	NS: 2* migraine	Raw	Oral/topical
				USD: 1* urinary tract infection/inflammation	Decoction	Oral
	Laurus nobilis L. (LRSBG/AB/20/078)	الرند	Leaves	GISD: 1* ulcers	Decoction	Oral

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
Linaceae	*Linum usitatissimum* L. (LRSBG/AB/20/130)	زريعة الكتان	Seeds	GISD: 1* IBS	Decoction	Oral
				RTD: 1* chest and lung diseases	Maceration	Topical
				ESD: 1* goiter	Raw	Oral
				GH: 1* hoarseness and sore throat	Raw	Oral
				Can: 1* Skin pimples and tumors	Decoction	Topical
Lythraceae	*Lawsonia inermis* L. (LRSBG/AB/20/079)	الحناء	Leaves	Can: 1* Skin pimples and tumors	Decoction	Topical
				SMSD: 1* fracture	Raw	Topical
				SD: 3* urticaria	Maceration	Topical
				Warts	Burned	Topical
				Head ulcers	Raw	Topical
				GH: 1* anxiety disorders and hypochondria	Raw	Topical
Malvaceae	*Hibiscus sabdariffa* L. (LRSBG/AB/20/131)	كركديه. او الورد الحر	Flowers	CVSD: 1* hypertension	Infusion	Oral
Moraceae	*Ficus carica* L. (LRSBG/AB/20/155)	التين	Fruits	RTD: 2* chest and lung diseases	Decoction	Oral
				Cough		
				GISD: 3* jaundice/icterus	Raw/infusion	Oral
				liver diseases	Maceration	Oral
Moringaceae	*Moringa oleifera* Lam. (LRSBG/AB/20/132)	المورينجا. او عشبة الحياة	Whole plant	GH: 1* incurable diseases	Decoction	Oral
				GISD: 1* IBS	Infusion	Oral
Myristicaceae	*Myristica fragrans* Houtt. (LRSBG/AB/20/114)	جوزة الطيب	Seeds	NS: 1* head problems	Raw	Topical
				Psychosis		
Myrtaceae	*Myrtus communis* L. (LRSBG/AB/20/151)	القمام	Leaves	RTD: 2* chest and lung diseases	Infusion	Topical
	Myrtus nivellei Batt. and Trab. (LRSBG/AB/20/167)	قمام الصحرا	Leaves	CVSD: 1* clogged arteries	Decoction	Oral
	Syzygium aromaticum (L.) Merr. and L.M.Perry. (LRSBG/AB/20/081)	القرنفل	Flower buds	NS: 1* migraine	Raw	Topical
				SRP: 3* infertility	Decoction/raw	Topical/oral
				RTD: 1* chest and lung diseases	Raw	Oral
				USD: 1* urinary tract infection/inflammation	Decoction	Oral
				GH: 1* earache and deafness	Decoction	Topical
				SD: 1* skin diseases, ulcer	Infusion	Topical
	Eucalyptus globulus Labill. (LRSBG/AB/20/082)	كالبتوس	Leaves	SRP: 1* infertility	Decoction	Topical
Nitrariaceae	*Nitraria retusa* (Forssk.) Asch. (LRSBG/AB/20/161)	شجرة ليهود	Leaves	USD: 1* bladder disease	Infusion	Oral
				Can: 1* tumors	Infusion	Oral
	Peganum harmala L. (LRSBG/AB/20/133)	الحرمل	Seeds	GISD: 1* IBS	Decoction	Oral
				SRP: 1* infertility	Raw	Topical
				RTD: 2* chest and lung diseases	Raw	Oral
				Nasal-lung inflammation	Decoction	Inhalation
				GH: 1* fever	Decoction	Oral
				USD: 1* urinary tract infection/inflammation	Decoction	Oral
Oleaceae	*Olea oleaster* Hoffmanns. and Link (LRSBG/AB/20/094)	الزبوج	Leaves	GH: 1* mouth ulcer	Decoction	Topical
	Olea europaea L. (LRSBG/AB/20/095)	الزيتون	Leaves fruits	GH: 1* mouth ulcer and halitosis	Decoction	Topical
				NS: 1* head problems	Raw	Topical
				Psychosis		
Orobanchaceae	*Cistanche tubulosa* (Schenk) Wight. (LRSBG/AB/20/134)	ذنون	Whole plant	GISD: 1* colitis	Raw	Oral
Papaveraceae	*Hypecoum procumbens* L. (LRSBG/AB/20/135)	زهيرة (الخشخاشية)	Aerial part	Can: 1* skin pimples and tumors	Raw	Topical
Parmeliaceae	*Evernia prunastri* L. (LRSBG/AB/20/158)	لحية شيخ	Lichens	Can: 1* cancer	Decoction	Oral
				GISD: 1* gastrointestinal diseases	Decoction	Oral
				NS: 1* epilepsy	Decoction	inhalation

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
Paronychioideae	*Telephium imperati* L. (LRSBG/AB/20/150)	تسمرغينت	Aerial part	GH: 1* mouth ulcer HSD: 1* anemia	Infusion	Topical/oral
Pedaliaceae	*Sesamum indicum* L. (LRSBG/AB/20/136)	السمسم أو. جلجلان	Seeds	SRP: 2* infertility Breast milk outage GH: 1* mouth ulcer	Raw Maceration Decoction	Oral Oral Oral
Phyllanthaceae	*Phyllanthus niruri* L. (LRSBG/AB/20/137)	الأملج	Leaves	Can: 1* cancer RTD: 1* cough	Raw/decoction	Oral
Pinaceae	*Pinus maritima* Aiton (LRSBG/AB/20/138)	الزنين	Fruits	Can: 2* blood cancer Stomach cancer Liver cancer RTD: 1* chest and lung diseases ESD: 1* goiter GH: 1* hoarseness and sore throat	Decoction Decoction Decoction Maceration Raw Raw	Oral Oral Oral Topical Topical Topical
	Pinus pinaster Aiton (LRSBG/AB/20/152)	تايدة لحاء شجرة الصنوبر البحري	Bark	GISD: 1* diarrhea	Raw	Oral
Piperaceae	*Piper cubeba* Bojer (LRSBG/AB/20/102)	الكبابة، حب العروس	Seeds	SRP: 1* infertility	Raw	Oral
	Piper nigrum L. (LRSBG/AB/20/103)	الفلفل الأسود	Seeds	GH: 1* earache and deafness	Decoction	Topical
Plantaginaceae	*Digitalis purpurea* L. (LRSBG/AB/20/115)	القمعية او الديجيتال	Flowers	CVSD: 1* cardiovascular diseases	Raw	Oral
Poaceae	*Cymbopogon schoenanthus* (L.) Spreng. (LRSBG/AB/20/091)	الإذخر أو الليمونية	Leaves	Can: 1* skin pimples and tumors	Decoction	Topical
	Stipa tenacissima L. (LRSBG/AB/20/092)	نبات الحلفـــــــاء	Leaves	CVSD: 1* cholesterol	Maceration	Oral
	Hordeum vulgare L. (LRSBG/AB/20/093)	الشعير الزرع	Seeds	SD: 1* burns	Frying	Topical
Poales	*Aristida pungens* Desf (LRSBG/AB/20/157)	الدرين	Stalk	HSD: 1* anemia	Decoction	Oral
Portulacaceae	*Portulaca oleracea* L. (LRSBG/AB/20/116)	البقلة او بندراق	Leaves	GISD: 1* stomach ache	Decoction	Oral
Punicaceae	*Punica granatum* L. (LRSBG/AB/20/080)	الرمان	Peels/fruits	GISD: 6* gastrointestinal diseases IBS Heartburn Stomach ache Diarrhea GH: 2* mouth ulcer Halitosis NS: 1* headache	Decoction Raw/decoction Stewing Raw Decoction Decoction Decoction	Oral Oral Oral Oral Oral Topical Topical
Ranunculaceae	*Nigella sativa* L. (LRSBG/AB/20/139)	حبة البركة. أو الحبة السوداء. او السانوج	Seeds	NS: 1* migraine RTD: 1* chest and lung diseases Can: 3* cancer SMSD: 3* acute arthritis and gout SD: 2* itchy skin Limb swelling	Decoction Raw Raw Raw Raw	Inhalation Oral Oral Oral Oral
Rhamnaceae	*Rhamnus alaternus* L. (LRSBG/AB/20/083)	آملیلس أو ملیلس أو عود الخير	Bark/leaves/flowers	GISD: 2* jaundice and icterus	Decoction	Oral
	Ziziphus spina-christi (L.) Desf. (LRSBG/AB/20/084)	النبق شجرة السدر	Fruits/leaves	GISD: 1* jaundice and icterus Can: 1* cancer SD: 1* skin diseases and ulcer	Raw Raw Decoction	Oral Oral Oral
Rosaceae	*Potentilla reptans* L. (LRSBG/AB/20/166)	حشيشة الخامسة	Leaves	SD: 2* itchy skin limb swelling	Raw Raw	Oral Oral
	Prunus persica (L.) Batsch. (LRSBG/AB/20/056)	الخوخ	Leaves	Can: 2* cancer SD: 1* limb swelling	Raw Infusion	Oral Topical
	Alchemilla vulgaris L. (LRSBG/AB/20/057)	رجل الأسد	Leaves	SD: 1* skin diseases and ulcer	Infusion	Topical
	Crataegus azarolus L. (LRSBG/AB/20/058)	الزعرور	Fruits/flowers	CVSD: 1* cardiovascular diseases NS: 2* headache	Raw Decoction	Oral Oral

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
				Dizziness		
	Eriobotrya japonica (Thunb.) Lindl (LRSBG/AB/20/059)	النيفلة او البشملة	Leaves	NS: 2* headache	Decoction	Oral
				Dizziness		
	Potentilla erecta (L.) Raeusch. (LRSBG/AB/20/060)	لنجبار	Roots	SRP: 1* breast milk outage	Maceration	Oral
				RTD: 1* chest and lung diseases	Raw	Oral
				GISD: 2* stomach ache	Infusion	Oral
				Ulcers	Decoction	Oral
	Prunus domestica L. (LRSBG/AB/20/061)	البرقوق	Fruits	GISD: 2* jaundice	Maceration	Oral
				Liver diseases		
	Prunus amygdalus L. (LRSBG/AB/20/062)	اللوز	Fruits	SRP: 1* infertility	Decoction	Oral
	Cydonia oblonga Mill. (LRSBG/AB/20/063)	السفرجل	Fruits	CVSD: 1* cardiovascular diseases	Raw	Oral
Rubiaceae	*Rubia tinctorum* L. (LRSBG/AB/20/117)	الفوة	Roots	NS: 1* sciatica	Raw	Oral
				SRP: 1* infertility	Raw	Oral
				HSD: 1* anemia	Maceration	Oral
				USD: 1* urinary tract infection/inflammation	Decoction	Oral
Rutaceae	*Citrus limon* (L.) Osbeck. (LRSBG/AB/20/105)	الليمون	Fruits	RTD: 3* asthma	Decoction	Oral
				Lung filtering/smoker	Decoction	Oral
				Pneumonia	Decoction	Oral
				NS: 1* dizziness	Decoction	Oral
				CVSD: 1* hypertension	Decoction	Oral
				GISD: 1* liver diseases	Decoction	Oral
				HSD: 1* spleen diseases	Decoction	Oral
	Ruta chalepensis L. (LRSBG/AB/20/106)	السذاب أو الفيجل	Aerial part	GISD: 2* IBS jaundice/ icterus,	Decoction	Oral
					Decoction/raw	Oral/topical
				SRP: 6* infertility	Decoction	Topical
				GH: 1* earache and deafness	Infusion	Topical
				SD: 2* limb swelling	Raw	topical
				NS: 1* headache	Decoction	Oral
Salvadoraceae	*Salvadora persica* L. (LRSBG/AB/20/140)	مسواك	Bark	RTD: 2* asthma	Decoction	Oral
				Lung filtering/smoker		
				SRP: 1* infertility	Decoction	Oral
Santalaceae	*Santalum album* L. (LRSBG/AB/20/118)	الصندل	Bark/fruits	NS: 1* migraine	Decoction	Topical/oral
Scrophulariaceae	*Verbascum sinuatum* L. (LRSBG/AB/20/141)	مصلح الأنظار أو البوصير أو تيسراو	Leaves	RTD: 5* pneumonia, chest and lung diseases, and asthma	Infusion	Topical
				GISD: 6* IBS and stomach pain	Decoction	Oral and steam
Solanaceae	*Lycium shawii* Roem. and Schult (LRSBG/AB/20/142)	العوسج	Roots/fruits/ leaves	SD: 5* skin ulcers	Decoction	Oral
				Leprosy		
				SRP: 4* uterine problems, infertility	Decoction	Oral
				SMSD: 2* osteoarthritis and gout	Decoction	Oral
	Nicotiana tabacum L. (LRSBG/AB/20/129)	الشمة	Leaves	GH: 1* tonsillitis	Infusion	Topical
Tamaricaceae	*Tamarix aphylla* (L.) H.Karst. (LRSBG/AB/20/143)	طحطاح	Leaves	NS: 1* headache	Decoction	Oral
Theaceae	*Camellia sinensis* (L.) Kuntze. (LRSBG/AB/20/120)	الشاي الأخضر	Leaves	SRP: 1* infertility	Maceration	Topical
				SD: 1* itchy skin	Maceration	Topical
Thymelaeaceae	*Daphne gnidium* L. (LRSBG/AB/20/107)	لازاز	Leaves	HC: 1* hair loss	Raw	Topical
				RTD: 1* sinusitis	Steaming	Topical
	Aquilaria malaccensis Lam. (LRSBG/AB/20/108)	العود الهندي أو عود غريس/أغريس	Bark	Can: 4* blood cancer	Decoction	Oral
				Stomach cancer	Decoction	Oral
				Liver cancer	Decoction	Oral
				Cancer	Raw	Topical
				HC: 1* alopecia areata	Raw	Oral
Ulmaceae	*Ulmus rubra* Muhl. (LRSBG/AB/20/144)	الدردار	Leaves	ID: 1* laryngitis	Decoction	Oral
				SMSD: 1* moving difficulty	Raw	Topical
Urticaceae		حريق أو القراص	Leaves		Decoction	Oral

(Continued on following page)

TABLE 7 | (*Continued*) List of medicinal plants used by traditional healers in the study areas.

Family	Scientific name (voucher number)	Local name	Part used	Ailments	Preparation methods	Administration
	Urtica dioica L. (LRSBG/AB/20/121)			USD: 1* urinary tract infection/inflammation		
				KD: 1* kidney problems	Decoction	Oral
				SMSD: 1* arthritis	Decoction	Oral
	Parietaria officinalis L. (LRSBG/AB/20/122)	فتات الحجر	Aerial part	KD: 1* urolithiasis	Decoction	Oral
Verbenaceae	*Verbena officinalis* L. (LRSBG/AB/20/146)	رعي الحمام	Aerial part	SRP: 1* uterine problems	Decoction	Oral
				USD: 1* bladder disease	Decoction	Oral
Vitaceae	*Vitis vinifera* L. (LRSBG/AB/20/147)	زبيب	Fruits	HSD: 1* anemia	Infusion	Oral
				SMSD: 2* back pain	Raw	Topical
				Moving difficulty	Raw	Oral
Xanthorrhoeaceae	*Aloe vera (L.)* Burm. f. (LRSBG/AB/20/164)	صبر	Leaves	SRP: 1* infertility	Decoction	Oral
				GISD: 1* stomach ache	Maceration	Oral
	Aloe perryi Baker (LRSBG/AB/20/125)	الصبر السقطري	Leaves	GISD: 2* colitis + flatulence	Raw	Oral
Zingiberaceae	*Zingiber officinale Roscoe* (LRSBG/AB/20/064)	زنجبيل او سكنجبير	Roots	Can: 3* breast cancer	Raw	Topical
				Legs cancer	Maceration	Topical
				RTD: 2* chest and lung diseases	Decoction	Oral
				USD: 1* urinary tract infection/inflammation	Raw	Oral
				GISD: 3* colitis	Raw	Oral
				Flatulence	Raw	Topical
				Jaundice/icterus	Raw	Oral
				GH: 1* hoarseness and sore throat	Raw	Oral
				NS: 1* head problems and psychosis	Raw	Topical
				SD: 1* skin diseases and ulcer	Maceration	Topical
				ESD: 1* goiter	Decoction	Oral
	Curcuma longa L. (LRSBG/AB/20/065)	الكركم	Roots	GISD: 2* jaundice	Infusion	Oral
				Liver diseases	Infusion	Oral
				GH: 2* anxiety disorders and hypochondria	Raw	Topical/oral
				NS: 1* head problems and psychosis	Raw	Topical
	Elettaria cardamomum (L.) Maton. (LRSBG/AB/20/066)	حب الهال	Seeds	GISD: 1* heartburn	Decoction	Oral
Zygophyllaceae	*Tetraena alba* (L.f.) Beier and Thulin. (LRSBG/AB/20/148)	العقاية	Leaves/seeds	ESD: 1* diabetes	Decoction	Oral

New Reports and New Uses

In the present study, 11% of the recorded 167 species have not been previously reported as medicinal plants in Algeria and neighboring countries in the Mediterranean basin. Moreover, more than 100 species reported here were previously reported to be used for therapeutic purposes in North-West (Benarba, 2015), South-West (Benarba, 2016), and North-East Algeria (Boual et al., 2020). Although each species had mostly the same therapeutic uses, for example, *A. herba-alba*, *Punica granatum* L., and *Senna alexandrina* Mill. were used mainly to treat gastrointestinal disorders, their vernacular names differed from one region to another such as *Aquilaria malaccensis* Lam. called Oud El-Rih in the West and A-ghriss in Sahara. These findings are in agreement with those reported in Algeria (Benarba, 2015; Bouasla and Bouasla, 2017), Morocco (Chaachouay et al., 2020; Merrouni and Elachouri, 2020; Yebouk et al., 2020), and other countries such as Yamen, Turkey, India, and China (Prabhu et al., 2014; Polat, 2019).

Interestingly, our findings report 47 new therapeutic uses for 20 known plant species. In the present study, we found that local populations living in the study areas used *Carlina gummifera* (L.) Less. to treat infertility, uterine problems, urinary tract infection, bladder disease, and osteoarthritis, whereas the plant uses previously reported included epilepsy, psoriasis, ulcers, and hemorrhage (Ahid et al., 2012; Hammiche et al., 2013). Likewise, leaves of *Cymbopogon schoenanthus* (L.) Spreng. were found to be used in the treatment of several types of cancer in the study areas. This use is reported for the first time since the plant was previously reported to be used mainly to treat termites and bruchid (Koba et al., 2007). *Prunus persica*, usually used against cough, constipation, and menstruation absent (Lin et al., 2021; Al-Fatimi., 2019), was reported by local populations to treat skin diseases.

Informant Consensus Factor and FL

Regarding the informant consensus factor, the highest F_{IC} value was recorded for cancer (F_{IC} = 0.49) with 44 medicinal species

used. This is the first study carried out in the three regions (West, Sahara, and Kabylia) of Algeria at the same time, calculating the informant consensus factor (F_{IC}). Our results revealed that cancer seems to be one of the most prevalent diseases in the study areas since no previous investigations had found cancer as the first ailment category according to their F_{IC} values. In fact, cancer has become a public health issue due to an increasing incidence, with 19.3 million new cases and about 10.0 million deaths worldwide in 2020 (Ferlay et al., 2021). Likewise, cancer incidence is increasing in Algeria. Actually, Algeria has the highest incidence of gastric (6%) (Behar et al., 2021) and liver cancer (Benarba and Meddah, 2014) when compared to North African countries. Moreover, breast and thyroid cancer incidence rose significantly in the last two decades (Mehemmai et al., 2020; Halfaoui et al., 2021). This pattern may be attributed to several causes, such as a westernized lifestyle, contaminated foods, pollution, and deteriorated living conditions. Furthermore, sexual-reproductive problems, gastrointestinal system diseases, skeletomuscular system disorders, and respiratory tract diseases were recorded to have higher F_{IC} values. In a previous study carried out in North-West Algeria, we found that gastrointestinal diseases had the highest F_{IC} value of 0.658, followed by general health (F_{IC} = 0.645) and respiratory diseases (0.642), while the cancer category was recorded to be the 4th highest (F_{IC} = 0.524) (Benarba et al., 2015). Moreover, a recent study carried out in the extreme North-West of Algeria reported that the reproductive and sexual disorders F_{IC} value were the highest score (0.98), and for the cancer category, they had an F_{IC} value of 0.77 with 6 species (Zatout et al., 2021). In disagreement with our findings, Bouasla and Bouasla (2017) indicated that cancer (F_{IC} = 0.25) was the least known ailment to be treated in the traditional medicine of the local population in North-East Algeria.

According to our results, *M. vulgare, A. herba-alba, Z. officinale,* and *J. phoenicia* had the absolute FL value of 100% in several ailment categories (SRD, cancer, respiratory diseases, and GISD). These findings are in agreement with those previously reported in different neighboring regions (Benarba et al., 2015; Bouasla and Bouasla, 2017; Chaachouay et al., 2020). Besides these species, *Parietaria officinalis* L. was found to possess an FL of 100% for kidney diseases which is consistent with findings previously reported in North-West Algeria (Benarba, 2016) and Morocco (Ammor et al., 2020). Inconsistent with our previous findings in both North-West (Benarba et al., 2015) and South-West Algeria (Benarba, 2016), *T. vulgaris* was the only species having the highest FL of 100% for skin diseases. This could be attributed to its antifungal and antimicrobial potentials demonstrated against the main pathogens causing skin diseases (Tadele et al., 2009; Vinciguerra et al., 2019). Recently, a facial phytocosmetic preparation from *T. vulgaris* was found to possess promising antiskin aging effects, as shown by enhanced adipogenesis through upregulation of PPAR-γ expression (Caverzan et al., 2021).

CONCLUSION

This is the first study carried out in three regions in Algeria (North, Center, and South) revealing an important botanical diversity and ethnobotanical knowledge held by local populations. The ethnobotanical survey allowed us to document 167 medicinal plants belonging to 70 families with their indigenous therapeutic uses (**Table 7**). Furthermore, 47 therapeutic uses for 20 known plant species were newly recorded, besides 25 species reported for the first time as medicinal plants in this study. On the other hand, *A. sativum, T. foenum-graecum, Z. officinale, R. chalepensis, A. herba-alba, P. anisum, M. chamomilla, O. basilicum,* and *T. vulgaris* had the highest UV. Moreover, some species had the absolute FL value of 100% in several ailment categories such as *M. vulgare*. These species could be further investigated to explore their curative proprieties and identify the possible active compounds.

Moreover, future ethnobotanical studies should adopt a multiple evidence-based approach that considers both the social-ecological-cultural context and local linguistic characteristics. In the same line, there is an urgent need for a clear strategy to include the local ethnobotanical knowledge in the conservation of biodiversity besides strong legislation aiming to protect the local medicinal species. Furthermore, establishing a unified local folk pharmacopeia based on different ethnobotanical and pharmacological investigations could be considered as one of the most important challenges in the future decade.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BB designed the study and prepared the questionnaire. KB carried out the ethnobotanical investigations. BB performed the identification of medicinal species. BB and KB verified the vernacular and scientific names of medicinal species. BB and KB analyzed the data and wrote the manuscript. BB revised the final version of the manuscript. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank all the traditional healers for sharing their valuable knowledge.

REFERENCES

Abdel-Hay, M. H., Saleh, A., El Ashry, E. S. H., Rashed, N., and Salama, O. (2002). Colorimetric Determination of Crude Powdered Myrrh, Purified Myrrh Extract, Oily Fraction, and its Different Pharmaceutical Dosage Forms. *Spectrosc. Lett.* 35 (2), 183–197. doi:10.1081/sl-120003804

Abdul-Ghani, R. A., Loutfy, N., and Hassan, A. (2009). Myrrh and Trematodoses in Egypt: an Overview of Safety, Efficacy and Effectiveness Profiles. *Parasitol. Int.* 58 (3), 210–214. doi:10.1016/j.parint.2009.04.006

Ahid, S., El Cadi, M. A., Meddah, B., and Cherrah, Y. (2012). Atractylis Gummifera: from Poisoning to the Analytic Methods. *Ann. Biol. Clin. (Paris)* 70 (3), 263–268. doi:10.1684/abc.2012.0699

Aissa, F. B. (1991). *Medicinal Plants in Algeria. Identification, Description of Active Ingredient Properties and Traditional Use of Common Plants in Algeria*.

Al-Fatimi, M. (2019). Ethnobotanical Survey of Medicinal Plants in central Abyan Governorate, Yemen. *J. Ethnopharmacol* 241, 111973. doi:10.1016/j.jep.2019.111973

Alami Merrouni, I., and Elachouri, M. (2020). Anticancer Medicinal Plants Used by Moroccan People: Ethnobotanical, Preclinical, Phytochemical and Clinical Evidence. *J. Ethnopharmacol* 266, 113435–435. doi:10.1016/j.jep.2020.113435

Alcaraz, C. (1989). Contribution à l'étude des groupements à Quercus ilex et Quercus faginea subsp. tlemcenensis, des monts de Tlemcen (Algérie). *ecmed* 15 (3), 15–32. doi:10.3406/ecmed.1989.1638

Allal, A., Bellifa, S., Benmansour, N., Selles, C., Semaoui, M., Hassaine, H., et al. (2019). Essential Oil and Hydrosol Extract Chemical Profile, Antioxidant and Antimicrobial Potential of Daphne Gnidium L. From Algeria. *J. Essent. Oil Bearing Plants* 22 (5), 1277–1288. doi:10.1080/0972060x.2019.1673832

Ammor, K., Mahjoubi, F., Bousta, D., and Chaqroune, A. (2020). Ethnopharmacological Survey of Medicinal Plants Used in the Traditional Treatment of Kidney Stones Realized in Fez-Morocco. *Ethnobotany Res. Appl.* 19, 1–2. doi:10.32859/era.19.50.1-12

Amri, E., and Kisangau, D. P. (2012). Ethnomedicinal Study of Plants Used in Villages Around Kimboza forest reserve in Morogoro, Tanzania. *J. Ethnobiol. Ethnomed* 8 (1), 1–9. doi:10.1186/1746-4269-8-1

Arnold, H. J., and Gulumian, M. (1984). Pharmacopoeia of Traditional Medicine in Venda. *J. Ethnopharmacol* 12 (1), 35–74. doi:10.1016/0378-8741(84)90086-2

BabaAissa, F. (1999). *Encyclopédie des plantes utiles*. Algiers: EDAS.

Barkaoui, M., Katiri, A., Boubaker, H., and Msanda, F. (2017). Ethnobotanical Survey of Medicinal Plants Used in the Traditional Treatment of Diabetes in Chtouka Ait Baha and Tiznit (Western Anti-atlas), Morocco. *J. Ethnopharmacol* 198, 338–350. doi:10.1016/j.jep.2017.01.023

Behar, D., Boublenza, L., Chabni, N., Hassaine, H., Dahmani, B., Masdoua, N., et al. (2021). Retrospective Epidemiological Study on Stomach Cancer in a Region of Western Algeria: about 394 Cases between 2011 and 2015. *J. Gastrointest. Canc* 52 (2), 706–710. doi:10.1007/s12029-020-00459-z

Bellakhdar, J. (1997). *Contribution à l'étude de la pharmacopée traditionnelle au Maroc: la situation actuelle, les produits, les sources du savoir (enquête ethnopharmacologique de terrain réalisée de 1969 à 1992)*. Metz, France: Doctoral dissertation, Université Paul Verlaine-Metz.

Benali, T., Khabbach, A., Ennabili, A., and Hammani, K. (2017). Ethnopharmacological Prospecting of Medicinal Plants from the Province of Guercif (NE of Morocco). *Moroccan J. Biol.* 14, 1–14.

Benarba, B., Belabid, L., Righi, K., Bekkar, A. A., Elouissi, M., Khaldi, A., et al. (2015). Ethnobotanical Study of Medicinal Plants Used by Traditional Healers in Mascara (North West of Algeria). *J. Ethnopharmacol* 175, 626–637. doi:10.1016/j.jep.2015.09.030

Benarba, B., and Meddah, B. (2014). Ethnobotanical Study, Antifungal Activity, Phytochemical Screening and Total Phenolic Content of Algerian Aristolochia Longa. *J. Intercult Ethnopharmacol* 3 (4), 150–154. doi:10.5455/jice.20140826030222

Benarba, B. (2016). Medicinal Plants Used by Traditional Healers from South-West Algeria: An Ethnobotanical Study. *J. Intercult Ethnopharmacol* 5 (4), 320–330. doi:10.5455/jice.20160814115725

Benarba, B. (2015). Use of Medicinal Plants by Breast Cancer Patients in Algeria. *EXCLI J.* 14, 1164–1166. doi:10.17179/excli2015-571

Benarba, B., Pandiella, A., and Elmallah, A. (2016). Anticancer Activity, Phytochemical Screening and Acute Toxicity Evaluation of an Aqueous Extract of Aristolochia Longa L. *Int. J. Pharm. Phytopharmacological Res.* 6 (1), 20–26.

Benítez, G., González-Tejero, M. R., and Molero-Mesa, J. (2010). Pharmaceutical Ethnobotany in the Western Part of Granada Province (Southern Spain): Ethnopharmacological Synthesis. *J. Ethnopharmacology* 129 (1), 87–105.

Bhattacharjee, R., and Sil, P. C. (2007). Protein Isolate from the Herb, Phyllanthus Niruri L. (Euphorbiaceae), Plays Hepatoprotective Role against Carbon Tetrachloride Induced Liver Damage via its Antioxidant Properties. *Food Chem. Toxicol.* 45 (5), 817–826. doi:10.1016/j.fct.2006.10.029

Boual, Z., Pierre, G., Kemassi, A., Mosbah, S., Benaoun, F., Delattre, C., et al. (2020). Chemical Composition and Biological Activities of Water-Soluble Polysaccharides from Commiphora Myrrha (Nees) Engl. GUM. *Analele Universităţii din Oradea, Fascicula Biologie*. 27 (1), 50–55.

Bouasla, A., and Bouasla, I. (2017). Ethnobotanical Survey of Medicinal Plants in Northeastern of Algeria. *Phytomedicine* 36, 68–81. doi:10.1016/j.phymed.2017.09.007

Boudjelal, A., Henchiri, C., Sari, M., Sarri, D., Hendel, N., Benkhaled, A., et al. (2013). Herbalists and Wild Medicinal Plants in M'Sila (North Algeria): An Ethnopharmacology Survey. *J. Ethnopharmacol* 148 (2), 395–402. doi:10.1016/j.jep.2013.03.082

Bouzabata, A. (2013). Traditional Treatment of High Blood Pressure and Diabetes in Souk Ahras District. *J. Pharmacognosy Phytotherapy* 5 (1), 12–20.

Calixto, J. B. (2019). The Role of Natural Products in Modern Drug Discovery. *Acad. Bras Cienc* 91 Suppl 3, e20190105. doi:10.1590/0001-3765201920190105

Caverzan, J., Mussi, L., Sufi, B., Padovani, G., Nazato, L., Camargo, F. B., et al. (2021). A New Phytocosmetic Preparation from Thymus Vulgaris Stimulates Adipogenesis and Controls Skin Aging Process: *In Vitro* Studies and Topical Effects in a Double-blind Placebo-controlled Clinical Trial. *J. Cosmet. Dermatol.* 20 (7), 2190–2202. doi:10.1111/jocd.13818

Çelik, G., Kılıç, G., Kanbolat, Ş., Özlem Şener, S., Karaköse, M., Yaylı, N., et al. (2021). Biological Activity, and Volatile and Phenolic Compounds from Five Lamiaceae Species. *Flavour Fragrance J.* 36 (2), 223–232.

Chaachouay, N., Benkhnigue, O., Khamar, H., and Zidane, L. (2020). Ethnobotanical Study of Medicinal and Aromatic Plants Used in the Treatment of Genito-Urinary Diseases in the Moroccan Rif. *J. Mater. Environ. Sci.* 11 (1), 15–29.

Chehma, A., and Djebbar, M. R. (2008). Les espèces médicinales spontanées du Sahara septentrional algérien: distribution spatio-temporelle et étude ethnobotanique. *Revue Synthèse* 17, 36–45.

Chelghoum, M., Khitri, W., Bouzid, S., and Lakermi, A. (2021). New Trends in the Use of Medicinal Plants by Algerian Diabetic Patients, Considerations of Herb-Drug Interactions. *J. Ethnopharmacology* 274, 113984. doi:10.1016/j.jep.2021.113984

Daoudi, A., Bammou, M., Zarkani, S., Slimani, I., Ibijbijen, J., and Nassiri, L. (2016). Étude ethnobotanique de la flore médicinale dans la commune rurale d'Aguelmouss province de Khénifra (Maroc). *Phytothérapie* 14 (4), 220–228. doi:10.1007/s10298-015-0953-z

El Ashry, E. S., Rashed, N., Salama, O. M., and Saleh, A. (2003). Components, Therapeutic Value and Uses of Myrrh. *Pharmazie* 58 (3), 163–168.

El-Hilaly, J., Hmammouchi, M., and Lyoussi, B. (2003). Ethnobotanical Studies and Economic Evaluation of Medicinal Plants in Taounate Province (Northern Morocco). *J. Ethnopharmacol* 86 (2-3), 149–158. doi:10.1016/s0378-8741(03)00012-6

Fakchich, J., and Elachouri, M. (2014). Ethnobotanical Survey of Medicinal Plants Used by People in Oriental Morocco to Manage Various Ailments. *J. Ethnopharmacology* 154 (1), 76–87.

Ferlay, J., Colombet, M., Soerjomataram, I., Parkin, D. M., Piñeros, M., Znaor, A., et al. (20211994). Cancer Statistics for the Year 2020: An overviewIn Vitro Experimental Infection of Primary Human Hepatocytes with Hepatitis B Virus. *Int. J. CancerGastroenterology* 149106 (43), 779664–789673. doi:10.1002/ijc.33588

Giday, M., Asfaw, Z., Woldu, Z., and Teklehaymanot, T. (2009). Medicinal Plant Knowledge of the Bench Ethnic Group of Ethiopia: an Ethnobotanical Investigation. *J. Ethnobiol. Ethnomed* 5, 34. doi:10.1186/1746-4269-5-34

Halfaoui, N. S., Majda, D., Nouria, D., Boulenouar, H., Behar, A., and Belhadj, M. (2021). Dietary and Female Reproductive Risk Factors for Thyroid Cancer: a Case-Control Study in Western Algeria. *WCRJ* 8, e1927.

Hammiche, V., and Maiza, K. (2006). Traditional Medicine in Central Sahara: Pharmacopoeia of Tassili N'ajjer. *J. Ethnopharmacol* 105 (3), 358–367. doi:10.1016/j.jep.2005.11.028

Hammiche, V., Merad, R., and Azzouz, M. (2013). *Plantes toxiques à usage médicinal du pourtour méditerranéen*. Paris: Springer.

Hoffman, B., and Gallaher, T. (2007). Importance Indices in Ethnobotany. *Ethnobot. Res. App.* 5, 201–218. doi:10.17348/era.5.0.201-218

Khan, I., AbdElsalam, N. M., Fouad, H., Tariq, A., Ullah, R., and Adnan, M. (2014). Application of Ethnobotanical Indices on the Use of Traditional Medicines against Common Diseases. *Evid Based. Complement. Altern. Med* 2014, 1–21. doi:10.1155/2014/635371

Koba, K., Poutouli, P. W., Nenonene, Y. A., Songai, M. S., Raynaud, C., and Sanda, K. (2007). Chemical Composition and Anti-termite Activity of Three Tropical Essential Oils against Termite Species Trinervitermes Geminatus (Wasmann). *J. Sci. Technol.* 5 (2), 39–46.

Kobayashi, H., Oguchi, H., Takizawa, N., Miyase, T., Ueno, A., Usmanghani, K., et al. (1987). New Phenylethanoid Glycosides from Cistanche Tubulosa (SCHRENK) HOOOK. F. I. *Chem. Pharm. Bull.* 35 (8), 3309–3314. doi:10.1248/cpb.35.3309

Kunkele, L., and Lohmeyer, T. R. (2007). *Plantes Médicinales*. London: Parragon Books Ltd.

Lahlah, Z. F., Meziani, M., and Maza, A. (2012). Silymarin Natural Antimicrobial Agent Extracted from Silybum marianum. *J. Acad.* 2, 164–169.

Lavagna, S. M., Secci, D., Chimenti, P., Bonsignore, L., Ottaviani, A., and Bizzarri, B. (2001). Efficacy of Hypericum and Calendula Oils in the Epithelial Reconstruction of Surgical Wounds in Childbirth with Caesarean Section. *Farmaco* 56 (5-7), 451–453. doi:10.1016/s0014-827x(01)01060-6

Leto, C., Tuttolomondo, T., La Bella, S., and Licata, M. (2013). Ethnobotanical Study in the Madonie Regional Park (Central Sicily, Italy)--medicinal Use of Wild Shrub and Herbaceous Plant Species. *J. Ethnopharmacol* 7146 (1), 90–112. doi:10.1016/j.jep.2012.11.042

Lievre, M., Marichy, J., Baux, S., Foyatier, J. L., and Perrot, J. (1992). Controlled Study of Three Ointments for the Local Management of 2nd and 3rd Degree burns. *Clin. trials meta-analysis* 28 (1), 9–12.

Lin, Y., Wang, S. P., Zhang, J. Y., Zhuo, Z. Y., Li, X. R., Zhai, C. J., et al. (2021). Ethnobotanical Survey of Medicinal Plants in Gaomi, China. *J. Ethnopharmacol* 265, 113228. doi:10.1016/j.jep.2020.113228

Massoud, A., El Sisi, S., Salama, O., and Massoud, A. (2001). Preliminary Study of Therapeutic Efficacy of a New Fasciolicidal Drug Derived from Commiphora Molmol (Myrrh). *Am. J. Trop. Med. Hyg.* 65 (2), 96–99. doi:10.4269/ajtmh.2001.65.96

Mattalia, G., Sõukand, R., Corvo, P., and Pieroni, A. (2020). Blended Divergences: Local Food and Medicinal Plant Uses Among Arbëreshë, Occitans, and Autochthonous Calabrians Living in Calabria, Southern Italy. *Plant Biosyst. - Int. J. Dealing all Aspects Plant Biol.* 154 (5), 615–626. doi:10.1080/11263504.2019.1651786

Mechaala, S., Bouatrous, Y., and Adouane, S. (2021). Traditional Knowledge and Diversity of Wild Medicinal Plants in El Kantara's Area (Algerian Sahara Gate): An Ethnobotany Survey. *Acta Ecologica Sinica*. doi:10.1016/j.chnaes.2021.01.007

Meddour, R., and Sahar, O. (2021). Floristic Inventory of Djurdjura National Park, Northern Algeria: a First Checklist of its Vascular flora. *Phytotaxa* 490 (3), 221–238. doi:10.11646/phytotaxa.490.3.1

Mehemmai, C., Cherbal, F., Hamdi, Y., Guedioura, A., Benbrahim, W., Bakour, R., et al. (2020). Correction to: BRCA1 and BRCA2 Germline Mutation Analysis in Hereditary Breast/Ovarian Cancer Families from the Aures Region (Eastern Algeria): First Report. *Pathol. Oncol. Res.* 26 (2), 2009–2010. doi:10.1007/s12253-019-00625-0

Menale, B., De Castro, O., Cascone, C., and Muoio, R. (2016). Ethnobotanical Investigation on Medicinal Plants in the Vesuvio National Park (Campania, Southern Italy). *J. Ethnopharmacol* 192, 320–349. doi:10.1016/j.jep.2016.07.049

Miara, M. D., Ait, H. M., Dahmani, W., Negadi, M., and Djellaoui, A. (2018). Nouvelles données sur la flore endémique du sous-secteur de l'Atlas tellien Oranais "O3" (Algérie occidentale). *abm* 43, 63–69. doi:10.24310/abm.v43i0.4453

Miara, M. D., Hammou, M. A., and Aoul, S. H. (2013). Phytothérapie et taxonomie des plantes médicinales spontanées dans la région de Tiaret (Algérie). *Phytothérapie* 11 (4), 206–218. doi:10.1007/s10298-013-0789-3

Missoun, F., Bouabdelli, F., Awatif, B., Amari, N., and Djebli, N. (2018). Antidiabetic Bioactive Compounds from Plants. *Mèd Tech. J.* 2 (2), 199–214. doi:10.26415/2572-004x-vol2iss2p199-214

Mrabti, H. N., Jaradat, N., Kachmar, M. R., Ed-Dra, A., Ouahbi, A., Cherrah, Y., et al. (2019). Integrative Herbal Treatments of Diabetes in Beni Mellal Region of Morocco. *J. Integr. Med.* 17 (2), 93–99. doi:10.1016/j.joim.2019.01.001

Msaddak, A., Durán, D., Rejili, M., Mars, M., Ruiz-Argüeso, T., Imperial, J., et al. (2017). Diverse Bacteria Affiliated with the Genera Microvirga, Phyllobacterium, and Bradyrhizobium Nodulate Lupinus Micranthus Growing in Soils of Northern Tunisia. *Appl. Environ. Microbiol.* 83 (6), e02820–16. doi:10.1128/AEM.02820-16

Namba, H., Narahara, K., Tsuji, K., Yokoyama, Y., Murakami, M., Matsubara, T., et al. (1994). "Developmental Change in Activity of Red Cell Porphobilinogen Deaminase and its Electrophoretic Variant in the Japanese Population," in *The Encyclopedia of Wakan-Yaku (Traditionol Sino-Japanese Medicines) with Color Picture* (Tokyo: Hoiku-sha), 36, 16–19. doi:10.1111/j.1442-200x.1994.tb03122.x

Obón, C., Rivera, D., Verde, A., Fajardo, J., Valdés, A., Alcaraz, F., et al. (2012). Árnica: a Multivariate Analysis of the Botany and Ethnopharmacology of a Medicinal Plant Complex in the Iberian Peninsula and the Balearic Islands. *J. Ethnopharmacol* 144 (1), 44–56. doi:10.1016/j.jep.2012.08.024

Ojah, E. O. (2020). Medicinal Plants: Prospective Drug Candidates against the Dreaded Coronavirus. *Iberoam J. Med.* 2, 314–321. doi:10.53986/ibjm.2020.0055

Ouelbani, R., Bensari, S., Mouas, T. N., and Khelifi, D. (2016). Ethnobotanical Investigations on Plants Used in Folk Medicine in the Regions of Constantine and Mila (North-East of Algeria). *J. Ethnopharmacol* 194, 196–218. doi:10.1016/j.jep.2016.08.016

Polat, R. (2019). Ethnobotanical Study on Medicinal Plants in Bingöl (City center) (Turkey). *J. Herbal Med.* 16, 100211. doi:10.1016/j.hermed.2018.01.007

Pooley, R. A. (2005). AAPM/RSNA Physics Tutorial for Residents: Fundamental Physics of MR Imaging. *Radiographics* 25 (4), 1087–1099. doi:10.1148/rg.254055027

Prabhu, S., Vijayakumar, S., Yabesh, J. E., Ravichandran, K., and Sakthivel, B. (2014). Documentation and Quantitative Analysis of the Local Knowledge on Medicinal Plants in Kalrayan hills of Villupuram District, Tamil Nadu, India. *J. Ethnopharmacol* 157, 7–20. doi:10.1016/j.jep.2014.09.014

Pranskuniene, Z., Bernatoniene, J., Simaitiene, Z., Pranskunas, A., and Mekas, T. (2016). Ethnomedicinal Uses of Honeybee Products in lithuania: the First Analysis of Archival Sources. *Evidence-Based Complement. Altern. Med.* 2016, 9272635. doi:10.1155/2016/9272635

Saravanan, R. (2016). *Evaluation of* In-Vitro *and* In-Vivo *Anticancer Activity of Leaf Extracts of Amaranthus Spinosus Linn (Doctoral Dissertation*. Chennai): College of Pharmacy Madras Medical College.

Sargin, S. A., Selvi, S., and Büyükcengiz, M. (2015). Ethnomedicinal Plants of Aydıncık District of Mersin, Turkey. *J. Ethnopharmacol* 174, 200–216. doi:10.1016/j.jep.2015.08.008

Sarri, M., Mouyet, F. Z., Benziane, M., and Cheriet, A. (2014). Traditional Use of Medicinal Plants in a City at Steppic Character (M'sila, Algeria). *J. Pharm. Pharmacognosy Res* 2 (2), 31–35.

Savić, J., Mačukanović-Jocić, M., and Jarić, S. (2019). Medical Ethnobotany on the Javor Mountain (Bosnia and Herzegovina). *Eur. J Integr. Med* 27, 52–64.

Sawadogo, W. R., Schumacher, M., Teiten, M. H., Dicato, M., and Diederich, M. (2012). Traditional West African Pharmacopeia, Plants and Derived Compounds for Cancer Therapy. *Biochem. Pharmacol.* 84 (10), 1225–1240. doi:10.1016/j.bcp.2012.07.021

Sekkoum, K., Cheriti, A., Taleb, S., Bourmita, Y., and Belboukhari, N. (2011). Traditional Phytotherapy for Urinary Diseases in Bechar District (South West of Algeria). *Electron. J. Environ. Agric. Food Chem.* 10 (8), 2616–2622.

Shao, Z. M., Wu, J., Shen, Z. Z., and Barsky, S. H. (1998). Genistein Exerts Multiple Suppressive Effects on Human Breast Carcinoma Cells. *Cancer Res.* 58 (21), 4851–4857.

Skalli, S., Hassikou, R., and Arahou, M. (2019). An Ethnobotanical Survey of Medicinal Plants Used for Diabetes Treatment in Rabat, Morocco. *Heliyon* 5 (53), e01421. doi:10.1016/j.heliyon.2019.e01421

Synowiec, A., Gniewosz, M. A. Ł. G. O. R. Z. A. T. A., Baczek, K., and Przybyl, J. L. (2014). Antimicrobial Effect of an Aqueous Extract of Potentilla Erecta Rhizome. *Herba Pol.* 60 (2). doi:10.2478/hepo-2014-0007

Tadele, A., Urga, K., Gemeda, N., Lemma, H., Melaku, D., and Mudie, K. (2009). Antimicrobial Activity of Topical Formulations Containing Thymus Vulgaris Essential Oil on Major Pathogens Causing Skin Diseases. *Ethiopian Pharm. J.* 26 (2), 103–110. doi:10.4314/epj.v26i2.43041

Taïbi, K., Abderrahim, L. A., Ferhat, K., Betta, S., Taïbi, F., Bouraada, F., et al. (2020). Ethnopharmacological Study of Natural Products Used for Traditional

Cancer Therapy in Algeria. *Saudi Pharm. J.* 28 (11), 1451–1465. doi:10.1016/j.jsps.2020.09.011

Taïbi, K., Abderrahim, L. A., Helal, F., and Hadji, K. (2021). Ethnopharmacological Study of Herbal Remedies Used for the Management of Thyroid Disorders in Algeria. *Saudi Pharm. J.* 29 (1), 43–52.

Teixidor-Toneu, I., Martin, G. J., Ouhammou, A., Puri, R. K., and Hawkins, J. A. (2016). An Ethnomedicinal Survey of a Tashelhit-Speaking Community in the High Atlas, Morocco. *J. Ethnopharmacol* 188, 96–110. doi:10.1016/j.jep.2016.05.009

Trabut, L. (2015). *Répertoire des noms des plantes du Maghreb*. Algiers: Livres Editions.

Tuttolomondo, T., Licata, M., Leto, C., Savo, V., Bonsangue, G., Letizia Gargano, M., et al. (2014). Ethnobotanical Investigation on Wild Medicinal Plants in the Monti Sicani Regional Park (Sicily, Italy). *J. Ethnopharmacol* 14153 (3), 568–586. doi:10.1016/j.jep.2014.02.032

Vinciguerra, V., Rojas, F., Tedesco, V., Giusiano, G., and Angiolella, L. (2019). Chemical Characterization and Antifungal Activity of Origanum Vulgare, Thymus Vulgaris Essential Oils and Carvacrol against Malassezia Furfur. *Nat. Prod. Res.* 33 (22), 3273–3277. doi:10.1080/14786419.2018.1468325

Yabesh, J. E., Prabhu, S., and Vijayakumar, S. (2014). An Ethnobotanical Study of Medicinal Plants Used by Traditional Healers in Silent valley of Kerala, India. *J. Ethnopharmacol* 154, 774–789. doi:10.1016/j.jep.2014.05.004

Yabesh, J. E., Prabhu, S., and Vijayakumar, S. (2014). An Ethnobotanical Study of Medicinal Plants Used by Traditional Healers in Silent valley of Kerala, India. *J. Ethnopharmacol* 154 (3), 774–789. doi:10.1016/j.jep.2014.05.004

Yebouk, C., Redouan, F. Z., Benítez, G., Bouhbal, M., Kadiri, M., Boumediana, A. I., et al. (2020). Ethnobotanical Study of Medicinal Plants in the Adrar Province, Mauritania. *J. Ethnopharmacol* 246, 112217. doi:10.1016/j.jep.2019.112217

Zatout, F., Benarba, B., Bouazza, A., Babali, B., Bey, N. N., and Morsli, A. (2021). Ethnobotanical Investigation on Medicinal Plants Used by Local Populations in Tlemcen National Park (Extreme North West Algeria). *Mediterr. Bot.* 15 (30), 1–12. doi:10.5209/mbot.69396

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 09 December 2021
doi: 10.3389/fphar.2021.777400

Ameliorative Effects of Dietary Ellagic Acid Against Severe Malaria Pathogenesis by Reducing Cytokine Storms and Oxidative Stress

Shilpa Mohanty[1,2†], *Amit Chand Gupta*[1†], *Anil Kumar Maurya*[1], *Karuna Shanker*[2,3], *Anirban Pal*[1,2] *and Dnyaneshwar Umrao Bawankule*[1,2*]

[1]In-vivo *Testing Laboratory, Molecular Bioprospection Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India,* [2]*Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India,* [3]*Analytical Chemistry Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India*

Ellagic acid (EA), a fruit- and vegetable-derived flavonoid, has been reported for multiple pharmacological activities, which encouraged us to examine its useful effect in severe malaria pathogenesis, especially malaria-induced cytokine storms and oxidative stress linked to damage in major organs. Malaria was induced by injecting *Plasmodium berghei*–infected RBCs intraperitoneally into the mice. EA was given orally (5, 10, and 20 mg/kg) following Peter's 4-day suppression test. EA exhibited the suppression of parasitemia, production of inflammatory cytokine storms and oxidative stress marker level quantified from vital organs significantly and an increase in hemoglobin, blood glucose, and mean survival time compared to the vehicle-treated infected group. EA administration also restored the blood–brain barrier integrity evidenced through Evans blue staining. Furthermore, we demonstrated the protecting effect of EA in LPS-induced inflammatory cytokine storms and oxidative stress in glial cells. The present study conclude that ellagic acid is able to alleviate severe malaria pathogenesis by reducing cytokine storms and oxidative stress–induced by malarial parasites. It also attributed promising antimalarial activity and afforded to improve the blood glucose and hemoglobin levels in treated mice. These research findings suggested the suitability of ellagic acid as a useful bioflavonoid for further study for the management of severe malaria pathogenesis.

Keywords: ellagic acid, malaria, inflammation, cytokine, storms, oxidative stress, mice

OPEN ACCESS

Edited by:
Massimo Lucarini, Council for Agricultural Research and Economics, Italy

Reviewed by:
Mavondo Greanious Alfred, National University of Science and Technology, Zimbabwe
Edson Roberto Silva, University of São Paulo, Brazil

***Correspondence:**
Dnyaneshwar Umrao Bawankule
du.bawankule@cimap.res.in

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *15 September 2021*
Accepted: *11 November 2021*
Published: *09 December 2021*

Citation:
Mohanty S, Gupta AC, Maurya AK, Shanker K, Pal A and Bawankule DU (2021) Ameliorative Effects of Dietary Ellagic Acid Against Severe Malaria Pathogenesis by Reducing Cytokine Storms and Oxidative Stress. Front. Pharmacol. 12:777400. doi: 10.3389/fphar.2021.777400

Abbreviations: BSA, bovine serum albumin; CMC, carboxymethylcellulose; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein; EA, ellagic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; LPS, lipopolysaccharide; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium); MPO, myeloperoxidase; MDA, malondialdehyde; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid; TMB, tetramethylbenzidine.

INTRODUCTION

According to the WHO, the incidence and death rates due to malaria have dropped down worldwide over the past 16 years; in 2018, there were 228 million new cases and 405,000 deaths from malaria. Still malaria is an important public health problem in many developing countries of the tropical and subtropical regions of the world, with high mortality in children and pregnant women (World Health Organization, 2019). Malaria is caused by *Plasmodium* parasites which are transmitted to humans through the bite of female mosquitoes of *Anopheles* species (Snow et al., 2005). Vaccination is the most effective method of preventing infectious diseases; however, producing an effective vaccine remains a challenge in malarial infection (Stanisic and Good, 2015). Current findings established that free radicals and its connection with oxidative stress in severe malaria can be responsible for several additional complications (Pabon et al., 2003; Becker et al., 2004). Clinical indications in severe malaria were found to be linked with the blood stage of infection with an excessive release of inflammatory cytokines (TNF-α, IL-6, and INF-γ) which contribute to further severity of infection such as organ damage and severe anemia (Saxena et al., 2016). Treatment of malaria has been complicated by the development of resistance to combination therapies based on artemisinin (Kumar et al., 2015).

Ellagic acid (EA) is a thermostable dilactone of hexahydroxydiphenic acid [2,3,7,8-tetrahydroxy-chromeno (5,4,3-cde)chromene-5,10-dione] with a molecular mass of 338.2 g/mol. EA is an important bioflavonoid present in several fruits, berries, and vegetables. It is also present as a primary constituent of various tannin-bearing antimalarial plants which are found in Africa (Vattem et al., 2005). EA has received considerable biomedical research attention due to their potent antioxidant activity and noticeable pharmacological effects on the prevention of various chronic pathological conditions linked with oxidative stress (Shakeri et al., 2018). EA possesses multiple health benefits including anti-inflammatory, antiviral, anticancer, antibacterial, hepatoprotective, cardioprotective, neuroprotective, gastroprotective, and antihyperlipidemic effects (Evtyugin et al., 2020). Considering the pharmacological importance of ellagic acid, several research groups are working on nanotechnology-related experimental approaches based on innovative oral drug carriers to improve its bioavailability (Ceci et al., 2020). In an attempt to evaluate the novel pharmacological activity of ellagic acid, we have explored the beneficial effect of EA on oxidative stress and inflammation-linked severe malaria pathogenesis in mice. The research findings of this study suggested the suitability of ellagic acid as a useful bioflavonoid for further study for the management of severe malaria pathogenesis.

MATERIALS AND METHODS

Chemicals

Ellagic acid (**Figure 1**), chloroquine diphosphate, sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4), LPS (*Escherichia coli* 055:B5), disodium hydrogen phosphate (Na_2HPO_4), DMEM, FBS, streptomycin, penicillin, potassium chloride, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), trichloroacetic acid, thiobarbituric acid, pyrogallol, TMB, Evans blue, and dimethylsulfoxide (DMSO) were procured from Sigma-Aldrich, United States. Mouse- and rat-specific ELISA kits were purchased from BD Biosciences.

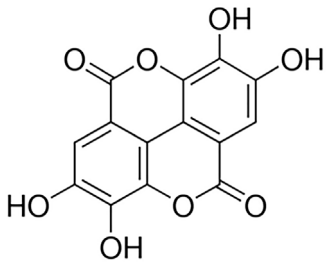


FIGURE 1 | Chemical structure of ellagic acid.

In Vivo Study

Experimental Animals and Ethical Approval

Swiss albino (male, 18–22 g) mice were obtained from the institutional animal facility and acclimatized for 7 days under standard environmental conditions (12:12 dark-to-light cycle, 23 ± 2°C). After the termination of experiments, the experimental mice were euthanized by cervical dislocation after anesthetizing the animals using ketamine (80 mg/kg) and xylazine (10 mg/kg) as per the approved protocol (CIMAP/IAEC/2016-19/09). The vital organs of the experimental mice were immediately isolated for quantification of inflammation and oxidative stress markers. Animal experiments were performed as per the approved protocol by the Institutional Animal Ethics Committee (IAEC).

Infection and Drug Treatment

Malaria was induced by giving intraperitoneal injection of *Plasmodium berghei* K-173–infected RBC's into the experimental mice. The antimalarial activity was assessed using the method described by Knight and Peters (Kalia et al., 2015). One hour after infection, the experimental mice were orally treated with EA (5, 10, and 20 mg/kg) for 4 days consecutively. Carboxymethylcellulose (CMC) was used as a vehicle. Thin smears were prepared from blood of the infected mice and stained with Giemsa stain every alternate day from 4th day up to 28th day to calculate parasitemia by microscopic examination. Parasitemia was counted based on parasitized RBCs counted per 100 normal RBCs. Suppression of parasitemia after treatment was calculated by using the formula [(A-B)/A]100, where A denotes the mean percent parasitemia of the vehicle-treated mice and B denotes the mean percent parasitemia of the treated group. Survival of the infected experimental mice was observed up to the 28th day to calculate the percent survival and mean survival time.

Quantification of Blood Glucose and Hemoglobin

On the peak day of parasitemia, blood glucose and hemoglobin were quantified to evaluate the possible effect of EA in malarial infection.

Hemoglobin was quantified using Drabkin's cyanmethemoglobin procedure and blood glucose using a glucometer.

Quantification of Inflammatory Mediators

Blood was collected from each mouse for serum, and whole brain was isolated on the peak day of infection. Serum and brain homogenate were used for the quantification of pro-inflammatory cytokine production by using ELISA reagents.

Quantification of Oxidative Stress Markers

To determine the effect of EA on organ damage caused by oxidative stress due to malaria infection in mice, a separate set of experiment was performed. The vital organs were harvested from the infected mice for oxidative stress marker quantification. In total, 10% (w/v) tissue homogenates were prepared and centrifuged at 5000 g for 10 min, and the collected supernatant was stored at -80°C immediately. The activity of superoxide dismutase was evaluated for its ability to inhibit pyrogallol autoxidation (Del Maestro and McDonald, 1985). The production of reactive oxygen species (ROS) was quantified using DCF-DA, a non-fluorescent cell-permeating compound, as described previously (Driver et al., 2000). Myeloperoxidase activity was quantified in tissue homogenates using TMB (3,3′5,5′ tetramethylbenzidine), as previously described (Suzuki et al., 1983). Lipid peroxidation was quantified by measuring the content of thiobarbituric acid reactive substances (TBARS) spectrophotometrically (Draper and Hadley, 1990).

Evans Blue Extravasation Assay

For Evans blue assay, 0.2 ml of 2% Evans blue solution in PBS was injected intraperitoneally into the infected mice to assess the blood–brain barrier permeability on the peak day of parasitemia. After 2 h, brain tissues were removed for dye extraction in 100% formamide. After 48 h, absorbance was measured at 620 nm and presented as micrograms of Evans blue stain per gram of brain tissue (Baptista et al., 2010).

In Vitro Study

Quantification of ROS Generation and Pro-Inflammatory Mediators in C6 Glial Cells

C6 glial cells, procured from the National Centre for Cell Science (NCCS), Pune, India, were cultured using Ham's F-10 media containing 10% FBS and placed in an incubator (5% CO2, 37°C). The cytotoxicity effect of EA was assessed using MTT assay on C6 cells, as described previously (Sharma et al., 2012). Intracellular ROS generation induced by H_2O_2 was calculated using DCFH-DA. The fluorescence intensity was measured with a spectrofluorometer (FLUO Star Omega, BMG Labtech), with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The intracellular reactive oxygen species production was also measured by flow cytometry (Vinoth et al., 2015). LPS-induced production of pro-inflammatory cytokines was quantified in the cell culture supernatant using rat-specific EIA reagents (Gupta et al., 2018).

Statistical Analysis

Experimental data were shown as means ± SEM. The statistical significance of vehicle vs treatment groups was calculated using ANOVA, followed by Tukey's multiple comparison tests. $p < 0.05$ was considered significant.

RESULTS

Effect of EA on Parasitemia and Mortality in Malaria Pathogenesis

Oral treatment with EA at a dose of 5, 10, and 20 mg/kg showed a significant inhibition of parasitemia ($p < 0.05$) in comparison to the vehicle-treated infected mice in a dose-dependent manner (**Figure 2A**). Mean survival time was also significantly improved in the EA-treated group compared to the vehicle-treated infected group (**Figure 2B**).

Effect of EA on Blood Glucose and Hemoglobin in Malaria Pathogenesis

Blood glucose and hemoglobin levels were found to be decreased significantly on the peak day of parasitemia in the vehicle-treated infected mice in compared to uninfected normal. Treatment with EA improved the blood glucose and hemoglobin in a dose-dependent manner (**Figure 3**).

Effect of EA on Oxidative and Antioxidative Markers in Malaria Pathogenesis

The MDA level, MPO activity, and ROS production were increased significantly (p‹0.05) in the brain, liver, and spleen of the vehicle-treated infected group in comparison to the uninfected normal group. EA treatment reduced the level of these oxidative stress markers compared with the vehicle-treated infected group. The SOD level was found to be significantly (p‹0.05) decreased in the vehicle-treated infected group compared to that in normal uninfected group, whereas EA-treated groups significantly increased the SOD level in comparison to the vehicle-treated infected group. The effect of EA treatment on the oxidative stress marker level in tissue homogenate is illustrated in **Table 1**.

Effect of EA on Pro-Inflammatory Markers in Malaria Pathogenesis

The production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IFN-γ) in serum and brain homogenate of the vehicle-treated infected mice was significantly increased compared to the normal group on the peak day of parasitemia. EA treatment showed a significant inhibition of pro-inflammatory cytokine production dose-dependently in malaria pathogenesis than in the vehicle-treated infected mice (**Figures 4**, **5**).

Effect of EA on Blood–Brain Barrier Permeability in Malaria Pathogenesis

An increase in the blood–brain barrier (BBB) permeability is a characteristic feature of severe malaria pathogenesis. Evans blue leakage assay showed that the normal mice have no disruption of BBB, whereas the brain isolated from the vehicle-treated infected mice has shown prominent dye accumulation, indicating the breakdown of the BBB during malaria pathogenesis. The brain isolated from the EA-treated mice has shown the significant ($p <$

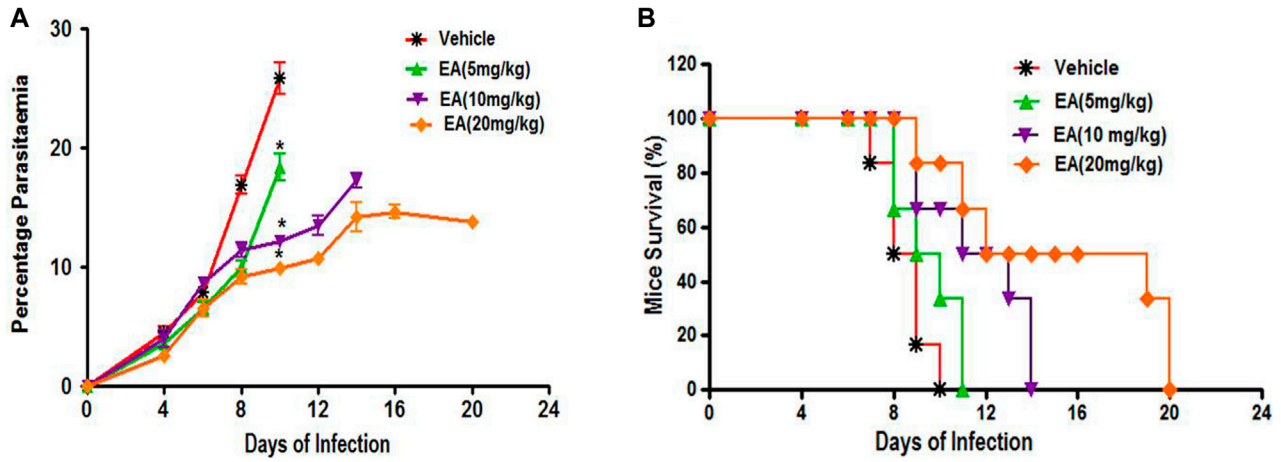


FIGURE 2 | Effect of *EA* on antimalarial activity in P. berghei included malaria in mice **(A)** Percent Parasiteman **(B)** Percent Survival. Data are expressed as Mean ± SEM. *$P < 0.05$; *Vehicle vs Treatment; n = 6.

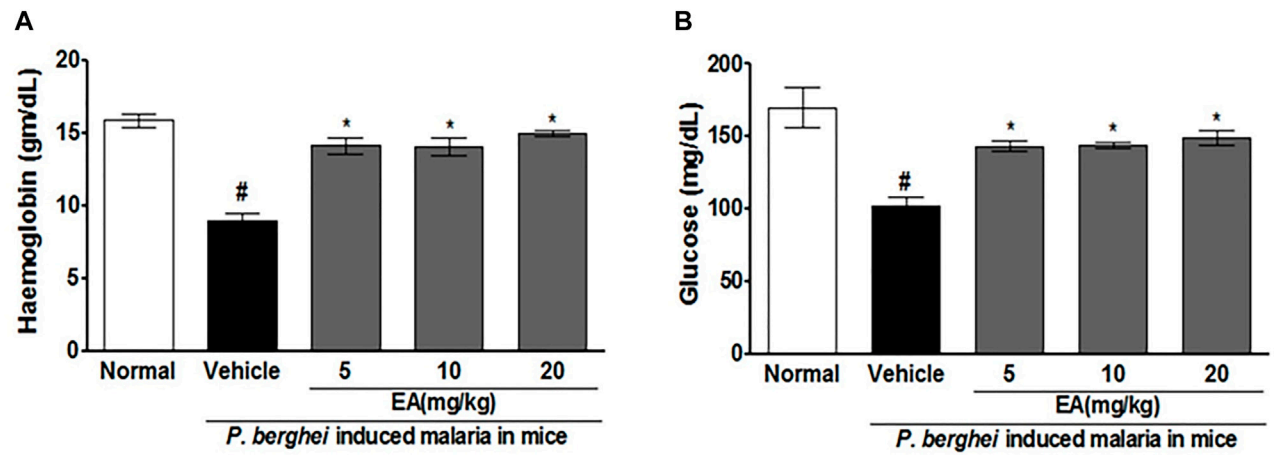


FIGURE 3 | Effect of *EA* on haemoglobin and blood glucose level on peak day of parasitaemia in mice. **(A)** Haemoglobin **(B)** Blood glucose. Data are expressed as Mean ± SEM: *P < 0.05; #Normal vs vehicle treated; *Vehicle vs Treatment; n = 3.

0.05) lower staining of dye than that from the vehicle-treated infected mice (**Figure 6**).

Cytotoxicity Profile of EA in Glial Cells

The effect of EA on cytotoxicity in C6 glial cells was studied using MTT assay. After EA treatment at 10, 30, and 100 µg/ml, percent live cell population was not significantly ($p < 0.05$) changed compared with normal untreated cells.

Effect of EA on ROS Generation and Neuroinflammation in C6 Glial Cells

To investigate the effect of EA on ROS generation, the spectrofluorometric and flow cytometric analyses were performed. The absorbance recorded spectrophotometrically showed that H_2O_2 alone significantly ($p < 0.05$) generated ROS in C6 glial cells after 1.5 h of incubation when compared with unstimulated cells. EA treatment (1, 10, 30 µg/ml) significantly attenuated H_2O_2-induced ROS generation in a dose-dependent manner when compared to H_2O_2-treated cells (**Figure 7A**). In agreement with the spectrophotometric observation, in the flow cytometric analysis, DCF-positive cells were found to decrease dose-dependently when treated with EA (1, 10, 30 µg/ml) (**Figure 7B**). Also, H_2O_2-induced cells exhibited higher DCF fluorescence intensities than the unstimulated cells. ROS generation was significantly ($p < 0.05$) decreased when the H_2O_2-induced cells were treated with EA (1, 10, 30 µg/ml) dose-dependently (**Figure 7C**). Experiments were performed on C6 glial cells stimulated with LPS to induce the production of pro-inflammatory cytokines. The production of pro-inflammatory cytokines (TNF-α and IL-6) in the cell culture supernatant was increased significantly in the LPS-stimulated cells compared with

TABLE 1 | Effect of EA on oxidative and antioxidant stress marker levels in major organs affected by malaria infection. Data are expressed as mean±SEM; $P < 0.05$ considered statistically significant; *vehicle *vs* treatment group; # control *vs* vehicle group; $n = 4$.

	Groups	*P. berghei* infection	Treatment (mg/kg)	Pro-oxidative stress markers		Anti-oxidative stress marker	
				ROS (µmol DCF/ mg protein)	LPO (mM/mg protein)	MPO (U/mg protein)	SOD (U/mg protein)
Brain	Control	-		263.56 ± 12.58	0.100 ± 0.029	0.393 ± 0.008	1.709 ± 0.069
	Vehicle	✓	-	11166.61 ± 44.85#	3.104 ± 0.040#	1.714 ± 0.011#	0.267 ± 0.032#
	EA	✓	5	938.46 ± 14.45*	3.093 ± 0.029*	0.757 ± 0.001*	0.581 ± 0.042*
		✓	10	917.29 ± 16.59*	3.047 ± 0.035*	0.749 ± 0.006*	0.836 ± 0.024*
		✓	20	887.94 ± 34.68*	2.910 ± 0.052*	0.736 ± 0.016*	0.998 ± 0.016*
Liver	Control	-		280.47 ± 13.79	0.653 ± 0.03	0.322 ± 0.002	1.09 ± 0.03
	Vehicle	✓	-	1965.74 ± 18.91#	3.065 ± 0.09#	1.420 ± 0.019#	0.30 ± 0.02#
	EA	✓	5	1818.45 ± 8.50*	2.840 ± 0.05*	0.577 ± 0.004*	0.60 ± 0.04*
		✓	10	1704.07 ± 30.54*	2.444 ± 0.04*	0.550 ± 0.006*	0.65 ± 0.03*
		✓	20	11612.47 ± 13.80*	2.245 ± 0.08*	0.535 ± 0.044*	0.69 ± 0.09*
Spleen	Control	-		571.17 ± 38.63	0.533 ± 0.058	0.618 ± 0.027	1.206 ± 0.085
	Vehicle	✓	-	3926.17 ± 51.27#	3.463 ± 0.031#	1.625 ± 0.025#	0.437 ± 0.029#
	EA	✓	5	3878.22 ± 26.54*	3.433 ± 0.026*	0.762 ± 0.009*	0.640 ± 0.112*
		✓	10	3791.05 ± 80.28*	3.371 ± 0.109*	0.752 ± 0.002*	0.840 ± 0.081*
		✓	20	3496.46 ± 110.26*	3.209 ± 0.139*	0.734 ± 0.033*	1.156 ± 0.093*

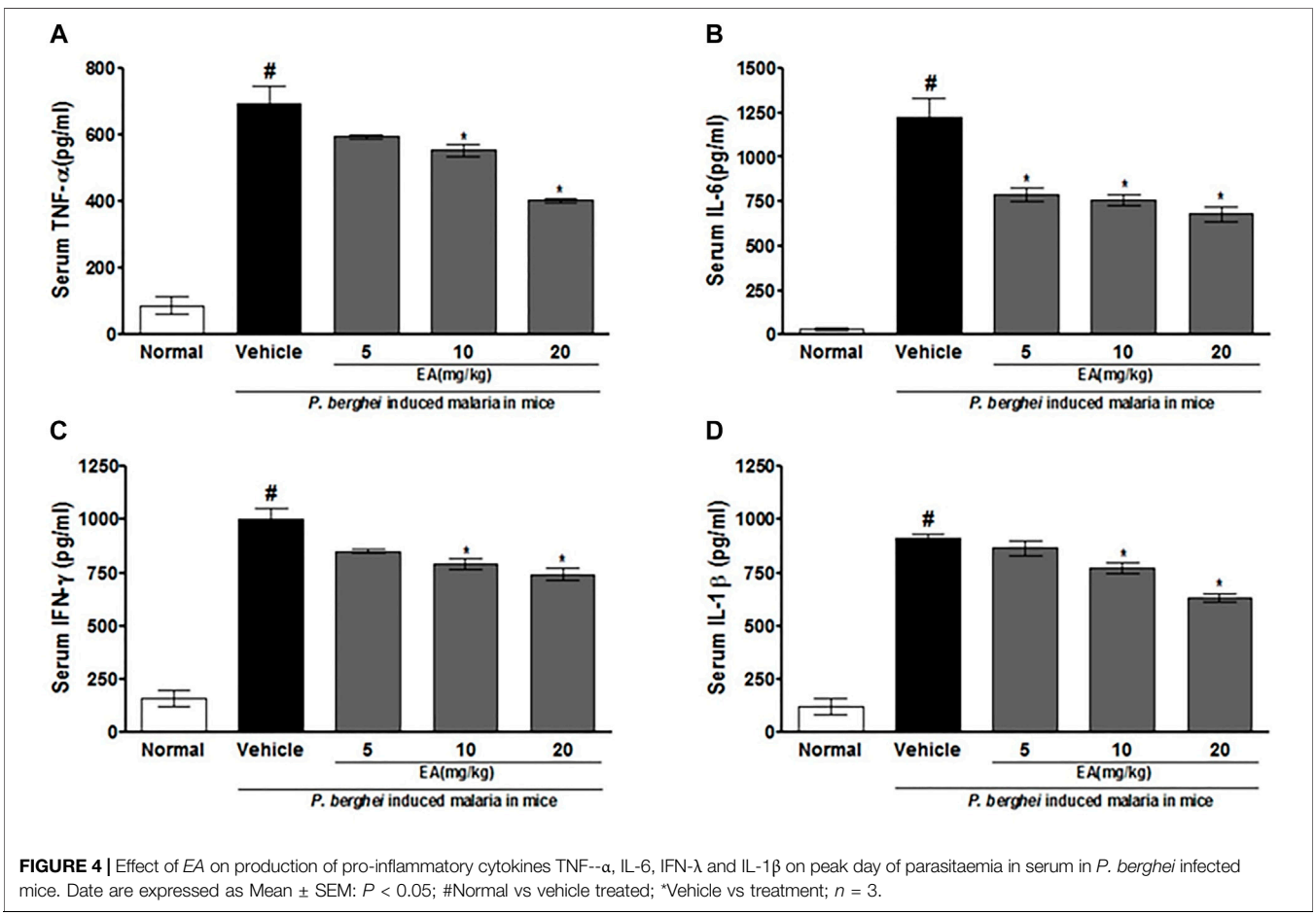


FIGURE 4 | Effect of *EA* on production of pro-inflammatory cytokines TNF--α, IL-6, IFN-λ and IL-1β on peak day of parasitaemia in serum in *P. berghei* infected mice. Date are expressed as Mean ± SEM: $P < 0.05$; #Normal vs vehicle treated; *Vehicle vs treatment; $n = 3$.

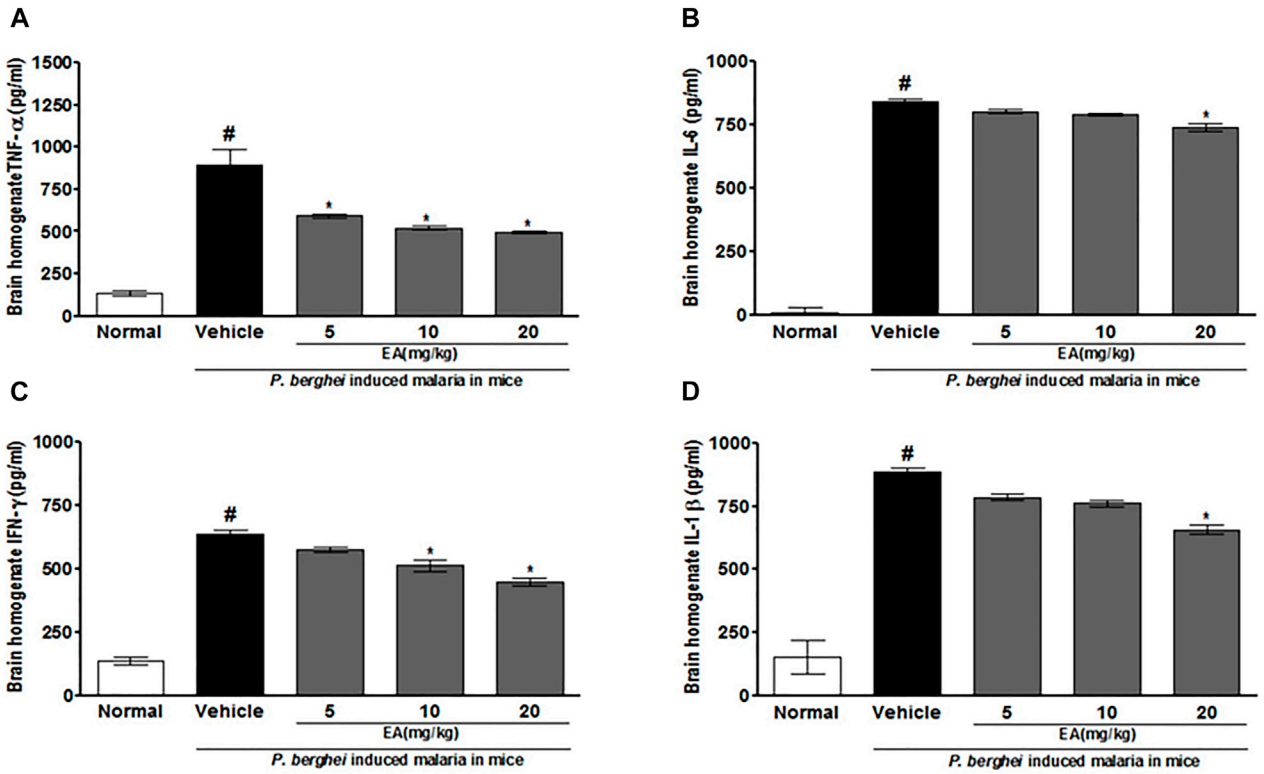


FIGURE 5 | Effect of *EA* on production of pro-inflammatory cytokines TNF-α, IL-6, IFN-λ and IL-1β on peak day of parasitaemia in brain homogenate in P. berghei infected mice. Data are expressed as Mean ± SEM: $P < 0.05$; #Normal vs vehicle treated; *Vehicle vs treatment; n = 3. #Normal vs vehicle treated; *$p < 0.05$; *Vehicle vs treatment; $n = 3$.

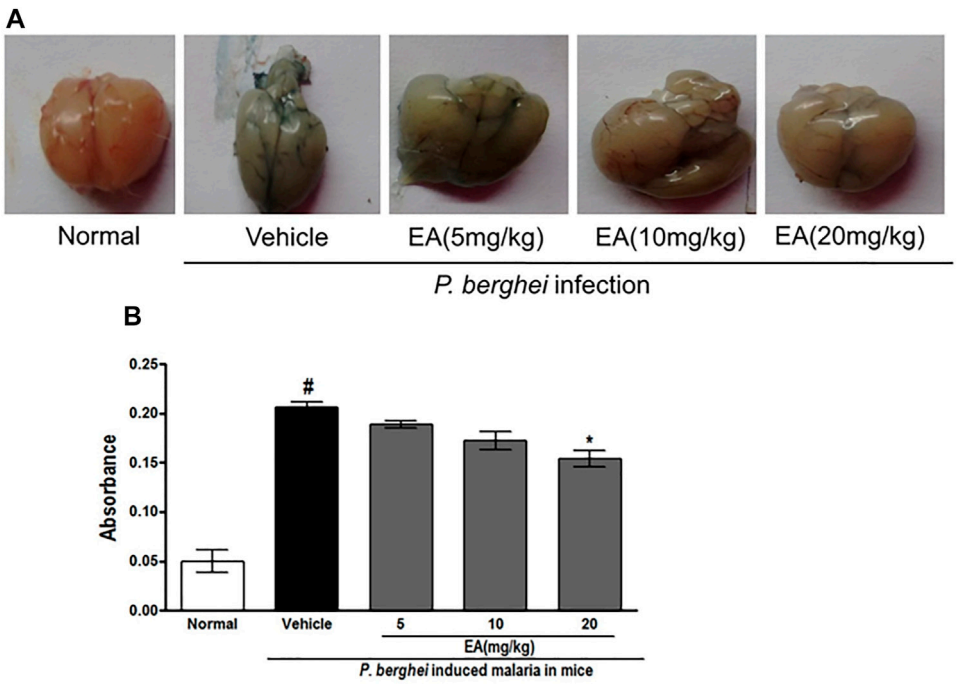


FIGURE 6 | Vascular permeability of brains was assessed by Evans Blue injection on peak day of parasitaemia **(A)** Vascular leakage was assessed by the blue discoloration of the brain tissue of the various treated groups **(B)** Quantification of Evans Blue extravasation in formamide, absorbance was measured at 620 nm; $n = 3$. $P < 0.05$; #Normal vs vehicle treated; *Vehicle vs treatment.

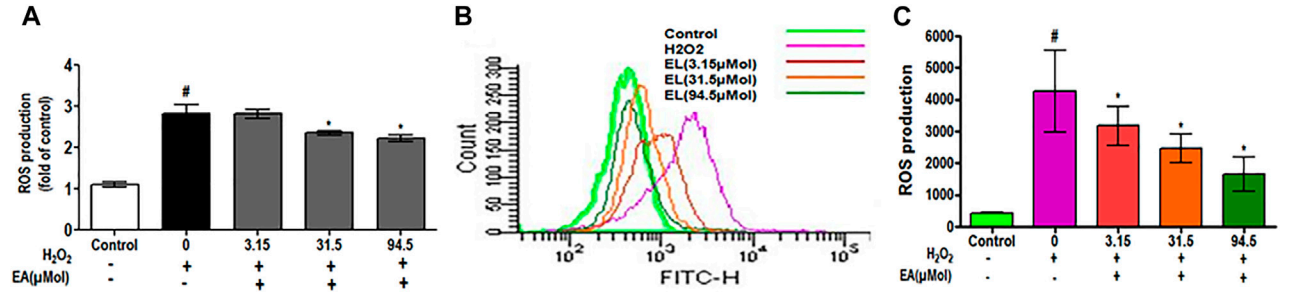


FIGURE 7 | Effect of *EA* on H2O2-simulated ROS production using **(A)** spectrofluorometer and **(B, C)** flowcytometric analysis in C6 microglial cells. Data are expressed as Mean ± SEM; *$p < 0.05$ *treatment vs H_2O_2 treated cells. #$p < 0.05$ #control vs H_2O_2 treated cells; $n = 3$.

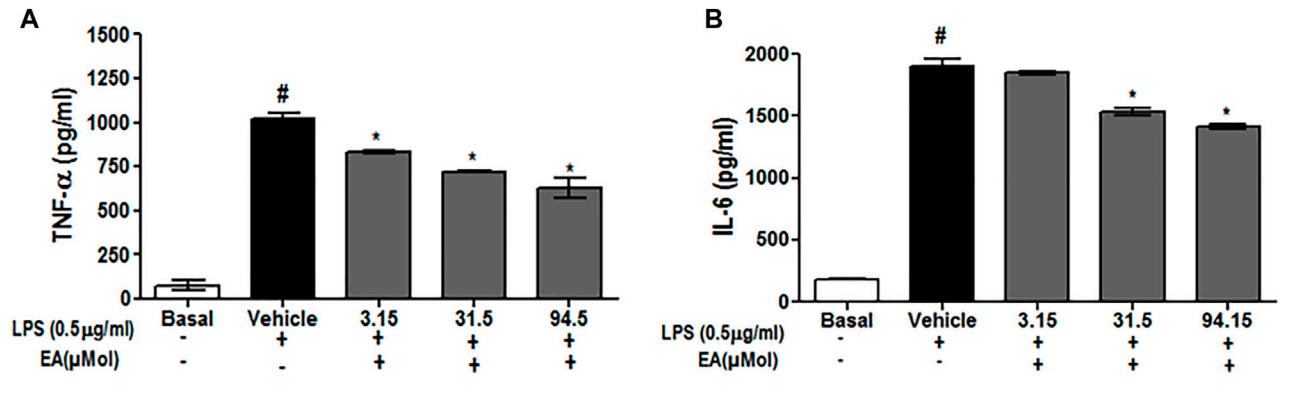


FIGURE 8 | Effect of EA on production of pro-inflammatory cytokine **(A)** TNF-α and **(B)** IL-6 in C6 microglial cells. Data are expressed as Mean ± SEM; $P < 0.05$; #Normal vs Vehicle treated; *Vehicle vs treatment; n = 3.

that in the normal cells. EA treatment (1, 10 and 30 µg/ml) exhibited a significant ($p < 0.05$) inhibition of pro-inflammatory cytokine production dose-dependently compared with the LPS-stimulated cells (**Figure 8**).

DISCUSSION

Malaria is a major public health problem in endemic countries with greater mortality in pregnant women and children, and severe malaria pathogenesis involves the series of host immune responses which eventually gets amplified, leading to severe organ injury and dysfunction (World Health Organization, 2019). Several research studies reported that malaria is a highly inflammatory condition characterized by acute periods of fever, headache, and nausea caused by *Plasmodium* infection of host red blood cells (Hunt, and Grau 2003; Togbe et al., 2007). Malaria-induced inflammatory cytokine storm circulating in the bloodstream of patients (Clark et al., 2008) is a critical pathological process which leads to severities like cerebral malaria, hypoglycemia, hyper-lactatemia, and acidosis (Schofield and Grau 2005) in malarial pathogenesis. Results of this study demonstrated that EA treatment is able to reduce parasitemia and increase the mean survival time in a dose-dependent manner in experimental malaria induced by *P. berghei*–infected RBCs in mice. Several findings suggest that plant-derived molecules exhibit antimalarial activity (Mohanty et al., 2013; Saxena et al., 2018; Gupta et al., 2018). Flavonoids are the natural molecules abundantly present in fruits and vegetables, showing beneficial effects against malarial pathogenesis (Puttappa et al., 2017; Amiri et al., 2018). Anemia and hypoglycemia are associated with severe malaria pathogenesis, which leads to mortality, especially in children and pregnant women (Boeuf et al., 2012). Oral treatment of EA is also able to restore hemoglobin and blood glucose in experimental malaria toward normal. Earlier published reports demonstrated that plant-derived natural molecules are able to restore hemoglobin and glucose levels in experimentally infected animals (Mohanty et al., 2015; Saxena et al., 2016). Oxidative stress due to malaria parasites if not checked by the host antioxidant mechanism can lead to oxidative damage in host tissues, which contributes to severe malarial pathogenesis (Becker et al., 2004). This study also revealed that EA treatment is able to protect from oxidative stress in vital organs during malarial pathogenesis. These findings are in agreement with the findings of recent reports

that ellagic acid alleviates clozapine-induced oxidative stress and mitochondrial dysfunction in cardiomyocytes (Ahangari et al., 2020) and that plant-derived molecules are able to reduce the level of oxidative stress and improve the level of antioxidant enzymes in *P. berghei*–infected mice (Singh et al., 2017; Gupta et al., 2018). Results of this study reported a very high level of pro-inflammatory cytokine production (cytokine storms) in serum and the brain homogenate of *P. berghei* vehicle-treated infected mice on the peak day of parasitemia. EA treatment significantly inhibited the production of pro-inflammatory cytokines in serum and brain homogenates of malaria-infected mice in a dose-dependent manner when compared to the vehicle-treated infected mice. Cerebral malaria is the most severe form of infection with *Plasmodium falciparum* characterized by a highly inflammatory response (IFN-γ, IL-1β, TNF-α, iNOS, and IL-6), which contributes to severity of the disease (Angulo and Fresno, 2002). There are several reports concluding that standard antimalarial drugs (chloroquine and artemisinin) and other plant-derived molecules exert antimalarial activity by reducing parasitemia and also modulating the pro-inflammatory cytokines (Mohanty et al., 2015; Gupta et al., 2018). Severe acidosis, anemia, hypoxia, and renal and hepatic insufficiencies are associated with BBB dysfunction in malarial pathogenesis (Clark et al., 2004). In this study, the BBB integrity was restored by EA-treated mice as evident by reduced Evans blue extravasation. To further substantiate results related to the beneficial effect of EA treatment in malarial pathogenesis by promoting antioxidant and anti-inflammatory effects, we performed the additional experiments using C6 glial cells. EA significantly reduced the H_2O_2-induced ROS generation in C6 cells and LPS-induced inflammatory mediators (TNF-α and IL-6) in a dose-dependent manner without any cytotoxic effect. This finding also supports the previous finding which indicated that the neuroprotective role of ellagic acid is due to its antioxidant and anti-inflammatory effects on brain tissue (Wang et al., 2020).

CONCLUSION

Taken together, the results of this study indicate that ellagic acid is able to alleviate severe malaria pathogenesis by reducing cytokine storms and oxidative stress induced by malaria parasites. It also attributed promising antimalarial activity and afforded to improve the blood glucose and hemoglobin levels in treated mice. These research findings suggested the suitability of ellagic acid as a useful bioflavonoid for further study for the management of severe malaria pathogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved, and the animal experiments were carried out as per the approved protocol by the Institutional Animal Ethics Committee (IAEC), followed by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), government of India (Registration No: 400/01/AB/ CPCSEA).

AUTHOR CONTRIBUTIONS

The authors listed in this article have contributed to perform the antimalarial, oxidative stress-, inflammation-related parameters of tissue and serum from the experimental mice, as well as to the analysis of data and preparation of the manuscript.

FUNDING

The study was financially supported by the Council of Scientific and Industrial Research (CSIR), New Delhi, under project HCP-0019. AG, an author is grateful to the University Grants Commission (UGC), New Delhi, India, for providing the fellowship. The authors are grateful to the director, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India, for providing essential research facilities and support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.777400/full#supplementary-material

REFERENCES

Ahangari, R., Khezri, S., Jahedsani, A., Bakhshii, S., and Salimi, A. (2020). Ellagic Acid Alleviates Clozapine-induced O-xidative S-tress and M-itochondrial D-ysfunction in C-ardiomyocytes. *Drug Chem. Toxicol.*, 1–9. doi:10.1080/01480545.2020.1850758

Amiri, M., Nourian, A., Khoshkam, M., and Ramazani, A. (2018). Apigenin Inhibits Growth of the Plasmodium Berghei and Disrupts Some Metabolic Pathways in Mice. *Phytother Res.* 32, 1795–1802. doi:10.1002/ptr.6113

Angulo, I., and Fresno, M. (2002). Cytokines in the Pathogenesis of and protection against Malaria. *Clin. Diagn. Lab. Immunol.* 9, 1145–1152. doi:10.1128/cdli.9.6.1145-1152.2002

Baptista, F. G., Pamplona, A., Pena, A. C., Mota, M. M., Pied, S., and Vigário, A. M. (2010). Accumulation of Plasmodium Berghei-Infected Red Blood Cells in the Brain Is Crucial for the Development of Cerebral Malaria in Mice. *Infect. Immun.* 78, 4033–4039. doi:10.1128/IAI.00079-10

Becker, K., Tilley, L., Vennerstrom, J. L., Roberts, D., Rogerson, S., and Ginsburg, H. (2004). Oxidative Stress in Malaria Parasite-Infected Erythrocytes: Host-Parasite Interactions. *Int. J. Parasitol.* 34, 163–189. doi:10.1016/j.ijpara.2003.09.011

Boeuf, P. S., Loizon, S., Awandare, G. A., Tetteh, J. K., Addae, M. M., Adjei, G. O., et al. (2012). Insights into Deregulated TNF and IL-10 Production in Malaria: Implications for Understanding Severe Malarial Anaemia. *Malar. J.* 11, 253. doi:10.1186/1475-2875-11-253

Ceci, C., Graziani, G., Faraoni, I., and Cacciotti, I. (2020). Strategies to Improve Ellagic Acid Bioavailability: From Natural or Semisynthetic Derivatives to Nanotechnological Approaches Based on Innovative Carriers. *Nanotechnology* 31, 382001. doi:10.1088/1361-6528/ab912c

Clark, I. A., Alleva, L. M., Budd, A. C., and Cowden, W. B. (2008). Understanding the Role of Inflammatory Cytokines in Malaria and Related Diseases. *Trav. Med Infect Dis* 6 (1-2), 67–81. doi:10.1016/j.tmaid.2007.07.002

Clark, I. A., Alleva, L. M., Mills, A. C., and Cowden, W. B. (2004). Pathogenesis of Malaria and Clinically Similar Conditions. *Clin. Microbiol. Rev.* 17, 509–contents. doi:10.1128/CMR.17.3.509-539.2004

Del Maestro, R., and McDonald, W. (1985), Oxidative Enzymes in Tissue Homogenates. *Handbook of Methods for Oxygen Radical Research*, 291–296.

Draper, H. H., and Hadley, M. (1990). Malondialdehyde Determination as index of Lipid Peroxidation. *Methods Enzymol.* 186, 421–431. Elsevier. doi:10.1016/0076-6879(90)86135-i

Driver, A. S., Kodavanti, P. R., and Mundy, W. R. (2000). Age-related Changes in Reactive Oxygen Species Production in Rat Brain Homogenates. *Neurotoxicol Teratol* 22, 175–181. doi:10.1016/s0892-0362(99)00069-0

Evtyugin, D. D., Magina, S., and Evtuguin, D. V. (2020). Recent Advances in the Production and Applications of Ellagic Acid and its Derivatives. A Review. *A ReviewMolecules* 25, 2745. doi:10.3390/molecules25122745

Gupta, A. C., Mohanty, S., Saxena, A., Maurya, A. K., and Bawankule, D. U. (2018). Plumbagin, a Vitamin K3 Analogue Ameliorate Malaria Pathogenesis by Inhibiting Oxidative Stress and Inflammation. *Inflammopharmacology* 26, 983–991. doi:10.1007/s10787-018-0465-1

Hunt, N. H., and Grau, G. E. (2003). Cytokines: Accelerators and Brakes in the Pathogenesis of Cerebral Malaria. *Trends Immunol.* 24 (9), 491–499. doi:10.1016/s1471-4906(03)00229-1

Kalia, S., Walter, N. S., and Bagai, U. (2015). Antimalarial Efficacy of Albizia Lebbeck (Leguminosae) against Plasmodium Falciparum *In Vitro* & P. Berghei *In Vivo*. *Indian J. Med. Res.* 142 Suppl, S101–S107. doi:10.4103/0971-5916.176635

Kumar, S., Kumari, R., and Pandey, R. (2015). New Insight-Guided Approaches to Detect, Cure, Prevent and Eliminate Malaria. *Protoplasma* 252, 717–753. doi:10.1007/s00709-014-0697-x

Mohanty, S., Maurya, A. K., Jyotshna, A., Saxena, A., Shanker, K., Pal, A., et al. (2015). Flavonoids Rich Fraction of Citrus Limetta Fruit Peels Reduces Proinflammatory Cytokine Production and Attenuates Malaria Pathogenesis. *Curr. Pharm. Biotechnol.* 16, 544–552. doi:10.2174/1389201016061504071140 23

Mohanty, S., Srivastava, P., Maurya, A. K., Cheema, H. S., Shanker, K., Dhawan, S., et al. (2013). Antimalarial and Safety Evaluation of Pluchea Lanceolata (DC.) Oliv. & Hiern: *In-Vitro* and *In-Vivo* Study. *J. Ethnopharmacol* 149, 797–802. doi:10.1016/j.jep.2013.08.003

Pabón, A., Carmona, J., Burgos, L. C., and Blair, S. (2003). Oxidative Stress in Patients with Non-complicated Malaria. *Clin. Biochem.* 36, 71–78. doi:10.1016/s0009-9120(02)00423-x

Puttappa, N., Kumar, R. S., and Yamjala, K. (2017). Artesunate-quercetin/luteolin Dual Drug Nanofacilitated Synergistic Treatment for Malaria: A Plausible Approach to Overcome Artemisinin Combination Therapy Resistance. *Med. Hypotheses* 109, 176–180. doi:10.1016/j.mehy.2017.10.016

Saxena, A., Yadav, D., Mohanty, S., Cheema, H. S., Gupta, M. M., Darokar, M. P., et al. (2016). Diarylheptanoids Rich Fraction of Alnus Nepalensis Attenuates Malaria Pathogenesis: *In-Vitro* and *In-Vivo* Study. *Phytother Res.* 30, 940–948. doi:10.1002/ptr.5596

Saxena, A., Upadhyay, H. C., Cheema, H. S., Srivastava, S. K., Darokar, M. P., and Bawankule, D. U. (2018). Antimalarial Activity of Phytol Derivatives: *In Vitro* and *In Vivo* Study. *Med. Chem. Res.* 27, 1345–1354. doi:10.1007/s00044-017-2132-2

Schofield, L., and Grau, G. E. (2005). Immunological Processes in Malaria Pathogenesis. *Nat. Rev. Immunol.* 5 (9), 722–735. doi:10.1038/nri1686

Shakeri, A., Zirak, M. R., and Sahebkar, A. (2018). Ellagic Acid: A Logical lead for Drug Development? *Curr. Pharm. Des.* 24, 106–122. doi:10.2174/1381612823666171115094557

Sharma, S., Chattopadhyay, S. K., Yadav, D. K., Khan, F., Mohanty, S., Maurya, A., et al. (2012). QSAR, Docking and *In Vitro* Studies for Anti-inflammatory Activity of Cleomiscosin A Methyl Ether Derivatives. *Eur. J. Pharm. Sci.* 47, 952–964. doi:10.1016/j.ejps.2012.09.008

Singh, D. K., Cheema, H. S., Saxena, A., Jyotshana, S., Singh, S., Darokar, M. P., et al. (2017). Fraxetin and Ethyl Acetate Extract from Lawsonia Inermis L. Ameliorate Oxidative Stress in P. Berghei Infected Mice by Augmenting Antioxidant Defence System. *Phytomedicine* 36, 262–272. doi:10.1016/j.phymed.2017.09.012

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y., and Hay, S. I. (2005). The Global Distribution of Clinical Episodes of Plasmodium Falciparum Malaria. *Nature* 434, 214–217. doi:10.1038/nature03342

Stanisic, D. I., and Good, M. F. (2015). Whole Organism Blood Stage Vaccines against Malaria. *Vaccine* 33, 7469–7475. doi:10.1016/j.vaccine.2015.09.057

Suzuki, K. T., Tanaka, Y., and Kawamura, R. (1983). Properties of Metallothionein Induced by Zinc, Copper and Cadmium in the Frog, *Xenopus laevis*. *Comp. Biochem. Physiol. C Comp. Pharmacol. Toxicol.* 75, 33–37. doi:10.1016/0742-8413(83)90007-5

Togbe, D., Schofield, L., Grau, G. E., Schnyder, B., Boissay, V., Charron, S., et al. (2007). Murine Cerebral Malaria Development Is Independent of Toll-like Receptor Signaling. *Am. J. Pathol.* 170 (5), 1640–1648. doi:10.2353/ajpath.2007.060889

Vattem, D. A., Ghaedian, R., and Shetty, K. (2005). Enhancing Health Benefits of Berries through Phenolic Antioxidant Enrichment: Focus on cranberry. *Asia Pac. J. Clin. Nutr.* 14, 120–130.

Vinoth, K. J., Manikandan, J., Sethu, S., Balakrishnan, L., Heng, A., Lu, K., et al. (2015). Differential Resistance of Human Embryonic Stem Cells and Somatic Cell Types to Hydrogen Peroxide-Induced Genotoxicity May Be Dependent on Innate Basal Intracellular ROS Levels. *Folia Histochem. Cytobiol* 53, 169–174. doi:10.5603/FHC.a2015.0016

Wang, W., Yang, L., Liu, T., Wang, J., Wen, A., and Ding, Y. (2020). Ellagic Acid Protects Mice against Sleep Deprivation-Induced Memory Impairment and Anxiety by Inhibiting TLR4 and Activating Nrf2. *Aging (Albany NY)* 12, 10457–10472. doi:10.18632/aging.103270

World Health Organization (2019). *World Malaria Report*.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 09 December 2021
doi: 10.3389/fphar.2021.755941

Is Our Natural Food Our Homeostasis? Array of a Thousand Effect-Directed Profiles of 68 Herbs and Spices

*Tamara Schreiner, Dorena Sauter, Maren Friz, Julia Heil and Gertrud Elisabeth Morlock**

Institute of Nutritional Science, Chair of Food Science, and TransMIT Center for Effect-Directed Analysis, Justus Liebig University Giessen, Giessen, Germany

OPEN ACCESS

Edited by:
Michael Heinrich, UCL School of Pharmacy, United Kingdom

Reviewed by:
Yusof Kamisah, Universiti Kebangaan Malaysia, Malaysia
David Morton, La Trobe University, Australia

***Correspondence:**
Gertrud Elisabeth Morlock
gertrud.morlock@uni-giessen.de

Dedicated to the 75th birthday of Prof. Dr. Teresa Kowalska, University of Silesia, Poland

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *09 August 2021*
Accepted: *03 November 2021*
Published: *09 December 2021*

Citation:
Schreiner T, Sauter D, Friz M, Heil J and Morlock GE (2021) Is Our Natural Food Our Homeostasis? Array of a Thousand Effect-Directed Profiles of 68 Herbs and Spices. Front. Pharmacol. 12:755941. doi: 10.3389/fphar.2021.755941

The beneficial effects of plant-rich diets and traditional medicines are increasingly recognized in the treatment of civilization diseases due to the abundance and diversity of bioactive substances therein. However, the important active portion of natural food or plant-based medicine is presently not under control. Hence, a paradigm shift from quality control based on marker compounds to effect-directed profiling is postulated. We investigated 68 powdered plant extracts (botanicals) which are added to food products in food industry. Among them are many plants that are used as traditional medicines, herbs and spices. A generic strategy was developed to evaluate the bioactivity profile of each botanical as completely as possible and to straightforwardly assign the most potent bioactive compounds. It is an 8-dimensional hyphenation of normal-phase high-performance thin-layer chromatography with multi-imaging by ultraviolet, visible and fluorescence light detection as well as effect-directed assay and heart-cut of the bioactive zone to orthogonal reversed-phase high-performance liquid chromatography–photodiode array detection–heated electrospray ionization mass spectrometry. In the non-target, effect-directed screening via 16 different on-surface assays, we tentatively assigned more than 60 important bioactive compounds in the studied botanicals. These were antibacterials, estrogens, antiestrogens, androgens, and antiandrogens, as well as acetylcholinesterase, butyrylcholinesterase, α-amylase, α-glucosidase, β-glucosidase, β-glucuronidase, and tyrosinase inhibitors, which were on-surface heart-cut eluted from the bioautogram or enzyme inhibition autogram to the next dimension for further targeted characterization. This biological-physicochemical hyphenation is able to detect and control active mechanisms of traditional medicines or botanicals as well as the essentials of plant-based food. The array of 1,292 profiles (68 samples × 19 detections) showed the versatile bioactivity potential of natural food. It reveals how efficiently and powerful our natural food contributes to our homeostasis.

Keywords: botanical, effect-directed analysis, 8D hyphenation, high-performance thin-layer chromatography, high-performance liquid chromatography, mass spectrometry

1 INTRODUCTION

Herbs and spices are widely used for nutrition, flavoring, cosmetics, dyeing, or fragrances (Guldiken et al., 2018). They are also applied in medicine due to their known beneficial effects on human health (Yuan et al., 2016; Caesar et al., 2019), inspired by traditional healers who have used botanical extracts since ancient times (Belwal et al., 2018b). The knowledge of biologically active plants, their harvesting, production, preparation, and administration has been passed down through thousands of years of traditional medicine (Yuan et al., 2016). Particularly phenols were reported to have antibacterial, antiviral, and antioxidant effects, as well as the ability to modulate enzyme activity and transduction pathways (Krüger et al., 2017; Tresserra-Rimbau et al., 2018). Some studies have quantified the total amount of healthful constituents in herbal extracts and calculated the recommended intake of antioxidants from culinary herbs (Halvorsen et al., 2002; Wojdylo et al., 2007). However, their multifactorial relevance in homeostasis is underexplored. It is evident that the use of the whole natural plant extract is more powerful for homeostasis due to the versatility of the gentle mechanisms of active compounds than the use of isolated compounds (Morlock and Heil, 2020).

In a typical screening for potential drug candidates, plant extracts are currently freed from assay-interfering tannins by solid-phase extraction, separated with an HPLC gradient (42 min/sample including equilibration), and collected in fractions, which are screened for bioactivity in a microtiter plate assay (Kongstad et al., 2015). Therefore, bioactivity can only be assigned to a fraction containing several analytes via a costly and time-consuming workflow, which subsequently requires analytical separation and testing of each peak to assign the individual bioactive compounds (Caesar et al., 2019). In routine, there has only been a little progress in non-target screening of food for bioactive compounds at an affordable price. Most methods deal with illicit additions, organic contaminants (Fu et al., 2017), adulterated foods (Díaz et al., 2012), and migrants from packaging (Rusko et al., 2020; Su et al., 2020). Also, generic chromatography-based high-resolution mass spectrometric methods were examined to cover as many substances as possible within a single analysis (Díaz et al., 2012). However, one drawback is the high load of interfering matrix caused by the diversity and abundance of substances in such natural products as spices and herbs (Caesar et al., 2019; Morlock and Heil, 2020). Elaborate sample preparation (which is selective and error-prone) would otherwise limit the validity and significance of the results. The state of the art is setting an intensity threshold and focusing on highly abundant signals (Wu et al., 2016). But even the smallest signal can have an important biological effect. Ignoring minor signals from the set instrumental threshold will produce grossly negligent results. Moreover, compounds may not ionize well or at all with standard settings of mass spectrometric recording. That is why routine analysis of natural extracts is still tailored and limited to marker compounds. However, the important active portion of natural food needs to be under (analytical) control, which is presently not the case.

To overcome these limitations and expand the analytical toolbox, a high-throughput eight-dimensional (8D) hyphenation was recently developed, and its proof of principle was shown for cinnamon samples detected with an antibacterial bioassay (Schreiner and Morlock, 2021). It demonstrated the information gained by combining effect-directed assays (EDA) with normal-phase high-performance thin-layer chromatography including multi-imaging by ultraviolet, visible, and fluorescence light detection (NP-HPTLC–UV/Vis/FLD) (Morlock, 2021). Heart-cut elution and transfer of the bioactive compound zone to an orthogonal reversed-phase high-performance liquid chromatography (RP-HPLC) system was exploited to separate potentially coeluting bioactive substances. The subsequent photodiode array detection (DAD) and heated electrospray ionization mass spectrometry (HESI-MS) were used for additional straightforward characterization of the bioactive substances. The advantage of NP-HPTLC–UV/Vis/FLD–EDA–heart-cut RP-HPLC–DAD–HESI-MS is that it prioritizes and reduces the thousands of compounds in such natural samples to the most important bioactive compounds. As the previously developed hyphenation was only shown for cinnamon and one antibacterial bioassay, this study intended to examine the influence of 68 different plant matrices and 16 different assays on the robustness of the new 8D hyphenation. It was of interest to prove its universal validity and significance, to figure out potential limitations, and to verify its suitability as generic activity screening. Such straightforward effect-directed profiling could be applied to reveal, understand, and control the mode of action of traditional medicines, botanicals, and plant-based food.

2 MATERIALS AND METHODS

2.1 Chemicals and Materials

Purity grades were listed when available. All salts were of p. a. quality and water free unless stated otherwise. Ethanol, toluene (all solvents of chromatography grade), bovine serum albumin (BSA, fraction V, ≥98%), dipotassium hydrogen phosphate (K_2HPO_4, ≥99%), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, ≥98%), glycerol (Rotipuran, 86%), potassium dihydrogen phosphate (KH_2PO_4, ≥99%), dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3\ H_2O$, ≥99%), sodium hydroxide (NaOH, ≥98%), disodium hydrogen phosphate (Na_2HPO_4, ≥99%), potassium chloride (KCl, 98.5%), polyethylene glycol (PEG) 8000 (Ph. Eur.), kojic acid (>98%), acetic acid (100%), sulfuric acid (96%), hydrochloric acid (37%, HCl, purest), citric acid (p. a.), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, ≥98%), 3-[(3cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS, ≥98%), dimethyl sulfoxide (DMSO), and tris(hydroxymethyl)aminomethane (TRIS, ≥99.9%) were obtained from Carl Roth, Karlsruhe, Germany. Diammonium hydrogen phosphate ($[NH_4]_2HPO_4$, ≥99%) was purchased from Acros Organics, Morris Plains, NJ, United States. Butyrylcholinesterase (BChE) from equine serum (≥140 U/mg) was provided by SERVA, Heidelberg, Germany. Acarbose (≥95%), α-glucosidase from *Saccharomyces cerevisiae*

TABLE 1 | Compilation of 68 botanicals, including botanical name, plant part, and sample weights (W) extracted with 5 ml methanol (*filtered through 0.45 µm PTFE filter).

No	Common name	Botanical name	Plant part	W [mg]
1	Acerola	*Malpighia glabra* L [Malphighiaceae]	fruits	501.5
2	Horehound, white	*Marrubium vulgare* L. [Lamiaceae]	herb	500.1
3	Apple*	*Malus sylvestris* (L.) Mill. [Rosaceae]	peel	500.7
4	Artichoke, globe	*Cynara cardunculus* subsp. *scolymus* (L.) [Asteraceae]	leaves	501.3
5	Basil	*Ocimum basilicum* L. [Lamiaceae]	herb	500.6
6	Fenugreek	*Trigonella foenum-graecum* L. [Fabaceae]	seeds	499.9
7	Stinging nettle*	*Urtica dioica* L. [Urticaceae]	leaves	501.5
8	Blackberry	*Rubus fruticosus* L. [Rosaceae]	leaves	500.6
9	*Eucalyptus*	*Eucalyptus globulus* Labill. [Myrtaceae]	leaves	499.7
10	Fennel	*Foeniculum vulgare* Mill. [Apiaceae]	fruits	499.9
11	Fruit tea, yellow	not available	unknown	501.3
12	Fruit tea, red	not available	unknown	502.6
13	Galangal	*Alpinia officinarum* Hance. [Zingiberaceae]	roots	501.8
14	Ginkgo	*Ginkgo biloba* L. [Ginkgoaceae]	leaves	502.7
15	Ginseng	*Panax ginseng* C.A.Mey. [Araliaceae]	roots	502.3
16	Guarana	*Paullinia cupana* Kunth [Sapindaceae]	seeds	498.8
17	Dog rose	*Rosa canina* L. [Rosaceae]	fruits	501.0
18	Blueberry, European	*Vaccinium myrtillus* L. [Eriaceae]	fruits	501.2
19	*Hibiscus*	*Hibiscus rosa-sinensis* L. [Malvaceae]	blossoms	499.6
20	Raspberry	*Rubus idaeus* L. [Rosaceae]	juice concentrate from fruits	503.0
21	Elderberry	*Sambucus nigra* L. [Adoxaceae]	fruits	501.4
22	Elder flower	*Sambucus nigra* L. [Adoxaceae]	blossoms	502.5
23	Honeybush*	*Cyclopia genistoides* (L.) R.Br. [Fabaceae]	leaves, branches, blossoms	499.3
24	Hop	*Humulus lupulus* L. [Cannabaceae]	blossoms	502.1
25	Ginger	*Zingiber officinale* Roscoe [Zingiberaceae]	roots	499.0
26	Jasmine*	*Jasminum officinale* L. [Oleaceae]	blossoms	499.2
27	Cassis	*Ribes nigrum* L. [Grossulariaceae]	juice concentrate from fruits	500.7
28	Chamomile	*Matricaria chamomilla* L. [Asteraceae]	blossoms	499.3
29	Cardamom*	*Elettaria cardamomum* (L.) Maton [Zingiberaceae]	fruits	499.6
30	Garlic	*Allium sativum* L. [Amaryllidaceae]	bulbs	499.9
31	Kola*	*Cola nitida* (Vent.) Schott and Endl. [Malvaceae]	seeds	500.8
32	Coriander	*Coriandrum sativum* L. [Apiaceae]	fruits	501.3
33	Caraway	*Carum carvi* L. [Apiaceae]	fruits	500.0
34	Lovage	*Levisticum officinale* W.D.J.Koch [Apiaceae]	roots	499.6
35	Marjoram	*Origanum majorana* L. [Lamiaceae]	herb	502.4
36	Yerba mate*	*Ilex paraguariensis* A.St.-Hil. [Aquifoliaceae]	leaves, roasted	499.6
37	Yerba mate	*Ilex paraguariensis* A.St.-Hil. [Aquifoliaceae]	leaves	500.2
38	Lemon balm	*Melissa officinalis* L. [Lamiaceae]	leaves	500.6
39	Clove*	*Syzygium aromaticum* (L.) Merr. and L.M.Perry [Myrtaceae]	flower buds	501.9
40	Orange	*Citrus* × *aurantium* L. [Rutaceae]	blossoms	499.7
41	Orange	*Citrus* × *aurantium* L. [Rutaceae]	peel	501.1
42	Oregano	*Origanum vulgare* L. [Lamiaceae]	herb	501.5
43	Passionflower	*Passiflora incarnata* L. [Passifloraceae]	blossoms	501.1
44	Peppermint	*Mentha* × *piperita* L. [Lamiaceae]	leaves	500.3
45	Rooibos*	*Aspalathus linearis* (Burm.f.) R.Dahlgren [Fabaceae]	leaves	500.7
46	Rosemary*	*Salvia Rosmarinus* Spenn. [Lamiaceae]	leaves	500.9
47	Sage	*Salvia officinalis* L. [Lamiaceae]	leaves	499.9
48	Sea buckthorn	*Hippophae rhamnoides* L. [Elaeagnaceae]	fruits	501.9
49	Horsetail	*Equisetum arvense* L. [Equisetaceae]	herb	499.3
50	Yarrow*	*Achillea millefolium* L. [Asteraceae]	herb	501.6
51	Celeriac	*Apium graveolens* L. [Apiaceae]	bulb	501.3
52	Coneflower	*Echinacea angustifolia* DC. [Asteraceae]	herb and roots	499.1
53	Plantain	*Plantago lanceolata* L. [Plantaginaceae]	leaves	500.5
54	Star anise	*Illicium verum* Hook.f. [Schisandraceae]	fruits	500.3
55	Licorice	*Glycyrrhiza glabra* L. [Fabaceae]	roots	500.3
56	Siberian ginseng	*Eleutherococcus senticosus* (Rupr. and Maxim.) Maxim. [Araliaceae]	roots	503.4
57	Thyme	*Thymus vulgaris* L. [Lamiaceae]	herb	499.6
58	Grape*	*Vitis vinifera* L. [Vitaceae]	seed	499.9
59	Grape	*Vitis vinifera* L. [Vitaceae]	peel	499.7
60	Juniper	*Juniperus communis* L. [Cupressaceae]	fruits	501.5
61	Grape	*Vitis vinifera* L. [Vitaceae]	leaves	501.2
62	Hawthorn	*Crataegus* sp. [Rosaceae]	leaves and blossoms	499.7
63	Hawthorn leaves (Batch 1)	*Crataegus* sp. [Rosaceae]	leaves	501.8
64	Hawthorn leaves (Batch 2)	*Crataegus* sp. [Rosaceae]	leaves	499.9
65	Chicory	*Cichorium intybus* L. [Asteraceae]	roots	501.1
66	Cinnamon	*Cinnamomum verum* J.Presl [Lauraceae]	bark	501.5
67	Lemon peel	*Citrus* × *limon* (L.) Osbeck [Rutaceae]	peel	500.7
68	Lemon verbena	*Aloysia citridora* Paláu [Verbenaceae]	leaves	500.4

(1,000 U/vial), tyrosinase from mushroom (≥1,000 U/mg, 25 kU/vial), β-glucuronidase from *Escherichia coli* (5,000 U/vial), acetylcholinesterase (AChE) from *Electrophorus electricus* (≥245 U/mg, 10 kU/vial), peptone from casein (for microbiology), sodium acetate, sodium chloride (NaCl), Müller-Hinton broth (for microbiology), D-(+)-glucose (99.5%), rivastigmine (≥98%), imidazole (≥99.5%), copper sulfate, 7-hydroxy-4-methylcoumarin (4-methylumbelliferone, >98%), yeast nitrogen base without amino acids (for molecular biology), quercitin-3-*O*-glucoside (≥90%), liquiritigenin (≥97%), naringenin (≥95%), syringic acid (≥95%), pinobanksin (≥95%), sodium hydrogen carbonate (99.7%), lysogeny broth (containing 5 mg/ml sodium chloride) powder, ampicillin sodium salt, α-amylase from hog pancreas (50 U/mg), Gram's iodine solution (for microscopy) and testosterone (≥99%) were delivered by Sigma-Aldrich, Steinheim, Germany. 2-Naphthyl-β-D-glucopyranoside (95%) and β-glucosidase from almonds (3,040 U/mg) were provided by ABCR, Karlsruhe, Germany. 1-Naphthyl acetate (≥98%) and 2-naphthyl-α-D-glucopyranoside were obtained from AppliChem, Darmstadt, Germany. Fast Blue B salt (95%) was purchased from MP Biomedicals, Eschwege, Germany. 5-Bromo-4-chloro-3-indolyl-β-D-glucopyranosid-uronic sodium salt was obtained from Carbosynth, Compton-Berkshire, United Kingdom. Methanol (MS quality) and formic acid (99%) were delivered from VWR, Darmstadt, Germany. D-Saccharolactone and (2S)-2-amino-3-(3,4-dihydroxyphenyl) propionic acid (levodopa) was obtained from Santa Cruz Biotechnology, Dallas, TX, United States. 17-β-Estradiol (98.5%) was obtained from Dr. Ehrenstorfer, Augsburg, Germany. Ethyl acetate (≥99.8%) and yeast extract powder (for microbiology) were purchased from Th. Geyer, Renningen, Germany. The medium for the Gram-negative, naturally luminescent marine *Aliivibrio fischeri* bacteria (DSM-7151, German Collection of Microorganisms and Cell Cultures, Berlin, Germany) is listed elsewhere (European Committee for Standardization, 2009). Gram-positive soil bacteria *Bacillus subtilis* subsp. *spizizenii* (DSM-618), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$, 99.5%), citric acid monohydrate (≥99.5%), 4-methyl-umbelliferyl-β-D-galactopyranoside, phosphate-buffered saline (without Ca^{2+}), soluble starch, as well as HPTLC plates silica gel 60 F_{254} MS-grade and HPTLC plates silica gel 60 (both 20cm × 10 cm) were provided by Merck, Darmstadt, Germany. Bidistilled water was prepared by a Heraeus Destamat Bi-18 E (Thermo Fisher Scientific, Dreieich, Germany). *Saccharomyces cerevisiae* BJ 1991, equipped with the human androgen receptor, S9 enzyme mixture (from rat liver), nicotinamide adenine dinucleotide phosphate (NADP), and glucose 6-phosphate were delivered by Xenometrix, Allschwil, Switzerland. Additional chemicals and reagents used for planar yeast ant-/agonistic androgen/estrogen screens were reported elsewhere (Klingelhöfer and Morlock, 2015; Klingelhöfer et al., 2020). The *Saccharomyces cerevisiae* cells equipped with the hERβ were obtained from the Erwin Herberle-Bors, University of Vienna, Austria (Kirchmayer, 2009). Reference substances acacetin (99%), eriocitrin (96%), naringin (92%), ginkgolide A (99%) and B (99%), isorhamnetin (99%), liquiritin apioside (≥95%), hesperidin (>96%), (−)-epicatechin (100%), (+)-catechin (98%), rutin (90%), and meranzin (98%) were obtained from PhytoLab, Vestenbergsgreuth, Germany. Rosmaric acid (≥98%), galangin (≥98%), chlorogenic acid, kaempferol, and daidzein were delivered by Cayman Chemical, Ann Arbor, MI, United States. Glycyrrhizic acid and 4-nitroquinoline-1-oxide (98%) were purchased from TCI, Eschborn, Germany. The strain TA1535 of *Salmonella typhimiurium* (genetically modified to contain the plasmid pSK1002) was purchased as cryostock from Trinova Biochem, Giessen, Germany. Resorufin-β-D-galactopyranoside was obtained from Toronto Research Chemicals, Toronto, Canada.

2.2 Standard Solutions and Sample Preparation

Standards solutions were prepared in methanol (1 mg/ml). Samples were obtained as dried, homogenized (mostly aqueous) extracts from Martin Bauer Group, Vestenbergsgreuth, Germany. For a 10% extract solution, an aliquot (0.5 g, **Table 1**) of each botanical powder was suspended in 5 ml methanol, ultra-sonicated for 30 min (Sonorex Digiplus, Bandelin, Berlin, Germany), and centrifuged at 3,000 × *g* for 15 min (Labofuge 400, Heraeus, Hanau, Germany). Each supernatant was transferred in an autosampler vial. Some extracts were additionally filtered (**Table 1**, marked*) through a 0.45 µm polytetrafluoroethylene filter (VWR, Darmstadt, Germany).

2.3 HPTLC–UV/Vis/FLD

Plates were pre-washed with methanol—water (4:1 *V/V*), dried in an oven (Memmert, Schwabach, Germany) for 20 min at 110°C (Morlock, 2014), and stored wrapped in aluminum foil. All botanical extracts (4 µL/band) were applied as 6 mm bands on a pre-washed plate (Automatic TLC Sampler 4, CAMAG, Muttenz, Switzerland). The plate was developed up to a migration distance of 70 mm with 7 ml ethyl acetate—toluene—formic acid—water (16:4:3:2 *V/V/V/V*) (Krüger et al., 2017). Separation was performed in a twin trough chamber (20cm × 10 cm, CAMAG) followed by drying for 4 min with a stream of cold air (hair dryer) and for 20 min in a laminar flow of air (Automated Development Chamber 2, CAMAG). The developed plates were documented at Vis, UV 254 nm, and FLD 366 nm (TLC Visualizer 2, CAMAG). The software winCATS (version 1.4.7.2018) or visionCATS (version 2.5.18262.1, both CAMAG) controlled the instruments.

2.4 HPTLC–EDA

For bioprofiling, 16 silica gel 60 F_{254} MS-grade chromatograms were prepared. The buffer and assay solutions were piezoelectrically sprayed (Derivatizer, CAMAG) if not stated otherwise. To remove acidic traces left on the planar chromatogram (which can interfere with pH-sensitive bioassays), the chromatogram was neutralized with 1.5 ml phosphate buffer (80 mg/ml Na_2HPO_4, pH 7.5 adjusted with NaOH; yellow/green nozzle, level 6) for enzymatic and bacterial assays, 1.4 ml citrate buffer (6 mg/ml citric acid monohydrate, 10 mg/ml Na_2HPO_4, adjusted to pH 12 with NaOH, yellow ultra-nozzle, level 2) (Klingelhöfer et al., 2020) for the hormonal-effective bioassays, 1.25 ml sodium bicarbonate buffer (2.5%, yellow nozzle, level 3) for

α-amylase bioassay or twice with 2.8 ml sodium bicarbonate buffer for SOS-Umu-C bioassay (neutralization procedure for SOS-Umu-C bioassay was investigated during this study, **Supplementary Figure S1**). The moist chromatogram was dried as mentioned in 2.3. A positive control was applied at three different concentrations at the top plate edge to verify the proper bioassay performance. The assay solutions/suspensions were applied as follows. For incubation, the plates were horizontally placed in a moistened polypropylene KIS box (26.5 cm × 16 cm × 10 cm, ABM, Wolframs-Eschenbach, Germany) pre-saturated with 30 ml water at 37°C (30°C for hormonal-effective bioassays) for 30 min. The procedure was documented at FLD 366 nm and white light illumination in transmission, reflection, and reflection/transmission mode.

2.4.1 *Bacillus subtilis* Bioassay

For the Gram-positive *B. subtilis* inhibition bioassay, 80 µl of stock solution was suspended in 20 ml Müller-Hinton Broth and incubated overnight at 37°C. Before usage, the cell number was determined using a spectrophotometer (M501, Camspec, Garforth, United Kingdom) at 600 nm. At an optical density (OD_{600}) between 0.8 and 1.1, the culture was ready to use for EDA. An aliquot of the bacteria suspension (2 ml) was sprayed on the planar chromatogram (red nozzle, level 6) (Morlock et al., 2021b). The plate was incubated at 37°C for 2 h. As substrate solution (2 mg/ml), MTT was freshly prepared in phosphate-buffered saline. After the application of 250 µl substrate solution (blue nozzle, level 6), the plate was incubated again for 30 min at 37°C. Inhibitory zones appeared colorless (white) on a formazan-purple background. The positive control was tetracycline (10 µg/ml in ethanol, 0.4, 0.8, and 1.2 µl/band).

2.4.2 *Aliivibrio fischeri* Bioassay

The bioluminescent marine Gram-negative bacteria *A. fischeri* were cultured according to DIN EN ISO 11348-1, Section 5 (European Committee for Standardization, 2009). Therefore, 200 µl of cryostock were suspended in 20 ml medium. The cultivation was performed overnight (18–24 h) in a 100 ml Erlenmeyer flask at room temperature by shaking at 75 rpm. Once the culture showed brilliant blue fluorescence by shaking in the dark, it was ready for use. An aliquot of the bacteria suspension (3 ml) was sprayed on the plate (blue nozzle, level 6) and directly recorded (BioLuminizer 2, CAMAG) (Morlock et al., 2021a; Morlock et al., 2021b). The native bioluminescence (depicted as a greyscale image) was documented in ten images at time intervals of 3 min. Exposure time was set to 120 s. Antibacterial components were detected as dark zones, whereas metabolism-enhancing substances appeared as bright zones on the bioluminescent background. The positive control was caffeine (1 mg/ml in methanol, 0.5, 1.5, and 3 µl/band).

2.4.3 Cholinesterase Inhibition Assays

The initial AChE and BChE inhibition assays (Marston et al., 2002) were modified (Hage and Morlock, 2017; Morlock et al., 2021b). The plates were pre-wetted with 0.5 ml TRIS-HCl buffer (7.55 mg/ml TRIS, pH 7.8 adjusted with HCl, green nozzle, level 6). Then, 1.5 ml of enzyme solution (AChE 6.66 U/ml, BChE 3.34 U/ml, and each 1 mg/ml BSA in TRIS-HCl buffer) were applied (green nozzle, level 6) and the chromatogram was subsequently incubated at 37°C for 30 min. For detection, 0.5 ml substrate mixture (1 mg/ml 1-naphthyl acetate, 2 mg/ml Fast Blue B salt) was sprayed (red nozzle, level 6) onto the plate to obtain colorless (white) inhibition zones on a purple background. The positive control was rivastigmine (0.1 mg/ml in methanol, 2, 4, and 8 µl/band).

2.4.4 Glucosidase Inhibition Assays

An improved version of Simões-Pires *et al.* (Simões-Pires et al., 2009) was used to detect α- and β-glucosidase inhibitors. The substrate solution (12 mg 2-naphthyl-α-D-glucopyranoside or 2-naphthyl-β-D-glucopyranoside in 9 ml ethanol and adding 1 ml of 10 mM NaCl solution) was sprayed (1 ml, red nozzle, level 6) onto the plate, followed by drying in a stream of cold air. Pre-wetting was carried out by spraying 0.5 ml sodium acetate buffer (41 mg/ml, pH 7.5 adjusted with 0.1 mM acetic acid, green nozzle, level 6). An aliquot of the respective enzyme solution (α-glucosidase 10 U/ml, β-glucosidase 1,000 U/ml in sodium acetate buffer) was applied (1 ml; green nozzle, level 6) and the plate was subsequently incubated at 37°C for 30 min. The antidiabetic effect was visualized by Fast Blue B salt staining (2 mg/ml in water, 0.5 ml, red nozzle, level 6), resulting in colorless (white) inhibitory zones on a purple background. The positive controls were acarbose (3 mg/ml in ethanol, 1, 3, and 6 µl/band) for the α-glucosidase assay and imidazole (1 mg/ml in ethanol, 3, 5, and 7 µl/band) for the β-glucosidase assay.

2.4.5 β-Glucuronidase Inhibition Assay

The β-glucuronidase inhibition assay was run as described recently (Mahran et al., 2020) The chromatogram was pre-wetted with potassium phosphate buffer (0.5 ml; 9.34 mg/ml K_2HPO_4 and 6.31 mg/ml KH_2PO_4; green nozzle, level 6). Then, 750 µL enzyme solution (25 U/ml in potassium phosphate buffer with 1 mg/ml BSA) were sprayed onto the chromatogram (green nozzle, level 6). Incubation followed for 15 min at 37°C. As substrate, 750 µl of a 2 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide sodium salt solution was sprayed. The plate was incubated again for 60 min for producing colorless (white) inhibitory zones on a blue background. The positive control was D-saccharolactone (0.1 mg/ml in water, 1, 1.5, and 2 µl/band).

2.4.6 Tyrosinase Inhibition Assay

The tyrosinase inhibitor potential was investigated according to an improved (Morlock et al., 2021b) workflow (Taibon et al., 2015). To prepare the substrate solution, 45 mg levodopa, 25 mg CHAPS, and 75 mg PEG 8000 were dissolved in 10 ml of phosphate buffer (1.4 mg/ml K_2HPO_4, 1.68 mg/ml Na_2HPO_4, pH 6.8) and stored at 4°C until use. The levodopa substrate solution was sprayed onto the chromatogram (1 ml, blue nozzle, level 6) and subsequently dried for 2 min in a stream of cold air. Then, 1 ml of enzyme solution (400 U/ml in phosphate buffer) was sprayed onto the plate (blue nozzle, level 6), followed by incubation at room temperature for 20 min. After incubation, the plate was immediately dried and documented. Tyrosinase

inhibition activity was apparent as colorless (white) zones on a greyish-brown background. The positive control was kojic acid (0.1 mg/ml in ethanol, 1, 3, and 6 µl/band).

2.4.7 Planar Yeast Androgen/Estrogen Screen (pYAS/pYES) Bioassay

The hormonal-effective bioassays were run as recently described (Klingelhöfer et al., 2020). Cryogenic YAS or YES cell culture (1 ml each) was suspended in 39 ml or 29 ml medium, respectively. The suspensions were cultivated by shaking at 70–75 rpm and 30°C overnight (20–22 h). The cell number was determined with a hemocytometer after diluting 50 µl culture in 950 µl 0.9% NaCl solution. The required cell count of 0.8×10^8 cells/ml was adjusted via centrifugation (2,500 × *g*, 5 min) of 5 ml yeast cell culture and resuspension in the required amount of medium plus 50 µl copper sulfate. This suspension was sprayed on the plate (1.4 ml, red nozzle, level 6), followed by incubation for 4 h (pYAS) or 3 h (pYES) at 30°C. Substrate solution (2 mg 4-methylumbelliferyl-β-D-galactopyranoside, 100 µl DMSO, 3 ml citrate buffer) was sprayed onto the chromatogram (1.5 ml, yellow ultra-nozzle, level 2). Subsequently, the plates were incubated for 1 h at 37°C. Bioautograms were recorded at FLD 366 nm. Endocrine agonists appeared as 4-methylumbelliferone-blue fluorescent zones on a dark blue background. As a positive control, testosterone (for pYAS: 0.5 µL, 1.5 µg/ml in methanol) or 17-β-estradiol (for pYES: 5 µl, 100 ng/ml in ethanol) were applied.

2.4.8 Metabolization via S9–pYES Bioassay

Potential estrogens resulting from liver metabolism were investigated by adding the S9 enzyme mixture (500 µl) and respective cofactors (166 µl NADP, 42 µl glucose 6-phosphate, 958 µl phosphate buffer) to 3,334 µl *Saccharomyces cerevisiae* cell culture (0.8×10^8 cells/ml). The assay was performed as described above.

2.4.9 Planar Yeast Antagonistic Androgen/Estrogen Screen (pYAS/pYES) Bioassay

To screen the antagonistic activity, the pYAS or pYES bioassays were extended by overspraying along the middle of each track a 1 mm × 70 mm area of testosterone (4 µl, 1.5 µg/ml in methanol) or 17-β-estradiol (5 µl, 2 ng/ml in ethanol), respectively, with the Freemode option of winCATS (Klingelhöfer et al., 2020). Endocrine antagonists appeared as fluorescence-reducing bands in the 4-methylumbelliferone-blue fluorescent testosterone or 17-β-estradiol track part.

2.4.10 SOS-Umu-C Bioassay

The planar SOS-Umu-C bioassay was run on HPTLC silica gel plates without a fluorescence indicator. After development, the plates were additionally scanned at 546/>580 nm using the TLC Scanner 3 (CAMAG). *Salmonella typhimurium* cells (50 µl cryostock) were suspended in 35 ml Lysogeny broth (20 mg/ml with 1 mg/ml D-(+)-glucose and 106 mg/L ampicillin sodium salt) and incubated overnight at 75 rpm and 37°C for 16 h. Before use, the cells were centrifuged (3,000 × *g*, 10 min). The pelleted cells were re-suspended in fresh medium to obtain the required OD_{660} of 0.2 (Meyer et al., 2020). The chromatogram was sprayed with *Salmonella* suspension (2.8 ml, yellow nozzle, level 3) and incubated at 37°C for 3 h. Substrate solution [15 µl resorufin-β-D-galactopyranoside solution (20 mg/ml in DMSO) in 2.1 ml phosphate buffer and 0.9 ml glycerol] was sprayed onto the plate (2.5 ml, red nozzle, level 6). Incubation followed at 37°C for 1 h. The plates were documented at white light illumination and 366 nm. The generated resorufin fluorescence was measured at 546/>580 nm. Genotoxic substances were detectable either as pink zones on the colorless background at white light illumination or as pink fluorescent zones on a brown-reddish background at 254 nm or 366 nm. The positive control was 4-nitroquinoline-1-oxide (1 µg/ml in methanol, 1 µl/spot).

2.4.11 α-Amylase Inhibition Assay

The latest α-amylase inhibition method, which used immersion of the plate into enzyme and substrate solutions (Agatonovic-Kustrin and Morton, 2017; Agatonovic-Kustrin et al., 2019), was adjusted and transferred to a piezoelectric spraying procedure, in which the enzyme solution (62.5 U/mL in sodium acetate buffer) was sprayed onto the chromatogram (1 ml, red nozzle, level 5), followed by 30 min incubation at 37°C. As substrate 2%-soluble starch solution was sprayed onto the wet plate (0.5 ml, red nozzle, level 5). After 20 min incubation at 37°C, Gram's iodine solution was sprayed (250 µl, yellow nozzle, level 5) for visualization. The α-amylase inhibition activity was observed as violet zones on a colorless background. The positive control was acarbose (0.1 mg/ml in methanol, 0.3, 0.6, and 0.9 µl/band).

2.5 Instrumental Setup of the 8D-Hyphenation

The multipotent bioactive zones were further characterized with RP-HPLC-DAD-HESI-MS directly after EDA. The UPLC system (Acquity H Class, Waters, Eschborn, Germany) was equipped with the quaternary solvent manager, solvent degasser, sample manager, column oven, photodiode array detector (DAD), and HESI-MS (single quadrupole QDa, Waters). The bioactive target zone was heart-cut eluted with an oval elution head (4 mm × 2 mm) of the TLC-MS Interface 2 (CAMAG) with 90% aqueous methanol. A standalone pump supplied the solvent (515 HPLC pump, Waters). Analytes were transferred through a biocompatible inline filter (IDEX Health and Science, Oak Harbor, WA, United States) containing a PEEK frit (0.5 µm, Techlab, Brunswick, Germany) to an online desalting RP pre-column/defender guard (Accucore RP-MS, 10 mm × 2.1 mm, 2.6 µm, Thermo Scientific, Bellefonte, PA, United States). The online desalting device was installed onto a two-position switching valve (MXT-Series PD715-000, Rheodyne IDEX Health and Science) and served as an analyte trap while discarding the bioassay salts as waste. By switching, controlled via remote control and Rheodyne TitanMX software, the analytes were transferred to the main RP column (Accucore RP-MS 100 mm × 2.1 mm, 2.6 µm, Thermo Scientific) and separated orthogonally. The column was thermostated at 40°C. The 13 min HPLC gradient consisted of (A) 2.5 mM ammonium acetate (pH 4.5 adjusted with acetic acid) and (B) methanol. Starting

conditions were 98% A at a flow rate of 0.6 ml/min for the first 2 min. The methanolic portion increased linearly to 20% within the following 2.5 min. At 8 min, a ratio of 10/90% A/B was reached and held for the next 2 min; then it fell to 98% A within 0.1 min, followed by 3 min equilibration time. Detection parameters were set to a wavelength scan from 190 to 400 nm for DAD. The MS was operated in polarity-switching mode, while the ESI probe was heated to 600°C and ESI source to 120°C. The sampling frequency was set to 5 Hz and cone voltage to ±10 V in both ionization modes (Schreiner and Morlock, 2021). The MassLynx V4.2 software (Waters) was used to evaluate and process the data.

3 RESULTS AND DISCUSSION

3.1 Outline of the Study

A total of 68 very different powdered plant extracts (botanicals added to food products in food industry) and 16 different effect-directed assays were selected to investigate and prove the suitability of the biological–physicochemical 8D hyphenation for generic screening (**Figure 1**). Among the plants (**Table 1**) were such ones that are commonly used as culinary spices and herbs or in traditional medicine. Their diverse and varying compositions represent different matrix loads for the analytical system. Moreover, the nine biological and seven biochemical assay media differed over a wide range in salt and nutrient composition. This represents the diversity of possible compositions of a bioactive zone (to be heart-cut and transferred to the next dimension) and was therefore considered a good worst-case scenario to test whether the developed generic hyphenation method is suitable for routine analyses. First, the bioactivity screening was evaluated per assay (**Figures 2–6**), whereby some botanicals were mentioned repeatedly, *i.e.* galangal (no. 13) yerba mate (no. 37), orange peel (no. 41), licorice (no. 55), and Siberian ginseng (no. 56). Then, these botanicals were subjected to heart-cut RP-HPLC–DAD–HESI-MS analysis (**Figures 7–11**). All botanicals were extracted and applied analogously. Thus, the effect profiles of each assay could directly be compared by their response pattern. The most effective and important botanicals were highlighted at a glance in side-by-side comparison. The band broadening (diffusion) depended on the assay incubation time. The most important bioactive compounds discovered were tentatively assigned based on information obtained about spectral (UV/Vis/FLD), polarity (hR_F values with a deviation of ±1), and molecular (mass signal) properties. Since there was no access to a high-resolution mass spectrometry system, the assignments were verified by comparing with reference standards.

Using the Gram-negative *Aliivibrio fischeri* and Gram-positive *Bacillus subtilis* bioassays, natural antibacterial compounds were detected that can subtly fight infections and contribute to longer shelf life and better preservation of products. Natural AChE and BChE inhibitors, which can provide symptomatic benefits for the cognitive decline of Alzheimer's patients (Tundis et al., 2016), were revealed by the respective planar enzyme inhibition assays. The tyrosinase inhibition assay was used to screen for plant-based skin whiteners or inhibitors of the enzymatic food browning. The β-glucuronidase inhibition assay was used to detect compounds that prevent the gut-bacterial reversion of detoxification via glucuronidation. Additional α-amylase, α- and β-glucosidase inhibition assays were employed to determine natural compounds with benefits for diabetes patients. Phytoandrogens and phytoestrogens were detected via the human estrogen receptor α (hERα) or hERβ or in combination with the S9 enzyme mixture simulating liver metabolization, and respective antagonists were investigated using recombinant yeast cells equipped with the human estrogen/androgen receptor. Genotoxins were detected with a recombinant *Salmonella typhimurium* strain equipped with the SOS-Umu-C repair mechanism. Hence, the spectrum of effects in the investigated botanicals may be linked to the mitigation of bacterial infects, the improvement of cholinergic transmission for Alzheimer's patients, the decrease of blood glucose levels in diabetics, the reduction of skin abnormalities, and to the balance of the steroid hormonal system, among other disorders.

3.2 Screening Results

3.2.1 Compounds Inhibiting Bacteria

In traditional medicines, plant-based extracts are used to assist in the treatment of bacterial infections (Brantner and Grein, 1994; Palombo and Semple, 2001). Antibacterial activities can be so effective that plant extracts are also used as preservatives in food products. For example, the ingredients carnosol and carnosic acid of rosemary extract are marketed as preservative E 392. Rosemary (no. 46) is also screened here, using non-pathogenic bacterial representatives which are easier to handle in the laboratory. The Gram-positive *B. subtilis* bioassay is based on an oxidoreductase enzyme reaction. Intact enzymes of viable *B. subtilis* cells reduce the tetrazolium salt MTT to the insoluble purple formazan (Marston, 2011). Cell death is visualized as colorless zones indicating antibacterial compounds. Most antibacterials detected were located at hR_F values ≥ 90 (**Figures 2–4C**). In eucalyptus (no. 9), marjoram (no. 35), yerba mate green (no. 37), Siberian ginseng (no. 56), thyme (no. 57), hawthorn leaves (nos. 63 and 64), and cinnamon bark (no. 66) additional antibacterials were detected in the lower hR_F range. Essential oils from herbs such as oregano and thyme are known for their antimicrobial activity, especially against Gram-positive bacteria (Soković et al., 2010).

The Gram-negative *A. fischeri* bacteria are able to convert metabolic energy into turquoise bioluminescence via luciferase. A change in bioluminescence intensity is correlated with substances enhancing or reducing the cell metabolism. Such effects are visualized as lightened or dark zones on the bioluminescent background of the bioautogram (depicted as greyscale image). Almost all botanical extracts showed antimicrobial activity against *A. fischeri* (**Figures 2–4D**). Intense antimicrobial zones were detected in yerba mate green (no. 37), passionflower (no. 43), peppermint (no. 44), rooibos (no. 45), licorice (no. 55), Siberian ginseng (no. 56), and cinnamon bark (no. 66). Most samples showed at least one dark antimicrobial zone, while more than half of all samples had two or more. The more universally and sensitively detecting *A. fischeri* bioassay proved to be a good starting assay to investigate complex mixtures.

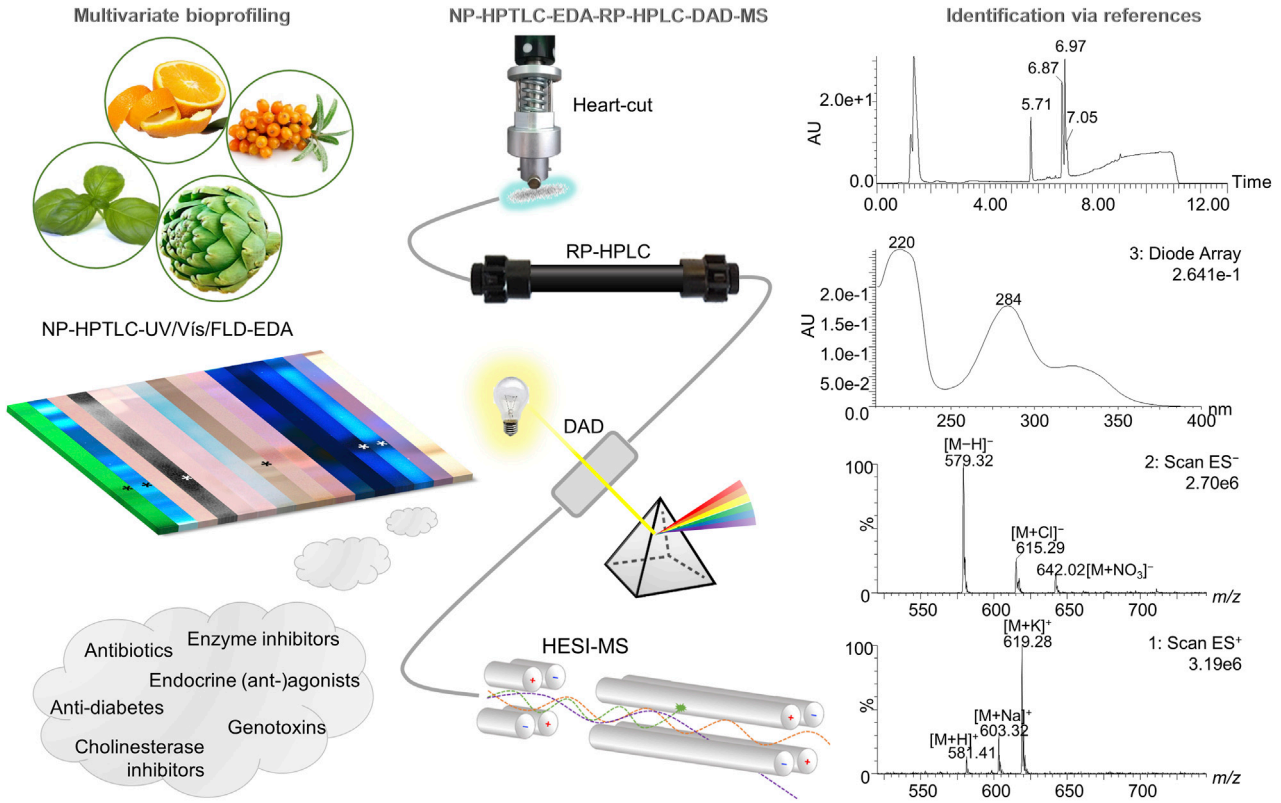


FIGURE 1 | Schematic overview of the hyphenation NP-HPTLC-UV/Vis/FLD-EDA-heart-cut RP-HPLC-DAD-HESI-MS. Botanical extracts were separated on NP-HPTLC plates followed by biochemical and microbiological detections. Bioactive zones were heart-cut eluted directly out of the (bio)autogram and transferred to the orthogonal RP-HPLC-DAD-HESI-MS, resulting in comprehensive information about a distinct bioactive compound.

3.2.2 Compounds Inhibiting AChE and BChE

In traditional or ayurvedic medicine, plants and their phytoconstituents are used to assist in the treatment of Alzheimer´s disease (Azadniya et al., 2021). The pathophysiology of Alzheimer's disease is often associated with cholinergic system dysfunction. Therefore, many synthetic drugs target the inhibition of cholinesterases. Both AChE and BChE catalyze the hydrolysis of the neurotransmitter acetylcholine into acetic acid and choline. While AChE is highly selective for acetylcholine, BChE can also convert other substrates, *e.g.*, butyrylcholine, succinylcholine, or organophosphates. Besides synthetic drugs, also natural compounds are able to inhibit this enzyme mechanism. Particularly polyphenols interact with amino acid residues of the active side of the enzymes terminating the splitting from acetylcholine into acetic acid and choline and thus maintaining colinergic neurotransmission and improve cognition of Alzheimer's patients (Jabir et al., 2018). The enzyme-inhibiting potential is revealed as colorless zones on a purple background. Acerola fruit (no. 1) showed a remarkably strong inhibition zone at hR_F 41 (**Figures 2E,F**). While roots and branches from the acerola tree are known to have anti-cholinesterase activity through the norfriedelins A-C (Liu et al., 2013), only cytotoxic, anti-HIV, antioxidant, antihyperglycemic, skin whitening, and antimicrobial activities are described for extracts from the fruits (Motohashi et al., 2004; Belwal et al., 2018a). This zone showed not only a strong response in the AChE and BChE assays, but also in most other assays (**Figure 2**). Other inhibitory zones were detected in ginger (no. 25, hR_F 99), kola (no. 31, hR_F 68), marjoram (no. 35, hR_F 99), yerba mate green (no. 37, hR_F 88), lemon balm (no. 38, hR_F 32), peppermint and rooibos (nos. 44 and 45, both hR_F 34), Siberian ginseng (no. 56, hR_F 93), and hawthorn (nos. 62–64, hR_F 60 and 85) (**Figures 2–4E,F**). Some typical traditional medicines used in the treatment of Alzheimer´s disease, such as *Panax notoginseng*, *Ginkgo biloba* (no. 14), *Melissa officinalis* (no. 38), and *Salvia officinalis* (no. 47), are also screened here. Among these, *Melissa officinalis* (no. 38) possess cholinesterase-inhibiting potential, the others operate according to a different mechanism to treat the neurodegenerative disease (Sharma et al., 2019).

3.2.3 Compounds Inhibiting β-Glucuronidase

The detoxification mechanism via glucuronidation can be reversed with β-glucuronidase from opportunistic *Enterobacteriaceae* such as *Escherichia coli*, resulting in gastrointestinal malfunction. This can be prevented by inhibiting the microbial β-glucuronidase, where such inhibitors address the extra loop in the bacterial enzyme (in contrast to the mammalian one) (Mahran et al., 2020). The enzyme inhibitors do not cleave the chromogenic substrate and are therefore detected

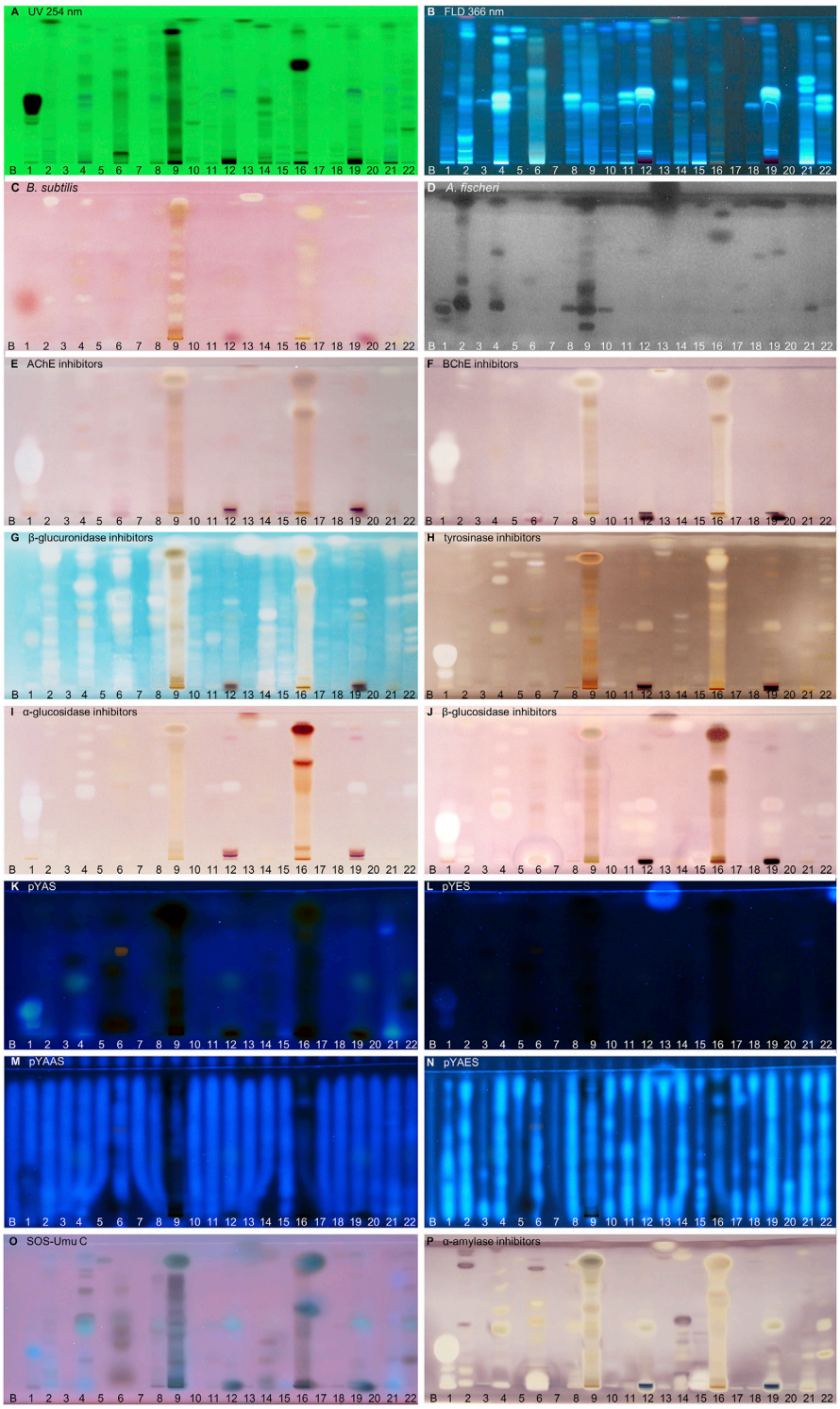


FIGURE 2 | NP-HPTLC–UV/Vis/FLD–EDA profiles of the plant extracts no. 1–22. Separation of the applied botanicals (4 µL/band, assignments in **Table 1**; solvent blank B for comparison) on HPTLC plate silica gel 60 F_{254} MS-grade with ethyl acetate—toluene—formic acid—water (16:4:3:2, *V/V/V/V*) up to 70 mm, detected at UV 254 nm **(A)**, FLD 366 nm **(B, K–O)** and white light illumination **(C, E–J, P)** after the *B. subtilis* bioassay **(C)**, *A. fischeri* bioassay with bioluminescence depicted as a greyscale image, **(D)** and AChE **(E)**, BChE **(F)**, β-glucuronidase **(G)**, tyrosinase **(H)**, α-glucosidase **(I)**, β-glucosidase **(J)** and α-amylase **(P)** inhibition assays, as well as pYAS **(K)**, pYES **(L)**, pYAAS **(M)**, pYAES **(N)**, SOS-Umu-C **(O)** bioassays.

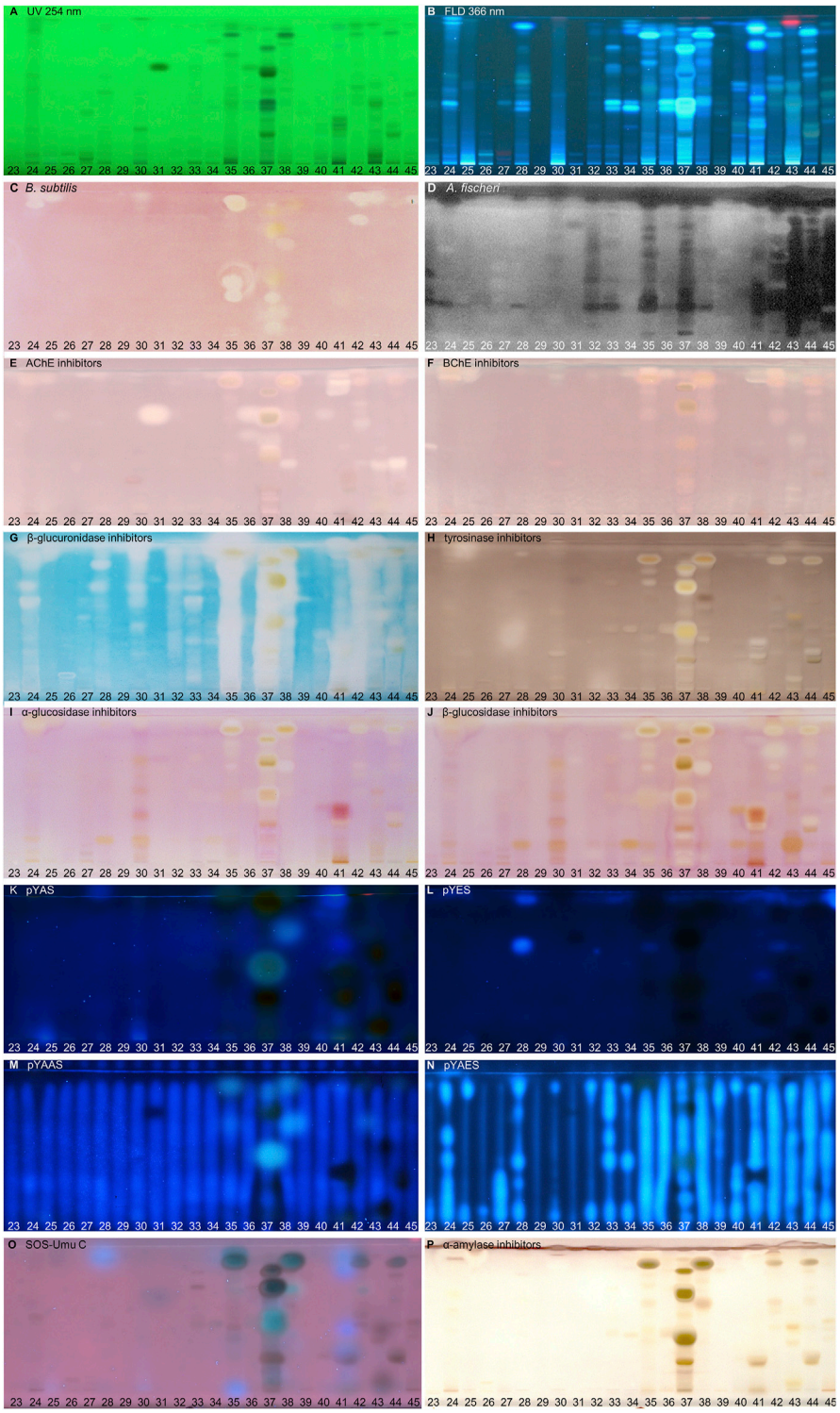


FIGURE 3 | NP-HPTLC–UV/Vis/FLD–EDA profiles of the plant extracts no. 23–45. Separation of the applied botanicals (4 µL/band, assignments in **Table 1**) on HPTLC plate silica gel 60 F_{254} MS-grade with ethyl acetate—toluene—formic acid—water (16:4:3:2, *V/V/V/V*) up to 70 mm, detected at UV 254 nm **(A)**, FLD 366 nm **(B, K–O)** and white light illumination **(C, E–J, P)** after the *B. subtilis* bioassay **(C)**, *A. fischeri* bioassay with bioluminescence depicted as a greyscale image, **(D)** and AChE **(E)**, BChE **(F)**, β-glucuronidase **(G)**, tyrosinase **(H)**, α-glucosidase **(I)**, β-glucosidase **(J)**, and α-amylase **(P)** inhibition assays, as well as pYAS **(K)**, pYES **(L)**, pYAAS **(M)**, pYAES **(N)**, SOS-Umu-C **(O)** bioassays.

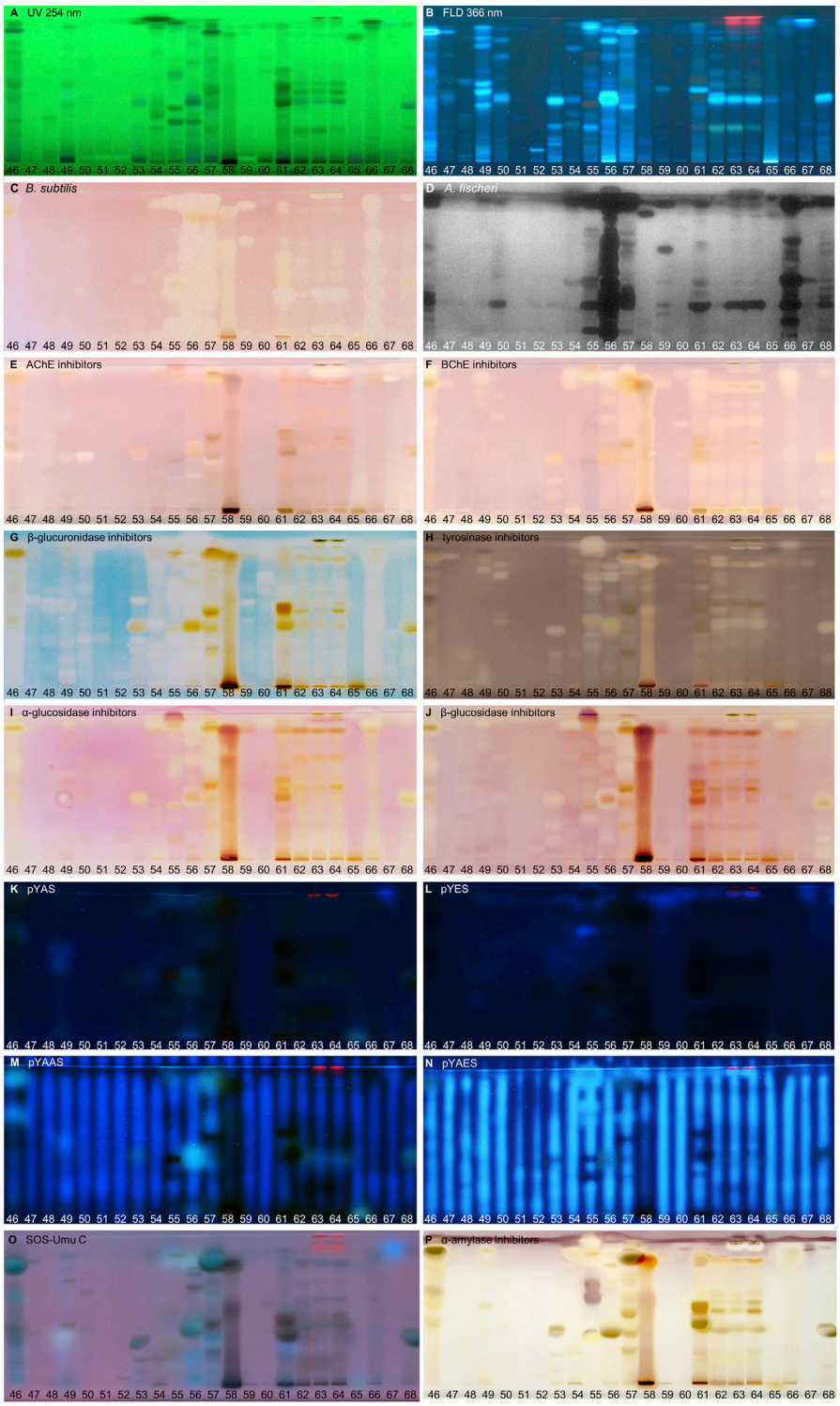


FIGURE 4 | NP-HPTLC–UV/Vis/FLD–EDA profiles of the plant extracts no. 46–68. Separation of the applied botanicals (4 µL/band, assignments in **Table 1**; solvent blank B for comparison) on HPTLC plate silica gel 60 F_{254} MS-grade with ethyl acetate—toluene—formic acid—water (16:4:3:2, *V/V/V/V*) up to 70 mm, detected at UV 254 nm **(A)**, FLD 366 nm **(B, K–O)**, and white light illumination **(C, E–J, P)** after the *B. subtilis* bioassay **(C)**, *A. fischeri* bioassay with bioluminescence depicted as a greyscale image, **(D)** and AChE **(E)**, BChE **(F)**, β-glucuronidase **(G)**, tyrosinase **(H)**, α-glucosidase **(I)**, β-glucosidase **(J)**, and α-amylase **(P)** inhibition assays, as well as pYAS **(K)**, pYES **(L)**, pYAAS **(M)**, pYAES **(N)**, SOS-Umu-C **(O)** bioassays.

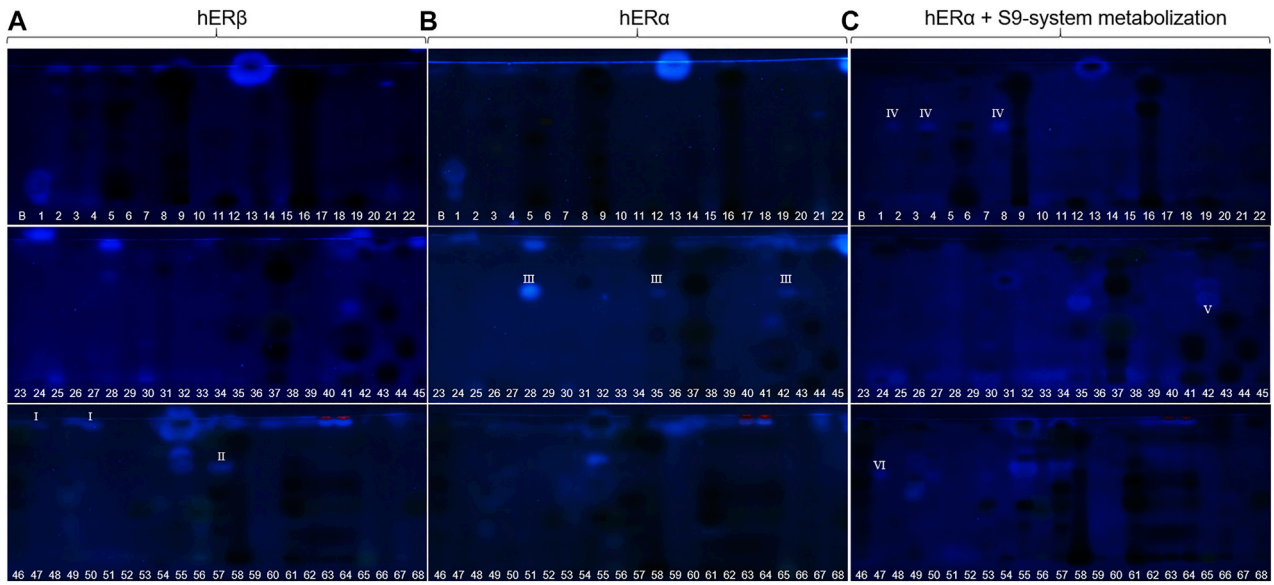


FIGURE 5 | Comparison and observed differences (I–VI) of the estrogenic activity via NP-HPTLC–pYES using the hERα versus hERβ used for the first time, or after metabolization with the S9 mixture. Separation of the applied botanicals (no. 1–68, 4 µl/band, assignments in **Table 1**; solvent blank B for comparison) on HPTLC plate silica gel 60 F_{254} MS-grade with ethyl acetate—toluene—formic acid—water (16:4:3:2, *V/V/V/V*) up to 70 mm, detected at FLD 366 nm after pYES. Cells were equipped either with hERβ **(A)**, hERα **(B)** or phytochemicals were metabolized with the S9 enzyme mixture, simulating liver metabolism **(C)**.

as colorless zones on an indigo-blue background. In each eucalyptus (no. 9), guarana (no. 16), marjoram (no. 35), yerba mate green (no. 37), oregano (no. 42), Siberian ginseng (no. 56), and cinnamon bark (no. 66), the whole sample track appeared white on the indigo-blue background due to the comparatively high abundance of β-glucuronidase inhibitors (**Figures 2–4G**). If the application volume is reduced by a factor of 4 for these botanicals, the individual inhibitors become evident (**Supplementary Figure S2**). All botanical extracts showed β-glucuronidase inhibitory potential at least in the solvent front (hR_F 99). For such screening results, repetition using a mobile phase of reduced solvent strength is recommended in order to better differentiate the individual inhibitors (**Supplementary Figure S3C,D**). Some isolated flavonoid standards, *i.e.,* isorhamnetin, kaempferol, liquiritigenin, daidzein, *etc.,* already proved to be active against β-glucuronidase (Sun et al., 2020). Their activity and also that of additional flavonoids have been confirmed by our study directly in herbs and spices.

3.2.4 Compounds Inhibiting Tyrosinase

Polyphenoloxidases are responsible for the browning of cut or injured fruits or plant tissues. In mammalian cells, the corresponding tyrosinase controls melanogenesis by catalyzing the hydroxylation of phenols with subsequent oxidation to quinones. An overproduction of melanin induces pigmentary abnormality, freckles, or age spots. Preferably, the cosmetics industry is interested in naturally derived tyrosinase inhibitors (Taibon et al., 2015). In this context, ethnobotanicals are brought into focus. In South Africa, herbal extracts are traditionally used as skin care products to treat burns, abcesses, wounds and acne (Lall and Kishore, 2014). Chinese herbal medicines with anti-tyrosinase activity are traditionally used as folk skin whiteners. Among the studied botanicals, *Ginkgo biloba* (no. 14), *Panax ginseng* (no. 15), and *Zingiber officinale* (no. 25) were reported to inhibit mushroom tyrosinase (Ye et al., 2010; Hu et al., 2020). Our screening results showed that ginseng (no. 15) and ginger (no. 25) played a minor role in tyrosinase inhibition compared to other botanicals. However, differences in effects can be caused for example by plant subspecies, climate, soil, environmental and agricultural conditions as well as extraction mode. In the planar assay, tyrosinase inhibitors are shown as colorless zones on a greyish-brown background. Many botanical extracts showed multiple tyrosinase inhibitors (**Figures 2–4H**). While acerola (no. 1) (Belwal et al., 2018a), ginkgo (no. 14) (Shu et al., 2020), licorice (no. 55) (Li et al., 2017), and hawthorn (nos. 62–64) (Rocchetti et al., 2020) are known for their anti-tyrosinase activity, the screening results proved similarly potent tyrosinase inhibitors in artichoke (no. 4), plantain (no. 35), yerba mate green (no. 37), rosemary (no. 46), and yarrow (no. 50).

3.2.5 Compounds Inhibiting α- and β-Glucosidase

The enzymes α- and β-glucosidase hydrolyze the saccharide dimers and oligomers, as well as glucosides dependent on the anomeric glycosidic bond, into resorbable monomers such as glucose and into aglycones. In the treatment of hyperglycemic blood levels of type 2 diabetes patients, enzyme inhibitors are of therapeutic interest by reducing postprandial glucose uptake (Simões-Pires et al., 2009; Turkiewicz et al., 2019). Screening results showed several α- and β-glucosidase inhibitors as a specific pattern (at hR_F 42, 48, 62, and 80) for artichoke (no. 4) in both assays (**Figures 2I,J**). Although the α- (Turkiewicz et al., 2019)

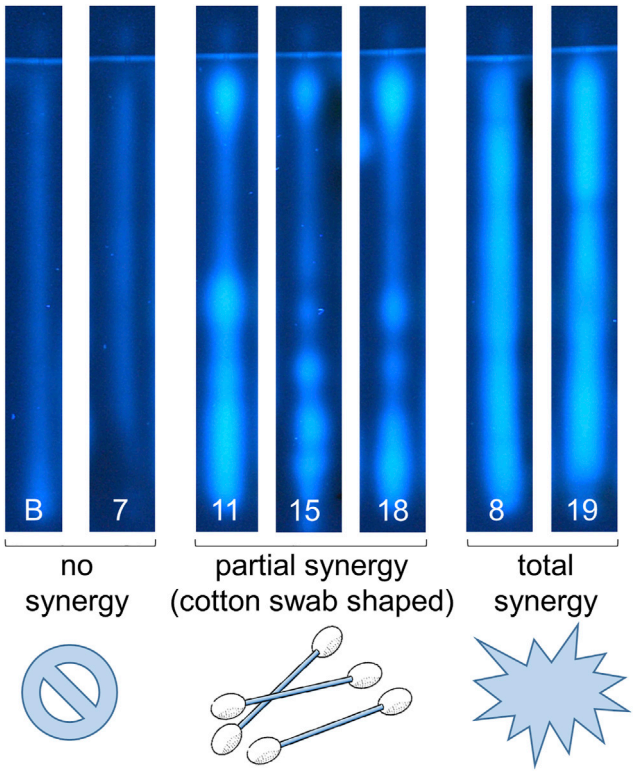


FIGURE 6 | First report of synergy profiles, exemplarily shown for the NP-HPTLC-pYAES bioassay. Separation of the applied botanicals (nos. 7–19, assigned in **Table 1**; 4 µl/band each; solvent blank B for comparison) on HPTLC plate silica gel 60 F_{254} MS-grade with ethyl acetate—toluene—formic acid—water (16:4:3:2, *V/V/V/V*) up to 70 mm; each separated track was oversprayed by an 1 mm × 70 mm area of 17-β-estradiol (10 ng/band) and after bioassay application detected at FLD 366 nm. Fortified estrogenic fluorescence was interpreted as synergistic effects, which were not observed in solvent blank B and no. 7 (only a slight anti-estrogenic effect in the lower hR_F range), partially observed as cotton-swab-shaped 17-β-estradiol band (nos. 11, 15 and 18) or as overall broadened estrogenic band (nos. 8 and 19), if compared to the solvent blank B, which shows reference 17-β-estradiol fluorescence.

and β-glucosidase (Morlock et al., 2021a) inhibitory potential and chemical composition of extracts of different artichoke cultivars have already been described, the distinct bioactive components have been scarcely assigned in literature. In both glucosidase inhibition assays, samples of blackberry leaves (no. 8), yellow fruit tea (no. 11), red fruit tea (no. 12), elderflower (no. 22), yerba mate green (no. 37), horsetail (no. 49), plantain (no. 53), Siberian ginseng (no. 56), and lemon verbena (no. 68) showed a positive response at hR_F 42 (**Figures 2–4I,J**). In all of them, the same bioactive compound was assumed. Another remarkably similar active compound was observed at hR_F 94 for basil (no. 5), ginkgo (no. 14), European blueberry (no. 18), elderberry (no. 21), hop (no. 24), yerba mate green (no. 37), lemon balm (no. 38), oregano (no. 42), peppermint (no. 44), rosemary (no. 46), star anise (no. 54), Siberian ginseng (no. 56), thyme (no. 57), and cinnamon bark (no. 66). All of them showed both α- and β-glucosidase inhibitory activities. Worldwide more than 1,000 herbal remedies were traditionally deployed for the maintenance and treatment of high blood glucose levels and thus diabetes. Both, the ethnobotanicals used and their mode of application (as tincture or extract, orally or as infusion) differ between local communities (Cock et al., 2021). Since western medical treatment methods for type 2 diabetes focus on hypoglycemic drugs, such as insulin, ethnopharmacological remedies are considered to be safe and to have less toxic side effects. According to the theory of traditional Chinese medicine, flavonoids (Bai et al., 2019) and polyphenols (Umeno et al., 2016) are attributed to have antidiabetic effects via several mechanisms. Glycyrrhizic acid from *Glycyrrhiza glabra* (no. 55), apigenin and its derivates as well as quercetin, found in many botanicals (**Table 2**), are known to target α-glucosidase (Bai et al., 2019).

3.2.6 Compounds With Agonistic/Antagonistic Hormonal Effects

Disrupting endocrine signaling pathways can have a severe impact on hormonal balance and cause feminization/masculinization, infertility, acne, and menstrual cycle disorders. Also phytochemicals present in food (Morlock and Klingelhöfer, 2014) or commodities in daily use (Klingelhöfer et al., 2020) can affect the human hormone system. HPTLC hyphenated to the planar yeast androgen/estrogen screens (pYAS/pYES) and their antagonistic versions (pYAAS/pYAES) showed positive reactions to a limited extent (**Figures 2–4K–N**). In the pYAS bioassay, a few 4-methylumbelliferone-blue and thus androgenic responses were detected in acerola (no. 1, hR_F 16), elderberry (no. 21, hR_F 72), chamomile (no. 28, hR_F 99), orange peel (no. 41, hR_F 67 and 98), horsetail (no. 49, hR_F 49 and 61), lemon peel (no. 67, hR_F 99), and for several samples at the application zone (nos. 4, 15, 25, 30, and 56). In the pYES bioassay, 15 botanicals showed estrogen-like activity. In some samples, phytoestrogens were known and expected, *e.g.,* 8-prenylnaringenin in hop (no. 24) (Prencipe et al., 2014; Mbachu et al., 2020), (iso-)liquiritigenin in licorice (no. 55) (Boonmuen et al., 2016), or pesticide residues from fruit surfaces (Schulte-Oehlmann et al., 2011) such as orange peel (no. 41), grape peel (no. 59), or lemon peel (no. 67). However, in acerola (no. 1, hR_F 15 and 27), galangal (no. 13, hR_F 99), chamomile (no. 28, hR_F 63 and 95), lovage (no. 34, hR_F 97), marjoram (no. 35, hR_F 62), oregano (no. 42, hR_F 45), juniper (no. 60, hR_F 89), grape leaves (no. 61, hR_F 92), and hawthorn (nos. 62–64, hR_F 94), estrogen-like responses were also detected. Since steroid hormone-like compounds are known to have a greater affinity to hERα, but several phytoestrogens (*e.g.,* daidzein or genistein) to hERβ (Mbachu et al., 2020), the pYES bioassay was also performed via the hERβ for the first time. The assay was analogously run for both receptors, but no remarkable difference was observed in the results obtained by both (**Figure 5A** versus **Figure 5B**). The hERα seemed to be less selective. However, a significant difference in the estrogenic pattern was observed after metabolization with the S9 mixture (**Figure 5C**). The use of genetically modified yeast cells containing the hERβ for the pYES bioassay and the simulated metabolization via the S9 liver enzyme system were reported here for the first time.

In the respective antagonistic assays, only a few zones were detected. For acerola (no. 1, hR_F 99), fenugreek (no. 6, hR_F 99),

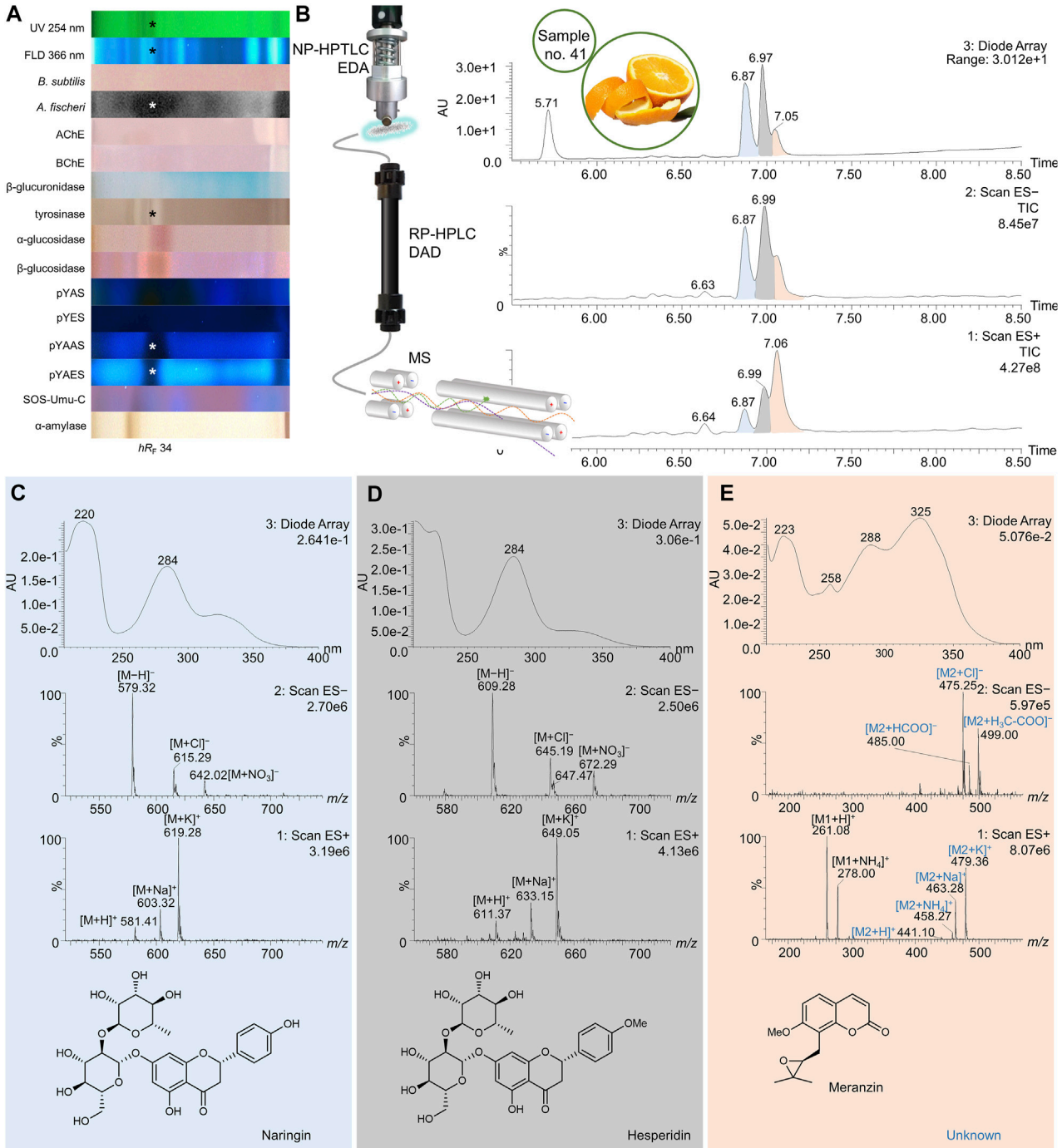


FIGURE 7 | NP-HPTLC–UV/Vis/FLD–EDA–heart-cut RP-HPLC–DAD–HESI-MS analysis of orange peel no. 41. NP-HPTLC–UV/Vis/FLD–EDA analysis as in **Figure 3 (A)**, followed by heart-cut elution of the bioactive zone at hR_F 34 (marked*) directly out of the pYAES bioautogram and online desalting to obtain the RP-HPLC–DAD–HESI-MS chromatograms **(B)** and respective UV and mass spectra of the peaks at RT 6.87 min **(C)**, 6.98 min **(D)**, and 7.05 min **(E)**.

eucalyptus (no. 9, hR_F 5 or 89), ginseng (no. 15, hR_F 96), guarana (no. 16, hR_F 60), kola (no. 31, hR_F 73), orange peel (no. 41, hR_F 25 or 32), licorice (no. 55, hR_F 27), thyme (no. 57, hR_F 24 or 50), and lemon verbena (no. 68, hR_F 99), the possibly antiandrogenic zones were also investigated using an overlayed area of the fluorescent 4-methylumbelliferone (**Supplementary Figure S4**) to exclude any false-positive response as observed for the physicochemical fluorescence reduction by pigments or dyes (Klingelhöfer et al., 2020). Seven botanical samples (nos. 6, 15, 16, 31, 41, 55, and 68) showed truly antiandrogenic activities. The same verification test was

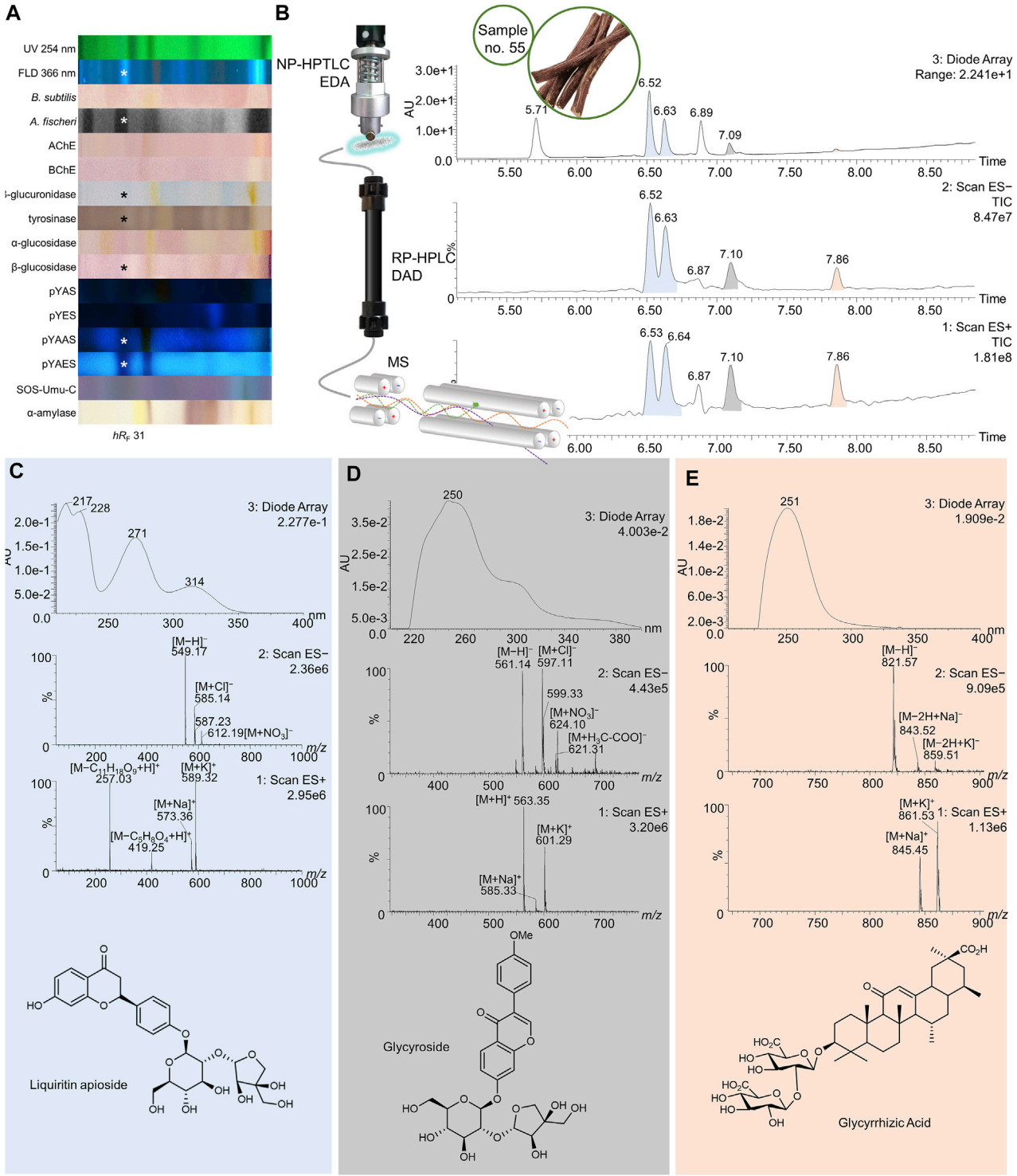
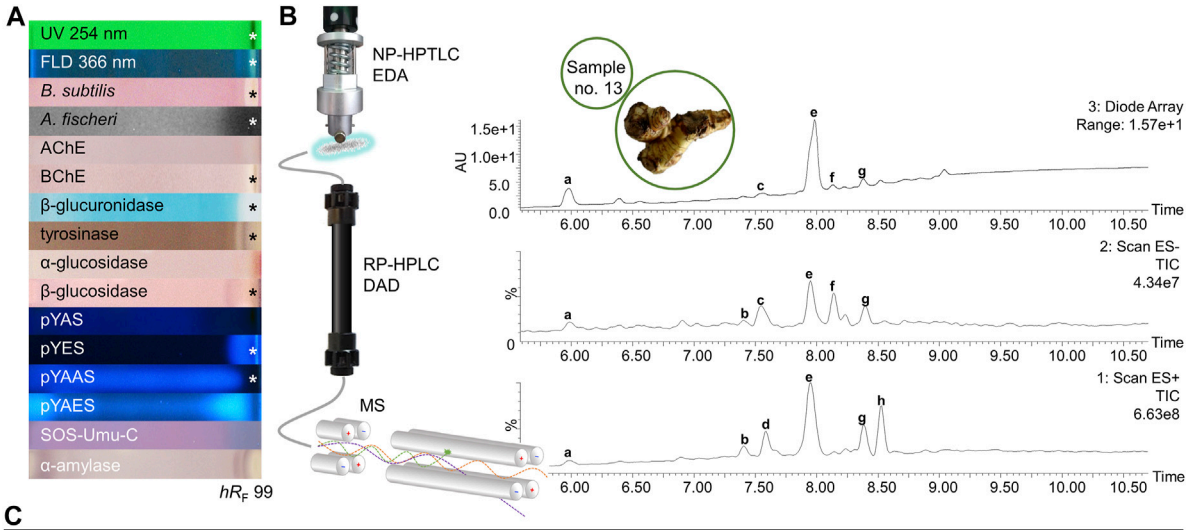


FIGURE 8 | NP-HPTLC–UV/Vis/FLD–EDA–heart-cut RP-HPLC–DAD–HESI-MS analysis of licorice no. 55. NP-HPTLC–UV/Vis/FLD–EDA analysis as in **Figure 4 (A)**, followed by heart-cut elution of the bioactive zone at hR_F 31 (marked*) directly out of the pYAES bioautogram and online desalting to obtain the RP-HPLC–DAD–HESI-MS chromatograms **(B)** and respective UV and mass spectra of the peaks at RT 6.53/6.64 min **(C)**, 7.10 min **(D)**, and 7.86 min **(E)**.

run for possible antiestrogens in galangal (no. 13, hR_F 99), guarana (no. 16, hR_F 72 or 91), garlic (no. 30, hR_F 99), kola (no. 31, hR_F 99), orange peel (no. 41, hR_F 34), licorice (no. 55, hR_F 31 or 43), thyme (no. 57, hR_F 31), grape seeds/leaves (nos. 58/61, hR_F 48, 61 or 92), and lemon verbena (no. 68, hR_F 99). Seven botanicals (nos. 16, 30, 41, 55, 57, 58, and 68) revealed true antiestrogenic properties



C

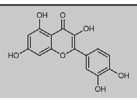
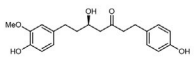
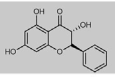
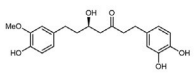
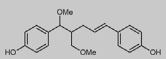
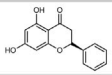
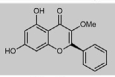
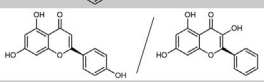
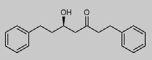
Signal	Tentative assignment	Structure	RT [min]	λ_{max} [nm]	*m/z*	Mass signal
a	quercetin		5.99	237, 284	301 320 325 341	$[M-H]^-$ $[M+NH_4]^+$ $[M+Na]^+$ $[M+K]^+$
b	5-hydroxy-1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone		7.40	-	343 379 389 403 345 362 367 383	$[M-H]^-$ $[M+Cl]^-$ $[M+HCOO]^-$ $[M+H_3C\text{-}COO]^-$ $[M+H]^+$ $[M+NH_4]^+$ $[M+Na]^+$ $[M+K]^+$
c	pinobanksin		7.54	236, 289	271	$[M-H]^-$
d	5-hydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone		7.59	-	359 395 405 419 361 378 383 399	$[M-H]^-$ $[M+Cl]^-$ $[M+HCOO]^-$ $[M+H_3C\text{-}COO]^-$ $[M+H]^+$ $[M+NH_4]^+$ $[M+Na]^+$ $[M+K]^+$
e	(4*E*)-1,5-bis(4-hydroxyphenyl)-1-methoxy-2-(methoxymethyl)-4-pentene		7.94	221, 280	327 363 329 346 351 367	$[M-H]^-$ $[M+Cl]^-$ $[M+H]^+$ $[M+NH_4]^+$ $[M+Na]^+$ $[M+K]^+$
f	pinocembrin		8.14	237, 289	255 257 279	$[M-H]^-$ $[M+H]^+$ $[M+Na]^+$
g	galangin-3-methyl ether		8.37	236	283 285	$[M-H]^-$ $[M+H]^+$
g	apigenin/galangin		8.37	235	269 271	$[M-H]^-$ $[M+H]^+$
h	dihydroyashabushiketol		8.52	-	283 300 305 321	$[M+H]^+$ $[M+NH_4]^+$ $[M+Na]^+$ $[M+K]^+$

FIGURE 9 | NP-HPTLC–UV/Vis/FLD–EDA–heart-cut RP-HPLC–DAD–HESI-MS analysis of galangal no. 13. NP-HPTLC–UV/Vis/FLD–EDA analysis as in **Figure 2 (A)**, followed by heart-cut elution of the bioactive zone at hR_F 99 (marked*) directly out of the BChE autogram and online desalting to obtain the RP-HPLC–DAD–HESI-MS chromatograms **(B)** and respective UV and mass spectral data of the peaks a–h **(C)**.

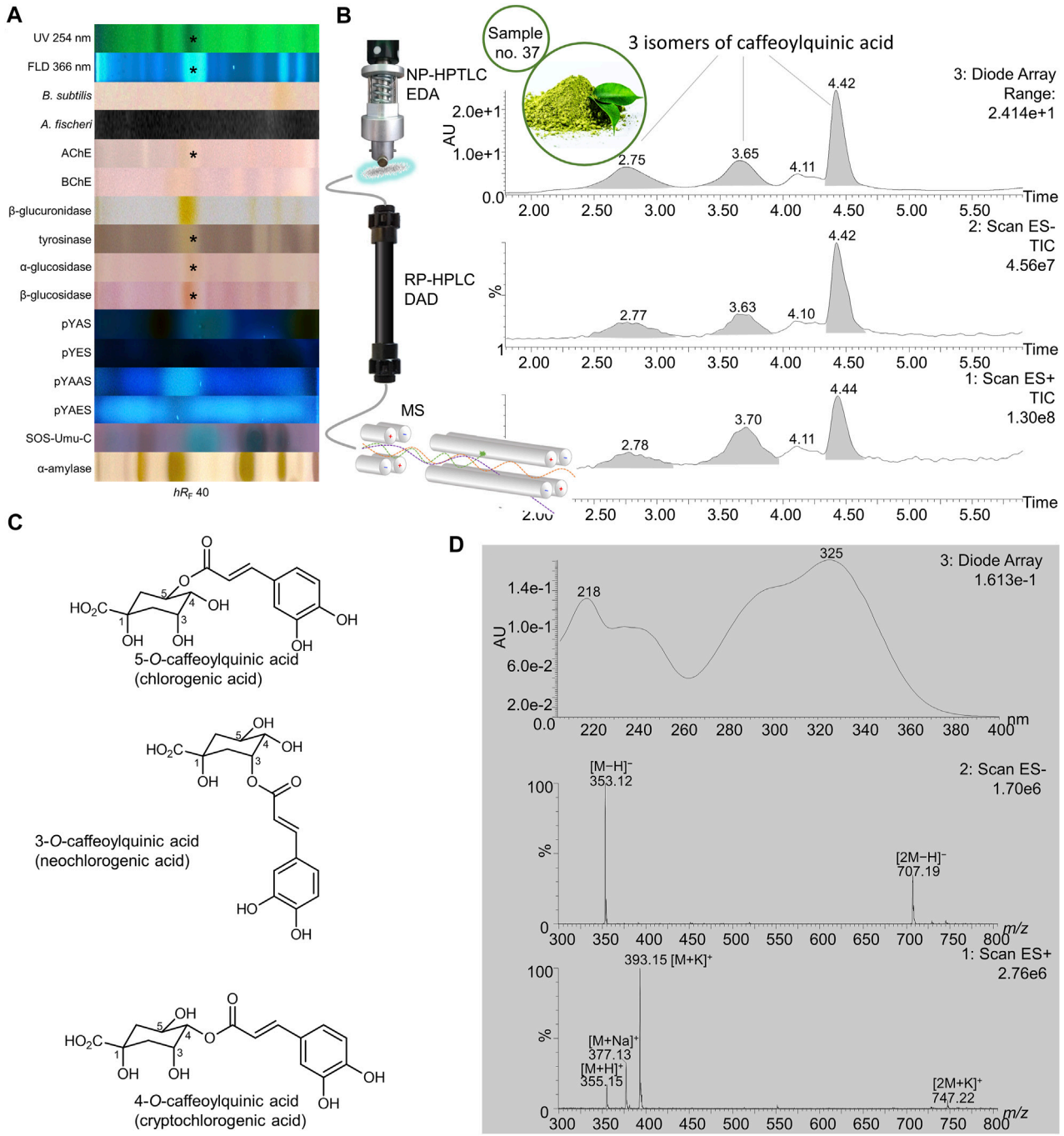


FIGURE 10 | NP-HPTLC–UV/Vis/FLD–EDA–heart-cut RP-HPLC–DAD–HESI-MS analysis of yerba mate green no. 37. NP-HPTLC–UV/Vis/FLD–EDA analysis as in **Figure 3 (A)**, followed by heart-cut elution of the bioactive zone at hR_F 40 (marked*) directly out of the BChE autogram and online desalting to obtain the RP-HPLC–DAD–HESI-MS chromatograms **(B)** and respective UV and mass spectra for the peaks at RT 2.78, 3.70, and 4.44 min, which were assigned to the isomeric structures of caffeoylquinic acid with the most predominant depicted, **(C)** as all three peaks provided the same signals **(D)**.

(**Supplementary Figure S5**). In the antiestrogenic assay, synergistic effects were evident. The overlapping 17-β-estradiol area (10 pg/70 mm area) is partially enhanced on sample tracks, apparent as a cotton swab shape if compared to the solvent blank (**Figures 2–4N**). This observation revealed synergistic effects between distinct botanical ingredients in most samples (except for nos. 7, 29, and 31) and 17-β-estradiol, resulting in fortified estrogenic activities (**Figure 6**). This overlapped experimental setup can be transferred to all other assays to identify synergy. Such a

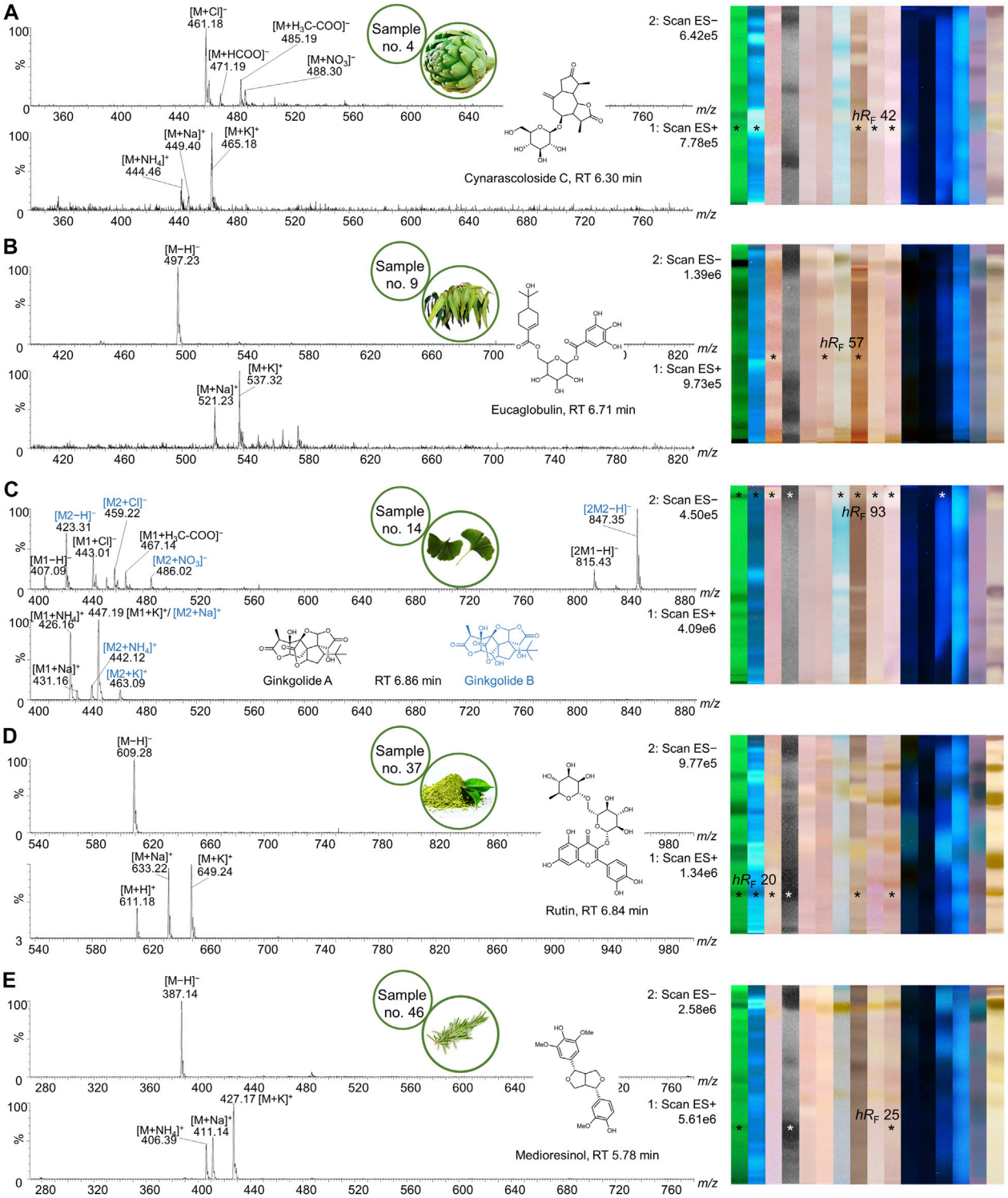


FIGURE 11 | Mass spectra of multipotent bioactive zones. NP-HPTLC–UV/Vis/FLD–EDA analysis as in **Figures 2–4**, followed by heart-cut elution of the multipotent bioactive zones of artichoke **(A)**, eucalyptus **(B)**, ginkgo **(C)**, yerba mate green **(D)**, and rosemary **(E)**. Pure mass spectra of respective HPLC peaks are shown with their associated structural formula and respective HPTLC fingerprints (bioactive zones marked*).

TABLE 2 | NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
2	Horehound, white	90	7.38	—	431	$[M–H]^-$	apigenin-*O*-glucoside	Amessis-Ouchemoukh et al. (2014)	X	X	X	X	X	X					X
					467	$[M + Cl]^-$													
					455	$[M + Na]^+$													
					471	$[M + K]^+$													
4	Artichoke	42	3.27/ 3.91/ 4.43	234, 322	353	$[M–H]^-$	**chlorogenic acid**[b]	Rejeb et al. (2020), Morlock et al. (2021a)			**X**	**X**		**X**					
					355	$[M + H]^+$													
					372	$[M + NH_4]^+$													
					377	$[M + Na]^+$													
					393	$[M + K]^+$													
			6.30	—	461	$[M + Cl]^-$	cynarascoloside C	Farag et al. (2013)			X	X	X	X					
					471	$[M + HCOO]^-$													
					485	$[M + H_3C\text{-}COO]^-$													
					444	$[M + NH_4]^+$													
					449	$[M + Na]^+$													
					465	$[M + K]^+$													
			6.71	232, 276	519	$[M–H]^-$	unknown caffeic acid conjugate	Schütz et al. (2004); El Senousy et al. (2014)			X	X	X	X					
					555	$[M + Cl]^-$													
					538	$[M + NH_4]^+$													
					543	$[M + Na]^+$													
					559	$[M + K]^+$													
		58	6.79	240	447	$[M–H]^-$	luteolin-7-*O*-glucoside (cynaroside)	Farag et al. (2013)		X	X	X	X						
					483	$[M + Cl]^-$													
		82	6.84	238, 280	433	$[M–H]^-$	naringenin-7-*O*-glucoside	Schütz et al. (2004)			X		X	X					
					469	$[M + Cl]^-$													
					496	$[M + NO_3]^-$													
9	*Eucalyptus*	57	6.71	222, 261	497	$[M–H]^-$	eucaglobulin	Boulekbache-Makhlouf et al. (2010)	X	X	X		X	X					
					521	$[M + Na]^+$													
					537	$[M + K]^+$													
			6.76	251, 352	447	$[M–H]^-$	methyl ellagic acid pentose	Boulekbache-Makhlouf et al. (2010); Santos et al. (2011)	X	X	X		X	X					
					449	$[M + H]^+$													
					466	$[M + NH_4]^+$													
					471	$[M + Na]^+$													
					487	$[M + K]^+$													
		78	7.54	240	519	$[M–H]^-$	cypellocarpin C	Boulekbache-Makhlouf et al. (2010)			X		X						
		90	5.23/ 6.18	—	185	$[M + H]^+$	methyl gallate	Santos et al. (2011)	X	X	X	X	X	X					
					202	$[M + NH_4]^+$													
					207	$[M + Na]^+$													
					223	$[M + K]^+$													
12	Fruit tea, red	41	3.74	—	353	$[M–H]^-$	**chlorogenic acid**[b]				**X**	**X**		**X**					
		46	3.22/ 3.91/ 4.58	233, 326	353	$[M–H]^-$	**chlorogenic acid**[b]				**X**	**X**		**X**					
					355	$[M + H]^+$													
					372	$[M + NH_4]^+$													
					377	$[M + Na]^+$													
					393	$[M + K]^+$													
13	Galangal	99	5.99	237, 284	301	$[M–H]^-$	quercetin	Zhou et al. (2018)	X	X	X	X	X	X		X	X		
					320	$[M + NH_4]^+$													
					325	$[M + Na]^+$													
					341	$[M + K]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
			7.40	—	343	$[M–H]^-$	5-hydroxy-1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone	Zhou et al. (2018)	X	X	X	X	X	X		X	X		
					379	$[M + Cl]^-$													
					389	$[M + HCOO]^-$													
					403	$[M + H_3C\text{-}COO]^-$													
					345	$[M + H]^+$													
					362	$[M + NH_4]^+$													
					367	$[M + Na]^+$													
					383	$[M + K]^+$													
			7.54	236, 289	271	$[M–H]^-$	**pinobanksin**	Zhou et al. (2018)			**X**	**X**		**X**					
			7.59	—	359	$[M–H]^-$	5-hydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone	Zhou et al. (2018)	X	X	X	X	X	X		X	X		
					395	$[M + Cl]^-$													
					405	$[M + HCOO]^-$													
					419	$[M + H_3C\text{-}COO]^-$													
					361	$[M + H]^+$													
					378	$[M + NH_4]^+$													
					383	$[M + Na]^+$													
					399	$[M + K]^+$													
			7.94	221, 280	327	$[M–H]^-$	(4 E)-1,5-bis(4-hydroxyphenyl)-1-methoxy-2-(methoxymethyl)-4-pentene	Zhou et al. (2018)	X	X	X	X	X	X		X	X		
					363	$[M + Cl]^-$													
					329	$[M + H]^+$													
					346	$[M + NH_4]^+$													
					351	$[M + Na]^+$													
					367	$[M + K]^+$													
			8.14	237, 289	255	$[M–H]^-$	pinocembrin	Zhou et al. (2018)	X	X	X	X	X	X		X	X		
					257	$[M + H]^+$													
					279	$[M + Na]^+$													
			8.37	236	283	$[M–H]^-$	galangin-3-methyl ether	Zhou et al. (2018)	X	X	X	X	X	X		X	X		
					285	$[M + H]^+$													
			8.37	235	269	$[M–H]^-$	**galangin**	Krüger et al. (2017); Zhou et al. (2018)	X	X	X	X	**X**	**X**		X	X		
					271	$[M + H]^+$													
			8.52	—	283	$[M + H]^+$	dihydroyashabushiketol	Zhou et al. (2018)	X	X	X	X	X	X		X	X		
					300	$[M + NH_4]^+$													
					305	$[M + Na]^+$													
					321	$[M + K]^+$													
14	Ginkgo	46	4.03	230, 267	344	$[M + NH_4]^+$	bilobalide	Mauri et al. (1999), Niu et al. (2017)					X	X					X
					349	$[M + Na]^+$													
					365	$[M + K]^+$													
		93	6.42	—	439	$[M–H]^-$	ginkgolide C	Chen et al. (2005); Ding et al. (2006); Niu et al. (2017)	X	X		X	X	X			X		
					475	$[M + Cl]^-$													
					458	$[M + NH_4]^+$													
					463	$[M + Na]^+$													
					479	$[M + K]^+$													
			6.86	—	407	$[M–H]^-$	**ginkgolide A**	Wang et al. (2016), Niu et al. (2017)	X	X		**X**	X	X			X		
					443	$[M + Cl]^-$													
					453	$[M + HCOO]^-$													
					467	$[M + H_3C\text{-}COO]^-$													
					815	$[2M–H]^-$													
					426	$[M + NH_4]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
					431	$[M + Na]^+$													
					447	$[M + K]^+$													
			6.86	—	423	$[M–H]^-$	**ginkgolide B**	Wang et al. (2016), Niu et al. (2017)	X	X		**X**	X	X			X		
					459	$[M + Cl]^-$													
					847	$[2M–H]^-$													
					442	$[M + NH_4]^+$													
					463	$[M + K]^+$													
15	Ginseng	16	7.60	243	836	$[M + Cl]^-$	ginsenoside Rg1/Rf	Du et al. (2018); Wilson and Sander (2018)					X				X		X
					846	$[M + HCOO]^-$													
					860	$[M + H_3C\text{-}COO]^-$													
					824	$[M + Na]^+$													
					840	$[M + K]^+$													
		24	8.08	—	800	$[M–H]^-$	ginsenoside Rg1/Rf	Du et al. (2018), Wilson and Sander (2018)					X				X		
					836	$[M + Cl]^-$													
					846	$[M + HCOO]^-$													
					860	$[M + H_3C\text{-}COO]^-$													
					824	$[M + Na]^+$													
					840	$[M + K]^+$													
16	Guarana	91	5.12/ 5.98	203, 279	289	$[M–H]^-$	**(epi)catechin**	da Silva et al. (2017), Morlock et al. (2021b)		X			X	**X**			X	**X**	
					325	$[M + Cl]^-$													
					291	$[M + H]^+$													
					329	$[M + K]^+$													
			4.23	205, 281	577	$[M–H]^-$	B-type procyanidin dimer	da Silva et al. (2017)		X			X	X			X	X	
					579	$[M + H]^+$													
					601	$[M + Na]^+$													
					617	$[M + K]^+$													
19	*Hibiscus*	45	4.57	234, 322	353	$[M–H]^-$	**chlorogenic acid**[b]				**X**	**X**	X	**X**					
22	Elder flower	40	3.80/ 4.67	233, 326	353	$[M–H]^-$	**chlorogenic acid**[b]				**X**	**X**		**X**					
					355	$[M + H]^+$													
					372	$[M + NH_4]^+$													
					377	$[M + Na]^+$													
					393	$[M + K]^+$													
24	Hop	92	7.05	264, 347	447	$[M–H]^-$	kaempferol-3-*O*-glucoside (astragalin)	Önder et al. (2013)	X	X			X						
					449	$[M + H]^+$													
					471	$[M + Na]^+$													
					487	$[M + K]^+$													
		94	7.83	254, 324	317	$[M–H]^-$	cohulupone	Önder et al. (2013), Sommella et al. (2018)	X	X	X	X	X	X		X			
					319	$[M + H]^+$													
					336	$[M + NH_4]^+$													
					341	$[M + Na]^+$													
					357	$[M + K]^+$													
25	Ginger	94	8.06	297	293	$[M–H]^-$	6-gingerol	Krüger et al. (2018)	X	X	X	X	X			X			
					295	$[M + H]^+$													
					312	$[M + NH_4]^+$													
					317	$[M + Na]^+$													
					333	$[M + K]^+$													
28	Chamomile	50	6.95	237, 266, 335	431	$[M–H]^-$	apigenin-7-*O*-glucoside	Lin and Harnly, (2012)					X	X					
					433	$[M + H]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
					455	$[M + Na]^+$													
					471	$[M + K]^+$													
		63	7.51	241,	473	$[M–H]^-$	apigenin-7-*O*-(2″-*O*-acetylglucoside)	Lin and Harnly, (2012)					X			X			
				257, 331	475	$[M + H]^+$													
					497	$[M + Na]^+$													
					513	$[M + K]^+$													
33	Caraway	43	7.04	—	447	$[M–H]^-$	luteolin-7-*O* glucoside						X	X					
					471	$[M + Na]^+$													
					487	$[M + K]^+$													
35	Marjoram	78	6.52	236, 278	433	$[M–H]^-$	quercetin arabinoside	Hossain et al. (2014)		X			X	X		X			
					469	$[M + Cl]^-$													
					452	$[M + NH_4]^+$													
					457	$[M + Na]^+$													
					473	$[M + K]^+$													
		93	6.20	219, 326	359	$[M–H]^-$	**rosmarinic acid**	Hossain et al. (2014), Çelik et al. (2017)		X	**X**	X	X	X					
					395	$[M + Cl]^-$													
					719	$[2M–H]^-$													
					378	$[M + NH_4]^+$													
					383	$[M + Na]^+$													
					399	$[M + K]^+$													
		97	7.85	235, 287	301	$[M–H]^-$	hesperetin/**quercetin**	Hossain et al. (2014); Erenler et al. (2016)	X	X	**X**	X	X	**X**					
					337	$[M + Cl]^-$													
					303	$[M + H]^+$													
37	Yerba mate green	6	3.90	—	343	$[M + H]^+$	dicaffeic acid	Souza et al. (2011)	X	X	X	X	X	X					
					365	$[M + Na]^+$													
					381	$[M + K]^+$													
			3.90	—	452	$[M + NH_4]^+$	quercetin arabinoside	Souza et al. (2011)	X	X	X	X	X	X					
					457	$[M + Na]^+$													
					473	$[M + K]^+$													
		14	7.07	—	482	$[M + NH_4]^+$	quercetin glucoside	Bravo et al. (2007); Souza et al. (2011), Mateos et al. (2018)	X	X	X	X	X	X					
					487	$[M + Na]^+$													
					503	$[M + K]^+$													
			4.94/ 5.32	236, 324	341	$[M–H]^-$	dicaffeic acid	Bravo et al. (2007); Souza et al. (2011)	X	X	X	X	X	X					
					364	$[M + Na]^+$													
					381	$[M + K]^+$													
		20	6.84	256, 356	609	$[M–H]^-$	**rutin**	Krüger et al. (2017), Mateos et al. (2018)	X	X	**X**	X	X	**X**					
					611	$[M + H]^+$													
					633	$[M + Na]^+$													
					649	$[M + K]^+$													
		40	2.75/ 3.65/ 4.42	217, 324	353	$[M–H]^-$	**chlorogenic acid**[b]	Mateos et al. (2018), Morlock et al. (2021a)	X	X	**X**	**X**	X	**X**					
					355	$[M + H]^+$													
					372	$[M + NH_4]^+$													
					377	$[M + Na]^+$													
					393	$[M + K]^+$													
		47	5.11	236, 322	367	$[M–H]^-$	feruloylquinic acid	Bravo et al., 2007, Souza et al. (2011), Mateos et al. (2018)		X		X	X	X					
					369	$[M + H]^+$													
					391	$[M + Na]^+$													
					407	$[M + K]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
		85	6.20/ 6.39/ 6.48	220, 240, 327	515 517 539 555	$[M–H]^-$ $[M + H]^+$ $[M + Na]^+$ $[M + K]^+$	dicaffeoylquinic acid	Krüger et al. (2017), Mateos et al. (2018)			X	X	X	X					
38	Lemon balm	92	6.21	219, 326	359 395 719 361 378 383 399	$[M–H]^-$ $[M + Cl]^-$ $[2M–H]^-$ $[M + H]^+$ $[M + NH_4]^+$ $[M + Na]^+$ $[M + K]^+$	**rosmarinic acid**	Krüger et al. (2017), Yilmaz (2020)		X	**X**	X	X	X					
41	Orange peel	21	5.74	229, 278	390 395 411	$[M + NH_4]^+$ $[M + Na]^+$ $[M + K]^+$	sinensetin/tangeretin	Anagnostopoulou et al. (2005); Li et al. (2006)		X	X	X	X	X					
			6.83	231,283	593 629 595 617 633	$[M–H]^-$ $[M + Cl]^-$ $[M + H]^+$ $[M + Na]^+$ $[M + K]^+$	didymin	Anagnostopoulou et al. (2005)		X	X	X	X	X					
			7.06	227, 268, 339	577 613 579 601 617	$[M–H]^-$ $[M + Cl]^-$ $[M + H]^+$ $[M + Na]^+$ $[M + K]^+$	apigenin-7-*O*-rutinoside (isorhoifolin)	Anagnostopoulou et al. (2005)		X	X	X	X	X					
			7.06	227, 268, 339	607 643 609 631 647	$[M–H]^-$ $[M + Cl]^-$ $[M + H]^+$ $[M + Na]^+$ $[M + K]^+$	diosmin	Anagnostopoulou et al. (2005)		X	X	X	X	X					
		34	6.63	232, 280	595 631 658 597 619 635	$[M–H]^-$ $[M + Cl]^-$ $[M + NO_3]^-$ $[M + H]^+$ $[M + Na]^+$ $[M + K]^+$	**eriocitrin**	Manthey and Grohmann (1996), Anagnostopoulou et al. (2005)			**X**			X			X	X	
			6.87	220, 284	579 615 642 581 603 619	$[M–H]^-$ $[M + Cl]^-$ $[M + NO_3]^-$ $[M + H]^+$ $[M + Na]^+$ $[M + K]^+$	**naringin**	Anagnostopoulou et al. (2005); Sawalha et al. (2009), Puranik et al. (2019)			X			X			X	**X**	
			6.98	284	609 645 672 611 633 649	$[M–H]^-$ $[M + Cl]^-$ $[M + NO_3]^-$ $[M + H]^+$ $[M + Na]^+$ $[M + K]^+$	**hesperidin**	Anagnostopoulou et al. (2005), Sawalha et al. (2009), Puranik et al. (2019)			X			X			X	**X**	

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
		[a]	7.05	—	261	$[M + H]^+$	meranzin	Dugo et al. (2000)			X			X			X	X	
					278	$[M + NH_4]^+$													
		92	8.23	249,	403	$[M + H]^+$	hexamethoxyflavone (nobiletin)	Anagnostopoulou et al. (2005), Li et al. (2006)			X		X		X			X	
				269, 335	425	$[M + Na]^+$													
					441	$[M + K]^+$													
					827	$[2M + Na]^+$													
			8.44	—	373	$[M + H]^+$	sinensetin/tangeretin/	Anagnostopoulou et al. (2005), Li et al. (2006)			X		X		X			X	
					395	$[M + Na]^+$													
					411	$[M + K]^+$													
42	Oregano	32[a]	2.88	219, 270	304	$[M + NH_4]^+$	luteolin/kaempferol	Hossain et al. (2010); Vallverdú-Queralt et al. (2014)		X			X	X					
					309	$[M + Na]^+$													
					325	$[M + K]^+$													
		62	7.38	241	331	$[M–H]^-$	carnosic acid	Hossain et al. (2010)		X	X	X	X						
					367	$[M + Cl]^-$													
					377	$[M + HCOO]^-$													
					391	$[M + H_3C\text{-}COO]^-$													
					333	$[M + H]^+$													
					350	$[M + NH_4]^+$													
					355	$[M + Na]^+$													
					371	$[M + K]^+$													
		81	5.81/	—	379	$[M + Cl]^-$	rosmadial	Hossain et al. (2010)	X		X								
			6.20		367	$[M + Na]^+$													
					383	$[M + K]^+$													
		93	5.99	238	331	$[M + H]^+$	carnosol	Hossain et al. (2010)	X	X	X	X	X	X					
					353	$[M + Na]^+$													
					369	$[M + K]^+$													
			7.97	221, 268	331	$[M–H]^-$	carnosic acid	Hossain et al. (2010)	X	X	X	X	X	X					
					367	$[M + Cl]^-$													
					333	$[M + H]^+$													
					371	$[M + K]^+$													
		99	7.51	238, 279	271	$[M–H]^-$	**naringenin**	Krüger et al. (2017), Mbachu et al. (2020)	X	X	**X**		X			X			
			7.79	239	269	$[M–H]^-$	**apigenin**	Hossain et al. (2010), Mbachu et al. (2020)	X	X	**X**		**X**			X			
					271	$[M + H]^+$													
			7.79	239	315	$[M–H]^-$	3-*O*-methylquercetin **(isorhamnetin)**	Hossain et al. (2010)	X	X	**X**		X			X			
					317	$[M + H]^+$													
					339	$[M + Na]^+$													
					355	$[M + K]^+$													
			8.01	221, 268	313	$[M–H]^-$	cirsimaritin	Hossain et al. (2010), Krüger et al. (2017)	X	X	X		X			X			
					315	$[M + H]^+$													
					337	$[M + Na]^+$													
					353	$[M + K]^+$													
44	Peppermint	20	7.05	249, 344	607	$[M–H]^-$	diosmin	Fecka et al. (2004)		X	X			X					
					609	$[M + H]^+$													
					631	$[M + Na]^+$													
					647	$[M + K]^+$													
		26	7.00	267,	577	$[M–H]^-$	apigenin-7-*O*-rutinoside (isorhoifolin)	Hawrył, (2014)		X	X	X	X						
				267, 339	613	$[M + Cl]^-$													
					579	$[M + H]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
					601	$[M + Na]^+$													
					617	$[M + K]^+$													
		93	6.23	235, 286	359	$[M–H]^-$	**rosmarinic acid**	Fecka et al. (2004)	X	X	X	X	X	X					
					395	$[M + Cl]^-$													
					383	$[M + Na]^+$													
					399	$[M + K]^+$													
			6.99	236	577	$[M–H]^-$	apigenin-7-*O*-rutinoside (isorhoifolin)	Hossain et al. (2010)	X	X	**X**	X	X	X					
					613	$[M + Cl]^-$													
					640	$[M + NO_3]^-$													
					579	$[M + H]^+$													
					601	$[M + Na]^+$													
					617	$[M + K]^+$													
46	Rosemary	25	4.89	232, 269	337	$[M + Na]^+$	cirsimaritin	Ezzat et al. (2016), Krüger et al. (2017), Pérez-Mendoza et al. (2020)		X	X	X	X	X					
					353	$[M + K]^+$													
			5.44	226, 285	362	$[M + NH_4]^+$	rosmadial	Mena et al. (2016), Pérez-Mendoza et al. (2020)		X	X	X	X	X					
					367	$[M + Na]^+$													
					383	$[M + K]^+$													
			5.78	224, 263	387	$[M–H]^-$	medioresinol	Mena et al. (2016)		X	X	X	X	X					
					406	$[M + NH_4]^+$													
					411	$[M + Na]^+$													
					427	$[M + K]^+$													
		[a]	6.06	233	315	$[M–H]^-$	3-*O*-methylquercetin (isorhamnetin)	Mena et al. (2016)		X	X	X	X	X					
					351	$[M + Cl]^-$													
					361	$[M + HCOO]^-$													
					375	$[M + H_3C\text{-}COO]^-$													
					317	$[M + H]^+$													
					339	$[M + Na]^+$													
					355	$[M + K]^+$													
			6.13	235	499	$[M + Cl]^-$	quercetin-3-*O*-hexoside (isoquercitrin)	Hossain et al. (2010)		X	X	X	X	X					
					503	$[M + K]^+$													
			6.99	236	577	$[M–H]^-$	apigenin-7-*O*-rutinoside (isorhoifolin)	Mena et al. (2016)		X	X	X	X	X					
					613	$[M + Cl]^-$													
					579	$[M + H]^+$													
					601	$[M + Na]^+$													
					617	$[M + K]^+$													
		41	4.05/ 5.05	220, 263	299	$[M–H]^-$	diosmetin/6-*O*-methylapigenin (hispidulin)	Pérez-Mendoza et al. (2020)			X	X	X						
					335	$[M + Cl]^-$													
					301	$[M + H]^+$													
					318	$[M + NH_4]^+$													
					323	$[M + Na]^+$													
					339	$[M + K]^+$													
		62[a]	6.48	—	319	$[M + Cl]^-$	7-*O*-methylapigenin (genkwanin)/ acacetin	Ezzat et al. (2016), Mena et al. (2016), Krüger et al. (2017), Pérez-Mendoza et al. (2020)		X	X	X	X	X					
					302	$[M + NH_4]^+$													
					307	$[M + Na]^+$													
					323	$[M + K]^+$													
			6.89	236	461	$[M–H]^-$	luteolin-7-*O*-glucuronide	Mena et al. (2016), Pérez-Mendoza et al. (2020)		X	X	X	X	X					
					463	$[M + H]^+$													
					485	$[M + Na]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
					501	$[M + K]^+$													
			7.23	—	377	$[M + Cl]^-$	caffeic acid hexoside	Hossain et al. (2010)		X	X	X	X	X					
					401	$[M + H_3C\text{-}COO]^-$													
					365	$[M + Na]^+$													
					381	$[M + K]^+$													
		92	6.19	326	359	$[M–H]^-$	**rosmarinic acid**	Hossain et al. (2010), Mena et al. (2016), Pérez-Mendoza et al. (2020)	X	X	**X**	X	X	X					
					395	$[M + Cl]^-$													
					719	$[2M–H]^-$													
					378	$[M + NH_4]^+$													
					383	$[M + Na]^+$													
					399	$[M + K]^+$													
					743	$[2M + Na]^+$													
			8.06	—	331	$[M–H]^-$	carnosic acid	Ezzat et al. (2016), Mena et al. (2016), Pérez-Mendoza et al. (2020)	X	X	X	X	X	X					
					333	$[M + H]^+$													
					355	$[M + Na]^+$													
					371	$[M + K]^+$													
		99	6.79	—	287	$[M + H]^+$	luteolin	Mena et al. (2016)	X	X	X	X	X	X					
					309	$[M + Na]^+$													
					325	$[M + K]^+$													
48	Sea buckthorn	21	6.74/ 7.02	254, 354	623	$[M–H]^-$	isorhamnetin-3-*O*-rutoside (narcissin)/ isorhamnetin-3-glucoside-7-rhamnoside (brassidin)	Zheng et al. (2016)					X	X					
					659	$[M + Cl]^-$													
					669	$[M + HCOO]^-$													
					625	$[M + H]^+$													
					647	$[M + Na]^+$													
					663	$[M + K]^+$													
50	Yarrow	40	3.80	221, 325	353	$[M–H]^-$	**chlorogenic acid**[b]	Giorgi et al. (2009)	X	X	**X**	**X**	X	**X**					
					355	$[M + H]^+$													
					377	$[M + Na]^+$													
					393	$[M + K]^+$													
55	Licorice	31	6.52/ 6.63	217, 228, 271	549	$[M–H]^-$	**liquiritin apioside**	Wong et al. (2018)	X	X	**X**	X	X	X			X	**X**	
					585	$[M + Cl]^-$													
			6.52/ 6.63	218, 276	257	$[M + H]^+$	2′,4′,4′-trihydroxychalcone	Li et al. (2016)	X				X	X			X	X	
			7.10	250	561	$[M–H]^-$	glycyroside	Montero et al. (2016); Wong et al. (2018)	X				X	X			X	X	
					597	$[M + Cl]^-$													
					621	$[M + H_3C\text{-}COO]^-$													
					563	$[M + H]^+$													
					585	$[M + Na]^+$													
					601	$[M + K]^+$													
			7.85	251	821	$[M–H]^-$	**glycyrrhizic acid**	Kong et al. (2014); Li et al. (2016), Krüger et al. (2017)				X		X			X	**X**	
					843	$[M–2H + Na]^-$													
					859	$[M–2H + K]^-$													
		49	6.64	—	549	$[M–H]^-$	**liquiritin apioside**	Krüger et al. (2017), Wong et al. (2018)	X	X	**X**	X	X	X			X	**X**	
					257	$[M–C_5H_8O_4+H]^+$													
					419	$[M–C_{11}H_{18}O_9+H]^+$													
					573	$[M + Na]^+$													
					589	$[M + K]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
		62	7.15	249	695	$[M–H]^-$	licorice glycoside B/D1/D2	Wong et al. (2018)						X					X
					719	$[M + Na]^+$													
					735	$[M + K]^+$													
		77	6.49/ 7.15/ 7.24	234, 268, 372	417	$[M–H]^-$	(iso)liquiritin	Krüger et al. (2017), Wong et al. (2018)		X	X			X	X	X			X
					453	$[M + Cl]^-$													
					419	$[M + H]^+$													
					441	$[M + Na]^+$													
					457	$[M + K]^+$													
		93	7.17	218, 276	255	$[M–H]^-$	**(iso)liquiritigenin**	Kong et al. (2014), Boonmuen et al. (2016), Li et al. (2016), Montero et al. (2016)	X	X	X	X	X	X		**X**			
					257	$[M + H]^+$													
					279	$[M + Na]^+$													
			7.41	—	253	$[M–H]^-$	**daidzein**	Liu et al. (2001), Nomura et al. (2002), Cornwell et al. (2004)	X	X	X	X	X	X		**X**			
					255	$[M + H]^+$													
					277	$[M + Na]^+$													
					293	$[M + K]^+$													
			7.41	—	283	$[M–H]^-$	biochanin A	Liu et al. (2001)	X	X	X	X	X	X					
					285	$[M + H]^+$													
					307	$[M + Na]^+$													
					323	$[M + K]^+$													
			7.91	—	267	$[M–H]^-$	coumestrol	Cornwell et al. (2004)	X	X	X	X	X	X		X			
					269	$[M + H]^+$													
					291	$[M + Na]^+$													
					307	$[M + K]^+$													
56	Siberian ginseng	14	6.48	—	499	$[M + Cl]^-$	quercetin-3-*O*-galactoside (hyperoside)/quercetin-3-*O*-glucopyranoside (isoquercitrin)	Wang et al. (2019)	X	X		X	X	X		X			
					423	$[M + H_3C\text{-}COO]^-$													
					487	$[M + Na]^+$													
					503	$[M + K]^+$													
		36	2.81/ 3.68/ 4.45/ 4.66	217, 324	353	$[M–H]^-$	**chlorogenic acid**[b]	Wang et al. (2019)	X	X	**X**	**X**	X	**X**					
					355	$[M + H]^+$													
					372	$[M + NH_4]^+$													
					377	$[M + Na]^+$													
					393	$[M + K]^+$													
			5.40	—	439	$[M–H]^-$	akebonoic acid	Ge et al. (2017)	X	X	**X**	**X**	X	**X**					
					475	$[M + Cl]^-$													
					499	$[M + H_3C\text{-}COO]^-$													
					463	$[M + Na]^+$													
					479	$[M + K]^+$													
			7.38	—	469	$[M + Cl]^-$	naringenin-7-*O*-glucoside (prunin)	Kuźniewski et al. (2018)	X	X	X	X	X	X					
					479	$[M + HCOO]^-$													
					493	$[M + H_3C\text{-}COO]^-$													
					452	$[M + NH_4]^+$													
					457	$[M + Na]^+$													
					473	$[M + K]^+$													
			7.78	—	483	$[M + Cl]^-$	kaempferol-3-*O*-glucoside (astragalin)/ quercetin-3-*O*-rhamnoside (quercitrin)	Kuźniewski et al. (2018), Wang et al. (2019)	X	X	X	X	X	X					
					493	$[M + HCOO]^-$													
					507	$[M + H_3C\text{-}COO]^-$													
					466	$[M + NH_4]^+$													
					471	$[M + Na]^+$													
					487	$[M + K]^+$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
57	Thyme	22	6.62	235	557	$[M + Cl]^-$	rosmarinyl glucoside (rosmarinic acid-*O*-hexoside)	Vallverdú-Queralt et al. (2014)	X	X	X	X	X	X					
					540	$[M + NH_4]^+$													
					545	$[M + Na]^+$													
					561	$[M + K]^+$													
		31	4.36	—	355	$[M + H]^+$	**chlorogenic acid**[b]	Hossain et al. (2010), Vallverdú-Queralt et al. (2014)	X	X	**X**	**X**	X	**X**					
			5.78	—	387	$[M–H]^-$	medioresinol	Hossain et al. (2010)	X	X	X	X	X	X					
		61	7.36	237, 293	355	$[M + Na]^+$	carnosic acid	Hossain et al. (2010), Vallverdú-Queralt et al. (2014)	X	X	X	X	X	X					
					371	$[M + K]^+$													
		64[a]	6.46	—	302	$[M + NH_4]^+$	7-*O*-methylapigenin (genkwanin)/	Hossain et al. (2010)	X	X	X	X	X	X					
					307	$[M + Na]^+$	**acacetin**												
					323	$[M + K]^+$													
		75	7.15	238	344	$[M + NH_4]^+$	coumaroyl hexoside (coumaric acid-*O*-hexoside)	Vallverdú-Queralt et al. (2014)	X	X	X	X	X	X					
					349	$[M + Na]^+$													
					365	$[M + K]^+$													
		91	6.13	326	359	$[M–H]^-$	**rosmarinic acid**	Krüger et al. (2017)	X	X	**X**	X	X	X					
					395	$[M + Cl]^-$													
					361	$[M + H]^+$													
					378	$[M + NH_4]^+$													
					383	$[M + Na]^+$													
					399	$[M + K]^+$													
58	Grape seed	92	5.13	204, 279	289	$[M–H]^-$	**(epi)catechin**	Yilmaz and Toledo (2004)	X	X		X	X	**X**			X	**X**	
					325	$[M + Cl]^-$													
					291	$[M + H]^+$													
					329	$[M + K]^+$													
62–64	Hawthorn	41	6.14	234, 280	359	$[M–H]^-$	lariciresinol/isolariciresinol/	Huang et al. (2018), Rocchetti et al. (2020)	X	X	X		X	X					
					395	$[M + Cl]^-$	cyclolariciresinol												
			6.54	—	421	$[M + Cl]^-$	dimethylmatairesinol	Rocchetti et al. (2020)	X	X	X		X	X					
					409	$[M + Na]^+$													
					425	$[M + K]^+$													
			6.80/ 7.13	230, 268, 336	619	$[M–H]^-$	4‴-acetylvitexin-2″-*O*-rhamnoside	Krüger et al. (2017)	X	X	X		X	X					
					655	$[M + Cl]^-$													
					621	$[M + H]^+$													
					643	$[M + Na]^+$													
					659	$[M + K]^+$													
		58	4.70/ 5.32	234, 310	337	$[M–H]^-$	3-*p*-coumaroylquinic acid/4-*p*-coumaroylquinic acid/5-*p*-coumaroylquinic acid/	Rocchetti et al. (2020)			X		X	X					
					339	$[M + H]^+$													
					356	$[M + NH_4]^+$													
					361	$[M + Na]^+$													
					377	$[M + K]^+$													
			6.82	255, 355	463	$[M–H]^-$	isorhamnetin-7-*O*-rhamnoside	Rocchetti et al. (2020)			X		X	X					
					465	$[M + H]^+$													
					487	$[M + Na]^+$													
					503	$[M + K]^+$													
			7.64	—	417	$[M–H]^-$	syringaresinol/oleoside dimethylester	Rocchetti et al. (2020)			X		X	X					
					453	$[M + Cl]^-$													

(Continued on following page)

TABLE 2 | (*Continued*) NP-HPTLC-EDA-RP-HPLC-DAD-HESI-MS signals of bioactive zones and respective activity (X) in *B. subtilis* **(A)**, *A. fischeri* **(B)**, AChE/BChE **(C)**, α-/β-glucosidase **(D)**, β-glucuronidase **(E)**, tyrosinase **(F)**, pYAS **(G)**, pYES **(H)**, pYAAS **(I)**, pYAES **(J)** and α-amylase **(K)**. In blue: analytes and respective bioactivity, which were confirmed by a standard; in rose: rebutted ones.

No	Botanical	hR_F (±1)	RT [min]	UV λ_{max} [nm]	*m/z*	Mass signal	Tentative assignment	Literature	A	B	C	D	E	F	G	H	I	J	K
					463	$[M + HCOO]^-$													
					477	$[M + H_3C\text{-}COO]^-$													
					441	$[M + Na]^+$													
					457	$[M + K]^+$													
		98	7.24	—	286	$[M–H]^-$	cyanidin	Rocchetti et al. (2020)	X	X	X			X		X			
					288	$[M + H]^+$													
					326	$[M + K]^+$													
			7.60	—	197	$[M–H]^-$	syringic acid	Rocchetti et al. (2020)	X	X	X			X		X			
					199	$[M + H]^+$													
					221	$[M + Na]^+$													
					237	$[M + K]^+$													
			8.09	—	305	$[M–H]^-$	(+)-gallocatechin/(–)-epigallocatechin	Rocchetti et al. (2020)	X	X	X			X		X			
					341	$[M + Cl]^-$													
					307	$[M + H]^+$													
					329	$[M + Na]^+$													
					345	$[M + K]^+$													
			8.65		517	$[M–H]^-$	6″-*O*-malonylgenistin	Rocchetti et al. (2020)	X	X	X			X		X			
					541	$[M + Na]^+$													
					557	$[M + K]^+$													
			8.65	—	487	$[M–H]^-$	luteolin-6-C-glucoside/6″-*O*-acetylglycitin	Rocchetti et al. (2020)	X	X	X			X		X			
					489	$[M + H]^+$													
					506	$[M + NH_4]^+$													
					511	$[M + Na]^+$													
					527	$[M + K]^+$													
			9.27	-	471	$[M–H]^-$	crataegolic acid (maslinic acid)	Vierling et al. (2003)	X	X	X			X		X			
					495	$[M + Na]^+$													
					511	$[M + K]^+$													
67	Lemon peel	95	7.46	—	469	$[M–H]^-$	limonin	Baldi et al. (1995)		X			X	X	X	X			X
					505	$[M + Cl]^-$													
					515	$[M + HCOO]^-$													
					529	$[M + H_3C\text{-}COO]^-$													
					471	$[M + H]^+$													
					488	$[M + NH_4]^+$													
					493	$[M + Na]^+$													
					509	$[M + K]^+$													
68	Lemon verbena	42	6.51	—	639	$[M–H]^-$	β-OH-(iso)verbascoside	Bilia et al. (2008)	X	X	X	X	X	X				X	
					663	$[M + Na]^+$													
					679	$[M + K]^+$													
			6.66/ 6.82/ 6.93	222, 330	623	$[M–H]^-$	(iso)verbascoside	Bilia et al. (2008), Quirantes-Piné et al. (2010), Krüger et al. (2017)	X	X	X	X	X	X				X	
					642	$[M + NH_4]^+$													
					647	$[M + Na]^+$													
					663	$[M + K]^+$													

[a]*Standard eluted at another* hR_F, *value.*

[b]*Or neochlorogenic or cryptochlorogenic acid.*

synergistic effect detection strategy was reported here for the first time.

3.2.7 Compounds With Genotoxic Effects

In recent decades, there has been a steady trend away from industrial medical care towards phytotherapy based on medicinal herbs, which have been used in traditional medicine for years. The common belief that phytochemicals are gentler than synthetic medicines may prove to be a fallacy, as most toxic compounds originate in nature (Efferth and Kaina, 2011). The SOS-Umu-C assay is a reporter gene assay indicating genotoxicity based on a genetically modified test organism *Salmonella typhimurium* TA1535 [pSK1002]. Under genotoxic stress, the SOS-DNA repair mechanism is induced. The SOS-Umu response activates the *lacZ* gene, encoding for β-galactosidase, which enables substrate cleavage into detectable products (Meyer et al., 2020). As the analyzed botanicals are commonly used as spices, in herbal medicine, or as tea, no genotoxic substances were expected. Nevertheless, pyrrolizidine alkaloids are known for genotoxic properties and their occurrence in herbal formulations (Habs et al., 2017), *e.g.*, only a few micrograms per liter of tea (6 µg/L) (Mulder et al., 2015). The European Food and Safety Authority calculated a margin of exposure to 237 g/kg body weight per day for pyrrolizidine alkaloids and their *N*-oxides (Knutsen et al., 2017; Kaltner et al., 2020). No genotoxicity was detected as pink fluorescent zone for the 68 botanical extracts applied at 400 µg/band (**Figures 2–4O**). This bioassay was repeated to prove for the absence of genotoxic effects at a 2.5-fold (**Supplementary Figure S6**) and 12.5-fold higher amount applied, which latter at 5 mg/band was closest to overloading the chromatographic system (**Supplementary Figure S7**).

3.2.8 Compounds Inhibiting α-Amylase

In the context of hypoglycemic drugs from nature, not only the mentioned α- and β-glucosidase inhibitors, but also α-amylase inhibitors play a role in ethnopharmacological remedies with less toxic side effects. Hyperglycemic blood levels could be reduced by inhibiting α-amylases of the saliva and pancreas. The inhibition reduces the cleavage of starch into oligo- and disaccharides, and so the release of glucose molecules absorbed postprandially into the blood. Plant-derived flavones as luteolin present in celery, parsley, broccoli, carrot, peppers, cabbage, and apple peel can inhibit α-amylase (Bai et al., 2019). HPTLC coupled to α-amylase assay (**Figures 2–4P**) showed only a few inhibiting signals in white horehound (no. 2, hR_F 43, 83, 91, and 93), artichoke (no. 4, hR_F 94), fenugreek (no. 6, hR_F 81), ginkgo (no. 14, hR_F 46), licorice (no. 55, hR_F 59 and 68), and lemon peel (no. 67, hR_F 93 and 95). An α-amylase inhibitory activity has already been described for artichoke extracts from bracts (Turkiewicz et al., 2019), but it was not assigned to any single compound. In *Ginkgo biloba* extracts the compound sciadopitysin ($C_{33}H_{24}O_{10}$) was found to potentially inhibit α-amylase (Petersen et al., 2019).

3.3 Assigning Bioactivity to Single Compounds

In previous work, the samples were applied twice (as two sets). After plate cut, the assay was performed on one plate part, and the positions of bioactive zones were analogously marked on the other plate part for zone elution and transfer into the MS (Krüger et al., 2017). The challenge of this parallel handling was the accurate positioning of the elution head on the zone. Instead, the elution directly from the bioassay plate would avoid any possible mismatch. In addition, the orthogonal HPLC separation would solve coelution. Hence, zones of interest were heart-cut eluted directly out of the bioassay plate and transferred through an online desalting device to RP-HPLC–DAD–HESI-MS. This reduced the interfering salts and nutrients from the bioassay, separated possibly coeluting substances via the orthogonal chromatographic system, and added value via spectral and mass spectrometric data (Schreiner and Morlock, 2021). Through this straightforward comprehensive workflow, the 60 most bioactive compounds in the botanicals were assigned (**Table 2**). Several plant extracts out of the 68 botanicals contained multipotent compounds which were active in different assays. Among striking botanicals, such as galangal (no. 13), ginkgo (no. 14), yerba mate green (no. 37), orange peel (no. 41), licorice (no. 55), or Siberian ginseng (no. 56), four botanicals were exemplarily highlighted to demonstrate the targeted assignment of bioactive compounds (orange peel, **Figure 7**; licorice, **Figure 8**; galangal, **Figure 9**; yerba mate green, **Figure 10**). By transferring residual bioassay salts, different ion species were formed for a molecule, which was found to be helpful as it confirmed the assignment made several times. The adduct $[M+62]^-$ has not been described in HPTLC-MS literature so far, but appeared frequently during this study (**Figures 7C,D**, **Figures 8C,D**, **Figures 11A,C**). It was proposed to be the nitrate adduct. Nitrate is taken up by the root and transported via the xylem to leaves, shoots, and grains. If too much nitrate is available in the short term, which is the case as it is discussed as a global environmental challenge (Zhang et al., 2021), it passes through the cytoplasm into the vacuoles to be stored and thus can be found in botanicals (Dechorgnat et al., 2011). Consequently, it may also appear as a pronounced adduct in the mass spectrum.

3.3.1 Hormonal Antagonists in Orange Peel (No. 41)

For orange peel, manifold positive responses across the different assays were observed at hR_F 34 (**Figure 7A**, marked*). Using the pYEAS bioautogram, this zone was heart-cut eluted directly to RP-HPLC–DAD–HESI-MS. In the DAD chromatogram, three compound peaks were evident in the range of RT 6.87–7.07 min, apart from the 4-methylumbelliferone background signal at RT 5.71 min (**Figure 7B**). The corresponding mass spectral data led to the tentative identification of naringin (**Figure 7C**), hesperidin (**Figure 7D**), and meranzin (**Figure 7E**, coeluting with an unknown marked blue) and is discussed as follows. The total ion current (TIC) peak at RT 6.87 min revealed mass signals in the positive ionization mode at *m/z* 581 $[M + H]^+$, 603 $[M + Na]^+$, and 619 $[M + K]^+$. Corresponding mass signals in the negative ion mode were detected at *m/z* 579 $[M–H]^-$, 615 $[M + Cl]^-$, and 642 $[M + NO_3]^-$ (**Figure 7C**). The resulting neutral mass of 580 Da together with the absorption spectrum and maximal wavelengths (λ_{max} = 220 and 284 nm) suggested naringin, which was confirmed via co-chromatography against a bought standard (**Supplementary Figure S8E**). The extracted mass spectra of the TIC peak at RT

6.99 min showed positive ions at *m/z* 611 $[M + H]^+$, 633 $[M + Na]^+$, and 649 $[M + K]^+$ and negative ions at *m/z* 609 $[M–H]^-$, 645 $[M + Cl]^-$, and 672 $[M + NO_3]^-$ (**Figure 7D**). The absorption spectrum revealed a maximum wavelength at 284 nm. Based on this data, hesperidin was assumed. The third peak at RT 7.06 min had a major response in TIC-HESI$^+$ and a minor response in TIC-HESI$^-$ and DAD chromatograms. The UV absorbance spectrum and respective mass spectra suggested two different constituents. The HESI$^+$ mass signals at *m/z* 261 $[M1+H]^+$ and 278 $[M1+NH_4]^+$ potentially belong to the coumarin derivate meranzin (**Figure 7E**). The HESI$^+$ mass signals at *m/z* 441 $[M2+H]^+$, 458 $[M2+NH_4]^+$, 463 $[M2+Na]^+$, and 479 $[M2+K]^+$ and corresponding HESI$^-$ mass signals at *m/z* 475 $[M2+Cl]^-$, 485 $[M2+HCOO]^-$, and 499 $[M2+H_3C\text{-}COO]^-$ were assigned to a component with the neutral mass of 440 Da, which is not yet known in orange peel. The UV maxima at 258 and 325 nm provided additional evidence for meranzin (Fan et al., 2012). Other UV maxima at 223 and 288 nm were possibly induced by the unknown.

Citrus sinensis is rich in beneficial secondary metabolites and therefore traditionally used in the treatment of gastrointestinal malfunction, diseases of the upper respiratory tracts, or menstrual disorders (Favela-Hernández et al., 2016). Both naringin and hesperidin have been shown to bind to the antagonist pocket (3ERT) of hERα, and thereby cause an antiestrogenic effect (Puranik et al., 2019). This effect was verified for both via co-chromatography of standards and samples (**Supplementary Figure S8E**, **Supplementary Table S1**), whereby an estrogenic activity was not observed for the applied amount (4 µg/band). Furthermore, naringin was reported to slightly bind to the androgen receptor (Fang et al., 2003). The pronounced antiandrogenic effects observed in the pYAAS bioautogram confirmed this (**Figure 3M**, no. 41). Tyrosinase activity attributed to naringin (Itoh et al., 2009) and hesperidin (Zhang et al., 2007) has already been demonstrated. The only bioactivity reported for meranzin was no effect (Smyth et al., 2009) or a minor (Rosselli et al., 2007) antibacterial effect against *B. subtilis*. After NP-HPTLC–FLD comparison to a standard, meranzin was located at hR_F 99 (**Supplementary Figure S8G**), where antibacterial activity against *B. subtilis* and *A. fischeri* was detected in orange peel (no. 41). Hence, the assumption that meranzin was co-eluting with hesperidin and naringin at hR_F 34 was discarded.

3.3.2 Enzyme Inhibition and Endocrine Activity in Licorice (No. 55) and Galangal (No. 13)

In licorice (no. 55) a few bioactive analytes were found in the zone at hR_F 31 with antibacterial, tyrosinase, and β-glucuronidase inhibitory, antidiabetic, and endocrine-antagonistic properties (**Figure 8A**, marked*). These results illustrate the diverse pharmacological activities of this root, which has long been used in traditional medicine as remedy to treat gastrointestinal problems (β-glucuronidase inhibition) and respiratory infections (antibacterial activity). Moreover, *Glycyrrhiza glabra* extracts were subject of many pharmacological studies showing neuroprotective, antimicrobial, estrogenic and skin-whitening activity (Pastorino et al., 2018). The second chromatography produced five individual signals with pure mass spectra (**Figures 8B–E**). The mass spectral data extracted from the peaks at RT 6.52/6.63 min (colored blue) were identical, indicating a single analyte in different configurations (**Figure 8C**). The ESI$^-$ signals at *m/z* 549 $[M–H]^-$, 585 $[M + Cl]^-$, and 612 $[M + NO_3]^-$ were correlated to the highly plant-specific liquiritin apioside with a neutral mass of 550 Da. In positive ion mode, the signals at *m/z* 573 and 589 were identified as sodium and potassium adducts, respectively. The ESI$^+$ mass signals at *m/z* 257 $[M–C_{11}H_{18}O_9+H]^+$ and 419 $[M–C_5H_8O_4+H]^+$ could be assigned to fragments with a loss of carbohydrates. Additionally, the experimental UV absorbance spectra were consistent with the ones of liquiritin apioside found in literature (Wong et al., 2018). The isomeric isoliquiritin apioside probably caused the second peak at RT 6.63 min. ESI$^+$ mass signals at RT 7.10 min were *m/z* 563 $[M + H]^+$, 585 $[M + Na]^+$, and 601 $[M + K]^+$ (**Figure 8D**). (Iso)liquiritin apioside was found to inhibit capsaicin-induced cough, confirming its traditional use (Pastorino et al., 2018). In negative ion mode, signals were detected at *m/z* 561 $[M–H]^-$, 597 $[M + Cl]^-$, 621 $[M + H_3C\text{-}COO]^-$, and 624 $[M + NO_3]^-$. The less abundant DAD signal showed a UV absorption maximum at 250 nm. The spectral data could indicate glycyroside found in licorice root extracts (Montero et al., 2016), which is not known for any bioactivity. The third signal at RT 7.86 min (colored orange) was identified as glycyrrhizic acid against a standard (**Supplementary Figure S8E**). The ESI$^-$ and ESI$^+$ ions at m/z 821 $[M–H]^-$, 843 $[M–2H + Na]^-$, 859 $[M–2H + K]^-$, 845 $[M + H]^+$, and 861 $[M + K]^+$ originated from the neutral molecule of 822 Da. Lacking a π-electron system and conjugated double bonds, the UV absorbance spectrum showed background absorbance maxima at 251 nm. Glycyrrhizic acid was reported to have anti-inflammatory effects (Yu et al., 2015) similar to those of glucocorticoids (Pastorino et al., 2018), antitussic activity through increased tracheal mucus secretion (Sharma et al., 2018), and neuroprotective (Kao et al., 2009) properties.

Galangal extract (no. 13) responded in almost all assays at hR_F 99 (**Figure 9A**, marked*). After separating this zone with RP-HPLC-DAD-HESI-MS, multiple signals were observed (**Figure 9B**). The spectral details and tentative identifications are listed in the table below (**Figure 9C**). Traditionally, *Alpinia officinarum* is used against cold, which antibacterial effects were confirmed by the Gram-negative *A. fischeri* and Gram-positive *B. subtilis* bioassays. Other traditional applications were described for gynecological disorders, diabetes treatments, and skin washing (Abubakar et al., 2018). All these bioactivities were verified by the respective assays. To assign the bioactivity to one single component out of the coeluting substances from HPTLC, fractionation after column separation is necessary. The fractions can be applied again on a new plate followed by EDA and MS characterization. Alternatively, the mobile phase for planar chromatography has to be optimized in order to separate the previously coeluting substances during HPTLC analysis.

3.3.3 Separating Multipotent Isomers in Yerba Mate Green (No. 37) via 8D-Hyphenation

Biologically active isomers were also separated and detected, shown for example in yerba mate green (no. 37). A multipotent bioactive compound zone (hR_F 40) was observed in AChE, tyrosinase, and

α-/β-glucosidase inhibition autograms (**Figure 10A**, marked*). This zone was separated into three distinct signals via RP-HPLC (**Figure 10B**), all providing the same absorbance and mass spectra (**Figure 10D**) and assigned to chlorogenic acid isomers, which differed only in quinic acid positioning (**Figure 10C**). The most common isomers of mono-caffeoylquinic acid are 3-*O*-caffeoylquinic acid (neochlorogenic acid), 4-*O*-caffeoylquinic acid (cryptochlorogenic acid), and 5-*O*-caffeoylquinic acid (5-QCA, chlorogenic acid). The mass signals at *m/z* 353 $[M-H]^-$ and 707 $[2M-H]^-$ in the negative ionization mode and at *m/z* 355 $[M + H]^+$, 377 $[M + Na]^+$, 393 $[M + K]^+$ and 747 $[2M + K]^+$ in the positive ionization mode matched to the neutral mass of mono-caffeoylquinic acids of 354 Da. The UV absorbance spectra with maxima at 218 and 325 nm that we obtained were also consistent with literature (Velkoska-Markovska et al., 2020). While chlorogenic acids are reported to have antioxidant (Huang et al., 2017), anti-inflammatory (Willems et al., 2016; Huang et al., 2017), and anti-HIV (Tamayose et al., 2019) properties, this screening revealed even more bioactive potential for these phenolics. For instance, the assigned bioactive compound zone also showed anti-Alzheimer, antidiabetic, and skin-whitening effects in this study. Co-chromatography against standards (**Supplementary Figure S8A,B** and **D**, **Supplementary Table S1**) confirmed that these beneficial health effects of yerba mate green (no. 37, **Figure 10**) come from the mono-caffeoylquinic acids. The various effects of *Ilex paraguariensis* traditionally consumed as herbal beverage qualify this botancial for its new role as functional food (Cardozo Junior and Morand, 2016).

3.3.4 Universal Potential of 8D-Hyphenation

The characterization of additional multipotent bioactive zones, partially plant-specific, is shown in **Figure 11**. Artichoke (no. 4) showed bioactivity at hR_F 42 in the tyrosinase and α-/β-glucosidase inhibition assays. Transferring this zone to RP-HPLC-DAD-HESI-MS provided five signals (**Table 2**), one of which is specific for artichoke. At RT 6.30 min, cynarascoloside C was assumed (**Figure 11A**). Spectral data showed no UV absorbance, referring to structural properties of cynarascoloside C which possess neither a π-electron system nor conjugated double bonds. The mass signals at *m/z* 444 $[M + NH_4]^+$, 449 $[M + Na]^+$, 465 $[M + K]^+$, 461 $[M + Cl]^-$, 471 $[M + HCOO]^-$, 485 $[M + H_3C\text{-}COO]^-$, and 488 $[M + NO_3]^-$ indicated a neutral mass of 426 Da. Cynarascoloside C was not suspected to have bioactive effects. The bioactivity was probably attributed to the coeluting derivates of chlorogenic acid as in yerba mate green (no. 37, **Figure 10**). Nevertheless, *Cynara scolymus* has been used since the 4th century B.C. as medicinal product due to its health benefits and bioactive constituents, responsible for the hypoglycemic, anti-inflammatory, antimicrobial, and antioxidant properties (Turkiewicz et al., 2019).

Eucalyptus (no. 9) is known for its beneficial health properties against infections of the upper respiratory tracts or as antiseptic and is therefore widely used in the pharmaceutical industry (Hasegawa et al., 2008; Ács et al., 2018). The species *Eucalyptus* is closely associated with herbal medicine and traditional health care in various human cultures. Its leaf extracts are administered to fight against cold and cough, bacterial infections, high blood glucose levels, and to boost the immune system and skin health (Salehi et al., 2019). Planar bioanalytical screening confirmed most of those traditional uses, showing many positive responses through the assays in a wide hR_F range (**Figure 11B**). Focusing on hR_F 57, highly plant-specific eucaglobulin was identified with UV (λ_{max} = 222 and 261 nm) and mass spectral data (Boulekbache-Makhlouf et al., 2010). Both the positive ion species at *m/z* 521 $[M + Na]^+$ and 537 $[M + K]^+$, and the deprotonated molecule $[M-H]^-$ confirmed this suspicion. Anti-melanogenesis activity was attributed to eucaglobulin (Hasegawa et al., 2008) and proved with a planar tyrosinase bioassay. Extracts of eucalyptus fruits were demonstrated to have antibacterial effects against *B. subtilis* (Boulekbache-Makhlouf et al., 2013), which so far have not been directly correlated to the monoterpene conjugate eucaglobulin. The anti-cholinesterase activity was only described for the whole methanolic extract of *Eucalyptus globulus* (Amat-ur-Rasool et al., 2020) but not directly correlated to eucaglobulin.

Extracts of *Ginkgo biloba* seeds and leaves were applied as herbal remedies all over the globe. Originating from traditional Chinese medicine, it is used to treat bacterial skin diseases, cognitive decline (Chassagne et al., 2019), and cardiovascular diseases (Shu et al., 2018). Affirming the ethnopharmacological usage, EDA of ginkgo leaf extract (no.14) demonstrated a variety of bioactivity at hR_F 93, *i.e.,* antibacterial and antidiabetic effects, as well as β-glucuronidase and tyrosinase inhibition, and antiandrogenic activity (**Figure 11C**). RP-HPLC-DAD-HESI-MS analysis revealed the three ginkgolides A–C incorporated in this bioactive zone. Ginkgolide C eluted earlier from RP column (RT 6.42 min). Despite the second chromatography, ginkgolides A and B were not separated (both RT 6.86 min) and were thus detected in the same UV and mass spectra. Structurally they only differ in one additional hydroxy group of ginkgolide B. The high abundance of different ion species describing the two compounds is listed in **Table 2** and displayed in **Figure 11C**. The wide bioactive spectrum of these two diterpenes was confirmed against standards (**Supplementary Figure S8B**, **Supplementary Table S1**).

As an alternative to coffee, the leaves of *Ilex paraguariensis* are widely consumed as beverage in Latin America. Due to its chemical composition, mainly alkaloids and polyphenols, yerba mate exhibits many bioactive effects, *e.g.,* antibacterial, cardiovascular-protective, neuroprotective, and antidiabetic activities (Gan et al., 2018). In yerba mate green (no. 37), a multipotent bioactive analyte zone was detected at hR_F 20 (**Figure 11D**). Showing signals at *m/z* 609 $[M-H]^-$ in ESI^- and at *m/z* 611 $[M + H]^+$, 633 $[M + Na]^+$, and 649 $[M + K]^+$ in ESI^+ at an RT of 6.84 min, the flavonoid rutin was assumed. Its antibacterial (Orhan et al., 2010) potential against *B. subtilis*, anti-tyrosinase activity (Kishore et al., 2018), and glucosidase inhibition (Li et al., 2009) have already been demonstrated. The assumption was verified against a standard (**Supplementary Figure S8A** and **D**, **Supplementary Table S1**).

Salvia rosmarinus, predominantly growing in Mediterranean regions, is independently used in Mexican and Spanish ethnopharmacology. As medicinal plant, it was used to fight bacterial skin diseases, colds, intestinal parasites, and headaches (Heinrich et al., 2006). A very broad spectrum of bioactivities was confirmed by the presented study. The comprehensive NP-

HPTLC-EDA-heart-cut RP-HPLC-DAD-HESI-MS analysis of rosemary (no. 46) showed antibacterial activity in the *A. fischeri* bioassay and antidiabetic properties in the β-glucosidase assay at hR_F 25. Several ions at *m/z* 387 $[M-H]^-$, 406 $[M + NH_4]^+$, 411 $[M + Na]^+$, and 427 $[M + K]^+$ in positive and negative ion mode were correlated to the neutral molecular weight of 388 Da (**Figure 11E**). The mass spectrometric data substantiate the suspicion that this bioactivity is related to medioresinol. In rosemary extracts, various bioactive compounds have been described. Carnosic acid and carnosol are known to inhibit the growth of human cancer cell lines and operate as anti-inflammatory agents (Bai et al., 2010; Wang et al., 2018) or antioxidants (Loussouarn et al., 2017). Rosmarinic acid and rosmanol were found to have antioxidant potential (Vallverdú-Queralt et al., 2014), but to our knowledge, there is no bioactivity reported for medioresinol.

Many more examples could be explained in detail. All have in common that the effect profiles we obtained explain why consuming green tea and using fresh herbs and spices as seasoning can reduce the risk of diabetes by inhibiting α- and β-glucosidases, why healthy nutrition can protect the intestinal flora from severe impairment caused by β-glucuronidases from *Enterobacteriaceae*, why plant-derived cosmetics can reduce skin abnormalities via its tyrosinase-inhibiting potential, and why Alzheimer's disease can be prevented by daily intake of chlorogenic acid or rosmarinic acid from herbs such as artichoke, lemon balm, peppermint, thyme and rosemary, and so on. The wealth of effect information obtained inspires the mind and could fuel further studies. An enormous diversity in bioactivity was revealed in the effect-profiles of the 68 botanicals, contributing to human health by drug-like properties. It clearly shows the potential and spectrum of nature as basis for alternative medicines.

4 CONCLUSION

Since the major part, and especially, the important active part of natural food and traditional medicines is presently not under analytical control, a paradigm shift from quality control based on marker compounds to effect profiles is postulated for plant-based samples. Considering the global production chain, whose influences on the product cannot be controlled, at least entries or changes regarding the effect should be kept under control. The Chemical Abstracts database (www.cas.org) contains over 190 million chemicals, and thousands of compounds are added daily. There is of little help, if we can measure some thousands of them with ever lower limits of determination. By doing so, this does not come close to doing justice to the complexity of plant extracts, nor to the metabolic networking of natural processes, nor to the contamination-prone global production chain. The implementation of effect-directed profiles would subtantially improve quality control, ensure the expected activites and detect unexpected activities. Even small amounts of compounds can be highly active. Disruptive thinking is essential to better control our natural food and traditional medicines. Sophisticated instrumentation does not solve pressing challenges, but combining orthogonal areas does. The complexity of plant extracts requires modern non-target methods that combine chromatography with effect-directed assays to prioritize active compounds that show an effect and thus require utmost attention. This combination is indispensable for routine quality control to prioritize substances among the thousands of individual compounds in a plant extract. The modular NP-HPTLC–UV/Vis/FLD–EDA-heart-cut RP-HPLC–DAD–HESI-MS coupling can be used in routine and makes it easy to recognize the essence. It is said that a picture is worth a thousand words, but the effect image is worth even more. It visualizes the impressive power of nature to supply the body with important building blocks (multipotent chemicals). Several innovations were demonstrated. The pYES equipped with the hERβ or in combination with the simulated S9 metabolization were applied for the first time. In the antagonistic hormonal assays, the proof for false-positive results was newly included. Synergistic effects were revealed in the hormonal effect-profiles for the first time. The developed workflows can be transferred to any other assays or samples. The array of effect-directed profiles clearly showed that natural food has the power to contribute to our homeostasis in various effective ways. The 1,292 profiles (68 samples x 19 detections) obtained within a few weeks showed the versatility of the activity potential of natural food. Artificial intelligence could help evaluate the wealth of information obtained. Exemplarily, the 60 most bioactive components were identified as proof of principle. The developed non-targeted effect-directed hyphenation highlights the advantages of analytical speed, efficiency, and economy. First, the samples were freed from the interfering matrix via planar chromatographic separation. Secondly, the focus was exclusively laid on bioactive compounds, providing targeted characterization. Calculated per sample, the robust profiling takes 3–15 min and costs 0.5–1 Euro, depending on incubation time and material consumption, respectively. One current limitation is the low resolution of the single quadrupole MS instrument. Potential drug candidates were only tentatively assigned and confirmed in an additional run against standards. At the same time, this limitation can be an opportunity for further research. Upgrading the MS instrument to a high-resolution MS with fractionation possibility enables unambiguous assignment of molecular formulas and structure elucidation through fragmentation. This makes effect profiling even more attractive for routine analysis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TS carried out all bioassays and mass spectrometry experiments, analyzed the data, and wrote the manuscript draft. DS and MF

prepared the *B. subtilis*, α-/β-glucosidase, β-glucuronidase, pYAS, pYES, pYAAS, pYAES (bio)autograms. JH prepared the samples and *A. fischeri*, tyrosinase, and α-amylase (bio) autograms. GM initiated the project, concept and methodology, obtained research funding, supervised the study, and revised the manuscript.

FUNDING

This work was supported by the Instrumentation was partially funded by the Deutsche Forschungsgemeinschaft (DFG; INST 162/536-1 FUGG).

ACKNOWLEDGMENTS

We would like to thank Merck, Darmstadt, Germany, for providing the plates, Waters, Eschborn, Germany, for technical support, and Prof. Dr. Erwin Herberle-Bors, University of Vienna, Austria, for donation of *Saccharomyces cerevisiae* cells equipped with the hERβ.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.755941/full#supplementary-material

REFERENCES

Abubakar, I. B., Malami, I., Yahaya, Y., and Sule, S. M. (2018). A Review on the Ethnomedicinal Uses, Phytochemistry and Pharmacology of Alpinia Officinarum Hance. *J. Ethnopharmacol* 224, 45–62. doi:10.1016/j.jep.2018.05.027

Ács, K., Balázs, V. L., Kocsis, B., Bencsik, T., Böszörményi, A., and Horváth, G. (2018). Antibacterial Activity Evaluation of Selected Essential Oils in Liquid and Vapor Phase on Respiratory Tract Pathogens. *BMC Complement. Altern. Med.* 18, 227. doi:10.1186/s12906-018-2291-9

Agatonovic-Kustrin, S., Kustrin, E., Gegechkori, V., and Morton, D. W. (2019). High-Performance Thin-Layer Chromatography Hyphenated with Microchemical and Biochemical Derivatizations in Bioactivity Profiling of Marine Species. *Mar. Drugs* 17. doi:10.3390/md17030148

Agatonovic-Kustrin, S., and Morton, D. W. (2017). High-performance Thin-Layer Chromatography HPTLC-Direct Bioautography as a Method of Choice for Alpha-Amylase and Antioxidant Activity Evaluation in marine Algae. *J. Chromatogr. A.* 1530, 197–203. doi:10.1016/j.chroma.2017.11.024

Amat-ur-Rasool, H., Symes, F., Tooth, D., Schaffert, L. N., Elmorsy, E., Ahmed, M., et al. (2020). Potential Nutraceutical Properties of Leaves from Several Commonly Cultivated Plants. *Biomolecules* 10, 1556. doi:10.3390/biom10111556

Amessis-Ouchemoukh, N., Abu-Reidah, I. M., Quirantes-Piné, R., Madani, K., and Segura-Carretero, A. (2014). Phytochemical Profiling, *In Vitro* Evaluation of Total Phenolic Contents and Antioxidant Properties of Marrubium Vulgare (Horehound) Leaves of Plants Growing in Algeria. *Ind. Crops Prod.* 61, 120–129. doi:10.1016/j.indcrop.2014.06.049

Anagnostopoulou, M. A., Kefalas, P., Kokkalou, E., Assimopoulou, A. N., and Papageorgiou, V. P. (2005). Analysis of Antioxidant Compounds in Sweet orange Peel by HPLC-Diode Array Detection-Electrospray Ionization Mass Spectrometry. *Biomed. Chromatogr.* 19, 138–148. doi:10.1002/bmc.430

Azadniya, E., Thomä, I., Baake, J., and Morlock, G. E. (2021). High-throughput Enzyme Inhibition Screening of 44 Iranian Medicinal Plants via Piezoelectric Spraying of Planar Cholinesterase Assays. *J. Chromatogr. B* 1184, 122956. doi:10.1016/j.jchromb.2021.122956

Bai, L., Li, X., He, L., Zheng, Y., Lu, H., Li, J., et al. (2019). Antidiabetic Potential of Flavonoids from Traditional Chinese Medicine: A Review. *Am. J. Chin. Med.* 47, 933–957. doi:10.1142/S0192415X19500496

Bai, N., He, K., Roller, M., Lai, C. S., Shao, X., Pan, M. H., et al. (2010). Flavonoids and Phenolic Compounds from Rosmarinus Officinalis. *J. Agric. Food Chem.* 58, 5363–5367. doi:10.1021/jf100332w

Baldi, A., Rosen, R. T., Fukuda, E. K., and Ho, C.-T. (1995). Identification of Nonvolatile Components in Lemon Peel by High-Performance Liquid Chromatography with Confirmation by Mass Spectrometry and Diode-Array Detection. *J. Chromatogr. A* 718, 89–97. doi:10.1016/0021-9673(95)00676-1

Belwal, T., Devkota, H. P., Hassan, H. A., Ahluwalia, S., Ramadan, M. F., Mocan, A., et al. (2018a). Phytopharmacology of Acerola (Malpighia Spp.) and its Potential as Functional Food. *Trends Food Sci. Technology* 74, 99–106. doi:10.1016/j.tifs.2018.01.014

Belwal, T., Ezzat, S. M., Rastrelli, L., Bhatt, I. D., Daglia, M., Baldi, A., et al. (2018b). A Critical Analysis of Extraction Techniques Used for Botanicals: Trends, Priorities, Industrial Uses and Optimization Strategies. *Trac Trends Anal. Chem.* 100, 82–102. doi:10.1016/j.trac.2017.12.018

Bilia, A. R., Giomi, M., Innocenti, M., Gallori, S., and Vincieri, F. F. (2008). HPLC-DAD-ESI-MS Analysis of the Constituents of Aqueous Preparations of Verbena and Lemon Verbena and Evaluation of the Antioxidant Activity. *J. Pharm. Biomed. Anal.* 46, 463–470. doi:10.1016/j.jpba.2007.11.007

Boonmuen, N., Gong, P., Ali, Z., Chittiboyina, A. G., Khan, I., Doerge, D. R., et al. (2016). Licorice Root Components in Dietary Supplements Are Selective Estrogen Receptor Modulators with a Spectrum of Estrogenic and Anti-estrogenic Activities. *Steroids* 105, 42–49. doi:10.1016/j.steroids.2015.11.006

Boulekbache-Makhlouf, L., Meudec, E., Chibane, M., Mazauric, J. P., Slimani, S., Henry, M., et al. (2010). Analysis by High-Performance Liquid Chromatography Diode Array Detection Mass Spectrometry of Phenolic Compounds in Fruit of Eucalyptus Globulus Cultivated in Algeria. *J. Agric. Food Chem.* 58, 12615–12624. doi:10.1021/jf1029509

Boulekbache-Makhlouf, L., Slimani, S., and Madani, K. (2013). Total Phenolic Content, Antioxidant and Antibacterial Activities of Fruits of Eucalyptus Globulus Cultivated in Algeria. *Ind. Crops Prod.* 41, 85–89. doi:10.1016/j.indcrop.2012.04.019

Brantner, A., and Grein, E. (1994). Antibacterial Activity of Plant Extracts Used Externally in Traditional Medicine. *J. Ethnopharmacol* 44, 35–40. doi:10.1016/0378-8741(94)90096-5

Bravo, L., Goya, L., and Lecumberri, E. (2007). LC/MS Characterization of Phenolic Constituents of Mate (Ilex Paraguariensis, St. Hil.) and its Antioxidant Activity Compared to Commonly Consumed Beverages. *Food Res. Int.* 40, 393–405. doi:10.1016/j.foodres.2006.10.016

Caesar, L. K., Kellogg, J. J., Kvalheim, O. M., and Cech, N. B. (2019). Opportunities and Limitations for Untargeted Mass Spectrometry Metabolomics to Identify Biologically Active Constituents in Complex Natural Product Mixtures. *J. Nat. Prod.* 82, 469–484. doi:10.1021/acs.jnatprod.9b00176

Cardozo Junior, E. L., and Morand, C. (2016). Interest of Mate (Ilex Paraguariensis A. St.-Hil.) as a New Natural Functional Food to Preserve Human Cardiovascular Health - A Review. *J. Funct. Foods* 21, 440–454. doi:10.1016/j.jff.2015.12.010

Çelik, S. E., Tufan, A. N., Bekdeser, B., Özyürek, M., Güçlü, K., and Apak, R. (2017). Identification and Determination of Phenolics in Lamiaceae Species by UPLC-DAD-ESI-MS/MS. *J. Chromatogr. Sci.* 55, 291–300. doi:10.1093/chromsci/bmw184

Chassagne, F., Huang, X., Lyles, J. T., and Quave, C. L. (2019). Validation of a 16th Century Traditional Chinese Medicine Use of Ginkgo Biloba as a Topical Antimicrobial. *Front. Microbiol.* 10, 775. doi:10.3389/fmicb.2019.00775

Chen, E., Ding, C., and Lindsay, R. C. (2005). Qualitative and Quantitative Analyses of Ginkgo Terpene Trilactones by Liquid Chromatography/sonic spray Ionization Ion Trap Mass Spectrometry. *Anal. Chem.* 77, 2966–2970. doi:10.1021/ac048510p

Cock, I. E., Ndlovu, N., and van Vuuren, S. F. (2021). The Use of South African Botanical Species for the Control of Blood Sugar. *J. Ethnopharmacol* 264, 113234. doi:10.1016/j.jep.2020.113234

Cornwell, T., Cohick, W., and Raskin, I. (2004). Dietary Phytoestrogens and Health. *Phytochemistry* 65, 995–1016. doi:10.1016/j.phytochem.2004.03.005

da Silva, G. S., Canuto, K. M., Ribeiro, P. R. V., de Brito, E. S., Nascimento, M. M., Zocolo, G. J., et al. (2017). Chemical Profiling of Guarana Seeds (Paullinia Cupana) from Different Geographical Origins Using UPLC-QTOF-MS Combined with Chemometrics. *Food Res. Int.* 102, 700–709. doi:10.1016/j.foodres.2017.09.055

de Souza, L. M., Dartora, N., Scoparo, C. T., Cipriani, T. R., Gorin, P. A., Iacomini, M., et al. (2011). Comprehensive Analysis of Maté (Ilex Paraguariensis) Compounds: Development of Chemical Strategies for Matesaponin Analysis by Mass Spectrometry. *J. Chromatogr. A.* 1218, 7307–7315. doi:10.1016/j.chroma.2011.08.047

Dechorgnat, J., Nguyen, C. T., Armengaud, P., Jossier, M., Diatloff, E., Filleur, S., et al. (2011). From the Soil to the Seeds: the Long Journey of Nitrate in Plants. *J. Exp. Bot.* 62, 1349–1359. doi:10.1093/jxb/erq409

Díaz, R., Ibáñez, M., Sancho, J. V., and Hernández, F. (2012). Target and Non-target Screening Strategies for Organic Contaminants, Residues and Illicit Substances in Food, Environmental and Human Biological Samples by UHPLC-QTOF-MS. *Anal. Methods* 4, 196–209. doi:10.1039/C1AY05385J

Ding, S., Dudley, E., Plummer, S., Tang, J., Newton, R. P., and Brenton, A. G. (2006). Quantitative Determination of Major Active Components in Ginkgo Biloba Dietary Supplements by Liquid Chromatography/mass Spectrometry. *Rapid Commun. Mass. Spectrom.* 20, 2753–2760. doi:10.1002/rcm.2646

Du, Z., Li, J., Zhang, X., Pei, J., and Huang, L. (2018). An Integrated LC-MS-Based Strategy for the Quality Assessment and Discrimination of Three Panax Species. *Molecules* 23, 2988. doi:10.3390/molecules23112988

Dugo, P., Mondello, L., Dugo, L., Stancanelli, R., and Dugo, G. (2000). LC-MS for the Identification of Oxygen Heterocyclic Compounds in Citrus Essential Oils. *J. Pharm. Biomed. Anal.* 24, 147–154. doi:10.1016/S0731-7085(00)00400-3

Efferth, T., and Kaina, B. (2011). Toxicities by Herbal Medicines with Emphasis to Traditional Chinese Medicine. *Curr. Drug Metab.* 12, 989–996. doi:10.2174/138920011798062328

El Senousy, A. S., Farag, M. A., Al-Mahdy, D. A., and Wessjohann, L. A. (2014). Developmental Changes in Leaf Phenolics Composition from Three Artichoke Cvs. (Cynara Scolymus) as Determined via UHPLC-MS and Chemometrics. *Phytochemistry* 108, 67–76. doi:10.1016/j.phytochem.2014.09.004

Erenler, R., Sen, O., Aksit, H., Demirtas, I., Yaglioglu, A. S., Elmastas, M., et al. (2016). Isolation and Identification of Chemical Constituents from Origanum Majorana and Investigation of Antiproliferative and Antioxidant Activities. *J. Sci. Food Agric.* 96, 822–836. doi:10.1002/jsfa.7155

European Committee for Standardization (2009). *Water Quality - Determination of the Inhibitory Effect of Water Samples on the Light Emission of Vibrio Fischeri (Luminescent Bacteria Test): Part 1: Method Using Freshly Prepared Bacteria*.

Ezzat, S. M., Salama, M. M., ElMeshad, A. N., Teaima, M. H., and Rashad, L. A. (2016). HPLC-DAD-MS/MS Profiling of Standardized Rosemary Extract and Enhancement of its Anti-wrinkle Activity by Encapsulation in Elastic Nanovesicles. *Arch. Pharm. Res.* 39, 912–925. doi:10.1007/s12272-016-0744-6

Fan, R., Huang, X., Wang, Y., Chen, X., Ren, P., Ji, H., et al. (2012). Ethnopharmacokinetic- and Activity-Guided Isolation of a New Antidepressive Compound from Fructus Aurantii Found in the Traditional Chinese Medicine Chaihu-Shugan-San: a New Approach and its Application. *Evid. Based Complement. Alternat Med.* 2012, 607584. doi:10.1155/2012/607584

Fang, H., Tong, W., Branham, W. S., Moland, C. L., Dial, S. L., Hong, H., et al. (2003). Study of 202 Natural, Synthetic, and Environmental Chemicals for Binding to the Androgen Receptor. *Chem. Res. Toxicol.* 16, 1338–1358. doi:10.1021/tx030011g

Farag, M. A., El-Ahmady, S. H., Elian, F. S., and Wessjohann, L. A. (2013). Metabolomics Driven Analysis of Artichoke Leaf and its Commercial Products via UHPLC-Q-TOF-MS and Chemometrics. *Phytochemistry* 95, 177–187. doi:10.1016/j.phytochem.2013.07.003

Favela-Hernández, J. M., González-Santiago, O., Ramírez-Cabrera, M. A., Esquivel-Ferriño, P. C., and Camacho-Corona, Mdel. R. (2016). Chemistry and Pharmacology of Citrus Sinensis. *Molecules* 21, 247. doi:10.3390/molecules21020247

Fecka, I., Kowalczyk, A., and Cisowski, W. (2004). Optimization of the Separation of Flavonoid Glycosides and Rosmarinic Acid fromMentha Piperitaon HPTLC Plates. *J. Planar Chromatogr. - Mod. TLC* 17, 22–25. doi:10.1556/JPC.17.2004.1.5

Fu, Y., Zhao, C., Lu, X., and Xu, G. (2017). Nontargeted Screening of Chemical Contaminants and Illegal Additives in Food Based on Liquid Chromatography-High Resolution Mass Spectrometry. *Trac Trends Anal. Chem.* 96, 89–98. doi:10.1016/j.trac.2017.07.014

Gan, R. Y., Zhang, D., Wang, M., and Corke, H. (2018). Health Benefits of Bioactive Compounds from the Genus Ilex, a Source of Traditional Caffeinated Beverages. *Nutrients* 10, 1682. doi:10.3390/nu10111682

Ge, Y. W., Zhu, S., Yoshimatsu, K., and Komatsu, K. (2017). MS/MS Similarity Networking Accelerated Target Profiling of Triterpene Saponins in Eleutherococcus Senticosus Leaves. *Food Chem.* 227, 444–452. doi:10.1016/j.foodchem.2017.01.119

Giorgi, A., Mingozzi, M., Madeo, M., Speranza, G., and Cocucci, M. (2009). Effect of Nitrogen Starvation on the Phenolic Metabolism and Antioxidant Properties of Yarrow (Achillea Collina Becker Ex Rchb.). *Food Chem.* 114, 204–211. doi:10.1016/j.foodchem.2008.09.039

Guldiken, B., Ozkan, G., Catalkaya, G., Ceylan, F. D., Ekin Yalcinkaya, I., and Capanoglu, E. (2018). Phytochemicals of Herbs and Spices: Health versus Toxicological Effects. *Food Chem. Toxicol.* 119, 37–49. doi:10.1016/j.fct.2018.05.050

Habs, M., Binder, K., Krauss, S., Müller, K., Ernst, B., Valentini, L., et al. (2017). A Balanced Risk-Benefit Analysis to Determine Human Risks Associated with Pyrrolizidine Alkaloids (PA)-The Case of Tea and Herbal Infusions. *Nutrients* 9, 717. doi:10.3390/nu9070717

Hage, S., and Morlock, G. E. (2017). Bioprofiling of Salicaceae Bud Extracts through High-Performance Thin-Layer Chromatography Hyphenated to Biochemical, Microbiological and Chemical Detections. *J. Chromatogr. A.* 1490, 201–211. doi:10.1016/j.chroma.2017.02.019

Halvorsen, B. L., Holte, K., Myhrstad, M. C., Barikmo, I., Hvattum, E., Remberg, S. F., et al. (2002). A Systematic Screening of Total Antioxidants in Dietary Plants. *J. Nutr.* 132, 461–471. doi:10.1093/jn/132.3.461

Hasegawa, T., Takano, F., Takata, T., Niiyama, M., and Ohta, T. (2008). Bioactive Monoterpene Glycosides Conjugated with Gallic Acid from the Leaves of Eucalyptus Globulus. *Phytochemistry* 69, 747–753. doi:10.1016/j.phytochem.2007.08.030

Hawrył, M. A. (2014). HPLC-diode Array Detector Fingerprints of Various Mentha Species. *J. AOAC Int.* 97, 1268–1273. doi:10.5740/jaoacint.SGEHawryl

Heinrich, M., Kufer, J., Leonti, M., and Pardo-de-Santayana, M. (2006). Ethnobotany and Ethnopharmacology-Iinterdisciplinary Links with the Historical Sciences. *J. Ethnopharmacol* 107, 157–160. doi:10.1016/j.jep.2006.05.035

Hossain, M. B., Camphuis, G., Aguiló-Aguayo, I., Gangopadhyay, N., and Rai, D. K. (2014). Antioxidant Activity Guided Separation of Major Polyphenols of Marjoram (Origanum Majorana L.) Using Flash Chromatography and Their Identification by Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry. *J. Sep. Sci.* 37, 3205–3213. doi:10.1002/jssc.201400597

Hossain, M. B., Rai, D. K., Brunton, N. P., Martin-Diana, A. B., and Barry-Ryan, C. (2010). Characterization of Phenolic Composition in Lamiaceae Spices by LC-ESI-MS/MS. *J. Agric. Food Chem.* 58, 10576–10581. doi:10.1021/jf102042g

Hu, Y., Zeng, H., Huang, J., Jiang, L., Chen, J., and Zeng, Q. (2020). Traditional Asian Herbs in Skin Whitening: The Current Development and Limitations. *Front. Pharmacol.* 11, 982. doi:10.3389/fphar.2020.00982

Huang, W. Y., Fu, L., Li, C. Y., Xu, L. P., Zhang, L. X., and Zhang, W. M. (2017). Quercetin, Hyperin, and Chlorogenic Acid Improve Endothelial Function by Antioxidant, Antiinflammatory, and ACE Inhibitory Effects. *J. Food Sci.* 82, 1239–1246. doi:10.1111/1750-3841.13706

Huang, X. X., Xu, Y., Bai, M., Zhou, L., Song, S. J., and Wang, X. B. (2018). Lignans from the Seeds of Chinese Hawthorn (Crataegus Pinnatifida Var. Major N.E.Br.) against β-amyloid Aggregation. *Nat. Prod. Res.* 32, 1706–1713. doi:10.1080/14786419.2017.1399378

Itoh, K., Hirata, N., Masuda, M., Naruto, S., Murata, K., Wakabayashi, K., et al. (2009). Inhibitory Effects of Citrus Hassaku Extract and its Flavanone Glycosides on Melanogenesis. *Biol. Pharm. Bull.* 32, 410–415. doi:10.1248/bpb.32.410

Jabir, N. R., Khan, F. R., and Tabrez, S. (2018). Cholinesterase Targeting by Polyphenols: A Therapeutic Approach for the Treatment of Alzheimer's Disease. *CNS Neurosci. Ther.* 24, 753–762. doi:10.1111/cns.12971

Kaltner, F., Rychlik, M., Gareis, M., and Gottschalk, C. (2020). Occurrence and Risk Assessment of Pyrrolizidine Alkaloids in Spices and Culinary Herbs from Various Geographical Origins. *Toxins (Basel)* 12, 155. doi:10.3390/toxins12030155

Kao, T. C., Shyu, M. H., and Yen, G. C. (2009). Neuroprotective Effects of Glycyrrhizic Acid and 18beta-Glycyrrhetinic Acid in PC12 Cells via Modulation of the PI3K/Akt Pathway. *J. Agric. Food Chem.* 57, 754–761. doi:10.1021/jf802864k

Kirchmayer, T. (2009). *Reconstitution of ERβ Transactivation in Yeast. Diploma Thesis*. Vienna: University of Vienna, Faculty of Life Science. Available at: https://othes.univie.ac.at/8017 (Accessed October 06, 2021).

Kishore, N., Twilley, D., Blom van Staden, A., Verma, P., Singh, B., Cardinali, G., et al. (2018). Isolation of Flavonoids and Flavonoid Glycosides from Myrsine Africana and Their Inhibitory Activities against Mushroom Tyrosinase. *J. Nat. Prod.* 81, 49–56. doi:10.1021/acs.jnatprod.7b00564

Klingelhöfer, I., Hockamp, N., and Morlock, G. E. (2020). Non-targeted Detection and Differentiation of Agonists versus Antagonists, Directly in Bioprofiles of Everyday Products. *Anal. Chim. Acta* 1125, 288–298. doi:10.1016/j.aca.2020.05.057

Klingelhöfer, I., and Morlock, G. E. (2015). Bioprofiling of Surface/Wastewater and Bioquantitation of Discovered Endocrine-Active Compounds by Streamlined Direct Bioautography. *Anal. Chem.* 87, 11098–11104. doi:10.1021/acs.analchem.5b03233

Knutsen, H. K., Knutsen, H. K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., et al. (2017). Risks for Human Health Related to the Presence of Pyrrolizidine Alkaloids in Honey, tea, Herbal Infusions and Food Supplements. *EFSA J.* 15, e04908. doi:10.2903/j.efsa.2017.4908

Kong, W., Wen, J., Yang, Y., Qiu, F., Sheng, P., and Yang, M. (2014). Simultaneous Targeted Analysis of Five Active Compounds in Licorice by Ultra-fast Liquid Chromatography Coupled to Hybrid Linear-Ion Trap Tandem Mass Spectrometry. *Analyst* 139, 1883–1894. doi:10.1039/c3an02209a

Kongstad, K. T., Özdemir, C., Barzak, A., Wubshet, S. G., and Staerk, D. (2015). Combined Use of High-Resolution α-glucosidase Inhibition Profiling and High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry-solid-phase Extraction-Nuclear Magnetic Resonance Spectroscopy for Investigation of Antidiabetic Principles in Crude Plant Extracts. *J. Agric. Food Chem.* 63, 2257–2263. doi:10.1021/jf506297k

Krüger, S., Bergin, A., and Morlock, G. E. (2018). Effect-directed Analysis of Ginger (Zingiber Officinale) and its Food Products, and Quantification of Bioactive Compounds via High-Performance Thin-Layer Chromatography and Mass Spectrometry. *Food Chem.* 243, 258–268. doi:10.1016/j.foodchem.2017.09.095

Krüger, S., Hüsken, L., Fornasari, R., Scainelli, I., and Morlock, G. E. (2017). Effect-directed Fingerprints of 77 Botanical Extracts via a Generic High-Performance Thin-Layer Chromatography Method Combined with Assays and Mass Spectrometry. *J. Chromatogr. A.* 1529, 93–106. doi:10.1016/j.chroma.2017.10.068

Kuźniewski, R., Załuski, D., Olech, M., Banaszczak, P., and Nowak, R. (2018). LC-ESI-MS/MS Profiling of Phenolics in the Leaves of Eleutherococcus Senticosus Cultivated in the West Europe and Anti-hyaluronidase and Anti-acetylcholinestarase Activities. *Nat. Prod. Res.* 32, 448–452. doi:10.1080/14786419.2017.1308369

Lall, N., and Kishore, N. (2014). Are Plants Used for Skin Care in South Africa Fully Explored? *J. Ethnopharmacol* 153, 61–84. doi:10.1016/j.jep.2014.02.021

Li, G., Nikolic, D., and van Breemen, R. B. (2016). Identification and Chemical Standardization of Licorice Raw Materials and Dietary Supplements Using UHPLC-MS/MS. *J. Agric. Food Chem.* 64, 8062–8070. doi:10.1021/acs.jafc.6b02954

Li, K., Ji, S., Song, W., Kuang, Y., Lin, Y., Tang, S., et al. (2017). Glycybridins A-K, Bioactive Phenolic Compounds from Glycyrrhiza Glabra. *J. Nat. Prod.* 80, 334–346. doi:10.1021/acs.jnatprod.6b00783

Li, S., Yu, H., and Ho, C. T. (2006). Nobiletin: Efficient and Large Quantity Isolation from orange Peel Extract. *Biomed. Chromatogr.* 20, 133–138. doi:10.1002/bmc.540

Li, Y. Q., Zhou, F. C., Gao, F., Bian, J. S., and Shan, F. (2009). Comparative Evaluation of Quercetin, Isoquercetin and Rutin as Inhibitors of Alpha-Glucosidase. *J. Agric. Food Chem.* 57, 11463–11468. doi:10.1021/jf903083h

Lin, L. Z., and Harnly, J. M. (2012). LC-PDA-ESI/MS Identification of the Phenolic Components of Three Compositae Spices: Chamomile, Tarragon, and Mexican Arnica. *Nat. Prod. Commun.* 7, 749-752. doi:10.1177/1934578X1200700615

Liu, J., Burdette, J. E., Xu, H., Gu, C., van Breemen, R. B., Bhat, K. P., et al. (2001). Evaluation of Estrogenic Activity of Plant Extracts for the Potential Treatment of Menopausal Symptoms. *J. Agric. Food Chem.* 49, 2472–2479. doi:10.1021/jf0014157

Liu, J. Q., Peng, X. R., Li, X. Y., Li, T. Z., Zhang, W. M., Shi, L., et al. (2013). Norfriedelins A-C with Acetylcholinesterase Inhibitory Activity from Acerola Tree (Malpighia Emarginata). *Org. Lett.* 15, 1580–1583. doi:10.1021/ol4003702

Loussouarn, M., Krieger-Liszkay, A., Svilar, L., Bily, A., Birtić, S., and Havaux, M. (2017). Carnosic Acid and Carnosol, Two Major Antioxidants of Rosemary, Act through Different Mechanisms. *Plant Physiol.* 175, 1381–1394. doi:10.1104/pp.17.01183

Mahran, E., Keusgen, M., and Morlock, G. E. (2020). New Planar Assay for Streamlined Detection and Quantification of β-glucuronidase Inhibitors Applied to Botanical Extracts. *Anal. Chim. Acta X* 4, 100039. doi:10.1016/j.acax.2020.100039

Manthey, J. A., and Grohmann, K. (1996). Concentrations of Hesperidin and Other Orange Peel Flavonoids in Citrus Processing Byproducts. *J. Agric. Food Chem.* 44, 811–814. doi:10.1021/jf950572g

Marston, A., Kissling, J., and Hostettmann, K. (2002). A Rapid TLC Bioautographic Method for the Detection of Acetylcholinesterase and Butyrylcholinesterase Inhibitors in Plants. *Phytochem. Anal.* 13, 51–54. doi:10.1002/pca.623

Marston, A. (2011). Thin-layer Chromatography with Biological Detection in Phytochemistry. *J. Chromatogr. A.* 1218, 2676–2683. doi:10.1016/j.chroma.2010.12.068

Mateos, R., Baeza, G., Sarriá, B., and Bravo, L. (2018). Improved LC-MSn Characterization of Hydroxycinnamic Acid Derivatives and Flavonols in Different Commercial Mate (Ilex Paraguariensis) Brands. Quantification of Polyphenols, Methylxanthines, and Antioxidant Activity. *Food Chem.* 241, 232–241. doi:10.1016/j.foodchem.2017.08.085

Mauri, P., Migliazza, B., and Pietta, P. (1999). Liquid Chromatography/electrospray Mass Spectrometry of Bioactive Terpenoids in Ginkgo Biloba L. *J. Mass. Spectrom.*, 34, 13612–13676. doi:10.1002/(SICI)1096-9888(199912)34:12<1361:AID-JMS895>3.0.CO;2-6

Mbachu, O. C., Howell, C., Simmler, C., Malca Garcia, G. R., Skowron, K. J., Dong, H., et al. (2020). SAR Study on Estrogen Receptor α/β Activity of (Iso) flavonoids: Importance of Prenylation, C-Ring (Un)Saturation, and Hydroxyl Substituents. *J. Agric. Food Chem.* 68, 10651–10663. doi:10.1021/acs.jafc.0c03526

Mena, P., Cirlini, M., Tassotti, M., Herrlinger, K. A., dall'Asta, C., and Del Rio, D. (2016). Phytochemical Profiling of Flavonoids, Phenolic Acids, Terpenoids, and Volatile Fraction of a Rosemary (Rosmarinus Officinalis L.) Extract. *Molecules* 21, 1576. doi:10.3390/molecules21111576

Meyer, D., Marin-Kuan, M., Debon, E., Serrant, P., Cottet-Fontannaz, C., Schilter, B., et al. (2020). Detection of Low Levels of Genotoxic Compounds in Food Contact Materials Using an Alternative HPTLC-SOS-Umu-C Assay. *Altex* 38, 387–397. doi:10.14573/altex.2006201

Montero, L., Ibáñez, E., Russo, M., Di Sanzo, R., Rastrelli, L., Piccinelli, A. L., et al. (2016). Metabolite Profiling of Licorice (Glycyrrhiza Glabra) from Different Locations Using Comprehensive Two-Dimensional Liquid Chromatography Coupled to Diode Array and Tandem Mass Spectrometry Detection. *Anal. Chim. Acta* 913, 145–159. doi:10.1016/j.aca.2016.01.040

Morlock, G. E., and Heil, J. (2020). HI-HPTLC-UV/Vis/FLD-HESI-HRMS and Bioprofiling of Steviol Glycosides, Steviol, and Isosteviol in Stevia Leaves and Foods. *Anal. Bioanal. Chem.* 412, 6431–6448. doi:10.1007/s00216-020-02618-4

Morlock, G. E., Heil, J., Bardot, V., Lenoir, L., Cotte, C., and Dubourdeaux, M. (2021a). Effect-Directed Profiling of 17 Different Fortified Plant Extracts by High-Performance Thin-Layer Chromatography Combined with Six Planar Assays and High-Resolution Mass Spectrometry. *Molecules* 26, 1468. doi:10.3390/molecules26051468

Morlock, G. E., Heil, J., Inarejos-Garcia, A. M., and Maeder, J. (2021b). Effect-Directed Profiling of Powdered Tea Extracts for Catechins, Theaflavins, Flavonols and Caffeine. *Antioxidants (Basel)* 10, 117. doi:10.3390/antiox10010117

Morlock, G. E. (2021). High-performance Thin-Layer Chromatography Combined with Effect-Directed Assays and High-Resolution Mass Spectrometry as an Emerging Hyphenated Technology: A Tutorial Review. *Anal. Chim. Acta* 1180, 338644. doi:10.1016/j.aca.2021.338644

Morlock, G. E., and Klingelhöfer, I. (2014). Liquid Chromatography-Bioassay-Mass Spectrometry for Profiling of Physiologically Active Food. *Anal. Chem.* 86, 8289–8295. doi:10.1021/ac501723j

Morlock, G. E. (2014). Background Mass Signals in Tlc/hptlc-Esi-Ms and Practical Advices for Use of the Tlc-Ms Interface. *J. Liquid Chromatogr. Relat. Tech.* 37, 2892–2914. doi:10.1080/10739149.2014.907000

Motohashi, N., Wakabayashi, H., Kurihara, T., Fukushima, H., Yamada, T., Kawase, M., et al. (2004). Biological Activity of barbados Cherry (Acerola Fruits, Fruit of Malpighia Emarginata DC) Extracts and Fractions. *Phytother Res.* 18, 212–223. doi:10.1002/ptr.1426

Mulder, P. P. J., Sánchez, P. L., These, A., Preiss-Weigert, A., and Castellari, M. (2015). Occurrence of Pyrrolizidine Alkaloids in Food. *EFSA Supporting Publications* 12. doi:10.2903/sp.efsa.2015.EN-859

Niu, X., Luo, J., Xu, D., Zou, H., and Kong, L. (2017). Hydrogen/deuterium Exchange, a Unique and Effective Method for MS Fragmentation Behavior Elucidation of Ginkgolides and its Application to Systematic Research in Ginkgo Biloba. *J. Pharm. Biomed. Anal.* 134, 181–186. doi:10.1016/j.jpba.2016.11.043

Nomura, T., Fukai, T., and Akiyama, T. (2002). Chemistry of Phenolic Compounds of Licorice (Glycyrrhiza Species) and Their Estrogenic and Cytotoxic Activities. *Pure Appl. Chem.* 74, 1199–1206. doi:10.1351/pac200274071199

Önder, F. C., Ay, M., and Sarker, S. D. (2013). Comparative Study of Antioxidant Properties and Total Phenolic Content of the Extracts of Humulus Lupulus L. And Quantification of Bioactive Components by LC-MS/MS and GC-MS. *J. Agric. Food Chem.* 61, 10498–10506. doi:10.1021/jf4031508

Orhan, D. D., Ozçelik, B., Ozgen, S., and Ergun, F. (2010). Antibacterial, Antifungal, and Antiviral Activities of Some Flavonoids. *Microbiol. Res.* 165, 496–504. doi:10.1016/j.micres.2009.09.002

Palombo, E. A., and Semple, S. J. (2001). Antibacterial Activity of Traditional Australian Medicinal Plants. *J. Ethnopharmacol* 77, 151–157. doi:10.1016/S0378-8741(01)00290-2

Pastorino, G., Cornara, L., Soares, S., Rodrigues, F., and Oliveira, M. B. P. P. (2018). Liquorice (Glycyrrhiza Glabra): A Phytochemical and Pharmacological Review. *Phytother Res.* 32, 2323–2339. doi:10.1002/ptr.6178

Pérez-Mendoza, M. B., Llorens-Escobar, L., Vanegas-Espinoza, P. E., Cifuentes, A., Ibáñez, E., and Villar-Martínez, A. A. D. (2020). Chemical Characterization of Leaves and Calli Extracts of Rosmarinus Officinalis by UHPLC-MS. *Electrophoresis* 41, 1776–1783. doi:10.1002/elps.201900152

Petersen, M. J., de Cássia Lemos Lima, R., Kjaerulff, L., and Staerk, D. (2019). Immobilized α-amylase Magnetic Beads for Ligand Fishing: Proof of Concept and Identification of α-amylase Inhibitors in Ginkgo Biloba. *Phytochemistry* 164, 94–101. doi:10.1016/j.phytochem.2019.04.016

Prencipe, F. P., Brighenti, V., Rodolfi, M., Mongelli, A., dall'Asta, C., Ganino, T., et al. (2014). Development of a New High-Performance Liquid Chromatography Method with Diode Array and Electrospray Ionization-Mass Spectrometry Detection for the Metabolite Fingerprinting of Bioactive Compounds in Humulus Lupulus L. *J. Chromatogr. A.* 1349, 50–59. doi:10.1016/j.chroma.2014.04.097

Puranik, N. V., Srivastava, P., Bhatt, G., John Mary, D. J. S., Limaye, A. M., and Sivaraman, J. (2019). Determination and Analysis of Agonist and Antagonist Potential of Naturally Occurring Flavonoids for Estrogen Receptor (ERα) by Various Parameters and Molecular Modelling Approach. *Sci. Rep.* 9, 7450. doi:10.1038/s41598-019-43768-5

Quirantes-Piné, R., Arráez-Román, D., Segura-Carretero, A., and Fernández-Gutiérrez, A. (2010). Characterization of Phenolic and Other Polar Compounds in a Lemon Verbena Extract by Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry. *J. Sep. Sci.* 33, 2818–2827. doi:10.1002/jssc.201000228

Rejeb, I. B., Dhen, N., Gargouri, M., and Boulila, A. (2020). Chemical Composition, Antioxidant Potential and Enzymes Inhibitory Properties of Globe Artichoke By-Products. *Chem. Biodivers* 17, e2000073. doi:10.1002/cbdv.202000073

Rocchetti, G., Senizza, B., Zengin, G., Mahomodally, M. F., Senkardes, I., Lobine, D., et al. (2020). Untargeted Metabolomic Profiling of Three Crataegus Species (Hawthorn) and Their *In Vitro* Biological Activities. *J. Sci. Food Agric.* 100, 1998–2006. doi:10.1002/jsfa.10216

Rosselli, S., Maggio, A., Bellone, G., Formisano, C., Basile, A., Cicala, C., et al. (2007). Antibacterial and Anticoagulant Activities of Coumarins Isolated from the Flowers of Magydaris Tomentosa. *Planta Med.* 73, 116–120. doi:10.1055/s-2006-951772

Rusko, J., Perkons, I., Rasinger, J. D., and Bartkevics, V. (2020). Non-target and Suspected-Target Screening for Potentially Hazardous Chemicals in Food Contact Materials: Investigation of Paper Straws. *Food Addit Contam. Part. A. Chem. Anal. Control. Expo. Risk Assess.* 37, 649–664. doi:10.1080/19440049.2020.1711969

Salehi, B., Sharifi-Rad, J., Quispe, C., Llaique, H., Villalobos, M., Smeriglio, A., et al. (2019). Insights into Eucalyptus Genus Chemical Constituents, Biological Activities and Health-Promoting Effects. *Trends Food Sci. Technology* 91, 609–624. doi:10.1016/j.tifs.2019.08.003

Santos, S. A., Freire, C. S., Domingues, M. R., Silvestre, A. J., and Pascoal Neto, C. (2011). Characterization of Phenolic Components in Polar Extracts of Eucalyptus Globulus Labill. Bark by High-Performance Liquid Chromatography-Mass Spectrometry. *J. Agric. Food Chem.* 59, 9386–9393. doi:10.1021/jf201801q

Sawalha, S. M. S., Arráez-Román, D., Segura-Carretero, A., and Fernández-Gutiérrez, A. (2009). Quantification of Main Phenolic Compounds in Sweet and Bitter orange Peel Using CE-MS/MS. *Food Chem.* 116, 567–574. doi:10.1016/j.foodchem.2009.03.003

Schreiner, T., and Morlock, G. E. (2021). Non-target Bioanalytical Eight-Dimensional Hyphenation Including Bioassay, Heart-Cut Trapping, Online Desalting, Orthogonal Separations and Mass Spectrometry. *J. Chromatogr. A.* 1647, 462154. doi:10.1016/j.chroma.2021.462154

Schulte-Oehlmann, U., Oehlmann, J., and Keil, F. (2011). Before the Curtain Falls: Endocrine-Active Pesticides-Aa German Contamination Legacy. *Rev. Environ. Contam. Toxicol.* 213, 137–159. doi:10.1007/978-1-4419-9860-6_5

Schütz, K., Kammerer, D., Carle, R., and Schieber, A. (2004). Identification and Quantification of Caffeoylquinic Acids and Flavonoids from Artichoke (Cynara Scolymus L.) Heads, Juice, and Pomace by HPLC-DAD-ESI/MS(n). *J. Agric. Food Chem.* 52, 4090–4096. doi:10.1021/jf049625x

Sharma, R., Kuca, K., Nepovimova, E., Kabra, A., Rao, M. M., and Prajapati, P. K. (2019). Traditional Ayurvedic and Herbal Remedies for Alzheimer's Disease: from Bench to Bedside. *Expert Rev. Neurother* 19, 359–374. doi:10.1080/14737175.2019.1596803

Sharma, V., Katiyar, A., and Agrawal, R. C. (2018). "Glycyrrhiza Glabra: Chemistry and Pharmacological Activity,"in. *Sweeteners*. Editors J.-M. Mérillon and K. G. Ramawat (Cham: Springer International Publishing), 87–100. doi:10.1007/978-3-319-27027-2_21

Shu, P., Fei, Y., Li, J., Liu, A., Zhang, L., Niu, H., et al. (2020). Two New Phenylethanoid Glycosides from Ginkgo Biloba Leaves and Their Tyrosinase Inhibitory Activities. *Carbohydr. Res.* 494, 108059. doi:10.1016/j.carres.2020.108059

Shu, Z., Hussain Sh, A., Shahen, M., Wang, H., Alagawany, M., Abd El-Hac, M. E., et al. (2018). Pharmacological Uses of Ginkgo Biloba Extracts for Cardiovascular Disease and Coronary Heart Diseases. *Int. J. Pharmacol.* 15, 1–9. doi:10.3923/ijp.2019.1.9

Simões-Pires, C. A., Hmicha, B., Marston, A., and Hostettmann, K. (2009). A TLC Bioautographic Method for the Detection of Alpha- and Beta-Glucosidase Inhibitors in Plant Extracts. *Phytochem. Anal.* 20, 511–515. doi:10.1002/pca.1154

Smyth, T., Ramachandran, V. N., and Smyth, W. F. (2009). A Study of the Antimicrobial Activity of Selected Naturally Occurring and Synthetic Coumarins. *Int. J. Antimicrob. Agents* 33, 421–426. doi:10.1016/j.ijantimicag.2008.10.022

Soković, M., Glamočlija, J., Marin, P. D., Brkić, D., and van Griensven, L. J. (2010). Antibacterial Effects of the Essential Oils of Commonly Consumed Medicinal Herbs Using an *In Vitro* Model. *Molecules* 15, 7532–7546. doi:10.3390/molecules15117532

Sommella, E., Pagano, F., Salviati, E., Chieppa, M., Bertamino, A., Manfra, M., et al. (2018). Chemical Profiling of Bioactive Constituents in Hop Cones and Pellets Extracts by Online Comprehensive Two-Dimensional Liquid Chromatography with Tandem Mass Spectrometry and Direct Infusion Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *J. Sep. Sci.* 41, 1548–1557. doi:10.1002/jssc.201701242

Su, Q. Z., Vera, P., and Nerín, C. (2020). Direct Immersion-solid-phase Microextraction Coupled to Gas Chromatography-Mass Spectrometry and Response Surface Methodology for Nontarget Screening of (Semi-) Volatile Migrants from Food Contact Materials. *Anal. Chem.* 92, 5577–5584. doi:10.1021/acs.analchem.0c00532

Sun, C. P., Yan, J. K., Yi, J., Zhang, X. Y., Yu, Z. L., Huo, X. K., et al. (2020). The Study of Inhibitory Effect of Natural Flavonoids toward β-glucuronidase and Interaction of Flavonoids with β-glucuronidase. *Int. J. Biol. Macromol* 143, 349–358. doi:10.1016/j.ijbiomac.2019.12.057

Taibon, J., Ankli, A., Schwaiger, S., Magnenat, C., Boka, V. I., Simões-Pires, C., et al. (2015). Prevention of False-Positive Results: Development of an HPTLC Autographic Assay for the Detection of Natural Tyrosinase Inhibitors. *Planta Med.* 81, 1198–1204. doi:10.1055/s-0035-1546250

Tamayose, C. I., Torres, P. B., Roque, N., and Ferreira, M. J. P. (2019). HIV-1 Reverse Transcriptase Inhibitory Activity of Flavones and Chlorogenic Acid Derivatives from Moquiniastrum Floribundum (Asteraceae). *South Afr. J. Bot.* 123, 142–146. doi:10.1016/j.sajb.2019.02.005

Tresserra-Rimbau, A., Lamuela-Raventos, R. M., and Moreno, J. J. (2018). Polyphenols, Food and Pharma. Current Knowledge and Directions for Future Research. *Biochem. Pharmacol.* 156, 186–195. doi:10.1016/j.bcp.2018.07.050

Tundis, R., Bonesi, M., Menichini, F., and Loizzo, M. R. (2016). Recent Knowledge on Medicinal Plants as Source of Cholinesterase Inhibitors for the Treatment of Dementia. *Mini Rev. Med. Chem.* 16, 605–618. doi:10.2174/1389557515666150709104731

Turkiewicz, I. P., Wojdyło, A., Tkacz, K., Nowicka, P., and Hernández, F. (2019). Antidiabetic, Anticholinesterase and Antioxidant Activity vs. Terpenoids and Phenolic Compounds in Selected New Cultivars and Hybrids of Artichoke Cynara Scolymus L. *Molecules* 24. doi:10.3390/molecules24071222

Umeno, A., Horie, M., Murotomi, K., Nakajima, Y., and Yoshida, Y. (2016). Antioxidative and Antidiabetic Effects of Natural Polyphenols and Isoflavones. *Molecules* 21. doi:10.3390/molecules21060708

Vallverdú-Queralt, A., Regueiro, J., Martínez-Huélamo, M., Rinaldi Alvarenga, J. F., Leal, L. N., and Lamuela-Raventos, R. M. (2014). A Comprehensive Study on the Phenolic Profile of Widely Used Culinary Herbs and Spices: Rosemary, Thyme, Oregano, Cinnamon, Cumin and bay. *Food Chem.* 154, 299–307. doi:10.1016/j.foodchem.2013.12.106

Velkoska-Markovska, L., Jankulovska, M. S., Petanovska-Ilievska, B., and Hristovski, K. (2020). Development and Validation of RRLC-UV Method for Determination of Chlorogenic Acid in green Coffee. *Acta Chromatographica* 32, 34–38. doi:10.1556/1326.2019.00547

Vierling, W., Brand, N., Gaedcke, F., Sensch, K. H., Schneider, E., and Scholz, M. (2003). Investigation of the Pharmaceutical and Pharmacological Equivalence of Different Hawthorn Extracts. *Phytomedicine* 10, 8–16. doi:10.1078/094471103321648601

Wang, L. C., Wei, W. H., Zhang, X. W., Liu, D., Zeng, K. W., and Tu, P. F. (2018). An Integrated Proteomics and Bioinformatics Approach Reveals the Anti-inflammatory Mechanism of Carnosic Acid. *Front. Pharmacol.* 9, 370. doi:10.3389/fphar.2018.00370

Wang, S., Ouyang, B., Aa, J., Geng, J., Fei, F., Wang, P., et al. (2016). Pharmacokinetics and Tissue Distribution of Ginkgolide A, Ginkgolide B, and Ginkgolide K after Intravenous Infusion of Ginkgo Diterpene Lactones in a Rat Model. *J. Pharm. Biomed. Anal.* 126, 109–116. doi:10.1016/j.jpba.2016.04.035

Wang, Y. H., Meng, Y., Zhai, C., Wang, M., Avula, B., Yuk, J., et al. (2019). The Chemical Characterization of Eleutherococcus Senticosus and Ci-Wu-jia Tea Using UHPLC-UV-QTOF/MS. *Int. J. Mol. Sci.* 20. doi:10.3390/ijms20030475

Willems, J. L., Khamis, M. M., Mohammed Saeid, W., Purves, R. W., Katselis, G., Low, N. H., et al. (2016). Analysis of a Series of Chlorogenic Acid Isomers Using Differential Ion Mobility and Tandem Mass Spectrometry. *Anal. Chim. Acta* 933, 164–174. doi:10.1016/j.aca.2016.05.041

Wilson, W. B., and Sander, L. C. (2018). Method Development for the Certification of a Ginsenoside Calibration Solution via Liquid Chromatography with Absorbance and Mass Spectrometric Detection. *J. Chromatogr. A.* 1574, 114–121. doi:10.1016/j.chroma.2018.09.011

Wojdylo, A., Oszmianski, J., and Czemerys, R. (2007). Antioxidant Activity and Phenolic Compounds in 32 Selected Herbs. *Food Chem.* 105, 940–949. doi:10.1016/j.foodchem.2007.04.038

Wong, Y. F., Cacciola, F., Fermas, S., Riga, S., James, D., Manzin, V., et al. (2018). Untargeted Profiling of Glycyrrhiza Glabra Extract with Comprehensive Two-Dimensional Liquid Chromatography-Mass Spectrometry Using Multi-Segmented Shift Gradients in the Second Dimension: Expanding the Metabolic Coverage. *Electrophoresis* 39, 1993–2000. doi:10.1002/elps.201700469

Wu, B., Song, H. P., Zhou, X., Liu, X. G., Gao, W., Dong, X., et al. (2016). Screening of Minor Bioactive Compounds from Herbal Medicines by In Silico Docking and the Trace Peak Exposure Methods. *J. Chromatogr. A.* 1436, 91–99. doi:10.1016/j.chroma.2016.01.062

Ye, Y., Chou, G. X., Mu, D. D., Wang, H., Chu, J. H., Leung, A. K., et al. (2010). Screening of Chinese Herbal Medicines for Antityrosinase Activity in a Cell Free System and B16 Cells. *J. Ethnopharmacol* 129, 387–390. doi:10.1016/j.jep.2010.04.009

Yilmaz, M. A. (2020). Simultaneous Quantitative Screening of 53 Phytochemicals in 33 Species of Medicinal and Aromatic Plants: A Detailed, Robust and Comprehensive LC-MS/MS Method Validation. *Ind. Crops Prod.* 149, 112347. doi:10.1016/j.indcrop.2020.112347

Yilmaz, Y., and Toledo, R. T. (2004). Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. *J. Agric. Food Chem.* 52, 255–260. doi:10.1021/jf030117h

Yu, J. Y., Ha, J. Y., Kim, K. M., Jung, Y. S., Jung, J. C., and Oh, S. (2015). Anti-Inflammatory Activities of Licorice Extract and its Active Compounds, Glycyrrhizic Acid, Liquiritin and Liquiritigenin, in BV2 Cells and Mice Liver. *Molecules* 20, 13041–13054. doi:10.3390/molecules200713041

Yuan, H., Ma, Q., Ye, L., and Piao, G. (2016). The Traditional Medicine and Modern Medicine from Natural Products. *Molecules* 21, 559. doi:10.3390/molecules21050559

Zhang, C., Lu, Y., Tao, L., Tao, X., Su, X., and Wei, D. (2007). Tyrosinase Inhibitory Effects and Inhibition Mechanisms of Nobiletin and Hesperidin from Citrus Peel Crude Extracts. *J. Enzyme Inhib. Med. Chem.* 22, 91–98. doi:10.1080/14756360600953876

Zhang, X., Zhang, Y., Shi, P., Bi, Z., Shan, Z., and Ren, L. (2021). The Deep challenge of Nitrate Pollution in River Water of China. *Sci. Total Environ.* 770, 144674. doi:10.1016/j.scitotenv.2020.144674

Zheng, J., Kallio, H., and Yang, B. (2016). Sea Buckthorn (Hippophaë Rhamnoides Ssp. Rhamnoides) Berries in Nordic Environment: Compositional Response to Latitude and Weather Conditions. *J. Agric. Food Chem.* 64, 5031–5044. doi:10.1021/acs.jafc.6b00682

Zhou, Y.-Q., Liu, H., He, M.-X., Wang, R., Zeng, Q.-Q., Wang, Y., et al. (2018). "A Review of the Botany, Phytochemical, and Pharmacological Properties of Galangal," in *Natural And Artificial Flavoring Agents and Food Dyes*. Editors A. M. Grumezescu and A. M. Holban (London: Academic Press, an imprint of Elsevier), 351–396. doi:10.1016/b978-0-12-811518-3.00011-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 20 December 2021
doi: 10.3389/fphar.2021.774674

Artesunate Combined With Metformin Ameliorate on Diabetes-Induced Xerostomia by Mitigating Superior Salivatory Nucleus and Salivary Glands Injury in Type 2 Diabetic Rats via the PI3K/AKT Pathway

Siqin Zhang[1†], Jiarui Li[1†], Xiaolin Nong[1,2†], Yuxiang Zhan[1], Jiazhi Xu[1], Danni Zhao[1], Chubin Ma[1], Yuchen Wang[1], Yixing Li[1], Zhan Li[1] and Jiaquan Li[3,4]*

[1]College of Stomatology, Guangxi Medical University, Nanning, China, [2]Guangxi Key Laboratory of Oral and Maxillofacial Surgery Disease Treatment, Nanning, China, [3]Medical Science Research Center, Guangxi Medical University, Nanning, China, [4]Life Science Institute, Guangxi Medical University, Nanning, China

OPEN ACCESS

Edited by:
Massimo Lucarini,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Fei Liu,
Texas A&M University, United States
Toshiyuki Matsuzaki,
Gunma University, Japan
Changyu Zheng,
National Institutes of Health (NIH), United States

***Correspondence:**
Xiaolin Nong
xnong@gxmu.edu.cn

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 12 September 2021
Accepted: 09 November 2021
Published: 20 December 2021

Citation:
Zhang S, Li J, Nong X, Zhan Y, Xu J, Zhao D, Ma C, Wang Y, Li Y, Li Z and Li J (2021) Artesunate Combined With Metformin Ameliorate on Diabetes-Induced Xerostomia by Mitigating Superior Salivatory Nucleus and Salivary Glands Injury in Type 2 Diabetic Rats via the PI3K/AKT Pathway. Front. Pharmacol. 12:774674. doi: 10.3389/fphar.2021.774674

Polydipsia and xerostomia are the most common complications that seriously affect oral health in patients with diabetes. However, to date, there is no effective treatment for diabetic xerostomia. Recent studies have reported that artesunate (ART) and metformin (Met) improve salivary gland (SG) hypofunction in murine Sjögren's syndrome. Therefore, aim of this study was to investigate the effect and underlying mechanism of artesunate (ART) alone and in combination with metformin (Met) on hyposalivation in type 2 diabetes mellitus (T2DM) rats. T2DM rats were induced using a high-fat diet and streptozotocin. SPF male Sprague–Dawley rats were divided into the following five groups: normal control group, untreated diabetic group, ART-treated diabetic group (50 mg/kg), Met-treated diabetic group (150 mg/kg), and ART/Met co-treated diabetic group (50 mg/kg ART and 150 mg/kg Met). ART and Met were intragastrically administered daily for 4 weeks. The general conditions, diabetes parameters and serum lipids were evaluated after drug treatment. Furthermore, we observed changes in the central superior salivatory nucleus (SSN) and SG, and changes in the AQP5 expression, parasympathetic innervation (AChE and BDNF expression), and PI3K/AKT pathway- (p-AKT, and p-PI3K), apoptosis- (Bax, Bcl-2, and Caspase3), and autophagy- (LC3 and P62) related markers expression in T2DM rats after treatment. Our results showed that ART or Met alone and ART/Met combination attenuated a range of diabetic symptoms, including weight loss, urine volume increase, water consumption increase, hyperglycemia, insulin resistance, glucose intolerance and dyslipidemia. More

Abbreviations: AB-PAS, Alcian Blue Periodic acid Schiff; AChE, Acetylcholinesterase; ANOVA, Analysis of variance; AQP5, Aquaporin5; ART, Artesunate; AUC, Area under the curve; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; BDNF, Brain-derived neurotrophic factor; FBG, Fasting blood glucose; HDL, High-density lipoprotein; H&E, Hematoxylin and eosin; LDL, Low-density lipoprotein; MET, Metformin; OGTT, Oral glucose tolerance; PI3K/AKT, Phosphatidylinositol 3′-kinase/protein kinase B; SG, Salivary gland; SMGs, Submandibular glands; SSN, Superior salivatory nucleus; STZ, Streptozotocin; SQSTM1/p62, Sequestosome 1; T2DM, Type 2 Diabetes mellitus; TC, Total cholesterol; TG, Triglycerides.

importantly, we found that these three treatments, especially ART/Met combination, mitigated hyposalivation in the T2DM rats via improving the central SSN and SGs damage in hyperglycemia. Our data also indicated that ART/Met attenuated SG damage though regulating the PI3K/Akt pathway to inhibit apoptosis and autophagy of SGs in the T2DM rats. Moreover, ART/Met preserved parasympathetic innervation (AChE and BDNF expression) in SGs to alleviate diabetes-induced hyposalivation likely through rescuing central SSN damage. Taken together, these findings might provide a novel rationale and treatment strategy for future treatment of diabetes-induced xerostomia in the clinic.

Keywords: artesunate, metformin, diabetic xerostomia, salivary gland, superior salivatory nucleus, PI3K/Akt pathway

INTRODUCTION

Diabetes mellitus (DM) is a chronic multiple disease characterized by polyuria, polydipsia, polyphagia and weight loss and usually causes a systemic and multi-organ damage. Among them, diabetic xerostomia is one of the most common oral manifestations that seriously impacts the quality of life of diabetes patients (López-Pintor et al., 2016; Molania et al., 2017; Huang et al., 2018b); however, only few of the current studies have explored its causes. Studies have reported that diabetic patients with xerostomia are more susceptible to dental caries, oral candidiasis, taste disorders, gingivitis, fissured tongue, and other neurosensory disorders (Kudiyirickal and Pappachan, 2015; Mauri-Obradors et al., 2017), particularly in elderly people (Albert et al., 2012; Lima et al., 2017). In addition, accumulating evidence suggests that long-term oral infections secondary to diabetic xerostomia, such as periodontitis and dental caries, affect glycaemic control in diabetic patients, and also facilitate onset or progression of diabetes and its complications (Darré et al., 2008; Liccardo et al., 2019). However, the available treatment strategies for relieving or improving the symptoms of dry mouth are generally unsatisfactory and remain palliative. Therefore, this calls for development of new treatment strategies for diabetic patients with xerostomia.

Metformin (Met), a synthetic biguanide, is a first-line drug used in the clinic to treat and prevent type 2 diabetes mellitus (T2DM) and its various complications (Sanchez-Rangel and Inzucchi, 2017). Decades of clinical applications have proved that Met is safe and well-tolerated (Hostalek et al., 2015). Artesunate (ART) is a water-soluble derivative of artemisinin that is characterized by low toxicity, excellent tolerance, and multiple routes of administration such as oral and intravenous injection (Hien and White, 1993; Zuo et al., 2016). Furthermore, several ART pharmacological studies have demonstrated that it possesses a wide range of biological activities, with exception of anti-malarial effects, including anti-tumor, anti-microbes, anti-inflammation, antioxidant, and regulation of the immune system (Yu et al., 2016; Våtsveen et al., 2018; Zhang et al., 2018; Li et al., 2019). It has been demonstrated that Met mitigates hyposalivation and salivary gland (SG) inflammation in non-obese diabetic (NOD)/ShiLtj mice, an animal model of murine Sjögren's syndrome, via activating 5′ adenosine monophosphate-activated protein kinase (Kim et al., 2019). In addition, one recent study has reported that ART ameliorates salivary secretion dysfunction in NOD/ShiLtj mice via regulating the TRAF6-mediated NF-κB signaling pathway (Zhan et al., 2021). Importantly, ART is not only able to pass through the blood-brain barrier and maintain a high concentration in the brain (Zhao and Song, 1989; Zuo et al., 2016), but is also able to adjust glucose homeostasis against diabetes and its complications (Sun et al., 2018; Xu et al., 2019; Maurya et al., 2021). Moreover, our previous study also showed that ART controlled blood glucose levels and prevented cardiovascular complications in diabetic rats (Chen et al., 2021). However, to date, no study has investigated effects of ART alone or in combination with Met against diabetic xerostomia and elucidated the underlying mechanisms.

Xerostomia, also known as dry mouth, is mainly characterized by reduced saliva secretion (Bhattarai et al., 2018). It is well known that salivary secretion is regulated by superior salivatory nucleus (SSN) located in the lateral reticular formation of the brainstem (Mitoh et al., 2017). Most of the currently available studies have primarily focused on pathophysiology of salivary gland injury caused by hyperglycemia (Huang et al., 2018b; Lee et al., 2020; Xiang et al., 2020), with few studies focusing on the pathological changes of SSN that regulates saliva secretion in hyperglycemia. Therefore, one of the primary objectives of this study was to fill the gap in the literature, which can aid in the development of therapeutic strategies to ameliorate diabetes-induced hyposalivation. We hypothesized that diabetic xerostomia may be associated with the SSN damage, SG injury, or both caused by hyperglycemia. Besides, the PI3K/AKT signaling pathway, which widely exists in eukaryotes, is associated with a variety of biological processes, including metabolism, cytoskeleton reorganization, macromolecular synthesis, cell proliferation, and differentiation (Keppler-Noreuil et al., 2016; Very et al., 2018). Recent studies have also demonstrated that deregulation of the PI3K/AKT pathway is a key feature of diabetes and its complications due to the pathway's involvement in the regulation of insulin and glucose homeostasis (Huang et al., 2016; Maffei et al., 2018). Therefore, this study further investigated the effects of administering ART alone or in combination with Met on

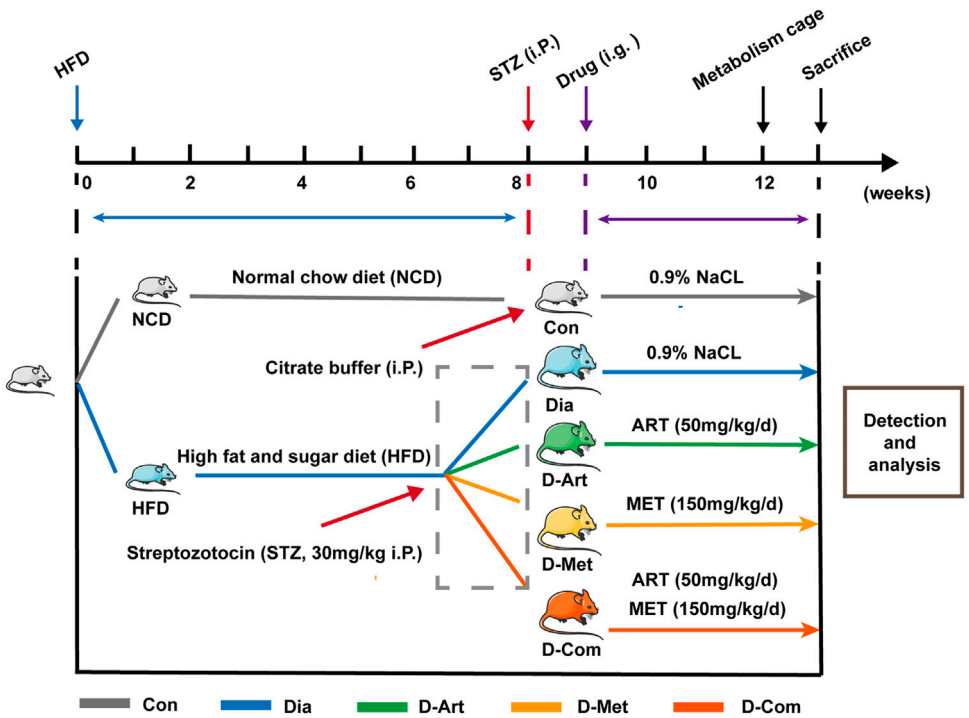


FIGURE 1 | Experimental design. STZ was intraperitoneally injected at 8 weeks after NCD or HFD feeding. Rats with HFD/STZ treatment respectively received intragastric administration of ART, Met, and ART/Met combination for four consecutive weeks at 9 weeks after feeding. Animals were placed in metabolism cages, followed by measurement of metabolic parameters (urine output, food consumption, and water intake) at 12 weeks after feeding. Finally, rats were sacrificed at 4 weeks post drug treatment for various analyses. ART, artesunate; Met, metformin. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

central SSN and SG in the T2DM rat model based on the PI3K/AKT pathway. Furthermore, we evaluated whether a combination of ART with Met would be more efficacious than administration of either drug alone with regard to diabetes-induced hyposalivation.

MATERIALS AND METHODS

Animals

SPF male Sprague–Dawley rats aged 5 weeks (150–180 g) were obtained from the animal center of Guangxi Medical University (Nanning, China). All rats were housed under controlled conditions with room temperature at 22 ± 2°C, 12/12 h light/dark cycle, and 55 ± 5% relative humidity. The rats were acclimatized for 1 week by providing food and water ad libitum. All procedures involving animal experiments were approved by the Animal Ethics Committee of Guangxi Medical University (No. 202006013).

Animal Grouping and Experimental Design

The high-fat high-sugar diet (HFD, boaigang-B1135DM, Boaigang Biotechnology Co. Ltd., Beijing, China) and low dose of streptozotocin (STZ; Sigma, St. Louis, MO) were used to establish a rat model of type 2 diabetes mellitus (T2DM) according to a previously described protocol (Kelany et al., 2016; Nie et al., 2018). After acclimation for 1 week, the rats were randomly assigned into two different dietary formulas: standard chow diet and HFD diet post (**Figure 1**). All animals were fasted overnight (16 h) after 8 weeks of HFD-feeding. Rats in the HFD dietary group received a single dose of STZ (30 mg/kg body weight, BW) dissolved in a freshly prepared pH 4.2 citrate buffer by intraperitoneal injection (i.p), while an equivalent volume of citrate buffer was administered to the rats fed with a normal standard diet. Fasting blood glucose (FBG) was determined at the tail vein using Accu-Chek Active test strips (Roche Diabetes Care GmbH, Mannheim, Germany) at 3 and 7 days after STZ administration. Notably, rats with a FBG of >11.1 mmol/L were considered as T2DM models (Zeng et al., 2019). Next, T2DM rats were randomly divided into four different experimental groups (n = 8/group): Untreated diabetic group (Dia), ART-treated diabetic group (D-Art), Met-treated diabetic group (D-Met), and ART/Met co-treated diabetic group (D-Com). In addition, rats fed on the standard chow diet were classified into the normal control group (Con). One week after STZ injection, the treatments were administrated by gavage to rats in each group, once a day, consecutively for 4 weeks (**Figure 1**). Rats in ART-treated, Met-treated, and ART/Met co-treated diabetic groups were treated with ART alone (50 mg/kg; Guilin Pharmaceutical Co., Ltd., Guilin, China),

Met alone (150 mg/kg; Shenzhen Neptune Pharmaceutical Co., Ltd., Shenzhen, China), and ART (50 mg/kg) combined with Met (150 mg/kg), respectively, whereas the non-diabetic control and untreated diabetic rats received equivalent doses of saline. Notably, the administered ART and Met doses were in accordance with previous studies (Cai et al., 2018; Bradley et al., 2019; Inyang et al., 2019; Chen et al., 2021).

Evaluation of General Conditions

BW was measured weekly after drug treatments. Moreover, the 24 h urine volume, daily food intake, and daily water intake were examined using metabolic cages at 4 weeks post drug treatments.

Measurement of FBG, Oral Glucose Tolerance and Insulin Resistance

FBG levels were detected post drug administration. Oral glucose tolerance test (OGTT) was performed to evaluate oral glucose tolerance of each group after 12 weeks. Animals were given intragastric administration of glucose solution (2 g/kg BW) following a 12 h fasting period. Blood glucose levels were respectively measured at 0, 30, 60, 90, and 120 min post glucose administration using the tail prick method. Serum insulin levels were assayed using the Rat Insulin (INS) ELISA Kit (Cat#CSB-E05070r, Cusabio Biotech Co. Ltd., Wuhan, China) according to the manufacturer's instructions. Finally, homeostatic model assessment of insulin resistance (HOMA-IR), used to quantify insulin resistance, was calculated using the HOMA-IR formula: HOMA-IR = FBG (mmol/L) × Fasting blood insulin (μU/mL)/22.5.

Measurement of Saliva Secretion

Saliva secretion was estimated at 4 weeks after drug treatment. The volume of saliva secretion was collected using several small degreased cotton balls and pieces according to a previously described protocol (Kojima et al., 2011). Briefly, rats were anesthetized with 1% sodium pentobarbital (40 mg/kg) and then subcutaneously injected with 0.2% pilocarpine (0.2 mg/kg). Next, pre-weighed small cotton balls were placed into the sublingual region of oral cavity post pilocarpine stimulation. After collection for 30 min, all cotton balls were removed from the oral cavity and immediately weighed using a weigh balance. Finally, saliva secretion was calculated and normalized to BW.

Evaluation of Biochemical Parameters

Levels of serum lipids, including triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C) were analyzed using the automatic biochemical analyzer (Mindray Co. Ltd., Shenzhen, China) at the end of experiment.

Sample Collection and Preparation

On the final day of the experiment, animals were anaesthetized and blood samples were collected from the inferior vena cava, followed by centrifugation at 3,000 rpm for 10 min at 4°C. After centrifugation, the serum was retained for use in further corresponding analyses. Next, submandibular glands (SMGs), brainstem, and pancreas tissue samples of rats were quickly isolated, weighed, and rinsed after euthanasia. The samples were immediately placed into liquid nitrogen and then stored at −80°C or fixed in 4% paraformaldehyde solution until further analysis.

Observation of Ultrastructure in SMG and SSN

At the end of the experiment, a transcardial perfusion using 2.5% glutaraldehyde and 4% paraformaldehyde mixed fixative in 0.1 M mol phosphate buffer (PB, pH 7.4) was performed on anesthetized rats. Next, SMG and SSG tissues were rapidly isolated and fixed in 2.5% glutaraldehyde at 4°C for 24 h, followed by 1% osmium tetroxide at 4°C for 3 h. After gradient alcohol dehydration, specimens were infiltrated and embedded using epoxy resin. A transmission electron microscope (TEM) (HITACHI H-7000, Tokyo, Japan) was then used to observe the ultrastructure in SMG and SSN tissues, including acinar cells, ductal cells, and secretory granules of the SMG and axon of the SSN. Moreover, at least 100 axon diameters of the SSN were determined in each group using ImageJ software in accordance with a previous study (Ito et al., 2016). Subsequently, the G-ratio was calculated using the following formula: G-ratio = inner axonal diameter (d)/(outer axonal diameter) (D), as shown in **Figure 5C**.

Histopathological Analysis

The tissues (SMGs, pancreas, and brain stem) were fixed in 4% paraformaldehyde solution for 48 h, dehydrated, and embedded in paraffin. Paraffin-embedded specimens were cut into 4 μm-thick sections using paraffin slicing machine (Leica, Braunschweig, German). Sections obtained from SMGs and pancreas tissues were stained with hematoxylin and eosin (H&E) and Alcian Blue Periodic acid Schiff (AB-PAS), while brain tissue sections were stained with H&E and Nissl. Finally, the sections were observed and images were captured using an optical microscope (Olympus, Tokyo, Japan). According to published studies (Sefi et al., 2011; Huang et al., 2018b; Tian et al., 2021), quantitative analyses of histology in the pancreas, SMGs and SSN were respectively performed using ImageJ software. Notably, five different images were captured per rat and at least three different rats were used from each group for comparative analysis.

Immunohistochemistry and Immunofluorescence Staining

Tissue sections (4 μm-thick) were dewaxed in dimethylbenzene, followed by high-pressure treated antigen retrieval for 5 min in a pH 6.0 citrate buffer heated at boiling temperature. Next, sections were immersed into 3% hydrogen peroxide to block endogenous peroxidase for 15 min and then

washed three times in a pH 7.4 phosphate buffer saline (PBS, 0.01 mol/L) for 5 min each time. After blocking in serum buffer at room temperature for 20 min, the sections were incubated with the primary antibodies, including anti-aquaporin5 (AQP5) (1:250, ab78486, Abcam, Cambridge, United Kingdom) and anti-acetylcholinesterase (AChE) (1: 200, Bioss Biological Technology Co., Ltd., Beijing, China) at 4°C overnight. Next, sections were rinsed in PBS three times for 5 min each time, followed by incubation with HRP-conjugated or fluorescence labeled (Alexa Fluor 594® anti-rabbit IgG, Cell Signaling Technology, Danvers, MA, United States) secondary antibodies at room temperature for 1 h. The sections were then respectively stained with 3,3′-diaminobenzidine (DAB) or counterstained with DAPI. The stained sections were observed and images captured using an optical microscope or a laser scanning confocal microscope (Olympus, Tokyo, Japan). Finally, the images were evaluated using Image-Pro Plus 6.0 Software (Media Cybernetics, CA, United States) according to a protocol described in a previous study (Li et al., 2020). The mean density was calculated as follows: mean density = integral optical density (IOD)/area. Notably, the reported values correspond to the average of at least five representative images obtained from three different animals from each group.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End Labeling

TUNEL staining in SMG was conducted using *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany), according to manufacturer's instruction. Apoptosis levels in each group were evaluated by counting TUNEL positive cells with ImageJ.

Quantification of Brain-Derived Neurotrophic Factor in SMG Using Enzyme-Linked Immunosorbent Assay

Tissue samples were centrifuged at 12,000 rpm for 10 min at 4°C to collect supernatants, followed by assessment of BDNF levels in SMG tissues using rat ELISA kits according to the manufacturer's instructions (Cat#D731142, Sangon Biotech Co. Ltd., Shanghai, China). Next, the resulting colors were quantified using a plate reader (Bio Tech Instruments, United States) at 450 nm.

Western Blot Analysis

Protein samples were extracted using RIPA lysis buffer with phosphatase and protease inhibitors according to the manufacturer's protocol. Western blot analysis was conducted according to a protocol described in a previous study (Ferenczyova et al., 2020). Briefly, non-closure sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) color preparation kits (Cat#C681100, C681101, and C681103, Sangon Biotech Co., Ltd., Shanghai, China) were used to resolve the protein samples extracted from all groups. Next, protein samples were transferred to polyvinylidene difluoride (PVDF) membranes using the wet electrotransfer method. Non-specific binding sites of the membranes were blocked with 5% non-fat milk, followed by incubation with primary antibodies at 4°C overnight. Membranes were then incubated with secondary antibodies in a swing bed at room temperature for 1 h. Finally, enhanced chemiluminescence substrate (ECL) (Cat#BL520, Lanjike Technology Co., Ltd., Biosharp, Hefei, China) was used to detect horseradish peroxidase (HRP) signal.

Molecular Docking of ART to Akt and PI3K Protein

Firstly, the three-dimensional structure of ART molecule in Conformer SDF format (Compound CID: 6917864) was obtained from the NCBI database (https://pubchem.ncbi.nlm.nih.gov/), followed by construction of the homology model of Akt (PDB ID: 2UZT) and PI3K (PDB ID: 1E7V) protein molecules based on the Protein Data Bank (PDB) database (https://www.rcsb.org/). Next, desolventization of above molecules was performed using the PyMOL software in accordance with the published literatures. The AutoDock1.5.6 software was then used to conduct molecular dockings, specifically setting the docking algorithm to Lamarckian Genetic Algorithm. Finally, the binding site was defined by selecting the appropriate size around the bound co-crystal ligand.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software Inc. San Diego CA, United States). The results were expressed as mean ± standard deviation (SD). One-/two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used to compare differences among groups. $p < 0.05$ was considered statistically significant.

RESULTS

ART or Met Alone and ART/Met Combination Alleviate General Conditions in the T2DM Rats

To investigate the effects of ART alone or in combination with Met on general conditions in T2DM rats, the BW of rats was measured every week after drug administration, and urine output, water intake, and food intake were measured 1 week before the terminal experiment. Results showed that BW in the untreated and drug-treated diabetic groups was significantly increased compared to the control group after 8 weeks of HFD feeding, while weight loss was observed in the untreated and drug-treated diabetic groups from 3 weeks post streptozotocin (STZ) administration (**Figure 2B**). ART/Met combination significantly increased BW of diabetic rats from 3 weeks after drug administration, while ART or Met alone significantly increased BW from 4 weeks after drug administration. In addition, BW was lower in rats receiving

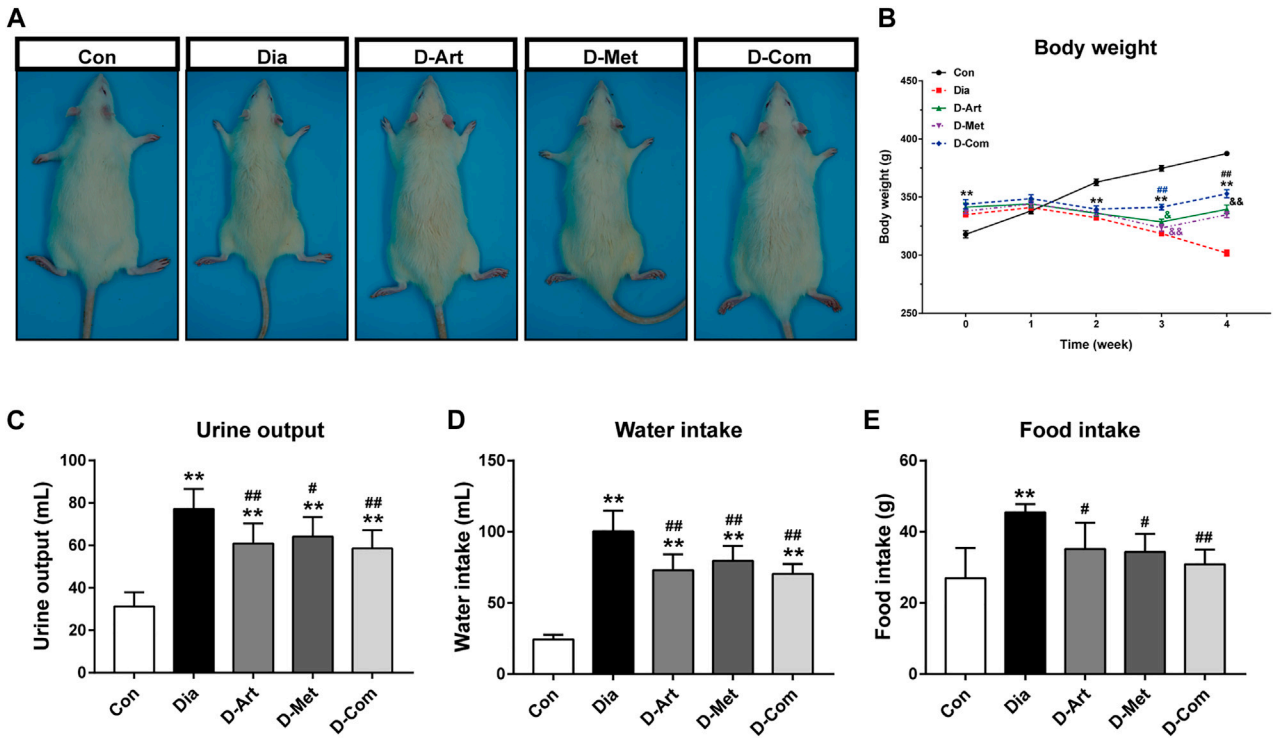


FIGURE 2 | ART or Met alone and ART/Met combination alleviate general conditions in the T2DM rats. **(A)** Gross appearance of each group of rats. **(B–E)** Body weight, urine output, water intake, and food intake in rats of each group (8 rats per group). Data are expressed as the mean ± SD. $^{**}p < 0.01$ versus Con group; $\#p < 0.05$ and $\#\#p < 0.01$ versus Dia group. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

ART or Met alone treatment than in rats with ART/Met combination at 3 weeks after drug administration, and no significant difference in BW was observed between the ART- and Met-treated groups. At the end of the experiment, an obvious decrease in BW (**Figures 2A,B**), and a significant increase in urine output (**Figure 2C**), and food and water intake (**Figures 2D,E**) were observed in the untreated diabetic rats compared to the control rats. Conversely, the drug-treated (ART, Met, and ART/Met combination) diabetic rats showed a significant decrease in urine output, food consumption, and water intake compared to the untreated diabetic rats, and there was no significant difference in these parameters among the three treatment groups.

ART or Met Alone and ART/Met Combination Alleviate Typical T2DM Characteristics and Pancreatic Injury in the T2DM Rats

To examine whether ART alone or in combination with Met could ameliorate hyperglycemia, hyperlipidemia, insulin resistance and pancreatic injury in T2DM rats, we assessed glycemic control, glucose tolerance (OGTT), insulin resistance (HOMA-IR), serum lipid profiles (TG, TC, LDL-C, and HDL-C), and pancreas histology post drug administration. Results showed that FBG levels were markedly increased in diabetic rats compared to the Con group from 1 week after STZ injection (**Figure 3A**). A significant decline over time in FBG level was observed in three drug treatment groups. The results also indicated that ART significantly diminished FBG levels compared to the untreated diabetic group 2 weeks post drug administration, while Met alone and ART/Met combination decreased FBG levels 1 weeks post drug administration. However, there was no significant difference in FGB level between the Met treated group and the ART/Met co-treated group. Moreover, the HOMA-IR index and glucose area under the curve (AUC) for the OGTT value were significantly higher in diabetic rats than in non-diabetic control rats (**Figures 3B–D**). In contrast, the HOMA-IR index and glucose AUC were significantly reduced after ART, Met, and ART/Met combination treatments compared to the untreated group. Notably, HOMA-IR index and glucose AUC were significantly higher in ART-treated group rats than in the ART/Met co-treated group, while no significant difference was observed between the Met-treated and ART/Met co-treated groups (**Figures 3B–D**). In addition, diabetic rats in the untreated and Met-treated groups exhibited an obvious elevation in serum TG, TC, and LDL-C levels (**Figures 3E,F,H**), whereas the only untreated diabetic rats showed a reduction in HDL-C level (**Figure 3G**) compared to the control

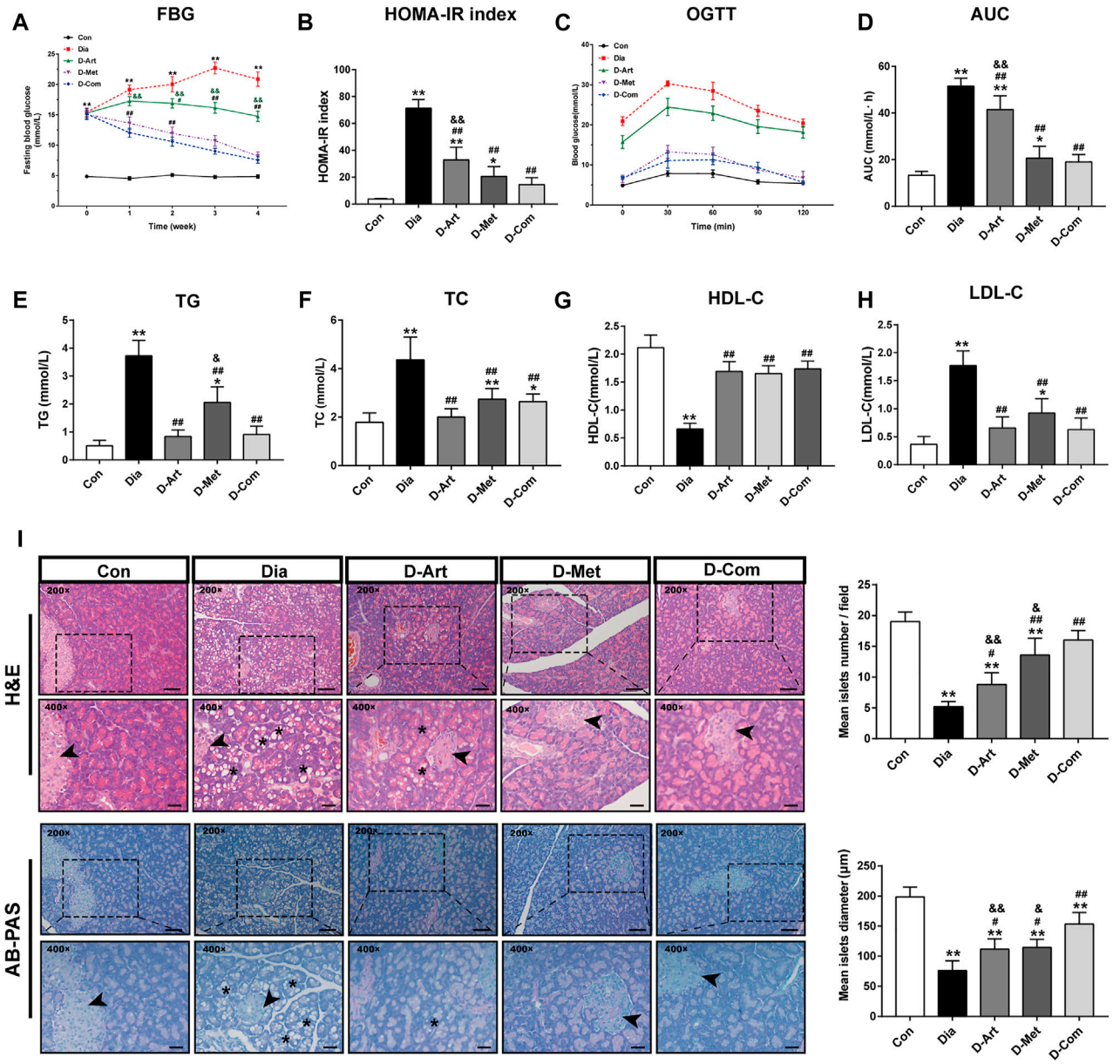


FIGURE 3 | ART or Met alone and ART/Met combination alleviate typical T2DM characteristics and pancreatic injury in the T2DM rats. **(A)** FBG, **(B)** HOMA-IR index, **(C)** OGTT, **(D)** AUC of the OGTT, **(E)** TG, **(F)** TC, **(G)** HDL-C, and **(H)** LDL-C of rats in each group (8 rats per group). **(I)** H&E and AB-PAS staining of pancreas tissues in each group (5 rats per group), scale bar = 50 μm (200×); scale bar = 20 μm (400×). Pancreas islet sites are labeled using black arrows, while vacuolation is labeled using black *. Data are expressed as the mean ± SD. *$p < 0.05$ and **$p < 0.01$ versus Con group; #$p < 0.05$ and ##$p < 0.01$ versus Dia group; &$p < 0.05$ and &&$p < 0.01$ versus D-Com group. OGTT, oral glucose tolerance test; AUC, area under the curve; TG, total glyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; H&E, Hematoxylin & eosin; AB-PAS, Alcian Blue Periodic acid Schiff. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

group rats. Conversely, rats from the three treatment groups showed a significant decrease in serum TG, TC, and LDL-C levels, and an increase in the serum HDL-C levels, but no significant difference was observed among the three treatment groups in serum TC, HDL-C, and LDL-C levels ($p > 0.05$).

Figure 3I showed the pancreatic histopathology results. Pancreatic histology in the untreated diabetic group showed a large number of diffuse vacuoles in pancreatic cells, a significant reduction in islet number and diameter, compared to the normal control group. However, these abnormal histopathological changes were significantly rescued by ART, Met, and ART/Met combination treatments, particularly in the ART/Met combination treatment group.

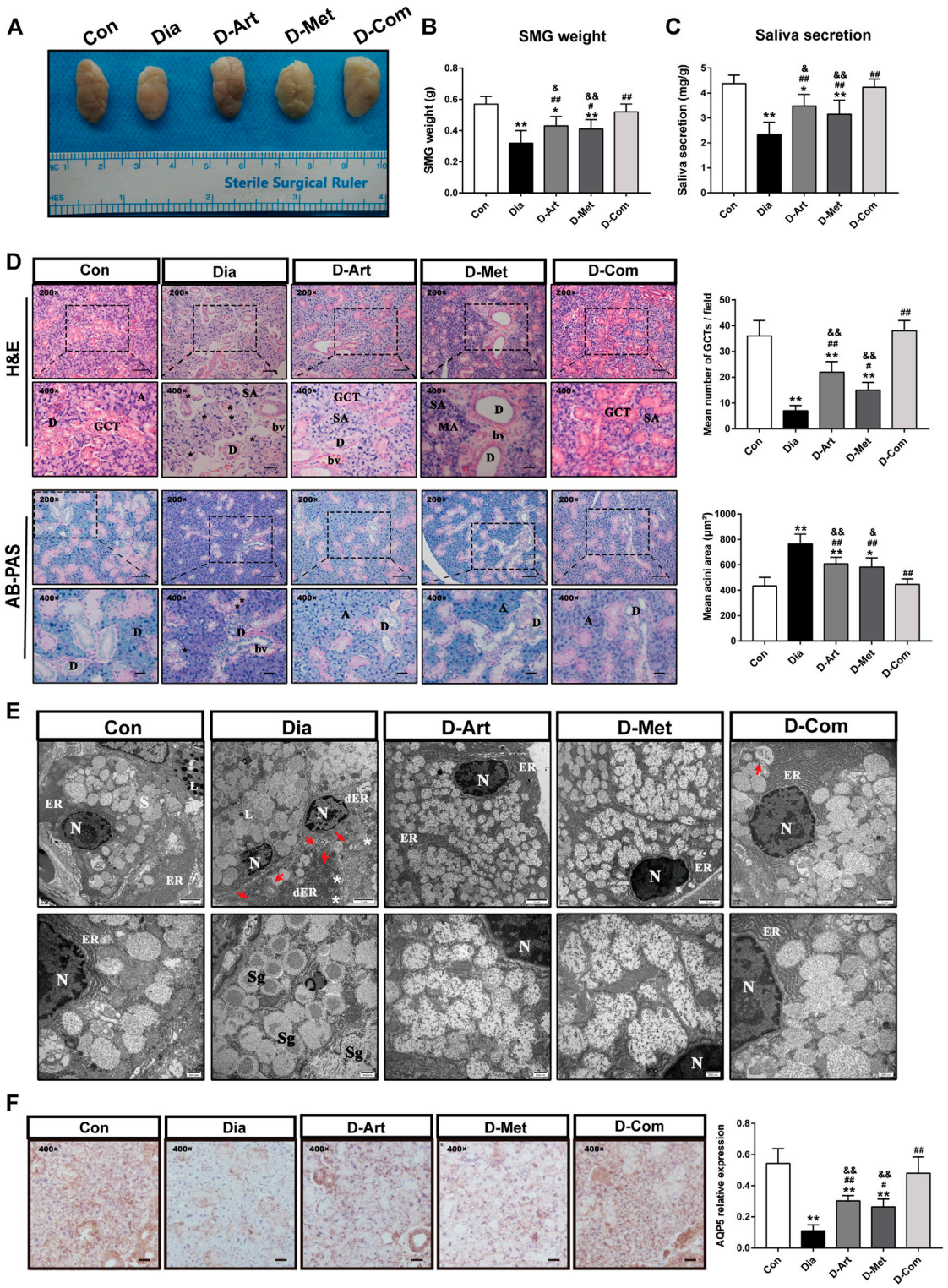


FIGURE 4 | ART or Met alone and ART/Met combination mitigate SGs' dysfunction and pathological alterations in the T2DM rats. **(A)** Gross morphology and **(B)** weight of SMGs in each group. **(C)** Saliva secretion of rats in each group (8 rats per group). **(D)** H&E and AB-PAS staining of SMGs in each group (5 rats per group), scale bar = 50 μm (200×); scale bar = 20 μm (400×). A, acini; SA, serous acini; MA, mucous acini; D, duct; GCT, granular convoluted tubules; bv, blood vessel. Vacuolation is labeled using black*. **(E)** Ultrastructure of SMGs in each group. N, nucleus; ER, endoplasmic reticulum; dER, disrupted endoplasmic reticulum; L, Lysosome; Sg, secretory granules. White * represent swollen and disrupted mitochondria and red arrows represent autophagosomes. **(F)** Immunohistochemistry analysis of AQP5

(*Continued*)

FIGURE 4 | expression in SMGs of each group, scale bar = 20 μm (400×). Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ versus Con group; #$p < 0.05$, ##$p < 0.01$ versus Dia group; &$p < 0.01$, &&$p < 0.01$ versus D-Com group. H&E, Hematoxylin & eosin; AB-PAS, Alcian Blue Periodic acid Schiff. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

ART or Met Alone and ART/Met Combination Mitigate SGs' Dysfunction and Pathological Alterations in the T2DM Rats

To investigate effects of ART/Met on SGs' function and pathological alterations in T2DM rats, we measured saliva secretion, SMG's weight, morphology, and ultrastructure of SMG. The rats from the Dia, D-ART and D-Met groups showed reduction of saliva secretion and SMG weight compared to the normal control group rats (**Figures 4A–C**). In addition, no obvious difference in saliva secretion and SMG weight was observed between the ART/Met co-treated and normal control groups. However, we noticed that treatment with ART or Met alone and the ART/Met combination markedly recovered saliva secretion and increased SMG weight when compared to the untreated diabetic group, among of which ART/Met combination exhibited the best effect.

H&E staining showed that SMG of the control group had a normal duct and acini structure, with neat arrangement. However, an obvious increase of acinus area and a decrease of granular convoluted tubules (GCT) number, and fibrosis around the ducts were observed in the SMG of T2DM rats (**Figure 4D**). The above abnormal changes were mitigated after ART, Met, and ART/Met combination treatments, with ART/Met combination exhibiting better effects than the other two treatment. Moreover, AB-PAS staining was used to determine changes in acidic mucin (colored blue) and neutral mucin (colored red) (**Figure 4D**). The AB-PAS staining results showed that the SMG of untreated diabetic group rats exhibited stronger staining with purple-red acini and deep blue nucleus than that of the control group, suggesting that more acidic and neutral mucin accumulated in the acini of diabetic SMG. However, the SMG of the three treatment groups were lightly stained by the AB-PAS reagents compared to SMG of the untreated diabetic group. In addition, the ultrastructure of SMG was examined using a TEM (**Figure 4E**). The GCT and acinar cells in the control group possessed round nucleus, clear nuclear membrane, round secretory granules, and well-developed organelles, such as rough endoplasmic reticulum, high electron density lysosomes, and mitochondria. In contrast, nuclear pyknosis, autophagosomes, disrupted endoplasmic reticulum, and swollen and disrupted mitochondria were observed in the untreated diabetic group compared to the control group. However, the three treatments alleviated the above impaired changes in the acinus of diabetic rats. Furthermore, more developed endoplasmic reticulum was observed in the ART/Met treated group compared to that in the ART- and Met-treated groups.

Besides, AQP5 is the most important aquaporin for saliva secretion (D'Agostino et al., 2020). Accordingly, we also evaluated AQP5 expression in SMGs (**Figure 4F**). Results also demonstrated that expression of AQP5 in the Dia group was obvious lower than the Con group. However, these three treatments upregulated AQP5 expression in the T2DM rats when compared with the Dia group. Furthermore, we noticed that the ART/Met combination had the best effect among the three treated group.

ART or Met Alone and ART/Met Combination Alleviate Central SSN Injury and Preserved Peripheral Parasympathetic Innervation of SGs in the T2DM Rats

It is well known that SSN innervates saliva and tear secretion (Ramos et al., 2020). To investigate the mechanism through which ART alone or in combination with Met mitigated diabetes-induced hyposalivation, we first conducted histological analysis (HE and Nissl staining) and ultrastructure examination in SSN. HE staining of SSN in the control group showed normal cell shape, well-defined and clear nuclei (**Figure 5A**). In contrast, markedly neuronal loss, and degenerated neurons with pyknosis and even vacuolization were observed in the untreated diabetic rats compared to that of the normal control group. However, the number of neurons was significantly increased after ART, Met, and ART/Met combination treatments, especially in the ART/Met combination group. Moreover, Nissl staining also confirmed that the density of Nissl bodies in the untreated diabetic group was obviously less than that in the normal control group. However, ART/Met combination treatment significantly increased density of Nissl bodies in the SSN of diabetic rats compared to the untreated diabetic rats and respective monotherapies. Furthermore, damaged axons, disrupted myeline, and decreased mitochondrion were observed in the diabetic rats (**Figures 5B,H**). In addition, mean axon diameter and G-ratio were significantly higher in the untreated diabetic group than those in the normal control group (**Figures 5C–G**). However, three drug treatments improved abnormal morphology of axons and myelin, significantly increased mitochondrion density, and reduced mean axon diameter and G-ratio, with ART/Met combination being superior to the other two monotherapies in increasing mitochondrion density.

Besides, evidence suggests that parasympathetic nerves from central SSN are closely associated with saliva secretion and regeneration of SGs after injury (Mitoh et al., 2017; Kim et al., 2020). It has been reported that BDNF promotes neural renewal (Knox et al., 2013), and AChE is known as an important marker of parasympathetic innervation. Therefore, we further examined the expression of AChEand BDNF in SGs. We observed that AChE and BDNF expression levels were reduced in the untreated diabetic group compared to the normal control group (**Figures 6A–C**). However, ART, Met, and ART/Met treatments significantly increased AChE and BDNF expression, and their

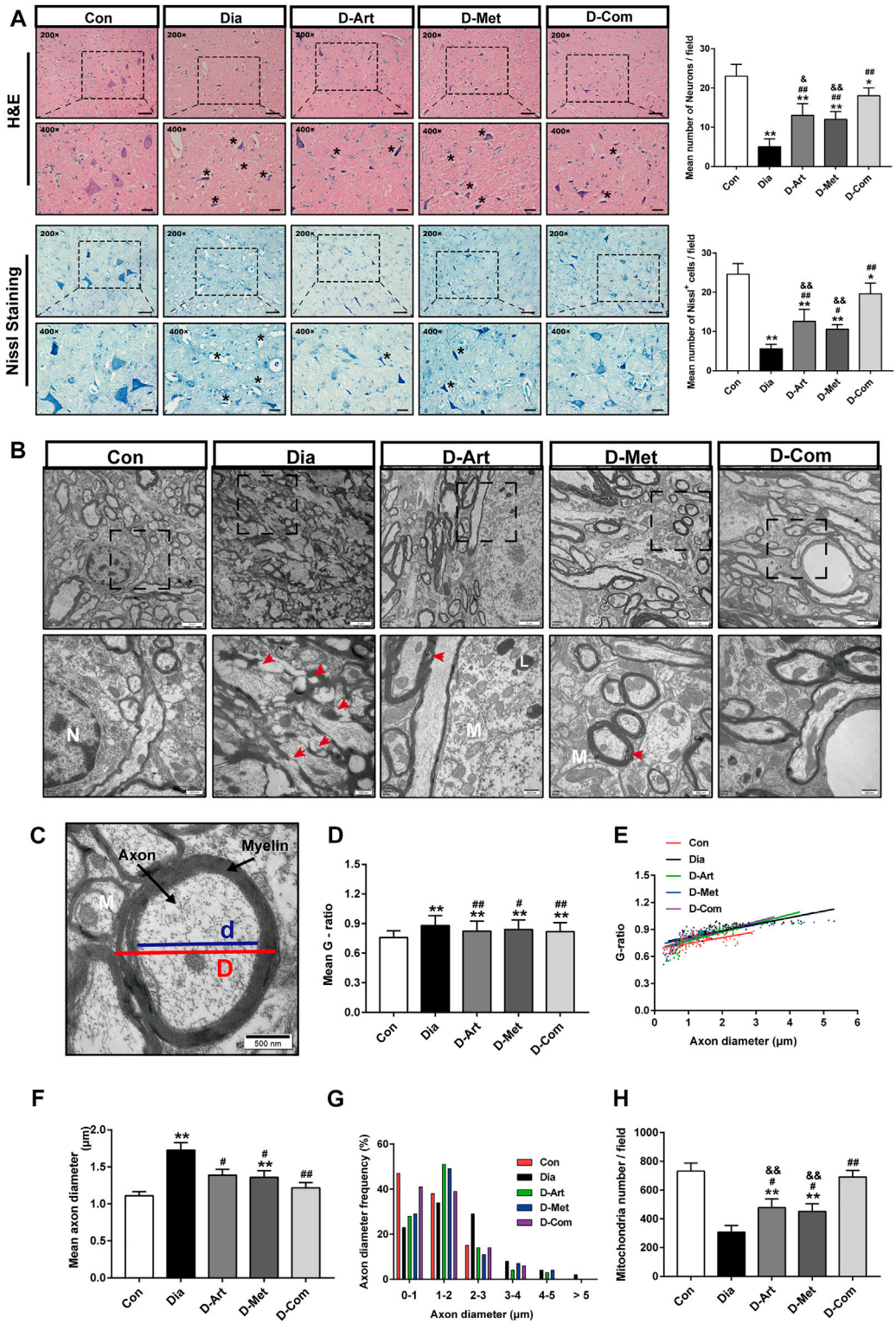


FIGURE 5 | ART or Met alone and ART/Met combination alleviate central SSN injury in the T2DM rats. **(A)** H&E and Nissl staining of SSN in each group (5 rats per group), scale bar = 50 μm (200×); scale bar = 20 μm (400×). Crumpled and vacuolated neurons were labeled using black *. **(B)** Ultrastructure of SSN in each group (3 rats per group). N, nucleus; L, Lysosome; M, mitochondria. Red arrows represent damaged and disrupted myelin. **(C)** Schematic diagram of G-ratio. **(D–H)** The mean g-ratios, individual g-ratios distribution, axonal diameters, and distributions of axonal diameters, and mitochondria number in different groups. SSN, superior salivatory nucleus; Data are expressed as the mean ± SD. *$p < 0.05$ and **$p < 0.01$ versus Con group; #$p < 0.05$, ##$p < 0.01$ versus Dia group; &$p < 0.01$ and &&$p < 0.01$ versus D-Com group. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

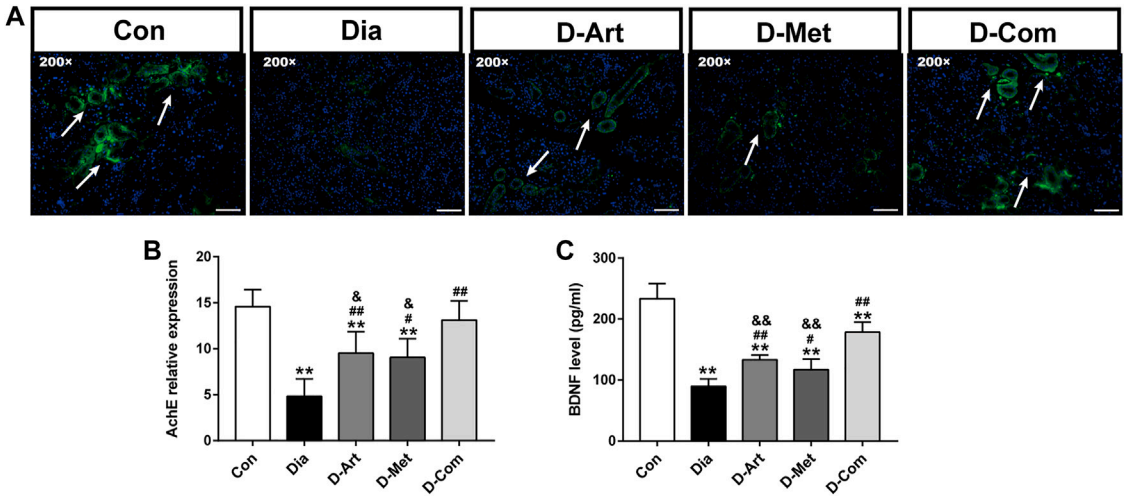


FIGURE 6 | ART or Met alone and ART/Met combination preserved peripheral parasympathetic innervation of SGs in the T2DM rats. **(A)** Immunofluorescence of AChE in SMGs of each group, scale bar = 50 μm (200×). **(B)** Level of AChE expression in SMGs of each group. **(C)** ELISA analysis of BDNF in SMGs of each group (5 rats per group). Data are expressed as the mean ± SD. $^{**}p < 0.01$ versus Con group; $\#p < 0.05$ and $\#\#p < 0.01$ versus Dia group; $\&p < 0.05$ and $\&\&p < 0.01$ versus D-Com group. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

expression levels were highest in the ART/Met co-treated group among the three treatment groups.

ART or Met Alone and ART/Met Combination Activate PI3K/AKT Pathway in SMGs of the T2DM Rats

To further explore the molecular mechanism of ART/Met effect on diabetes-induced SMGs damage, we assessed the proteins that are involved in the PI3K/AKT pathway, which has been shown to be highly associated with T2DM (Huang et al., 2018a). Molecular docking results showed that ART formed hydrogen bonds with amino acid residues at the active site of AKT (ARG-23 ARG-25, ARG-86, and LYS-14) and PI3K (LYS-382, ASP-367, and THR-362), with optimal conformational binding energies of −9.32 and −6.07 kcal/mol, respectively (**Figure 7A**). This suggested that the PI3K/AKT pathway had binding sites for ART. Moreover, western blot results showed that expression ratios of p-Akt/Akt and p-PI3K/PI3K in the untreated diabetic group was significantly lower than in the Con group (**Figure 7B**). However, the three treatment groups obviously upregulated p-AKT/AKT and p-PI3K/PI3K expression when compared to the Dia group. In addition, we noticed that expression of p-AKT/AKT and p-PI3K/PI3K in the D-ART and D-Met groups were markedly lower than that of the D-Com group.

ART or Met Alone and ART/Met Combination Inhibit Apoptosis and Autophagy in the SMGs of T2DM Rats

Previous studies have reported that the PI3K/Akt pathway can inhibit apoptosis and autophagy (Yang et al., 2018; Wang et al., 2019). Therefore, we employed TUNEL staining and Western blot to investigate whether these drug treatments could restrain apoptosis and autophagy in the SMGs with T2DM. TUNEL staining displayed that number of $TUNEL^+$ cells in the Dia group were obviously more than that in the Con group (**Figure 8A**). However, increased $TUNEL^+$ cells in the Dia group were inhibited by treatments with ART or Met alone and ART/Met combination, among of which ART/Met combination exhibited the best suppressive effect in $TUNEL^+$ cells increase ($p < 0.01$; **Figure 8A**). Furthermore, expression levels of typical apoptosis-related markers (Bax, Caspase3, and Bcl-2) (He et al., 2021), were evaluated using Western blot. The results showed that the expression levels of Bax and Caspase3 protein in the untreated diabetic group were upregulated ($p < 0.01$; **Figure 8B**), while expression level of Bcl-2 protein was downregulated compared to the normal control group ($p < 0.01$; **Figure 8B**). Conversely, a significant increase in expression of Bcl-2 protein, and a reduction in Bax and Caspase3 expression were observed after ART, Met, and ART/Met combination treatments ($p < 0.05$ or 0.01; **Figure 8B**). Notably, ART/Met combination was superior to the other two treatments in upregulating expression of Bcl-2 protein.

Furthermore, the two main indicators of autophagy (P62 and LC3) were also detected using western blot analysis. Results showed that the expression ratio of LC3I/LC3II protein in the untreated diabetic group were markedly increased compared to that in the normal control group, whereas the expression level of P62 protein was significantly reduced ($p < 0.01$; **Figure 8C**). In contrast, ART, Met, and ART/Met combination treatments significantly upregulated the expression of P62 protein, while only ART/Met combination treatment markedly downregulated the expression ratio of LC3I/LC3II protein in diabetes rats ($p <$

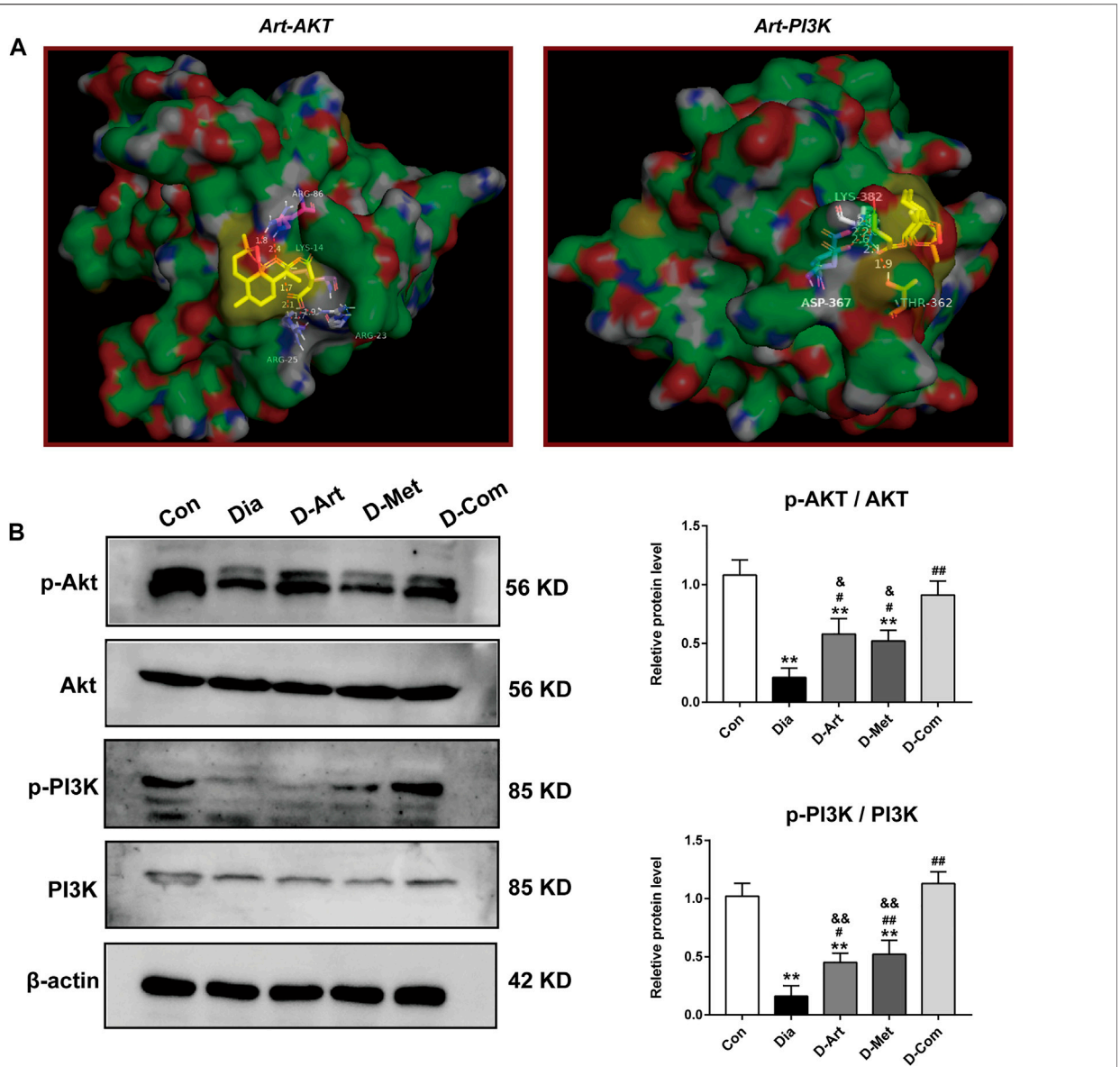


FIGURE 7 | ART or Met alone and ART/Met combination activate PI3K/AKT pathway in SMGs of the T2DM rats. **(A)** Molecular docking of ART to Akt and PI3K protein. **(B)** Western blot analysis of p-AKT/AKT and p-PI3K/PI3K (5 rats per group). Protein expression is normalized to β-actin. Data are expressed as the mean ± SD. $^{**}p < 0.01$ versus Con group; $\#p < 0.05$ and $\#\#p < 0.01$ versus Dia group; $\&p < 0.05$ and $\&\&p < 0.01$ versus D-Com group. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

0.01; **Figure 8C**). Moreover, the expression ratio of LC3I/LC3II protein was significantly lower in the ART- and Met-treated groups than that in the ART/Met co-treated group.

DISCUSSION

In our present study, the T2DM rat model was successfully established by using HFD and STZ, and was characterized by weight loss, increased water intake and urine output, hyperglycemia, hyperlipemia, insulin resistance, and pancreatic injury. Results showed that ART, Met, and ART/Met combination treatments significantly attenuated these typical characteristics of T2DM. Besides, we noticed that ART/Met combination had a superior effect than ART alone in controlling blood glucose levels, regulating glucose tolerance, and improved insulin resistance. Interestingly, we also observed that the above typical T2DM parameters in the rats with ART/Met combination treatment were comparable to those of the control group, whereas this effect was not achieved in the monotherapies. These results revealed that ART/Met combination treatment might possess more effective in the future in preventing development and progression of T2DM than ART or Met alone. In addition, we observed that saliva

secretion and SMG weight in the Dia, D-ART and D-Met groups were significantly reduced compared to the Con group. We also employed HE staining, AB-PAS staining and TEM analysis to evaluate histology and ultrastructure of SMGs. As shown in the **Figures 4D,E**, diabetes-induced SMGs' pathological alterations. Taken together, our data demonstrated that DM contributed to SGs' hyposalivation and damage, which is consistent with results reported by Xiang et al. (2020). As main transporter protein mediating saliva secretion, AQP5 is recognized as a potential target for the treatment of xerostomia (D'Agostino, C. 2020). Accordingly, we used immunohistochemistry staining of AQP5 to further confirm that hyperglycemia induced SGs' dysfunction (**Figure 4F**). Subsequently, it was observed that after treatments with ART, Met and ART/Met combination reduction of saliva production and injury of SMGs were alleviated in the T2DM rats. Notably, ART/Met combination administration was not only superior to monotherapies but also comparable to rats in the control group in restoring saliva secretion and alleviating SMGs damage. These findings suggest that ART/Met could ameliorate diabetes-induced dysfunction. However, the underlying mechanisms were not elucidated.

DM is a chronic metabolic disease characterized by hyperglycemia (Öztürk et al., 2017) and can damage multiple organs, among which, the brain is one of most common target organs in DM complications (Hamed, 2017; Zhao et al., 2019). Thus, SSN in the brainstem is unlikely to be immune to the damage caused by chronic hyperglycemia. It has been reported that the expression level of AQP5 in SMG is regulated by the parasympathetic nerve (Hosoi et al., 2020). Parasympathetic nerves are closely associated with SGs regeneration, and intact parasympathetic innervation facilitates SMGs regeneration (Wang et al., 2021). Given that the origin of the parasympathetic preganglionic is neurons, SSN plays a key role in innervating lacrimal gland and SMG (Mitoh et al., 2017). Previous studies have reported that parasympathetic denervation give rise to loss in SMG weight and decrease in AQP5 expression (Azlina et al., 2010; Wang et al., 2021), which was also observed in our study (**Figures 4B,F**). Therefore, we speculated that hyperglycemia may not only cause SMG injury, but also induce SSN damage, both of which lead to diabetes xerostomia.

To further clarify our speculation, we first carried out histological staining (HE and Nissl staining) in SSN. HE staining showed that SSN of diabetic rats had obvious pathological alterations, such as neurons vacuolization, neurons loss and karyopycnosis, which were consistent with Nissl staining result (**Figure 5A**). These results revealed that hyperglycemia resulted in SSN pathological injury, further confirmed by the ultrastructure observation of SSN. As shown in **Figures 5B–H**, a significant reduction in mitochondrion number, and an increase in axon diameter and G-ratio of untreated diabetic rats were observed through TEM analysis of SSN, suggesting that diabetes led to injury of SSN neurons and axons. Axons are the crucial output channels of excitatory impulses between a neuron and its target effector, such as muscle and salivary gland cells (Zhou et al., 2020). Notably, we observed that these pathological alterations of SSN in the T2DM rats were markedly mitigated after treatments with ART, Met and ART/Met combination, with ART/Met combination outperforming ART and Met alone in terms of alleviating neurons and mitochondrion loss.

Besides, previous studies have reported the important effects of BDNF, as a salivary trophic factor, on preservation of parasympathetic innervation in SGs post irradiation (Hu et al., 2018; Kim et al., 2020). Nevertheless, diabetes and its various complications generally lead to a decrease in BDNF level (Chan et al., 2019; Kim and Song, 2020). AChE has also been reported to be a marker of parasympathetic innervation (Hu et al., 2018). Thus, we detected expression levels of AChE and BDNF to evaluate peripheral parasympathetic innervation in SMGs of T2DM rats following drug administration respectively using immunofluorescence and ELISA (**Figure 6**). Results showed that AChE and BDNF levels were significantly decreased in SMG of the untreated diabetes rats compared to the control group. As expected, reduction of these indicators in the SMG of diabetic rats were improved by the ART, Met, and ART/Met combination therapies. Interestingly, treatment with ART/Met combination displayed superior effects in inhibiting reduction of AChE and BDNF expression in SMG with T2DM than the respective monotherapies. Collectively, these data suggested that ART/Met might preserve peripheral SGs' parasympathetic innervation to attenuate hyperglycemia-induced SG dysfunction through rescuing SSN injury.

Next, the possible molecular mechanisms through which ART/Met ameliorates the SGs injury induced by diabetes were further explored. Evidence suggests that the PI3K/Akt signaling pathway can regulate glucose homeostasis, inhibit apoptosis, and promote cell survival through multiple pathways (Huang et al., 2018a). The regulatory role of ART on the PI3K/Akt pathway has also been reported in previous studies (Feng and Qiu, 2018; Zhang et al., 2020), and it was further demonstrated by the molecular docking analysis of ART with PI3K and Akt conducted in this study. Huang et al. (2018b) reported that high glucose induced autophagy, thereby leading to AQP5 degradation in the diabetic SMG via the PI3K/AKT pathway, which is consistent with results obtained in this study (**Figures 4F**, **7**, **8C**). Subsequent western blot analysis results showed that the relative expressions of p-Akt, and p-PI3K proteins were upregulated in SGs after ART or Met alone and ART/Met combination, with ART/Met combination exhibiting the best effect among the three therapies. This suggested that the PI3K/Akt pathway was activated in the SGs of diabetic rats by the three treatments, especially ART/Met combination therapy. Previous studies have reported that the PI3K/Akt pathway is a vital pathway in the suppression of downstream apoptosis and autophagy (Tan et al., 2020; Tungsukruthai et al., 2021). Subsequently, we carried out TUNEL staining to determine whether ART alone and in combination with Met could mitigate apoptosis in SMGs with T2DM. As shown in TUNE staining (**Figure 8A**), ART, Met and ART/Met combination prominently ameliorated increase of $TUNEL^+$ cells when compared to that of the untreated diabetic group. Besides,

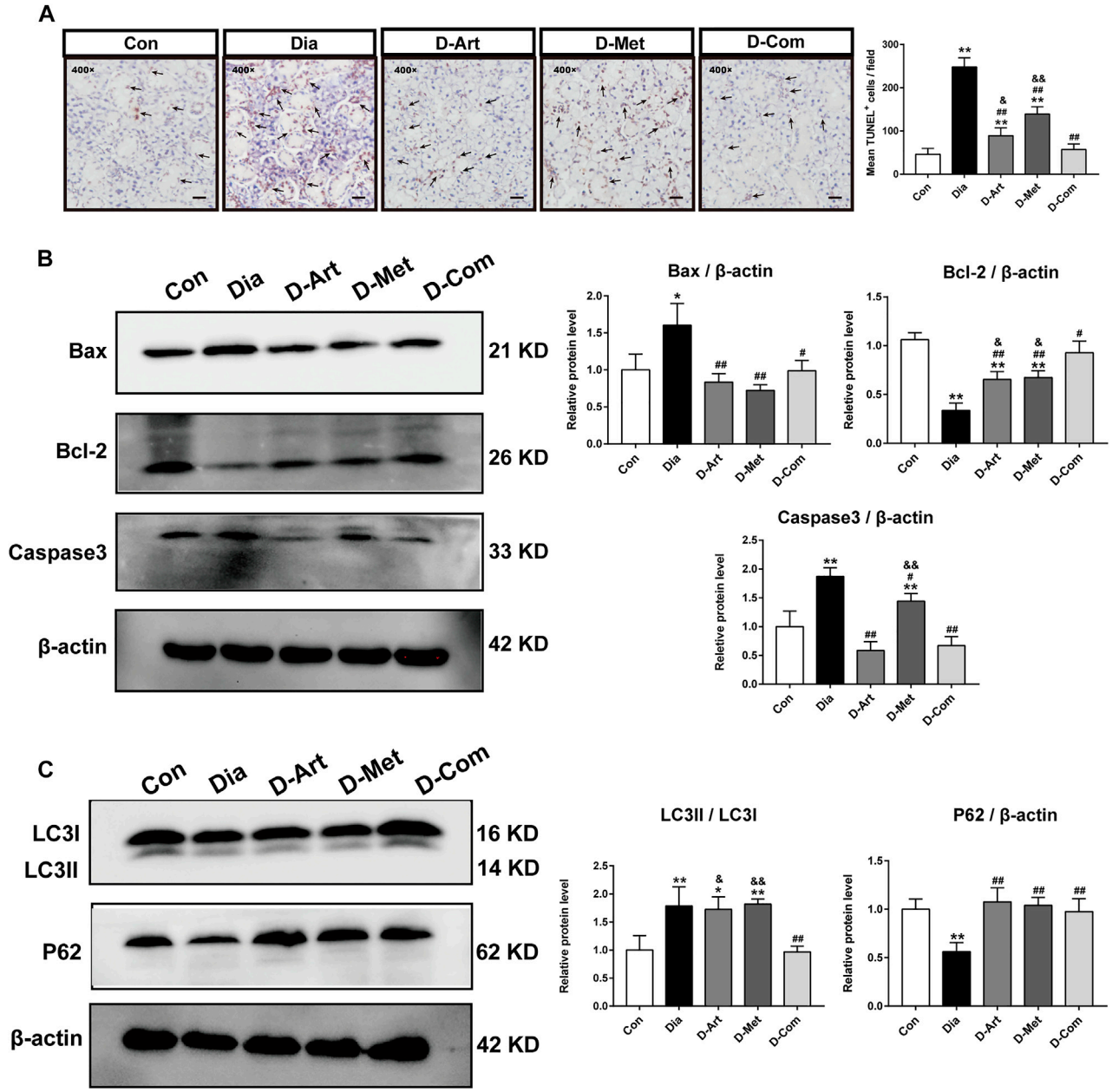
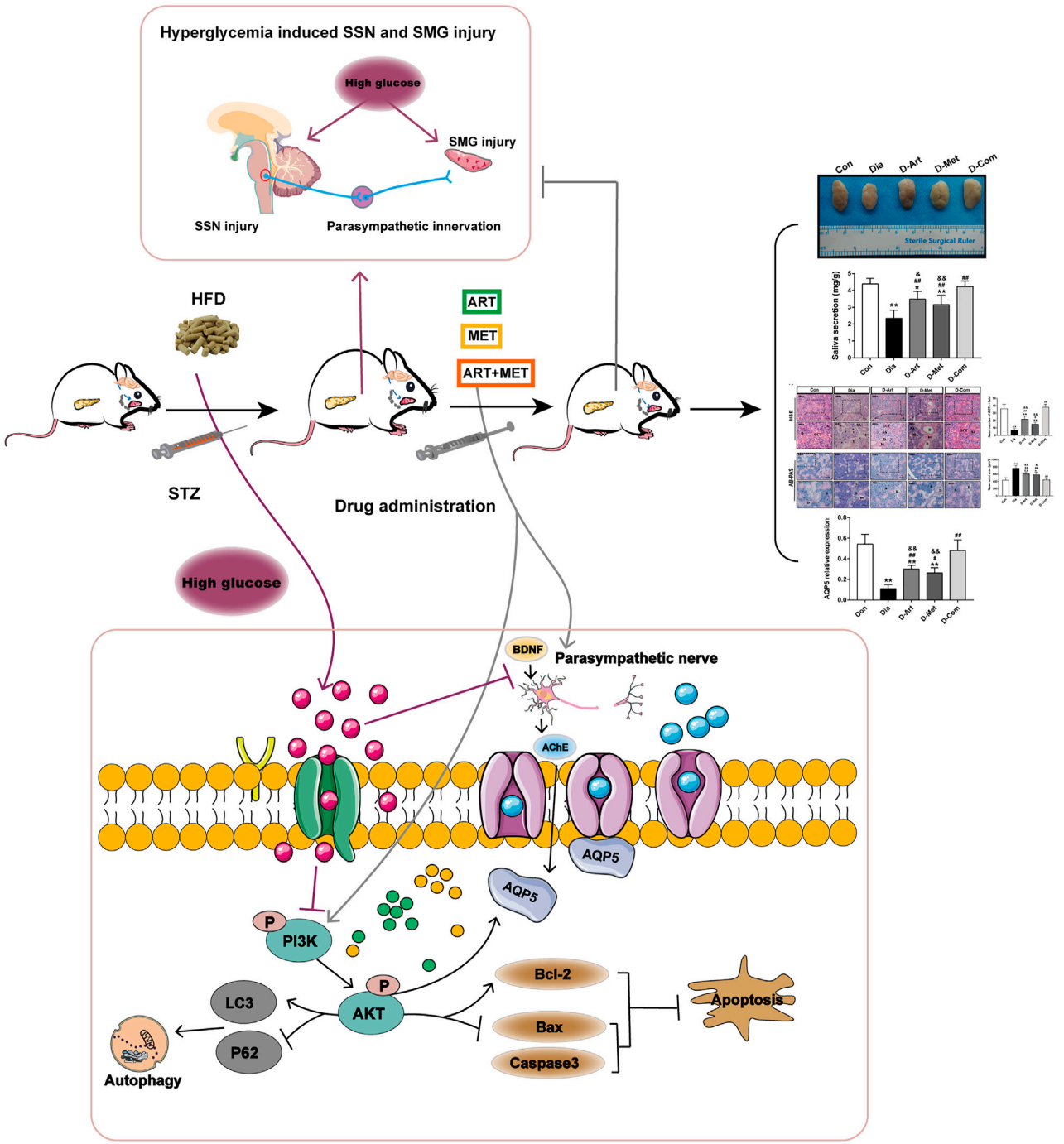


FIGURE 8 | ART or Met alone and ART/Met combination inhibit apoptosis and autophagy in the SMGs of T2DM rats. **(A)** TUNEL staining in SMGs. Black arrows display TUNEL positive cells. **(B)** Western blot analyses for Bax, Bcl-2, and Caspase3 protein expression in SMGs. **(C)** Western blot analyses for LC3 and P62 protein expression in SMGs (5 rats per group). Protein expression is normalized to β-actin. Data are expressed as the mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Con group; $\#p < 0.05$ and $\#\#p < 0.01$ versus Dia group; $\&p < 0.05$ and $\&\&p < 0.01$ versus D-Com group. Con, Normal control group; Dia, Untreated diabetic group; D-Art, ART-treated diabetic group; D-Met, Met-treated diabetic group; D-Com, ART/Met co-treated diabetic group.

we found that number of TUNEL^{+} cells in SMG of the D-Com group was significantly lower than the two other monotherapy groups. Notably, Bax (apoptosis regulator), Bcl-2 (anti-apoptotic protein), and Caspase3 (pro-apoptotic protein) are key indicators of apoptosis (Ji et al., 2021), while LC3II/LC3I and P62 are markers of autophagy (Komatsu and Ichimura 2010; Yue et al., 2019). Thus, this study also examined expression of these apoptosis and autophagy-related proteins in SMGs using the western blot method. Results demonstrated that treatments with ART, Met and ART/Met combination upregulated protein expression of Bcl-2 and P62 compared to those of the Dia group, and reduced protein expression of Bax, Caspase3. We noticed that ART/Met combination treatment group had higher expression level of Bcl-2 protein in comparison with the D-ART and D-Met groups. However, simply administration



ART and MET synergistically alleviated hyperglycemia-induced xerostomia in the HFD and STZ-induced T2DM Rats

FIGURE 9 | Schematic diagram of the proposed mechanisms through which ART and Met synergistically ameliorate xerostomia in HFD/STZ-induced T2DM rats via rescuing central SSN and SMG damage induced by hyperglycemia. On the one hand, ART/Met attenuated SMG damage though regulating the PI3K/Akt pathway to inhibit apoptosis and autophagy of SMGs. On the other hand, ART/Met treatment preserved peripheral parasympathetic innervation (AChE and BDNF) in SGs to alleviate diabetes-induced hyposalivation likely through alleviating central SSN damage. T2DM, type 2 diabetes mellitus.

with ART/Met combination significantly expression of LC3II/LC3I protein compared with the Dia group. Collectively, these results suggest that ART/Met combination treatment mitigated diabetes-induced SMG damage via activating the PI3K/AKT pathway to inhibit apoptosis and autophagy in SMGs.

In conclusion, this study has shown that combining ART (first-line antimalarial drug) and Met (frontline drug for T2DM treatment), two relatively non-toxic drugs, exhibited synergistic effects in improvement of xerostomia of diabetic rats induced by HFD/STZ (**Figure 9**). Both central SSN and SMG damage mediated by hyperglycemia contributed to SGs' dysfunction. Consequently, our data demonstrated that ART/Met treatment attenuated SMG damage though regulating the PI3K/Akt pathway to inhibit apoptosis and autophagy of SMGs in T2DM rats. Moreover, ART/Met treatment preserved parasympathetic innervation (AChE and BDNF) in SGs to alleviating diabetes-induced hyposalivation likely through rescuing central SSN damage. Taken together, our results suggested that ART in combination with Met treatment might be a viable strategy to ameliorate the detrimental effect of hyperglycemia on SGs' function, probably via mechanisms mentioned above (**Figure 9**). However, further in-depth studies and experimental evidence are required to validate and expand the results in the present. In short, the findings of this study may provide a novel insight and theoretical basis for future treating diabetes-induced xerostomia in the clinic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Welfare Committee of Guangxi Medical University (No. 202006013).

AUTHOR CONTRIBUTIONS

SZ and JL carried out the research and wrote the manuscript; JL provided experimental technique support. YZ, JX, DZ, CM, YW, YL, and ZL performed data collection and analysis. XN acquired fund supports, designed and supervised the study, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was funded by the Natural Science Foundation of China (82160187, 81860726) and the Natural Science Foundation Key Project of Guangxi (2019GXNSFDA245016).

ACKNOWLEDGMENTS

The authors appreciated professor Li Wei in Department of Human Anatomy of Guangxi Medical University, professor Bo Zhu in Cancer Hospital of Guangxi Medical University professor, Yingfen Qin in Department of Metabolic Endocrinology, the First Affiliated Hospital of Guangxi Medical University for technique support; and Kechen Ban in MD Anderson Cancer Center for his help with manuscript preparation. The authors also acknowledged Home for Researchers (www.home-for-researchers.com) for language correction.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.774674/full#supplementary-material

REFERENCES

Albert, D. A., Ward, A., Allweiss, P., Graves, D. T., Knowler, W. C., Kunzel, C., et al. (2012). Diabetes and Oral Disease: Implications for Health Professionals. *Ann. N. Y Acad. Sci.* 1255, 1–15. doi:10.1111/j.1749-6632.2011.06460.x

Azlina, A., Javkhlan, P., Hiroshima, Y., Hasegawa, T., Yao, C., Akamatsu, T., et al. (2010). Roles of Lysosomal Proteolytic Systems in AQP5 Degradation in the Submandibular Gland of Rats Following Chorda Tympani Parasympathetic Denervation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 299 (5), G1106–G1117. doi:10.1152/ajpgi.00194.2010

Bhattarai, K. R., Junjappa, R., Handigund, M., Kim, H. R., and Chae, H. J. (2018). The Imprint of Salivary Secretion in Autoimmune Disorders and Related Pathological Conditions. *Autoimmun. Rev.* 17 (4), 376–390. doi:10.1016/j.autrev.2017.11.031

Bradley, E. A., Premilovac, D., Betik, A. C., Hu, D., Attrill, E., Richards, S. M., et al. (2019). Metformin Improves Vascular and Metabolic Insulin Action in Insulin-Resistant Muscle. *J. Endocrinol.* 243 (2), 85–96. doi:10.1530/JOE-19-0067

Cai, Y., Wang, Y., Zhi, F., Xing, Q. C., and Chen, Y. Z. (2018). The Effect of Sanggua Drink Extract on Insulin Resistance through the PI3K/AKT Signaling Pathway. *Evid Based. Complement. Altern. Med. eCAM* 2018, 9407945. doi:10.1155/2018/9407945

Chan, C. B., Ahuja, P., and Ye, K. (2019). Developing Insulin and BDNF Mimetics for Diabetes Therapy. *Curr. Top. Med. Chem.* 19 (24), 2188–2204. doi:10.2174/1568026619666191010160643

Chen, Y., Li, W., Nong, X., Liang, C., Li, J., Lu, W., et al. (2021). Role of Artesunate on Cardiovascular Complications in Rats with Type 1 Diabetes Mellitus. *BMC Endocr. Disord.* 21 (1), 19. doi:10.1186/s12902-021-00682-0

D'Agostino, C., Elkashty, O. A., Chivasso, C., Perret, J., Tran, S. D., and Delporte, C. (2020). Insight into Salivary Gland Aquaporins. *Cells* 9 (6), 1547. doi:10.3390/cells9061547

Darré, L., Vergnes, J. N., Gourdy, P., and Sixou, M. (2008). Efficacy of Periodontal Treatment on Glycaemic Control in Diabetic Patients: A Meta-Analysis of Interventional Studies. *Diabetes Metab.* 34 (5), 497–506. doi:10.1016/j.diabet.2008.03.006

Feng, F. B., and Qiu, H. Y. (2018). Effects of Artesunate on Chondrocyte Proliferation, Apoptosis and Autophagy through the PI3K/AKT/mTOR Signaling Pathway in Rat Models with Rheumatoid Arthritis. *Biomed. Pharmacother.* 102, 1209–1220. doi:10.1016/j.biopha.2018.03.142

Ferenczyova, K., Kalocayova, B., Kindernay, L., Jelemensky, M., Balis, P., Berenyiova, A., et al. (2020). Quercetin Exerts Age-dependent Beneficial Effects on Blood Pressure and Vascular Function, but Is Inefficient in Preventing Myocardial Ischemia-Reperfusion Injury in Zucker Diabetic Fatty Rats. *Molecules* 25 (1), 187. doi:10.3390/molecules25010187

Hamed, S. A. (2017). Brain Injury with Diabetes Mellitus: Evidence, Mechanisms and Treatment Implications. *Expert Rev. Clin. Pharmacol.* 10 (4), 409–428. doi:10.1080/17512433.2017.1293521

He, L. L., Wang, Y. C., Ai, Y. T., Wang, L., Gu, S. M., Wang, P., et al. (2021). Qiangji Decoction Alleviates Neurodegenerative Changes and Hippocampal Neuron

Apoptosis Induced by D-Galactose via Regulating AMPK/SIRT1/NF-κB Signaling Pathway. *Front. Pharmacol.* 12, 735812. doi:10.3389/fphar.2021.735812

Hien, D., and White, N. J. (1993). Qinghaosu. *The Lancet* 341 (8845), 603–608. doi:10.1016/0140-6736(93)90362-k

Hosoi, K., Yao, C., Hasegawa, T., Yoshimura, H., and Akamatsu, T. (2020). Dynamics of Salivary Gland AQP5 under Normal and Pathologic Conditions. *Int. J. Mol. Sci.* 21 (4), 1182. doi:10.3390/ijms21041182

Hostalek, U., Gwilt, M., and Hildemann, S. (2015). Therapeutic Use of Metformin in Prediabetes and Diabetes Prevention. *Drugs* 75 (10), 1071–1094. doi:10.1007/s40265-015-0416-8

Hu, L., Zhu, Z., Hai, B., Chang, S., Ma, L., Xu, Y., et al. (2018). Intragland Shh Gene Delivery Mitigated Irradiation-Induced Hyposalivation in a Miniature Pig Model. *Theranostics* 8 (16), 4321–4331. doi:10.7150/thno.26509

Huang, G., Lv, J., Li, T., Huai, G., Li, X., Xiang, S., et al. (2016). Notoginsenoside R1 Ameliorates Podocyte Injury in Rats with Diabetic Nephropathy by Activating the PI3K/Akt Signaling Pathway. *Int. J. Mol. Med.* 38 (4), 1179–1189. doi:10.3892/ijmm.2016.2713

Huang, X., Liu, G., Guo, J., and Su, Z. (2018a). The PI3K/AKT Pathway in Obesity and Type 2 Diabetes. *Int. J. Biol. Sci.* 14 (11), 1483–1496. doi:10.7150/ijbs.27173

Huang, Y., Shi, X., Mao, Q., Zhang, Y., Cong, X., Zhang, X., et al. (2018b). Aquaporin 5 Is Degraded by Autophagy in Diabetic Submandibular Gland. *Sci. China Life Sci.* 61 (9), 1049–1059. doi:10.1007/s11427-018-9318-8

Inyang, K. E., Szabo-Pardi, T., Wentworth, E., McDougal, T. A., Dussor, G., Burton, M. D., et al. (2019). The Antidiabetic Drug Metformin Prevents and Reverses Neuropathic Pain and Spinal Cord Microglial Activation in Male but Not Female Mice. *Pharmacol. Res.* 139, 1–16. doi:10.1016/j.phrs.2018.10.027

Ito, Y., Ofengeim, D., Najafov, A., Das, S., Saberi, S., Li, Y., et al. (2016). RIPK1 Mediates Axonal Degeneration by Promoting Inflammation and Necroptosis in ALS. *Science* 353 (6299), 603–608. doi:10.1126/science.aaf6803

Ji, H., Xiao, F., Li, S., Wei, R., Yu, F., and Xu, J. (2021). GRP78 Effectively Protect Hypoxia/reperfusion-Induced Myocardial Apoptosis via Promotion of the Nrf2/HO-1 Signaling Pathway. *J. Cel Physiol* 236 (2), 1228–1236. doi:10.1002/jcp.29929

Kelany, M. E., Hakami, T. M., Omar, A. H., and Abdallah, M. A. (2016). Combination of Sitagliptin and Insulin against Type 2 Diabetes Mellitus with Neuropathy in Rats: Neuroprotection and Role of Oxidative and Inflammation Stress. *Pharmacology* 98 (5-6), 242–250. doi:10.1159/000448043

Keppler-Noreuil, K. M., Parker, V. E., Darling, T. N., and Martinez-Agosto, J. A. (2016). Somatic Overgrowth Disorders of the PI3K/AKT/mTOR Pathway & Therapeutic Strategies. *Am. J. Med. Genet. C Semin. Med. Genet.* 172 (4), 402–421. doi:10.1002/ajmg.c.31531

Kim, J. H., Jeong, B. K., Jang, S. J., Yun, J. W., Jung, M. H., Kang, K. M., et al. (2020). Alpha-Lipoic Acid Ameliorates Radiation-Induced Salivary Gland Injury by Preserving Parasympathetic Innervation in Rats. *Int. J. Mol. Sci.* 21 (7), 2260. doi:10.3390/ijms21072260

Kim, J. W., Kim, S. M., Park, J. S., Hwang, S. H., Choi, J., Jung, K. A., et al. (2019). Metformin Improves Salivary Gland Inflammation and Hypofunction in Murine Sjögren's Syndrome. *Arthritis Res. Ther.* 21 (1), 136. doi:10.1186/s13075-019-1904-0

Kim, O. Y., and Song, J. (2020). The Importance of BDNF and RAGE in Diabetes-Induced Dementia. *Pharmacol. Res.* 160, 105083. doi:10.1016/j.phrs.2020.105083

Knox, S. M., Lombaert, I. M., Haddox, C. L., Abrams, S. R., Cotrim, A., Wilson, A. J., et al. (2013). Parasympathetic Stimulation Improves Epithelial Organ Regeneration. *Nat. Commun.* 4, 1494. doi:10.1038/ncomms2493

Kojima, T., Kanemaru, S., Hirano, S., Tateya, I., Suehiro, A., Kitani, Y., et al. (2011). The Protective Efficacy of Basic Fibroblast Growth Factor in Radiation-Induced Salivary Gland Dysfunction in Mice. *Laryngoscope* 121 (9), 1870–1875. doi:10.1002/lary.21873

Komatsu, M., and Ichimura, Y. (2010). Physiological Significance of Selective Degradation of P62 by Autophagy. *FEBS Lett.* 584 (7), 1374–1378. doi:10.1016/j.febslet.2010.02.017

Kudiyirickal, M. G., and Pappachan, J. M. (2015). Diabetes Mellitus and Oral Health. *Endocrine* 49 (1), 27–34. doi:10.1007/s12020-014-0496-3

Lee, H. Y., Gu, M., Cheng, J., Suh, J. W., and Chae, H. J. (2020). Ixeris Dentata and Lactobacillus Gasseri Extracts Improve Salivary Secretion Capability in Diabetes-Associated Dry Mouth Rat Model. *Nutrients* 12 (5), 1331. doi:10.3390/nu12051331

Li, H., Fang, Q., Nie, Q., Hu, J., Yang, C., Huang, T., et al. (2020). Hypoglycemic and Hypolipidemic Mechanism of Tea Polysaccharides on Type 2 Diabetic Rats via Gut Microbiota and Metabolism Alteration. *J. Agric. Food Chem.* 68 (37), 10015–10028. doi:10.1021/acs.jafc.0c01968

Li, Z., Shi, X., Liu, J., Shao, F., Huang, G., Zhou, Z., et al. (2019). Artesunate Prevents Type 1 Diabetes in NOD Mice Mainly by Inducing Protective IL-4-producing T Cells and Regulatory T Cells. *FASEB J.* 33 (7), 8241–8248. doi:10.1096/fj.201900146R

Liccardo, D., Cannavo, A., Spagnuolo, G., Ferrara, N., Cittadini, A., Rengo, C., et al. (2019). Periodontal Disease: A Risk Factor for Diabetes and Cardiovascular Disease. *Int. J. Mol. Sci.* 20 (6), 1414. doi:10.3390/ijms20061414

Lima, D. L. F., Carneiro, S. D. R. M., Barbosa, F. T. S., Saintrain, M. V. L., Moizan, J. A. H., and Doucet, J. (2017). Salivary Flow and Xerostomia in Older Patients with Type 2 Diabetes Mellitus. *PloS one* 12 (8), e0180891. doi:10.1371/journal.pone.0180891

López-Pintor, R. M., Casañas, E., González-Serrano, J., Serrano, J., Ramírez, L., de Arriba, L., et al. (2016). Xerostomia, Hyposalivation, and Salivary Flow in Diabetes Patients. *J. Diabetes Res.* 2016, 4372852. doi:10.1155/2016/4372852

Maffei, A., Lembo, G., and Carnevale, D. (2018). PI3Kinases in Diabetes Mellitus and its Related Complications. *Int. J. Mol. Sci.* 19 (12), 4098. doi:10.3390/ijms19124098

Mauri-Obradors, E., Estrugo-Devesa, A., Jané-Salas, E., Viñas, M., and López-López, J. (2017). Oral Manifestations of Diabetes Mellitus. A Systematic Review. *Med. Oral Patol Oral Cir Bucal* 22 (5), e586–e594. doi:10.4317/medoral.21655

Maurya, A., Mohan, S., and Verma, S. C. (2021). Antidiabetic Potential of Naturally Occurring Sesquiterpenes: A Review. *Curr. Top. Med. Chem. Eng.* 21 (10), 851–862. doi:10.2174/1568026621666210305102500

Mitoh, Y., Ueda, H., Ichikawa, H., Fujita, M., Kobashi, M., and Matsuo, R. (2017). Effects of Cevimeline on Excitability of Parasympathetic Preganglionic Neurons in the superior Salivatory Nucleus of Rats. *Auton. Neurosci.* 206, 1–7. doi:10.1016/j.autneu.2017.05.010

Molania, T., Alimohammadi, M., Akha, O., Mousavi, J., Razvini, R., and Salehi, M. (2017). The Effect of Xerostomia and Hyposalivation on the Quality of Life of Patients with Type II Diabetes Mellitus. *Electron. Physician* 9 (11), 5814–5819. doi:10.19082/5814

Nie, Q., Chen, H., Hu, J., Gao, H., Fan, L., Long, Z., et al. (2018). Arabinoxylan Attenuates Type 2 Diabetes by Improvement of Carbohydrate, Lipid, and Amino Acid Metabolism. *Mol. Nutr. Food Res.* 62 (20), e1800222. doi:10.1002/mnfr.201800222

Öztürk, E., Arslan, A. K. K., Yerer, M. B., and Bishayee, A. (2017). Resveratrol and Diabetes: A Critical Review of Clinical Studies. *Biomed. Pharmacother.* 95, 230–234. doi:10.1016/j.biopha.2017.08.070

Ramos, J. M. J., Castillo, M. E., and Puerto, A. (2020). RELATIONSHIP between Prandial Drinking Behavior and Supersensitivity of Salivary Glands after superior Salivatory Nucleus Lesions in RATS. *Physiol. Behav.* 224, 113022. doi:10.1016/j.physbeh.2020.113022

Sanchez-Rangel, E., and Inzucchi, S. E. (2017). Metformin: Clinical Use in Type 2 Diabetes. *Diabetologia* 60 (9), 1586–1593. doi:10.1007/s00125-017-4336-x

Sefi, M., Fetoui, H., Lachkar, N., Tahraoui, A., Lyoussi, B., Boudawara, T., et al. (2011). Centaurium Erythrea (Gentianaceae) Leaf Extract Alleviates Streptozotocin-Induced Oxidative Stress and β-cell Damage in Rat Pancreas. *J. Ethnopharmacol* 135 (2), 243–250. doi:10.1016/j.jep.2011.02.029

Sun, Z., Ma, Y., Chen, F., Wang, S., Chen, B., and Shi, J. (2018). Artesunate Ameliorates High Glucose-Induced Rat Glomerular Mesangial Cell Injury by Suppressing the TLR4/NF-Kb/nlrp3 Inflammasome Pathway. *Chem. Biol. Interact* 293, 11–19. doi:10.1016/j.cbi.2018.07.011

Tan, X. H., Zhang, K. K., Xu, J. T., Qu, D., Chen, L. J., Li, J. H., et al. (2020). Luteolin Alleviates Methamphetamine-Induced Neurotoxicity by Suppressing PI3K/Akt Pathway-Modulated Apoptosis and Autophagy in Rats. *Food Chem. Toxicol.* 137, 111179. doi:10.1016/j.fct.2020.111179

Tian, D., Guo, Y., Zhang, D., Gao, Q., Liu, G., Lin, J., et al. (2021). Shenzhi Jiannao Formula Ameliorates Vascular Dementia *In Vivo* and *In Vitro* by Inhibition Glutamate Neurotoxicity via Promoting Clathrin-Mediated Endocytosis. *Chin. Med.* 16 (1), 65. doi:10.1186/s13020-021-00477-4

Tungsukruthai, S., Reamtong, O., Roytrakul, S., Sukrong, S., Vinayanwattikun, C., and Chanvorachote, P. (2021). Targeting AKT/mTOR and Bcl-2 for Autophagic and Apoptosis Cell Death in Lung Cancer: Novel Activity of a Polyphenol Compound. *Antioxidants (Basel, Switzerland)* 10 (4), 534. doi:10.3390/antiox10040534

Våtsveen, T. K., Myhre, M. R., Steen, C. B., Wälchli, S., Lingjærde, O. C., Bai, B., et al. (2018). Artesunate Shows Potent Anti-tumor Activity in B-Cell Lymphoma. *J. Hematol. Oncol.* 11 (1), 23. doi:10.1186/s13045-018-0561-0

Very, N., Vercoutter-Edouart, A. S., Lefebvre, T., Hardivillé, S., and El Yazidi-Belkoura, I. (2018). Cross-Dysregulation of O-GlcNAcylation and PI3K/AKT/mTOR Axis in Human Chronic Diseases. *Front. Endocrinol. (Lausanne)* 9, 602. doi:10.3389/fendo.2018.00602

Wang, X., Li, Z., Shao, Q., Zhang, C., Wang, J., Han, Z., et al. (2021). The Intact Parasympathetic Nerve Promotes Submandibular Gland Regeneration through Ductal Cell Proliferation. *Cell Prolif* 54 (7), e13078. doi:10.1111/cpr.13078

Wang, X., Pan, J., Liu, D., Zhang, M., Li, X., Tian, J., et al. (2019). Nicorandil Alleviates Apoptosis in Diabetic Cardiomyopathy through PI3K/Akt Pathway. *J. Cel Mol Med* 23 (8), 5349–5359. doi:10.1111/jcmm.14413

Xiang, R. L., Huang, Y., Zhang, Y., Cong, X., Zhang, Z. J., Wu, L. L., et al. (2020). Type 2 Diabetes-Induced Hyposalivation of the Submandibular Gland through PINK1/Parkin-Mediated Mitophagy. *J. Cel Physiol* 235 (1), 232–244. doi:10.1002/jcp.28962

Xu, J., Zhao, C., Huang, X., and Du, W. (2019). Regulation of Artemisinin and its Derivatives on the Assembly Behavior and Cytotoxicity of Amyloid Polypeptides hIAPP and Aβ. *ACS Chem. Neurosci.* 10 (11), 4522–4534. doi:10.1021/acschemneuro.9b00385

Yang, J., Pi, C., and Wang, G. (2018). Inhibition of PI3K/Akt/mTOR Pathway by Apigenin Induces Apoptosis and Autophagy in Hepatocellular Carcinoma Cells. *Biomed. Pharmacother.* 103, 699–707. doi:10.1016/j.biopha.2018.04.072

Yu, L., Chen, J. F., Shuai, X., Xu, Y., Ding, Y., Zhang, J., et al. (2016). Artesunate Protects Pancreatic Beta Cells against Cytokine-Induced Damage via SIRT1 Inhibiting NF-Kb Activation. *J. Endocrinol. Invest.* 39 (1), 83–91. doi:10.1007/s40618-015-0328-1

Yue, Z., Guan, X., Chao, R., Huang, C., Li, D., Yang, P., et al. (2019). Diallyl Disulfide Induces Apoptosis and Autophagy in Human Osteosarcoma MG-63 Cells through the PI3K/Akt/mTOR Pathway. *Molecules* 24 (14), 2665. doi:10.3390/molecules24142665

Zeng, Z., Yuan, Q., Yu, R., Zhang, J., Ma, H., and Chen, S. (2019). Ameliorative Effects of Probiotic Lactobacillus Paracasei NL41 on Insulin Sensitivity, Oxidative Stress, and Beta-Cell Function in a Type 2 Diabetes Mellitus Rat Model. *Mol. Nutr. Food Res.* 63 (22), e1900457. doi:10.1002/mnfr.201900457

Zhan, T., Wang, B., Fu, J., Shao, Y., Ye, L., Shi, H., et al. (2021). Artesunate Inhibits Sjögren's Syndrome-like Autoimmune Responses and BAFF-Induced B Cell Hyperactivation via TRAF6-Mediated NF-Kb Signaling. *Phytomedicine* 80, 153381. doi:10.1016/j.phymed.2020.153381

Zhang, J., Sun, X., Wang, L., Wong, Y. K., Lee, Y. M., Zhou, C., et al. (2018). Artesunate-induced Mitophagy Alters Cellular Redox Status. *Redox Biol.* 19, 263–273. doi:10.1016/j.redox.2018.07.025

Zhang, K., Yang, Y., Ge, H., Wang, J., Chen, X., Lei, X., et al. (2020). Artesunate Promotes the Proliferation of Neural Stem/progenitor Cells and Alleviates Ischemia-Reperfusion Injury through PI3K/Akt/FOXO-3a/p27kip1 Signaling Pathway. *Aging (Albany NY)* 12 (9), 8029–8048. doi:10.18632/aging.103121

Zhao, K. C., and Song, Z. Y. (1989). Distribution and Excretion of Artesunate in Rats. *Proc. Chin. Acad. Med. Sci. Peking Union Med. Coll.* 4 (4), 186–188.

Zhao, S. Y., Liao, L. X., Tu, P. F., Li, W. W., and Zeng, K. W. (2019). Icariin Inhibits AGE-Induced Injury in PC12 Cells by Directly Targeting Apoptosis Regulator Bax. *Oxid Med. Cel Longev* 2019, 7940808. doi:10.1155/2019/7940808

Zhou, L. Y., Han, F., Qi, S. B., Ma, J. J., Ma, Y. X., Xie, J. L., et al. (2020). Inhibition of PTEN Activity Promotes IB4-Positive Sensory Neuronal Axon Growth. *J. Cel Mol Med* 24 (18), 11012–11017. doi:10.1111/jcmm.15648

Zuo, S., Li, Q., Liu, X., Feng, H., and Chen, Y. (2016). The Potential Therapeutic Effects of Artesunate on Stroke and Other Central Nervous System Diseases. *Biomed. Res. Int.* 2016, 1489050. doi:10.1155/2016/1489050

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 21 December 2021
doi: 10.3389/fpls.2021.772389

Natural Bioactive Compounds of *Sechium* spp. for Therapeutic and Nutraceutical Supplements

María Isabel Iñiguez-Luna[1,2†], *Jorge Cadena-Iñiguez*[1,2*†], *Ramón Marcos Soto-Hernández*[2,3*†], *Francisco Javier Morales-Flores*[1,2], *Moisés Cortes-Cruz*[2,4‡] *and Kazuo N. Watanabe*[2,5]

[1] *Postgrado de Innovación en Manejo de Recursos Naturales, Colegio de Postgraduados, San Luis Potosí, Mexico,* [2] *Interdisciplinary Research Group at Sechium edule in Mexico, A.C., Texcoco, Estado de México, Mexico,* [3] *Programa de Botánica, Colegio de Postgraduados, Montecillo, Mexico,* [4] *Centro Nacional de Recursos Genéticos-INIFAP, Tepatitlán, Mexico,* [5] *Tsukuba Plant Innovation Research Center, University of Tsukuba, Tsukuba, Japan*

Natural products are in great demand because certain secondary metabolites (SMs) are sources of antioxidants, flavorings, active substances, or anticancer agents with less aggressiveness and selectivity, among which triterpenes and flavonoids are of importance because they inhibit carcinogenesis. For *Sechium* spp. P. Br. (chayotes), there is scientific evidence of antiproliferative activity that has occurred when cancer cell lines have been treated with this fruit. In order to compare future therapeutic designs and identify new and ancestral characteristics, triterpenes and flavonoids were determined in contrasting *Sechium* genotypes. The obtained data were analyzed *via* a cladistics approach, with the aim of identifying the characteristics and state of phytochemicals and genetic variables. The concentrations of flavonoids and triterpenes were determined, and a more complex composition of secondary metabolites was found in the wild types as compared to their domesticated genotypes. Bitter fruits contained a higher number of SMs, followed by those with a neutral and sweet flavor. A cladogram showed the differentiation of the three groups based on the flavor of the fruits. The diversity of SMs decreases in evolutionary terms, in response to domestication and environmental adaptation. Therefore, genotypes can be feasibly selected based on fruit flavor for gross-breeding, and cytotoxicity can be reduced without losing possible therapeutic effects.

Keywords: cucurbitacins, flavonoids, fruit, cancer, diabetes, endemic species

OPEN ACCESS

Edited by:
Alessandra Durazzo, Research Centre for Food and Nutrition, Council for Agricultural Research and Economics, Italy

Reviewed by:
Umakanta Sarker, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh
Arpita Roy, Sharda University, India
Rosalinda Gonzalez, Universidad Autónoma de Querétaro, Mexico

***Correspondence:**
Jorge Cadena-Iñiguez
jocadena@gmail.com
Ramón Marcos Soto-Hernández
msoto@colpos.mx

†These authors have contributed equally to this work and share first authorship

‡Deceased

Specialty section:
This article was submitted to Plant Metabolism and Chemodiversity, a section of the journal Frontiers in Plant Science

Received: *08 September 2021*
Accepted: *11 November 2021*
Published: *21 December 2021*

Citation:
Iñiguez-Luna MI, Cadena-Iñiguez J, Soto-Hernández RM, Morales-Flores FJ, Cortes-Cruz M and Watanabe KN (2021) Natural Bioactive Compounds of Sechium spp. for Therapeutic and Nutraceutical Supplements. Front. Plant Sci. 12:772389. doi: 10.3389/fpls.2021.772389

INTRODUCTION

The use of medicinal plants is based on empirical knowledge that has been preserved for generations, with the aim of improved or recovered health (Palma-Tenango et al., 2017). In Mexico, it is estimated that there are 5,000 species of plants with healing properties (Villaseñor, 2016); similarly, different active substances have been isolated from these, sometimes obtaining more

Abbreviations: AFLP, amplified fragment length polymorphism; BANGE*Se*, National Germplasm Bank of *Sechium edule*; CuD, cucurbitacin D; CuI, cucurbitacin I; CuB, cucurbitacin B; CuE, cucurbitacin E; Ru, rutin; Fz, florizidin; Mi, myricetin; Qu, quercetin; Na, naringenin; Ft, floretin; Ap, apigenin; Ga, galangin; P388, leukemia in mice; L929, mouse fibroblast cell line; J774, mouse macrophage cell line; HeLa, Henrietta Lacks, human cervical cancer cell line; WEHI-3, leukemic cells.

than one per plant (Molina-Mendoza et al., 2012). These substances correspond to secondary metabolites (SMs), which are biomolecules that enable plants to survive, adapt, and reproduce when they are threatened by predators or stress conditions (Piasecka et al., 2015).

Recently, SMs have become increasingly important in the food, industrial, cosmetic, textile, and pharmaceutical sectors (Bourgaud et al., 2001), and approximately 200,000 SMs have been identified (Croteau et al., 2000). Some species of the genus *Sechium* P. Browne (Cucurbitaceae) are found among plants with broad diversity and complex composition as reported in Mexico, whose fruits are known as chayotes (Lira et al., 1999; Cadena-Iñiguez et al., 2011). The SMs confer particular characteristics to this species, thereby creating morpho-biochemical differences that affect the phenotype, and there is evidence in *Sechium* of morpho-biochemical changes in edible types. Among the phyto compounds that have aroused particular interest are triterpenes, due to their antiproliferative properties. These have been evaluated with different *in vitro* and *in vivo* models, in an attempt to define their mechanism of action and effects when they are used as cancer cell treatments (Vega et al., 2006; Petronelli et al., 2009; Bishayeen et al., 2011; Aguiñiga-Sánchez et al., 2017). Flavonoids are metabolites identified in *Sechium*, and there are reports of their antineoplastic activities (Deschner et al., 1993; Manach et al., 1996; Salazar-Aguilar et al., 2017).

In the development of anticancer compounds from natural sources, various active substances have been extracted and commercialized, such as vincristine, camptothecin, and taxol. However, some of these have shown toxic effects or non-selective activities, resulting in the elimination of every cell with a high proliferation rate, such as lymphocytes and hair cells (Vega et al., 2006; Jacobo-Herrera et al., 2016). Under this premise, genetic improvement has begun with certain types of chayote because antineoplastic activity has been demonstrated through bioassays in the WEHI-3, HeLa, P388, J774, and L929 cell lines, among other principal ones (Cadena-Iñiguez et al., 2013; Aguiñiga-Sánchez et al., 2015, 2017; Salazar-Aguilar et al., 2017). In bone marrow mononuclear cells, DNA fragmentation of malignant cells was found, revealing the selective activity of chayote extracts. The advantages cited, in addition to being a genus that integrates cultivable genotypes having production yields exceeding 64 t ha^{-1} per year (SIAP, 2019), confer importance to chayote as a source of biomass for active substances with pharmacological objectives.

Agrobiodiversity includes wild relatives of domesticated genotypes or in the process of domestication (Convenio de Diversidad Biológica [CDB], 2016), however, there is a risk of genetic loss or erosion due to changes in consumer preference, displacement by new cultivars and habitat fragmentation. The intra- and interspecific complexes of agrobiodiversity contain biological variants with great potential for the extraction of SMs for diverse uses and are economically important for the pharmaceutical, health and food industries (Roskov et al., 2015). It is estimated that the diversity of vascular plants in Mexico ranges from 22,000 to 31,000 species, of which about 4,000 have medicinal use (Leon-Lobos et al., 2012).

Based on the differences in the phytochemical profiles of *Sechium sp*., an attempt has been made to help explain the causes of the morphobiochemical changes observed in the morphotypes. However, there is interest in developing genotypes with medium-term yields in the field (biomass) to recover SMs of therapeutic interest that increases the pharmacological options and dietary supplements. Starting from the hypothesis that domesticated genotypes have lost many SMs, while wild types can be highly toxic, an evaluation of contrasting and underutilized genotypes of *Sechium sp*. was carried out, in order to identify SMs for pharmacological and nutraceutical use, in order that it contributes to the design of new varieties by genetic crossing to obtain bioactive natural products, and to favor their revaluation and importance as components of agrobiodiversity that reduce the risk of loss.

MATERIALS AND METHODS

Materials

Sechium spp. fruits of genotypes with contrasting flavors were chosen that were considered representative of the contrasts (Cadena-Iñiguez et al., 2011; **Table 1**; **Figure 1**). The fruits evaluated were harvested at a stage of horticultural maturity (**Table 1**), and all of them came from the national gene bank of *Sechium edule* in Mexico (BANGE*Se*) (19° 08′ 48″ N, 97° 57′ 00″ W). All accessions registered their entry into the germplasm bank in 2005 and have been maintained under agroclimatic conditions and equal management.

Extraction Process

The fruits were washed, cut, weighed, and then placed in a drying oven (BLUE-M, Electronic Company/Blue Island, IL, United States) with an air flow at 45°C. After drying for 72 h, all parts of the fruits (exocarp, mesocarp, spines, and seed) were ground in a mill (Hamilton Beach, United States). A continuous extraction of the dry ground material was carried out with methanol (Cadena-Iñiguez et al., 2013; Aguiñiga-Sánchez et al., 2017). From the ground material, 2.5 g were weighed for each accession and submerged in 80% methanol at a 1:10 ratio in 50 mL Falcon tubes. The samples were homogenized, placed in an ultrasonic bath (Branson B-220 50/60 Hz) at room temperature,

TABLE 1 | Main characteristics of the accessions of *Sechium* spp. based on fruit color and flavor (n = 27).

Genotype	Accession	Color	Flavor	Status
S. edule var. albus levis	769	yellow	sweet	Semi-domesticated
S. edule var. albus levis	761	yellow	sweet	Semi-domesticated
S. edule var. albus minor	330	yellow	sweet	Semi-domesticated
S. edule var. nigrum minor	681	dark green	neutral	Semi-domesticated
S. edule var. virens levis	273	light green	neutral	Domesticated
S. edule var. nigrum xalapensis	530-a	dark green	neutral	Domesticated
Sechium edule	653	dark green	bitter	Wild type
Sechium compositum	11	dark green	bitter	Wild type
Sechium compositum	751	dark green	bitter	Wild type

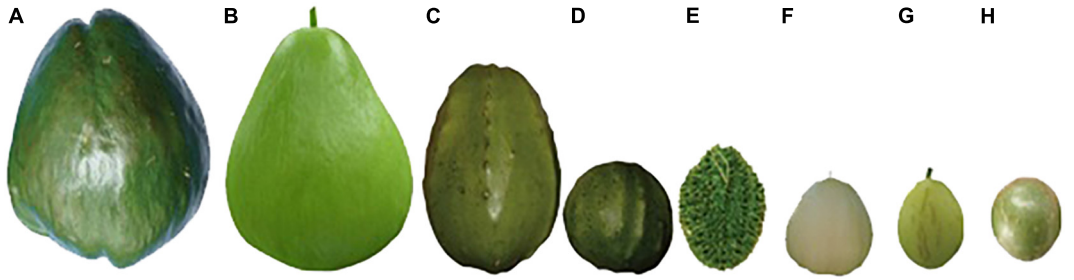


FIGURE 1 | Morphotypes of *Sechium* spp. evaluated. **(A)** *S. edule* var. *nigrum xalapensis*, **(B)** *S. edule* var. *virens levis*, **(C)** *S. compositum* (accession 11), **(D)** S. *compositum* (accession 751), **(E)** *S. edule* wild-type, **(F)** *S. edule* var. *albus levis*, **(G)** *S. edule* var. *albus minor*, **(H)** *S. edule* var. *nigrum minor*. The reference metric scale is 1 cm per square.

and were then subjected to two cycles of 10 min sonication and 5 min of rest between each cycle. Subsequently, the tubes were centrifuged at 3000 *g* for 5 min. and the supernatant was collected in 2 mL amber vials, and stored in the fridge before analysis by HPLC (Aguiñiga-Sánchez et al., 2017).

Determination of Cucurbitacins and Flavonoids

Based on a previously described methodology (Salazar-Aguilar et al., 2017), cucurbitacins and flavonoids were analyzed by high-performance liquid chromatography (HPLC), where 20 mg of extract was weighed per sample, dissolved with 1 mL of HPLC-grade methanol (Sigma-Aldrich, St. Louis, MO, United States), and then filtered using a nylon membrane acrodisc with a diameter of 0.45 μm (Merck, Millipore, Germany).

Cucurbitacins were analyzed through a symmetry shield C18 column (4.6 × 250 mm) (Waters, Spain) *via* an isocratic analysis. The mobile phase used was in isocratic mode with water, methanol, and acetonitrile (50:30:20 v/v), and the injection flow rate was 1 mL min^{-1} at a pressure of 179 bars, with all the samples being at a temperature of 25°C. The total volume injected per accession evaluated was 20 μL. Finally, the identification of cucurbitacins was performed using two wavelengths (λ1 235 nm and λ2 254 nm). As a reference standard, cucurbitacins D, I, B, and E were used (Sigma-Aldrich, United States).

In the case of flavonoids, a hypersil ODS column (125 × 40 mm) was used (Hewlett-Packard, United States). with a gradient of (A) H_2O adjusted at pH 2.5 with trifluoroacetic acid, and (B) acetonitrile 0–10 min, in the following mixtures A:B 85:15 for 20 min, and A:B 65:35 for 25 min. The following parameters were used: flow at 1 mL/min at 30°C, detection wavelengths, 254, 316, and 365 nm; injection volume, 20 μL and analysis time, 25 min. The standards used were rutin, florizidin, myricetin, quercetin, naringenin, florentin, and galangin (Sigma-Aldrich, United States).

DNA Extraction for Amplified Fragment Length Polymorphism Bands

In order to demonstrate an approach to the relationship between secondary metabolites and genetic expression, DNA was extracted from the young leaves of previously evaluated accessions (Shagai-maroof et al., 1984) and characterized by AFLPs (Vos et al., 1995). A double digestion was applied to 500 ng of DNA with *Eco*RI/*Mse*I restriction enzymes (10 and 2.5 U/μg, respectively) at 37°C for 4 h. After evaluating the restriction fragments by electrophoresis in 0.8% agarose for 70 min at 80 V, the ligation began by adding 10 μL of a mixture of adaptors at 5 mg kg^{-1} to the T4 DNA ligase enzyme at a final concentration of 1 U.

For the pre-amplification, 4 μL of the ligation product was combined with 21 μL of a mixture containing starters *Mse*I + C (5 μM), *Eco*RI + A (5 μM), and 10.5 μL of Sigma REDTaq® ReadyMix™ PCR Reaction Mix and was finally adjusted to a volume of 25 μL with Type I water. The thermocycler was programmed at 94°C for 30 s, 56°C for 60 s, and 72°C, for 25 cycles. The result of this PCR was corroborated in 0.8% agarose gel for 60 min at 80 V, and then, the pre-amplification reaction was diluted with 80 μL of Type I water.

For the selective amplification, 3.0 μL of the aforementioned dilution were taken, adding 3 μM *Eco*RI + ACC marked with FAM and 5 μM *Mse*I + CAT, and adjusting to a final volume of 10 μL with 1× SIGMA REDTaq® ReadyMix™ PCR Reaction Mix. The thermocycler was programmed at 95°C for 5 min, followed by 35 cycles (95°C for 40 s, 54°C for 40 s, and 72°C for 90 s) and final elongation at 72°C for 40 min.

The product of the amplification was observed in a 6% polyacrylamide gel that underwent electrophoresis at 200 V for 3 h. The gels were dyed with silver nitrate as soon as the fragments were visualized, and then, a dilution (1:10) of the PCR-selective and formamide products (Hi-Di™ Formamide, United States) was prepared and evaluated in a capillary sequencer 3500 XL Genetic Analyzer (ThermoFisher Scientific, United States) along with the fragment analysis running model (FragmentAnalysis50_POP7xl_2, Fragment_Analysis_PA_Protocol). Using the amplification data (size of the fragment), a binary matrix of presence and absence (1/0) was generated through the Gene Mapper 4.1 software (GeneMapper, Applied Biosystems).

Statistical Analysis

The data were analyzed *via* a cladistics approach with WinClada 1.00.08 (2002) (WinClada v.1.00.08, 2020). The applied analysis

was heuristic, with the Bootstrap/Jackknife tests, which generated the index of consistency and stability, respectively (Felsenstein, 1985; Lanyon, 1985).

RESULTS

Phytochemical Screening

The relationship between fresh and dry weight and extract yield showed that on average, the genotypes evaluated were composed of 73.85% water. As previously noted, fruits with a yellow epidermis (*albus minor*, *albus levis*) showed a lower percentage (52.27%), and those with the highest water content were the green fruits with a neutral flavor (82.40%), followed by the wild types accounting for 79.76%. In the case of the extract yield, 52.98% was found for genotypes of neutral flavor, while the *albus* (yellow) yielded 39.26%, and finally the wild fruits of bitter flavor accounted for 37.27% (**Table 2**).

High-performance liquid chromatography analysis revealed the presence of cucurbitacins I, D, B, and E, and in terms of flavonoids, rutin, myricetin, and floretin were found in all the genotypes, while galangin was present in all except for *virens levis* and *nigrum xalapensis* (neutral flavor). Quercetin was found in six of the nine genotypes, and was absent in *albus levis*, *virens levis*, and *nigrum xalapensis*. Naringenin was observed in *albus minor* and the two wild ones of *S. compositum*. Finally, florizidin was identified in *virens levis* and in accession 11 of *S. compositum* (**Tables 3**, **4**).

The genotypes with higher concentrations of cucurbitacin D (CuD) and CuE were yellow fruits (*albus minor, albus levis*), while higher amounts of CuB and CuI were found in bitter chayotes (*S. compositum* and *S. edule*). In the case of CuD, the highest concentration was found in *nigrum xalapensis*, followed by *albus minor* and *S. compositum*. In terms of CuI, the highest value was identified in *S. compositum* and *nigrum minor*, and in the case of CuB, the highest concentration was for *S. compositum* and *albus minor*. The only material that contained CuE was *albus levis* (accession 761).

High-performance liquid chromatography analysis for flavonoids showed that there were seven to eight standards in the evaluated genotypes, and no apigenin was reported. Rutin, myricetin, quercetin, and galangin were identified from the groups of flavonols, florizidin and floretin were identified from the chemical group of dihydrochalcones, and only naringenin came from flavones. In general, the highest concentration of flavonoids was quantified in bitter genotypes, followed by chayotes of neutral flavor. Only rutin and naringenin flavonoids were found in higher quantities in yellow fruits (*albus levis* and *albus minor*) (**Table 4**).

TABLE 3 | Yield of cucurbitacins obtained from fruits at horticultural maturity stage of contrasting flavor of *Sechium* spp. (*n* = 27).

Genotype	mg g^{-1}			
	CuD	CuI	CuB	CuE
S. edule var. albus levis	1.17	4.27	1.27	0
S. edule var. albus levis	4.77	3.52	0.46	1.73
S. edule var. albus minor	12.71	1.93	1.52	0
S. edule var. nigrum minor	1.06	5.84	0.27	0
S. edule var. virens levis	0.95	4.04	0.62	0
S. edule var. nigrum xalapensis	13.44	5.60	0.73	0
Sechium compositum	3.00	4.70	1.96	0
Sechium compositum	8.35	17.47	4.37	0
Sechium edule wild type	1.04	3.10	0.18	0

CuD = cucurbitacin D; CuI = cucurbitacin I; CuB = cucurbitacin B; CuE = cucurbitacin E.

TABLE 4 | Flavonoid content obtained from fruits at horticultural maturity stage of contrasting flavor of *Sechium* spp. (*n* = 27).

Genotype	mg g^{-1}							
	Ru	Fz	My	Qu	Na	Ft	Ap	Ga
S. edule var. albus levis	0.26	0.00	0.78	0.00	0.00	0.09	0	0.46
S. edule var. albus levis	0.13	0.00	0.55	0.00	0.00	0.10	0	0.43
S. edule var. albus minor	0.25	0.00	0.69	0.01	0.16	0.05	0	0.48
S. edule var. nigrum minor	0.13	0.00	0.27	0.01	0.00	0.06	0	0.44
S. edule var. virens levis	0.23	0.08	1.09	0.00	0.00	0.11	0	0.00
S. edule var. nigrum xalapensis	0.08	0.00	0.77	0.00	0.00	0.15	0	0.00
Sechium compositum	0.48	0.11	0.19	0.03	0.56	1.42	0	5.80
Sechium compositum	1.20	0.00	1.46	0.21	1.59	2.19	0	12.32
Sechium edule wild type	0.82	0.00	1.10	0.09	0.00	0.06	0	0.51

Ru = rutin; Fz = florizidin; My = myricetin; Qu = quercetin; Na = naringenin; Ft = floretin; Ap = apigenin; Ga = galangin.

TABLE 2 | Relationship between fresh, dry weight, and percentage yield of extracts from fruits at horticultural maturity stage of *Sechium* spp. (*n* = 27).

Variable	Fruit flavor/accession								
	Sweet			Neutral			Bitter		
	769	761	330	681	273	530-a	11	751	653
Fresh weight (g)	84.94	34.3	10.44	9.63	360	390	555	147.24	119.41
Dry weight (g)	27.6	19.1	4.7	3.3	37.9	29.7	80.6	27.3	33.00
Water (%)	67.5	55.68	54.98	65.33	89.5	92.38	85.47	81.45	72.36
Dry matter (%)	32.49	44.31	45.02	34.26	10.5	7.61	14.52	18.54	27.63
Extract (g)	1.15	1.08	0.71	1.07	1.29	1.61	0.94	1.01	0.85
Total yield (%)	46.04	43.29	28.47	42.94	51.6	64.37	37.44	40.52	33.91

Combination of Phytochemical Variables and Polymorphic Bands of Amplified Fragment Length Polymorphisms

Figure 1 shows the parsimonious arrangement of the combination of phytochemical variables and polymorphic bands of AFLPs (one thousand repetitions), signaling a reconstruction of inheritable similarities, where bitter chayotes of dark green color with spines were the genotypes most proximal to the root of the monophyletic tree, corresponding to ancestral characteristics. The cladogram shows the three groups distinguished by the flavor of the fruits. The first was formed by bitter genotypes, placing *S. edule* as the root taxon, followed by the two accessions of *S. compositum*. Similarly, the second group was formed by green chayotes of neutral flavor, and the last group was formed by those of yellow fruit of sweet flavor. Accordingly, and under a cladistics view, an evolutionary arrangement was marked, suggesting that the bitter flavor is an ancestral characteristic that changes (decreases) due to differences in secondary metabolites and associated polymorphic bands. The first case was observed in the group of bitter chayotes, where an absence of the 16 (77 pb) band was found in the ancestral traits (dark circles) of *S. edule* and *S. compositum* (accession 11), as well as the mean concentration of CuD in accession 751 of *S. compositum*.

It is difficult to consider abrupt changes in the genotypes of a species in the same agroclimatic region of distribution. However, small differences attributed to the same environment can possibly be identified. For example, even when accession 11 and 751 are *S. compositum* and come from the same region (Chiapas, Mexico), the first one comes from the evergreen tall forest, and the second comes from ruderal conditions without vegetation, which can timely mark modifications in the phenotype. Plasticity is a trait that allows plants to carry out morphological and biochemical adjustments, and especially for this case, accession 11 is of pyriform fruit, while accession 751 is round and small (**Figure 1**). The second group included genotypes with neutral-flavor fruits and corresponded to those with the highest consumption and commercial manipulation, indicating the presence of cucurbitacin B, rutin, and myricetin, which are classified as apomorphic traits (new). Finally, the genotypes *albus minor*, *albus levis*, and even *nigrum minor*, considered as semi-domesticated, of low consumption, and conserved in family backyards, exhibited six traits (**Table 5**), half of which corresponded to phytochemicals with mean values in terms of concentration of cucurbitacins D and E, such as the symplesiomorphic trait (ancestral and shared with the other genotypes), and the presence of band 54 (426 pb), as an autapomorphic trait (new).

With regard to polymorphic bands associated with phytochemical traits (**Figure 2** and **Table 5**), it is assumed that they are non-coding DNA, neutral to selection. This is why, it is necessary to understand what type of information these bands contain and why they need to be isolated, cloned, and sequenced, and then make an alignment ("blast") to identify possible associations with biological activity. Although these SMs do not completely determine the flavor of the fruits, they are of great influence. **Tables 6**, **7** show the concentrations of cucurbitacins and flavonoids per group of genotypes. The sweet fruits showed a sum of 12.59 mg g^{-1}, neutral showed 12.00 mg g^{-1}, and bitter showed 24.78 mg g^{-1}.

DISCUSSION

The type of metabolite present in chayote gives each fruit its characteristic flavor. For example, in a previous study (Tallamy and Krischik, 1989), it was mentioned that the qualitative composition of cucurbitacins among species of *Cucurbita* spp. was mutually exclusive. That is to say, the species produces cucurbitacins B and D, or cucurbitacins E and I, with additional qualitative variation in the production of bitter glycosides, where B and D are synthesized mainly as aglicones, while E and I are regularly produced in considerable amounts of glucosides. With *Sechium*, there was no synthesis excluding cucurbitacins per

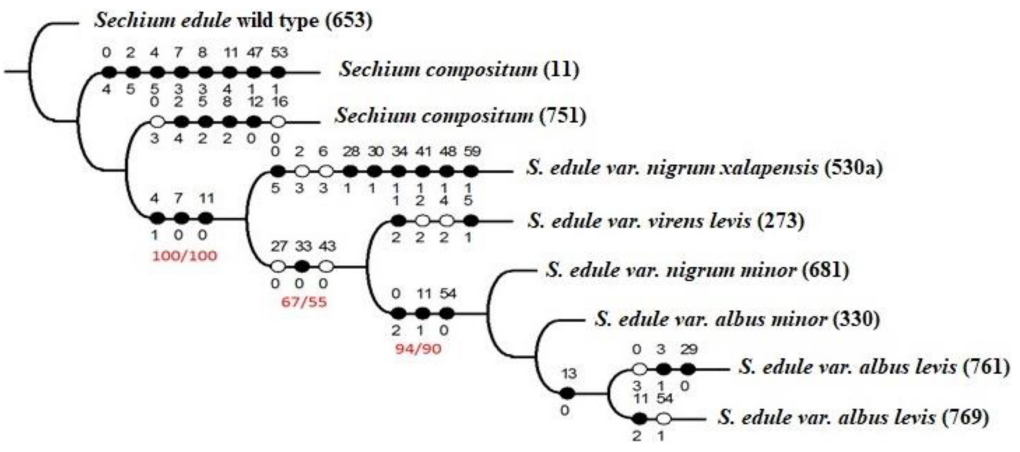


FIGURE 2 | Cladogram with nine genotypes (accessions) of *Sechium* spp., with phytochemical profile values (cucurbitacins and flavonoids), as well as AFLP polymorphic bands in combination with *Eco*RI *ACC and Mse1 CAT*. The white circle corresponds to the new traits, the black circle to the ancestral traits, and the values in the higher part of the circle reveal the trait and lower value of it, in its state. *L* = 87, *Ci* = 72, *Ri* = 38, and values 100/100, 67/55, and 94/90 for the bootstrap/jack knife tests, respectively.

TABLE 5 | Plesiomorphic and apomorphic traits, from the analysis of 12 biochemical variables and 48 polymorphic bands of AFLPs, from *Sechium* spp. (Phytochemical trait 0–11; AFLP bands: 12–59).

Genotype/accession	Trait/status of trait per clade	Internal clade
Sechium edule wild type 653	0/4, 2/5, 4/5, 7/3, 8/3, 11/4, 47/1, 53/1	4/1, 7/0, 11/0,
Sechium compositum 11	0/2, 2/4, 5/2, 8/2, 12/0, 16/0	
Sechium compositum 751	0/5, 2/3, 6/3, 28/1, 30/1, 31/1, 41/1, 48/1, 59/1	
S. edule var. nigrum xalapensis 530a	1/2, 2/2, 4/5, 5/1	
S. edule var. virens levis 273	0/2, 11/1, 54/0	27/0, 33/0,43/0
S. edule var. nigrum minor 681		0/2, 11/1, 54/0
S. edule var. albus minor 330		
S. edule var. albus levis 761	0/3, 3/1, 29/0	13/0
S. edule var. albus levis 769	11/2, 54/1	

TABLE 6 | Mean concentration of cucurbitacins related to the flavor of the fruit from *Sechium* spp. Values obtained from $n = 27 \pm$ standard error.

Group	CuD	CuI	CbB	CuE	Σ SMs
Sweet	6.22 ± 3.4	3.24 ± 0.69	1.08 ± 0.32	0.58 ± 0.58	11.12
Neutral	5.15 ± 4.14	5.16 ± 0.57	0.54 ± 0.14	0.00	10.85
Bitter	4.13 ± 2.18	8.43 ± 4.55	2.17 ± 1.21	0.00	14.73

CuD = cucurbitacin D; CuI = cucurbitacin I; CuB = cucurbitacin B; CuE = cucurbitacin E.

genotype, because D, I, and B appeared in all genotypes, and only E was identified in a sweet accession. This suggests that there is no exclusion of synthesis, but there are differences in terms of concentration.

A previous study (Ríos et al., 2012) mentioned cucurbitacin I (also called JSI-124) as a selective inhibitor of phosphorylate tyrosine JAK3/STAT3, and may be considered a potential anticancer agent in addition to cucurbitacins E and B. It has been previously reported (Takahashi et al., 2009) that cucurbitacin D acts as an inducer of apoptosis in hepatic carcinoma. The evaluated extracts revealed the presence of compounds with possible therapeutic interest, such as cucurbitacins and flavonoids, as reported in different fruits of chayote of separate genetic lineage (Cadena-Iñiguez et al., 2013; Aguiñiga-Sánchez et al., 2017; Salazar-Aguilar et al., 2017), whose antineoplastic and antiproliferative activity has also been previously determined (Petronelli et al., 2009; Bishayeen et al., 2011; Soto-Hernández et al., 2015).

Plants are the excellent source of SM and/or antioxidants. There are compounds that are not of direct use for the survival of plants but help organisms to function more optimally in their environment (Piasecka et al., 2015), such as SMs (O'Connor, 2015) which include phenolics and flavonoids, hydroxybenzoic acids (Sarker and Oba, 2019a), hydroxycinnamic acids (Sarker et al., 2020c), flavanols (Sarker et al., 2020b; Sarker and Oba, 2020b), flavones (Sarker and Oba, 2020e), flavanones (Sarker and Oba, 2020f), tocopherols (Sarker and Oba, 2020d), betalains (Sarker and Oba, 2021), ascorbic acid (Sarker and Oba, 2020c), carotenoids (Sarker and Oba, 2020a), betacyanin (Sarker et al., 2020a), betaxanthin (Sarker and Oba, 2019b), chlorophyll a and b (Sarker et al., 2018a; Sarker and Oba, 2019c), and beta-carotene (Sarker et al., 2018b).

Their production is frequently associated with conditions of biotic and abiotic stress, with the most harmful being the high incidence of light and nutritional deficiencies (Schlaepfer and Mendoza-Espinoza, 2010), thus leading to their carbon-based biosynthesis (phenolic). According to the carbon nutrient balance (CNB) hypothesis, in the case of nitrogen limitation, the SMs lean toward carbon-rich metabolites (phenols and terpenes), and when there is carbon limitation, there is an increase in nitrogen-rich metabolites (alkaloids) (Hamilton et al., 2001; Palumbo et al., 2007).

The literature has shown that any stresses like drought and salinity create reactive oxygen species (ROS) (Sarker and Oba, 2018b), osmotic stress (Sarker and Oba, 2020g), decrease in photosynthetic activities (Sarker and Oba, 2018d), nutrient imbalance (Sarker et al., 2018), and ultimately cause oxidative damage in plant cells (Sarker and Oba, 2018c), that affects the growth and productivity of crops (Sarker and Oba, 2019d). However, to detoxify ROS and cope with stresses, plant evolved mechanisms to augment the concentration of these SMs and/or antioxidants (Sarker and Oba, 2018a).

In this regard, it should be noted that the domesticated and semi-domesticated genotypes of *Sechium* provide nutrients (preferably nitrogenous) in their life history, suggesting an influence on the lower concentration of flavonoids and triterpenes, and, contrary to bitter types, these genotypes may exhibit deficiencies in their wild states, thereby increasing the amount of triterpenes and flavonoids. Hence, stress augments SMs content that can be used in food, cosmetic and pharmaceutical industry.

The probable movement that gave rise to the chayote varieties of *Sechium* spp. highlights the environmental variables and the manipulation that work together to select edible forms. This would promote scenarios for adaptation, where the plasticity of the genus would have mainly allowed morphological and biochemical variations. The semi-domesticated genotypes of yellow fruit express metabolites that can provide carotenoids with greater protection from solar radiation. These fruits are relatively small, which may decrease the area of incidence of

TABLE 7 | Mean concentration of flavonoids related to the flavor of fruit from *Sechium* spp. Values obtained from $n = 27 \pm$ standard error.

Group	Ru	Fz	My	Qu	Na	Ft	Ga	Σ SMs
Sweet	0.21 ± 0.04	0.00	0.67 ± 0.07	0.00	0.05 ± 0.05	0.08 ± 0.02	0.46 ± 0.02	1.47
Neutral	0.15 ± 0.05	0.03 ± 0.03	0.71 ± 0.24	0.0037	0.00	0.11 ± 0.03	0.15 ± 0.15	1.15
Bitter	0.83 ± 0.21	0.36 ± 0.04	0.92 ± 0.37	0.11 ± 0.05	0.72 ± 0.46	1.23 ± 0.62	6.21 ± 3.42	10.05

Ru = rutin; Fz = florizidin; Mi = myricetin; Qu = quercetin; Na = naringenin; Ft = floretin; Ga = galangin.

radiation and lower the amount of water compared to the domesticated materials, thereby modifying the expression of metabolites that provide protection against predators, such as cucurbitacins (Piasecka et al., 2015).

This is relevant because flavonols show higher protection activity at the level of the cell membrane (López-Revuelta et al., 2006), acting as antiradicals, antichelants, lipid antioxidants, antimutagens, and having an antiproliferative effect, thereby inhibiting carcinogenesis (Martínez-Flórez et al., 2002).

Considering the change in diversity and type of SMs found in the genotypes of *Sechium* spp., it is possible that certain current changes, such as flavor, color, and size, are a direct product of adjustments in the biosynthesis of the mevalonic acid pathway. For example, when photoprotective pigments are required in the fruit, the amount of triterpenes decreases, and with it, the bitter flavor of the fruits, in addition to gradually showing changes in color from dark green to light green with yellow colors of the epidermis. In addition to this, a previous report (Cadena-Iñiguez et al., 2011) described how the ascorbic acid in yellow fruits was attributed to the need for photoprotection, which coincides with the fact that the fruits of edible genotypes of *Sechium* spp. present stomas found in the epidermis (Cadena-Iñiguez et al., 2006). These results assist in explaining the causality of certain morphological variables in *Sechium* spp., in addition to evidencing relevant phytochemical variables that may guide the selection of sweet, neutral, and bitter genotypes, to design gross-breeding.

The cucurbitacins and flavonoids found have demonstrated anti-proliferative, antioxidant, and chelating activity, DNA fragmentation, and apoptosis induction (previously mentioned). However, the evaluated genotypes show contrasts in terms of content and diversity. Some flavonoid and terpenoid derivatives, such as cucurbitacins in one genotype, are at risk of generating intrinsic toxicity (Marinoff et al., 2009), thereby causing long-term intoxication. Therefore, it is important to understand the main phytochemicals and obtain new varieties of *Sechium* showing therapeutic and nutraceutical activity values that are easier to manage for human health and nutrition.

An important fact about the search and identification of natural compounds from species and genotypes little used and that are part of agrobiodiversity, is that when they are not used the trend is their loss in the medium term (Food and Agriculture Organization [FAO], 2019), therefore, this work highlights the opportunity to have alternative sources of inputs for health, pharmacology, and food industry.

CONCLUSION

The cladistic analysis can identify and contrast the diversity of cucurbitacins and flavonoids, suggesting that they are related to the polymorphic genetic bands in nine genotypes of *Sechium*, where the bitter flavors (wild) showed a greater number of ancestral traits, which makes them genetically and phytochemically more complex. Flavonoids were present in higher concentrations in genotypes as compared to those of cucurbitacins. However, both groups of metabolites are associated with therapeutic and nutraceutical activity, highlighting genotypes as an important source of biomass and metabolites of interest.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JC-I and RS-H: conceptualization. MI-L, RS-H, and KW: investigation. FM-F and MC-C: software, formal analysis, and data curation. MI-L, JC-I, and RS-H: writing – original draft.

FUNDING

This research was supported in part by Plant Transgenic Design Initiative (PTraD) at Tsukuba Plant Innovation Research Center, University of Tsukuba, Japan.

ACKNOWLEDGMENTS

The authors thank the Interdisciplinary Research Group of *Sechium edule* in Mexico (GISeM) for providing the biological material and the laboratory staff of the Phytochemical Lab of Colegio de Postgraduados for their technical support. The research was conducted in part as an extension of SATREPS (Diversity Assessment and Development of Sustainable Use of Mexican Genetic Resources 2012-2018) and DIGEM. Also, the research was supported in part by the Plant Transgenic Design Initiative (P-TraD) at Ksukuba Plant Innovation Research Center, University of Tsukuba, Japan.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.772389/full#supplementary-material

REFERENCES

Aguiñiga-Sánchez, I., Cadena-Íñiguez, J., Santiago-Osorio, E., Gómez-García, G., Mendoza-Núñez, V. M., Rosado-Pérez, J., et al. (2017). Chemical analyses and in vitro and in vivo toxicity of fruit methanol extract of *Sechium edule* var. nigrum spinosum. *Pharm. Biol.* 55, 1638–1645. doi: 10.1080/13880209.2017.1316746

Aguiñiga-Sánchez, I., Soto-Hernández, R. M., Cadena-Iñiguez, J., Ruiz-Posadas, L., Cadena-Zamudio, J. D., González-Ugarte, A. K., et al. (2015). Fruit extract from a *Sechium edule* hybrid induce apoptosis in leukaemic cell lines but

not in normal cells. *Nutr. Cancer* 67, 250–257. doi: 10.1080/01635581.2015.989370

Bishayeen, A., Ahmed, S., Brankov, N., and Perloff, M. (2011). Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. *Front. Biosci.* 16:980. doi: 10.2741/3730

Bourgaud, F., Gravot, A., Milesi, S., and Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Sci.* 161, 839–851. doi: 10.1016/S0168-9452(01)00490-3

Cadena-Iñiguez, J., Arévalo-Galarza, M. L., Ruiz-Posadas, L. M., Aguirre-Medina, J. F., Soto-Hernández, R. M., Luna-Cavazos, M., et al. (2006). Quality evaluation and influence of 1-MCP on *Sechium edule* fruit during postharvest. *Postharvest Biol. Technol.* 40, 170–176. doi: 10.1016/j.postharvbio.2005.12.013

Cadena-Iñiguez, J., Soto-Hernández, R. M., Arévalo-Galarza, M. L., Avendaño-Arrazate, C. H., Aguirre-Medina, J. F., and Ruíz-Posadas, L. M. (2011). Caracterización bioquímica de variedades domesticadas de chayote *Sechium edule* (Jacq.) Sw. comparadas con parientes silvestres. *Rev. Chapingo. Ser. Hortic.* 17, 45–55.

Cadena-Iñiguez, J., Soto-Hernández, R. M., Torres, A., Aguiñiga-Sánchez, I., Ruíz-Posadas, L. M., Rivera-Martínez, A. R., et al. (2013). The antiproliferative effect of chayote varieties (*Sechium edule* (Jacq.) Sw.) on tumour cell lines. *J. Med. Plants Res.* 7, 455–460. doi: 10.5897/JMPR12.866

Convention on Biological Diversity (2020). *Recommendation Adopted by the Open-Ended Working Group on the Post 2020 Global Biodiversity Framework*. United Nations. Available online at: https://www.cbd.int/doc/recommendations/wg2020-02/wg2020-02-rec-01-en.pdf

Croteau, R., Kutchan, T. M., and Lewis, N. G. (2000). "Natural products (secondary metabolites)," in *Biochemistry and Molecular Biology of Plants*, eds B. Buchanan, W. Grussem, and R. Jones (Rockville, MD: American Society of Plant Physiologists), 1250–1318.

Deschner, E. E., Ruperto, J., Wong, W., and Newmark, H. L. (1993). The effect of dietary quercetin and rutin on AOM-induced acute colonic epithelial abnormalities in mice fed a high-fat diet. *Nutr. Cancer* 20, 199–204. doi: 10.1080/01635589309514287

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using thebootstrap. *Evolution* 39, 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x

Food and Agriculture Organization [FAO] (2019). "The state of the world's biodiversity for food and agriculture," in *FAO Commission on Genetic Resources for Food and Agriculture Assessments*, eds J. Bélanger and D. Pilling (Rome: FAO), 572.

Hamilton, J. G., Zangerl, A. R., DeLucia, E. H., and Berenbaum, M. R. (2001). The carbon-nutrient balance hypothesis: it rise and fall. *Ecol. Lett.* 4, 86–95. doi: 10.1046/j.1461-0248.2001.00192.x

Jacobo-Herrera, N. J., Jacobo-Herrera, F. E., Zentella-Dehesa, A., Andrade-Cetto, A., Heinrich, M., and Pérez-Plasencia, C. (2016). Medicinal plants used in Mexican traditional medicine for the treatment of colorectal cancer. *J. ethnopharmacol.* 179, 391–402. doi: 10.1016/j.jep.2015.12.042

Lanyon, S. M. (1985). Detecting internal inconsistencies in distances data. *Syst. Zool.* 34, 397–403. doi: 10.2307/2413204

Leon-Lobos, P., Way, M., Aranda, D. P., and Lima-Junior, M. (2012). The role of ex situ seed banks in the conservation of plant diversity and in ecological restoration in Latin America. *Planta Ecol. Divers.* 5, 245–258.

Lira, R., Castrejón, J., Zamudio, S., and Rojas-Zenteno, C. (1999). Propuesta de ubicación taxonómica para los chayotes silvestres (*Sechium edule*, Cucurbitaceae) de México. *Acta Bot. Mex.* 49, 47–61.

López-Revuelta, A., Sánchez-Gallego, J. I., Hernández-Hernández, A., Sánchez-Yagüe, J., and Llanillo, M. (2006). Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress. *Chem. Biol. Interact.* 161, 79–91. doi: 10.1016/j.cbi.2006.03.004

Manach, C., Regerat, F., Texier, O., Agullo, G., Demigne, C., and Remesy, C. (1996). Bioavailability, metabolism and physiological impact of 4-oxo-flavonoids. *Nutr. Res.* 16, 517–544. doi: 10.1016/0271-5317(96)00032-2

Marinoff, M. A., Martínez, J. L., and Urbina, M. A. (2009). Precauciones en el empleo de plantas medicinales. *Bol. Latinoam. Caribe Plantas Med. Aromát.* 8, 184–187.

Martínez-Flórez, S., González-Gallego, J., Culebras, J. M., and Tuñón, M. (2002). Los flavonoides: propiedades y acciones antioxidantes. *Nutr. Hosp.* 17, 271–278.

Molina-Mendoza, J. L., Galván-Villanueva, R., Patiño-siciliano, A., and Fernández-Nava, R. (2012). Plantas medicinales y listado florístico preliminar del municipio de Huasca de Ocampo, Hidalgo, México. *Polibotánica* 34, 259–291.

O'Connor, S. E. (2015). Engineering of secondary metabolism. *Annu. Rev. Genet.* 49, 71–94. doi: 10.1146/annurev-genet-120213-092053

Palma-Tenango, M., San Miguel-Chávez, R., and Soto-Hernández, R. M. (2017). "Aromatic and medicinal plants in mexico," in *Aromatic and Medicinal Plants Back to Nature*, ed. H. A. El-Shemy (London: InTechOpen).

Palumbo, M. J., Putz, F. E., and Talcott, S. T. (2007). Nitrogen and gender effects on the secondary metabolism of yaupon, a caffeine-containing North American holly. *Oecologia* . 151, 1–9. doi: 10.1007/s00442-006-0574-1

Petronelli, A., Pannitteri, G., and Testa, U. (2009). Triterpenoids as new promising anticancer drugs. *Anticancer Drugs* 20, 880–892. doi: 10.1097/CAD.0b013e328330fd90

Piasecka, A., Jedrzejczak-Rey, N., and Bednarek, P. (2015). Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. *New Phytol.* 206, 948–964. doi: 10.1111/nph.13325

Ríos, L. J., Andújar, I., Escandell, M. J., Giner, M. R., and Recio, C. M. (2012). Cucurbitacins as inducers of cell death and a rich source of potential anticancer compounds. *Curr. Pharm. Des.* 18, 1663–1676. doi: 10.2174/138161212799958549

Roskov, Y., Abucay, L., Orrell, T., Nicolson, D., Kunze, T., Flann, C., et al. (eds) (2015). *Species 2000 & ITIS Catalogue of Life, 28th September 2015*. Leiden: Species 2000.

Salazar-Aguilar, S., Ruiz-Posadas, L. D. M., Cadena-Iñiguez, J., Soto-Hernández, M., Santiago-Osorio, E., Aguiñiga-Sánchez, I., et al. (2017). *Sechium edule* (Jacq.) swartz, a new cultivar with antiproliferative potential in a human cervical cancer HeLa cell line. *Nutrients* 9:798. doi: 10.3390/nu9080798

Sarker, U., and Oba, S. (2018a). Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected *Amaranthus tricolor* under salinity stress. *Sci. Rep.* 8:12349. doi: 10.1038/s41598-018-30897-6

Sarker, U., and Oba, S. (2018b). Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. *Sci. Rep.* 8:16496. doi: 10.1038/s41598-018-34944-0

Sarker, U., and Oba, S. (2018d). Response of nutrients, minerals, antioxidant leaf pigments, vitamins, polyphenol, flavonoid and antioxidant activity in selected vegetable amaranth under four soil water content. *Food Chem.* 252, 72–83. doi: 10.1016/j.foodchem.2018.01.097

Sarker, U., and Oba, S. (2018c). Drought stress effects on growth, ROS markers, compatible solutes, phenolics, flavonoids, and antioxidant activity in *Amaranthus tricolor*. *Appl. Biochem. Biotechnol.* 186, 999–1016. doi: 10.1007/s12010-018-2784-5

Sarker, U., and Oba, S. (2019b). Nutraceuticals, antioxidant pigments, and phytochemicals in the leaves of *Amaranthus spinosus* and *Amaranthus viridis* weedy species. *Sci. Rep.* 9:20413. doi: 10.1038/s41598-019-50977-5

Sarker, U., and Oba, S. (2019c). Protein, dietary fiber, minerals, antioxidant pigments and phytochemicals, and antioxidant activity in selected red morph *Amaranthus* leafy vegetable. *PLoS One* 14:e0222517. doi: 10.1371/journal.pone.0222517

Sarker, U., and Oba, S. (2019d). Salinity stress enhances color parameters, bioactive leaf pigments, vitamins, polyphenols, flavonoids and antioxidant activity in selected *Amaranthus* leafy vegetables. *J. Sci. Food Agric.* 99, 2275–2284. doi: 10.1002/jsfa.9423

Sarker, U., and Oba, S. (2019a). Antioxidant constituents of three selected red and green color *Amaranthus* leafy vegetable. *Sci. Rep.* 9:18233. doi: 10.1038/s41598-019-52033-8

Sarker, U., and Oba, S. (2020b). Nutraceuticals, phytochemicals, and radical quenching ability of selected drought-tolerant advance lines of vegetable amaranth. *BMC Plant Biol.* 20:564. doi: 10.1186/s12870-020-02780-y

Sarker, U., and Oba, S. (2020e). Phenolic profiles and antioxidant activities in selected drought-tolerant leafy vegetable amaranth. *Sci. Rep.* 10:18287. doi: 10.1038/s41598-020-71727-y

Sarker, U., and Oba, S. (2020f). Polyphenol and flavonoid profiles and radical scavenging activity in leafy vegetable *Amaranthus gangeticus*. *BMC Plant Biol.* 20:499. doi: 10.1186/s12870-020-02700-0

Sarker, U., and Oba, S. (2020d). Nutritional and bioactive constituents and scavenging capacity of radicals in *Amaranthus hypochondriacus*. *Sci. Rep.* 10:19962. doi: 10.1038/s41598-020-71714-3

Sarker, U., and Oba, S. (2020c). Nutrients, minerals, pigments, phytochemicals, and radical scavenging activity in *Amaranthus blitum* leafy vegetables. *Sci. Rep.* 10:3868. doi: 10.1038/s41598-020-59848-w

Sarker, U., and Oba, S. (2020a). Leaf pigmentation, its profiles and radical scavenging activity in selected *Amaranthus tricolor* leafy vegetables. *Sci. Rep.* 10:18617. doi: 10.1038/s41598-020-66376-0

Sarker, U., and Oba, S. (2020g). The response of salinity stress-induced A. tricolor to Growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. *Front. Plant Sci.* 11:559876. doi: 10.3389/fpls.2020.559876

Sarker, U., and Oba, S. (2021). Color attributes, betacyanin, and carotenoid profiles, bioactive components, and radical quenching capacity in selected *Amaranthus gangeticus* leafy vegetables. *Sci. Rep.* 11:11559. doi: 10.1038/s41598-021-91157-8

Sarker, U., Oba, S., and Daramy, M. A. (2020c). Nutrients, minerals, antioxidant pigments and phytochemicals, and antioxidant capacity of the leaves of stem amaranth. *Sci. Rep.* 10:3892. doi: 10.1038/s41598-020-60252-7

Sarker, U., Hossain, M. N., Iqbal, M. A., and Oba, S. (2020b). Bioactive components and radical scavenging activity in selected advance lines of salt-tolerant vegetable amaranth. *Front. Nutr.* 7:587257. doi: 10.3389/fnut.2020.587257

Sarker, U., Hossain, M. M., and Oba, S. (2020a). Nutritional and antioxidant components and antioxidant capacity in green morph *Amaranthus* leafy vegetable. *Sci. Rep.* 10:1336. doi: 10.1038/s41598-020-57687-3

Sarker, U., Islam, M. T., and Oba, S. (2018). Salinity stress accelerates nutrients, dietary fiber, minerals, phytochemicals and antioxidant activity in *Amaranthus tricolor* leaves. *PLoS One* 13:e0206388. doi: 10.1371/journal.pone.0206388

Sarker, U., Islam, T., Rabbani, G., and Oba, S. (2018a). Antioxidant leaf pigments and variability in vegetable amaranth. *Genetika* 50, 209–220. doi: 10.2298/GENSR1801209S

Sarker, U., Islam, T., Rabbani, G., and Oba, S. (2018b). Variability in total antioxidant capacity, antioxidant leaf pigments and foliage yield of vegetable amaranth. *J. Integr. Agric.* 17, 1145–1153. doi: 10.1016/S2095-3119(17)61778-7

Schlaepfer, L., and Mendoza-Espinoza, J. A. (2010). Las plantas medicinales en la lucha contra el cáncer, relevancia para México. *Rev. Mex. Cienc. Farm.* 41, 18–27.

Shagai-maroof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. (1984). Ribosomal spacer length polymorphisms in barley: mendelian inheritance, chromosomal location and population dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 81, 8014–8019. doi: 10.1073/pnas.81.24.8014

SIAP (2019). *Servicio de Información Agroalimentaria y Pesquera. Estadísticas.* Available online at: http://infosiap.siap.gob.mx/gobmx/datosAbiertos.php (accessed August 27, 2020).

Soto-Hernández, R. M., Cadena-Iñiguez, J., Arevalo-Galarza, L. C., Santiago-Osorio, E., Aguiñiga- Sánchez, I., and Ruíz-Posada, L. M. (2015). "Lead compound from Cucurbitaceae for the treatment of cancer," in *Chapter 12 Phytochemical Isolation, Characterization and Role in Human Health*, Vol. 12, eds A. Venket Rao and L. G. Rao (London: IntechOpen), 289–303. doi: 10.5772/60048

Takahashi, N., Yoshida, Y., Sugiura, T., Matsuno, K., Fujino, A., and Yamashita, A. (2009). Cucurbitacin D isolated from *Trichosanthes kirilowii* induces apoptosis in human hepatocellular carcinoma cells in vitro. *Int. immunopharmacol.* 9, 508–513. doi: 10.1016/j.intimp.2009.01.006

Tallamy, D. W., and Krischik, V. A. (1989). Variation and function of cucurbitacins in Cucurbita: an examination of current hypotheses. *Am. Nat.* 133, 766–786.

Vega, ÁE., Velasco-Lezama, R., and Jiménez-Estrada, M. (2006). Las plantas como fuente de compuestos antineoplásicos. *Rev. Bioquím.* 31, 97–111.

Villaseñor, J. L. (2016). Checklist of native vascular plants of México. *Rev. Mex. Biodivers.* 87, 559–902. doi: 10.1016/j.rmb.2016.06.017

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T. V. D., Hornes, M., et al. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23, 4407–4414.

WinClada v.1.00.08 (2020). *WinClada – FREE Download WinClada 1.00.08 Components & Libraries Development.* Available online at: https://www.scienceopen.com/document?vid=3c4c21f5-f2fc-49b1-a18e-4fba2f1337d7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 03 January 2022
doi: 10.3389/fnut.2021.787351

Development of Carrot Nutraceutical Products as an Alternative Supplement for the Prevention of Nutritional Diseases

Nadia Riaz[1,2], Zubaida Yousaf[1], Zarina Yasmin[3], Muneeb Munawar[4], Afifa Younas[1], Madiha Rashid[1,5], Arusa Aftab[1], Bushra Shamsheer[1], Hamna Yasin[1], Muhammad Najeebullah[5] and Philipp W. Simon[2,6]**

[1] Department of Botany, Lahore College for Women University, Lahore, Pakistan, [2] Department of Horticulture, University of Wisconsin-Madison, Madison, WI, United States, [3] Post-Harvest Research Centre, Ayub Agricultural Research Institute, Faisalabad, Pakistan, [4] Vegetable Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan, [5] Department of Botany, Division of Science and Technology, University of Education Lahore, Lahore, Pakistan, [6] Vegetable Crops Research Unit, US Department of Agriculture—Agricultural Research Service, Madison, WI, United States

OPEN ACCESS

Edited by:
Alessandra Durazzo,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Umakanta Sarker,
Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh
Saeid Doaei,
Shahid Beheshti University of Medical Sciences, Iran

***Correspondence:**
Zubaida Yousaf
zubaida.yousaf@lcwu.edu.pk
Philipp W. Simon
Philipp.simon@ars.usda.gov

Specialty section:
This article was submitted to Food Chemistry, a section of the journal Frontiers in Nutrition

Received: 30 September 2021
Accepted: 25 November 2021
Published: 03 January 2022

Citation:
Riaz N, Yousaf Z, Yasmin Z, Munawar M, Younas A, Rashid M, Aftab A, Shamsheer B, Yasin H, Najeebullah M and Simon PW (2022) Development of Carrot Nutraceutical Products as an Alternative Supplement for the Prevention of Nutritional Diseases. Front. Nutr. 8:787351. doi: 10.3389/fnut.2021.787351

Nutraceuticals can serve as an alternative supplement to overcome nutritional deficiency for a healthy lifestyle. They can also play a key role in disease management. To develop carrot nutraceutical products, 64 genotypes from four different continents were evaluated for a range of morpho-nutrition variables. Genetic variability, heritability, strength and direction of association among variables, and direct and indirect relationships among physiochemical and nutritional traits with β-carotene content were evaluated. Core diameter, foliage weight, root weight and shoulder weight showed significant association with β-carotene accumulation. Principal component analysis for physiochemical and nutritional assessment divided these genotypes into two distinctive groups, Eastern carrots and Western carrots. Caloric and moisture content had high positive associations with β-carotene content while carbohydrate content was negatively associated. Five genotypes (T-29, PI 634658, PI 288765, PI 164798, and Ames 25043) with the highest β-carotene contents were selected for making three nutraceutical supplements (carrot-orange juice, carrot jam and carrot candies). These nutraceutical supplements retained high β-carotene content coupled with antioxidant properties. Carrot jam (6.5 mg/100 g) and carrot candies (4.8 mg/100 g) had greater concentrations of β-carotene than carrot-orange juice (1.017 mg/100 g). Carrot jam presented high antioxidant activity with the highest values in T-29 (39% inhibition of oxidation) followed by PI 634658 (37%), PI 164798 (36.5%), Ames 25043 (36%) and PI 288765 (35.5%). These nutraceutical products, with 4–6.5 mg/100 g β-carotene content, had higher values than the USDA recommended dietary intake of 3–6 mg β-carotene/day can be recommended for daily use to lower the risk of chronic disease.

Keywords: antioxidant, β-carotenoid, genetic diversity, nutraceutical, heritability, morpho-nutritional, physiochemical

INTRODUCTION

Malnutrition is one of the main health problems emerge widely in the world. Malnutrition and protein-energy coupled with mineral deficiency is among the major causes of global illness and death. While the incidence of extreme hunger has reduced in the last several decades, 821 million people were estimated to be chronically undernourished in 2017 and according to the World Health Organization (WHO), 210 million children and women suffer from vitamin A deficiency (1) and 45% of children below the age of five are malnourished in developing countries with high prevalence in South Asia and Africa (2). Vitamin A deficiency (VAD) is among the main manifestations of malnutrition (3). For example, 51.5% of children in Pakistan are facing VAD with a slightly higher incidence in boys than girls (4). VAD can damage the eye's photoreceptors, leading to vision problems including xerophthalmia. Dietary sources of vitamin A assist in the protection of vision, reduce macular degeneration and suppress the development of senile cataracts, a main cause of blindness (5, 6). Beyond vision impairment, VAD reduces immune function, and it contributes to infertility, morbidity and mortality There are two approaches to overcome these non-communicable diseases (NCDs): prevention and treatment. Prevention can be less expensive than treatment since it involves changing lifestyle habits. For NCDs to be alleviated with dietary changes, researchers are investigating compounds naturally present in nutrient-fortified food including "nutraceuticals" to cure NCDs (7).

Nutraceuticals are described as food or a part of food which can provide health benefits including prevention against chronic diseases (8, 9). Nutraceuticals are indigenous to Indian, Roman, Sumerian and Chinese civilizations (10). Frequent consumption of nutraceuticals is very common in people of every age. In the USA ∼ 55% of children often consume nutraceuticals as vitamins and mineral supplements (11). The use of dietary supplements has increased over the past 20 years. Hence, dietary supplements comprise an estimated ∼$30 billion in the United States and ∼$100 billion globally (12, 13). In 2020, the U.S dietary supplements sale has significantly increased by 255% and 415% due to the pandemic COVID-19 (14). Nutraceuticals range from isolated nutrients to complex dietary supplements. Probiotics, prebiotics, dietary fibers, fatty acids and antioxidants are all categorized as dietary supplements (15).

Human beings and all animals are ultimately dependent on carotenoid pigments in plants to supply their vitamin A needs. A subset of the 600 to 800 carotenoids includes provitamin A carotenoids such as α-carotenes, β-carotenes (16), different xanthophyll like zeaxanthin, neoxanthin, violaxanthin, lutein (Sakar and Oba, 2020) and β-cryptoxanthin which, when consumed by animals, are converted to retinol (17). Carotenes and antioxidants (18) with anti-cancer, anti-cataract, anti-urinary tract infection, lowered blood pressure and reduced muscular degeneration properties (10, 17). Vegetables are an inexpensive source of carotenoids and carrot is one of the few plants of the family Apiaceae with massive carotenoid levels occurring in storage roots that have been estimated to provide 67% of the α-carotene and 28% of the β-carotene in the US diet (19) and 60–82% of the α-carotene and 60–90% of the β-carotene in several European diets (20).

Carrot (*Daucus carota* L.) is an economically important crop with diverse range of phenotypic and genotypic variation and with global production having been adapted for production in Europe, Asia and the Americas (21). Carrot is a biennial crop, having favorable cultivation from September to November in tropical and subtropical regions whereas, temperate regions can have an extended cultivation period throughout the year (22). Globally, carrot production has risen progressively in the last 50 years (23), with a three-fold increase in production area (383,965–1,166,885 ha) and two-fold increase in yield (166,893–329,021 hg/ha) to result in a six-fold increase in total production. With these increases, the average global increase in per capita carrot production has risen 2.7-fold in the last 50 years (24).

The domestication of carrot included increased carotenoid, anthocyanin and sugar content, loss of lateral root branching, biennial growth habit, and increased size and variation of root shape (25). Carrot colors include white, orange, yellow, red, and purple with each color comprising nutritionally valuable phytochemicals including carotenoids, anthocyanins, and other phenolic compounds. This makes the vegetable a good source of dietary antioxidants (26). The most abundant antioxidant compounds found in carrots are α- and β-carotene, vitamin E, and anthocyanin. Interestingly, the levels of these antioxidant pigments found in different cultivars are responsible for the colors of carrots. The carotenoids α- and β-carotene, lycopene, and lutein account for the orange, red, and yellow colors, respectively (27). Orange-colored carrots are unusual for their high α-carotene fraction ranging from 13 to 40% of their total carotenoids, with β-carotene accounting for most of the rest. Red carrots always contain lycopene and usually also contain α- and β-carotene along with lutein (26, 28, 29). Besides high bioavailability of carotenoids, carrots have also a unique combination of three flavonoids: kaempferol, quercetin and luteolin (30) and other phenolic derivatives stimulating cancer-fighting mechanisms in the human body (31). Carrot is also a good source of dietary fibers and trace mineral elements. Molybdenum, magnesium and manganese found in carrots help in carbohydrate metabolism, energy production, absorption of iron (32), insulin secretion (33, 34) and coordination of antioxidant enzymes in the body whereas, potassium helps in functioning of muscles (5, 6). Carrot extracts have been reported in experimental studies to possess cardio- and hepatoprotective (35–37), to reduce Alzheimer's and other dementia disorders (38–40), and to provide anti-bacterial and anti-fungal properties (41–43), anti-inflammatory and analgesic benefits (38, 44) and fertility benefits.

As carrot contributes 28–90% of β-carotene taken by humans (19, 20), several attempts have been made to utilize carrot in the form of its value-added raw, cooked or processed products (45). Carrot juice enriched with α- and β-carotenes has become the regular part of diet by some people throughout the world possessing high vitamin C content (46). Concomitant with the variation in carrot color, nutrient composition in diverse germplasm helps to characterize cultivars where darker color is typically associated with higher nutritional value (19, 47).

Moreover, information on nutritional properties of genotypes is very important to compliment basic phenotypic and genetic characterization. So far, no such work has been done in Pakistan to develop new carrot products. As market-based nutraceuticals and nutrient-fortified foods are quite expensive and out of the reach of the poor people of developing countries, this project was designed to develop carrot products in the form of carrot-orange mix juice, carrot candies and carrot jam as a nutritionally acceptable snacks for poor communities, especially children. This project also provides nutraceutical products useful to further explore nutrient bioavailability in clinical studies.

MATERIALS AND METHODS

The current project was planned in the Department of Botany, Lahore College for Women University, Lahore and executed in collaboration with Vegetable Research Institute and Post-Harvest Center, Ayub Agricultural Research Institute Faisalabad, Pakistan (48).

Plant Material

A panel of 64 genotypes consisting of 62 cultivated and 02 wild genotypes was obtained from Genetic Resource Information Network, United States Department of Agriculture, USA representing carrots from seventeen (17) countries of Asia, North America, Africa and Europe.

Field Experiment

Field trials were carried out in fall 2018, 2019, and 2020. The experiment was arranged in a randomized complete block design with three replications. The soil was sandy loam that was prepared by three ploughings followed by three finer cultivations to break up soil clods and the soil was leveled. Seed was treated with Imidacloprid WS 70% at 3 g kg^{-1} before sowing. Individual plot size was 5.0 × 2.5 m with inter-row distance of 40 cm. Nitrogen (N) from urea, phosphorus (P) from P_2O_5, and potassium (K) from muriate of potash were applied at 150:85:60 kg ha^{-1}. All the P and K and 1/3 of the N were applied before sowing. Successive irrigations were applied depending upon weather conditions to keep soil at field capacity. During this experiment F_1 and F_2 progenies seed was developed by selfing and roots were further used for phenotypic, nutritional evaluation and development of nutraceutical products. Roots were harvested 90–100 d after planting and stored at 4°C until analysis.

Characterization of Important Morpho-Nutritive Parameters

Morphological parameters add color characteristics related to nutrient content of carrots were evaluated in F_1 and F_2 generations. Root weight (g), shoot weight (g), root diameter (mm), root shoulder width (mm), petiole thickness (mm), lateral root growth, root shape, green color on shoulder, red color on shoulder, root tapering, root tip shape, root surface pigmentation, core color and cortex color were noted according to IPGRI (49) descriptor of wild and cultivated carrots. Passport data of 64 carrot genotypes along with recorded morpho-nutritive parameters is also provided (**Supplementary Table 1**).

Evaluation of Physiochemical and Nutritional Parameters

Sample Collection and Preparation

Mature carrots of different colors from F_2 generation were sent to the Post Harvest Institute for nutritional profiling based upon 2 years phenotypic screening. Selected roots of 64 genotypes were thoroughly washed, trimmed and air dried to make a fine powder. This powdered material was further used for testing proximate analysis (50), root color analysis and β-carotene.

Determination of Total Soluble Solids

Total soluble solids were determined through hand refractometer with automatic temperature compensation and the values were expressed as degrees Brix.

Determination of pH

pH of the carrot juices was determined with a pH meter and display reading was noted.

Determination of Acidity

Acidity was estimated through titration against 0.1 N NaOH in the presence of phenolphthalein indicator until reaching the light pink color end point.

Determination of Vitamin C Content

A titrimetric method estimation was performed by using 2–6 dichlorophenolindophenol dye for samples based on comparison to standard value.

Determination of Moisture Contents

2.0 g of root ground sample was dried in crucibles at 105°C for 72 h. The difference in weight was estimated as the moisture content.

Determination of Ash Contents

2.0 g samples were placed in crucibles and ashed at 500°C for 3 h to burn organic matter leaving inorganic residue. Ash was cooled in a desiccator and weighed to measure weight loss.

Determination of Crude Fats

Crude fats were determined by the Soxhlet extraction method. Five grams moisture sample was put into a thimble along with 150 mL n-hexane as solvent for the extraction and heated at 60°C for 2 h under reflux on a heating mantle. The extract was then air dried at 100°C, cooled and weighed for fats estimation.

Determination of Crude Fibers

Approximately 2.0 g of the sample was poured into a volumetric flask (1 L preheated) followed by the addition of 150 mL, 0.128 M H_2SO_4. The solution was refluxed for 30 min and cooled to room temperature. This was filtered through an ashless filter paper, and the residue washed with hot water (10 mL × 3). Exactly 150 mL of preheated 0.22 M KOH was added to the residue and refluxed for 30 min. It was cooled, filtered, and the residue was washed with acetone. The residue was oven-dried at 130°C for an h, weighed,

and the dried residue was ashed at 500°C for 3 h cooled and weighed. The loss in weight was used to estimate fiber content.

Determination of Protein Contents

Crude protein contents was determined by the micro Kjeldahl method (51). Protein content was estimated using the relationship: % protein content $= N \times 6.25$, where N is the nitrogen content, and 6.25 is the protein conversion factor. Approximately 1.0 g carrot powder of each variety was poured into a Kjeldahl digestion flask and followed by the addition of a catalyst (2.0 g of potassium sulfate, 1.0 g of copper sulfate and 0.1 g selenium powder) and 10 mL concentrated H_2SO_4. The flask was heated continuously in a fume hood until a green solution was obtained. The heating continued for about 30 min before cooling. Distilled water (10 mL) was added to the cooled digest and vigorously shaken. The digest was transferred into 100 mL volumetric flask and topped up to the mark with water. A mixture of 10 mL aliquot of the digest and 10 mL of 40% NaOH was distilled for 5 min into a receiver containing boric acid (10 mL, 2% w/v) using a Markham distillation unit. The distillate was titrated with 0.01 M HCl to determine the nitrogen content. Percent of crude protein was calculated by multiplying percent of Kjeldahl nitrogen with 6.25.

Determination of Carbohydrates

Percentage of carbohydrates was estimated by following formula:

$$\%\text{Carbohydrates} = 100(\%\text{ash} + \%\text{moisture} + \%\text{fat} + \%\text{fiber} + \%\text{protein})$$

Determination of Calories

Calories were estimated using the following formula.

$$\text{Calories} = (\%\text{protein} \times 4) + (\%\text{fat} \times 9) + (\%\text{carbohydrates} \times 4)$$

Determination of Color

Color properties of selected carrot roots were measured using a spectrophotometer. The color of the specimen was measured with a spectrophotometer (SP820λ; Techkon Gmbh, Konig-Stein, Germany) against a black background to stimulate the absence of light. All samples were chromatically measured in triplicate and each color parameter was averaged. The CIE (1976) L*a*b* color system was used for the determination of color. The measured parameters were L* (lightness/darkness), a* (red/green) and b* (yellow/blue). Then color was calculated by the below given formula:

$$\Delta Eab* = [(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2]^{1/2}$$

Whereas, L* is for lightness from white to black, a* red/green (a = green, +a = red), b* is blue /yellow (-b = blue, +b = yellow).

Analysis of Carotenoids

Carotenoid Extraction

Carotene extracted from carrots for "Reversed phased HPLC system" by the method of Khalil and Varananis (52). Ten grams of sample was homogenized in 30 mL of acetone. 0.1% (BHT) solution in acetone was added as an antioxidant. The resulting extract was filtered through Buchnar's funnel. The residue was washed twice with acetone until it become colorless. The filtrate was combined with 20 g of anhydrous sodium sulfate and anhydrous sodium sulfate removed through filtration and the volume of extract reduced by rotatory evaporator. The extract was transferred quantitatively to 100 mL volumetric flask and the volume was made up to the mark with acetone and water, so that the final extract contained 80% of acetone. Standard of β-carotene was supplied by Sigma-Aldrich (Germany). Stock solution of β-carotene was prepared dissolving 100 mg in 100 mL n-hexane. Chromatographic was analysis was performed with a Perkin Elmer HPLC programme using a LC-1000 pump (Isocratic), C18 column and LC 250 UV/VIS detector was used. Peak identification and quantification were made by "CSW 32 software" for the HPLC system. The HPLC was calibrated by running mobile phase (acetonitrile, dichloromethane and methanol volume ratio of 70:20:10, respectively) at a rate of 2 mL per min. Wavelength was fixed at 452 nm. The pressure of the column was kept 1800-2000 PSI. Each standard solution (20 μL) of β-carotene was injected when the injector was in load mode. The standard β-carotene peak was achieved at the retention time of 4.7 min (Rt = 4.7). The concentrations of the β-carotene standards were plotted against the peak area to obtain a straight line.

Sample Assay

Each sample of carotenoid extract in 80% acetone was used for HPLC assay like the standard; each carrot sample (20 μL) was taken by micro liter syringe. The peak was automatically identified and quantified.

Antioxidant Activity by DPPH Scavenging Assay

Sample Preparation and Analysis

Antioxidant capacity of carrot products was evaluated monthly up to 3 months. DPPH method (26) was used for the determination of antioxidant capacity of biofortified carrots was followed. The products were dried to make powder. Aliquot of extracted products were added to 1 mL of DDPH in ethanol solution and kept at room temperature for 30 min. After that, the absorbance was determined at 517 nm on spectrophotometer. The reduction of the absorbance was calculated according to the following equation:

$$\text{Inhibition}\% = [\text{Abs}_{t=0} - \text{Abs}_{t=30\,\text{min}})/\text{Abs}_{t=0} \times 100$$

Where $\text{Abs}_{t=0\,\text{min}}$ and $\text{Abs}_{t=30\,\text{min}}$ were the absorbance of DPPH solution at 0 and 30 min, respectively. The calculated inhibition percentage was used to express antioxidant capacity. The reduction of the absorbance against the amount of sample. Butylated hydroxytoluene (BHT) and α-tocopherol were used as a standard.

Development of Carrot Products

Sample Collection and Preparation

Five genotypes were selected for the development of carrot products. The selection criteria were based upon proximate evaluation. After washing and trimming, selected carrot roots were utilized to make juice, candies and jam. The product shelf life was evaluated monthly for 90–100 d with further evaluation of TSS, pH, acidity, vitamin C, color values, mineral matter content, dry matter content, carotenoid, and antioxidant activities to safeguard quality assurance. Occurrence of spoilage was noted.

Carrot Mix Juice

Juice was extracted by using a local juice blender. Market purchased fresh oranges were washed. Orange juice was extracted and filtered with muslin cloth to remove large fiber particles. Carrot juice was extracted by using juice extractor and 50% carrot- 50% orange juice was prepared according to the standard formulation methods of (50) which includes addition of sodium benzoate to prevent spoilage, carboxymethyl cellulose (CMC) powder for viscosity enhancement and citric acid to stabilize carrot and orange concentration as preservatives and stored at room temperature (±27°C) in the dark.

Carrot Candies

Carrots were blanched for 5–7 min then cooled. Sugar syrup of 30 brix was used for osmo-dehydration. Roots were dipped in the sugar syrup for 24 h, liquid drained, and carrots cut into round chewable pieces. A tunnel dehydrator was used to dry the pieces up to 30–35% moisture content. The samples were packed in transparent ziploc bags to make it visible for any contamination and stored in the dark at room temperature (±27°C).

Carrot Jam

One kg peeled carrot was cut into small chunks and dipped in 8% salt solution to maintain its color consistency. After 2 h plain water was added into the salt solution and the mixture was boiled for five min for blending. After fine blending 500 g sugar and 2 g citric acid was added and the mixture cooked until thickening. The cooked jam was removed from heat and 1 g sodium benzoate was added. After cooling down to 85°C jam was transferred into glass jars for storage in a dry place at room temperature.

Data Analysis

The nutritional data for 64 genotypes was assessed for variance analysis (53) and significance was tested at $p < 0.05$, $p < 0.01$, and $p < 0.001$ *via* F-test.

The broad sense heritability (h^2b) was calculated as σ_g^2/σ_p^2 (54). The heritability was classified as low (<50%), medium (50–80%) and high (>80%) (55, 56).

The genetic advance was computed as, $GA = h^2b \times \sigma_p \times K$, where σ_p and K represent phenotypic standard deviation and standardized selection differential constant) at 5% selection intensity, respectively (57). The GCV (genotypic coefficient of variation) and PCV (phenotypic coefficient of variation) were computed by Ogunniyan and Olakojo (57) and heat map was constructed for respective comparison.

$$PCV(\%) = \sigma_p/\bar{x} \times 100$$
$$GCV(\%) = \sigma_g/\bar{x} \times 100$$

σ_p = phenotypic standard deviation σ_g = genotypic standard deviation.

rp = phenotypic correlation and rg = genotypic correlation was calculated following the formula of Sarker et al. (58), Sarker et al. (59), and Sarker et al. (60). Path coefficient analysis was performed following the formula of Sarker et al. (58), Sarker et al. (59), Keles et al. (61) to evaluate the direct and indirect effects of the most highly correlated nutrients on β-carotene content.

To measure variation, minimize the dimensionality of variables and to assess the contribution of each genotype with phenotypic and nutritional parameters, principal component analysis was performed (62). The PCA was generated by using R statistical package "ggbiplot" in R studio version 2020. The K-means clustering algorithm was used as per following function to define k centroids, one for each cluster (63). Here, k is the number of cluster centers, n is number of data points from respective cluster center. The data was subjected to default K means function.

$$J = \sum_{j=1}^{k} \sum_{i=1}^{n} \| x^{2\,i\,c}_{i-j} \|$$

where J is objective function, k is number of clusters predefined and n is number of case. $\| x^{2\,i\,c}_{i-j} \|$ is distance function of Euclidean distance, x^i is case, i and c_j is centroid for cluster j.

Further Silhouette plot analysis function was carried out in the R programming software (R Studio 2020) for validation of consistency within clusters of data and to determine how well an observation is clustered. Cluster analysis Silhouette coefficient was calculated by using the following formula:

$$S_{(i)} = b_i - a_i/\max(a_i,\ b_i)$$

The S_i varies between $-1 \leq Si \leq 1$

All tests and analyses for carrot products were performed in triplicate with sample preparation and handling being performed twice, and the obtained data were averaged. The differences between mean and standard error were calculated by SPSS 16.0 statistical software.

RESULTS

Variation and Genetic Parameters of Variability, Heritability, and Genetic Advance for Nutrition

Genotype of carrots evaluated affected all the physiochemical and nutritional parameter (**Table 1**). Considerable variation existed among the genotypes able to be exploited for nutritional crop breeding was used to select more nutritious carrots for product development. Environmental variation was relatively small but slightly higher for moisture, calories, carbohydrates, and carotene. This variation was also confirmed by those same

TABLE 1 | ANOVA and genetic parameters of variability for fifteen (15) important nutritional traits of 64 *Daucus carota* L. genotypes.

Characters	TSS	pH	Acidity	Vit. C	L*	a*	b*	Moisture	Ash	Crude fat	Crude protein	Crude fiber	Calories	Carbs	β-carotene µg/100 g
DF	63	63	63	63	63	63	63	63	63	63	63	63	63	63	63
Mean square	12.413	0.473	0.273	474.42	295.23	26.670	210.609	2,089.99	0.0174	0.0067	0.016	0.020	2,102.65	2,095.59	444.559
sum square	782.05	29.761	17.173	29,888.4	18,599.3	53.3	13,268.4	131,670	1.093	0.42542	1.039	1.252	132.47	132.022	280,071.654
S.E of Mean	0.059	0.034	0.009	0.345	0.277	0.172	0.233	0.732	0.003	0.003	0.003	0.002	0.732	0.767	33.757
σ^2e	0.010	0.004	0.0002	0.356	0.230	0.088	0.162	1.605	0	0	0	0	1.608	1.615	3,418.625
σ^2_g	4.134	0.156	0.0908	158.020	98.330	37.380	70.150	696.130	0.006	0.002	0.005	0.007	697.993	700.347	1,480,721.063
σ^2_p	4.145	0.1598	0.091	158.377	98.562	37.466	70.311	697.735	0.006	0.002	0.005	0.007	699.601	701	1,484,139.689
GCV	29.480	6.0366	19.782	61.833	24.100	45.565	42.188	34.676	6.971	15.143	7.382	6.864	123.532	137.843	21.422
PCV	29.516	6.104	19.804	61.903	24.128	45.619	42.233	34.716	6.971	15.143	7.382	6.864	123.674	138.003	21.447
h^2b	0.997	0.9781	0.998	0.998	0.998	0.998	0.998	0.998	1	1	1	1	0.998	0.998	0.998
GA	4.183	0.805	0.620	25.866	20.404	12.579	17.234	54.289	0.156	0.097	0.153	0.167	54.362	54.453	2,503.819
GA % of mean	30.652	12.297	40.709	127.232	49.588	93.753	86.808	71.351	14.362	31.188	15.210	14.143	254.183	283.631	44.079

DF, degree of freedom; S.E, standard error; σ^2e, Environmental variance; σ^2_p, phenotypic variance; σ^2_g, genotypic variance; PCV, phenotypic coefficient of variation; GCV, genotypic coefficient of variance; h^2b, heritability broad sense; GA, genetic advance at 5% selection. Phenotypic and genotypic variation values were significant at the 95% value for all traits.

values as measured in the analysis of genotypic coefficient of variation, phenotypic coefficient of variation and broad sense heritability. These results indicate that genetic variation plays a more important role in expression of these traits than environment. Important for this study, genetic variation for β-carotene was found to be slightly higher than phenotypic variation. The GCV depicted true genetic potential of genotypes under study. High genetic advance values were observed for vitamin C, color, moisture, calories, and carbohydrates.

Correlation Analysis

A heat map of genotypic and phenotypic correlation coefficients was constructed among the nutritional parameters (**Figure 1**). The genotypic correlation coefficients were very closely correlated to the phenotypic correlation coefficients. Among genotypic correlation coefficients, TSS and moisture (0.59) were positively correlated, and both factors were negatively correlated with CIE color parameter a* (−0.33, −0.32), vitamin C (−0.48), carbohydrates (−0.59) and calories (−0.59). The latter three factors were highly correlated among each other. Crude protein was significantly correlated with moisture (0.41), ash (0.63), crude fat (0.58) and crude fiber (0.68) while moisture was negatively correlated with carbohydrates (−1) and calories (0.98). β-carotene was found to be non-significant with all parameters for both correlation coefficients.

Genotypic Path Analysis

Genotypic path analysis provided an evaluation of direct and indirect effects of the eight most highly correlated proximate parameters on β-carotene content (**Figure 2**; **Table 2**). In a similar pattern observed with those correlation values, β-carotene content was most directly associated with calories (33.0), moisture content (24.4) followed distantly by crude protein (0.12). vitamin C (−0.214), carbohydrates (−8.5) and crude fat (−0.12) showed negative direct effects. TSS affected β-carotene positively through vitamin C, moisture, crude protein, and carbohydrates except for its non-significant direct effect. Vitamin C had negative effects with all parameters except for calories. Moisture interacted negatively with crude fat, crude fiber, and calories while vitamin C and crude fat increased with β-carotene content and with vitamin C, crude protein, and calories. Crude fat interacted positively with vitamin C, moisture, crude protein, and carbohydrates, while negatively with calories. Crude protein interacted positively with vitamin C, moisture, and carbohydrates while negatively with calories and fat. Crude fiber had a positive relationship with vitamin C, moisture, crude fat, protein, and carbohydrates. TSS negatively interacted with β-carotene, carbohydrates, and calories (**Table 2**).

Principal Component Analysis of Morpho-Nutritional Parameters

Principle component analysis (PCA) was used to evaluate the relationships of individual carrot genotypes with quantitative morphological parameters associated with yield and market quality (**Figures 3A,B**). Principal components 1 and 2 (PC1 and PC2) had eigenvalues of 2.084 and 1.361, respectively, with a combined variance percentage of 49.2 (**Table 3**). PCA

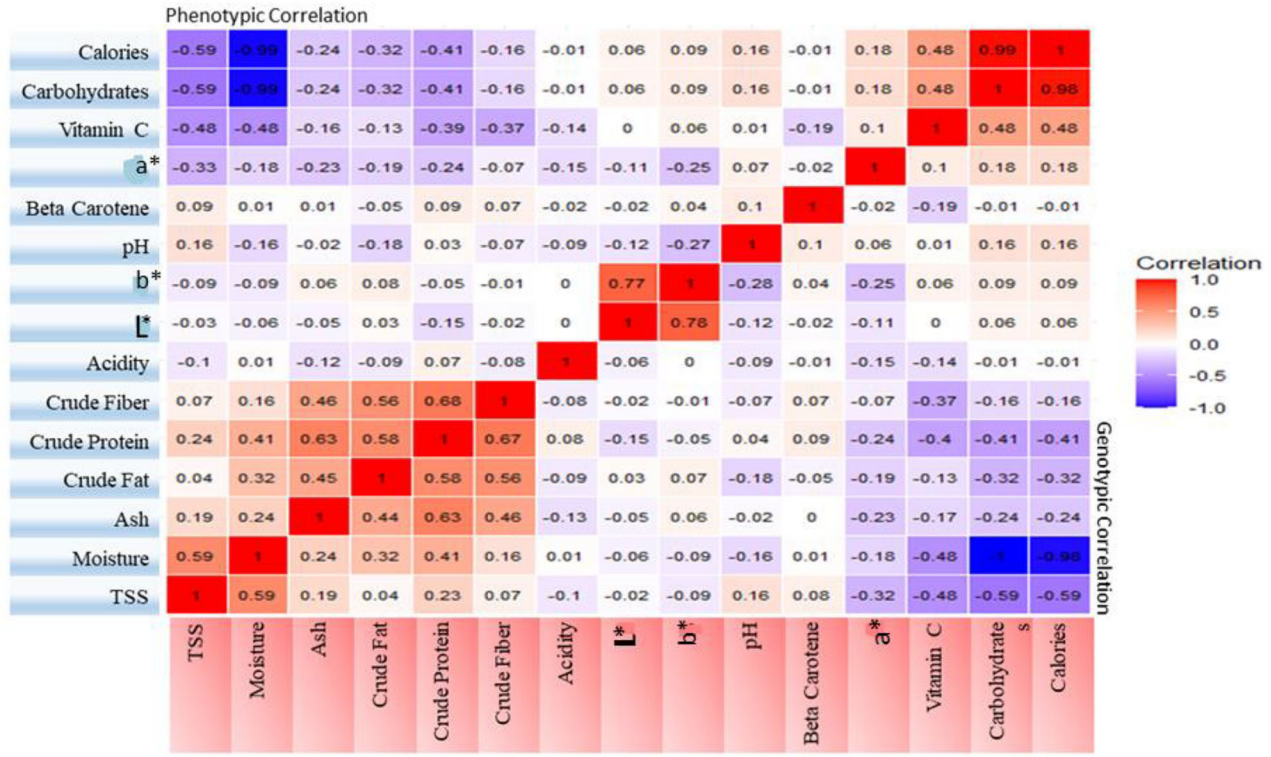


FIGURE 1 | Heat map showing phenotypic and genotypic correlation coefficients among fifteen nutritional characters.

analysis demonstrated the maximum variation in genotypes belonging to Turkey, Afghanistan, Pakistan and the United States for core diameter, foliage weight, root weight, and petiole thickness parameters (**Figure 3B**). Petiole thickness, root and foliage weight were most significantly correlated, accounting for 20% of the variation while, root diameter was negatively significant with least contribution (**Figure 3B**). Root length was strongly negatively correlated with petiole thickness. Foliage weight revealed no significant relationship with root length. Five genotypes (T29, PI 164798, PI 634658, PI 288765, and Ames 25043) were considered the best portraying considerable root characteristics based upon root color, root length, core size and sweetness which contribute to the yield enhancement, consumer attraction and genetic diversity.

Genotypes from Russia, Serbia, and France were closely associated based upon nutritional character profiles measured in this study (**Figure 4**). While one genotype belonging to Ukraine was positioned in the Asian group. Similarly, many American genotypes were associated with Asia, which is carrot's center of origin. PCA biplot showed the most significant positive associations with pH, vitamin C, carbohydrates, calories, and color. In contrast, crude fat, crude protein, moisture, TSS and ash were most negatively correlated. β-carotene had the minimal variation among genotypes.

The K-means cluster results were validated with portraying Silhouette plots to determine the similarity of genotypes within each cluster and differences with other clusters (**Figure 5A**). The silhouette coefficient width (Si) grouped these genotypes into 7 clusters. The five genotypes selected for nutraceutical products development, PI 164798, PI 634685, PI 288765, Ames 25043, and T29, were positioned in clusters 5, 7, and 8, respectively. The Si ranged in variation from 0.11 to 0.38. Cluster 5 had the highest Si average width (38), medium with cluster 7 (0.19) and the lowest with cluster 8 (0.11). Si average width was directly proportional to the genetic diversity among genotypes (**Figure 5B**).

Proximate Composition and Antioxidant Activity of Carrot Nutraceutical Products

Carrot candies, carrot juice and carrot-orange mix juice were prepared (**Figure 6**) and their proximate analyses for TSS, moisture, crude fat, crude fiber, and protein varied significantly. In the present study mineral content, dry matter, β-carotene and antioxidant activities were evaluated for 3 months (March, April and May) for products prepared in February. Variation among genotypes and variation during storage were both small for all products.

The highest value of mineral content was recorded for candies (especially PI 288765 and PI 164798) followed by jam similar for all genotypes except lower for Ames 25043). and least in mix juice (PI 634658 and PI 164798) (**Figure 7A**). Dry matter was calculated only for candies where, PI 634658 and PI 164798 products had the most promising results followed by Ames 25043, T-29 and PI 288765 (**Figure 7B**). β-carotene

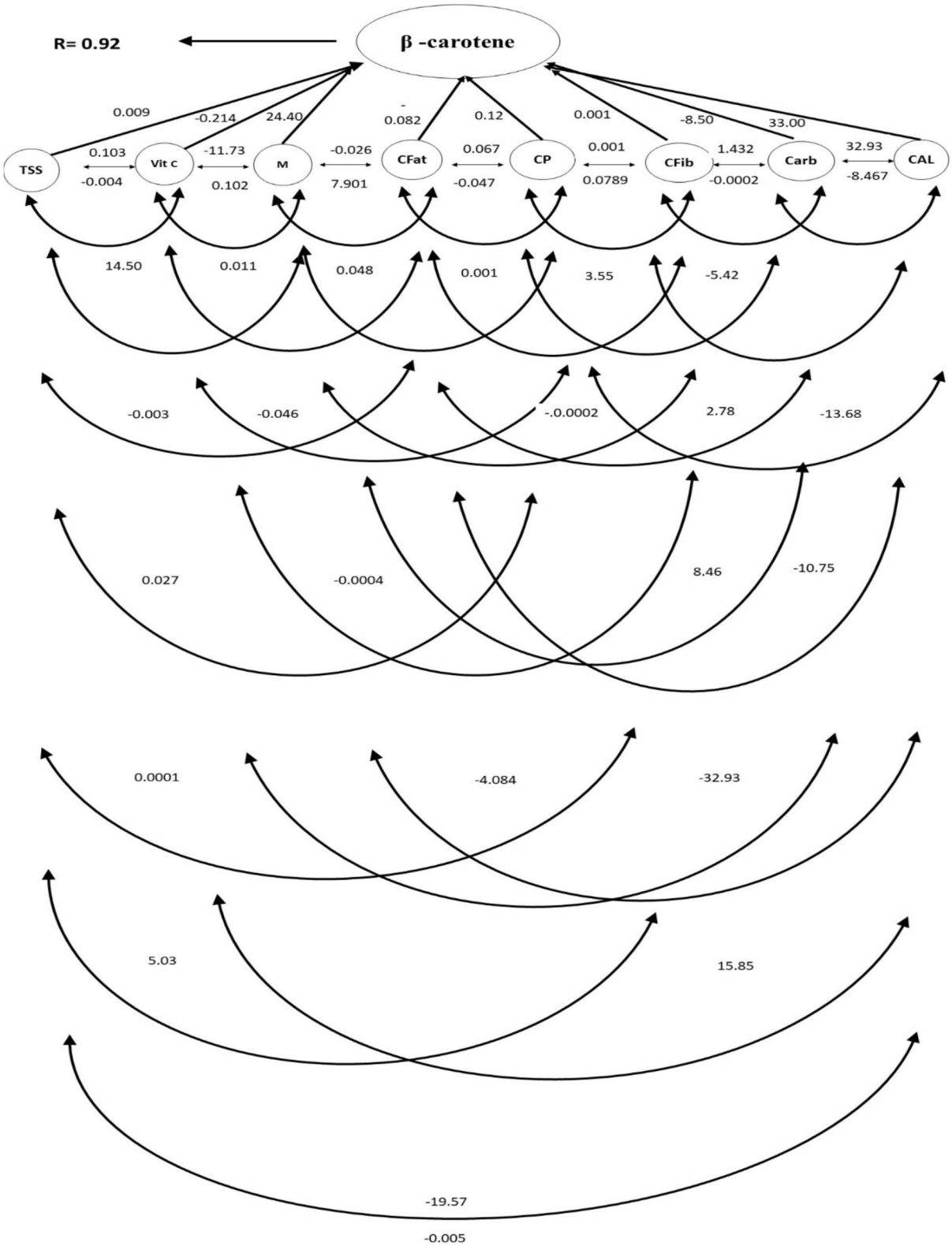


FIGURE 2 | Path diagram showing direct and indirect effects of eight significantly important nutritional characters of 64 *Daucus carota* L. genotypes on β-carotene content. TSS, total soluble solids; Vit. C, vitamin C; M, moisture; CFat, crude fat; CP, crude protein; Cfib, crude fiber; Carb, carbohydrates; Cal, calories; R, residual effects.

TABLE 2 | Estimation of direct and (bold numbers) indirect effects of nutritional parameters of 64 *Daucus carota* L. genotypes on β-carotene content.

Character	TSS	Vitamin C	Moisture	Crude fat	Crude protein	Crude fiber	Carbohydrate	Calories	β-carotene
TSS	**0.009**	0.103	14.50	−0.003	0.027	0.0001	5.03	−19.57	0.089
Vitamin C	−0.004	**−0.214**	−11.73	0.011	−0.046	−0.0004	−4.084	15.85	−0.228
Moisture	0.005	0.102	**24.40**	−0.026	0.048	−0.0002	8.46	−32.93	0.052
Crude fat	0.0004	0.029	7.901	**−0.082**	0.067	0.001	2.78	−10.74	−0.045
Crude Protein	0.002	0.085	10.078	−0.047	**0.12**	0.001	3.55	−13.68	0.104
Crude Fiber	0.0007	0.079	3.964	−0.046	0.0789	**0.001**	1.432	−5.42	0.092
Carbohydrates	−0.005	−0.103	−24.395	0.027	−0.049	−0.0002	**−8.50**	32.93	−0.053
Calories	−0.005	−0.103	−24.396	0.026	−0.048	−0.0002	−8.464	**33.00**	−0.052

content was higher in all genotypes when used for jam with the highest in T-29 (6.5 mg/100 g) followed by Ames 25043 (6.0 mg/100 g), PI 164798 (5.6 mg/100 g), PI 288765 (5.5 mg/100 g), and PI 634658 (5.4 mg/100 g) (**Figure 8A**). Carotene content in candies was lower than in jams and was much lower in juice. Similar antioxidant values were observed in all genotypes and products ranging from 27 to 39% (**Figure 8B**). The highest antioxidant values were observed in T29 carrot jam.

DISCUSSION

Plants are essential source of secondary metabolites, such as phenolic and flavonoids including hydroxycinnamic acids (64), hydroxybenzoic acids (65), flavones (66), flavanols, flavanones (66), tocopherols (67), betalain (16), ascorbic acids (67), carotenoids (27), betacyanin, betaxanthin (68), chlorophyll a (69), chlorophyll b (70) etc. that have high radical quenching ability (71). These secondary metabolites and some antioxidant enzymes associated with some physiological activities, such as reduce reactive oxygen species (ROS) (72), osmotic stress (18), oxidative damage (73), decrease in photosynthetic activities (71), improve nutrient imbalance (71), in plant cells, protect plants from drastic reduction in growth and productivity (74) and ultimately enhance the concentration of antioxidants (75) that can be used in human diet. The properties of some plants used historically as medicines have been investigated but relatively little has been reported for carrot. Before domestication, carrot seed was used as a traditional medicine in Iranian culture for healing gynecological disorders (76). With the discovery of vitamins in the 1900s carrots became recognized as a rich source of β-carotene along with some protein, carbohydrates, fiber, and fat.

PCA for nutritional revealed new insights for the domestication of carrot. PCA divided selected 64 genotypes into two groups, Asia, and Europe. This finding is in agreement with the statements of Baranski et al. (77), Iorizzo et al. (78), and Grzebelus et al. (79) that cultivated germplasm could be divided into eastern and western gene pools and the carrot evolution was based on morphological markers (80). Moderate to high variations in morphological parameters were observed. There was wide range of core and cortex pigmentation found having red, yellow, orange, and white colors. These variations can be attributed to the genetic and environmental effects (81) and agrees the hypothesis by Arif et al. (82), carrot genotypes have the great potential of variability for yield relating traits (root traits) associated with its nutrition.

Correlation is considered as a helping tool in the selection of desirable traits for breeding programs. Nutritional parameters in this study displayed a range of genetic variation and similar trends were observed to measure genetic variability among diverse genotypes of carrot (83). The significant genotypic control of β-carotene accumulation observed in this research supports the findings of Buishand and Gabelman (84), Ellison (85), Sarker (58), and Simon (47), where regulatory genes are responsible for the biosynthesis and accumulation of β- carotenoids content and hence, breeding has doubled carotenoid levels for the last 60 years (47). Genotypic control of carbohydrates calories and vitamin C also contributes to improvement of the crop. Total sugar content ranged from 3 to 8%, in this study and both genotype and environment have been reported to influence sugar content in previous research (86, 87) so all these multiple factors must be monitored to improve carrot quality (88). The relative magnitude of vitamin C, moisture, crude fat, crude protein and carbohydrates for β-carotene synthesis observed with path analysis support reports by Cavagnaro (89) and Yadav et al. (90) where the authors explained that these factors also contribute to the enhancement of total dissolved solids and dry matter and also influences the processing quality of carrot.

Moisture content is an important factor of food before consumption. Moisture affects physical and chemical properties of food associated with shelf life. All genotypes had 70–80% moisture content except for several from Indian, North American and Europe, with a range between 10-88-90%. A negative correlation of vitamin C, carbohydrates and calories with moisture content is an important factor in controlling microbial growth and increasing shelf life of the products according to Chukwu and Abdullahi (91) who found that lower moisture is beneficial for storage and better shelf life.

Total soluble solids (TSS) include sugars, vitamins, and minerals. In the present study, the majority of genotypes had average to high TSS in carrots from in or near carrot's Central Asian region of domestication. TSS content increases during maturation of storage root and increased TSS content of carrots was also observed during storage period by Lingaiah and Huddar (92) and Jitender et al.

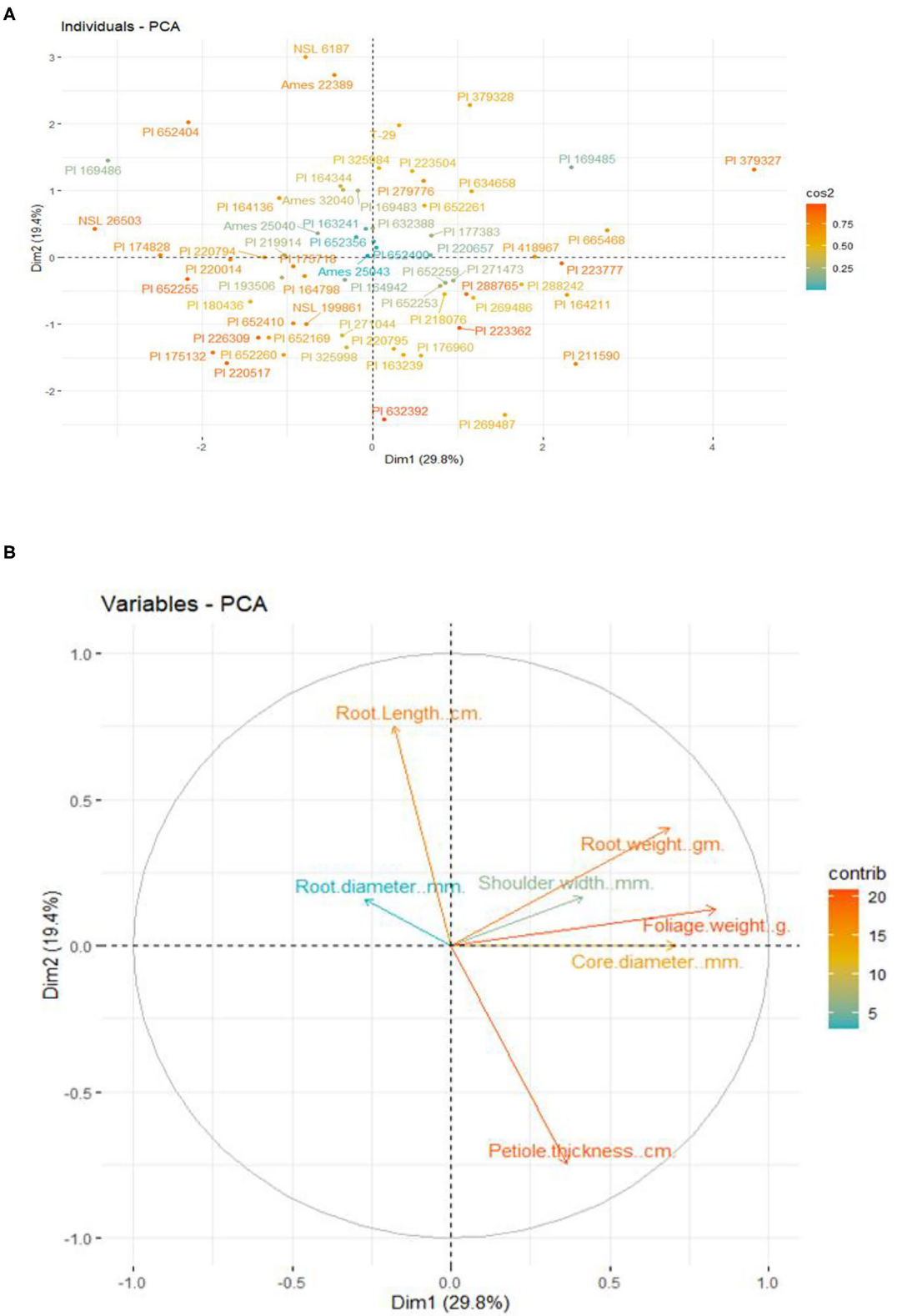


FIGURE 3 | (A) Classification of 64 *Daucus carota* L. genotypes for morpho-nutritional parameters based on the principal component analysis **(B)**. PCA analysis depicting the relationship between variables and their contribution in total variation of carrot morphological traits.

(93). It was also observed that carrot packaging significantly affected the changes in TSS content of carrots during storage period (94). Low temperature carrot storage (1°C) maintained TSS content better than carrots stored at room temperature (92, 93, 95, 96).

In most of the genotypes, pH was slightly acidic as observed in carrot puree by Abbas and Khoudi (97) and Arqha (98) reported an average pH of the carrot between 4.9 and 5.2. Indeed, according to Anonyme (99), pH of some carrot may vary with varietal characteristics and growing conditions. The relationship between the slightly acidic nature of carrot and its organoleptic profile warrants additional study.

β-carotene exhibited higher values in fresh cut carrots than carrot products in this study because carrots were mixed with other colorless or lower carotene ingredients. This was especially noted in the carrot juice product which had much lower carotene content than other products. The low mineral content of the carrot juice product was also likely attributable to it mixture with low mineral ingredients. Vitamin C present in carrot-orange mix juice may also have lowered β-carotene content as was observed with the involvement of vitamin C in pineapple juice blended with orange and carrot which was attributed to reduced β-carotene during storage for 2 months (100). Carrot jam had the highest concentrations of β-carotene (6.5 mg/100 g) followed by carrot candies (4.8 mg/100 g) and carrot-orange mix juice (1.2 mg/100 g). This difference could be due to processing along with the addition of sugar, preservatives in jam, and oranges in the carrot juice. This

TABLE 3 | Principal component analysis of quantitative traits of 64 *Daucus carota* L. genotypes.

Parameters	PC1	PC2	PC3	PC4
Eigenvalue	2.084	1.361	0.986	0.908
Variance percentage	29.8	19.4	14.087	12.976
Cumulative variance percentage	29.784	49.158	63.245	76.221

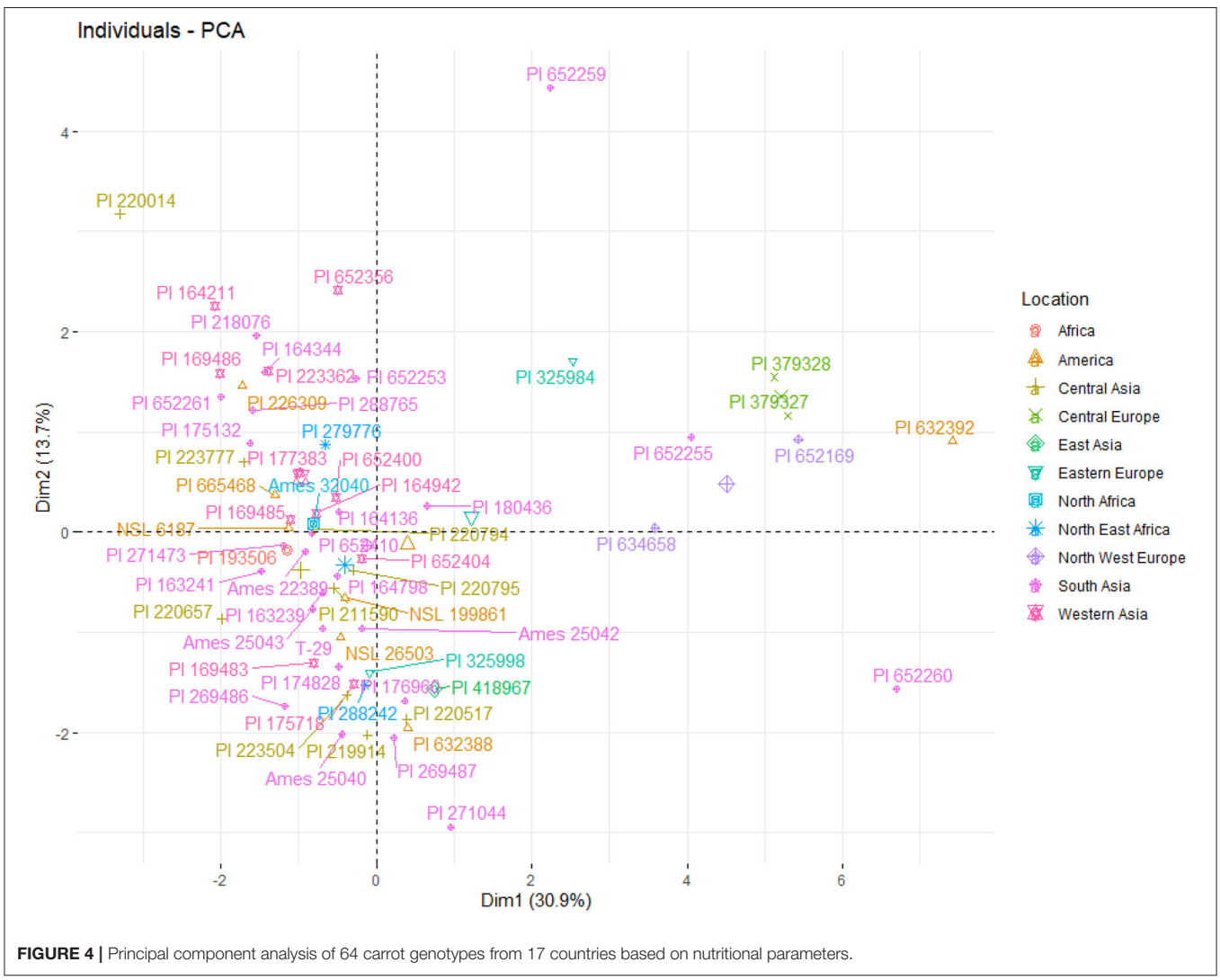


FIGURE 4 | Principal component analysis of 64 carrot genotypes from 17 countries based on nutritional parameters.

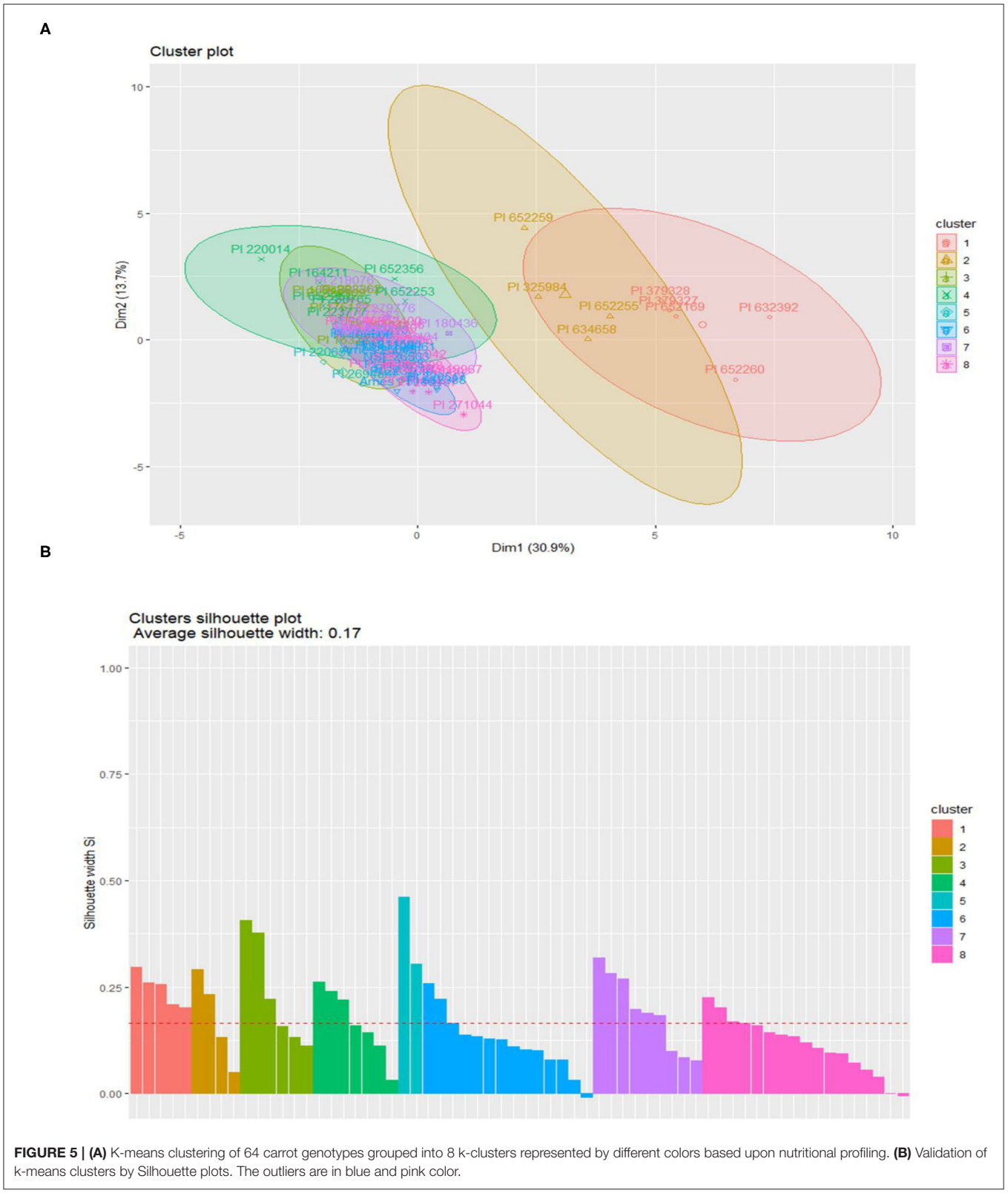


FIGURE 5 | (A) K-means clustering of 64 carrot genotypes grouped into 8 k-clusters represented by different colors based upon nutritional profiling. **(B)** Validation of k-means clusters by Silhouette plots. The outliers are in blue and pink color.

significant increase in carrot jam agrees with Sant'Ana et al. (7) and Miglio et al. (101) where the authors reported the highest stability of β-carotene after boiling carrot for food preparation rather than any other cooking methods. In this context the boiling of carrots probably improved β-carotene in carrot jam.

FIGURE 6 | Carrot nutraceutical products i.e., **(A)** Carrot Jam, **(B)** Carrot mix juice, and **(C)** Carrot candies.

Increased antioxidant capacity of carrot jam was also observed by Renna et al. (102), where they observed a 44% increase in antioxidant activity with yellow type carrot relative to other colors. No significant differences in antioxidant activity was observed among carrot products or among genotypes in this study but yellow carrots were not included. According to Sarker et al. (64) vegetables possessing high antioxidant quenching property can be consumed as nutraceutical products for nutrient deficient community.

While all products retained their nutrition for 3 months in this study candies stored at (±27°C started to degrade in the third month due to fungal attack. This storage temperature was used to reflect typical storage and handling practices of candies. This degradation is attributable to this high storage temperature, but the shelf life of carrot candies could be extended up to 6 months by storing at low temperature (1–3°C) (103). Kaur et al. (104) also observed carrot products treated with chemical additives improved physiochemical, phytochemical, antioxidant and shelf life while in this research carrot candies were only dipped into sugar syrup and no chemical treatment was applied. Based on these studies it is possible to stabilize carrot candies shelf-life, and with those studies either refrigerated storage or additives extend storage are recommended for long-sored carrot candies prepared by methods used in this study.

The studies by Sethi and Anand (103), Renna et al. (102), Kaur et al. (104), and Owolade et al. (100) noted above note a relatively

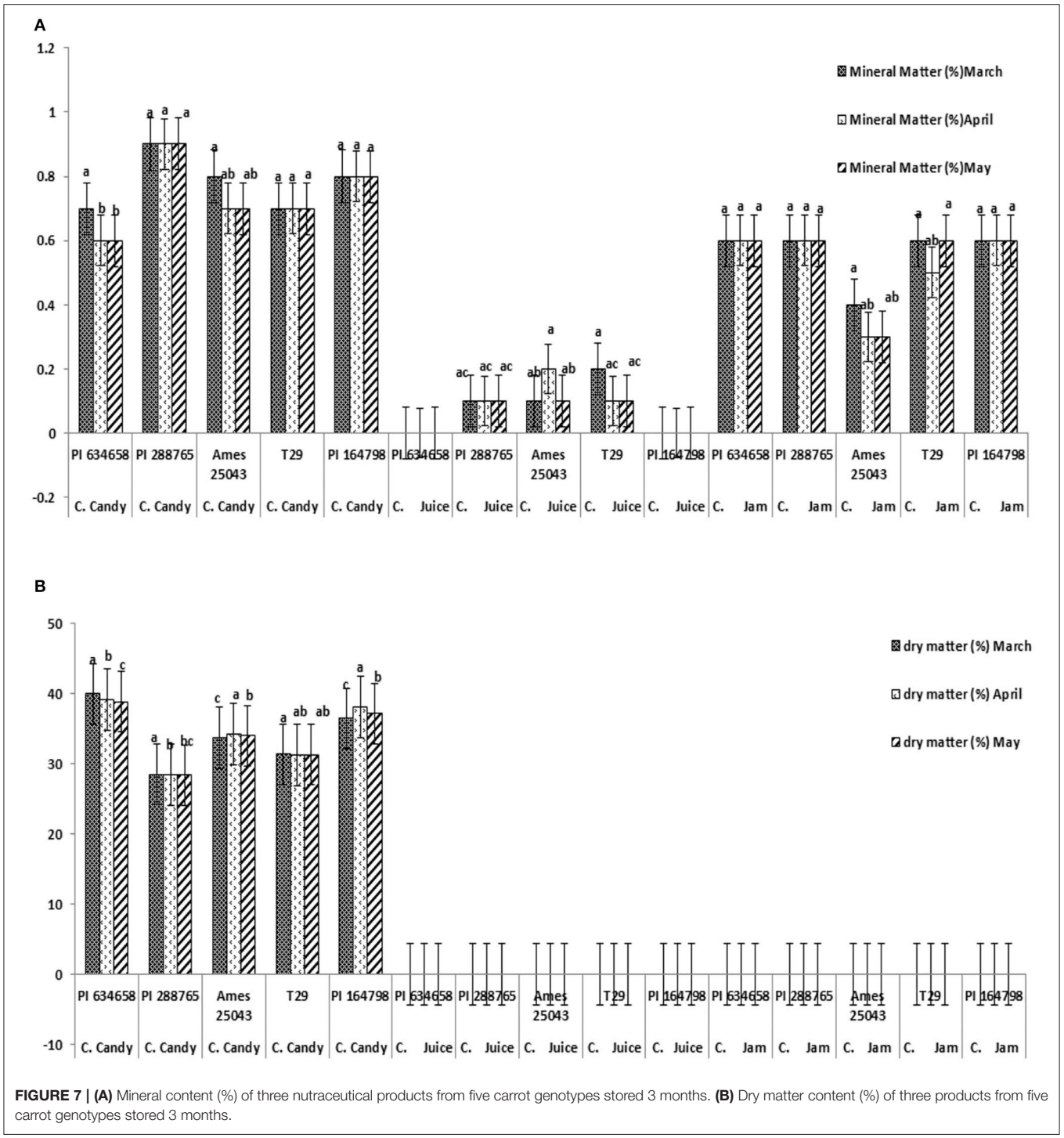


FIGURE 7 | (A) Mineral content (%) of three nutraceutical products from five carrot genotypes stored 3 months. **(B)** Dry matter content (%) of three products from five carrot genotypes stored 3 months.

few examples of carrot use for nutraceutical application. Given the extent of carrot crop production globally, the development of more long-storage products like jams, candies with preservatives, and "fruit leathers" would be valuable for global regions with minimal refrigerated long-storage. While carrot roots have relatively long post-harvest storage life than most fruit and vegetable crops, additional products could not only extend storage bust also increase overall consumption of this nutrient-rich crop.

CONCLUSION

Correlation among various carrot attributes, particularly those for yield and nutritional contributing traits, is important

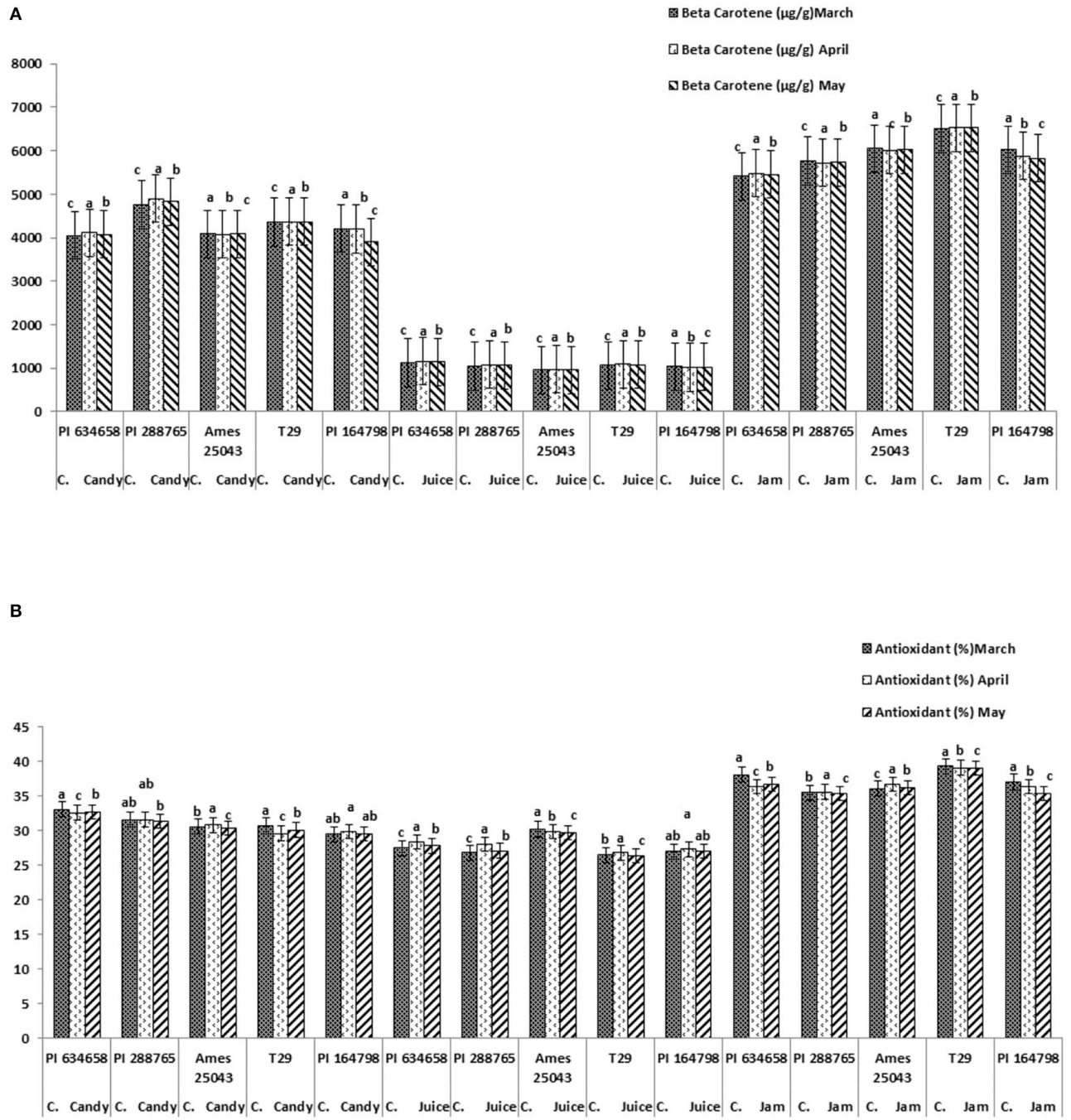


FIGURE 8 | (A) β-carotene content (µg/100 g) of three nutraceutical products from five carrot genotypes stored 3 months. **(B)** Antioxidant activity (%) of three products from five carrot genotypes stored 3 months.

for guiding future improvement of carrots. The wide range of variability recorded for various morphological and nutritional characters was important in identifying superior raw products genotypes in the development of nutritionally fortified nutraceutical products for this study. Among all the prepared products, carrot jam had the highest β-carotene and antioxidant content, while carrot candies also contained high levels of β-carotene and antioxidants and also demonstrated highest mineral and dry matter content. These results support the goal of this study in developing products attractive to consumers that can be used to alleviate vitamin A deficiency related disease. Consumption of these products can help in reducing xerophthalmia and other vision disorders like macular degeneration, increasing fertility, cognitive skills

enhancement and providing protection against communicable and non-communicable diseases and oxidative stress. The incidence of these diseases is high in rural areas where no firsthand β-carotene supplements are available. This study proposed carrot-based products enriched in β-carotene and antioxidants that could be available to rural community at lower cost. Carrot jam and candies can be an alternative source to fulfill β-carotene requirement of 4 mg/100 g to 6.5 mg/100 g β-carotene per day along with significant antioxidant capacity which is in accordance with the recommended intake level by the USDA. Based on these studies, extended research is required to create additional high-quality products and to develop novel carrot hybrids which combine the blend of crop productivity with nutritional and functional attributes suitable for sustainably and locally produced nutraceutical products.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

NR: investigated, visualized, and wrote this original draft. ZYo: conceptualized, supervised, and helped in editing/reviewing. ZYa: post-harvest nutritional evaluation and carrot products development. MM: morphology data collection and statistical data analysis. AY, MR, AA, BS, and HY: review and edited. MN: crop cultivation. PS: conceptualized and assisted in editing/reviewing. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by Punjab Agricultural Research Board (PARB) under CGS grant No. 929, project "Phenotypic and Genotypic exploration of worldwide carrot germplasm to enhance its value-added applications in Pakistan", and United States Department of Agriculture (USDA), Agricultural Research Service (ARS), under grant No. 5090-21000-069-00D, project, "Trait Discovery, Genetics, and Enhancement of Allium, Cucumis, and Daucus Germplasm".

ACKNOWLEDGMENTS

We acknowledge the germplasm shared by Germplasm Resources Information Network (GRIN) and United States Department of Agriculture (USDA).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.787351/full#supplementary-material

REFERENCES

1. World Health Organization. *Global Prevalence of Vitamin A Deficiency in Populations at Risk 1995-2005: WHO Global Database on Vitamin A Deficiency*. (2009). Available online at: http://apps.who.int/iris/bitstream/handle/10665/44110/9789241598019_eng.pdf?sequence=1F (accessed October 21, 2021).
2. World Health Organization. *World Health Statistics 2021: Monitoring Health for the SDGs, Sustainable Development Goals* (2021).
3. Müller O, Krawinkel M. Malnutrition and health in developing countries. *CMAJ*. (2005) 173:279–86. doi: 10.1503/cmaj.050342
4. National Nutrition Survey. Final Key Findings Report 2019.pdf (2018). Available online at: unicef.org (accessed September 24, 2021).
5. Dias JS. Major Classes of phytonutriceuticals in vegetables and health benefits: a review. *J Nutr Ther*. (2012) 1:31–62. doi: 10.6000/1929-5634.2012.01.01.5
6. Dias JS. Nutritional quality and health benefits of vegetables: a review. *Food Nutr Sci*. (2012) 3:1354–74. doi: 10.4236/fns.2012.310179
7. Sant'Ana-Gálvez J, Cisneros-Zevallos L, Jacobo-Velázquez DA. A practical guide for designing effective nutraceutical combinations in the form of foods, beverages, and dietary supplements against chronic degenerative diseases. *Trends Food Sci Technol*. (2019) 88:179–93. doi: 10.1016/j.tifs.2019.03.026
8. Cicero FGA, Colletti A. Nutraceuticals and dietary supplements to improve quality of life and outcomes in heart failure patients. *Curr Pharm Design*. (2017) 23:1265–72. doi: 10.2174/1381612823666170124120518
9. Garg M, Ahuja V. Development and evaluation of a nutraceutical herbal summer drink. *Int J Pharm Pharm Sci*. (2015) 9:581–4. doi: 10.5281/zenodo.1109093
10. Maurya AP, Chauhan J, Yadav DK, Gangwar R, Maurya VK. Nutraceuticals and their impact on human health. In: Egbuna C, Mishra AP, Goyal MR, editors. *Preparation of Phytopharmaceuticals for the Management of Disorders*. Cambridge, MA: Academic Press (2021). p. 229–54.
11. Balluz LS, Kieszak SM, Philen RM, Mulinare J. Vitamin and mineral supplement use in the United States: results from the third National Health and Nutrition Examination Survey. *Arch Fam Med*. (2000) 9:258. doi: 10.1001/archfami.9.3.258
12. Shahbandeh M. US sales of vitamins and nutritional supplements manufacturing 2018-2019. *Statista*. (2019) 2019. (accessed May 13, 2020).
13. Mikulic M. *Dietary Supplements Market Size Worldwide 2022 Forecast*. Statista (2018).
14. Grebow J. Healthy sales, dietary supplements sales skyrockets during the Covid-19 pandemic. *Nutr Outlook*. (2020) 23:3–6. Available online at: nutritionaloutlook.com (accessed October 13, 2021).
15. Kokate CK, Purohit AP, Gokhale SB. Nutraceutical and cosmaceutical. In: *Pharmacognosy*. 21st ed. Pune, India: Nirali Prakashan. p. 542–9 (2002).
16. Sarker U, Oba S. Color attributes, betacyanin, and carotenoid profiles, bioactive components, and radical quenching capacity in selected *Amaranthus gangeticus* leafy vegetables. *Sci Rep*. (2021) 11:1–14. doi: 10.1038/s41598-021-91157-8
17. Tanumihardjo S. *Carotenoids and Human Health*. Totowa, NJ: Humana Press. 331 p (2013).
18. Sarker U, Oba S. The response of salinity stress-induced *A. tricolor* to growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. *Front Plant Sci*. (2020) 11:10–3389. doi: 10.3389/fpls.2020.559876
19. Simon PW, Pollak LM, Clevidence BA, Holden JM, Haytowitz DB. Plant breeding for human nutritional quality. *Plant Breed Rev*. (2009) 31:325–92. doi: 10.1002/9780470593783.ch7

20. Maiani G, Cast MJP, Catasta C, Toti E, Cambrod IG, Bysted A, et al. Carotenoids: actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. *Mol Nutri Food Res.* (2009) 53:S194–218. doi: 10.1002/mnfr.200800053
21. Simon PW, Freeman RE, Vieira JV, Boiteux LS, Briard M, Nothnagel T, et al. Carrot. In: Prohens J, Carena MJ, Nuez F, editors. *Handbook of Crop Breeding*, Vo.l 1. Vegetable Breeding. Heidelberg: Springer. p. 327–57 (2008).
22. Haq R, Prasad K. Nutritional and processing aspects of carrot (*Daucus carota*)-A review. *South Asian J Food Technol Environ.* (2015) 1:1–4. doi: 10.46370/sajfte.2015.v01i01.01
23. FAO (2017). Available online at: www.fao.org/statistics (accessed September 30, 2021).
24. Simon PW. Economic and academic importance. In: *The Carrot Genome*. Cham: Springer (2019). p. 1–8.
25. Ellison S. Carrot domestication. In: *The Carrot Genome*. Cham: Springer (2019). p. 77–91.
26. Sun T, Simon PW, Tanumihardjo SA. Antioxidant phytochemicals and antioxidant capacity of biofortified carrots (*Daucus carota L.*) of various colors. *J Agric Food Chem*. (2009). 57:4142–7. doi: 10.1021/jf9001044
27. Simon PW, Geoffriau E, Ellison S, Iorizzo M. Carrot carotenoid genetics and genomics. In: *The Carrot Genome*. Cham: Springer (2019). p. 247–60.
28. Arscott SA, Tanumihardjo SA. Carrots of many colors provide basic nutrition and bioavailable phytochemicals acting as a functional food. *Compr Rev Food Sci Food Saf.* (2010) 9:223–39. doi: 10.1111/j.1541-4337.2009.00103.x
29. Grassmann J, Schnitzler WH, Habegger R. Evaluation of different coloured carrot cultivars on antioxidative capacity based on their carotenoid and phenolic contents. *Int J Food Sci Nutr.* (2007) 58:603–11. doi: 10.1080/09637480701359149
30. Ching LS, Mohamed S. Alpha-tocopherol content of 62 edible tropical plants. *J Agric Food Chem.* (2001) 49:3101–5. doi: 10.1021/jf000891u
31. Hansen L, Hammershoy O, Boll PM. Allergic contact dermatitis from falcarinol isolated from *Schaffera arboricola*. *Contact Dermatitis.* (1986) 14:91–3. doi: 10.1111/j.1600-0536.1986.tb01167.x
32. Singh MN, Srivastava R, Yadav I. Study of different varietis of carrot and its benefits for human health: a review. *J Pharmacogn Phytochem*. (2021) 10:1293–9. doi: 10.22271/phyto.2021.v10.i1r.13529
33. Bartlett HE, Eperjesi F. Nutritional supplementation for type 2 diabetes: a systematic review. *Ophthalmic Physiol Optics.* (2008) 28:503–23. doi: 10.1111/j.1475-1313.2008.00595.x
34. Kim DJ, Xun P, Liu K, Loria C, Yokota K, Jacobs DRJr, et al. Magnesium intake in relation to system inflammation, insulin resistance, and the incidence of diabetes. *Diabetes Care.* (2010) 33:2604–10. doi: 10.2337/dc10-0994
35. Muralidharan P, Balamurugan G, Kumar P. Inotropic and cardioprotective effects of *Daucus carota* Linn. on isoproterenol-induced myocardial infarction Bangladesh. *J Pharmacol.* (2008) 3:74–9. doi: 10.3329/bjp.v3i2.849
36. Singh K, Singh N, Chandy A, Maniganha A. *In vivo* antioxidant and hepatoprotective activity of methanolic extracts of *Daucus carota* seeds in experimental animals. *Asian Pacific J Trop Biomed.* (2012) 2:385–8. doi: 10.1016/S2221-1691(12)60061-6
37. Aydin AF, Kiraz ZK, Abbasoghu SD, Gulluoglu M, Uysal M, Toker NK. Effect of carnosine against thioacetamide-induced liver cirrhosis in rat. *Peptides.* (2010) 31:67–71. doi: 10.1016/j.peptides.2009.11.028
38. Vasudevan M, Gunnam KK, Parle M. Anticonceptive and anti-inflammatory properties of *Daucus carota* seeds extract. *J Health Sci.* (2006) 52:598–606. doi: 10.1248/jhs.52.598
39. Wehbe K, Mroueh M, Daher CF. The potential role of daucus carota aqueous and methanolic extracts on inflammation and gastric ulcers in rats. *J Complement Integr Med*. (2009) 6. doi: 10.2202/1553-3840.1159
40. Gambhir SS, Sanyal AK, Sen SP, Das PK. Studies on *Daucus carota* Linn. II Cholinergic activity of the quaternary base isolated from water-soluble fraction of alcoholic extracts of seeds. *Indian J Med Res.* (1966) 54:1053–6.
41. Rossi PG, Bao L, Luciani A, Panighi J, Desjobert JM, Costa J, et al. (*E*)-Methylisoeugenol and Elemicin: Antibacterial Components of *Daucus carota* L. Essential Oils against *Campylobacter jejuni. J Agric Food Chem.* (2007) 55:7332–6. doi: 10.1021/jf070674u
42. Misiaka IJ, Lipoka J, Nowakowska EM, Wieczoreka PP, Mlynarz P, Kafarski P. Antifungal activity of carrot seed oil and its major sesquiterpene compounds. *Zeitschrift für Naturforschung.* (2004) 59:791–6. doi: 10.1515/znc-2004-11-1205
43. Kumarasamy Y, Nahar L, Byres M, Delazar A, Sarker SD. The assessment of biological activities associated with the major constituents of the methanol extract of "wild carrot" (*Daucus carota* L.) seeds. *J Herb Pharmacother.* (2005) 5:61–72. doi: 10.1300/J157v05n01_07
44. Mornin RA, De Witt DL, Nair MG. Inhibition of cyclooxygenase (COX) enzymes by compounds from *Daucus carota* L. *Seeds Phytother Res.* (2003) 17:976–9. doi: 10.1002/ptr.1296
45. Araya XIT, Smale N, Zabaras D, Winley E, Forde C, Stewart CM, et al. Sensory perception and quality attributes of high pressure processed carrots in comparison to raw, sous-vide and cooked carrots. *Innov Food Sci Emerg Technol.* (2009) 10:420–33. doi: 10.1016/j.ifset.2009.04.002
46. Potter AS, Foroudi S, Stamatikos A, Patil BS, Deyhim F. Drinking carrot juice increases total antioxidant status and decreases lipid peroxidation in adults. *Nutr J.* (2011) 10:1–6. doi: 10.1186/1475-2891-10-96
47. Simon P. Carrots and other horticultural crops as a source of provitamin a carotenes. *HortScience.* (1990) 25:1495–9. doi: 10.21273/HORTSCI.25.12.1495
48. Munawar M, Hammad G, Nadeem K, Raza MM, Saleem M. Assessment of genetic diversity in Tinda gourd through multivariate analysis. *Int J Veg Sci.* (2015) 21:157–66. doi: 10.1080/19315260.2013.853721
49. IPGRI. Descriptors for wild and cultivated carrots (Daucus carota L.). Rome: *International Plant Genetic Resources Institute* (1998).
50. AOAC. *Official Methods of Analysis*. Washington, DC: Association of Official Analytical Chemists Ed. (2012). 684p.
51. Hussain J, Khan AL, Rehman N, Hamayun M, Shah T, Nisar M. Proximate and nutrient analysis of selected vegetable species: a case study of Karak region. Pakistan. *Afr J Biotechnol.* (2009) 8:2725–9. doi: 10.3923/pjn.2009.620.624
52. Khalil IA, Varananis FR. Carotenoid extraction and analysis by reversed phase HPLC system. *Sarhad J Agric.* (1996) 105:15–21.
53. Steel RGD, Torrie JH, Dicky DA. *Principles and procedures of Statistics. A Biometrical Approach.* 3rd ed. New York, NY: McGraw Hill Book Co. Inc. (1997). p. 400–28.
54. Piepho HP, Möhring J, Melchinger AE, Büchse A. BLUP for phenotypic selection in plant breeding and variety testing. *Euphytica.* (2008) 161:209–28. doi: 10.1007/s10681-007-9449-8
55. Dey SS, Dey RB, Sharma S, Sharma K, Kumar R, Sharma VK, et al. Genetic divergence and interaction among CUPRAC, FRAP and total phenolics content in cauliflower (*Brassica oleracea* var. *botrytis L). Indian J Genet Plant Breed.* (2015) 75:79–85. doi: 10.5958/0975-6906.2015.00010.3
56. Singh D, Wang X, Kumar U, Gao L, Noor M, Imtiaz M, et al. High-throughput phenotyping enabled genetic dissection of crop lodging in wheat. *Front Plant Sci.* (2019) 10:394. doi: 10.3389/fpls.2019.00394
57. Ogunniyan DJ, Olakojo SA. Genetic variation, heritability, genetic advance and agronomic character association of yellow elite inbred lines of maize (Zea mays L) Nigerian. *J Genet.* (2014) 28:24–8. doi: 10.1016/j.nigjg.2015.06.005
58. Sarker U, Islam T, Rabbani G, Oba S. Genotype variability in composition of antioxidant vitamins and minerals in vegetable amaranth. *Genetika.* (2015) 47:85–96. doi: 10.2298/GENSR1501085S
59. Sarker U, Islam MS, Rabbani MG, Oba S. Variability, heritability and genetic association in vegetable amaranth (*Amaranthus tricolor* L). *Spanish J Agric Res.* (2015) 13:17. doi: 10.5424/sjar/2015132-6843
60. Sarker U, Oba S, Daramy MA. Nutrients, minerals, antioxidant pigments and phytochemicals, and antioxidant capacity of the leaves of stem amaranth. *Sci Rep*. (2020) 10:3892. doi: 10.1038/s41598-020-60252-7
61. Keleş D, Özgen S, Saraçoglu O, Ata A, Özgen M. Antioxidant potential of Turkish pepper (*Capsicum annuum* L.) genotypes at two different maturity stages. *Turkish J Agric Forestry*. (2016) 40:542–51. doi: 10.3906/tar-1601-24
62. Jolliffe IT, Cadima J. Principal component analysis: a review and recent developments. *Philos Trans R Soc A Math Phys Eng Sci.* (2016) 374:20150202. doi: 10.1098/rsta.2015.0202
63. MacQueen J. Some methods for classification and analysis of multivariate observations. *Proc Fifth Berkeley Symp Math Statistics Probability.* (1967) 1:281–97.

64. Sarker U, Hossain MM, Oba S. Nutritional and antioxidant components and antioxidant capacity in green morph Amaranthus leafy vegetable. *Sci Rep.* (2020) 10:1–10. doi: 10.1038/s41598-020-57687-3
65. Sarker U, Oba S. Salinity stress enhances color parameters, bioactive leaf pigments, vitamins, polyphenols, flavonoids and antioxidant activity in selected Amaranthus leafy vegetables. *J Sci Food Agric.* (2019) 99:2275–84. doi: 10.1002/jsfa.9423
66. Sarker U, Oba S. Polyphenol and flavonoid profiles and radical scavenging activity in leafy vegetable *Amaranthus gangeticus*. *BMC Plant Biol.* (2020) 20:1–12. doi: 10.1186/s12870-020-02700-0
67. Sarker U, Oba S. Phenolic profiles and antioxidant activities in selected drought-tolerant leafy vegetable amaranth. *Sci Rep.* (2020) 10:1–11. doi: 10.1038/s41598-020-71727-y
68. Sarker U, Hossain MN, Iqbal MA, Oba S. Bioactive components and radical scavenging activity in selected advance lines of salt-tolerant vegetable amaranth. *Front Nutr.* (2020) 7:587257. doi: 10.3389/fnut.2020.587257
69. Sarker U, Oba, S. Nutraceuticals, antioxidant pigments, and phytochemicals in the leaves of *Amaranthus spinosus* and *Amaranthus viridis* weedy species. *Sci Rep.* (2019) 9:1–10. doi: 10.1038/s41598-019-50977-5
70. Sarker U, Islam MT, Oba S. Salinity stress accelerates nutrients, dietary fiber, minerals, phytochemicals and antioxidant activity in *Amaranthus tricolor* leaves. *PLoS ONE.* (2018) 13:e0206388. doi: 10.1371/journal.pone.0206388
71. Sarker U, Oba S. Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected *Amaranthus tricolor* under salinity stress. *Sci Rep.* (2018) 8:10–1038. doi: 10.1038/s41598-018-30897-6
72. Sarker U, Oba S. Catalase, superoxide dismutase, and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. *Sci Rep.* (2018) 8:16496. doi: 10.1038/s41598-018-34944-0
73. Achten WM, Maes W, Reubens B, Mathijs E, Singh VP, Verchot L, et al. Biomass production and allocation in *Jatropha curcas* L. seedlings under different levels of drought stress. *Biomass Bioenergy*. (2010) 34:667–676. doi: 10.1016/j.biombioe.2010.01.010
74. Sarker U, Oba S. Antioxidant constituents of three selected red and green color Amaranthus leafy vegetable. *Sci Rep.* (2019) 9:1–11. doi: 10.1038/s41598-019-52033-8
75. Sarker U, Oba S. Protein, dietary fiber, minerals, antioxidant pigments and phytochemicals, and antioxidant activity in selected red morph Amaranthus leafy vegetable. *PLoS ONE.* (2019) 14:e0222517. doi: 10.1371/journal.pone.0222517
76. Molkara T, Akhlaghi F, Ramezani MA, Salari R, Vakili V, Kamalinejad M, et al. Effects of a food product (based on *Daucus carota*) and education based on traditional Persian medicine on female sexual dysfunction: a randomized clinical trial. *Electronic Phys.* (2018) 10:6577. doi: 10.19082/6577
77. Baranski R, Allender C, Klimek-Chodacka M. Towards better tasting and more nutritious carrots: carotenoid and sugar content variation in carrot genetic resources. *Food Res Int.* (2012) 47:182–7. doi: 10.1016/j.foodres.2011.05.006
78. Iorizzo M, Senalik DA, Ellison SL, Grzebelus D, Cavagnaro PF, Allender C, et al. Genetic structure and domestication of carrot (*Daucus carota* subsp. *sativus) (Apiaceae). Am J Bot.* (2013) 100:930–8. doi: 10.3732/ajb.1300055
79. Grzebelus D, Iorizzo M, Senalik D, Ellison S, Cavagnaro P, Macko-Podgórni A, et al. Diversity, genetic mapping, and signatures of domestication in the carrot (Daucus carota L) genome, as revealed by Diversity Arrays Technology (DArT) markers. *Mol Breed.* (2014) 33:625–37. doi: 10.1007/s11032-013-9979-9
80. Small E. A numerical taxonomic analysis of the *Daucus carota* complex. *Can J Bot.* (1978) 56:248–76. doi: 10.1139/b78-033
81. Manosa NA. *Influence of temperature on yield and quality of carrots (Daucus carota var. sativa).* (M. Sc. Thesis), University of The Free State Bloemfontein, South Africa (2011).
82. Arif U, Amir S, Siddiqui MFKSU, Jatoi SA. Agro-morphological assessment and seed protein profiling in carrot (*Daucus Carota* L.) Germplasm. *Pak J Bot.* (2020) 52:1595–603. doi: 10.30848/PJB2020-5(7)
83. Santhi VP, Priya PA, Anita B, Selvaraj N. Genetic variability, heritability and genetic advance in varieties of carrot (*Daucus carota* L.). *Int J Plant Sci.* (2015) 10:136–41. doi: 10.15740/HAS/IJPS/10.2/136-141
84. Buishand J, Gabelman W. Investigations on the inheritance of color and carotenoid content in phloem and xylem of carrot roots (*Daucus carota* L.). *Euphytica.* (1979) 28:611–32. doi: 10.1007/BF00038928
85. Ellison SL, Luby CH, Corak KE, Coe KM, Senalik D, Iorizzo M, et al. Carotenoid presence is associated with the *Or* gene in domesticated carrot. *Genetics.* (2018) 210:1497–508. doi: 10.1534/genetics.118.301299
86. Simon PW, Peterson CE, Lindsay RC. Genetic and environmental influences on carrot flavor. *J Am Soc Hortic Sci.* (1980) 105:416–20.
87. Simon PW. Carrot flavor: effects of genotype, growing conditions, storage, and processing. Ch. 11. In: Pattee HE, editors. *Evaluation of Quality of Fruits and Vegetables*. Wesport, CT: AVI Publishing. p. 315–28 (1985).
88. Simon PW, Peterson CE, Lindsay RC. Genotype, soil, and climate effects on sensory and objective components of carrot flavor. *J Am Soc Hortic Sci.* (1982) 107:644–8.
89. Cavagnaro PF. Genetics and genomics of carrot sugars and polyacetylenes. In: Simon P, Iorizzo M, Grzebelus D, Baranski R, editors. *The Carrot Genome*. Cham: Springer (2019). p. 295–315.
90. Yadav M, Tirkey S, Singh DB, Chaudhary R, Roshan RK, Pebam N. Genetic variability, correlation coefficient and path analysis in carrot. *Indian J Horticult.* (2009) 66:315–8.
91. De Corato U. Improving the shelf-life and quality of fresh and minimally-processed fruits and vegetables for a modern food industry: a comprehensive critical review from the traditional technologies into the most promising advancements. *Crit Rev Food Sci Nutr.* (2020) 60:940–75. doi: 10.1080/10408398.2018.1553025
92. Lingaiah HB, Huddar AG. Influence of pre-cooling, waxing and prepackaging on the shelf life and quality of carrots (*Daucus carota* L.). *Mysore J Agric Sci.* (1991) 25:102–23.
93. Jitender K, Mangal JL, Tewatia AS, Kumar J. Effect of storage conditions and packaging materials on shelf life of carrots cv. *Hisar Gairic Veg Sci.* (1999) 26:196–7.
94. Workneh TS, Osthoff G, Steyn MS. Physiological and chemical quality of carrots subjected to pre-and postharvest treatments. *Afr J Agric Res.* (2011) 6:2715–24. doi: 10.5897/AJAR10.1078
95. Pal RK, Roy SK. Zero-energy cool chamber for maintaining postharvest quality of carrot (*Daucus carota* var. *sativa). Indian J Agric Sci.* (1988) 58:665–7.
96. Waskar DP, Khedkar RM, Garande VK. Effect of post-harvest treatments on shelf life and quality of pomegranate in evaporative cool chamber and ambient conditions. *J Food Sci Technol.* (1999) 36:114–7.
97. Abbas S, Khoudi A. *Essai de formulation d'une boisson à base de fruits (orange, citron et pomme) et légumes (concombre et carotte) au niveau de NCA Rouïba*. Mémoire de Master, Université M'Hamed Bougara Boumerdes, République Algérienne Démocratique et Populaire. (2016). 68 p.
98. Arqha GF. *pH des aliments*. (2016). 8p. Available online at: https://www.agridea.ch/fileadmin/thematic/Exploitation__Famille__Diversification/pH_aliments_Lebensmittel_FR_DE.pdf (accessed September 30, 2021).
99. Anonyme. *Mannuel d'inspection des produits*. (2009). 24p.
100. Owolade SO, Akinrinola AO, Popoola FO, Aderibigbe OR, Ademoyegun OT, Olabode IA. Study on physico-chemical properties, antioxidant activity and shelf stability of carrot (*Daucus carota*) and pineapple (*Ananas comosus*) juice blend. *Int Food Res J.* (2017) 1:24.
101. Miglio C, Chiavaro E, Visconti A, Fogliano V, Pellegrini N. Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. *J Agric Food Chem.* (2008) 56:139–47. doi: 10.1021/jf072304b

102. Renna M, Pace B, Cefola M, Santamaria P, Serio F, Gonnella M. Comparison of two jam making methods to preserve the quality of colored carrots. *LWT-Food Sci Technol.* (2013) 53:547–54. doi: 10.1016/j.lwt.2013.03.018
103. Sethi V, Anand JC. Studies on the preparation, quality and storage of intermediate moisture vegetables. *J Food Sci Technol.* (1982) 19:168–70.
104. Kaur G, Aggarwal P. Effect of thermal processing and chemical preservatives on the physicochemical and phytochemical parameters of carrot juice. *Asian J Dairy Food Res.* (2016) 35:71–5. doi: 10.18805/ajdfr.v35i1.9255

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 10 January 2022
doi: 10.3389/fphar.2021.787125

Extracts of *Andrographis paniculata* (Burm.f.) Nees Leaves Exert Anti-Gout Effects by Lowering Uric Acid Levels and Reducing Monosodium Urate Crystal-Induced Inflammation

Eldiza Puji Rahmi[1]*, *Endang Kumolosasi*[2], *Juriyati Jalil*[2], *Fhataheya Buang*[2] and *Jamia Azdina Jamal*[2]*

[1]Pharmacy Program, Faculty of Medicine, Universitas Pembangunan Nasional Veteran Jakarta, Jakarta, Indonesia, [2]Drug and Herbal Research Centre, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

OPEN ACCESS

Edited by:
Massimo Lucarini,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Ashok K. Shakya,
Al-Ahliyya Amman University, Jordan
Sheikh Fayaz Ahmad,
King Saud University, Saudi Arabia

***Correspondence:**
Eldiza Puji Rahmi
eldizapr@upnvj.ac.id
Jamia Azdina Jamal
jamia@ukm.edu.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 30 September 2021
Accepted: 16 December 2021
Published: 10 January 2022

Citation:
Rahmi EP, Kumolosasi E, Jalil J, Buang F and Jamal JA (2022) Extracts of Andrographis paniculata (Burm.f.) Nees Leaves Exert Anti-Gout Effects by Lowering Uric Acid Levels and Reducing Monosodium Urate Crystal-Induced Inflammation. Front. Pharmacol. 12:787125. doi: 10.3389/fphar.2021.787125

Andrographis paniculata (Burm.f.) Nees has been found to have anti-inflammatory and immunostimulatory effects. This study was to investigate antihyperuricemic and anti-inflammatory effects of *A. paniculata* leaf extracts. Andrographolide, 14-deoxy-11,12-didehydroandrographolide, and neoandrographolide were quantified in 80% ethanol (EtOH80) and water extracts using High Performance Liquid Chromatography (HPLC) analysis. Antihyperuricemic activity was evaluated using a spectrophotometric *in vitro* inhibitory xanthine oxidase (XO) assay. The most active extract and andrographolide were further investigated in a hyperuricemic rat model induced by potassium oxonate to determine serum uric acid levels, liver XO activity, followed by Western blot analysis for renal urate transporter URAT1, GLUT9, and OAT1 to investigate the excretion of uric acid *via* kidney. Anti-inflammatory activity was assessed by *in vitro* interleukin assay for interleukin (IL-1α, IL-1β, IL-6, IL-8), and tumor necrosis factor (TNF-α) in monosodium urate (MSU) crystal-induced human fibroblast-like synoviocyte (HFLS) cells using ELISA-kits, followed by Western blot analysis for the expression of MyD88, NLRP3, NF-κB p65, and caspase-1 proteins to investigate the inflammation pathway. *In vivo* assay of the most active extract and andrographolide were performed based on the swelling rate and inhibition of pro-inflammatory mediator release from synovial fluid of a rat knee joint induced by MSU crystals. The results showed that the EtOH80 extract had a greater amount of andrographolide (11.34% w/w) than the water extract (1.38% w/w). In the XO inhibitory activity, none of the samples exhibited greater than 50% inhibition. However, in a rat model, EtOH80 extract (200 mg/kg/day) and andrographolide (30 mg/kg/day) decreased serum uric acid levels and reduced liver XO activity, reduced the protein expression levels of URAT1 and GLUT9, and restored the decrease in OAT1 levels. In the *in vitro* anti-inflammatory study, EtOH80 extract and andrographolide significantly decreased production of IL-1α, IL-1β, IL-6, and TNF-α, as well as inhibited the synthesis of MyD88, NLRP3, NF-κB p65, and caspase-1 in a concentration-dependent manner, almost comparable to dexamethasone. The EtOH80 extract (200 mg/kg/day) and andrographolide (30 mg/kg) significantly decreased swelling rate

and IL-1α, IL-1β, IL-6, and TNF-α in the synovial fluid of rat models in a time-dependent manner, comparable to indomethacin (3 mg/kg/day). In conclusion, the findings show that EtOH80 extract has a substantial anti-gout effect by lowering uric acid levels and suppressing pro-inflammatory mediator production due to the andrographolide content, that might be beneficial in the treatment of gouty-inflammation.

Keywords: andrographolide, xanthine oxidase, uricosuric, pro-inflammatory cytokines, MyD88, NLRP3

INTRODUCTION

Andrographis paniculata (Burm.f.) Nees (The Plant List, 2021) (Acanthaceae), also known as "King of Bitter," sambiloto (Indonesia) or hempedu bumi (Malaysia), has long been used in traditional medicine to treat respiratory diseases, skin infections, herpes, dysentery, fever, sore throat, lower urinary tract infections and diarrhea, as well as to reduce inflammation (Jarukamjorn and Nemoto 2008). It is also used to treat snake bites, insect bites, diabetes, and malaria (Burkill et al., 1966). Many medicinal plants, including *A. paniculata*, have been used as spices or food for thousands of years. *A. paniculata* is widely consumed in Indonesia as *Jamu* (herbal drink) and *Lalapan* (fresh vegetable) (Putra, 2003).

Pharmacological activities such as antiplatelet aggregation activity, immunomodulatory activity, hepatoprotective activity, cytotoxicity against cancer cells, anti-inflammatory and antiangiogenic activities, antimalaria, antidiabetic, cardiovascular activity, and antivenom activity have been reported for *A. paniculata* (Puri et al., 1993; Melchior et al., 1997; Deshpande et al., 2014). Diterpene lactones, such as andrographolide, neoandrographolide, and 14-deoxy-11,12 didehydroandrographolide, are active phytochemicals found in *A. paniculata* (Thisoda et al., 2006; Ren et al., 2008). Andrographolide, a major active constituent, is responsible for the most of pharmacological effects, including anti-inflammatory, antibacterial, antitumor, antidiabetic, antimalaria, and hepatoprotective properties (Jarukamjorn and Nemoto 2008). Other diterpene lactones (neoandrographolide and 14-deoxyandrographolide), flavonoids, quinic acids, and xanthones are also mentioned as having a significant effect.

A. paniculata extract has been shown to reduce inflammation by inhibiting the expression of iNOS, TNF-α, IL-1β, IL-6, and IL-12, as well as NO production, through the downregulation of p38MAPKs signaling pathways (Liu et al., 2008; Wang et al., 2013). Andrographolide potently modulated the level of LPS-induced TNF-α, IL-6, IL-1β, and IL-10 secretion in human blood in a concentration-dependent manner by selectively down-regulating cytokines and cytokine receptors (TNFSF14, TNF, TNFRSF6, and IL1A), chemokines (CCL8 and CXCL11), JAK/STAT signaling (JAK3 and STAT5A), TLRs family (TLR4 and TLR8) and nuclear factor kappa B (NF-κB) (Parichatikanond et al., 2010).

Salim et al. (2014) discovered that the methanol extract of *A. paniculata* and andrographolide inhibited IL-1α, an important regulatory cytokine whose release after an injury can activate transcription factors NF-kB and activator protein (AP-1), promoting expression of genes involved in cell survival, proliferation, and angiogenesis (Wolf et al., 2001). To our knowledge, there is limited information about the action of *A. paniculata* on gouty arthritis, such as its effects on lowering uric acid levels and MSU-induced inflammatory in synovial cells.

Gout is an ancient inflammatory arthritis that has been documented for thousands of years. The pathogenesis of gout is started with the high level of uric acid in blood and the deposition of MSU crystals in the joint. MSU crystal is a very important factor for gouty inflammation as a pro-inflammatory stimulus by stimulating cells via toll-like receptor signaling (Cronstein and Terkeltaub 2006). MSU crystals can interact with almost all of the synovial cell types including neutrophils, monocytes/macrophages and fibroblast-like synoviocytes. In monocytes, microcrystals stimulate the synthesis of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor alpha (TNF-α) and prostaglandin E_2 (Pauliot et al., 1998). Previous study on MSU-induced cell activation showed that MSU deposition in the joints was often caused by leucocyte infiltration that leads to inflammation. Long-term inflammation would result in *tophy*, deformation of joints and thickening of synovial walls (Shi et al., 2012).

Interleukin (IL)-1β has been reported to have an important role in MSU-induced inflammation in gout. Thus, the inhibition of IL-1β is able to downregulate the inflammatory responses related to gout (Shi et al., 2012; Malawista et al., 2011). MSU crystals also up-regulate IL-6 and TNF-α secretion when they interact with cells, especially monocytes (Busso and So 2010). TNF-α has been reported to play a complex rote in joint inflammation and tophi formation (Ansari et al., 2021). Moreover, chemokine such as IL-8 (CXCL8) and cyclooxygenase (COX)-2 also play an important role in gouty inflammation (Choi et al., 2005). Prostaglandin E_2 (PGE_2) is an eicosanoid that is, biosynthesised from arachidonic acid precursor by the action of cyclooxygenase-2 (COX-2) enzyme and PGE_2 synthase in endothelial cells.

Based on this information, it is hypothesized that *A. paniculata* extract maybe useful as an anti-inflammatory and anti-hyperuricemic agent in the treatment of gouty arthritis. Thus, the aim of this study was to determine the anti-hyperuricemic effect of *A. paniculata* leaf extracts by exploring the xanthine oxidase (XO) inhibitory and uricosuric effects, asl well as the anti-inflammatory effect on the release of pro-inflammatory markers via the MyD88, NLRP3, NF-κB p65 and caspase-1 pathways in MSU-induced HFLS.

MATERIALS AND METHODS

Chemicals and Reagents

All analytical grade solvents and HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Hesse, Germany) while pure water for HPLC was obtained from ultrapure water system machine (PureLab, United States). Andrographolide (AP, 99.7%), 14-deoxy-11,12-didehydroandrographolide (DAP, < 99.6%) and neoandrographolide (NAP, ≥ 95%) were HPLC grade and acquired from Chromadex (California, United States). Dexamethasone was purchased from CCM Duopharma Biotech Bhd. Xanthine, Tween 20, potassium oxonate, 20 mM HEPES, FBS, pen-strep, RPMI-1640 medium containing L-glutamine, MTT, LPS from *Salmonella abortusequii*, uric acid, DMSO, and tryphan blue® were purchased from Sigma-Aldrich (Missouri, United States). Synoviocyte growth medium was procured from Cell Applications (California, United States). 100× Halt Protease and Phosphatase Inhibitor Cocktail were purchased from Thermo Fisher Scientific (Massachusetts, United States). Xanthine oxidase from bovine milk (20 U/mL) was purchased from Roche Diagnostic GmbH (Mannheim, Baden-Württemberg, Germany). Lymphoprep was purchased from Axis-Shield PoC AS (Oslo, Norway). Phosphate buffer saline (PBS) was purchased from MP Biomedicals (USA). Uric acid and xanthine oxidase kits were purchased from BioVision (Milpitas, California, United States). The concentration of cytokine was tested using appropriate ELISA kits for human. IL-8 ELISA kits were purchased from Abnova, Germany, while all other kits were purchased from Cayman, USA. Limit of detection of these kits were: 2.0 ρg/mL for IL-8, 7.8 ρg/mL for IL-6, and 3.9 ρg mL for IL-1α, IL-1β, and TNF-α. IL-1α, IL-1β, IL-6, and TNF-α in rats were measured using a multi-cytokine bead array detection system (ProcartaPlex, eBioscience, United States).

Preparation of *Andrographis paniculata* Extracts

Andrographis paniculata was cultivated at Field 2 Universiti Putra Malaysia (UPM) in Serdang, Selangor. A voucher specimen (No. SK965/04) was deposited at the Herbarium of the Laboratory of Natural Products, Institute of Bioscience, UPM. The leaves were dried in an oven dryer at 40°C for 3 days before being ground in an electric grinder. Dried powder was extracted with ethanol (80%) at a 1:10 ratio by rigorous maceration for 3 days at room temperature and repeated five times. To eliminate any remaining organic solvent, the organic filtrates were collected and concentrated under reduced vacuum pressure. The solvent-free extract was mixed with water and then freeze-dried (Labconco's FreeZone 4.5 L Freeze Dry Systems) to get the crude ethanol 80% (EtOH80) extract. Meanwhile, the water extract was obtained from the UPM laboratory of Prof. Dr. Johnson Stanslas. All crude extracts were kept at 4°C until further usage.

Animals

Male Sprague-Dawley rats (6–8 weeks old, 200–300 g) were procured from Universiti Kebangsaan Malaysia's Laboratory Animal Resource Unit (LARU-UKM). The animals were kept in plastic cages on a 12 h/12 h light/dark cycle. The temperature and relative humidity were maintained at 25 ± 2°C and 50%, respectively. They were fed a commercial laboratory diet and were given access to food and drink ad libitum during the study. They were given 1 week to acclimate to their surroundings before the experiment. All procedures followed the A CIOMS Ethical Code for Animal Experimentation (Howard-Jones, 1985) and were approved by the Universiti Kebangsaan Malaysia-Animal Ethics Committee (approval reference: FF/2016/JAMIA/27-JULY/772-AUG.-2016-JULY.2019).

Quantitative Analysis of *Andrographis paniculata* Extracts Using HPLC

Phytochemical analysis of ethanol 80% (EtOH80) and water extracts of *A. paniculata* leaves was carried out using RP-HPLC based on a slightly modified method described by Xu et al. (2008) to quantify the amount of chemical markers, namely andrographolide (AP), 14-deoxy-11,12-didehydroandrographolide (DAP), and neoandrographolide (NAP). The EtOH80 and water extracts were individually prepared by dissolving 10 mg of crude extracts in 1 ml of HPLC-grade methanol, while the chemical markers solution was made by mixing 200 g of each of the three standard diterpene lactones in 1 ml of HPLC-grade methanol. Before analysis, all extract and standard solutions were filtered through a 0.45 m Millipore Millex PTFE membrane. Separate analyses of the extract and standard solutions were performed under the following conditions; column: reversed phase, C-18 column (250 mm × 4.6 mm i.d., 5 µm, Xbridge, Waters, Ireland), detector: PDA (Waters 2998), wavelength: 205 nm, flow rate: 0.8 ml/min, mobile phase: A. methanol, B. water isocratically eluted with 55% A for 30 min with a 10-min equilibration period before injection. The injection volume of the solution was 10 µL.

Determination of Anti-Hyperuricemic Effect

In Vitro Xanthine Oxidase Inhibitory Assay

XO inhibitory activity of the extracts (400 µg/ml), chemical markers (100 µg/ml) and allopurinol (100 µg/ml) as a positive control was measured in 96-well plates using a spectrophotometric technique previously published by Rahmi et al. (2020).

In Vivo Anti-Hyperuricemic Assay of Potassium Oxonate-Induced Rats

Only the most active EtOH80 extract (50, 100, and 200 mg/kg/day, p.o.) and AP (30 mg/kg/day, p.o.) were investigated to assess the anti-hyperuricemic effect compared to the positive control allopurinol (5 mg/kg, p.o.). The *in vivo* anti-hyperuricemic assay extract was carried out for 14 days in accordance with a previous study by Rahmi et al. (2020). Using a uric acid assay kit (BioVision, Milpitas, CA. United States), the serum uric acid levels were determined by an enzymatic-colorimetric technique on a 96-well plate (Greiner Bio One, Germany). The XO colorimetric assay kit from BioVision (Milpitas, CA, United States) was used to measure enzyme and liver XO levels.

Protein Expressions of Renal URAT1, GLUT9, and OAT1 in Rats by Western Blotting

Kidney samples from each treatment group were homogenized in RIPA lysis buffer with protease and phosphatase inhibitors, and chilled for 30 min. The lysate was then centrifuged at 13,000 rpm for 10 min at 4°C to extract total proteins, which were then quantified using the Bradford assay. Total proteins were incubated in boiling water for 7 min with loading buffers. On a 10% SDS-PAGE, an equal quantity of total proteins was isolated and transferred onto a PVDF membrane. Membranes were blocked in TBST containing 5% skimmed milk powder for 1 h. They were then incubated with the primary antibodies againts rabbit anti-SLC22A12 (URAT1) antibody (1: 2000, abx003918; Abbexa, UK), rabbit anti-GLUT9 antibody (1: 5,000, ab223470; Abcam, UK), rabbit anti-OAT1 antibody (1: 3,000, abx218709; Abbexa, UK), and rabbit anti- β-actin (13E5) antibody (1: 1,000, 34970; Cell Signaling, United States) in TBST containing 5% skimmed milk powder overnight at 4°C. After three TBST washes of the membranes, immunoreactive bands were identified using HRP conjugated goat anti-rabbit IgG (ab205718; Abcam, UK) as a secondary antibody that was diluted in a ratio of 1:5,000 in TBST for 1 h at room temperature. ECL detection reagent (Bio-Rad) was used to view the proteins, while ChemiDoc XRS + was used to evaluate the density of bands, which was then normalized to β-actin.

Determination of Anti-Inflammatory Effect

In Vitro Cytokine Assay Using HFLS Cells

Human fibroblast-like synoviocyte cells (HFLS) were purchased from Cell Applications (California, United States). HFLS were grown in synoviocyte growth medium with 10% synoviocyte growth supplement in 5% CO_2 at 37°C. Every other day, the medium was changed. When HFLS cells achieved 80% confluence, they were sub-cultured.

The MTT assay was used to determine HFLS cell viability following a method described by Rahmi et al. (2020). In short, HFLS were seeded at a density of 1×10^6 per mL in 96-well plates with the same volume of extracts (i.e., EtOH80 and water), chemical markers (i.e., AP, DAP, and NAP) and dexamethasone as a positive control at concentrations of 5 and 10 μg/ml and 0.5% DMSO as a negative control (5 μg/ml), and pre-incubated for 27 h at 37°C with 5% CO_2. After adding 20 μL of MTT reagent (5 mg/ml), the plates were incubated for an additional 4 h at 37°C with 5% CO_2. The supernatant was discarded carefully, and the cells' formazan blue crystals were dissolved in 100 ml DMSO. Finally, the absorbance was determined using Tecan's Infinite 200PRO NanoQuant microplate reader at a wavelength of 570 nm.

The concentrations of IL-1α, IL-1β, IL-6, and TNF-α were measured in the HFLS culture supernatant using the ELISA technique described before (Rahmi et al., 2020). The HFLS (1×10^6 per mL) were pre-treated with extracts, chemical markers and dexamethasone at a concentration of 10 μg/ml, as well as complete medium with 0.5% DMSO for 3 h at 37°C in 5% CO_2, and then stimulated for 24 h with MSU crystal suspension (200 mg/ml). Cells were centrifuged for 10 min at 300 × g and 4°C following incubation. The supernatant was carefully transferred to a sterile tube, and the concentration of cytokines was determined using suitable human ELISA kits. The amounts of cytokine secretion were compared to a negative control, which was assumed to have 100% cytokine secretion. The IC_{50} values for the active extracts were obtained from five different concentrations (0.625–10 μg/ml).

Protein Expressions of MyD88, NLRP3, NF-κB, and Caspase-1 in MSU-Induced HFLS Cells by Western Blotting

Cells were seeded in 6-well plates (1×10^6 cells/well) for 24 h before being pre-treated for 3 h with 1.25, 2.5, and 5 μg/ml concentrations of extracts and chemical markers. MSU crystals were then used to stimulate cells, which were then incubated for 24 h. The cells were rinsed with cold PBS and disrupted in a lysis buffer containing protease and phosphatase inhibitors for the radioimmunoprecipitation assay (RIPA). On ice, harvested cells were lysed. The lysate was then centrifuged at 13,000 rpm for 10 min at 4°C to extract total proteins, which were then quantified using the Bradford assay. Total proteins were incubated in boiling water for 5 min with loading buffers. Equal quantity of total proteins was then separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in TBST (Tris-buffered saline with 0.1 percent Tween-20) with 5% skimmed milk powder for 1 h. They were then incubated overnight at 4°C in 3% BSA with primary antibodies from Cell Signalling, USA against MyD88 (D80F5) (1:3,000, #4283), NLRP3 (D2P5E) (1:3,000, #13158), NF-κB p-p65 (D14E12) (1:3,000, #3033), caspase 1 (D7F10) (1:3,000, #3866), and β-actin (13E5) (1:3,000, #4970S). After washing the membrane three times with TBST, immunoreactive bands were detected using a secondary antibody, anti-rabbit IgG HRP-linked antibody (Cell Signaling, United States) that was diluted in a ratio of 1:3,000 in BSA and incubated for 1 h at room temperature. ECL detection reagent (Bio-Rad) was used to visualize the proteins and ChemiDoc XRS+ was used to analyze the density of bands, which was normalized to β-actin.

In Vivo Anti-Inflammatory Assay of MSU-Induced Rats

Only the most active EtOH80 extract and AP were investigated to assess the anti-gouty inflammatory properties. An experimental model of gouty inflammation was used with MSU crystals as the inducer. The *in vivo* assay was carried out for 14 days in accordance with the previously published protocol (Rahmi et al., 2020). Briefly, 36 rats were randomly assigned to one of six experimental groups (n = 6 per group): normal control, hyperuricemia-induced control, indomethacin treatment (3 mg/kg/day) as a positive control, andrographolide treatment (30 mg/kg/day) and extract treatment of three different doses (50, 100, and 200 mg/kg/day). Feeding was stopped 2 h before oral administration of the allopurinol, andrographolide, or extract. Except for the normal control group, all rats were anaesthetized with isoflurane on the 11th day of the experiment, and inflammation was induced by intra-articular injection of 50 μL of MSU crystals suspension (100 mg/ml) into a rat's right knee

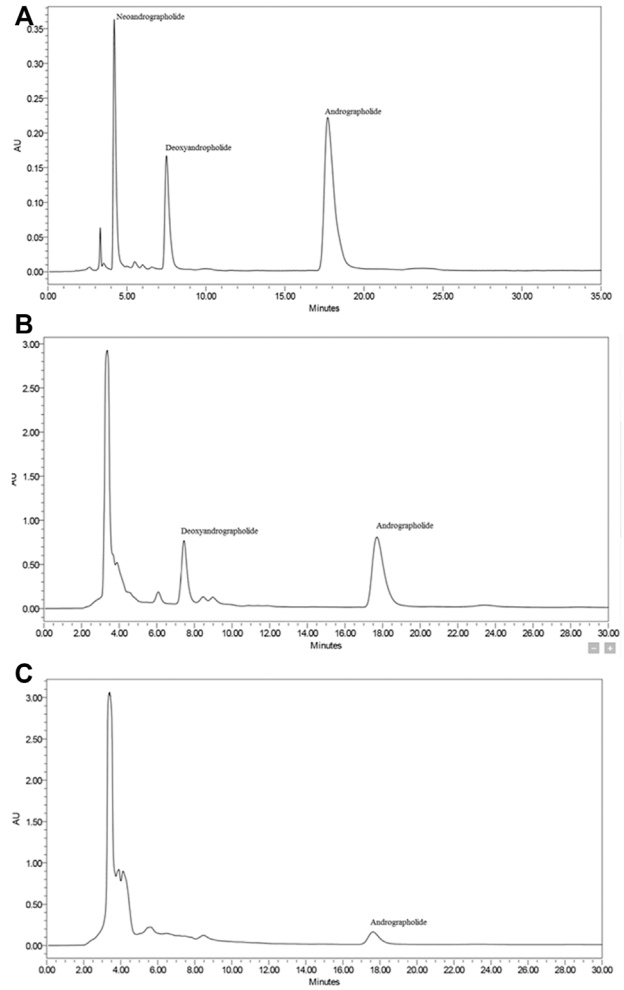


FIGURE 1 | Representative HPLC chromatograms of diterpene lactones (i.e., andrographolide, 14-deoxy-11,12-didehydroandrographolide, and neoandrographolide) **(A)**, ethanol (80%) extract **(B)** and water extract **(C)** of *Andrographis paniculata* leaves.

joint 1 h after oral treatment. A caliper was used to determine the swelling rate of a rat's knee joint at 2, 4, 8, 12, 24, 48, and 72 h after induction. Rats were anaesthetized with isoflurane and killed by cervical dislocation at the end of experiment. After shaving the skin around the knee, 100 µL sterile saline was injected into the synovial cavity using a 27 G needle, followed by the insertion of a second needle to collect synovial fluid. Inflammation was determined by measuring the IL-1α, IL-1β, IL-6, and TNF-α levels in synovial fluid according to the manufacturer's instructions using a multi-cytokine bead array detection system (Procarta, eBioscience, United States). The PGE_2 level was quantified using a single ELISA assay as directed by the manufacturer. The liver, kidneys, spleen, heart, and lung were promptly removed, rinsed in cold saline (0.9%), dried with a paper towel, and weighed.

TABLE 1 | Amount of andrographolide (AP), 14-deoxy-11,12-didehydroandrographolide (DAP), and neoandrographolide (NAP) in 80% of ethanol (EtOH80) and water extracts of *Andrographis paniculata* leaves analyzed by HPLC.

Standard	Concentration (mg/g)		Amount (% w/w)	
	EtOH80	Water	EtOH80	Water
AP	113.44 ± 1.63	13.788 ± 1.61	11.344 ± 0.16	1.379 ± 0.16
DAP	74.350 ± 1.88	ND	7.435 ± 0.19	ND
NAP	ND	ND	ND	ND

Data are presented as mean ± SEM (n = 3). ND-not detected.

TABLE 2 | *In vitro* xanthine oxidase inhibitory activity (%) of ethanol (80%) and water extracts of *Andrographis paniculata* leaves, diterpene lactones, and allopurinol.

Test substances	% Inhibition
EtOH80 extract (400 µg/ml)	28.28 ± 2.80
Water extract (400 µg/ml)	20.71 ± 1.96
AP (100 µg/ml)	4.39 ± 0.15
DAP (100 µg/ml)	15.19 ± 1.73
NAP (100 µg/ml)	6.35 ± 0.21
Allopurinol (positive control) (100 µg/ml)	99.87 ± 0.09

Data are presented as mean ± SEM (n = 3). Data were analyzed by one-way ANOVA followed by post hoc Tukey. Percent inhibition >2.5% was significant at $p < 0.05$ *when compared with negative control (0% inhibition). All percent inhibition values of extracts were statistically different compared with allopurinol* ($p < 0.01$).

Statistical Analysis

GraphPad Prism 5 was used to analyse all of the data and to calculate the IC_{50} values. Every experiment was performed three times ($n = 3$) in this study, and the results were presented as mean ± SEM. The test samples and controls were compared using one-way analysis of variance (ANOVA) and post hoc Tukey. If the p value was less than 0.05 ($p < 0.05$), the variance was considered significant.

RESULTS

Quantitative Analysis of *Andrographis paniculata* Extracts Using HPLC

Three diterpene lactones previously discovered in *A. paniculata* leaf extracts were detected using HPLC in this work, namely andrographolide (AP), 14-deoxy-11,12-didehydroandrographolide (DAP), and neoandrographolide (NAP) (**Figure 1**). Chemical markers of pure AP, DAP, and NAP were used as reference standards to identify and quantify the peaks found in the extracts. **Table 1** summarizes the amount of diterpene lactones found in *A. paniculata* leaves. In general, the EtOH80 extract contained more diterpene lactones than the water extract. AP was identified at a concentration of 113.44 mg/g (11.34% w/w) in EtOH80 extract, which was greater than the concentrations of the other identifiable phytochemicals.

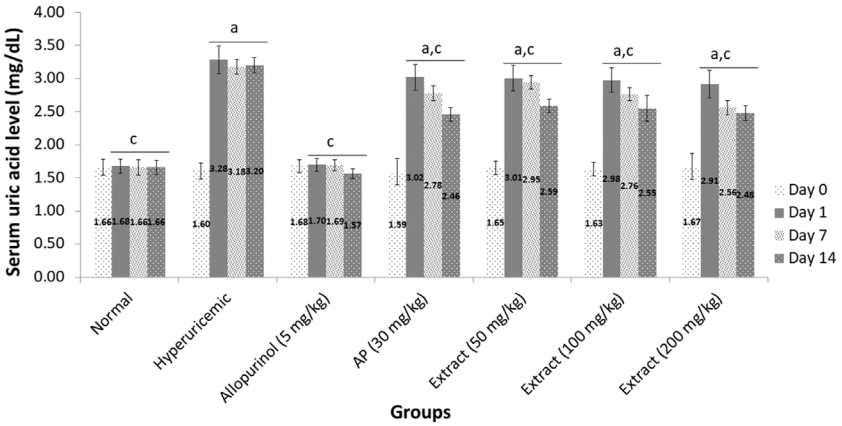


FIGURE 2 | Effect of ethanol (80%) extract of *Andrographis paniculata* leaves, andrographolide and allopurinol on serum uric acid levels in hyperuricemic-induced rats. Data are presented as mean ± SEM ($n = 6$). Data were analyzed using one-way ANOVA followed by post hoc Tukey. [a]Significantly different compared to normal group ($p < 0.05$). [b]Not significantly different compared to normal group ($p > 0.05$). [c]Significantly different compared to hyperuricemic group ($p < 0.05$). [d]Not significantly different compared to allopurinol group ($p > 0.05$).

TABLE 3 | Effect of ethanol (80%) extract of *Andrographis paniculata* leaves, andrographolide and allopurinol on xanthine oxidase activity in rat's liver.

Treatment	XO activity ± SEM (nmole uric acid/min per mg protein)	% Inhibition
Normal	2.36 ± 0.31[b,c]	—
Hyperuricemic	2.88 ± 0.42[a,c]	—
Allopurinol (5 mg/kg)	1.48 ± 0.11[a,b]	48.61 ± 0.22
Andrographolide (30 mg/kg)	2.31 ± 0.50[b,c]	19.79 ± 0.61[c]
Extract (50 mg/kg)	2.46 ± 0.42[b,c]	14.58 ± 0.58[c]
Extract (100 mg/kg)	2.42 ± 0.39[b,c]	15.97 ± 0.42[c]
Extract (200 mg/kg)	2.39 ± 0.64[b,c]	17.01 ± 0.59[c]

Data are presented as mean ± SEM (n = 6). Data were analysed by using one-way ANOVA followed by post hoc Tukey.
[a]Significantly different compared to normal group (p ≤ 0.05).
[b]Significantly different compared to hyperuricemic group (p ≤ 0.05).
[c]Significantly different compared to allopurinol (p ≤ 0.05).

Effect of *Andrographis paniculata* Leaves on Xanthine Oxidase Inhibitory Activity *In Vitro*

Table 2 shows that all extracts and chemical markers at the tested concentrations had lower XO inhibitory activity than 50%. Thus, IC_{50} values were not determined.

Effect of Ethanol (80%) Extract of *Andrographis paniculata* Leaves on Serum Uric Acid Levels, Liver Xanthine Oxidase and Protein Expression of Renal URAT1, GLUT9 and OAT1 in Hyperuricemic Rats

As indicated in **Figure 2**, the baseline serum uric acid levels of each group tested on Day 0 ranged from 1.59 to 1.68 mg/dl. When hyperuricemic rats were given EtOH80 extract at dosages of 50, 100, and 200 mg/kg for 14 days, serum uric acid levels were significantly ($p < 0.05$) decreased to 2.59, 2.54, and 2.48 mg/dl, respectively, compared to the hyperuricemic control group. However, none of the extract treatments could lower serum uric acid levels to the baseline value. When compared to the hyperuricemic control group, allopurinol (5 mg/kg) resulted in a significant ($p < 0.05$) reduction in serum uric acid levels. This decrease was seen even after 1 day of allopurinol treatment, and normal serum uric acid levels were maintained throughout the 14-days experiment with values of 1.70, 1.69, and 1.57 mg/dl on days 1, 7, and 14, respectively.

The inhibitory action of liver XO in rats was investigated to determine the antihyperuricemic efficacy of the EtOH80 extract. As demonstrated in **Table 3**, the hyperuricemic control group had significantly increased liver XO activity compared to the normal control group ($p < 0.05$). Treatment with EtOH80 extract at a dose of 200 mg/kg and AP reduced XO activity in the liver by only 17.01 and 19.79%, respectively, which were not comparable to the 48.61% inhibition observed with allopurinol.

Figure 3 shows the effects of EtOH80 extract, AP and allopurinol on protein expressions of URAT1, GLUT9, and OAT1 in hyperuricemic rats. The protein expression levels of renal urate transporters URAT1 and GLUT9 were reduced in potassium oxonate-induced hyperuricemic rats but were not significantly different from allopurinol. The hyperuricemic rat's renal OAT1 protein levels were significantly lower than in the control group, which were recovered by EtOH80 extract, AP, and allopurinol.

Effect of *Andrographis paniculata* Leaves and Diterpene Lactones on Cytokine Secretion in MSU-Induced HFLS

The MTT assay, which is based on the conversion of MTT to purple colored formazan by mitochondrial dehydrogenase from

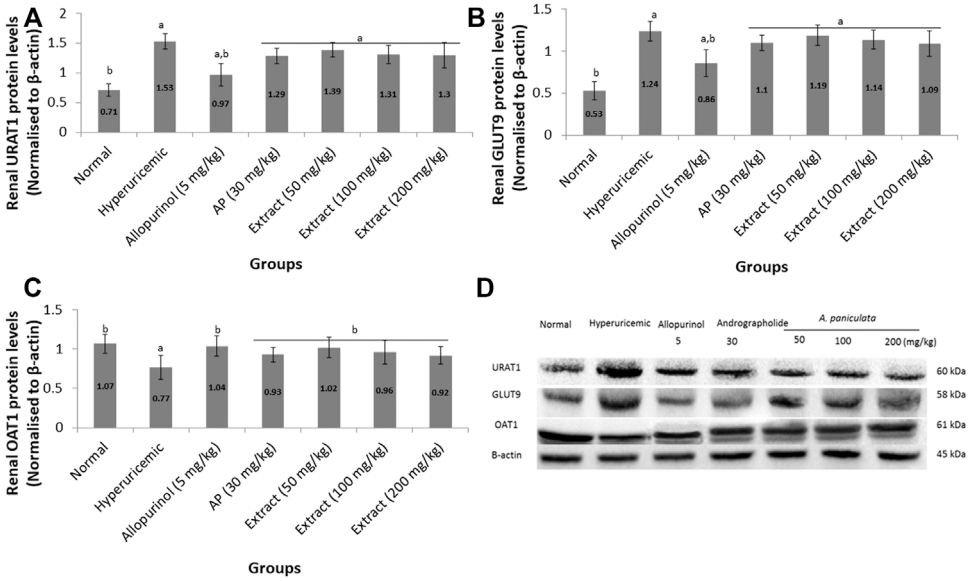


FIGURE 3 | Effect of ethanol (80%) extract of *A. paniculata* leaves, andrographolide and allopurinol on protein expressions of renal URAT1 **(A)**, GLUT9 **(B)** and OAT1 **(C)** in hyperuricemic-induced rats obtained from Western blot analysis **(D)**. Data are presented as mean ± SEM ($n = 6$). Data were analyzed using one-way ANOVA followed by post hoc Tukey. [a]Significantly different compared to normal group ($p < 0.05$). [b]Significantly different compared to hyperuricemic group ($p < 0.05$).

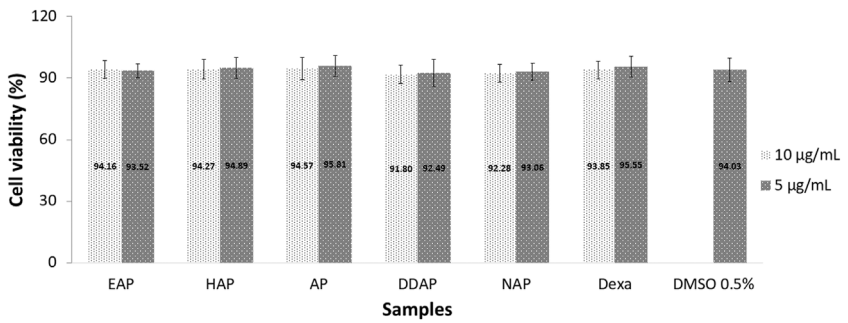


FIGURE 4 | Viability of human fibroblast-like synoviocyte (HFLS) cells after 27 h of exposure to 80% ethanol (EAP) and water (HAP) extracts of *Andrographis paniculata* leaves, andrographolide (AP), 14-deoxy-11,12-didehydroandrographolide (DAP), neoandrographolide (NAP), dexamethasone (DEXA), and 0.5% DMSO. Data are presented as mean ± SEM ($n = 3$).

viable cells, was used to assess HFLS cell viability. More than 90% of viable cells were found at 5 and 10 µg/ml concentrations of all *A. paniculata* extracts, chemical markers, and dexamethasone, showing that such concentrations had no influence on HFLS viability after 27 h of exposure (**Figure 4**). As a result, in this experiment, the 10 µg/ml concentration was used as the maximum concentration. The cell viability assay was crucial in demonstrating that the inhibition of cytokine production was not related to cell death.

Table 4 summarizes the inhibitory action of EtOH80 and water crude extracts, as well as three diterpene lactones at a concentration of 10 g/ml, on IL-1α, IL-1β, IL-6, and TNF-α. Only EtOH80 extract, AP and DAP were shown to suppress cytokine release actively (> 50% inhibition). EtOH80 extract inhibited the production of four cytokines most effectively, including IL-1α (54.34%), IL-1β (79.92%), IL-6 (71.23%), and TNF-α (69.29%). Additionally, AP reduced the production of four cytokines, including IL-1α (70.45%), IL-1β (86.34%), IL-6 (74.59%), and TNF-α (72.37%). Meanwhile, DAP suppressed just two cytokines actively, namely IL-1α (50.34%) and IL-1β (50.93%). The positive control, dexamethasone effectively suppressed the production of five cytokines, i.e., IL-1α (79.36%), IL-1β (69.37%), IL-6 (70.36%), IL-8 (53.28%), and TNF-α (65.13%). However, only AP possessed a comparable inhibitory activity to dexamethasone for all cytokines ($p \geq 0.05$). Meanwhile, EtOH80 extract possessed a comparable inhibitory activity to dexamethasone for IL-1β, IL-6 and TNF- α ($p \geq 0.05$).

TABLE 4 | Percentage of inhibition of ethanol (80%) and water extracts of *Andrographis paniculata* leaves, diterpene lactones and dexamethasone at a concentration of 10 μg/ml and IC_{50} values (μg/ml) on cytokine secretion in MSU-induced human fibroblast-like synoviocyte (HFLS).

Test substances and drug	% Inhibition and (IC_{50} values in μg/mL)				
	IL-1α	IL-1β	IL-6	IL-8	TNF-α
EtOH80 extract	54.34 ± 8.63 (7.26 ± 1.21)[b]	79.92 ± 7.34[a] (2.76 ± 0.23)[b]	71.23 ± 4.21[a] (3.03 ± 0.69)[b]	49.28 ± 3.48[a] (-)	69.29 ± 24.12[a] (3.95 ± 0.97)[b]
Water extract	37.59 ± 5.46 (-)	48.23 ± 6.83 (-)	40.34 ± 3.45 (-)	33.29 ± 2.84 (-)	41.02 ± 3.48 (-)
AP	70.45 ± 7.36[a] (3.09 ± 1.02)[b]	86.34 ± 5.34[a] (1.19 ± 0.29)[a]	74.59 ± 4.35[a] (2.97 ± 0.37)[b]	48.66 ± 4.92[a] (-)	72.37 ± 5.11[a] (3.12 ± 1.04)[b]
DAP	50.34 ± 6.64 (8.16 ± 1.26)[b]	50.93 ± 4.35 (8.39 ± 1.11)[b]	47.88 ± 3.13 (-)	32.38 ± 4.11 (-)	44.21 ± 3.41 (-)
NAP	44.67 ± 5.23 (-)	47.34 ± 3.25 (-)	44.63 ± 4.87 (-)	29.39 ± 2.63 (-)	38.23 ± 2.94 (-)
Dexamethasone (positive control)	79.36 ± 6.92 (0.71 ± 0.09)	69.37 ± 6.14 (0.57 ± 0.17)	70.36 ± 5.46 (0.81 ± 0.16)	53.28 ± 3.28 (0.76 ± 0.25)	65.13 ± 3.65 (0.49 ± 0.10)

Data are presented as mean ± SEM (n = 3). Data were analysed using one-way ANOVA followed by post hoc Tukey. Percentage inhibition >2.5% was significant at p ≤ 0.05 when compared with negative control. (-) = IC_{50} was not determined as none of tested concentration exceeded 50% inhibition.

[a]*Not significantly different compared to dexamethasone (p ≥ 0.05).*

[b]*Significantly different compared to dexamethasone (p < 0.01).*

Effect of *Andrographis paniculata* Leaves and Diterpene Lactones on Protein Expressions of MyD88, NLRP3, NF-κB and Caspase-1 in MSU-Induced HFLS Cells

To elucidate the mechanisms of action by which *A. paniculata* and its main constituents reduced MSU-induced inflammation in HFLS, western blot analysis was used to determine the MyD88, NLRP3, NF-κB, and caspase-1 protein levels. The results (**Figure 5**) demonstrated that MSU-induced HFLS significantly ($p < 0.05$) increased protein expression levels for all protein targets when compared to untreated cells. The treatment of EtOH80 extract, AP, DAP, and NAP significantly ($p < 0.05$) reduced the expression of all target proteins following MSU induction in a concentration dependent manner. However, treatment with water extract had no significant effect on the expression of MyD88, NF-κB, or caspase-1 in MSU-induced HFLS. In fact, it reduced NLRP3 expression considerably only at concentrations of 5 and 2.5 μg/ml.

Effect of *Andrographis paniculata* Leaf Ethanol (80%) Extract and Andrographolide on Swelling Rate, Cytokine Secretion and PGE_2 Secretion of MSU-Induced Inflammation in Rats

After 72 h, EtOH80 extract (200 mg/kg) and AP (30 mg/kg) reduced swelling to normal levels, equivalent to the indomethacin (3 mg/kg) group (**Figure 6**). In addition, treatment with EtOH80 extract (200 mg/kg) and AP (30 mg/kg) significantly ($p < 0.05$) decreased levels of IL-1α, IL-1β, IL-6, TNF-α, and PGE_2 in a dose-dependent manner, equivalent to indomethacin and normal control groups ($p > 0.05$) (**Figure 7**).

Body and Organ Weight Observation

During the 14-days experiment, changes in body weight (**Supplementary Figure S1**) and organ weight (**Supplementary Tables S1, S2**) were noted. The findings revealed that there were no significant changes in body and organ weight for any of the groups when compared to their baseline values ($p > 0.05$). Physical observations revealed no changes in the rats' skin, hair, eyes, mucous membranes, behavioral pattern, tremor, salivation, or diarrhea. There was no death or substantial weight loss in the rats at the tested dosages ($p > 0.05$).

DISCUSSION

In Indonesia and Malaysia, *A. paniculata* is a widely used traditional medicine. Despite its unpleasant bitter taste, the plant is often consumed as a herbal drink and fresh salad among other vegetables. *A. paniculata* has been extensively used empirically to treat a variety of illnesses, including inflammation, allergic reactions, and as an immunomodulator. Numerous publications have reported on the anti-inflammatory effects of *A. paniculata* and its bioactive components. Although *A. paniculata* has many anti-inflammatory chemicals, andrographolide is the most prevalent (Dong et al., 2009; Parichatikanond et al., 2010).

Gout develops because of the crystallization of uric acid in tissues, tendons, and joints, triggering inflammatory reactions (Chen et al., 2011). Uric acid is a byproduct of human purine metabolism that is, insoluble in blood. When uric acid levels exceed 7 mg/dl, the physiologic fluid becomes saturated, resulting in crystal formation in joints, tendons, and tissues. This syndrome hastens the onset of gout, which is characterized by repeated attacks of acute inflammation (Nuki 2006).

MSU crystals can initiate, intensify, and maintain an inflammatory attack by promoting the production and release of humoral and cellular inflammatory mediators (Choi et al., 2005). Toll-like receptor 2/4 (TLR 2/4) and CD16, which are components of the innate immune system, are the first to recognize MSU crystals. By binding with membrane antigen-presenting cells (APCs), it stimulates the spleen tyrosine kinase

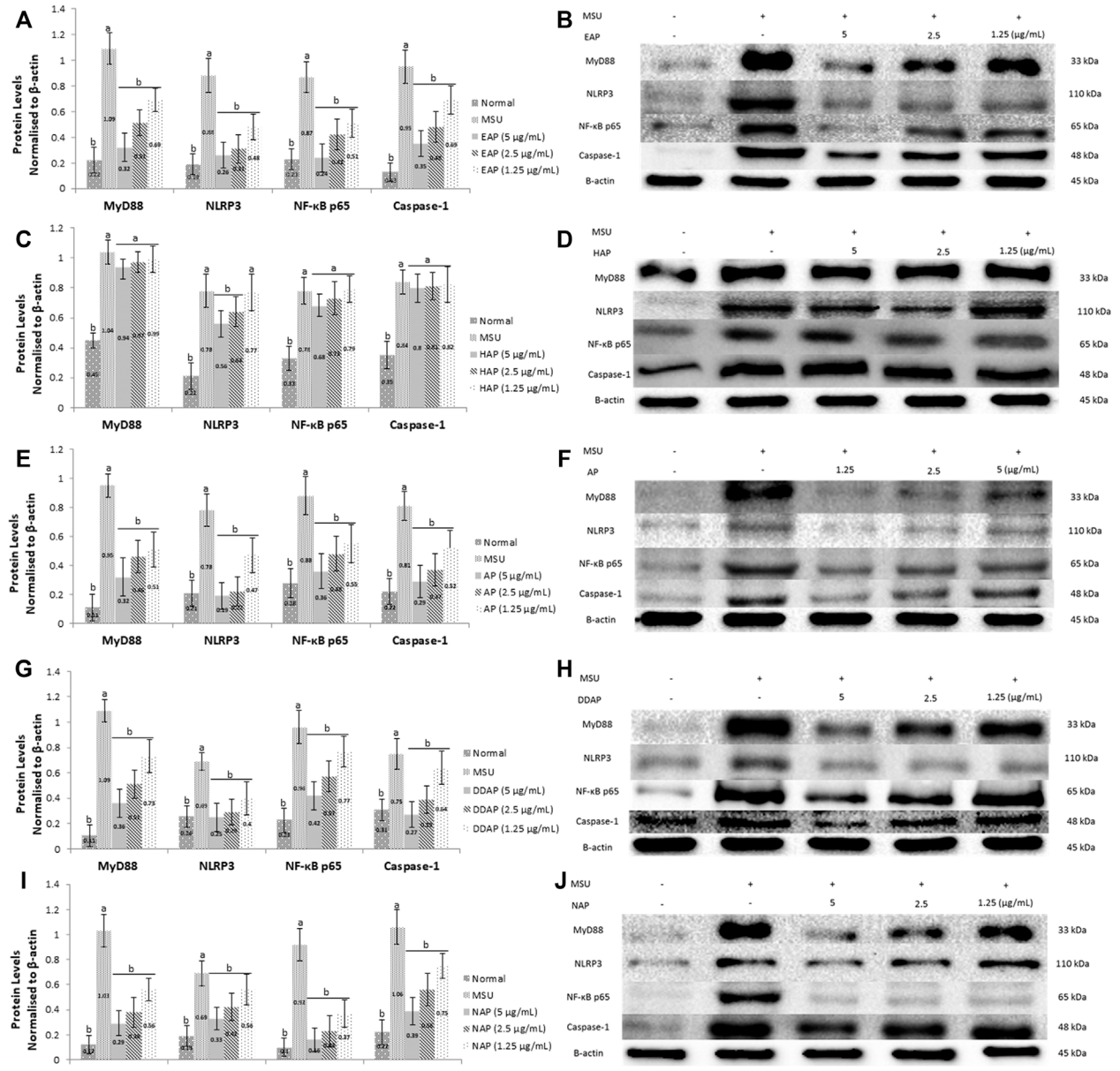


FIGURE 5 | Effect of ethanol (80%) extract **(A,B)**, water extract **(C,D)** of *A. paniculata* leaves, andrographolide **(E,F)**, 14-deoxy-11,12-didehydroandrographolide **(G,H)** and neoandrographolide **(I,J)** on protein expressions of MyD88, NLRP3, NF-κB, and caspase-1 in MSU-induced HFLS. Data are presented as mean ± SEM (n = 3). Data were analyzed using one-way ANOVA followed by post-hoc Tukey. [a]Significantly different compared to normal group ($p < 0.05$). [b]Significantly different compared to MSU-induced group ($p < 0.05$).

(Syk) pathway, causing lipid sorting and the aggregation of immunoreceptor tyrosine-based activation motifs (ITAMs). The downstream of phosphoinositide 3-kinases (PI3Ks), which are crucial in phagocytosis, is then activated by Syk. Phagocytosis causes lysosomal damage and activates the inflammasome via two distinct signaling pathways: 1) a signal from Syk activates pro-IL-1β production *via* CARD9, while ROS production and K+ efflux activates the NLRP3 inflammasome and leads to IL-1β activation; and 2) a signal from TLR activates NF-κB, which activates pro-IL-1β production *via* the MyD88/TRIF pathway. MSU phagocytosis results in cathepsin B leaking into the cytoplasm, which activates the inflammasome. Finally, non-hematopoetic cells or IL-1R produce mature IL-1β as an inflammatory mediator (Shi et al., 2012).

When MSU crystals interact with cells, particularly monocytes, they upregulate IL-6 and TNF-α secretion (Busso and So 2010). In addition, chemokines including IL-8 (CXCL8) and cyclooxygenase-2 (COX-2) have a role in gouty inflammation (Choi et al., 2005). The action of the cyclooxygenase-2 (COX-2) enzyme and PGE_2 synthase in endothelial cells biosynthesizes

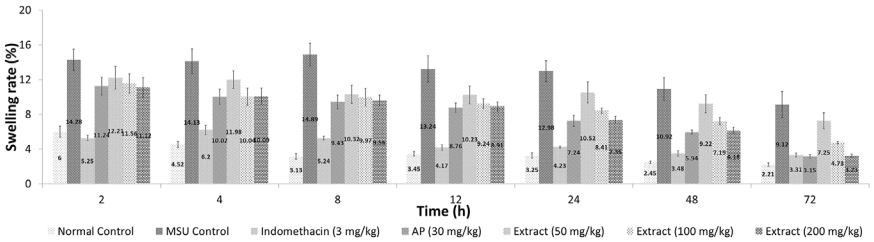


FIGURE 6 | Effect of ethanol (80%) extract of *Andrographis paniculata* leaves, andrographolide, and indomethacin on swelling rate in MSU-induced inflammation in rat's knee joint.

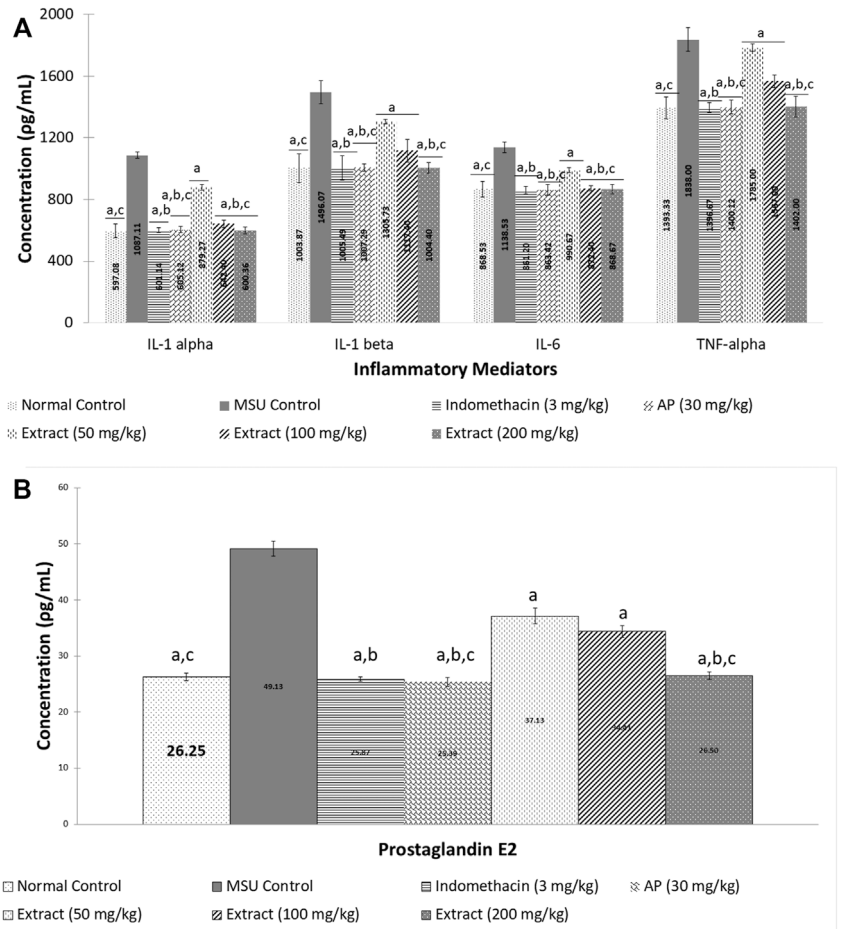


FIGURE 7 | Effect of ethanol (80%) extract of *Andrographis paniculata* leaves and andrographolide on MSU-induced inflammatory mediator secretion in rat's knee joint synovial fluid: **(A)** cytokines and **(B)** prostaglandin E_2. Data are presented as mean ± SEM ($n = 6$). [a]Significantly different compared to MSU control group ($p < 0.05$). [b]Not significantly different compared to normal control group ($p > 0.05$). [c]Not significantly different compared to indomethacin group ($p > 0.05$).

prostaglandin E_2 (PGE_2) from arachidonic acid precursor. PGE_2 plays a crucial role as a pro-inflammatory mediator in inflammation. As a result, a PGE_2 production inhibitor might be useful as a treatment for inflammation (Ward, 2010). One of the modes of action of inflammation in gout is the production of eiconasoids, particularly PGE_2, *via* MSU crystals formation (Joosten et al., 2010). Various inflammatory mediators, such as cytokine and prostanoids, are produced when MSU crystals are stimulated in joint tissues. The inflammatory characteristics of intense pain, oedema, and erythema in the joints are all caused by these mediators. Prostanoids, particularly PGE_2, are essential for the development of pain, vasodilation, oedema, and leukocyte

migration. According to Pauliot et al. (1998), MSU crystals increased COX-2 production in monocytes, which was linked to PGE_2 and tromboxaneA_2 (TXA_2) secretion. As a result, inhibiting PGE_2 production is crucial in the therapy of gouty inflammation.

The kidneys excrete uric acid with the aid of uric acid transporters. Recent studies have demonstrated that hyperuricemia is related with alterations in the expression and function of urate transporters. Uric acid transporters are classified into two types: those that promote urate reabsorption, such as urate anion transporter 1 (URAT1), organic anion transporter 4 (OAT4), and glucose transporter 9 (GLUT9), and those that promote urate excretion, such as OAT1, OAT3, urate transporter (UAT), multidrug resistance protein 4 (MRP4/ABCC4), ABCG-2, and sodium-dependent phosphate transport protein.

The effects of *A. paniculata* extracts and its bioactive compounds, such as andrographolide, 14-deoxy-11,12-didehydroandrographolide, and neoandrographolide, on anti-gout activity in terms of lowering uric acid and reducing crystal-induced inflammation were investigated in this study. The anti-inflammatory effect of this study was focused on the MyD88 pathway, which regulates the synthesis of NLRP3, NF-κB, caspase-1, and IL-1β. Meanwhile, the anti-hyperuricemic effect was attributed to its ability to modulate uric acid transporters, including URAT1, GLUT9, and OAT1.

Our findings indicated that 80% ethanol extract of *A. paniculata* and one of its primary constituents, andrographolide, inhibited expression of MyD88, NLRP3, NF-κB, and caspase-1 proteins in MSU-induced HFLS. Additionally, the extract and andrographolide reduced MSU-induced cytokine production of IL-1α, IL-1β, IL-6, and TNF-α in HFLS and hyperuricemic rats, as well as PGE_2 in rats. Furthermore, the extract and andrographolide decreased the protein expression levels of the renal urate transporters URAT1 and GLUT9 in hyperuricemic rats induced by potassium oxonate, but the reduction was not comparable to that of allopurinol. Meanwhile, renal OAT1 protein levels were considerably reduced in potassium oxonate-induced hyperuricemic rats compared to the normal control rats, which were significantly recovered by the extract, andrographolide and allopurinol.

CONCLUSION

Overall, the results indicated that an ethanol (80%) extract of *A. paniculata* leaves and its main diterpene lactone compound, andrographolide, may have antihyperuricemic and anti-inflammatory properties and therefore be used to treat gout. *In vivo*, the antihyperuricemic action was mediated by xanthine oxidase inhibition or maybe in synergy with other mechanisms such as uricosuric effect. Meanwhile, anti-inflammatory action was demonstrated by inhibitory activity of monosodium urate crystals-induced cytokines and prostaglandin E_2 secretion. *In vitro* assays with HFLS may indicate that EtOH extract of *A. paniculata* leaves and andrographolide inhibited gouty inflammation by inhibiting the production of cytokines and protein expression of MyD88, NLRP3, NF-κB, and caspase-1. This research establishes a foundation for the development of a novel anti-inflammatory phytotherapy. It is believed that well characterized standardised *A. paniculata* herbal product gives an excellent opportunity to further investigate its potential treatment of gouty-inflammatory conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Universiti Kebangsaan Malaysia-Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

ER was a M.Sc. candidate who conducted the experimental works and drafted the manuscript. JaJ is the project leader responsible for the research design and editing of the manuscript. EK is a project team member who contributed ideas for the research design and reviewed the manuscript. JuJ is project team member who contributed ideas for the research design. FB is veterinarian who helped with the *in vivo* assay.

FUNDING

The study was funded by the Ministry of Agriculture and Agro-Based Industry, Malaysia for the NKEA research grant scheme (NRGS/NH1014D028).

ACKNOWLEDGMENTS

We would like to express our gratitude to Prof. Dr. Johnson Stanslas from Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, for valuable assistance on water extraction of the plant.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.787125/full#supplementary-material

REFERENCES

Ansari, M. A., Nadeem, A., Bakheet, S. A., Attia, S. M., Shahid, M., Alyousef, F. S., et al. (2021). Chemokine Receptor 5 Antagonism Causes Reduction in Joint Inflammation in a Collagen-Induced Arthritis Mouse Model. *Molecules* 26 (7), 1839. doi:10.3390/molecules26071839

Burkill, I. H. (1966). A Dictionary Of the Economic Product Of the Malay Peninsula. *Vol I (A-H). Goverment of Malaysia and Singapore by the Ministry of Agriculture and Co-operatives*. Malaysia: Kuala Lumpur.

Busso, N., and So, A. (2010). Mechanisms of Inflammation in Gout. *Arthritis Res. Ther.* 12, 206–213. doi:10.1186/ar2952

Chen, L., Yin, H., Lan, Z., Ma, S., Zhang, C., Yang, Z., et al. (2011). Anti-hyperuricemic and Nephroprotective Effects of Smilax china L. *J. Ethnopharmacol.* 135, 399–405. doi:10.1016/j.jep.2011.03.033

Choi, H. K., Mount, D. B., and Reginato, A. M. (2005). Pathogenesis of Gout. *Ann. Intern. Med.* 143, 499–516. doi:10.7326/0003-4819-143-7-200510040-00009

Cronstein, B. N., and Terkeltaub, R. (2006). The Inflammatory Process of Gout and its Treatment. *Arthritis Res. Ther.* 8 (Suppl. 1), S3. doi:10.1186/ar1908

Deshpande, P. K., Pathak, A. K., and Gothalwal, R. (2014). Pharmacological Properties of. *Andrographis paniculataIJRAMR* 01 (04), 105–110.

Dong, H. J., Zhang, Z. J., Yu, J., Liu, Y., and Xu, F. G. (2009). Chemical Fingerprinting of *Andrographis Paniculata* (Burm. F.) Nees by HPLC and Hierarchical Clustering Analysis. *J. Chromatogr. Sci.* 47 (10), 931–935. doi:10.1093/chromsci/47.10.931

Howard-Jones, N. (1985). A CIOMS Ethical Code for Animal Experimentation. *WHO Chron.* 39, 51–56.

Jarukamjorn, K., and Nemoto, N. (2008). Pharmacological Aspects of *Andrographis Paniculata* on Health and its Major Diterpenoid Constituent Andrographolide. *J. Health Sci.* 54 (4), 370–381. doi:10.1248/jhs.54.370

Joosten, L. A., Netea, M. G., Mylona, E., Koenders, M. I., Malireddi, R. K., Oosting, M., et al. (2010). Engagement of Fatty Acids with Toll-like Receptor 2 Drives Interleukin-1β Production via the ASC/caspase 1 Pathway in Monosodium Urate Monohydrate crystal-induced Gouty Arthritis. *Arthritis Rheum.* 62 (11), 3237–3248. doi:10.1002/art.27667

Liu, J., Wang, Z. T., and Ge, B. X. (2008). Andrograpanin, Isolated from *Andrographis Paniculata*, Exhibits Anti-inflammatory Property in Lipopolysaccharide-Induced Macrophage Cells through Down-Regulating the P38 MAPKs Signaling Pathways. *Int. Immunopharmacol* 8, 951–958. doi:10.1016/j.intimp.2007.12.014

Malawista, S. E., de Boisfleury, A., and Naccache, P. H. (2011). Inflammatory Gout: Observations over a Half-century. *FASEB J.* 25, 4073–4078. doi:10.1096/fj.11-1201ufm

Melchior, J., Palm, S., and Wikman, G. (1997). Controlled Clinical Study of Standardized Andrographis Paniculata Extract in Common Cold - a Pilot Trial. *Phytomedicine* 3, 315–318. doi:10.1016/S0944-7113(97)80002-5

Nuki, G. (2006). Gout. *Medicine* 34 (10), 417–423. doi:10.1053/j.mpmed.2006.07.012

Parichatikanond, W., Suthisisang, C., Dhepakson, P., and Herunsalee, A. (2010). Study of Anti-inflammatory Activities of the Pure Compounds from *Andrographis Paniculata* (Burm.f.) Nees and Their Effects on Gene Expression. *Int. Immunopharmacol.* 10 (11), 1361–1373. doi:10.1016/j.intimp.2010.08.002

Pouliot, M., James, M. J., McColl, S. R., Naccache, P. H., and Cleland, L. G. (1998). Monosodium Urate Microcrystals Induce Cyclooxygenase-2 in Human Monocytes. *Blood* 91 (5), 1769–1776. doi:10.1182/blood.v91.5.1769

Puri, A., Saxena, R., Saxena, R. P., Saxena, K. C., Srivastava, V., and Tandon, J. S. (1993). Immunostimulant Agents from *Andrographis Paniculata*. *J. Nat. Prod.* 56, 995–999. doi:10.1021/np50097a002

Putra, I. P. E., and Marianto, L. A. (1998). *Khasiat & Manfaat Sambiloto: Raja Pahit Penakluk Aneka Penyakit*. Jakarta: Agromedia, 13.

Rahmi, E. P., Kumolosasi, E., Jalil, J., Husain, K., Buang, F., Abd Razak, A. F., et al. (2020). Anti-hyperuricemic and Anti-inflammatory Effects of *Marantodes Pumilum* as Potential Treatment for Gout. *Front. Pharmacol.* 11, 289. doi:10.3389/fphar.2020.00289

Ren, X., Du, G., Zhou, B., Zong, K., and Ma, B. (2008). Study on Spectroscopy of Two Flavonyl Glycosides from *Andrographis Paniculata*. *Front. Chem. China* 3 (2), 178–181. doi:10.1007/s11458-008-0034-z

Salim, E., Kumolosasi, E., and Jantan, I. (2014). Inhibitory Effect of Selected Medicinal Plants on the Release of Pro-inflammatory Cytokines in Lipopolysaccharide-Stimulated Human Peripheral Blood Mononuclear Cells. *J. Nat. Med.* 68, 647–653. doi:10.1007/s11418-014-0841-0

Shi, Y. W., Wang, C. P., Wang, X., Zhang, Y. L., Liu, L., Wang, R. W., et al. (2012). Uricosuric and Nephroprotective Properties of Ramulus Mori Ethanol Extract in Hyperuricemic Mice. *J. Ethnopharmacol.* 143, 896–904. doi:10.1016/j.jep.2012.08.023

The Plant List (2021). *Theplantlist.Org. The Plant List*. Version 1.1.

Thisoda, P., Rangkadilok, N., Pholphana, N., Worasuttayangkurn, L., Ruchirawat, S., and Satayavivad, J. (2006). Inhibitory Effect of *Andrographis Paniculata* Extract and its Active Diterpenoids on Platelet Aggregation. *Eur. J. Pharmacol.* 553, 39–45. doi:10.1016/j.ejphar.2006.09.052

Wang, Q., Kuang, H., Su, Y., Sun, Y., Feng, J., Guo, R., et al. (2013). Naturally Derived Anti-inflammatory Compounds from Chinese Medicinal Plants. *J. Ethnopharmacol* 146, 9–39. doi:10.1016/j.jep.2012.12.013

Ward, P. A. (2010). in *"Acute and Chronic Inflammation," in Fundamentals of Inflammation*. Editors C. N. Serhan, P. A. Ward, and D. W. Gilroy (New York: Cambridge University Press), 1–16.

Wolf, J. S., Chen, Z., Dong, G., Sunwoo, J. B., Bancroft, C. C., Capo, D. E., et al. (2001). IL (Interleukin)-1alpha Promotes Nuclear Factor-kappaB and AP-1-Induced IL-8 Expression, Cell Survival, and Proliferation in Head and Neck Squamous Cell Carcinomas. *Clin. Cancer Res.* 7 (6), 1812–1820.

Xu, T., Pan, J., and Zhao, L. (2008). Simultaneous Determination of Four Andrographolides in Andrographis Paniculata Nees by Silver Ion Reversed-phase High-Performance Liquid Chromatography. *J. Chromatogr. Sci.* 46 (8), 747–750. doi:10.1093/chromsci/46.8.747

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 10 January 2022
doi: 10.3389/fphar.2021.790567

Ethnobotanical Documentation of Medicinal Plants Used by the Indigenous *Panay Bukidnon* in Lambunao, Iloilo, Philippines

Cecilia Salugta Cordero[1,2]*, *Ulrich Meve*[3] and *Grecebio Jonathan Duran Alejandro*[1,4]

[1]The Graduate School, University of Santo Tomas, Manila, Philippines, [2]Biology Department, School of Health Science Professions, St. Dominic College of Asia, City of Bacoor, Philippines, [3]Department of Plant Systematics, University of Bayreuth, Bayreuth, Germany, [4]College of Science and Research Center for the Natural and Applied Sciences, University of Santo Tomas, Manila, Philippines

OPEN ACCESS

Edited by:
Alessandra Durazzo,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Bahar Gürdal,
Istanbul University, Turkey
Emin Ugurlu,
Bursa Technical University, Turkey
Wajid Rashid,
Beijing Normal University, China
Esezah Kakudidi,
Makerere University, Uganda
Mohammad Sadat-Hosseini,
University of Jiroft, Iran

***Correspondence:**
Cecilia Salugta Cordero
cecilia.cordero@sdca.edu.ph

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 06 October 2021
Accepted: 18 November 2021
Published: 10 January 2022

Citation:
Cordero CS, Meve U and Alejandro GJD (2022) Ethnobotanical Documentation of Medicinal Plants Used by the Indigenous Panay Bukidnon in Lambunao, Iloilo, Philippines. Front. Pharmacol. 12:790567. doi: 10.3389/fphar.2021.790567

The *Panay Bukidnon* is a group of indigenous peoples living in the interior highlands of Panay Island in Western Visayas, Philippines. Little is known about their ethnobotanical knowledge due to limited written records, and no recent research has been conducted on the medicinal plants they used in ethnomedicine. This study aims to document the medicinal plants used by the indigenous *Panay Bukidnon* in Lambunao, Iloilo, Panay Island. Semi-structured interviews were conducted with 75 key informants from June 2020 to September 2021 to determine the therapeutic use of medicinal plants in traditional medicine. A total of 131 medicinal plant species distributed in 121 genera and 57 families were used to address 91 diseases in 16 different uses or disease categories. The family Fabaceae was best represented with 13 species, followed by Lamiaceae with nine species and Poaceae with eight species. The leaf was the most frequently used plant part and decoction was the most preferred form of preparation. To evaluate the plant importance, use value (UV), relative frequency citation (RFC), relative important index (RI), informant consensus factor (ICF), and fidelity level (FL) were used. *Curcuma longa* L. had the highest UV (0.79), *Artemisia vulgaris* L. had the highest RFC value (0.57), and *Annona muricata* L. had the highest RI value (0.88). Diseases and symptoms or signs involving the respiratory system and injury, poisoning, and certain other consequences of external causes recorded the highest ICF value (0.80). *Blumea balsamifera* (L.) DC. and *Chromolaena odorata* (L.) R.M. King & H. Rob were the most relevant and agreed species for the former and latter disease categories, respectively. *C. odorata* had the highest FL value (100%) and was the most preferred medicinal plant used for cuts and wounds. The results of this study serve as a medium for preserving cultural heritage, ethnopharmacological bases for further drug research and discovery, and preserving biological diversity.

Keywords: ethnobotany, ethnomedicine, Panay Bukidnon, Panay Island, Philippines

INTRODUCTION

About 370–500 million indigenous peoples live in 90 countries worldwide, making up 5% of the global population. They represent 5,000 distinct and diverse cultures, but they also account for 15% of the extremely poor and deprived communities from social services and economic resources (United Nations Development Programme, 2019).

In the Philippines, there are 110 ethnolinguistic groups with more than 14 million people spread across the archipelago, with the highest population in Mindanao (63%), followed by Luzon (32%) and Visayas (3%) (National Commission on Indigenous

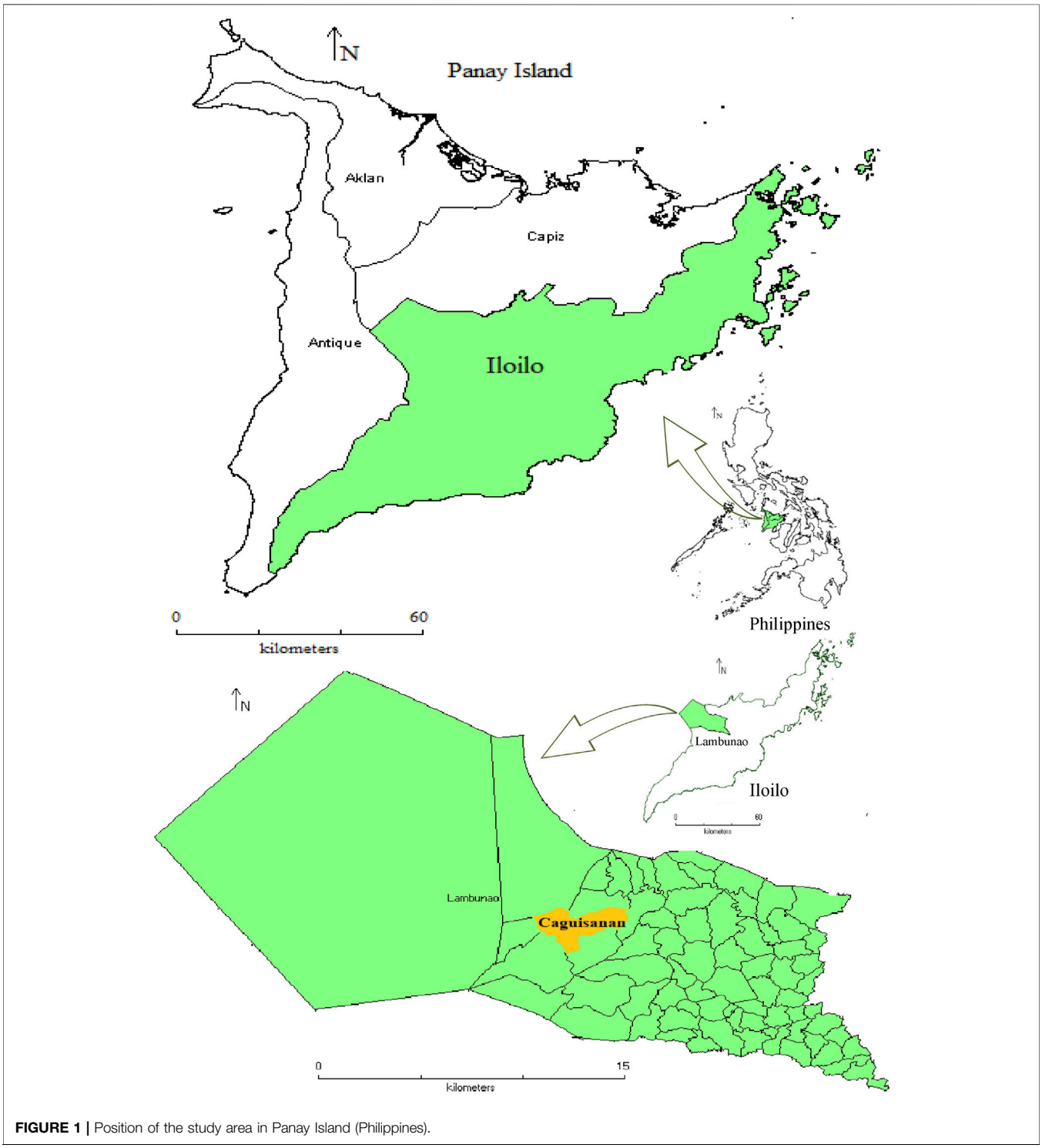


FIGURE 1 | Position of the study area in Panay Island (Philippines).

Peoples, 2011), who occupy around 13 million hectares (ha) (45%) of the national land territory (National Economic Development Agency, 2017). The *Bukidnon* is the major indigenous group in the Central and Western Visayas in terms of population size, followed by the *Ati/Ata* (Negritoes). In Panay Island in Western Visayas, the *Bukidnon* population is about 112,000. The province of Iloilo is one of the four provinces in Panay Island, together with Aklan, Antique, and Capiz. It has the highest *Bukidnon* population with 62,245 individuals (National Commission on Indigenous Peoples, 2019). *Bukidnon,* which literally translates to the "mountain people," were once coastal dwellers, but due to the piratical raids from Mindanao and political suppressions during the reign of the Spanish government, they moved to the hinterlands of the island (Magos, 2004). This was depicted in their epic tradition of chanting called *Sugidanon* (Magos, 1999). To distinguish the *Bukidnon* in Panay from the other *Bukidnon* groups in Mindanao, Negros, and other neighboring islands, the "*Panay*" is added (Gowey, 2016). Other authors used *Mundo* (Beyer, 1917), *Monteses* "mountaineer" (Ealdama, 1938), *Sulod* or *Sulodnon* "enclosed by the mountains" (Jocano, 1968; German, 2010), *Tumandok* "native of the place" (Talledo, 2004), and *Bukidnon* (Smith, 1915; Magos, 1999) to designate the *Panay Bukidnon* people.

The *Panay Bukidnon* primarily utilized the forest resources, rivers, and streams for their food and livelihood. They also engaged in slash and burn farming and building boats to transport their goods to the lowlands. In the 1970s, when logging activities were prohibited by the Philippine government, they shifted to other means of living, including farming various crops (Magos, 1999). Their social organization is relatively similar to the lowlanders. Their community membership pattern is composed of the *baylan/babaylan, mirku* (herb doctor), *parangkutun* (advisor), and the *husay* (arbiter). The *baylan* is considered the most important status in society and regarded with high respect. The *baylan* is the one who communicates with the spiritual world, interprets dreams, and handles religious performances. He or she may also administer herb medicine to the sick and practice folk medicine and physical therapy. Their language is *Kiniray-a*, a dialect that is similar to Ilonggo/Hiligaynon. Today the *Panay Bukidnon* settled in the interior "barangays" (villages) of at least 24 municipalities of the four provinces of Panay (Provincial Planning Development Office, 2018; National Commission on Indigenous Peoples, 2020) and most of their communities are located in the mountainous areas of the Central Panay Mountain Range.

Iloilo province is situated in the southeastern part of Panay Island in Western Visayas. It is geographically located at the center of the archipelago, and it is known as the "Heart of the Philippines." Its excellent port facility and strategic location made the province the center of trade during the 1890s when the sugar industry was booming and it was once given the title of "Queen City of the South." It is also known for the "*Dinagyang Festival,*" one of the most spectacular religious and cultural celebrations in the country in honor of Senior Sto. Nino (Child Jesus) (Province of Iloilo, 2015). The province has a total land area of 491,940 ha, 24% of which is classified as forestland, while 76% is classified as alienable and disposable land (Department of Environment and Natural Resources, 2019).

Several ethnobotanical surveys in Panay Island have been conducted on the *Ati* (Negritoes) indigenous groups (Madulid et al., 1989; Ong and Kim, 2015; Cordero et al., 2020; Cordero and Alejandro, 2021), but there is no study focused exhaustively on the medicinal plants used by the *Panay Bukidnon* in ethnomedicine. Nevertheless, several plants were listed with medicinal purposes in the anthropological case studies documented in the interior barangays of Tapaz, Capiz in Central Panay in 1945–1959 (Jocano, 1968). Given the absence of recent research about the ethnobotanical knowledge of *Panay Bukidnon*, it is therefore urgent to document this indigenous knowledge before it is forgotten. The documentation of traditional knowledge will serve as a medium for preserving cultural heritage, ethnopharmacological bases of drug research and discovery, and preserving biological diversity. Thus, this study is the first attempt to extensively survey the ethnobotanical knowledge in one of the indigenous *Panay Bukidnon* communities in the province of Iloilo in Panay Island, Western Visayas, Philippines.

MATERIALS AND METHODS

Study Area and Permits

The town of Lambunao is a first-class municipality in the third district of Iloilo province, with a population of 81,236 individuals as of May 2020 (Philippine Statistics Authority, 2021). It is the largest municipality in the province in terms of land area (40, 709 ha), about 26.12% of which are forestlands, and the rest are alienable and disposable land. It is bounded by the municipalities of Calinog in the North, Duenas and Pototan in the East, Janiuay and Badiangan in the South, and Janiuay and Valderrama, Antique, in the west (**Figure 1**). It is a mountainous municipality and has the highest elevation (194 m a.s.l.) in the province. The climate of the area has two pronounced seasons: dry from the months of November to April and wet for the rest of the year. Seven out of its 73 barangays are inhabited by the *Panay Bukidnon* people. Brgy. Caguisanan, which lies between 11°05′37.6″N and 11°04′42.3″N latitude and 122°24′26.6″E 122°26′51.6″E longitude, has a land area of about 5.20 km^2. According to the recent survey, it is one of the seven indigenous *Panay Bukidnon* barangays with a population of 1,842 in 394 households. The main source of livelihood in the barangay is farming of various crops such as rice, banana, corn, and other vegetables. Some of the younger generations are professionals working in various private and government sectors and some are working as overseas Filipino workers abroad. The landscape of the study site is dominated by hills and mountains with scattered rice terraces, grasslands, and patch forests.

Certification Precondition was acquired from the National Commission on Indigenous Peoples (NCIP)-Region VI/VII and the researchers have satisfactorily complied with the requirements for securing the Indigenous Knowledge and Systems Practices (IKSPs) and Customary Laws (CLs). It was issued in compliance with Section 59 of the Republic Act No.

TABLE 1 | Demographic profile of the *Panay Bukidnon* informants in Lambunao, Iloilo, Philippines.

Social group	Variables	No. of informants (75)	Percentage (%)
Sex	Male	31	41
	Female	44	59
Age	24–39	9	12
	40–55	29	39
	56–70	25	33
	71–89	12	16
Civil Status	Married	71	95
	Single	2	3
	Widowed	2	3
Education	No formal education	5	7
	Elementary	37	49
	Secondary	16	21
	Tertiary	17	23
Occupation	Farmer	19	25
	Housewife	29	39
	Self-employed	11	15
	Employed	5	7
	Barangay officials	7	9
	Albularyo (herb doctor)	2	3
	Paltera (midwife)	1	1
	IP leader	1	1

8371 "The Indigenous Peoples Rights Act (IPRA) of 1997." Several meetings were conducted: Pre-FPIC (Free and Prior Informed Consent) Conference; Disclosure Conference with the Indigenous Peoples (IP) Community and Presentation of Application; Community Decision Meeting; Memorandum of Agreement Preparation and Signing; and Output Validation Meeting. A Wildlife Gratuitous Permit was issued by the Department of Natural Resources Region (DENR) VI before conducting the study.

Data Collection

Fieldworks and interviews were conducted from June 2020 to September 2021. Interviews were carried out using semi-structured questionnaires, ethically reviewed, and approved (**Supplementary Material S1**). A purposive sampling technique was used, and the principal key informants were determined during the community decision meeting in the presence of the NCIP officers, barangay officials, IP leader, and council of elders. The informants were composed of the tribal leader, council of elders, herb doctors (*mirku/surhano/albularyo*), midwife (*paltera*), and other members of the community who have indigenous knowledge of using medicinal plants in treating and addressing health problems and conditions. A total of 75 informants, 31 males and 44 females, aged between 24 and 89 years old, were interviewed at their own convenience in their community during the study. Questions regarding personal information and the medicinal plants they used when they experienced any health-related problems were asked during the surveys. The information about the demographic profile of the participants, such as age, gender, civil status, educational attainment, and occupation, is shown in **Table 1**. The plant part used, mode of preparation, and administration were also recorded during the interviews. A focus group discussion was conducted with the 10 members of the council of elders to verify the acquired data among the informants during the output validation meeting. The meeting was facilitated by the NCIP officers, IP leader, and Brgy. Captain.

Plant Collection and Identification

Collecting medicinal plant samples was carried out with the help of the informants, if available in their immediate surroundings or their home gardens right after the interview. Some field collections were assisted by the informants who have the knowledge of the location of some plants that were not available in the home gardens. Plants were also photographed for documentation purposes. Voucher specimens were prepared using three to five branches with preferably reproductive parts (flowers and fruits), inserted in newspapers, and positioned in a way that best represents the plant in the wild. The plants were poisoned with a generous amount of denatured alcohol in polyethylene bags. Poisoned specimens were then transferred to a new newspaper and placed in a presser. Pressed and dried plant specimens were then mounted on herbarium sheets with proper documentation labels. Voucher specimens were accessioned and deposited in the Herbarium of the Northwestern University Luzon (HNUL). Identification of the collected medicinal plants was made using different online databases such as Co's Digital Flora of the Philippines, (https://www.philippineplants.org/), Phytoimages (http://www.phytoimages.siu.edu), Stuartxchange (http://www.stuartxchange.org/), and Plants of the World Online (http://plantsoftheworldonline.org/), then verified by Mr. Danilo Tandang, a botanist at the Philippine National Museum Herbarium and Mr. Michael Calaramo of the Herbarium of Northwestern University Luzon (HNUL). For the validation of the family and scientific names, Tropicos (Tropicos, 2021), World Flora Online (World Flora Online, 2021), and International Plant Names Index (International Plant Names Index, 2021) were used. To identify the geographical distribution and endemicity of the medicinal plants, Co's Digital Flora of the Philippines (Pelser et al., 2011) and Plants of the World Online (Plants of the World Online, 2021) were used.

Data Analyses

There were five values used to quantify the plant importance: use value (UV), relative frequency of citation (RFC), relative importance index (RI), informant consensus factor (ICF), and fidelity level (FL). The UV was calculated to determine the relative importance of the medicinal plant species using the following formula: $UVs = Ui/N$, where U_i is the number of use reports cited or mentioned by each informant for a particular species and N is the total number of informants (Phillips and Gentry, 1993). A use report was considered every time an informant cited or mentioned a plant being used for any medical condition or purpose. RFC was used to determine the culturally important medicinal plants using the following formula: $RFCs = FCs/N$, where FC_s is the number of informants who cited or mentioned a plant species (frequency citation) and N is the total number of informants who

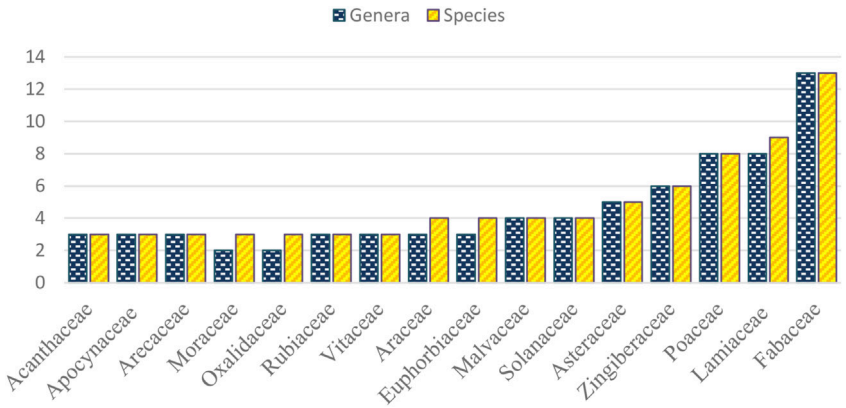


FIGURE 2 | Family of medicinal plants with a high number of genera and species.

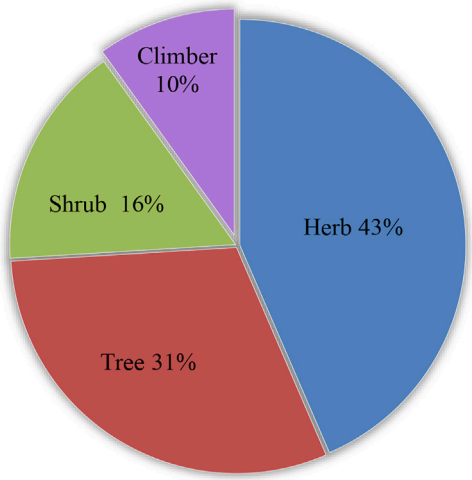


FIGURE 3 | Medicinal plant growth habit.

participated in the study. The values range from 0 to 1, 1 being the highest and indicating that all informants cited or mentioned a particular plant species (Tardío and Pardo-De-Santayana, 2008). RI was used to assess the relative importance of medicinal plants by use category using the following formula: $RIs = [RFCs(max) + RNUs(max)]/2$, where $RFC_{s(max)}$ ($RFC_{s(max)} = FC_s/FC_{max}$) is the relative frequency of citation of the species and is obtained by dividing the frequency citation of informant/s for a particular species (FC_s) by the number of informants citation of the species that has the maximum or highest frequency citation (FC_{max}). $RNU_{s(max)}$, ($RN_{s(max)} = NU_s/NU_{max}$) is the relative number of the use categories and is obtained by dividing the number of use categories of a particular species (NU_S) by the number of use categories of the species with the highest use categories (NU_{max}). Values closest to 1 indicate that the medicinal plants are most frequently cited as useful in different use categories (Tardío and Pardo-De-Santayana, 2008). ICF was used to evaluate the consensus or homogeneity of the ethnobotanical information from the participating informants using the following formula: $ICF = (Nur - Nt)/(Nur - 1)$, where N_{ur} is the number of use reports for each disease category and N_t is the number of species used in that category (Heinrich et al., 1998). FL was used to assess the percentage of the most preferred medicinal plant for a particular disease category using the following formula: $FL = (Np/N)\,x100$, where N_p is the number of informants who cited or mentioned the use of a medicinal plant for a particular disease category and N is the total number of informants who cited that plant for any other use or purpose (Friedman et al., 1986). A high value indicates that a medicinal plant has the highest use report and the most preferred species within a particular disease category. There were 16 different use or disease categories adapted and modified from the ICD-11 International Classification of Diseases 11th Revision of the World Health Organization (World Health Organization, 2021), which is used in this ethnopharmacological documentation.

RESULTS

Medicinal Plants Characteristics

The present study documented a total of 131 medicinal plant species distributed in 121 genera and 57 families. The family Fabaceae was best represented with 13 species, followed by Lamiaceae with nine species and Poaceae with eight species (**Figure 2**). Fabaceae are used to treat 28 diseases in 13 different use or disease categories, Lamiaceae in 24 diseases in ten disease categories, and Poaceae in 21 diseases in 12 disease categories.

The medicinal plants recorded possess different growth forms such as herbs (43%), trees (31%), shrubs (16%), and climbers (10%) (**Figure 3**). The plants were collected within the vicinity of the barangay mostly cultivated in the informant's home gardens or backyards that serve as ornamentals and vegetables and used for medicinal purposes; some were cultivated as crops in the farmland; some were grown on the riverbanks and forest; others

TABLE 2 | Top ten medicinal plants with the highest UV, RFC, and RI values.

Rank	Medicinal plants	UV	Medicinal plants	RFC	Medicinal plants	RI
1	*Curcuma longa*	0.79	*Artemisia vulgaris*	0.57	*Annona muricata*	0.88
2	*Blumea balsamifera*	0.64	*Curcuma longa*	0.47	*Curcuma longa*	0.87
3	*Artemisia vulgaris*	0.59	*Blumea balsamifera*	0.44	*B lumea balsamifera*	0.80
4	*Annona muricata*	0.56	*Zingiber officinale*	0.43	*Zingiber officinale*	0.78
5	*Jatropha curcas*	0.55	*Psidium guajava*	0.40	*Artemisia vulgaris*	0.77
6	*Psidium guajava* L	0.55	*Pseuderanthemum carruthersii*	0.40	*Jatropha curcas*	0.72
7	*Justicia gendarussa*	0.52	*Musa balbisiana* cv	0.40	*Moringa oleifera*	0.72
8	*Pseuderanthemum carruthersii*	0.52	*Plectranthus scutellarioides*	0.40	*Plectranthus scutellarioides*	0.62
9	*Musa balbisiana* cv	0.52	*Annona muricata*	0.39	*Psidium guajava*	0.62
10	*Plectranthus scutellarioides/Zingiber offinale*	0.49	*Justicia gendarussa*	0.37	*Carica papaya/Justicia gendarussa*	0.60

do grow as weeds pervasively around the community. Of all the 131 medicinal plants listed, 91 species were collected as cultivated plants and 40 species were collected in the wild. Out of 127 plants identified up to the species level, 78 species are not native (introduced, naturalized, cultivated) in the Philippines and 49 species are native. Three species (*Areca catechu* L., *Musa textilis* Née, and *Mussaenda philippica* A. Rich.) of the native plants are considered endemic and their occurrence is widespread in the country. Information about the plant growth habit, collection sites, and geographical distribution and endemicity are found in = **Supplementary Table S1**.

The medicinal plant details are summarized in **Supplementary Table S2**. The scientific, local, and family names are also listed along with the plant part used, disease or purpose, quantity, mode of preparation, the form of administration, adverse or side effects, use value, relative frequency citation, and relative importance index.

Plant Part Used and Mode of Preparation and Administration

Sixteen different medicinal plant parts were used to address 94 diseases and health-related conditions documented in this study. The most frequently used plant parts for the preparation of the remedy were leaf (51%), followed by bark (8%), fruit (6%), and rhizome (6%) (**Figure 4**). Root, stem, whole plant, flower, seed, bulb, shoot, sap, and aerial root were also used but less frequently. The least utilized plant parts were tuber, petiole, and tendril. There were ten different ways to prepare the medicinal plants and the most common forms were decoction (35%), followed by crushing or pounding (23%) and direct application (20%) (**Figure 5**). Eat/chew/drink, heat/roast, soak in water, and grate/slice were also practiced. The least forms of preparation were cooking, processing into vinegar or oil, and burning for smoke or ash. The plant parts used and the mode of preparation of the medicinal plants depend on the ailments to be addressed and to whom they will be administered. Occasionally, some of the preparations include animal parts and products such as blood, egg, beeswax (*kabulay*), slaked lime (*apog*), minerals like salt, and chemicals like kerosene but in minute amounts. Sugar or breastmilk was also added to reduce or mask the bitterness of plant extracts to be taken orally by infants and children.

More than half of the medicinal plant preparations (52%) recorded were administered externally or topically by applying plant parts directly on the body, rubbing plant extracts, bathing, and burning for smoke and ash. The rest were taken orally (48%) by drinking decoction, eating, chewing, drinking extracts or liquids and used as a mouthwash.

Quantity and Dosage

The quantity of the medicinal plants used is influenced by the guided cultural and religious beliefs of the *Panay Bukidnon* and should be prepared or administered in odd numbers (3, 5, or 7). For example, in treating headaches, three different medicinal plants such as *Pseuderanthemum carruthersii* (Seem.) Guillaumin (3, 5, or 7 leaves depending on the leaf size), *Curcuma longa* L. (7 thinly sliced rhizomes), and *Zingiber officinale* Roscoe (7 thinly sliced rhizomes) were applied on the forehead. The frequency of the administration was dependent on the disease to be treated. For decoction, it was often administered by drinking a full glass to be taken twice or thrice a day or as a replacement for water intake. The detailed quantity and frequency of administration of medicinal plants are shown in **Supplementary Table S2**.

Use Value and Relative Frequency of Citation

The use value was used to evaluate the relative importance of the medicinal plants: high values indicate high use report, while relative frequency citation determined the usefulness of the plant by high FC or being mentioned by all the informants.

The top three medicinal plants with the highest use value were *Curcuma longa* L. (0.79), *Blumea balsamifera* (L.) DC. (0.64), and *Artemisia vulgaris* L. (0.59) (**Table 2**). *C. longa* is used to treat 13 diseases in nine disease categories and recorded a high use report in suppressing fever, headache, and *sinda*. It is usually prepared with two or four medicinal plants. The preparation and mode of administration for headache and fever were the same and with few modifications for *sinda*. *C. longa* is also used for muscle pain, stomachache, bloated stomach, tooth decay, typhus, typhoid fever, memory loss, cancer, cuts/wounds, and tetanus. It is cultivated in the informant's home gardens for medicinal purposes.

B. balsamifera was used to treat nine conditions or purposes under eight different disease categories and is widely known to cure cough, used in postpartum care and recovery, and relieved headache. It is also used for muscle pain, bloated

TABLE 3 | Use or disease category, reported disease or uses, ICF, and FL of the most cited species.

Use or disease- category	Reported diseases or uses under each category*	No. of used taxa	Use report	ICF	Most cited species for each category	Fidelity level (%)	Use or purpose of the most cited taxa
Infectious and parasitic diseases	Amoebiasis (4), athletes foot (1), boil (11), chicken pox (2), dengue (2), helminthiases (2), measles (3), mumps (6), oral thrush (1), shingles (3), ringworm (2), tetanus (2) Pityriasis versicolor (2), tuberculosis (3), typhus (2), typhoid fever (11), wart (2)	59	153	0.62	*Carica papaya*	60	Typhoid fever
Neoplasms	Cancer (12)	12	27	0.58	*Annona muricata*	31	Cancer
Endocrine, nutritional and metabolic diseases	Diabetes (5), goiter (1), high uric acid (1)	7	8	0.14	*Morinda citrifolia*	15	Diabetes
Diseases and symptoms or signs involving the nervous system	Headache (35), migraine (1), dizziness (8), cramps/spasm (4)	48	178	0.73	*Pseuderanthemum carruthersii*	93	Headache
Diseases of the ear or mastoid process, visual system, and symptoms or signs involving speech or voice	Hearing impairment (2), conjunctivitis (1), hoarseness (2)	5	9	0.50	*Moringa oleifera*	15	Conjunctivitis
Diseases of the circulatory system and blood or blood-forming organs	Anemia (6), hypertension (11)	17	28	0.41	*Annona muricata*	24	Hypertension
Diseases and symptoms or signs involving the respiratory system	Cough (24), pneumonia (1), rhinorrhea (2), itchy throat (1), chest pain (1), tonsilitis (1)	30	148	0.80	*Blumea balsamifera*	91	Cough
Diseases and symptoms or signs involving the digestive system or abdomen	Abdominal pain (2), angular cheilitis (1), bleeding gums (1), bloated stomach/ gas pain (17), blood in feces (1), constipation (2), diarrhea (17), gastric ulcer (1), halitosis (1), indigestion (1) intestinal cleansing (1), nausea (1), stomachache (39), teething syndrome (1), tooth decay (11) vomiting blood (7)	103	252	0.59	*Chrysophyllum cainito*	67	Diarrhea
Diseases and symptom or signs involving the skin	Dandruff (1), eczema (1), lump (5), Pityriasis rosea (2), rashes (2), skin lesion (5)	16	35	0.56	*Psidium guajava*	20	Skin lesion
Diseases and symptoms or signs of the musculoskeletal system or connective tissue	Lower back pain (2), limb pain (3), muscle pain (8), muscle swelling (2), rheumatoid arthritis (8)	22	51	0.58	*Artemisia vulgaris*	26	Muscle pain
Diseases of the genitourinary system	Induce period/menstruation (1), kidney stones (7), kidney trouble (13), swelling of male genitalia (2), urinary tract infection (22)	45	92	0.52	*Annona muricata*	55	UTI
Pregnancy, childbirth and the puerperium	Breast engorgement (3), birth control (4), induce labor (2), lactation support (1), postpartum bleeding (2), postpartum care and recovery (22)	34	118	0.72	*Bambusa spinosa*	93	Postpartum care and recovery
Injury, poisoning and certain other consequences of external causes	Animal bite (2), bruise (1), burn (1), caterpillar dermatitis (1), circumcision (1), cuts and wounds (21), fracture (7), splinter (2)	36	176	0.80	*Chromolaena odorata*	100	Cuts and wounds
General symptoms and signs	Chill (2), fever (19), malaise (1)	21	59	0.66	*Curcuma longa*	43	Fever
Mental or behavioral symptoms, signs or clinical findings	Memory loss	1	1		*Curcuma longa*	2	Memory loss
Other cultural uses	*Doklong* (14), *hiwit* (2), *inaswang* (5), *kolebra* (3), *sinda* (10)	34	89	0.63	*Curcuma longa*	46	*Sinda*

**Number inside the parenthesis denotes the number of species in each reported disease or use.*

stomach/gas pain, goiter, urinary tract infection (UTI), vomiting blood, and *inaswang*. It was collected growing in the farmland, but some informants also cultivated it in their backyards.

A. vulgaris was used to treat seven ailments in six disease categories and is the best-known therapy for cough, fever, headache, and body pains. It is also used for the remedy of chest pain, fracture, and hearing problems. It is grown in the

TABLE 4 | Medicinal plants used to strengthen immunity against infection.

Medicinal plants	Preparation and administration
Capsicum annuum	Eat one fruit before dinner
Vitex trifolia	Boil handful of leaves and drink decoction like water
Euphorbia hirta	Boil handful of whole plant and drink decoction as water intake
Musa balbisiana cv	Eat ripe fruits three times a day
Peperomia pellucida	Soak a handful of whole plants in warm water then drink like water
Curcuma longa	Pound a handful of rhizomes then rub extract on the body three times a day
Zingiber officinale	Boil rhizome and drink one glass of decoction three times a day
Areca catechu, *Piper betle*, and *Nicotiana tabacum*	Wrap a pinch of *A. catechu* seeds in a *P. betle* leaf daubed with slaked lime (*apog*) and plug cut dried leaf of *N. tabacum* then chew (chewing betle quid)

home gardens as medicinal plants for future use. Seven medicinal plants were recorded with only one use report and FC for each species: *Cheilocostus speciosus* (J.Koenig) C.D.Specht, *Luffa aegyptiaca* Mill., *Syzygium cumini* (L.) Skeels, *Lygodium circinnatum* (Burm. f.) Sw., *Solanum melongena* L., *Nauclea orientalis* (L.) L., and *Leea guineensis* G. Don, which garnered the lowest value for UV (0.01) and RFC (0.01).

Medicinal plants with the highest RFC were *Artemisia vulgaris* (0.57), followed by *Curcuma longa* (0.47) and *Blumea balsamifera* (0.44) (**Table 2**). Out of the 75 informants who participated in the survey, *A. vulgaris* had the highest informant citation or FC.

Relative Importance Index

The RI was used to assess the relative importance of the medicinal plants by use or disease categories. A high value indicates that a particular medicinal plant species is most frequently cited as useful with a high number of use categories or having multiple uses. The top three plants with the highest RI values were *Annona muricata* L. (0.88), *C. longa* (0.87), and *B. balsamifera* (0.80) (**Table 2**). *A. muricata* is used in 11 different use or disease categories: diseases of the genitourinary system; neoplasms; diseases of the circulatory system and blood or blood-forming organs; endocrine, nutritional and metabolic diseases; diseases and symptoms or signs involving the respiratory system; diseases and symptoms or signs involving the nervous system; diseases and symptoms or signs involving the digestive system or abdomen; infectious and parasitic diseases; diseases and symptoms or signs involving the skin; diseases and symptoms or signs of the musculoskeletal system or connective tissue; and other cultural uses. It is used to address 13 diseases or purposes and recorded the high use report in treating UTI, cancer, and hypertension by drinking the leaf decoction or soaking the young leaves in warm water and drink or by just eating a medium sliced fruit three times a day. *A. muricata* is also used for the remedy of high uric acid, pneumonia, dizziness, intestinal cleansing, kidney trouble, itchy throat, amoebiasis, lump, arthritis, and *doklong*. *C. longa* was used in nine categories: injury, poisoning and certain other consequences of external causes; neoplasms; diseases and symptoms or signs involving the nervous system; general symptoms and signs; diseases and symptoms or signs involving the digestive system or abdomen; infectious and parasitic diseases; diseases and symptoms or signs of the musculoskeletal system or connective tissue; mental or behavioral symptoms, signs, or clinical findings; other cultural uses. *B. balsamifera* is used in eight disease categories: diseases and symptoms or signs involving the digestive system or abdomen; endocrine, nutritional, and metabolic diseases; diseases of the genitourinary system; diseases and symptoms or signs of the musculoskeletal system or connective tissue; diseases and symptoms or signs involving the nervous system; diseases and symptoms or signs involving the respiratory system; pregnancy, childbirth, and the puerperium; other cultural uses. The top ten medicinal plants with the highest use value, relative frequency citation, and relative importance index are shown in **Table 2**.

Informant Consensus Factor

There were 91 diseases and purposes in 16 different use or disease categories recorded in this study (**Table 3**). ICF was used to evaluate the consensus in the medicinal plant information among the informants. High ICF values indicate one or few medicinal plants mentioned by a high number of informants within a particular disease category, and low values indicate that more species are being used and the informants differ in their preference on which plant to use. The highest ICF value (0.80) was in the diseases and symptoms or signs involving the respiratory system and in injury, poisoning, and certain other consequences of external causes. The diseases and symptoms or signs involving the respiratory system were cough, pneumonia, rhinorrhea, itchy throat, chest pain, and tonsillitis. *B. balsamifera* had the highest use report within the category and was frequently used plant for treating cough by consuming the young leaves or by drinking leaf or root decoction or by rubbing leaf extract on the head of the afflicted ones. A high proportion of informants mentioned and agreed upon the use of *B. balsamifera* in treating cough within the category. Injuries, poisoning, and certain other consequences of external causes recorded, such as animal bite, bruise, burn, caterpillar dermatitis, circumcision, cuts and wounds, fracture, and splinter, were the reported purposes or medical uses. *Chromolaena odorata* (L.) R.M.King & H. Rob. had the highest use report within the category and was the most preferred medicinal plant used to treat cuts/wounds by applying crushed leaves on the affected area. The next highest value was in the diseases and symptoms or signs involving the nervous system (0.73) with headache, migraine, and dizziness as the reported medical condition. *Pseuderanthemum carruthersii* had the highest use report and was widely used for treating headaches by applying leaves on the forehead alone or with *C. longa* and *Z. officinale*. The lowest ICF value was recorded in mental disorder (0.00) with memory loss

TABLE 5 | Comparative presence-absence matrix of the medicinal plants used by the *Panay Bukidnon* with other ethnobotanical studies.

Scientific name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	New medicinal plant uses/purpose
Andrographis paniculata (Burm.f.) Nees	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	1	0	1	0	0	1	1	
Justicia gendarussa Burm.f.*	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	1	1	1	0	1	
Pseuderanthemum carruthersii (Seem.) Guillaumin	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	
Acorus calamus L.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	
Amaranthus viridis L.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Anemia
Alternanthera sessilis (L.) R. Br. ex DC.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Anemia, vomiting blood
Allium sativum L.	1	1	0	1	1	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	
Allium fistulosum L.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Teething syndrome
Mangifera indica L.	1	1	0	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	0	0	1	0	1	Stomachache, cough, kidney trouble,
Spondias pinnata (L.f.) Kurz	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	
Annona muricata L.	0	1	1	0	1	0	1	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1	Pneumonia, lumps
Annona squamosa L.	0	0	1	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	Postpartum care and recovery
Centella asiatica (L.) Urb	1	0	1	0	0	1	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	
Alstonia scholaris (L.) R.Br.*	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	
Catharanthus roseus (L.) G.Don	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	1	
Tabernaemontana pandacaqui Poir.*	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	
Alocasia macrorrhizos (L.) G.Don *Alocasia* cultivar	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	
Colocasia esculenta (L.) Schott	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	Breast engorgement
Homalomena philippinensis Engl	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	
Schefflera elliptica (Blume) Harms	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Areca catechu L.	1	0	1	1	1	0	1	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	
Cocos nucifera L.	0	1	1	1	1	1	1	0	1	0	0	1	1	0	0	1	0	0	0	1	0	0	1	
Corypha utan Lam.	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	
Cordyline fruticosa (L.) A.Chev.*	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	
Aloe vera (L.) Burm.f.	0	0	0	0	0	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	1	
Artemisia vulgaris L.	1	1	0	0	1	1	1	0	1	0	0	1	1	0	1	1	1	0	1	1	1	1	1	
Bidens pilosa L.	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	Toothache
Blumea balsamifera (L.) DC.*	1	0	1	1	1	1	1	0	1	0	0	1	0	0	0	1	1	1	1	1	1	1	1	
Chromolaena odorata (L.) R.M.King and H.Rob	0	1	0	0	1	1	1	0	1	1	0	0	1	1	0	1	1	1	1	1	1	0	1	
Elephantopus tomentosus L.*	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	1	
Impatiens balsamina L.	0	0	1	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	1	
Basella alba L.	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	Mumps
Bixa orellana L.	0	0	1	0	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	Bloated stomach/gas pain
Cordia dichotoma G.Forst.	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	0	0	1	
Brassica rapa L.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Kidney problem, anemia
Ananas comosus (L.) Merr.	1	0	0	1	1	0	1	0	1	0	0	1	1	0	0	1	1	0	0	1	0	0	1	Bleeding gums
Carica papaya L.	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	0	1	1	1	0	1	
Ipomoea batatas (L.) Lam	0	0	1	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	Breast engorgement
Decalobanthus peltatus (L.) A.R.Simões and Staples*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	
Cheilocostus speciosus (J.Koenig) C.D.Specht	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	1	
Kalanchoe pinnata (Lam.) Pers.*	1	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	1	0	1	0	1	1	1	
Cucurbita maxima Duchesne	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Fever, swelling of female genitalia

(Continued on following page)

TABLE 5 | (*Continued*) Comparative presence-absence matrix of the medicinal plants used by the *Panay Bukidnon* with other ethnobotanical studies.

Scientific name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	New medicinal plant uses/purpose
Luffa aegyptiaca Mill	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
Momordica charantia L	1	0	0	1	0	0	1	0	1	1	0	1	1	0	1	1	0	0	0	1	1	0	1	
Cyperus mindorensis (Steud.) Huygh	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
Dioscorea esculenta (Lour.) Burkill	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Mumps, ringworm
Euphorbia hirta L.*	1	1	1	0	0	1	1	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	1	UTI, angular cheilitis
Euphorbia tirucalli L.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Tooth decay
Jatropha curcas L.	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	Tetanus
Manihot esculenta Crantz	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	Fracture, gas pain, lower back pain
Caesalpinia sappan L.*	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	1	Typhoid fever
Cajanus cajan (L.) Huth	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	
Clitoria ternatea L.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Cancer, hypertension
Desmodium triflorum (L.) DC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	
Gliricidia sepium (Jacq.) Kunth ex Walp	0	0	1	1	1	0	1	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	1	
Indigofera tinctoria L	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	
Leucaena leucocephala (Lam.) de Wit	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	
Mimosa pudica L.*	0	0	1	1	1	1	1	0	1	1	0	1	1	1	0	0	1	1	1	1	0	1	1	
Phaseolus lunatus L.	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	
Pithecellobium dulce (Roxb.) Benth.	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	
Senna alata (L.) Roxb.*	1	0	0	1	0	0	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	1	
Tamarindus indica L.	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	1	
Vigna unguiculata (L.) Walp	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Bloated stomach/gas pain, gas pain
Cratoxylum sumatranum (Jack) Blume*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	
Eleutherine palmifolia (L.) Merr.	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	Rashes, headache
Clerodendrum quadriloculare (Blanco) Merr.	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	
Gmelina arborea Roxb. ex Sm.	0	0	0	0	1	1	0	0	0	1	0	0	0	1	0	1	1	0	0	1	1	0	1	
Hyptis capitata Jacq.	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1	Helminthiasis
Mentha arvensis L.	1	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	1	
Orthosiphon aristatus (Blume) Miq.	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	Cancer
Plectranthus amboinicus (Lour.) Spreng.	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	1	
Plectranthus scutellarioides (L.) R.Br.	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	1	1	0	1	1	0	1	1	
Tectona grandis L.f.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	Cuts, wounds, rheumatoid arthritis
Vitex trifolia L.*	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	
Persea americana Mill	1	1	0	1	1	1	1	0	1	1	0	1	1	1	0	1	0	0	1	0	1	1	1	
Barringtonia asiatica (L.) Kurz	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	
Lygodium circinnatum (Burm. f.) Sw.*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Headache
Lagerstroemia speciosa (L.) Pers.	1	0	0	1	1	0	1	0	1	0	0	1	1	1	0	0	1	0	1	1	1	0	1	Cancer
Abelmoschus esculentus (L.) Moench	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	UTI, cancer, hypertension
Corchorus olitorius L.	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Birth control
Hibiscus acetosella Welw. ex Hiern*	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	Cuts, wounds, boils, anemia, hypertension, diabetes, rhinorrhea, cough
Urena lobata L.*	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	1	0	1	0	0	0	1	
Sandoricum koetjape (Burm.f.) Merr.	0	0	1	0	1	0	1	0	1	1	0	0	1	1	1	1	1	0	1	0	1	0	1	Kidney trouble
Swietenia mahagoni (L.) Jacq	0	1	1	0	1	0	1	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	1	
Tinospora crispa (L.) Hook. f. and Thomson*	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	1	1	0	0	1	1	0	1	
Artocarpus heterophyllus Lam	0	1	1	0	1	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	
Ficus benjamina L. HNUL 0021383	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	1	1	0	0	1	Postpartum care and recovery, stomachache
Ficus septica Burm.f.	0	0	0	1	0	0	1	1	0	0	0	1	0	1	0	1	1	0	0	1	1	0	1	

(Continued on following page)

TABLE 5 | (*Continued*) Comparative presence-absence matrix of the medicinal plants used by the *Panay Bukidnon* with other ethnobotanical studies.

Scientific name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	New medicinal plant uses/purpose
Moringa oleifera Lam.	0	1	1	1	1	1	1	0	1	0	0	1	1	0	0	1	0	0	1	1	1	1	1	
Muntingia calabura L.	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1	0	1	
Musa balbisiana cv. Colla	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	
Musa textilis Née	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	
Musa x *paradisiaca* L.	0	1	0	1	1	1	1	0	1	1	0	0	1	0	1	1	0	0	0	1	1	0	1	
Psidium guajava L.	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	
Syzygium cumini (L.) Skeels	0	0	1	0	1	0	1	0	1	1	0	0	1	1	0	1	0	0	0	1	0	0	1	
Averrhoa bilimbi L.	0	0	1	1	1	0	1	0	1	0	0	1	1	0	1	1	0	0	1	0	0	0	1	Kidney trouble
Averrhoa carambola L.	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Kidney trouble, postpartum care and recovery
Oxalis triangularis A.St.-Hil	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	Cuts/wounds
Peperomia pellucida (L.) Kunth	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0	1	1	1	
Piper betle L	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0	1	0	0	1	1	0	0	1	Mumps
Antidesma bunius (L.) Spreng	0	0	1	1	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	1	0	0	1	
Bambusa spinosa Roxb	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	Cancer
Chrysopogon aciculatus (Retz.) Trin	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Stomachache, tooth decay, wart
Cymbopogon citratus (DC.) Stapf	1	1	0	0	0	1	0	0	1	1	0	1	1	1	0	1	0	0	0	1	1	1	1	Halitosis
Eleusine indica (L.) Gaertn.*	0	1	0	0	1	1	1	0	1	1	0	0	1	1	0	0	1	1	0	1	1	1	1	
Imperata cylindrica (L.) Raeusch	1	1	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	Cancer
Oryza sativa L	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	Postpartum care and recovery
Saccharum officinarum L.	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	Hoarseness
Zea mays L.	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	
Chrysophyllum cainito L.	0	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	
Capsicum annuum L.	1	1	1	1	1	1	1	0	1	0	0	0	0	1	1	1	1	0	1	0	1	0	1	
Solanum lycopersicum L.	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	
Nicotiana tabacum L.	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	
Solanum melongena L.	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	
Nauclea orientalis (L.) L.	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	
Morinda citrifolia L.	0	0	1	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	1	
Mussaenda philippica A.Rich	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	
Citrus maxima (Burm.) Merr.	0	0	0	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	0	1	1	0	1	Kidney trouble, chicken pox
Citrus microcarpa Bunge	1	1	1	0	0	1	1	0	1	0	0	1	0	1	1	1	0	0	0	1	1	0	1	
Pipturus arborescens (Link) C.B. Rob.*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	Breast engorgement
Stachytarpheta jamaicensis (L.)*	0	1	0	1	1	1	0	1	0	1	0	0	0	0	1	1	1	1	1	1	1	0	1	
Cissus sp.																								
Leea guineensis G. Don	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	
Tetrastigma sp. Planch.																								
Alpinia galanga (L.) Willd	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	
Amomum sp.																								
Curcuma longa L.	0	1	0	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	Typhus, memory loss, tetanus
Etlingera philippinensis (Ridl.) R.M.Sm.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	Typhoid fever, tuberculosis, headache
Kaempferia galanga L.	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	1	1	
Zingiber officinale Roscoe	1	1	0	1	1	1	1	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	1	Tetanus

as the

reported condition and *Curcuma longa* was used for the treatment.

Fidelity Level

The FL was used to determine the relative importance of a medicinal plant within each category. Medicinal plants with the highest FL values were *Chromolaena odorata* (100%), *Bambusa spinosa* Roxb. (93%), and *Pseuderanthemum carruthersii* (93%) (**Table 3**). *C. odorata* is exclusively used to treat cuts and wounds and can be seen growing invasively along the paths and roadsides in the community. *B. spinosa* recorded the highest use report for postpartum care and recovery under the pregnancy, childbirth, and puerperium category. It is preferably used and highly suggested by many informants for postpartum care and recovery therapy. Decoction of at least three up to ten different medicinal plants was used for drinking (1–2 glasses), body steaming, and bathing to be performed nine days after a mother gave birth. *B. spinosa* has also been used to treat cancer, UTI, and kidney stones but with only one citation recorded for each ailment *P. carruthersii* has the highest use report and is the most preferred medicinal plant for relieving headaches under the diseases and symptoms or signs involving the nervous system. The lowest FL value was recorded for *C. longa* in treating memory loss under the mental disorder category with only one informant mentioned for its curative effect.

Comparing Different Indices

Table 2 shows the top 10 medicinal plants with the highest UV, RFC, and RI values. High UV and RFC values indicate the high number of use reports and frequency citations (FC) from the informants, while high RI values consider the multiplicity of uses or the high number of uses in different disease categories. This implies that medicinal plants with high UV, RFC, and RI values are the most important and valued medicinal plants in the community. There are a few considerable differences in species ranking yielded by the three indices set out in **Table 2**. The ranks of the first three species (*C. longa, Blumea balsamifera,* and *Artemisia vulgaris*) are nearly the same in all indices except in RI where *Annona muricata* had the highest value but ranked 4th in UV and only 9th in RFC. *C. longa, B. balsamifera,* and *A. vulgaris* had the highest use reports and FC from the informants and only next to *A. muricata* in terms of multiple uses in different disease categories. *A. muricata* had the highest number of uses or purposes in different disease categories (11 disease categories); however, its use reports and FC are not quite as high as those of the first three species mentioned above. *A. muricata* is the most frequently used medicinal plant in a wide range of diseases (13 diseases). Another noticeable difference is the inclusion of *Moringa oleifera Lam.* and *Carica papaya* L. in the RI index, which are not shown in the top ten species with high UV and RFC values. *M. oleifera* ranks 12th in UV and 13th in RFC, while *C. papaya* ranks 17th in UV and 14th in RFC (values are not shown in **Table 2** but available in **Supplementary Table S2**). Their UV and RFC values are not quite as high, but they

1-Balangcod and Balangcod, 2011.
2-Olowa et al., 2012.
3-Tantiado, 2012.
4-Abe and Ohtani, 2013.
5-Ragragio et al., 2013.
6-Gruyal et al., 2014.
7-Ong and Kim, 2014.
8-Raterta et al., 2014.
9-Balangcod and Balangcod, 2015.
10-Pizon et al., 2016.
11-Odchimar et al., 2017.
12-Baddu and Ouano, 2018.
13-Tantengco et al., 2018.
14-Agapin, 2019.
15-Pablo, 2019.
16-Cordero et al., 2020.
17-Dapar et al., 2020.
18-Paraguison et al., 2020.
19-Belgica et al., 2021.
20-Cordero and Alejandro, 2021.
21-Nuñeza et al., 2021.
22-Montero and Geducos, 2021.
23-PTKD (Philippine Traditional Knowledge Digital Library on Health).
**Medicinal plants recorded in Panay Bukidnon communities in the Province of Capiz (Jocano, 1968).*
Plants in bold are the newly recorded species with medicinal values.
Values in bold indicate the absence of medicinal value of the newly recorded species compared with the other existing ethnobotanical studies in the Philippines.

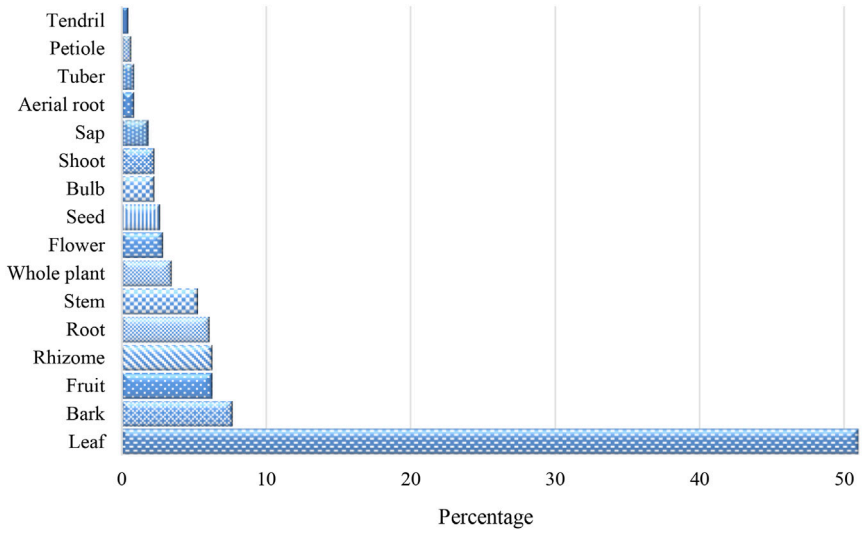


FIGURE 4 | Medicinal plant parts used.

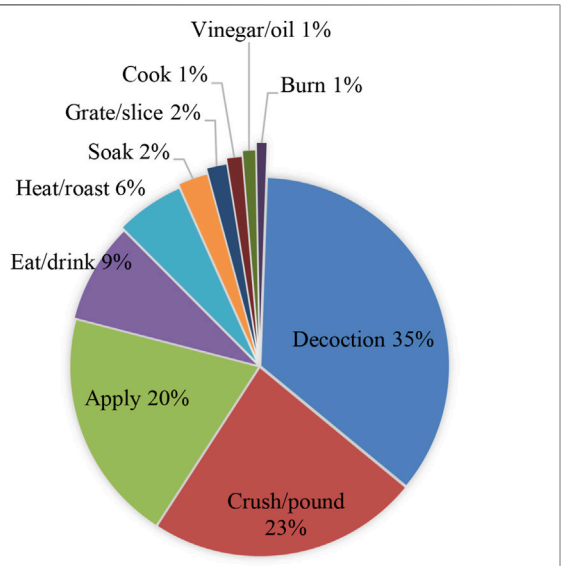


FIGURE 5 | Medicinal plant preparation.

have multiple uses in different disease categories. Low UV, RFC, and RI values indicate a low number of use reports, FC, and have one or few uses in disease categories. For example, *Andrographis paniculata* (Burm.f.) has low UV (0.04), RFC (0.03), and RI (0.07) values, indicates low use report (2) and FC (2 informants), and is only used in one disease category (diseases and symptoms or signs involving the digestive system or abdomen). This implies that *A. paniculata* is a less important and less preferred medicinal plant species in the *Panay Bukidnon* community. On the other hand, **Table 3** shows the use or disease categories and ICF and FL values. High ICF values are considered the most culturally relevant medicinal plants and the agreement of its use within a disease category in the community, while FL highlights the most preferred species for a particular disease. Medicinal plants with the high ICF values were *Blumea balsamifera* (0.80), *Chromolaena odorata* (0.80), and *Pseuderanthemum carruthersii* (0.73) and these species are highly agreed upon by most informants for the therapy of diseases in their respective disease categories. Medicinal plants with high FL values were *C. odorata* (100%), *P. carruthersii* (93%), and *Bambusa spinosa* (93%) and these are the most preferred species to a particular disease in each disease category. Though *B. spinosa* is not included in the top medicinal plants with high UV, RFC, and RI values, its curative effect for postpartum care and recovery is preferred by a high proportion of informants. Medicinal plants with high UV, RFC, RI, ICF, and FL values are the most culturally important, relevant, preferred, and agreed on species in the *Panay Bukidnon* communities.

Cultural Important Medicinal Plants

Indigenous peoples are strongly tied with their spiritual beliefs and practices. Interestingly, up to date, the *Panay Bukidnon* still believe that some of the illnesses and diseases are caused by spirits, supernatural beings, and sorcery. Some diseases were mentioned that were caused by *aswang* (witch), *hiwit* (sorcery), *sinda* (charm of spirits) and some health conditions like *doklong* and *kolebra* with more complicated and sometimes unexplained symptoms. *Sinda* is a condition with symptoms like dizziness and fever caused by spirits or supernatural beings, while *kolebra* has symptoms like chills, stomachache, nausea, shortness of breath, and paleness. *Doklong* is somewhat similar to "relapse" and sometimes accompanied by other symptoms like headache, muscle pain, and weakness. For *inaswang*, five medicinal plants were recorded for the therapy and a cultivar of *Alocasia* is frequently used by applying the heated leaf to the stomach area. For the treatment of *hiwit*, a species of *Amomum* is used by rubbing the stem extract on the body or by crushing the

stem with the inner bark of *Pipturus arborescens* (Link) C.B. Rob. and taking the extract orally. *Curcuma longa* is the most used medicinal plant used to cure *sinda* by rubbing the rhizome's extract on the head or applying the sliced rhizome along with other plants on the forehead. *Jatropha curcas* L. is frequently used as a remedy for *kolebra* by drinking the extract of the inner bark and for *doklong*, drinking the leaf decoction of *Citrus maxima* (Burm.) Merr. alone or with other medicinal plants is the most preferred.

Medicinal Plants Used to Strengthen Immunity Against Infection and for Potential COVID-19 Therapy

With the current situation of the novel coronavirus disease (COVID-19) in the country and the resurgences of the infection waves, communities in the far-flung areas tend to explore different medical plants as an alternative for potential therapy while waiting for the vaccine. There were ten medicinal plants mentioned by the council of elders that they used to boost their immunity against COVID-19 infection (**Table 4**). If someone is suspected of having a COVID-19 infection or exhibits symptoms related to COVID-19, they use the available medicinal plants (**Table 2**) available to alleviate their condition. Medicinal plants such as *Curcuma longa, Zingiber officinale, Capsicum annuum* L., and *Peperomia pellucida* (L.) Kunth are traditionally used by the *Panay Bukidnon* to treat fever, headache, cough, and body pains which were also the symptoms of COVID-19 and influenza. They also believed that chewing betel quid which is composed of *Areca catechu* L., *Piper betle* L., *Nicotiana tabacum* L., and slaked lime (*apog*) can help them fight the infection and help them feel substantially better.

Comparative Review of the Medicinal Plants With Other Ethnobotanical Studies

The anthropological study conducted in the *Panay Bukidnon* communities in the Province of Capiz in the 1950s recorded a total of 54 medicinal plant species and 21 of which are cited in this current ethnobotanical study. An additional 109 taxa were documented to the medicinal flora used by the *Panay Bukidnon* in Panay Island from this present study.

To identify the new medicinal plants and plant use, a comprehensive comparison was performed with 22 ethnobotanical studies published from 2011 up to the present and with one online database. Only the scientific names and their synonyms were used for the comparative review; local names were not considered because they are arbitrary within different cultures and dialects. Of the 127 medicinal plants identified up to the species level, three species (*Eleutherine palmifolia* (L.) Merr., *Hibiscus acetosella* Welw. ex Hiern, and *Oxalis triangularis* A.St.-Hil.) show some novel medicinal uses that were not documented in other existing ethnobotanical studies conducted in the country. These medicinal plant species are not native to the Philippines. *E. palmifolia* is used for the therapy of rashes and headaches. Its red bulb is the preferred plant part for the treatment. It is usually used as an ornamental plant grown in the home gardens and community center. *H. acetosella* is used to cure cuts/wounds, boils, anemia, hypertension, diabetes, rhinorrhea, and cough. Filipinos also used this medicinal plant as a vegetable and normally use it to "sour" the dishes. With its deep red-purple foliage, it is also served as an ornamental plant. *O. triangularis* is used to treat cuts/wounds by the *Panay Bukidnon* and serves as a hanging ornamental plant for its striking deep maroon trifoliate leaves. Forty-seven medicinal plants were recorded to have an additional therapeutic use or purpose not mentioned in the other previous ethnobotanical studies, while 80 species have the same medicinal values as mentioned in the existing literature. Some of the additional uses or purposes of the medicinal plants that are rarely listed in other studies are angular cheilitis, breast engorgement, and promoting teething in toddlers. The detailed information about the comparative review of the medicinal plants and the additional plant uses is shown in **Table 5**.

DISCUSSION

The documentation of 131 medicinal plant species used in the indigenous health care practices showed the extensive usage of *Panay Bukidnon* ethnobotanical knowledge and indicative importance for their rich cultural heritage. The families of Fabaceae, Lamiaceae, and Poaceae were represented with a high number of medicinal plant species. Fabaceae as the most preferred medicinal plant family used by the *Panay Bukidnon* is parallel to the other folkloric studies conducted in Western Visayas (Madulid et al., 1989; Tantiado 2012; Ong and Kim, 2014; Cordero and Alejandro, 2021) and other indigenous communities in the country (Ragragio et al., 2013; Obico and Ragrario, 2014; Tangtengco et al., 2018; Pablo, 2019). Fabaceae is highly used by the *Panay Bukidnon* to treat infectious and parasitic diseases and diseases and symptoms or signs involving the digestive system or abdomen. The family constitutes phytochemicals that have antibacterial, antifungal, antioxidant, and insecticidal activities (Wanda et al., 2015).

The use of leaves as the most preferred medicinal plant part to address medical conditions is comparable to other ethnobotanical surveys conducted throughout the archipelago (Balangcod and Balangcod, 2011; Olowa et al., 2012; Abe and Ohtani, 2013; Gruyal et al., 2014; Ong and Kim, 2014; Raterta et al., 2014; Balangcod and Balangcod, 2015; Pizon et al., 2016; Odchimar et al., 2017; Baddu and Ouano, 2018; Tantengco et al., 2018; Agapin, 2019; Pablo, 2019; Cordero et al., 2020; Dapar et al., 2020; Belgica et al., 2021; Cordero and Alejandro, 2021; Madjos and Ramos, 2021; Montero and Geducos, 2021; Nuñeza et al., 2021). As a tropical country, leaves are always available for most plant species at all seasons and are readily accessible in case of emergencies. The collection of leaves is more sustainable than gathering other plant parts such as barks and roots that can cause damaging effects and even mortality to a plant if harvested in large quantities. Leaves contain the highest secondary metabolites with an antimicrobial effect (Chanda and Kaneria, 2011),

antioxidant property, antibiotic activity, and antidiabetic potential compared with other plant parts (Jain et al., 2019).

Decoction is the most common form of preparation and preferably to be taken orally and occasionally used for body steaming, bathing, and washing. It is also an evident form of preparation in other indigenous communities in the country (Balangcod and Balangcod, 2015; Pizon et al., 2016; Odchimar et al., 2017; Baddu and Ouano, 2018; Tantengco et al., 2018; Cordero et al., 2020; Cordero and Alejandro, 2021; Madjos and Ramos, 2021; Nuñeza et al., 2021). Decoction is done with the use of one medicinal plant species or in a combination of two or more. The *Panay Bukidnon* are culturally used to combine three, five, or seven (colloquially known as *pito-pito*) different medicinal plants for higher efficacy. Each plant constitutes phytochemical compounds and is sometimes present in small quantities and inadequate to achieve desirable therapeutic effects. To yield better results and effectiveness, the combination of different medicinal plants demonstrates the synergistic effects. Some bioactive chemicals work significantly when combined with other plants rather than used singly (Parasuraman et al., 2014).

Curcuma longa recorded the highest use value and is used as therapy for headache, fever, body pain, stomachache, bloated stomach, tooth decay, typhus, typhoid fever, anti-tetanus, memory loss, cancer, and *sinda*. The rhizome's extract is usually used for the treatment. It is also used by other indigenous groups in the country for diarrhea, abdominal pain, flatulence, arthritis, and hypertension by the *Higaonon* tribe in Iligan City (Olowa et al., 2012); arthritis, cough, and cuts and wounds by the *Ivatan* tribe in Batan Island (Abe and Ohtani, 2013); fever, burn, dizziness, and abdominal pain by the *Ati* tribe in Guimaras Island (Ong and Kim, 2014); arthritis by the *Subanen* tribe in Zamboanga del Sur (Pizon et al., 2016); flatulence, headache, numbness, rheumatism, stomachache, and vomiting by the *Aetas* tribe in Bataan (Pablo, 2019); cancer by the *Manobo* tribe in Bukidnon (Pucot et al., 2019); skin eruptions and gastric pain by the *Ati* tribe in Aklan (Cordero et al., 2020); ten different diseases by the *Manobo* tribe in Agusan del Sur (Dapar et al., 2020); myoma, hepatitis, relapse, sore eyes, and stye by the eight ethnolinguistic groups in Zamboanga Peninsula (Madjos and Ramos, 2021); bruise and boils by the *Mamanwa* tribe in Surigao del Norte and Agusan del Norte (Nuñeza et al., 2021). In India, the use of *C. longa* dates back to 4,000 years ago not only as a culinary spice but also for religious and medicinal importance. It contains bioactive compounds that have antioxidant, antimutagenic, antimicrobial, antimutagenic, antimicrobial, antifungal, anticancer, and other countless medicinal uses (Prasad and Aggarwal, 2011).

Artemisia vulgaris has the highest relative frequency citation. It is a cosmopolitan weed and is available nearly everywhere. It thus does not surprise that it is commonly used for cough, fever, headache, body pains, chest pain, fracture, and hearing problems. Other ethnobotanical surveys mentioned its efficacy against cough and scabies by the *Kalanguya* tribe in Ifugao (Balangcod and Balangcod, 2011); stomachache (Olowa et al., 2012); sore eyes, ear infection, and cough by the *Ayta* tribes in Pampanga (Ragragio et al., 2013); cough with phlegm, fever, abdominal pain, body pains, and headache (Ong and Kim, 2014); colds by the *Talaandig* tribe in Bukidnon (Odchimar et al., 2017); dysmenorrhea by the *Y'Apayaos* in Cagayan arthritis (Baddu and Ouano, 2018); fever, sore throat, colds, cough, and phlegm *Ayta* in Bataan (Tantengco et al., 2018); fever, headache, dizziness, stomachache, bloated stomach, and cough (Cordero et al., 2020); 11 different folkloric uses (Madjos and Ramos, 2021); cough and gas pain (Cordero and Alejandro, 2021); fever, cough, and cough with phlegm (Nuñeza et al., 2021). In medieval times, it was known as the "mother of herbs" due to its beneficial effects. Studies have been conducted worldwide for its antioxidant, bronchodilatory, hepatoprotective analgesic, antihypertensive, estrogenic, cytotoxic, antifungal and antibacterial, anti-inflammatory, anti-allergenic, antimalarial, and anthelmintic activities (Ekiert et al., 2020). *Artemisia,* per se, is an extremely important plant genus, pharmacologically as well as economically. *A. annua* L. makes the most important example, famous for its many pharmacologically active substances but especially for Artemisin (Tu, 2011), an effective remedy against malaria.

Annona muricata, an important, widely grown fruit tree, has the highest relative importance index value (0.88) and is used to treat 13 diseases in 11 different use or disease categories. It recorded the high use report for treating UTI, cancer, and hypertension by drinking the leaf decoction or eating just the ripe fruit. It is also used by the *Panay Bukidnon* for high uric level, pneumonia, dizziness, intestinal cleansing, kidney trouble, *doklong*, itchy throat, amoebiasis, lump, and arthritis. In traditional medicine across the country, it is also used for the treatment of diarrhea (Olowa et al., 2012); dermatological diseases (Tantiado, 2012); fever, insect repellent, headache, and stomachache (Ragragio et al., 2013); tetanus (Pizon et al., 2016); gastrointestinal cleansing and tumors (Odchimar et al., 2017); fever and arthritis (Baddu and Ouano, 2018); stomachache and dizziness (Tantengco et al., 2018); diabetes, high blood, stomachache, UTI, and vertigo (Pablo, 2019); cancer (Agapin, 2019); 12 different diseases (Dapar et al., 2020); kidney problems, urinary tract infection, goiter, and anthelmintic (Cordero et al., 2020); at least 16 medical problems (Madjos and Ramos, 2021); cuts and wounds, stomach ulcer, intestinal cleansing, UTI, cough, and cancer (Cordero et al., 2020); cancer (Montero and Geducos, 2021); cough, wound, asthma, and UTI (Nuñeza et al., 2021); cancer, stomach acidity, hypertension, and cough (Belgica et al., 2021). Phytochemical constituents investigated on *A. muricata* exhibited antiarthritic, anticancer, anticonvulsant, antidiabetic, anti-inflammatory, antioxidant, antihypertensive, antiparasitic, antiplasmodial, cytotoxic, gastroprotective, and wound healing activity (Moghadamtousi et al., 2015; Coria-Téllez et al., 2018).

The highest ICF value is in the diseases of the respiratory system category and *Blumea balsamifera* is the most frequently used medicinal plant to treat cough. A high number of informants agreed on the effectiveness of *B. balsamifera* against the diseases on the respiratory system, particularly for treating cough in the community. However, this therapeutic claim must be seriously considered for further pharmacological investigations to determine its efficacy. *B. balsamifera* is one of the ten medicinal plants endorsed by the Philippine Department of Health (DOH) as part of basic healthcare and clinically proven

to have diuretic and antiurolithiasis properties. It is manufactured in the country for national distribution and marketing by the National Drug Formulary (World Health Organization, 1998). It also contains compounds (monoterpenes, diterpenes, sesquiterpenes) that have antitumor, antioxidant, antimicrobial and anti-inflammation, antiplasmodial, antityrosinase, wound healing, anti-obesity, disease and insect resistance, and hepatoprotective effects and radical scavenging activities (Pang et al., 2014).

The medicinal plant with the highest FL was the *Chromolaena odorata* under the injury, poisoning, and certain other consequences of external causes category. All informants who cited *C. odorata* preferred to use it as first aid for cuts and wounds. This suggests that *C. odorata* might contain valuable bioactive compounds with pharmacological effects for cuts and wounds that must be proven scientifically. Several ethnobotanical studies also recorded the use of *C. odorata* for cuts and wounds in the country (Olowa et al., 2012; Ong and Kim, 2014; Pizon et al., 2016; Odchimar et al., 2017; Tantengco et al., 2018; Cordero et al., 2020; Dapar et al., 2020; Belgica et al., 2021; Cordero and Alejandro 2021; Madjos and Ramos, 2021). The leaves of *C. odorata* are rich in flavonoids and have the highest concentration of allelochemicals. They have antimalarial, anti-inflammatory, antibacterial, analgesic, antipyretic, antioxidant, anticancer, and wound healing properties (Vijayaraghavan et al., 2017).

Traditional medical practices in the indigenous groups in the Philippines are generally influenced by their cultural, spiritual, and religious beliefs of supernatural beings. *Curcuma longa* is the most preferred medicinal plant administered by the *Panay Bukidnon* to a sick person with conditions caused by unseen beings. In Hindu worship rights, *C. longa* has been used for offerings and magic (Velayudhan et al., 2012).

Plant-based compounds have been in constant use since ancient times for any emerging disease. There were several bioactive compounds extracted from medicinal plants with promising antiviral properties against the novel coronavirus (COVID-19) (Adhikari et al., 2020). In Thailand, 60 medicinal plant species were used to treat mild symptoms of COVID-19 (Phumthum et al., 2021). In Nepal, there were also 60 medicinal plants used (Khadka et al., 2021) and 23 plants in Morroco (El Alami et al., 2020) for potential COVID-19 therapy and *Zingiber officinale* is one of the common species used. In Bangladesh, phytochemicals extracted from *Calotropis gigantea* exhibited positive inhibitory effects against the COVID-19 virus (Dutta et al., 2021), as well as the alkaloids and terpenoids isolated from plants of African origin (Gyebi et al., 2021). *Curcumin* from *C. longa* also showed promising effects against the virus (Adhikari et al., 2020).

In the Philippines, the Department of Science and Technology (DOST) has been conducted clinical trials and explored the therapeutic effects of the virgin coconut oil (VCO), *Euphorbia hirta* (tawa-tawa), and *Vitex trifolia* (lagundi) for their potential efficacy against COVID-19 infection (Arayata, 2021). In the recent updates published in the Global Media Arts (GMA) news articles, clinical trials for *V. trifolia* and *E. hirta* have been proven to decrease mild-to-moderate symptoms of COVID-19. Mild-to-moderate symptoms of 172 random COVID-19 patients disappeared within 3–5 days after taking a 1,950 mg capsule of *E. hirta* thrice a day for ten days as a food supplement. *V. trifolia* also showed a positive result in decreasing mild symptoms of COVID-19. Community trials of VCO as an adjuvant for mild symptoms of COVID-19 patients showed a positive result in decreasing the virus count by 60–90%. A clinical trial of VCO on mild and severe symptoms of COVID-19 conducted in Philippine General Hospital is still ongoing (Global Media Arts News, 2021a; Global Media Arts News, 2021b).

V. trifolia, *C. longa, Z. officinale, Capsicum annuum, E. hirta,* and *Peperomia pellucida* were used by the *Panay Bukidnon* as an alternative medicine to strengthen their immunity and they have claimed that these species can alleviate the symptoms of the COVID-19 infection. They used these plants traditionally to treat fever, headache, cough, and body pains, which were also the common indications of COVID-19. Further pharmacological research and investigations are highly suggested for these medicinal plants to explore their potential uses and therapeutic effects against COVID-19 infection especially for *Zingiber officinale, Capsicum annuum*, and *Peperomia pellucida* species. The *Panay Bukidnon* also believed that chewing betel quid could give them the strength to fight the virus. Chewing betel quid has been a customary practice of Filipinos since the pre-Spanish colonial period throughout the Philippines. It is part of the social undertakings and ceremonies and is believed to increase stamina, good health, and longevity (Valdes, 2004). In India, the practice of chewing betel dates back to around 75 AD and it is known for centuries for its therapeutic properties (Toprani and Patel 2013). A review was conducted on the synergistic prophylaxis effects of *Piper betle* and gold ash can hypothetically limit and manage the COVID-19 infection (Sharma and Malik, 2020).

For the comparative review performed on the medicinal plants with other ethnobotanical studies conducted in the country, three species (*Eleutherine palmifolia*, *Hibiscus acetosella*, and *Oxalis triangularis*) showed no record of medicinal value in the previous studies. *E. palmifolia* is used by the *Dayaks* in Indonesia to treat a variety of diseases such as diabetes, cancer, hypertension, stroke, and sexual disorders and as a galactagogue. Bioactive compounds from this species contain various pharmacological activities such as antibacterial, anti-inflammatory, anticancer, and antidiabetic (Kamarudin et al., 2021). *H. acetosella* is used as therapy for anemia in Southern Uganda (Ssegawa and Kasenene, 2007) and its phenolic compounds exhibit antioxidant and antibacterial properties (Lyu et al., 2020). Limited literature is available for *O. triangularis,* but its medicinal uses include remedies for fever, UTI, mouth sores, cuts, rashes, and skin infections (Arakelyan and Arakelyan, 2020). The comparison of the medicinal plants and their uses was performed with five ethnobotanical studies that were previously conducted in the rural and urban communities and villages (Tantiado, 2012; Gruyal et al., 2014; Agapin, 2019; Belgica et al., 2021; Montero and Geducos, 2021), 17 studies conducted in the IP communities (Balangcod and Balangcod, 2011; Olowa et al., 2012; Abe and Ohtani, 2013; Ragragio et al., 2013; Ong and Kim, 2014; Raterta et al., 2014; Balangcod and Balangcod, 2015; Pizon et al., 2016; Odchimar et al., 2017; Baddu and Ouano, 2018; Tantengco et al., 2018; Pablo, 2019; Cordero et al., 2020; Dapar et al., 2020; Paraguison et al., 2020; Cordero and Alejandro, 2021; Nuñeza

et al., 2021) all over the country, and one online database: the Philippine Traditional Knowledge Digital Library on Health (Philippine Traditional Knowledge Digital Library on Health, 2021). The PTKDL is an electronic library that documented 16,690 enumerations of medicinal plant preparations and 66 healing rituals and practices mentioned by 509 traditional healers in 43 different research sites in the country (World Health Organization, 2019). The database (https://www.tkdlph.com/) recorded about 1,200 medicinal plants used by the local and indigenous communities from different ethnobotanical studies, lexicographic and linguistic texts, and current researches conducted in selected indigenous communities nationwide.

CONCLUSION

The ethnobotanical use of many different plant species is an important predominating practice in the Philippines. It is an integral part of Filipino custom and tradition and has been culturally accepted for ages. The results of this ethnobotanical documentation of 131 medicinal plants used in addressing 91 diseases across 16 different disease categories portray the strong dependence of the *Panay Bukidnon* in the medicinal flora in their area. This could be attributed to the great distance of the study site to the town and the health centers or well-functioning hospitals. The most culturally relevant and important species recorded in this study in terms of UV, RFC, RI, ICF, and FL are *Curcuma longa*, *Blumea balsamifera*, *Artemisia vulgaris, Annona muricata,* and *Chromolaena odorata,* respectively. The efficacy and effectivity of the therapeutic claims of these species must be further pharmacologically investigated and validated. These species have been used for centuries by many people worldwide and have proven to cure a myriad of diseases. The comparative study of the medicinal plants with other ethnobotanical studies revealed some novel and additional therapeutic uses that are valuable to the immense body of traditional knowledge and practices in the country. The traditional knowledge and practices from indigenous peoples add more treatment opportunities for potential therapy of pre-existing and novel diseases. The indigenous knowledge on the medicinal plants used by the *Panay Bukidnon* is passed from one generation to the other mostly in oral forms with the influence of their religious and cultural beliefs. Furthermore, it is urgent to document the indigenous knowledge before it is forgotten because of environmental and social challenges such as species extinction, climate change, acculturation, modernization, availability and accessibility of prescribed medicines, and lack of interest of the younger generations. The results of this study also serve as a medium for preserving cultural heritage, ethnopharmacological bases for further drug research and discovery, and preserving biological diversity. The ethnobotanical study on the *Panay Bukidnon* communities in Panay Island is limited by the expensive and lengthy process of acquiring government permits and by the fact that some communities are infested by leftists (New People's Army) that could risk the safety of researchers and there are no access roads in the upland areas. Lastly, it is strongly recommended to conduct further comprehensive surveys on other *Panay Bukidnon* communities in other provinces of the Panay Island and to conduct pharmacological studies and investigations on the important medicinal plants, especially the ones that have high ICF and FL values for potential drug development and formulation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Graduate School–Ethics Review Committee, University of Santo Tomas. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CSC processed and acquired the necessary government permits, conducted the field works, and drafted the manuscript. All authors designed the study, GJDA and UM supervised, reviewed, and made the final revision of the manuscript.

FUNDING

The research had partial funding from the scholarship of the first author from the Commission on Higher Education K-12 Transition Program (CHED K-12) and the Digital Cooperation Fellowship from the Alexander von Humboldt Foundation awarded to GJDA and UM.

ACKNOWLEDGMENTS

The authors are forever grateful to the *Panay Bukidnon* IP leader, the council of elders, members, and Brgy. officials of Brgy. Caguisanan, Lamunao, Iloilo; DENR Region VI for the Wildlife Gratuitous Permit; NCIP Iloilo-Guimaras Service Center officers for facilitating the community meetings; NCIP Region VI/VII for the issuance of the Certification Precondition. Heartfelt gratitude is also given to the Ethics Review Committee of the University of Santo Tomas Graduate School, Danilo Tandang of the Philippine

National Museum, and Michael Calaramo of the Herbarium of the Northwestern University Luzon. CSC would like to thank CHED K-12 for her scholarship. GJDA and UM acknowledge the Alexander von Humboldt Foundation for the Digital Cooperation Fellowship.

REFERENCES

Abe, R., and Ohtani, K. (2013). An Ethnobotanical Study of Medicinal Plants and Traditional Therapies on Batan Island, the Philippines. *J. Ethnopharmacol.* 145, 554–565. doi:10.1016/j.jep.2012.11.029

Adhikari, B., Marasini, B. P., Rayamajhee, B., Bhattarai, B. R., Lamichhane, G., Khadayat, K., et al. (2020). Potential Roles of Medicinal Plants for the Treatment of Viral Diseases Focusing on COVID-19: A Review. *Phytother. Res.* 5, 1298–1312. doi:10.1002/ptr.6893

Agapin, J. S. F. (2019). Medicinal Plants Used by Traditional Healers in Pagadian City, Zamboanga del Sur, Philippines. *Philipp. J. Sci.* 149, 83–89. doi:10.2139/ssrn.3625332

Arakelyan, S. S., and Arakelyan, H. S. (2020). *Beauty for Health – Oxalis Triangularis*. Available at: https://www.researchgate.net/publication/340829095_Beauty_For_Health_-Oxalis_Triangularis (Accessed November 5, 2021).

Arayata, M. C. (2021). *PH Researchers Study Herbal Plants' Benefit in Fight vs. Covid-19*. Philippine News Agency. Available at: https://www.pna.gov.ph/articles/1104955 (Accessed September 03, 2021).

Baddu, V., and Ouano, N. (2018). Ethnobotanical Survey of Medicinal Plants Used by the *Y'Apayaos* of Sta. Praxedes in the Province of Cagayan, Philippines. *Mindanao J. Sci. Technol.* 16, 128–153.

Balangcod, T., and Balangcod, A. K. (2011). Ethnomedical Knowledge of Plants and Healthcare Practices Among the Kalanguya Tribe in Tinoc, Ifugao, Luzon, Philippines. *Indian J. Tradit Knowl.* 10, 227–238.

Balangcod, T., and Balangcod, A. K. (2015). Ethnomedicinal Plants in Bayabas, Sablan, Benguet Province, Luzon, Philippines. *E J. Biol.* 11, 63–73.

Belgica, T. H., Suba, M., and Alejandro, G. J. (2021). Quantitative Ethnobotanical Study of Medicinal flora Used by Local Inhabitants in Selected Barangay of Malinao, Albay, Philippines. *Biodiversitas* 22, 2711–2721. doi:10.13057/biodiv/d220720

Beyer, O. H. (1917). *Population of the Philippine islands in 1916 (población de las islas Filipinas en 1916) prepared under the direction of, preparado bajo la dirección de H. Otley Beyer*. Manila, Philippines: Philippine Education Co. Inc.

Chanda, S., and Kaneria, M. (2011). in *Indian Nutraceutical Plant Leaves as a Potential Source of Natural Antimicrobial Agents in Science against Microbial Pathogens: Communicating Current Research and Technological Advances*. Editor M. Vilas (Badajoz, Spain: Formatex Research Center), 1251–1259.

Cordero, C., and Alejandro, G. J. D. (2021). Medicinal Plants Used by the Indigenous Ati Tribe in Tobias Fornier, Antique, Philippines. *Biodiversitas* 22, 521–536. doi:10.13057/biodiv/d220203

Cordero, C., Ligsay, A., and Alejandro, G. (2020). Ethnobotanical Documentation of Medicinal Plants Used by the *Ati* Tribe in Malay, Aklan, Philippines. *J. Complement. Med. Res.* 11, 170–198. doi:10.5455/jcmr.2020.11.01.20

Coria-Téllez, A. V., Montalvo-GónzalezYahia, E. E. M., and Obledo-Vázquez, E. N. (2018). *Annona Muricata*: A Comprehensive Review on its Traditional Medicinal Uses, Phytochemicals, Pharmacological Activities, Mechanisms of Action and Toxicity. *Arab. J. Chem.* 11, 662–691. doi:10.1016/j.arabjc.2016.01.004

Dapar, M. L. G., Alejandro, G. J. D., Meve, U., and Liede-Schumann, S. (2020). Quantitative ethnopharmacological documentation and molecular confirmation of medicinal plants used by the Manobo tribe of Agusan del Sur, Philippines. *J. Ethnobiol. Ethnomed* 16, 14. doi:10.1186/s13002-020-00363-7

Department of Environment and Natural Resources (2019). *Regional Profile: Historical Background of the Region*. DENR Region. Available at: https://r6.denr.gov.ph/index.php/about-us/regional-profile (Accessed September 02, 2021).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.790567/full#supplementary-material

Dutta, M., Nezam, M., Chowdhury, S., Rakib, A., Paul, A., Sami, S. A., et al. (2021). Appraisals of the Bangladeshi Medicinal Plant *Calotropis Gigantea* Used by Folk Medicine Practitioners in the Management of COVID-19: A Biochemical and Computational Approach. *Front. Mol. Biosci.* 8, 625391. doi:10.3389/fmolb.2021.625391

Ealdama, E. (1938). *The Monteses of Panay*, 35. Manila, Philippines: Philippine Magazine.

Ekiert, H., Pajor, J., Klin, P., Rzepiela, A., Ślesak, H., and Szopa, A. (2020). Significance of Artemisia Vulgaris L. (Common Mugwort) in the History of Medicine and its Possible Contemporary Applications Substantiated by Phytochemical and Pharmacological Studies. *Molecules* 25, 4415. doi:10.3390/molecules25194415

El Alami, A., Fattah, A., and Chait, A. (2020). Medicinal Plants Used for the Prevention Purposes during the Covid-19 Pandemic in Morocco. *J. Anal. Sci. Appl. Biotechnol.* 2, 4–11. doi:10.48402/IMIST.PRSM/jasab-v2i1.21056

Friedman, J., Yaniv, Z., Dafni, A., and Palewitch, D. (1986). A Preliminary Classification of the Healing Potential of Medicinal Plants, Based on a Rational Analysis of an Ethnopharmacological Field Survey Among Bedouins in the Negev Desert, Israel. *J. Ethnopharmacol.* 16, 275–287. doi:10.1016/0378-8741(86)90094-2

German, M. A. (2010). *(Re)searching Identity in the highlands of Central Panay*. Quezon City, Philippines: Ugnayang Pang-Aghamtao, Inc. Vol. 19, 20–37.

Global Media Arts News (2021a). DOST Exec Bares Positive Results of Lagundi, Tawa-Tawa Trials. Available at: https://www.gmanetwork.com/news/scitech/science/807308/dost-exec-bares-positive-results-of-lagundi-tawa-tawa-trials/story/(Accessed October 30, 2021).

Global Media Arts News (2021b). VCO Trials Show Big Drop in Virus Count of Mild COVID-19 Cases. Available at: https://www.gmanetwork.com/news/scitech/science/807310/vco-trials-show-big-drop-in-virus-count-in-mild-covid-19-cases/story/(Accessed October 30, 2021).

Gowey, D. (2016). *Palawod, Pa-Iraya: A Synthesis of Panay Bukidnon Inland Migration Models*. Available at: https://www.academia.edu/29928804/Palawod_Pairaya_A_Synthesis_of_Panay_Bukidnon_Inland_Migration_Models (Accessed September 03, 2021).

Gruyal, G., del Rosario, R., and Palmes, N. (2014). Ethnomedicinal Plants Used by Residents in Northern Surigao del Sur,Philippines. *Nat. Prod. Chem. Res. 2014* 2, 1–5. doi:10.4172/2329-6836.1000140

Tantengco, O. A. G., Condes, M. L. C., Estadilla, H. H. T., and Ragragio, E. M. (2018). Ethnobotanical Survey of Medicinal Plants Used by Ayta Communities in Dinalupihan, Bataan, Philippines. *Pharmacog. J.* 10, 859–870. doi:10.5530/pj.2018.5.145

Gyebi, G. A., Ogunro, O. B., Adegunloye, A. P., Ogunyemi, O. M., and Afolabi, S. O. (2021). Potential Inhibitors of Coronavirus 3-chymotrypsin-like Protease (3CLpro): an In Silico Screening of Alkaloids and Terpenoids from African Medicinal Plants. *J. Biomol. Struct. Dyn.* 39, 1–13. doi:10.1080/07391102.2020.1764868

Heinrich, M., Ankli, A., Frei, B., Weimann, C., and Sticher, O. (1998). Medicinal Plants in Mexico: Healers' Consensus and Cultural Importance. *Soc. Sci. Med.* 47, 1859–1871. doi:10.1016/s0277-9536(98)00181-6

International Plant Names Index (2021). *The Royal Botanic Gardens, Kew, Harvard University Herbaria & Libraries and Australian National Botanic Gardens*. Available at: http://www.ipni.org (Accessed September 04, 2021).

Jain, C., Khatan, S., and Vijayvergia, R. (2019). Bioactivity of Secondary Metabolites of Various Plants: a Review. *Int. J. Pharm. Sci. Res.* 10, 494–504. doi:10.13040/IJPSR.0975-8232.10(2).494-04

Jocano, L. (1968). *Sulud Society. A Study in the Kinship System and Social Organization of a Mountain People of Central Panay. Diliman*. Quezon City: University of the Philippines Press.

Kamarudin, A. A., Sayuti, N. H., Saad, N., Razak, N. A. A., and Esa, N. M. (2021). *Eleutherine Bulbosa* (Mill.) Urb. Bulb: Review of the Pharmacological Activities and its Prospects for Application. *Int. J. Mol. Sci.* 22, 6747. doi:10.3390/ijms22136747

Khadka, D., Dhamala, M. K., Li, F., Aryal, P. C., Magar, P. R., Bhatta, S., et al. (2021). The Use of Medicinal Plants to Prevent COVID-19 in Nepal. *Ijms* 22, 26. doi:10.1186/s13002-021-00449-w

Lyu, J. I., Ryu, J., Jin, C. H., Kim, D. G., Kim, J. M., Seo, K. S., et al. (2020). Phenolic Compounds in Extracts of *Hibiscus acetosella* (Cranberry Hibiscus) and Their Antioxidant and Antibacterial Properties. *Molecules* 25 (18), 4190. doi:10.3390/molecules25184190

Madjos, G., and Ramos, K. (2021). Ethnobotany, Systematic Review and Field Mapping on Folkloric Medicinal Plants in the Zamboanga Peninsula, Mindanao, Philippines. *J. Complement. Med. .Res.* 12. doi:10.5455/jcmr.2021.12.01.05

Madulid, D. A., Gaerlan, F. J. M., Romero, E. M., and Agoo, E. M. G. (1989). Ethnopharmacological Study of the Ati Tribe in Nagpana, Barotac Viejo, Iloilo. *Acta Manil* 38, 25–40.

Magos, A. (2004). *Balay Turun-An: An Experience in Implementing Indigenous Education in Central Panay*, 13. Quezon City, Philippines: Ugnayang Pang-Aghamtao, Inc., Vol. 19, 95–102.

Magos, A. (1999). Sea Episodes in the Sugidanon (Epic) and Boat-Buildng Tradition in Central Panay, Philippines. *Danyag (UP Visayas Journal of the Social Sciences and the Humanities)* 4, 5–29.

Moghadamtousi, S. Z., Fadaeinasab, M., Nikzad, S., Mohan, G., Ali, H. M., and Kadir, H. A. (2015). *Annona Muricata* (Annonaceae): A Review of its Traditional Uses, Isolated Acetogenins and Biological Activities. *Int. J. Mol. Sci.* 16, 15625–15658. doi:10.3390/ijms160715625

Montero, J. C., and Geducos, D. T. (2021). Ethnomedicinal plants used by the local folks in two selected villages of San Miguel and Surigao del Sur, villages of San Miguel, Surigao del Sur, Mindanao, Philippines. *Int. J. Agric. Technol.* 17, 193–212.

National Commission on Indigenous Peoples (2019). *Community Population (Census) Province of Antique and Aklan*. San Jose Buenavista, Antique: National Commission on Indigenous Peoples Antique/Aklan Community Service Center.

National Commission on Indigenous Peoples (2011). *Indigenous Peoples MasterPlan (2012-2016)*. Available at: https://www.ombudsman.gov.ph/UNDP4/wp-content/uploads/2013/02/Indigenous-Peoples-Master-Plan-2012-2016.pdf (Accessed August 21, 2021).

National Commission on Indigenous Peoples (2020). *Region VI Statistical Report 2020*. Available at: https://www.ncipro67.com.ph/statistics-corner/(Accessed September 01, 2021).

National Economic Development Agency (2017). *Philippine Development Plan 2017-2022*. Available at: http://pdp.neda.gov.ph/(Accessed August 02, 2021).

Nuneza, O., Rodriguez, B., and Nasiad, J. G. (2021). Ethnobotanical survey of medicinal plants used by the Mamanwa tribe of Surigao del Norte and Agusan del Norte, Mindanao, Philippines. *Biodiversitas* 22, 3284–3296. doi:10.13057/biodiv/d220634

Obico, J. J. A., and Ragrario, E. M. (2014). A Survey of Plants Used as Repellants against Hematophagous Insects by the Ayta People of Porac, Pampanga Province. *Phil. Sci. Lett.* 7, 179–186.

Odchimar, N. M., Nuñeza, O., Uy, M., and Senarath, W. T. P. S. (2017). Ethnobotany of Medicinal Plants Used by the Talaandig Tribe in Brgy. Lilingayon, Valencia City, Bukidnon, Philippines. *Asian J. Biol. Life Sci.* 6, 358–364.

Olowa, L., Torres, M. A., Aranico, E., and Demayo, C. (2012). Medicinal Plants Used by the Higaonon Tribe of Rogongon, Iligan City, Mindanao, Philippines. *Adv. Environ. Biol.* 6, 1442–1449. doi:10.3923/erj.2012.164.174

Ong, H. G., and Kim, Y.-D. (2015). Herbal Therapies and Social-Health Policies: Indigenous Ati Negrito Women's Dilemma and Reproductive Healthcare Transitions in the Philippines. *Evidence-Based Complement. Altern. Med.* 2015, 1–13. doi:10.1155/2015/491209

Ong, H. G., and Kim, Y. D. (2014). Quantitative Ethnobotanical Study of the Medicinal Plants Used by the Ati Negrito Indigenous Group in Guimaras Island, Philippines. *J. Ethnopharmacol.* 157, 228–242. doi:10.1016/j.jep.2014.09.015

Pablo, C. G. (2019). Botika Sa Kalikasan: Medicinal Plants Used by Aetas of Sitio Parapal Hermosa Bataan, Philippines. *J. Soc. Health* 2, 101–127.

Pang, Y., Wang, D., Fan, Z., Chen, X., Yu, F., Hu, X., et al. (2014). *Blumea Balsamifera*--a Phytochemical and Pharmacological Review. *Molecules* 19, 9453–9477. doi:10.3390/molecules19079453

Paraguison, L. D. R., Tandang, D. N., and Alejandro, G. J. D. (2021). Medicinal Plants Used by the Manobo Tribe of Prosperidad, Agusan Del Sur, Philippinesan Ethnobotanical Survey. *Ajbls* 9, 326–333. doi:10.5530/ajbls.2020.9.49

Parasuraman, S., Thing, G. S., and Dhanaraj, S. A. (2014). Polyherbal Formulation: Concept of Ayurveda. *Pharmacogn. Rev.* 8, 73–80. doi:10.4103/0973-7847.134229

Pelser, P. B., Barcelona, J. F., and Nickrent, D. L. (2011). *Co's Digital Flora of the Philippines*. Available at: www.philippineplants.org (Accessed September 02, 2020).

Philippine Statistics Authority (2021). *2020 Census of Population and Housing (2020 CPH) Population Counts Declared Official by the President*. Available at: https://psa.gov.ph/content/2020-census-population-and-housing-2020-cph-population-counts-declared-official-president (Accessed November 11, 2021).

Philippine Traditional Knowledge Digital Library on Health (2021). *Philippine Plants and Natural Products Used Traditionally*. Available at: https://www.tkdlph.com/index.php/ct-menu-item-3/ct-menu-item-7 (Accessed October 30, 2021).

Phillips, O., and Gentry, A. H. (1993). The Useful Plants of Tambopata, Peru: I. Statistical Hypotheses Tests with a New Quantitative Technique. *Econ. Bot.* 47, 15–32. doi:10.1007/bf02862203

Phumthum, M., Nguanchoo, V., and Balslev, H. (2021). Medicinal Plants Used for Treating Mild Covid-19 Symptoms Among Thai Karen and Hmong. *Front. Pharmacol.* 12, 699897. doi:10.3389/fphar.2021.699897

Pizon, J. R. L., Nuneza, O. M., Uy, M. M., and Senarath, W. T. P. S. K. (2016). Ethnobotany of Medicinal Plants Used by the Subanen Tribe of Lapuyan, Zamboanga del Sur. *Bull. Env. Pharmacol. Life Sci.* 5, 53–67.

Plants of the World Online (2021). *Facilitated by the Royal Botanic Gardens, Kew*. Available at: http://plantsoftheworldonline.org/(Accessed September 03, 2021).

Prasad, S., and Aggarwal, B. B. (2011). "Turmeric, the Golden Spice: From Traditional Medicine to Modern Medicine," in *Herbal Medicine: Biomolecular and Clinical Aspects*. Editors I. F. F. Benzie and S. Wachtel-Galor (Boca Raton, FL: CRC Press/Taylor & Francis).

Province of Iloilo (2015). *About Iloilo*. Available at: https://www.iloilo.gov.ph/about-iloilo (Accessed September 01, 2021).

Provincial Planning Development Office (2018). *Iloilo Provincial Annual Profile*. Available at: https://www.iloilo.gov.ph/iloilo-provincial-annual-profile (Accessed August 20, 2021).

Pucot, J. R., Manting, M. M. E., and Demayo, C. G. (2019). Ethnobotanical Plants Used by Selected Indigenous Peoples of Mindanao, the Philippines as Cancer Therapeutics. *Pharmacophore* 10, 61–69.

Ragragio, E., Zayas, C. N., and Obico, J. J. A. (2013). Useful Plants of Selected Ayta Communities from Porac, Pampanga, Twenty Years after the Eruption of Mt. Pinatubo. *Philipp. J. Sci.* 142, 169–181.

Raterta, R., de Guzman, G. Q., and Alejandro, G. J. D. (2014). Assessment, Inventory and Ethnobotanical Survey of Medicinal Plants in Batan and Sabtang Island (Batanes Group of Islands, Philippines). *Int. J. Pure App. Biosci.* 2, 147–154.

Sharma, S., and Malik, J. (2020). Synergistic Prophylaxis on COVID-19 by Nature Golden Heart (*Piper Betle*) & Swarna Bhasma. *Asian J. Res. Dermat. Sci.* 3, 21–27.

Smith, W. (1915). *Notes on the Geology of Panay. From the Division of Mines*. Manila, Philippines: Bureau of Science.

Ssegawa, P., and Kasenene, J. M. (2007). Medicinal Plant Diversity and Uses in the Sango bay Area, Southern Uganda. *J. Ethnopharmacol* 113 (3), 521–540. doi:10.1016/j.jep.2007.07.014

Talledo, T. (2004). Construction of Identity in Central Panay: A Critical Examination of the Ethnographic Subject in the Works of Jocano and Magos. *Asian Stud.* 40, 111–123.

Tantiado, R. (2012). Survey on Ethnopharmacology of Medicinal Plants in Iloilo, Philippines. *Inter. J. Bio-sci. Bio-tech.* 4, 11–26.

Tardío, J., and Pardo-De-Santayana, M. (2008). Cultural Importance Indices: a Comparative Analysis Based on the Useful Wild Plants of Southern Cantabria (Northern Spain)1. *Econ. Bot.* 62, 24–39. doi:10.1007/s12231-007-9004-5

Toprani, R., and Patel, D. (2013). Betel Leaf: Revisiting the Benefits of an Ancient Indian Herb. *South. Asian J. Cancer* 2, 140–141. doi:10.4103/2278-330X.114120

Tropicos (2021). *Missouri Botanical Garden*. Available at: https://tropicos.org (Accessed September 02, 2021).

Tu, Y. (2011). The Discovery of Artemisinin (Qinghaosu) and Gifts from Chinese Medicine. *Nat. Med.* 17, 1217–1220. doi:10.1038/nm.2471

United Nations Development Programme (2019). *10 Things to Know about Indigenous Peoples*. Available at: https://stories.undp.org/10-things-we-all-should-know-about-indigenous-people (Accessed August 06, 2021).

Valdes, C. O. (2004). Betel Chewing in the Philippines. *Arts of Asia* 34, 104–115.

Velayudhan, K. C., Dikshit, N., and Nizar, M. A. (2012). Ethnobotany of Turmeric (*Curcuma Longa* L.). *Indian J. Tradit. Knowl.* 11, 607–614.

Vijayaraghavan, K., Rajkumar, J., Bukhari, S. N., Al-Sayed, B., and Seyed, M. A. (2017). *Chromolaena Odorata*: A Neglected weed with a Wide Spectrum of Pharmacological Activities (Review). *Mol. Med. Rep.* 15, 1007–1016. doi:10.3892/mmr.2017.6133

Wanda, J. M., Gamo, F., and Njamen, D. (2015). "Medicinal Plants of the Family of Fabaceae Used to Treat Various Ailments in Fabaceae," in *Classification, Nutrient Composition and Health Benefits Series*. Editor W. Garza (NY: Nova Science Publishers, Inc.).

World Flora Online (2021). *World Flora Online*. Available at: http://www.worldfloraonline.org/(Accessed September 02, 2021).

World Health Organization (2021). *ICD-11 International Classification of Diseases 11th Revision*. Available at: https://icd.who.int/en (Accessed September 03, 2021).

World Health Organization (2019). *WHO Global Report on Traditional and Complementary Medicine*. Available at: https://www.who.int/traditionalcomplementary-integrative medicine/Who Global Report On TraditionalAndComplementaryMedicine2019.pdf?ua=1 (Accessed October 28, 2021).

World Health Organization (1998). *Working Group on Herbal Medicines*. Manila: WHO Regional Office for the Western Pacific.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 11 January 2022
doi: 10.3389/fphar.2021.714434

Analytical Challenges and Metrological Approaches to Ensuring Dietary Supplement Quality: International Perspectives

Alessandra Durazzo[1], Barbara C. Sorkin[2], Massimo Lucarini[1], Pavel A. Gusev[3], Adam J. Kuszak[2], Cindy Crawford[4,5], Courtney Boyd[4,5], Patricia A. Deuster[4,5], Leila G. Saldanha[2], Bill J. Gurley[6], Pamela R. Pehrsson[3], James M. Harnly[3], Aida Turrini[1], Karen W. Andrews[3], Andrea T. Lindsey[4,5], Michael Heinrich[7] and Johanna T. Dwyer[2]**

[1]CREA - Research Centre for Food and Nutrition, Rome, Italy, [2]Office of Dietary Supplements, National Institutes of Health, US Department of Health and Human Services, Bethesda, MD, United States, [3]Beltsville Human Nutrition Research Center, Agricultural Research Service, US Department of Agriculture, Bethesda, MD, United States, [4]Consortium for Health and Military Performance, Department of Military & Emergency Medicine, F. Edward Hebert School of Medicine, Uniformed Services University, Bethesda, MD, United States, [5]Henry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, MD, United States, [6]National Center for Natural Products Research, University of Mississippi, Bethesda, MD, United States, [7]UCL School of Pharmacy, Pharmacognosy and Phytotherapy, London, United Kingdom

OPEN ACCESS

Edited by:
Lie-Fen Shyur,
Academia Sinica, Taiwan

Reviewed by:
Pavel Klouček,
Czech University of Life Sciences Prague, Czechia
Angela Calderon,
Auburn University, United States

***Correspondence:**
Alessandra Durazzo
alessandra.durazzo@crea.gov.it
Johanna T. Dwyer
dwyerj1@od.nih.gov

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 25 May 2021
Accepted: 16 December 2021
Published: 11 January 2022

Citation:
Durazzo A, Sorkin BC, Lucarini M, Gusev PA, Kuszak AJ, Crawford C, Boyd C, Deuster PA, Saldanha LG, Gurley BJ, Pehrsson PR, Harnly JM, Turrini A, Andrews KW, Lindsey AT, Heinrich M and Dwyer JT (2022) Analytical Challenges and Metrological Approaches to Ensuring Dietary Supplement Quality: International Perspectives. Front. Pharmacol. 12:714434. doi: 10.3389/fphar.2021.714434

The increased utilization of metrology resources and expanded application of its' approaches in the development of internationally agreed upon measurements can lay the basis for regulatory harmonization, support reproducible research, and advance scientific understanding, especially of dietary supplements and herbal medicines. Yet, metrology is often underappreciated and underutilized in dealing with the many challenges presented by these chemically complex preparations. This article discusses the utility of applying rigorous analytical techniques and adopting metrological principles more widely in studying dietary supplement products and ingredients, particularly medicinal plants and other botanicals. An assessment of current and emerging dietary supplement characterization methods is provided, including targeted and non-targeted techniques, as well as data analysis and evaluation approaches, with a focus on chemometrics, toxicity, dosage form performance, and data management. Quality assessment, statistical methods, and optimized methods for data management are also discussed. Case studies provide examples of applying metrological principles in thorough analytical characterization of supplement composition to clarify their health effects. A new frontier for metrology in dietary supplement science is described, including opportunities to improve methods for analysis and data management, development of relevant standards and good practices, and communication of these developments to researchers and analysts, as well as to regulatory and policy decision makers in the public and private sectors. The promotion of closer interactions between analytical, clinical, and pharmaceutical scientists who are involved in research and product development with metrologists who develop standards and methodological guidelines is critical to advance research on dietary supplement characterization and health effects.

Keywords: dietary supplements, food supplements, analytical methodologies, metrological approaches, data management, infrastructures, institutional efforts, case studies

INTRODUCTION

Definition

Metrology is the science of measurement and its practice emphasizes an assessment of traceability and measurement uncertainty, concepts that are not always given the attention they deserve in analytical chemistry (King, 1997). It is responsible for the development of internationally agreed upon reference points so that the accuracy, precision, and repeatability of measures of doses or activity can be compared. Metrologists work with research and industry scientists who are making measurements to call attention to standards and to develop and disseminate best practices and new methods.

Present Context

Dietary supplements (abbreviated as DS throughout this article using the regulatory definitions and framework of the United States) include a variety of ingredients in different countries. They include food supplements and some botanical and herbal medicines in Europe and other regions, listed medicines in Australia, and Natural Health Products in Canada. DS are widely consumed in many countries, purchased not only through brick-and-mortar stores, but also through a variety of online and other marketing channels[1] (Binns et al., 2018). The products sold often contain dozens of ingredients that vary greatly in their chemical composition, as well as in the information provided about their contents on packaging labels (Dwyer et al., 2018). Both consumers and practitioners expect that supplements contain the ingredients and amounts listed on their labels, and researchers require well-characterized, authenticated products to obtain replicable results. Recommendations and guidance have been issued by regulatory authorities[2]. Nevertheless, when supplements are evaluated, their contents often do not match label claims and quality problems are common, including the presence of prescription drugs, other pharmaceuticals, contaminants (e.g., pesticides, heavy metals, mycotoxins, microbes), misbranded herbal and botanical ingredients, adulterants caused by economic motivations, fillers, dyes, and filth. In other instances, ingredients listed on the label are absent, raising further questions about the quality of DS (Martinez-Sanz et al., 2017).

A great deal of attention was devoted to DS and their sales rose rapidly during the COVID-19 epidemic. Mullin et al. (2021) have recently reviewed commonly used immune-modulating DS, including vitamin D, ascorbic acid, zinc, and melatonin, highlighting the biological plausibility for salutary benefit against COVID-19. However, another recent review on DS in the time of COVID 19 issued by the US National Institutes of Health concluded that currently, data are insufficient to support recommendations for or against the use of any vitamin, mineral, herb or other botanical, fatty acid, or other dietary supplement ingredient to prevent or treat COVID-19[3].

The safety, quality, and efficacy of supplement ingredients are highly relevant, especially for the more chemically complex DS with beneficial but also adverse health effects (Di Lorenzo et al., 2015; Roytman et al., 2018). They include botanicals as well as products with blends of botanicals and other non-vitamin, non-mineral ingredients and various nutrients. Many such products marketed for sexual enhancement, weight loss, pre-workout, and body building purposes have been found to be spiked with synthetic and often unlicensed drugs. Structure-function claims do not guarantee efficacy or approval by regulatory authorities in the United States[3,4]. In Italy as well (Ministero della Salute, 2020), product registration does not correspond to a scientific evaluation[4,5]. It is crucial to understand exactly what the claims on DS mean (American Cancer Society, 2021). The claims should be true and not misleading[2] (Muela-Molina et al., 2021). When they are not, there is good reason for concern about consumer safety (Binns et al., 2018; Crawford et al., 2020). This is particularly true because many adults but also some children are supplement users (Smith et al., 2005; Parnell et al., 2006; O'Brien et al., 2017).

If the analytical problems outlined above, coupled with control and compliance issues, were brought to the attention of the public, consumer and healthcare provider trust in DS would be reduced. Therefore, all stakeholders should pay greater attention to dietary supplement quality through analytical chemistry and better characterization, since these are critical steps in developing and maintaining trust and providing safe, high quality supplement products.

OBJECTIVES

This article discusses the utility of applying rigorous analytical techniques and adopting metrological principles more widely in the study of dietary supplement products and ingredients. With

[1]https://www.rakutenintelligence.com/blog/2016/online-vitamin-sales-growing-faster-rest-e-commerce.

[2]https://www.fda.gov/food/dietary-supplements-guidance-documents-regulatory-information/dietary-supplement-labeling-guide https://www.fda.gov/food/dietary-supplements-guidance-documents-regulatory-information/dietary-supplement-labeling-guide-chapter-iv-nutrition-labeling; https://www.teknoscienze.com/Contents/Riviste/PDF/DIET_SUPP_AF4_2013_RGB_16-17.pdf; http://www.ahpa.org/Portals/0/PDFs/Policies/Guidance-Documents/AHPA_Herbal_Supplements_Labeling_Guide.pdf.

[3]Dietary supplements in the time of Covid19 https://ods.od.nih.gov/factsheets/COVID19-HealthProfessional/.

[4]https://www.fdareader.com/blog/tag/structure+function+claims.

[5]http://www.salute.gov.it/portale/temi/p2_6.jsp?lingua=italiano&id=3668&area=Alimenti%20particolari%20e%20integratori&menu=registri.

respect to analytical chemistry, some of the newer direct, objective, rapid, and inexpensive quality control methods that could be used internationally to identify ingredients are briefly discussed. The present status of efforts to develop new methodologies and approaches for data analysis, evaluation and management that can be applied to evaluate dietary supplement quality is reviewed, and the need for the techniques and methods to be modified and further improved is examined. A brief discussion of the problems presented by DS follows. The article concludes with case studies of examples involving the analytical characterization and application of metrological principles to botanical DS that has enriched our understanding of the health effects of these products.

The New Frontiers

At present, the utility of metrology in applying analytical methods with a higher degree of accuracy, sensitivity, standardization and a harmonized system to verify food integrity (Rychlik et al., 2018) is underappreciated, especially in dealing with the many challenges botanical DS present. The new frontiers include opportunities in the development of additional standards, new or better analytical and data management methods, and international engagement and dissemination of these developments.

Metrology and Dietary Supplement Science

The first new challenge is a need for greater focus of metrology on bioanalytical chemistry and the methods used in studying complex mixtures of ingredients that are often present in dietary supplement research. Metrology has a long and well-established history in physics and engineering, but less so in dietary supplement science. In part, this is because of the lack of validated methods and/or reference standards for the measurement of many dietary supplement constituents. The development of such resources is made challenging by the inherently variable chemistry of many natural products. Heterogeneity in products that share the same general designation (e.g., curcumin), but differ in their chemistry is a recipe for confusion in research involving comparisons between studies, and even more importantly heterogeneity in health outcomes associated with the products (Nelson, et al., 2017; Durazzo et al., 2018; Simmler et al., 2018; Sorkin B. C. et al., 2020).

Metrology in Data Analysis and Management

The production of reference standards, development of new methodology, and dissemination of best practices in chemical analysis has been the traditional role for metrologists. An emerging challenge is how best to handle and manage the increasingly large amount of data resulting from analytical studies. Often these data are analyzed using modelling, bioinformatics tools, and other software programs. All too often, automated results are accepted uncritically with little attention paid to how the results are generated and how replicable they are. Metrologists have much to offer in assisting analysts in discovering how and why results differ and in quantifying the uncertainty in these measurements (Sene et al., 2017).

Metrology and International Collaboration

A second new frontier is the need for increased international collaboration on dietary supplement ingredient descriptions (Dwyer et al., 2021). The chemical characterization and quality control of DS and natural health products are important because they easily cross international borders in an increasingly global marketplace. DS manufacturers and analysts in all countries should utilize the resources produced by metrology institutes and the international metrology system to a greater extent than they do at present. This will enable reproducible measurements of DS to be made with a quantified degree of uncertainty. Additionally, metrology institutes should increase their efforts to disseminate their resources and collaborate with DS stakeholders at national and international levels.

Efforts to develop methods and other resources to address quality and safety problems are in progress in several countries. Currently a few bodies such as the European Medicines Agency, AOAC International, the U.S. Department of Commerce's National Institutes of Standards and Technology (NIST), the National Institutes of Health's Office of DS (NIH ODS) and the U.S. Pharmacopeia are working in various ways to promote the development and validation of innovative methods to characterize botanicals and other natural products and to disseminate this information internationally. Related groups, such as the food metrology global community are also dealing with many of the same issues[6,7]. Particularly, the IMEKO[5] (International Measurement Confederation[5]) has been active. The World Metrology Day was celebrated on 20th May with the theme of Measurement for Health, and in August 2021 the 23rd IMEKO World Congress was held in Yokohama, Japan[8]. Importantly, IMEKO FOODS[9] was established and is devoted to metrology as it applies to food and nutrition. The first IMEKO FOODS conference was held in 2014 by the ENEA[10], and the ENEA is currently coordinating a project to build the research infrastructure. It is called "Infrastructure for promoting metrology in food and nutrition" (METROFOOD-RI)[6]. This RI is part of the Health and Food Area of the European Strategy Forum on Research Infrastructure (ESFRI)[11].

Of course, better regulation and compliance than that which currently exists are also needed (FAO, 2021). It is the regulator's responsibility to take appropriate steps to ensure that those who produce products respect quality while continuing to improve rigor in new and better methods applications. The absence of an "ideal" *modus operandi* at present should not prevent appropriate regulatory steps to be taken immediately to ensure quality. Communication amongst those engaging in these efforts may

[6]https://www.imeko.org.
[7]http://www.metrofood.eu/.
[8]https://imeko2021.org/.
[9]https://www.imeko.org/index.php/tc23-homepage.
[10]https://www.enea.it/en.
[11]https://www.esfri.eu/.

facilitate more efficient division of labor and allocation of resources in the future.

ANALYTICAL CHALLENGES AND APPROACHES IN CHARACTERIZING DS

The rigorous characterization of DS and natural products with accurate, precise, and reliable analytical methods to ensure the reproducibility and quality of preparations is clearly needed to underpin scientific advancement. Metrology has already had a positive impact on DS science through the generation of certified reference materials that anchor analytical results to well characterized standards (Betz et al., 2007).

The major challenges in dietary supplement science with respect to analytical measurements at the metrology interface are providing accurate, reliable, and timely measures of DS quality and applying them to improve dietary supplement quality assessment. Even in the absence of a regulatory or scientific consensus around minimum quality criteria, reference materials and standards can be used in developing and validating analytical methods. This is an important step towards achieving a consensus around understanding and defining quality. Metrology efforts can support those working with botanical supplements by generating a series of botanical reference materials. Matrix-based reference materials for dietary supplement ingredients and products, as well as calibration solutions of key chemical constituents, are powerful tools for making measurements of quality against a regulatory standard or pharmacopeial monograph.

This perspective provides an assessment of current and emerging dietary supplement characterization data analysis and evaluation approaches. This includes both targeted and non-targeted approaches, with a focus on chemometrics, toxicity, dosage performance, and data management for medicinal plants and botanical quality assessment. In addition, statistical methods, and optimized data management methods are considered.

Analytical Techniques

It is critical to have in-depth knowledge of the major analytical methods in the investigation of "functional" foods and DS (Betz et al., 2018; Spagnuolo et al., 2019). The pros, cons, and data gaps of some conventional and non-conventional techniques are described briefly below, with attention to emerging and innovative analytical methods, including some "green" methodologies.

Genomic Analyses for Authentication

DNA barcoding is a taxonomic method using a short genetic marker from a standard part of the genome of an organism's DNA to identify it as belonging to a particular individual, breed/cultivar, or species (Galimberti et al., 2013; Barcaccia et al., 2016). It has been portrayed as a universal tool that can be linked to any kind of biological or biodiversity information (Galimberti et al., 2013). DNA barcoding systems utilize a short, standardized region (between 400 and 800 base pairs) to identify species (Kress & Erickson, 2012). The technique is based on the assumption that *inter*species variation should exceed *intra*species variation: this difference (i.e. the barcode gap) of a standardized region is exploited for species level identification. This is now a popular method, as revealed by a search in Scopus, with a search for "DNA Barcoding" returning 10,078 publications covering the time period from 2002 to 2020 (see **Figure 1**).

DNA barcoding may be used to determine whether additional tests for an adulterant or contaminant are needed, but the absence of a signal does not indicate absence of the material originating from that genotype. DNA bar coding cannot be used for authentication except with intact plant material, as standard extracts may not contain intact DNA from the starting material and may contain DNA from excipients.

One goal in the dietary supplement industry is the identification and confirmation of species for processed raw materials that are affected by drying and milling at many points throughout a multilevel supply chain before the finished product is reached. Several studies report on the authentication and identification of plant materials in herbal supplements by means of barcoding. For instance, several reviews have discussed the strengths and limitations in the use of DNA barcoding for identifying botanicals in herbal medicine and DS (Parveen et al., 2016; Raclariu et al., 2018). Further technological advances such as mini-barcodes, digital polymerase chain reaction, and next generation sequencing, genomic skimming approach would provide additional tools for the authentication of herbs and might help in identifying processed ingredients used in finished herbal products (Parveen et al., 2016).

Pawar et al. (2017) compared chemical and DNA barcoding methods when assessing the authenticity of herbal DS. Twelve samples of frequently consumed botanical DS of ginkgo, soy, valerian, yohimbe, and St. John's wort obtained from the market were evaluated. The results suggested that newer chemical analytical approaches coupled with barcoding or next-generation sequencing (NGS) were promising as a means of analyzing complex botanical supplement products (Ivanova et al., 2016; Haynes et al., 2019; Lo and Shaw, 2019; Handy et al., 2021).

Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique used in quality control and research for determining the content and purity of a sample as well as its molecular structure. Wu et al. (2020) described the use of chemometric analysis of low-field 1H NMR Spectra for unveiling adulteration of slimming DS by pharmaceutical compounds. Avula et al. (2021) described an integrated workflow for the analysis of bio-macromolecular supplements, including NMR profiling. Simmler et al. (2018) proposed an NMR-based untargeted metabolomic model as a rapid, systematic, and complementary screening for the discrimination of authentic vs potentially adulterated botanicals. Further, Lee et al. (2020) reported a validation study of a proton NMR method for the determination of L-arginine, L-citrulline, and taurine contents in DS.

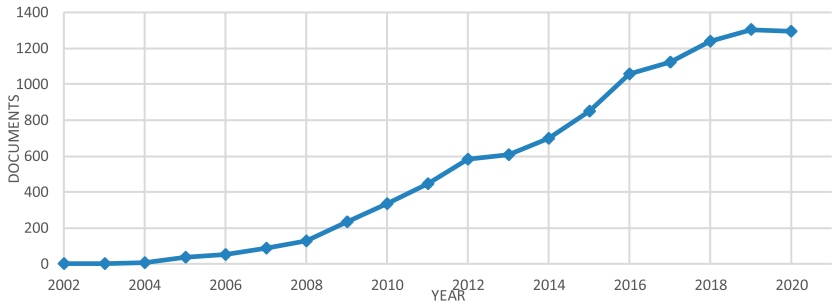


FIGURE 1 | Trends in the number of publications utilizing DNA barcoding (Data from Scopus Database).

Near-Infrared and Mid-infrared Spectroscopy

Infrared Spectroscopy (Small, 1996; Griffiths, and De Haseth, 2007) is "a chemically specific analysis technique that identifies the chemical bonding or molecular structure of materials, based on absorption in the infrared region of the electromagnetic spectrum" (Jadhav et al., 2013). Mid-infrared (MIR) and near-infrared (NIR) spectroscopy techniques combined with chemometrics reflect another emerging procedure, which is being applied in authentication processes to verify if the product contents are in accordance with the label or if it complies with the current legislation.

The fundamental vibrational modes of MIR are in the mid-infrared spectral range (200–4000 cm^{-1}), whereas the near-infrared range from 4000 to 13,333 cm^{-1} of NIR contains overtone and combination bands. Sources and detectors in these ranges are required. Spectral dispersion of the signal is normally reached through an interferometric analysis by using a Michelson interferometer. The interferogram is Fourier transformed to yield the spectrum in the frequency domain, which leads to the common name Fourier-transform infrared (FTIR) spectroscopy. The most common instruments are FTIR spectrometers.

Fourier-transform infrared-attenuated total reflection (ATR) spectroscopy is a common and easier to use technique. FTIR spectroscopy represents a rapid, less destructive, and high-throughput method for the analysis of food products, DS and nutraceuticals (Durazzo et al., 2020). It provides simplified handling and enables the samples to be examined directly in their original state. In addition to being more environmentally friendly, FTIR spectroscopy is also a more environmentally friendly procedure, rapid, fast and non-destructive technique, that is simple to perform and requires minimal sample preparation. FTIR spectroscopy is as an innovative analytical technique for determining the "fingerprint" of organic compounds because their functional groups exhibit characteristic signatures in specific infrared regions. Thus, the IR spectra can be used to identify or differentiate between samples. Recent developments and advances in instrumentation as well as chemometric pattern recognition techniques have amplified the range of the IR spectroscopy applications, including evaluating and determining components, monitoring contaminants and adulterants, classification, discrimination, authentication, and other uses. Applications of IR spectroscopy joined with chemometrics are discussed below.

Liquid Chromatography/Mass Spectrometry and LC/Tandem Mass Spectrometry

Mass Spectrometry (MS) is an analytical chemistry technique used to identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions. It is useful for establishing the chemistry of botanical DS (van Breemen, 2020). Over 15 years ago, Sherma, (2003) described qualitative and quantitative analysis by high-performance column liquid chromatography/mass spectrometry (LC/MS) and LC/tandem mass spectrometry (LC/MS/MS) of botanical drugs, drug substances or preparations, and finished botanical products. He also described LC/MS and LC/MS/MS techniques and commercial instruments as well as examples of applications (Sherma, 2003). Currently in the U.S., the NIH and USDA collaborate on the Dietary Supplement Ingredient database (DSID), which provides analytically determined information about the ingredient content in DS commonly sold in the U.S. The analytical contents of vitamins, minerals and botanicals in DS were quantified using HPLC, LC/MS and ICP/MS (Andrews et al., 2017). DS samples were tested along with matrix-matched NIST Standard Reference Materials with known values and uncertainties to monitor laboratory performance. Analytical results were also compared to the United States Pharmacopeia (USP) monographs for multivitamin and mineral content in various dosage forms, monographs for plant extracts and, where available, for finished botanical products (Andrews, Roseland, Gusev et al., 2017). Performance quality standards for dietary supplements should be better harmonized across all major pharmacopoeias: European Pharmacopoeia (Ph Eur), British Pharmacopoeia, USP, and Japanese Pharmacopoeia (JP) (Al-Gousous and Langguth, 2015). Vargas Medina et al. (2020) have more recently described the state-of-the-art and future trends on chip-based LC–MS, including an overview of the commercially available chip-based LC–MS platforms as well as main chip substrates and

microfabrication technologies. Rocco et al. (2018) focused on miniaturized separation techniques as analytical methods to ensure quality and safety of DS. LC/MS and LC/MS/MS are extremely sensitive and highly specific techniques for the determination of contaminants and illicit compounds even if present at very low levels.

Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique used in chemical analysis to separate molecules in an electric field according to size and charge. Gotti (2011) has summarized studies on capillary electrophoresis of phytochemical substances, including alkaloids, polyphenols, carbohydrates, lipids, terpene in herbal drugs and medicinal plants, and provides a short description of the basic principles of capillary electrophoretic techniques. Several previous papers focused on applications of CE toward a specific class of DS or a target biological function. For example, Cianchino et al. (2008) analyzed the potential adulteration of herbal medicines and DS promoted for weight loss by using capillary electrophoresis. Other recent applications of capillary electrophoresis include fatty acid determination in encapsulated vegetable oils supplements (Amorim et al., 2019) and docosahexaenoic and eicosapentaenoic acids determination in marine oil omega-3 supplements (Amorim et al., 2020).

CE with other analytical techniques i.e., mass spectrometry, contactless conductivity detection, evaporative light scattering detection, etc. For example, Restaino et al. (2020) developed a high-performance CE method to determine intact keratan sulfate and hyaluronic acid in chondroitin sulfate samples and food supplements of animal origin. Nguyen et al. (2019) used dual-channeled CE coupled with contactless conductivity detection for rapid determination of choline and taurine in energy drinks and DS. Dos Santos et al. (2016) analyzed amphetamine and its derivatives in "natural" weight loss pills and DS by using CE-tandem mass spectrometry. Duong et al. (2020) proposed using CE coupled with contactless conductivity detection for the determination of 10-hydroxy-2-decenoic acid and free amino acids in royal jelly supplements. CE coupled with evaporative light scattering detection has been used by Bouri et al. (2013) to directly quantitate underivatized amino acids in tea samples.

Data Analysis and Evaluation

Innovative approaches for data analysis are now available, including sophisticated procedures used in concert with the technologies listed above. Advanced statistical techniques can be applied for data evaluation today. Hypothesis testing and assessment of the likelihood of false positives or negatives are required for analyzing and assessing the significance of "differences" reported for non-targeted or chemometric data. Procedures for dosage design and the toxicity reports as tools for data analysis are also presented in this section.

Targeted and Non-Targeted Approaches

In the past, the main approaches for quality control of herbal medicines were the 'component-based' and 'pattern-based' (Mok and Chau, 2006; Zeng et al., 2008). Component-based studies addressed specific characteristic compounds (also known as the marker approach and multi-compound approach), whereas pattern-based studies investigated all detectable compounds (also known as the pattern approach and the multi-pattern approach). Both approaches have limitations. Herbal ingredients used for testing are complex, and single markers do not allow for an adequate evaluation of quality (Simmler et al., 2018). Generally, one or two markers should be considered insufficient for authenticity and quality control of herbs and medicinal plants. More than a few markers are needed to give a total overview of an herbal product and to qualitatively differentiate between products (Simmler et al., 2018).

A current strategy is the use of fingerprint analysis and chemometrics. These approaches compare overall chemical compositions (including any bioactive compounds) across multiple samples by using chromatograms acquired from spectroscopy, gas chromatography, liquid chromatography, or mass spectrometry. **Figure 2** presents a graphical representation of this new approach covering the workflow for chemistry, manufacturing, quality assessment and controls of botanical drugs.

As shown in **Figure 2**, the identification of new markers is required and, in this direction, multi-compound, multi-target and multi-pathway studies are being carried out. Harnly et al. (2017) explain how to detect the transition of chemical composition from botanical ingredients to resulting products by means of chemometrics, which permits them to be differentiated quickly. Wallace et al. (2020) gives a current example of identification of adulteration identification in botanical samples with untargeted metabolomics.

Gas chromatography (GC) and liquid chromatography (LC), in conjunction with mass spectrometry (MS), are appropriate for targeted analysis compared to general untargeted analysis; however, results require comparison of full-scan spectra to preestablished libraries (Wu et al., 2012). Using a full screen spectrum reduces the sensitivity of identification of components and may show unknown components. The need for standardization of analytical methods for analysis of dietary supplements is needed, especially methods which are more specific than TLC and LC-UV. Technical difficulties like to the complexity of LC-MS data and annotation of metabolites using untargeted LC-MS is emerging (Chaleckis et al., 2019) and the need for new software tools and a platform that can gather all the existing free mass spectral libraries for metabolite annotation is necessary (Misra, 2021).

Pferschy-Wenzig and Bauer (2015) explored emerging directions in the area pharmacognosy and pharmacological research on herbal medicinal products and suggested that each stage of production should be followed by adequate quality assessment measures (Pferschy-Wenzig and Bauer, 2015). Different methodologies ranging from macroscopic, microscopic, and DNA-based authentication to chromatographic methods i.e., HPLC, TLC, LC-UV, GC–MS and LC–MS and spectroscopic methods i.e., NMR, FTIR-ATR, would be applied, depending on the purpose. The quality of finished preparations would then be evaluated either by means of chemical or bio-marker constituents and/or analytical fingerprints.

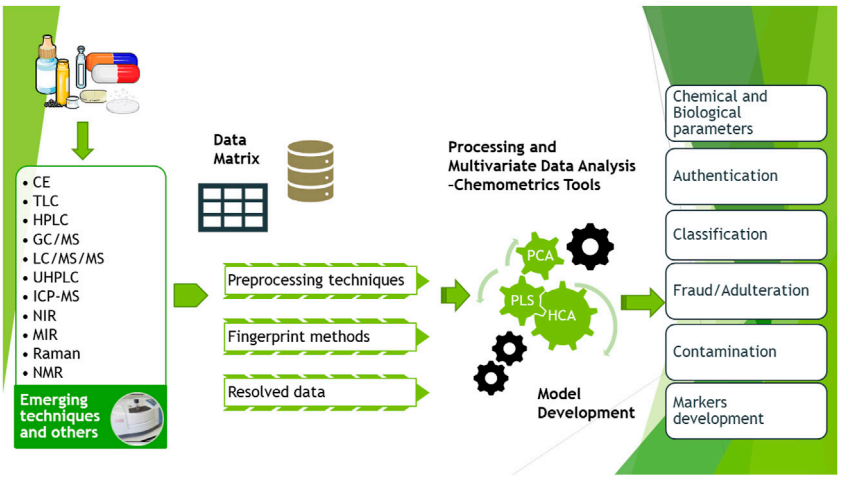


FIGURE 2 | An approach workflow based on fingerprint and chemometrics.

Chemometrics

Hibbert (2016) define chemometrics as "the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods." Chemometrics uses mathematical and statistical methods to obtain relevant information from collected data (Otto, 2016). Analytical technologies provide complex information. The data treated by chemometrics are often multivariate and such large datasets require multivariate data analytic methods. The statistical techniques are useful for extracting and providing qualitative and quantitative information from complex data, highlighting trends, investigating relationships, building models for defining characteristics of studies and/or for predicting outcomes and drawing conclusions form the experimental data. Chemometrics deal primarily with the extraction of useful chemical information from measured data, rather than theoretical calculations. It has applications in many fields, not only in chemistry (Hibbert, 2016).

The main chemometric pattern recognition techniques are designated as unsupervised and supervised ones. Unsupervised methods aim to identify groups of samples with related features, so they can be separated into different classes. They include principal component analysis (PCA) (Abdi and Williams, 2010), hierarchical clustering analysis (HCA), and partial least squares regression (PLSR). Supervised techniques are designed to extract the information in the mathematical model in order to assign a new sample to an already known class. Supervised procedures include linear discriminant analysis (LDA), PLS-discriminant analysis (PLS-DA), artificial neuronal network (ANN), and soft independent modelling of class analogy (SIMCA), among others.

Chemometrics opened a new scenario for botanicals (Bansal et al., 2014; Durazzo et al., 2018). The chemometric approach is a tool that can be applied in several aspects of the quality control of herbs and medicinal plants used in DS. This can include authentication of individual ingredients, monitoring of the quality of ingredients, identification of chemical constituents, detection of adulteration or contamination, and/or production of standardized formulations. As noted in Simmler et al. (2018), a great advantage of the use of agnostic or non-targeted analyses is that identification of the chemical entities which differ between products or batches is not required, but is facilitated where a novel constituent is associated with biological activity, for example, toxicity.

Figure 3 shows a search conducted on the Scopus platform by using the string "chemometric*" AND "dietary supplement*" OR "food supplement*". The "full records and cited references" were exported to VOSviewer software (version 1.6.16, VOSviewer software websitewww.vosviewer.com) for further bibliometric analyses and additional processing. The VOSviewer, 2020 software (v.1.6.16, 2020) analyzes the terms/words used in the titles and abstracts of publications, by breaking down the paragraphs into words and phrases, linking them with the citation data of the publications, and visualizes the results in the form of a bubble map by using a term map with default settings (Van Eck and Waltman, 2009; Van Eck and Waltman, 2010; Van Eck and Waltman, 2011; Van Eck, 2011a; b; Waltman et al., 2010). The search led to 111 documents covering a period from 1970 to 2021 with an H index of 22 and 13,18 Citations Per Publication (CPP). A total of 97 terms were derived from the quantitative literature research consisting of 111 publications and depicted as a term map in **Figure 3**. The top 15 recurring keywords are listed in **Table 1**. It is interesting to note that "chemometric", "principal component analysis" and "chemometric analysis" appear among the top 15 keywords, thereby highlighting the integrated approach between analysis and statistics today.

Examples of infrared spectroscopy joined with chemometrics are described here in greater detail. Deconinck et al. (2019), evaluated the feasibility of using infrared spectroscopy combined with attenuated total reflectance to screen plant-based preparations for nine specific plants (five regulated and four common plants used in herbal supplements). They showed that

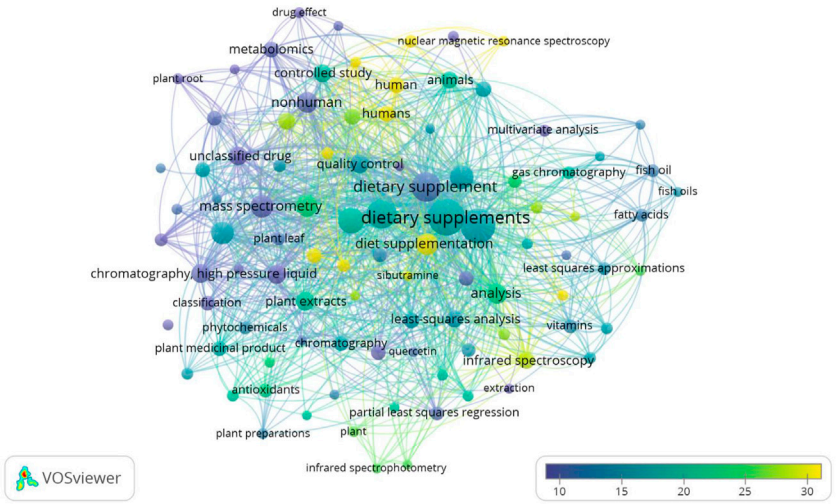


FIGURE 3 | Map of terms for search on: chemometrics and dietary supplement/food supplements research. Bubble map visualizing words from titles, abstracts and keywords of the 111 publications. Bubble size represents the number of publications. Bubble color represents the citations per publication (CPP). Two bubbles are closer to each other if the terms co-appeared more frequently (Based on data from Scopus and elaborated by VOSviewer software).

TABLE 1 | The top 15 recurring keywords on a chemometrics and dietary supplement/food supplements research web search (Based on data from Scopus and elaborated by VOSviewer software).

Term	Occurrence	Total link strength
Dietary supplements	61	746
Chemometric	54	550
Chemistry	41	571
Dietary supplement	41	528
Principal component analysis	30	390
Procedures	27	417
Mass spectrometry	24	327
High performance liquid chromatography	23	324
Chemometric analysis	22	333
Diet supplementation	22	294
Non-human	21	286
Analysis	19	318
Chromatography, high pressure liquid	17	248
Plant extracts	17	237
Quality control	17	247

the best model was obtained with the MIR data by using SIMCA as the modeling technique. They reported that MIR combined with SIMCA could be applied as a first step in the screening of unknown samples, before applying more sophisticated fingerprint approaches or identification tests that are described in several national and international pharmacopeia. In a small study comprising 35 DS on the market for slimming and 34 for male sexual potency enhancements, Deconinck et al. (2017) also applied a strategy based on fingerprinting and chemometrics for the detection of regulated plants.

An example of an investigation that used delayed luminescence combined with PCA is provided by Sun et al. (2019). They proposed simple, direct, rapid, and inexpensive measurements of delayed luminescence for the identifying herbal materials as a first step toward rapid quality control for DS. They also highlighted the importance of developing and establishing a valid and perhaps developing a novel digital tool for the quality control of herbal materials. Sima et al. (2018) used applied electropherograms and chromatograms and chemometric tools, i.e., PCA, cluster analysis and a combination of PCA and linear discriminant analysis (PCA-LDA) and applied them for the authentication of fruit-based extract herbal medicines.

Dosage Form Performance

Dosage form performance is the ability of a tablet, capsule, or liquid gel capsule to disintegrate and release its functional ingredient(s) into gastrointestinal fluids in a timely manner. Readily disintegrating dosage forms with good release characteristics generally have better oral bioavailability than those with inferior performance profiles. Together, dosage form disintegration and release comprise the process of dissolution. More specifically, dissolution in the context of DS, is an *in vitro* assessment of the rate and extent of phytochemical release into simulated gastric or intestinal fluids using standardized conditions and equipment (Azarmi et al., 2007). Phytochemical dissolution, like that of active pharmaceutical ingredients is a function of a molecule's physicochemical properties (e.g., molecular weight, polarity, water solubility and their subsequent dissolution into the aqueous gastrointestinal fluids is often greatly diminished, such as when the molecule is hydrophobic. Therefore, inadequate phytochemical dissolution can adversely affect oral bioavailability (Azarmi et al., 2007).

The efficacy of a DS is determined not by only by the active ingredient amount but also the formulation's design. Supplement formulation can greatly influence the fraction of the ingested bioactive amount that is absorbed, how much reaches the target site within a defined period, and, ultimately, the benefits it might

provide for the users. The USP develops performance standards such as *in vitro* disintegration and dissolution tests to detect problems with active ingredient release from dosage forms. The problems may occur due to formulation design and/or manufacturing processes. Disintegration testing demonstrates how a tablet or capsule breaks apart under agitation within 30 min in specific solutions by mimicking gastric or intestinal fluid. A dissolution test measures the amount of a marker compound released from a dosage unit into a stirred solution of simulated gastric or intestinal fluid in a form that is absorbable by the body. A product passes the test if, after 1 h, ≥ 75% of the amount of marker compound is released into the simulated fluid.

Although *in-vitro* disintegration and dissolution tests do not directly predict ingredient absorption and bioavailability (unless such a link has been established for a product experimentally), they are important tools for assuring dietary supplement quality and consistency. Products that failed to pass USP tests may fail to properly disintegrate and release ingredients, and so their active ingredients cannot be absorbed, and expected health benefits may not be achieved. Recently, researchers have shown widespread and inconsistent performance for green tea dietary supplement dosage forms. Based on these results, a recommendation was made to the National Institutes of Health's National Center for Complementary and Integrative Health policy for "Natural Product Integrity" to consider including satisfactory performance quality of dosage forms as a requirement for funding to avoid inconsistent results in clinical trials due to variability of performance quality (Gusev et al., 2020).

At present, most botanicals do not have published methods in USP for dosage form performance and many existing methods need improvement. DS may have very complex compositions, and there could be more than one option for selecting applicable USP testing protocols. Also, the biological relevance of the hydrodynamics, media, and mechanical stresses in USP disintegration and dissolution testing needs to be evaluated further (Gusev et al., 2020). In this regard, the use of biorelevant dissolution media should be considered. Biorelevant media emulate either fed or fasted conditions by incorporating appropriate electrolytes, enzymes and natural surfactants found in gastric or intestinal fluids in the context of a meal (Dresman, 2014). Since food can markedly influence DS bioavailability, an assessment of dosage form performance in biorelevant media is prudent.

Data Management

The management of data on dietary supplement quality is of concern nationally and globally because of the enormous expense involved in maintaining and updating very large amounts of data on dietary supplement ingredients and ensuring that the data are valid. Monitoring and surveillance of products is required because many manufacturers in the dietary supplement industry lack transparent methods or adequate audit trails. Many resources are required to maintain and constantly update the increasing number of DS. Dietary/food supplement databases are dynamic and must be constantly updated owing to the frequent changes in the formulation of ingredients and products, introduction of new ingredients and/or possible adulterants.

Big Data Processing Infrastructures

The term "big data" refers to any data exhibiting unusual features of any of five dimensions: volume, variety, velocity, volatility, and veracity (Fuller et al., 2017). It is important to encourage the use of big data techniques in collection, processing, storage, and analysis to allow for several types of research to go forward, including in-depth investigations of correlations between several types of data at a single point in time.

An example of using big data to deal with knowledge bases relating to DS information is the iDISK, an integrated DIetary Supplement Knowledge base (iDISK). The iDISK integrates and standardizes DS-related information from four existing resources: The Natural Medicines Comprehensive database, the "About Herbs" page on the Memorial Sloan Kettering *Cancer* Center website, the Dietary Supplement Label database, and the Natural Health Products database (Rizvi et al., 2020).

According to Zeb and Soininen (2021) data harmonisation is one of the keys that enable digitalization for foods, and the same methods may be useful for DS. Foods and DS are similar in that a large amount of data is produced; data harmonization produces compatible and comparable datasets consisting of interoperable and widely usable data. Several recent papers (Cade, 2017; Pehrsson, and Mitchell, 2020; Sorkin B. et al., 2020) summarize technologies and new tools and infrastructures for data management, by emphasizing the importance of findability, accessibility, interoperability, reusability of data and related metadata.

Analysis of extremely large "big datasets" computationally may reveal patterns, trends, and associations of interest, such as those relating to human behavior and interactions affecting human health. Cloud-based solutions are on-demand services, computer networks, storage, applications or resources that are accessed via the internet and through a third party's shared cloud computing infrastructure. The benefits of cloud-based solutions in dealing with big datasets for the end users include increased capacity, scalability, functionality, and reduced maintenance and cost for a computer infrastructure. Some of the newer cloud solutions allow the dynamic allocation of computer resources. They include *Hadoop*, a Java-based, open-source framework for software development that supports the storage and processing of massive data sets, *Spark* is a fast and general engine for large-scale data processing which can quickly perform processing tasks on very large data sets and can also distribute data processing tasks across multiple computers, either on its own or in tandem with other distributed computing tools. The differences between *Hadoop* and *Spark* technologies are that while *Hadoop* is designed to handle batch processing efficiently, *Spark* is designed to handle real-time data efficiently. *Hadoop* is a high latency computing framework, which does not have an interactive mode whereas *Spark* is a low latency computing and can process data interactively.

Orchestration of the different steps is also key in big data ecosystems. Mansouri et al. (2020) describe automated implementation for performance evaluation of a hybrid cloud for distributed databases that integrate resources between private and public clouds.

Hybrid Database Approaches by Using Graph and Relational Databases

Database relationships are associations between tables that are created using join statements to retrieve data. A relational database stores and organizes data points that are related to one another. Based on the relational database model, a relational database presents data sets as a collection of tables and provides relational operators to manipulate the data in tabular form. A distributed database is one database that is one that is spread over different sites, i.e., on multiple computers or over a network of computers that do not need to share the same site or physical components, such as when a database is accessed by various users globally. However, it is managed so that it seems to be a single database to users. Traditionally, relational databases have been used for dealing with dietary supplement ingredients. Data were entered into tables with columns and rows. However, files in such a format are cumbersome, slow, and expensive to manipulate or to join with other databases. When data are likely to need to be connected or joined with other databases, graph databases have the advantage of speedy data retrieval for such connected data. Integrated or hybrid database structure/architecture uses a hybrid of both graph and relational databases and are useful for managing data and improving dietary supplement quality by better dealing with data from integrated approaches. Vyawahare et al. (2018) proposed a hybrid database approach for integrating relational and graph databases in a single system, reasoning that the relational database provided a good structure for storing, managing and querying information, while it was possible to take advantage of the features graph-structured databases as well, thus capturing the strengths of both systems. Structured Query Language (SQL) databases are primarily relational databases whereas "not only" or NoSQL databases are non-relational or distributed database.

Bjeladinovic et al. (2020) proposed an architecture for integration and uniform use of hybrid SQL/NoSQL database components: the SQL managed data contains the fixed or rarely changeable structure, whereas the NoSQL databases are used for vast quantities of data that change rapidly.

METROLOGY APPLICATIONS TO ENSURE DIETARY SUPPLEMENT QUALITY: CONSIDERATIONS AND CASE STUDIES

This section includes examples of metrological approaches and resources that have been successfully applied to characterize and study several botanical DS in the United States. They serve as an example of how metrology can be used in efforts to improve supplement quality. It could be equally argued that similar approaches are needed in Traditional Chinese Medicine, Kampo medicine or many other systems. The discussion begins with general considerations applying to any chemically complex set of dietary supplement ingredients. It then turns to phytochemical examples while recognizing that many of the same considerations apply to nutrients, other ingredients in supplements, and toxic elements.

The first case study describes the authentication of black cohosh, a commonly used botanical supplement, using a standard reference material. A second describes the measurement of DMAA, a prohibited "natural" substance found in DS. The final case relates the development and fate of one of the first dietary supplement reference materials for complex botanical mixtures produced by the U.S. National Institute of Standards and Technology (NIST). Each of these cases discuss the problems, the potential implications for human health, ingredients measured and what was achieved by the measurement, challenges in quantification and their resolution, and lessons learned. Taken together, they attest to the important role that metrology and analytical chemistry have to play in dietary supplement science.

Considerations Applying to Measurements of Nutrients and Phytochemicals

The chemical composition of ingredients and formulations used in DS can present considerable challenges for analysts when attempting to separate and measure the chemically diverse nutrients (e.g., vitamins, fatty acids, minerals, phytochemicals) and contaminants such as toxic elements (e.g., Cd, Hg, As, Pb). This significant chemical diversity is especially true for ingredients derived from botanicals and other natural products (e.g., spirulina, fish oils). In this context, it can be very difficult to measure and maintain consistency in the DS products found in the international marketplace and available for study to biomedical researchers.

In response to these chemical measurement challenges, the US National Institutes of Health Office of Dietary Supplements (NIH-ODS) Analytical Methods and Reference Materials Program and the NIST partnered to establish laboratory quality assurance programs (QAPs) with a purpose to promote and support enhanced capabilities for the analytical characterization of DS (Phillips et al., 2011; Sander et al., 2013). These voluntary QAPs, as well as NIST certified reference materials for common dietary ingredients (Rimmer et al., 2013; Eichner et al., 2019; Wise and Phillips, 2019) are designed to help laboratories establish and improve their analytical accuracy, precision, and repeatability of measurements for analytes found in DS. Participant laboratories measure target nutrients and/or phytochemicals, as well as potential contaminants (e.g., pesticides, toxic elements) in samples distributed by NIST, and subsequent data identify analytical challenges, and describe methodological advances.

Through participation in the Health Assessment Measurements QAP[12] (and in the past the Dietary Supplements Laboratory QAP[13]), industry, academic, and government laboratories from around the world can assess

[12]https://www.nist.gov/programs-projects/dietary-supplement-laboratory-quality-assurance-program.

[13]https://www.nist.gov/programs-projects/health-assessment-measurements-quality-assurance-program.

and improve their analytical measurements of botanical dietary ingredients that are popular in supplement products and are of interest to researchers who study dietary supplement health effects. For example, NIH-ODS/NIST QAP exercises have helped the analytical community reduce calibration errors in green tea catechin measurements (DSQAP Ex E; DSQAP Ex I; LG Saldanha et al., 2015), quantify the significance of utilizing a hydrolysis step in the extraction and measurement of soy isoflavones (DSQAP Ex F; Zhang et al., 2015), and validate rigorous methods for the determination of curcuminoids (Mudge et al., 2020).

Considerations Applying to Investigations of Activities for Chemically Complex DS

Many DS products are chemically complex, in particular those derived from botanicals and other natural products whose chemical compositions are inherently variable, the result of numerous combinations of variables including genetic, environment, and preparative (e.g., harvesting, extraction, formulation) factors. Moreover, the molecular mechanisms of action responsible for their biological effects of interest, including both the specific chemical constituent(s) in the product which contribute to the activity/ies of interest, and their physiological, cellular, or molecular biological target(s), are often unclear (Sorkin B. C. et al., 2020). In some cases, there is a dearth of relevant data, while in others the existing data are heterogeneous. Often rigorous, comprehensive chemical characterization of the materials studied is not available. Documentation of marker compounds may be useful to assess whether the source of a product is likely to be correctly identified, but if characterization relies entirely on a single marker compound it is subject to the following caveats: 1) economic adulteration of the products by addition of a synthetic marker compound can give the appearance that the correct material is present when it is not, or it can deceptively inflate the amount apparently present, 2) in the absence of clear evidence that the marker compound is solely responsible for the biological effects of interest, different products may have identical amounts of marker compound while having very different biological effects, 3) analysis for a single marker compound may fail to detect the presence of adulterants or contaminants.

Agnostic, non-targeted, analytical approaches may address many of these issues with a single method (Urban, 2016). Hight et al. (2019) described a method for computational integration of data from three different high-content, high-throughput analyses (UPLC-MS-MS, gene expression analysis, and detailed cell phenotyping) of the same sets of chemically complex natural product fractions. This allowed the simultaneous generation of strong hypotheses regarding the chemical species contributing to the biological activity and the cellular and molecular substrates through which those compounds exert their effects. Notably, unlike the traditional approach of fractionating a product prior to conducting assays for biological activity, this approach should facilitate the detection of biological activities which require multiple chemical constituents acting on multiple targets, or where multiple constituents are needed for action through the same target. The accompanying UPLC-MS-MS analyses can provide a comprehensive chemical description of the product, as well as of the constituents associated with biological activities of interest.

This approach has human health implications. It can address the problem of contamination or adulteration of products with unexpected compounds through its use of untargeted chemical analyses. Further, by rapidly identifying which constituents are likely to be responsible for the observed activities, the approach facilitates targeted testing of the molecular mechanism of action, in turn setting the stage for future targeted product assessments based on the chemistry critical for the product application or safety.

Although this approach requires a variety of advanced instrumentation and methodology, the US NIH-supported Center for High-Content Functional Annotation of Natural Products is working to develop on-line resources to be used by the community for biological screening, metabolomics and discovery of compound-activity associations[14].

Quantification of Metabolites and Human Health Implications

A thorough understanding of DS health effects requires a knowledge of dietary intake and chemical composition as well as the resulting metabolite profiles present in clinical samples. To advance understanding of their health implications, the NIH-ODS/NIST Health Assessment Measurements QAP approach is unique in its pairing of studies that measure the chemical composition of dietary ingredients/supplements representing human intake with measurements of nutrient, phytochemical, and toxic metabolites in serum, plasma, or urine. These clinically focused QAP studies are designed to promote the measurement of physiologically relevant analytes in addition to measurement of traditional biomarkers that may or may not have a known biological activity.

Many methodological resources available can help research scientists and industry analysts measure myriad constituents in DS. A major issue, however, is that established good practices in analytical chemistry and pharmaceutical sciences may not be uniformly accepted or applied within the larger dietary supplement scientific community. It is important that rigorous and fit for purpose methods be applied to support the development of domestic and international standards, regulations, and legislation.

Case Studies

Case Study 1: Authentication of Black Cohosh Using the National Institutes of Standards and Technology Standard Reference Material

Challenges in the Use of Black Cohosh

Advances in metrology science have traditionally been passed to researchers in the form of certified reference standards. These

[14]https://hifan2.sites.ucsc.edu.

standards allow verification of in-house quality control standards and assure the accuracy of quantitative analytical results worldwide. However, qualitative analysis, specifically authentication of botanical supplements, introduces new demands on standards that are not necessarily in agreement with the intended purpose of certified reference materials. Metrology standards still have a useful role in validating the chemical components of supplements, but a lesser role in authentication.

Authentication of botanical supplements is most effectively accomplished using non-targeted methods in conjunction with pattern recognition programs (chemometrics) such as principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA). Of primary importance is establishing the natural range of biological variation of a species or sub-species. Using these approaches it is possible to establish if a sample is abnormal (adulterated, contaminated, or substituted) without knowing what is normal and the range of normality.

Establishing normality and its range is usually accomplished by accumulating authentic materials that encompass all the expected variables such as genetics, environment, management, and processing. An appropriate collection of authentic materials can be analyzed and then used to build a model and to determine or whether an unknown sample is authentic (lies within the model) or different (lies outside the model). Questions regarding how many authentic samples are necessary, what variables to cover, and what method of analysis to use are critical and beyond the scope of this case study and addressed elsewhere (LaBudde and Harnly, 2012).

In 2014, a study was initiated to develop a method for authentication of black cohosh (*Actaea racemosa*) (Harnly et al., 2016). For this study, four species of vouchered, authentic *Actaea* samples (*A. dahurica*, *A. pachypoda*, *A. podocarpa*, and *A, racemosa* (black cohosh) were obtained from four sources and four black cohosh candidate standard reference materials (SRMs) were obtained from the National Institute of Standards and Technology (NIST). Samples were analyzed by flow injection mass spectrometry (FIMS), nuclear magnetic resonance spectrometry (NMR), and DNA barcoding. This case study presents the FIMS results for the four sets of authentic black cohosh samples analyzed by principal components analysis (PCA) and soft independent modeling of class analogy (SIMCA).

Samples were acquired from the American Herbal Pharmacopoeia (AHP, Scotts Valley, CA, United States), North Carolina Arboretum Germplasm Repository (NCAGR, Asheville, NC, United States), Strategic Sourcing, Inc (SSI, Banner Elk, NC, United States), and NIST (Gaithersburg, MD, United States).

Results

Figure 4A provides perspective by showing the PCA score plot for three genera: four species of *Actaea* (*A. dahurica*, *A. pachypoda*, *A. podocarpa*, and *A. racemosa*), two species (*E. purpurea* and *E. angustifolia*) and two plant parts (aerial and rhizome) of *Echinacea*, and two species of *Panax* (*P. ginseng* and *P. quinquefolius*). The three genera were easily separated and identified by FIMS.

The 2-dimensional PCA score plot for four species of *Actaea* (**Figure 4B**). The 2-dimensional plot suggests the four species can be separated and identified. Discrimination at the 95% confidence limit was verified by SIMCA (data not shown).

Figure 4C shows the PCA score plot for authentic black cohosh materials from the four sources listed above. Samples from NCAGR (green) were rhizome materials hand-collected from 22 sites in the eastern US. AHP (red) and SSI (blue) each collected and vouchered seven rhizome materials from unidentified sources. The NIST SRMs were obtained from an unidentified commercial company producing a black cohosh supplement. The company provided rhizome (SRM 3295), leaf (SRM 3296), extracted rhizome (SRM 3297), and extracted solid dosing form (SRM 3298) samples. Each sample was analyzed 4 times.

Each organization provided authentic, vouchered black cohosh samples. The PCA score plot was expected to show all rhizome samples in a homogeneous cluster with only the leaf material (SRM 3296) and the extracted solid dosing form (SRM 3298) lying outside the cluster. The rhizome and leaf, different physical parts of the plant, were anticipated to have different compositions and the extracted solid dosing form would have lost and gained components during the preparation process.

The PCA score plot in **Figure 4C**, however, shows relatively clear separation of the AHP and NCAGR rhizomes with the SSI samples spread between the two clusters. Further SIMCA studies (**Figure 4D**) confirmed the difference in the chemical composition of the authentic black cohosh samples. **Figure 4D** is a complicated double SIMCA plot showing the residuals for independent models of the AHP (*X* axis) and SSI rhizomes (*Y* axis). The AHP rhizomes are primarily located to the left of the vertical 95% confidence limit and are significantly different from the other rhizomes. The SSI rhizomes are located below the horizontal 95% confidence limit and are significantly different from most of the other rhizomes. The NCAGR rhizomes to the right and above the 95% confidence limits can be judged as significantly different from the other rhizomes.

The NIST rhizome standard (SRM 3295) can be grouped with either the SSI or NCAGR rhizomes but appears statistically different from the AHP rhizomes. In general, however, the carefully collected and processed NIST rhizome standard (SRM 3295) falls roughly in the middle of the other vouchered rhizome samples while the leaf (SRM 3296) and extracted dosing form (SRM 3298) are distant from the rhizomes in the upper right corner. Thus, despite the variation between authentic samples, SRM 3295 validates their identification as black cohosh.

The failure of the rhizome samples from the different sources to form a single cluster can be attributed to a number of factors such as growing location, harvest year, handling, preparation, or storage conditions. With respect to growing location, a detailed examination of the NCAGR samples showed distinct compositional differences between some of the samples from the 22 growing locations (Harnly et al., 2016). Differences were attributed to soil, weather, endophytes, and local genetic variations. Unfortunately, there is a lack of similar data (metadata) from the other sources.

These data serve to illustrate two major points. First, there is considerable compositional variation between all plants (indeed, all living organisms) despite their morphological similarity. This

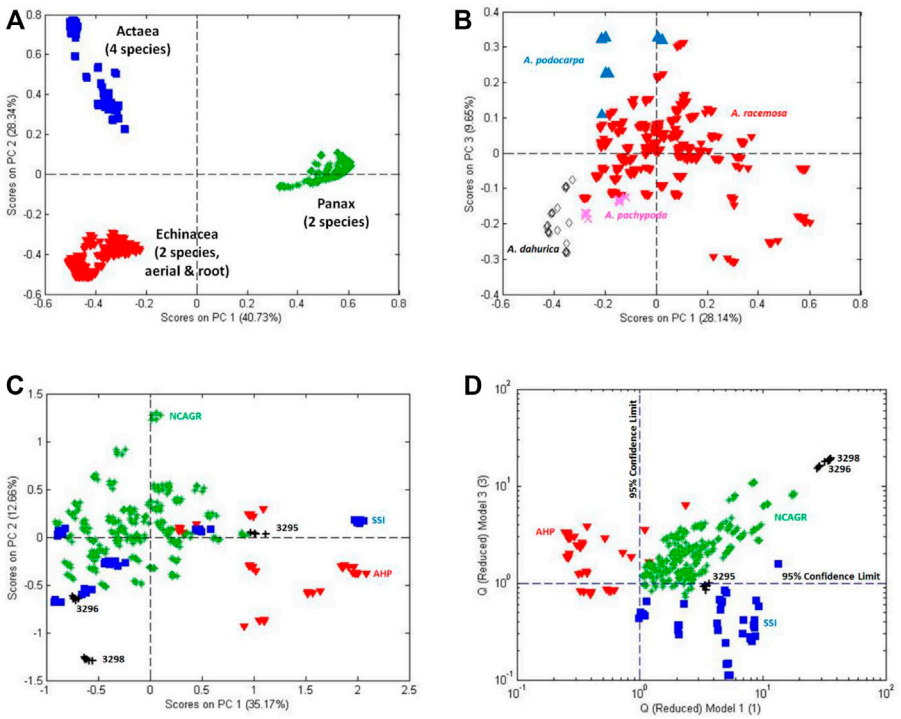


FIGURE 4 | Authentication of Actaea racemosa: **(A)** PCA score plots of three genera (*Actaea*, *Echinacea*, and *Panax*), **(B)** PCA score plot of four species of Actaea (*A. dahurica, A. pachypoda, A. podocarpa*, and *A. racemosa*), **(C)** PCA score plot of vouchered A. racemosa rhizomes from three sources (American Herbal Pharmacopoeia, North Carolina Arboretum Germplasm Repository, and Strategic Sourcing, Inc.) and NIST SRM 3295 (rhizome), 3296 (leaves), and 3298 (extracted dosing form), and **(D)** Double SIMCA Q residuals plot for the same samples shown in plot C.

variation must be accounted for by any model that seeks to establish authenticity. Second, a reference standard from a metrology organization can validate the identification of the genus, species, and plant part. This case study shows that, although a single NIST SRM could not provide information regarding the biological, environmental, and processing variability, it did confirm the accuracy of the non-targeted mass spectral profile.

Case Study 2: DMAA: A Prohibited "Natural" Substance

Challenges With DMAA

1,3-dimethylamylamine (also methylhexanamine or DMAA) is an amphetamine derivative added to dietary supplement products with claims of being a "natural" stimulant derived from geranium (Cohen, 2012; Eliason et al., 2012), although there is no conclusive evidence to support the claim of being from a botanical (Zhang et al., 2012). DMAA, promoted for weight loss, bodybuilding, and performance enhancement, was first introduced as a drug for nasal decongestion in 1948 before being voluntarily removed from the market in 1983. In 2006 it was re-introduced in 2006 as Geranamine, an extract of *Geranium* and constituent of geranium flower oil. In 2013, the U.S. Food and Drug Administration (2004) stated that "DMAA is not a dietary ingredient, and DMAA-containing products marketed as DS are illegal and their marketing violates the law." (U.S. Food and Drug Administration, 2018). Nevertheless, despite the FDA ruling, DMAA continues to appear in dietary supplement products offered on the market.

What Is Measured and what Is Achieved

Approximate concentrations of DMAA in popular DS range from 0.11 to 673% of what is noted on the label, and products typically provide between 4.6–25 mg in a single serving. This wide range may reflect differences in extraction/analysis methodologies, insufficient quality control in dietary supplement production, as well as shifting formulations (Austin et al., 2014). In fact, inconsistencies were noted between ingredients listed on product labels and those found within the product when analyzing products by using analytical methods: some products also contain DMAA despite it not being listed on the Supplement Facts label (Cohen et al., 2017). Further, some DS contain DMAA in combination with other stimulants and ingredients either included/excluded on the label or, which could further amplify the stimulatory effects of DMAA. The serving size of DMAA is also not always specified on the label, particularly when included as part of a proprietary blend. As such, without a clear understanding of the presence of DMAA and in what amounts, it is difficult to be certain what effects, if any, can be attributed to DMAA (McCarthy et al., 2011; Powers, 2015). Regardless, DMAA is not a DS ingredient and the marketing

of products with DMAA according to the FDA "...violates the law". The only way to understand what is in any product is through applying quality testing and analysis.

Problems With the Quantification of Metabolites and Human Health Implications

As a synthetic stimulant and vasoconstrictor, DMAA narrows blood vessels and arteries and increases blood pressure, which could lead to cardiovascular (e.g., heart attack, shortness of breath, arrhythmias, tightening of the chest, heart attack), neurological, and psychological problems. In fact, numerous case reports have linked DMAA-containing products to several serious adverse events including liver injury, cardiac arrest, stroke, brain hemorrhage, and death (following physical exertion) (Eliason et al., 2012; Col et al., 2013). Animal toxicology studies, moreover, have shown that the systematic toxicity of DMAA in animals is greater than ephedrine, another restricted stimulant that is not only linked to several side effects but can also lead to abuse and addiction (Palamar, 2010).

Scientific research concerning the safety of DMAA-containing products is limited at best given the regulatory framework for DS. In addition, DMAA is not a legitimate dietary ingredient. Based on the available literature, serious adverse events may be due to consuming quantities of DMAA and what can be tolerated by human consumption, both of which are actually unknown (Schilling et al., 2013). In some case reports describing significant reactions, the person had ingested almost 15 times the DMAA serving size reported on the label in addition to other stimulants (Gee et al., 2010; Gee et al., 2012). Only a handful of DMAA pharmacokinetic studies have been conducted in humans, and these suggest only a fraction of an orally administered DMAA dose is even metabolized (Bloomer et al., 2018). Because DMAA is often combined with other ingredients, the concomitant administration of other ingredients (e.g., caffeine) may inhibit DMAA clearance and lead to excessive concentrations (Bloomer et al., 2018). This remains to be demonstrated and has not been confirmed through analytical methods.

Resolution of Issue/Problem

Given the numerous reports of serious adverse events associated with DMAA-containing products and concerns about potential abuse, as noted above, the FDA declared DMAA a potential health risk and illegal for use as a DS ingredient in 2013 (U.S. Food and Drug Administration, 2018). DMAA is currently banned by multiple countries (e.g., United States, Ireland, Sweden, Denmark) and organizations (e.g., World Anti-Doping Agency, Department of Defense's Operation Supplement Safety) (Cohen, 2012; Col et al., 2013; Operation Safety Supplement, 2021).

The FDA continues to identify and remove DMAA-containing DS products from the market as quickly as possible by either giving companies the opportunity to voluntarily recall and destroy products or, in instances of non-compliance, administratively detaining and/or seizing products. Despite being prohibited since 2013, however over 268 products on the market in 2021 clearly listed DMAA on the Supplements Facts label even though it is illegal for use in DS (Crawford and Deuster., 2020).

As the Food and Drug Administration FDA continues its efforts to remove these violative products from the market, consumers should not buy or use any product containing DMAA. Because some retailers still have stocks of discontinued or reformulated products with DMAA, consumers should carefully read the product's Supplement Facts label to verify it does not contain DMAA. Although DMAA is most commonly listed as 1,3-dimethylamylamine; 2-amino-4-methylhexane; dimethylpentylamine; geranamine, or methylhexanenamine, it can also be noted under several different names, including but not limited to 1,3-DMAA; 1,3-dimethylpentylamine; 2-hexanamine, 4-methyl-(9CI); 4-methyl-2-hexanamine; 4-methyl-2-hexylamine, or; dimethylamylamine. Further, some products that list *Pelargonium* graveolens extract or *Geranium* extract may also contain DMAA and should be reviewed with caution; a full list of the various terms and synonyms for DMAA can be found on the Operation Supplement Safety website[15,16]. Despite these efforts, these products continue to be on the market, whether the consumer knows it or not. Metrology and analytical methods can pre-emptively establish what is contained in these products and avoid dangerous or misleading labels from entering the marketplace. Such methods should be established and implemented for DMAA.

For now, consumers are also encouraged to check OPSS for a list of current, discontinued, and reformulated products noting DMAA on their product label/website. Consumers should be encouraged to use only DS that have evidence of third-party testing, which confirms the product's label ingredients[17].

Lessons Learned and Other Comments

Numerous case reports have documented adverse events associated with DMAA containing products (Eliason et al., 2012, Department of Defense 2013). Studies that have reported its safety, moreover, may be biased as they were supported by companies responsible for marketing DMAA-containing supplements (Zovico et al., 2016). Although some confusion about DMAA's classification as a natural vs synthetic simulant has emerged, the no reliable, definitive evidence substantiaties DMAA's status as being derived naturally from a botanical; DMAA should not be sold as a DS ingredient. Finally, most studies on DMAA are based on results gathered from products that include other ingredients in addition to DMAA. Research to investigate the safety of DMAA as a single ingredient is not available.

Rigorous study designs developed to evaluate the safety of DMAA, especially in populations with concomitant use of other substances and high frequency of use, are needed to understand the magnitude of the association between DMAA and serious

[15]OPSS.org.

[16]https://www.opss.org/sites/default/files/downloadable/OPSS%20DS%20Products%20Containing%20DMAA_508.pdf.

[17]https://www.opss.org/article/dmaa-prohibited-stimulant.

adverse effects. Until then, because it is difficult to ensure their safety, DMAA-containing products should continue to be prohibited.

Case Study 3: Development of Ephedra Standard Reference Materials in the United States

Challenges Associated With Ephedra

Ephedra sinica (Ma huang) is the only extant genus of gymnosperm shrubs in its family (Ephedraceae) and order (Ephedrales). In DS, ephedra (ES) is usually either a formulation of powdered stems and aerial portions or a dried extract (Natural Medicines Database, 2018). The plant contains multiple chemical compounds, but the major effects of ES are likely caused by ephedrine (Ibragic and Sofić, 2015); although possibly other bioactives are also contributors. The principal alkaloid constituents are ephedrine, pseudoephedrine, and sometimes small amounts of phenylpropanolamine along with smaller amounts of other constituents (e.g. norpseudoephedrine, methylephedrine, and norephedrine and tannins) (Gurley et al., 2015). Ephedrine is also sold as a drug used in stimulants in the 1930s and World War II, and for weight loss in the 1970s. During the late 1990s to mid 2000s ES was included a dietary ingredient usually in combinations of ephedrine alkaloids, caffeine and other phytochemicals in widely popular over-the-counter DS advertised primarily for weight loss and sports performance in the United States and Canada.

Identification and Characterization

Assays for ephedrine and related alkaloids were available in the 1970s and 80s, but the analytical models were mostly titrations, not suitable for measuring alkaloid in complex multi-botanical finished products. It woud have been difficut to assess the amounts in DS and compare them against a standard.

The amounts and constituents in ES containing DS varied greatly, not only in ephedrine but in many other alkaloids (Natural Medicines Database, 2018). According to the Natural Medicines database, in studies for the treatment of obesity, the typical dose of ES was 15–40 mg of the ES alkaloids, calculated as ephedrine, taken up to three times daily. In some studies, this dose of ES was taken with caffeine 100–210 mg daily in divided doses. In other studies, other combinations were used that included 12 mg of ES with 40 mg of guarana three times daily plus 17 other vitamins, minerals, damiana, bee pollen, Ma huang and other ingredients and 60 mg of ES daily with cola nut and willow bark (Natural Medicines Database, 2018). Different brands used different amounts in their DS products. For the larger brands sold commercially, batch to batch uniformity of the same brand was usually good, while for the smaller brands that used the herb rather than the extract results were less consistent. Labeled amounts were generally fairly accurate because the products were often spiked with ephedrine alkaloid, and determination was by quantities added. There was little demand for ephedrine alkaloid reference materials in DS. By the late 1970s pharmaceutical scientists clearly recognized the potentially adverse effects of certain formulations of ES products seemed to be greater when combined with caffeine (Gurley et al., 2015). Therefore, reference materials with the inclusion of caffeine were needed. Also, DS also existed in several matrices, each of which required different reference materials.

Issues and Human Health Implications

Reports from Europe about the efficacy of ephedrine as a prescription weight loss drug stimulated great interest in the nutrition community and public, ES products sold over the counter without prescription had the potential for being used to treat obesity based (Astrup et al.,. 1985). US law views DS as foods so they are considered inherently safe, unlike drugs for which benefit and risk are weighed. However, during the early 1990s ES use became increasingly associated with adverse side events that were troubling, including drug interactions, blood pressure and circulatory problems (stroke, fainting, heart rhythm disorders) and depression. By the early 2000s private individuals and attorneys general in various states around the country were bringing many lawsuits against manufacturers. Also, state public health authorities attempted to limit the sale of products containing large amounts of ephedrine. However, federal law required clear evidence of harm before a DS product could be seized or banned. Compelling evidence was needed because the legal climate was anti-regualtory, and in the 1990s US Food and Drug Administration prosecutors had lost several important agency cases in federal courts and FDA's regulatory powers were subsequently restricted. FDA's original response to ES related adverse events was publication in 1997 of proposed rule embodying a regulatory approach od limiting the dose and permitting ES-containing products to remain on the market while limiting their total alkaloid amounts (Porter 2007). Thus FDA needed reference standards so that when the contemplated ceiling on the amounts of ephedrine that woud be allowed in DS products, manufacturers and regulators would have a common standard to use for assessing compliance. Other agency efforts to mitigate risk under DSHEA that had been used successfully for other ingredients continued as well. These included a 2001 FDA request for voluntary recall of aristolochic acid (AA) containing ingredients by manufacturers and distributors coupled with an import alert on AA containing botanical dietary ingredients. FDA posted warnings for consumers about pyrrolizidine alkaloids in comfrey in 2001. Also in 2001 FDA warned health care practitioners that kava might be a serious health risk It accompanied the warning with a request for them to review and report cases of adverse effects. Congress continued to be supportive of the Dietary Supplement and Health Education Act (DSHEA) of 1994, and so a Congressional action to ban ES was unlikely. In the early 2000s the Secretary of the Department of Health and Human Services had the ability to declare ES an imminent hazard, but this step was not taken, and FDA lawyers were concerned about losing another regulatory battle. To make an adulteration charge, FDA needed evidence (Porter 2002). However, in the face of DSHEA's new provisions, there was still uncertainty about the required level of evidence on the dangers to human health of ES containing DSs that would be conclusive enough for FDA to remove products from the market. Therefore, until regulators deemed the adverse events and scientific evidence sufficient to defend FDA's action to completely remove ES containing products from

the market, FDA continued to pursue what might be a practical and politically acceptable way to minimize harm to users by regulating the amount of ephedrine in the supplements. This effort was difficult to implement technically because the limit on total alkaloids pushed the limits of analytical reproducibility for the minor alkaloids. Also, FDA's adverse event data did not indicate that a predictable dose/response existed.

At the same time there was a growing public outcry over a number of high-profile ES associated deaths. In 2001, the NIH's Office of Dietary Supplements (ODS) received Congressional language encouraging it to enhance clinical research on the safety and efficacy of DSs. This move was spurred by concerns over the increasing use of ES and ephedrine for weight loss and athletic performance and the deaths. ODS sponsored an Agency for Healthcare Research on Quality (AHRQ) review conducted by the Rand Corporation's Southern California Evidence-based Practice Center, and it was completed in 2003 (Agency for Healthcare Research on Quality 2003; Shekelle et al., 2003a) The review concluded that DS containing ES/ephedrine (usually in combination with caffeine) had modest short-term effects on weight loss, although long term effects on weight were unavailable and unknown. Ephedrine plus caffeine was associated with a boost in immediate physical performance for fit young men. However, there was no evidence that ES or ephedrine improved long-term physical performance of athletes or that it would do so for most members of the public. ES and ephedrine increased the risk of nausea, vomiting, jitteriness, and palpitations. Moreover, there were more serious risks linking the products to catastrophic events such as sudden death, heart attack, or stroke. While individuals with a history of cardiovascular disease, those taking high doses of ES containing supplements, and those taking it in combination with other stimulants such as caffeine were be expected to be at increased risk, some of the severe adverse events occurred in individuals with no preexisting medical problems, those taking relatively low doses of ES, or taking ES alone (Shekelle et al., 2003b). Although, when the study was first published it was still unclear whether these data were sufficient to remove the products from the market, the RAND evidence-based review contributed needed information for generating the FDA ban on ES in DS in February 2004.

Resolution of the Problem

Metrology became involved early dealing with ES. The initial 1997 FDA dose limiting regulatory approach for mitigating the harms caused by ES DSs was hampered because analytical resources (methods, and matrix reference materials) were insufficient to support a proposed rule (Betz et al., 1997; Hurlbut and Carr 1998). NIST resolved the problems involving lack of reference materials necessary for enforcing possible regulations involving ceilings for the amounts of ephedrine in DS by beginning development of a suite of ES reference materials. Many cases of heart problems and some deaths linked to ES containing DS continued to be reported, but public outcries and calls for action mushroomed after the death of a sports celebrity, the 23-year-old Baltimore Orioles baseball pitcher Steve Belcher, from a stroke after taking diet pills containing ES.

Although pure ephedrine alkaloids for use as calibration standards had long been commercially available from fine chemical companies, NIST's contribution of matrix reference materials with certified values for ephedrine alkaloids and caffeine was critical. The reference materials were intended primarily for methods validation and use as control materials to support the analysis of DS and related botanical materials. In 2006 a suite of five ES-containing dietary supplement Standard Reference Materials (SRMs) was issued by the National Institute of Standards and Technology (NIST) with certified values for ephedrine alkaloids, synephrine, caffeine, and selected toxic trace elements. The materials represented a variety of natural, extracted, and processed sample matrixes that provided different analytical challenges. The content of constituents was determined by multiple independent methods with measurements performed by NIST and by three collaborating laboratories. The methods utilized different sample extraction and cleanup steps in addition to different instrumental analytical techniques and approaches to quantification. (Sander et al., 2005). Because ES containing supplements were frequently produced as blends with other ingredients which appeared to enhance cardiovascular effects (e.g., caffeine and other botanicals), values for these were also included in the reference suite (Brown et al., 2007).

However, by 2006, since FDA had banned ES in DS in 2004, reference materials for quality control centering on dose to limit the amounts of actives in marketed products were no longer needed. By the time the methods and materials became available, the regulatory goal had changed to developing methods suitable for enforcing a limit of zero tolerance (Roman 2004; Sharpless et al., 2006). Since ES was banned shortly after the development of the NIST reference materials. they were never used as much as was originally intended. In the first years after the 2004 ban the furor against ES was so great that at one point, officials at the US Drug Enforcement Agency who feared that the matrix reference materials might be purchased by unscrupulous individuals and diverted to produce methamphetamine requested NIST to stop selling them.

Soon after the 2004 ban of ES containing products, "ES free" weight loss products began to appear in increasing numbers. The first was bitter orange (also called Seville orange or sour orange), which contains synephrine, the main bioactive in ES, as substitutes for ES. NIST developed a standard reference material (SRM) suite for Bitter Orange which provided three forms representing different challenges analytically: ground fruit, extract, and a solid dose oral table form. The SRM provided certified concentration values for synephrine, octopamine, tyramine, N-methylytramine, hordenine, total alkaloids and caffeine.

Lessons Learned and Other Comments

The first lesson learned from the ES experience was the important role of metrology in improving the quality of complex botanical DS like ES, caffeine and bitter orange containing DS products by providing certified SRM to

validate measurements of the major bioactives. This stimulated the later development of a new category of SRM for DS by NIST (Sander et al., 2006). These SRM are used primarily in method development, as control materials, and to assist manufacturers of DS in characterizing raw materials for potency, authenticity, and contamination or adulteration. SRM are also valuable in assisting in assessment of consistency and quality in finished products and can be used by the DS industry and measurement experts to improve DS quality and ultimately reduce public health risks that may be associated with such products (Sander et al., 2006).

A second lesson was that CRM are helpful in improving quality *only* if they are used. A DS industry with higher quality standards ideally would lessen safety risks. Although some large DS manufacturers use the CRM and call for stronger quality enforcement, other DS producers place a low premium on improving DS product quality. There was little regulatory incentive to improve quality under current law and enforcement measures, but in recent years the FDA has issued Good Manufacturing Practice guidance for DS that may be helpful.

The third lesson is that although ES SRMs were not used as widely as was originally intended, the experience amassed in developing them has been helpful in development of other SRM for DS. Since 2006 NIST has issued SRM for dietary supplement matrices including: ES, bitter orange, *Ginkgo biloba*, saw palmetto, St. John's wort, green tea, yerba mate, kelp, turmeric, ginger, multivitamin/multielement tablets, botanical oils, and fish oils. All are characterized with values assigned for the content of active ingredients and/or marker compounds and toxic elements. Two experts predict that in the next decade the focus in development of food and DS CRMs will be on nutrients in food and DS matrices and use of isotope dilution (ID) LC-MS and ID LC-MS/MS methods for the determination of vitamins and other organic nutrients, all with matrices with potentially lower (<3%) expanded uncertainties. (Wise and Phillips 2019). From a metrological standpoint, the values assigned for all vitamins in food- and DS-matrix CRMs should be determined using MS-based methods with ID quantification, if feasible, rather than with microbiological assays. DS-matrix SRMs now have values assigned for over 80 organic and inorganic nutrients, toxic elements, proximates (e.g., protein, fat, moisture, ash and carbohydrates), and contaminants.

A fourth lesson is that only a small number of DS containing ES or ephedrine alkaloids at low doses are still being sold on the US market today compared to the higher doses and larger number before the 2004 ban although new threats to the public health emerge when a product is banned. In 2021 the NIH Dietary Supplement Label database listed only 94 products with mentions of ES on the label and 15 contained varying amounts of ES extracts. ES in DSs was banned in the European Union (EU) in 2006, and it is illegal in the United States, Australia and Canada and several other countries. ES was 'in the marketplace', mostly as *Ma Huang*, pre-DSHEA, and that herb is still sold in traditional Chinese pharmacies and also appears in some foods. It was "grandfathered in" by law and not subject to FDA's new drug ingredient (NDI) notification rules.

A fifth lesson is that irrational DS product formulations that fly in the face of pharmaceutical science are still being marketed by manufacturers who fail to seek the advice of knowledgeable scientists who could warn against using potentially risky combinations of multi-ingredient botanical products with caffeine and other chemicals. Premarket clinical testing and approval are not legally required and so their adverse effects may go undetected. More evaluation of these complex mixtures and their physiological effects is needed to protect the health of the public. In spite of the ES experience, DS of complex botanical mixtures with serious adverse cardiovascular effects became popular after the ES ban and are still being marketed for weight loss, sports performance and energy enhancement. The tablets and pills are devoid of ephedrine alkaloids but containing bitter orange, with the synephrine usually present as a natural part of the plant, and less frequently as the synthetic form, or as a purified form from the plant. In one study, concentrations varied from 5 to 14 mg/g in five different DSs (Santana et al., 2008). The amounts of the caffeine containing ingredients often exceeded a serving of caffeinated beverages) and other botanicals with many different pharmacological activities were present in addition to the *Citrus aurantium* extracts. The product labels made it difficult to determine exactly how much caffeine was present, since it was often in proprietary blends or only the botanical sources were listed (Gurley et al., 2014). More recently, DS and "energy drinks" containing very high amounts of caffeine have also been associated with cardiovascular problems (Institute of Medicine 2014; Gurley et al., 2015). Although little evidence exists that bitter orange is safer than ES, many products are sold with it listed as an ingredient, usually as an extract. In 2021 the DSLD listed 474 product labels with bitter orange as an ingredient, usually as an extract, and some also contained caffeine although they probably contained lower amounts of both ingredients than in the early 2000s.

All that has been learned from the unfortunate history of ES containing DS does not guarantee that another DS might not cause serious health problems in the future. The United States lacks a registration system for DS and continues to allow "natural" botanical sources of caffeine, ephedrine alkaloids and propanolamine combinations in DS. Since the early 1980s FDA has restricted the marketing of their synthetic forms in "amphetamine look-alikes" because of the cardiovascular risks they impose (Gurley et al., 2015). FDA focuses on safety risks but monitoring the myriad products now on the market exceeds their resources. DS product labeling for non-nutrient ingredients is confusing because the amounts of these bioactives are not required to be listed in a standard manner by DSHEA and also many are in proprietary blends, which do not disclose amounts. Some of these problems created by DSHEA remain and legislators have not yet revisited the law and resolved the issues Until the law changes, it is unlikely that such information will be disclosed.

RECOMMENDATIONS

More Involvement of Analytical Chemists Pharmaceutical Scientists and Metrologists in Research on Dietary Supplement Quality

This article has focused on expanding the role of analytical chemistry and metrology in assuring dietary supplement quality and safety to the extent that the bioactive dose affects it. Guidelines for evaluating herbs, medicinal plants and other ingredients using these tools should be disseminated more widely.

Clearly, metrology and a comprehensive and accurate understanding of the chemistry of complex products are necessary first for quality, which is key to beginning the process of addressing the safety and efficacy of products for replicable research. However, the safety and efficacy of DS also depends on issues involving design, replicability, and interpretability of clinical trials (especially of botanical supplements). These are additional important matters that need attention but are outside of the scope of this paper (Sorkin B. et al., 2020). Metrologists must become familiar with these issues and work with other experts to deal with them.

Greater Awareness of the Regulatory and Communications Context of Methods Development, Metrology and Analysis

Metrologists and analytical chemists working in the field of DS can maximize their impact on public health if their plans are designed to be relevant to regulators both at home and abroad. More communication is needed between, metrologists and analytical chemists and pharmacovigilance experts within and between countries on ingredients and new compounds such as excipients that while safe in drugs consumed over a few days may not be so in supplements consumed over weeks or years, that may be causing adverse events. Finally, there is the issue of international communication which at present appears to be on an ad hoc basis rather than through regular interactions between experts in various countries. While the current efforts of various agencies (AOAC International, US Pharmacopoeia, the European Medicines Agency, and others) to disseminate information on dietary supplement ingredients internationally are helpful, the advisability of launching complementary efforts to optimize outcomes is appealing. The US NIH's National Center for Complementary and Integrative Health (NCCIH) regularly updates its guidance on compliance with its natural product integrity policy, and both NCCIH and the ODS now have formalized policies to help ensure appropriate characterization and reproducibility of reagents and experimental interventions used in funded research[18]. This policy establishes guidance on the information required by ODS and NCCIH for different types of products used in both mechanistic and clinical research including complex botanical and animal products, probiotics, refined products, and placebos. Importantly, the NIH Policy notes that multiple, orthogonal analytical methods may be needed to appropriately characterize natural products and DS, noting that techniques to confirm the authenticity of a product ". . .may include, but are not limited to, HPLC, UV, MS, IR, NMR, and should, when taken together, generate a unique profile, or "fingerprint," which can be diagnostic for that substance". However, although research grants involving DS are now reviewed for product integrity, many federally funded studies still appear to lack rigor in this regard in the United States.

Communicate Findings to Other Disciplines Nationally and Internationally

Another area requiring further work is in the dissemination of findings. In contrast to well established channels for disseminating analytical methods internationally, the communication of findings about dietary supplement quality to the public is usually confined within national boundaries and are not widely publicized internationally. Broader dissemination may be in order since many Web e-marketing ventures involving problematic DS often bypass some or all country-level regulatory control efforts. Although some valuable informal and irregular "cross talk" goes on among some of the pharmacovigilance experts across countries more regular communication may be in order. Metrologists, pharmacists, toxicologists, natural product chemists, pharmacognosists, and food and nutrition scientists could work more effectively with regulatory authorities and others to disseminate their findings both nationally and globally. Present efforts to deal with regulatory problems and international communications and recommendations for enhancing them will be addressed in a future communication.

Conclusion

Awareness is increasing on the importance of analytical replicability and rigorous chemical characterization in DS, and powerful methods are now available for such characterization. However, metrology is often underappreciated and underutilized in addressing the many challenges presented by complex botanical DS. Greater use of metrology resources and expanded application of metrology approaches are needed, and current best practices should be disseminated more widely. These steps will ensure that academic researchers, regulators, and scientists in industry are familiar with and apply metrology in addition to other analytical resources to improve the quality, safety, and effectiveness of DS. A new frontier for metrology is promoting interactions between the analytical, clinical, and pharmaceutical scientists who are making products and conducting research with metrologists to develop standards and establish methodological guidelines. This is a critical step to advancing research on DS. A second new frontier for metrology is in developing improved analytical methods, standards and data management methods in research on DS and communicating these developments to academic

[18]https://www.nccih.nih.gov/research/nccih-policy-natural-product-integrity.

and industry researchers and analysts, as well as to decision makers in the public and private sectors. Finally, more international collaborative efforts are needed to speed the development of internationally agreed upon measurements that can enhance the basis for regulatory harmonization, support reproducible research, and advance scientific understanding.

AUTHOR CONTRIBUTIONS

AD Conceptualization of manuscript and wrote several sections of main manuscript (i.e., Analytical techniques, Data Analysis and Evaluation, Data Management), reviewed and coordinated manuscript. BS Contributed content to Metrology Applications section and overall editing. ML Wrote sections (i.e., Analytical techniques, Data Analysis and Evaluation), reviewed and edited manuscript. PG Contributed content to sections on Analytical techniques and Dosage form performance, reviewed, and edited manuscript. AK Wrote case studies section, reviewed, and edited manuscript. CC, CB, PD and AL: Wrote case studies section, reviewed and edited manuscript. LS: Reviewed all versions of the manuscript and commented and edited the introduction and recommendations sections. BG: Wrote case studies section and reviewed and edited manuscript. PP: Reviewed and edited manuscript. JH: Wrote case studies section and reviewed and edited manuscript. AT: Reviewed and edited manuscript and indicated references to prior food and nutrition themes in metrology. KA Contributed content to sections on Analytical techniques and Dosage form performance, reviewed and edited manuscript. MH: Discussed the overarching perspective and strategy, reviewed and edited manuscript JD: Conceptualization of manuscript, data analyses and evaluation, and wrote several sections including case study 3, reviewed and edited all versions, coordinated and consolidated comments on the final manuscript.

FUNDING

Office of Dietary Supplements, National Institutes of Health, Beltsville Human Nutrition Research Center, US Department of Agriculture.

ACKNOWLEDGMENTS

We thank Stephen Wise of the NIH Office of Dietary Supplements for expert review of the section of the manuscript dealing with Analytical Challenges and Approaches in Characterizing Dietary Supplements, and Joseph Betz, acting Director of the NIH Office of Dietary Supplements for his critical review and comments, particularly on the ES case study.

REFERENCES

Abdi, H., and Williams, L. J. (2010). Principal Component Analysis. *Wires Comp. Stat.* 2 (4), 433–459. doi:10.1002/wics.101

Agency for Healthcare Research on Quality (2003). *Ephedra and Ephedrine for Weight Loss and Athletic Performance Enhancement Evidence Report Technical Assessment No 76 Agency for Health Care Research on Quality Washington DC 2003*.

Al-Gousous, J., and Langguth, P. (2015). Oral Solid Dosage Form Disintegration Testing - the Forgotten Test. *J. Pharm. Sci.* 104 (9), 2664–2675. Epub 2014 Dec 24. PMID: 25546430. doi:10.1002/jps.24303

American Cancer Society (2021). Understanding the Claims on Dietary Supplements Labels. Available at: https://www.cancer.org/treatment/treatments-and-side-effects/complementary-and-alternative-medicine/dietary-supplements/reading-labels.html.

Amorim, T. L., Duarte, L. M., de la Fuente, M. A., de Oliveira, M. A. L., and Gómez-Cortés, P. (2020). Fast Capillary Electrophoresis Method for Determination of Docosahexaenoic and Eicosapentaenoic Acids in marine Oils omega-3 Supplements. *J. Chromatogr. A.* 1613, 460641. doi:10.1016/j.chroma.2019.460641

Amorim, T. L., Duarte, L. M., Chellini, P. R., and de Oliveira, M. A. L. (2019). A Validated Capillary Electrophoresis Method for Fatty Acid Determination in Encapsulated Vegetable Oils Supplements. *Lwt* 114, 108380. doi:10.1016/j.lwt.2019.108380

Andrews, K. W., Roseland, J. M., Gusev, P. A., Palachuvattil, J., Dang, P. T., Savarala, S., et al. (2017). Analytical Ingredient Content and Variability of Adult Multivitamin/mineral Products: National Estimates for the Dietary Supplement Ingredient Database. *Am. J. Clin. Nutr.* 105 (2), 526–539. doi:10.3945/ajcn.116.134544

Astrup, A., Lundsgaard, C., Madsen, J., and Christensen, N. J. (1985). Enhanced Thermogenic Responsiveness during Chronic Ephedrine Treatment in Man. *Am. J. Clin. Nutr.* 42 (1), 83–94. PMID: 4014068. doi:10.1093/ajcn/42.1.83

Austin, K. G., Travis, J., Pace, G., and Lieberman, H. R. (2014). Analysis of 1,3 Dimethylamylamine Concentrations in *Geraniaceae*, geranium Oil and Dietary Supplements. *Drug Test. Anal.* 6 (7-8), 797–804. doi:10.1002/dta.1491

Avula, B., Parveen, I., Zhao, J., Wang, M., Techen, N., Wang, Y. H., et al. (2021). A Comprehensive Workflow for the Analysis of Bio-Macromolecular Supplements: Case Study of 20 Whey Protein Products. *J. Diet. Suppl.*, 1–19. doi:10.1080/19390211.2021.1897724

Azarmi, S., Roa, W., and Löbenberg, R. (2007). Current Perspectives in Dissolution Testing of Conventional and Novel Dosage Forms. *Int. J. Pharm.* 328, 12–21. doi:10.1016/j.ijpharm.2006.10.001

Bansal, A., Chhabra, V., Rawal, R. K., and Sharma, S. (2014). Chemometrics: A New Scenario in Herbal Drug Standardization. *J. Pharm. Anal.* 4, 223–233. doi:10.1016/j.jpha.2013.12.001

Barcaccia, G., Lucchin, M., and Cassandro, M. (2016). DNA Barcoding as a Molecular Tool to Track Down Mislabeling and Food Piracy. *Diversity* 8, 2. doi:10.3390/d8010002

Betz, J. M., Gay, M. L., Mossoba, M. M., and Adams, S. (1997). Chiral Gas Chromatographic Determination of Ephrine-type Alkaloids in Dietary Supplements Containing Ma Huang. *J. AOAC Int.* 80 (2), 303. doi:10.1093/jaoac/80.2.303

Betz, J. M., Fisher, K. D., Saldanha, L. G., and Coates, P. M. (2007). The NIH Analytical Methods and Reference Materials Program for Dietary Supplements. *Anal. Bioanal. Chem.* 389, 19–25. doi:10.1007/s00216-007-1342-8

Betz, J. M., Rimmer, C. A., Saldanha, L. G., Phillips, M. M., Andrews, K. W., Wise, S. A., et al. (2018). Challenges in Developing Analytically Validated Laboratory-Derived Dietary Supplement Databases. *J. Nutr.* 148 (Suppl. 2), 1406S–1412S. doi:10.1093/jn/nxy134

Binns, C. W., Lee, M. K., and Lee, A. H. (2018). Problems and Prospects: Public Health Regulation. *Annu. Rev. Public Health* 39, 403–420. doi:10.1146/annurev-publhealth-040617-013638

Bjeladinovic, S., Marjanovic, Z., and Babarogic, S. (2020). A Proposal of Architecture for Integration and Uniform Use of Hybrid SQL/NoSQL

Database Components. *J. Syst. Soft.* 168, 110633. doi:10.1016/j.jss.2020.110633

Bloomer, R., Smith, N. J. G., Moore, D., and Yates, C. (2018). 1,3-Dimethylamylamine (DMAA): a Brief History and Review of Anecdotal and Laboratory Findings. *J. Altern. Complement. Integr. Med.* 4. doi:10.24966/ACIM-7562/100057

Bouri, M., Salghi, R., Zougagh, M., and Ríos, A. (2013). Capillary Electrophoresis Coupled to Evaporative Light Scattering Detection for Direct Determination of Underivatized Amino Acids: Application to tea Samples Using Carboxyled Single-Walled Carbon Nanotubes for Sample Preparation. *Electrophoresis* 34 (18), 2623–2631. doi:10.1002/elps.201300145

Brown, J., Sharpless, K., Mitvalsky, S., Roman, M., Yen, J., and Satterfield, M. (2007). Determination of Caffeine, and Caffeine-Related Metabolites in Ephedra-Containing Standard Reference Materials Using Liquid Chromatography with Absorbance Detection and Tandem Mass Spectrometry. *J. AOAC Int.* 90 (4), 934–940. doi:10.1093/jaoac/90.4.934

Cade, J. E. (2017). Measuring Diet in the 21st century: Use of New Technologies. *Proc. Nutr. Soc.* 76 (3), 276–282. doi:10.1017/S0029665116002883

Chaleckis, R., Meister, I., Zhang, P., and Wheelock, C. E. (2019). Challenges, Progress and Promises of Metabolite Annotation for LC-MS-based Metabolomics. *Curr. Opin. Biotechnol.* 55, 44–50. doi:10.1016/j.copbio.2018.07.010

Cianchino, V., Acosta, G., Ortega, C., Martínez, L. D., and Gomez, M. R. (2008). Analysis of Potential Adulteration in Herbal Medicines and Dietary Supplements for the Weight Control by Capillary Electrophoresis. *Food Chem.* 108 (3), 1075–1081. doi:10.1016/j.foodchem.2007.11.042

Cohen, P. (2012). DMAA as a Dietary Supplement Ingredient. *Arch. Intern. Med.* 172 (13), 1038–1039. doi:10.1001/archinternmed.2012.1677

Cohen, P., Travis, J., Keizers, P., Deuster, P., and Venhus, B. (2017). Four Experimental Stimulants Found in Sports and Weight Loss Supplements: 2-Amino-6-Methylheptane (Octodrine), 1,4-dimethylamylamine (1,4-DMAA), 1,3-dimethylamylamine (1,3-DMAA) and 1,3-dimethylbutylamine (1,3-DMBA). *Clin. Toxicol.* 56 (6), 421–426. doi:10.1080/15563650.2017.1398328

Crawford, C., Boyd, C., Avula, B., Wang, Y. H., Khan, I. A., and Deuster, P. A. (2020). A Public Health Issue: Dietary Supplements Promoted for Brain Health and Cognitive Performance. *J. Altern. Complement. Med.* 26 (4), 265–272. doi:10.1089/acm.2019.0447

Crawford, C., and Deuster, P. (2020). Be in the Know: Dietary Supplements for Cognitive Performance. *J. Spec. Op. Med.* 20 (2), 132–135.

Deconinck, E., Sokeng Djiogo, C. A., Bothy, J. L., and Courselle, P. (2017). Detection of Regulated Herbs and Plants in Plant Food Supplements and Traditional Medicines Using Infrared Spectroscopy. *J. Pharm. Biomed. Anal.* 142, 210–217. doi:10.1016/j.jpba.2017.04.051

Deconinck, E., Vanhamme, M., Bothy, J. L., and Courselle, P. (2019). A Strategy Based on Fingerprinting and Chemometrics Forthe Detection of Regulated Plants Inplant Food Supplements from the Belgian Market: Two Case Studies. *J. Pharm. Biomed. Anal.* 166, 189–196. doi:10.1016/j.jpba.2019.01.015

Di Lorenzo, C., Ceschi, A., Kupferschmidt, H., Lüde, S., De Souza Nascimento, E., Dos Santos, A., et al. (2015). Adverse Effects of Plant Food Supplements and Botanical Preparations: a Systematic Review with Critical Evaluation of Causality. *Br. J. Clin. Pharmacol.* 79 (4), 578–592. doi:10.1111/bcp.12519

dos Santos, V. B., Daniel, D., Singh, M., and do Lago, C. L. (2016). Amphetamine and Derivatives in Natural Weight Loss Pills and Dietary Supplements by Capillary Electrophoresis-Tandem Mass Spectrometry. *J. Chromatogr. B* 1038, 19–25. doi:10.1016/j.jchromb.2016.10.019

Duong, H. A., Vu, M. T., Nguyen, T. D., Nguyen, M. H., and Mai, T. D. (2020). Determination of 10-Hydroxy-2-Decenoic Acid and Free Amino Acids in Royal Jelly Supplements with Purpose-Made Capillary Electrophoresis Coupled with Contactless Conductivity Detection. *J. Food Comp. Anal.* 87, 103422. doi:10.1016/j.jfca.2020.103422

Durazzo, A., D'Addezio, L., Camilli, E., Piccinelli, R., Turrini, A., Marletta, L., et al. (2018). From Plant Compounds to Botanicals and Back: a Current Snapshot. *Molecules* 23(8):1844. doi:10.3390/molecules23081844

Durazzo, A., Lucarini, M., Kiefer, J., and Mahesar, S. A. (2020). State-of-the-Art Infrared Applications in Drugs, Dietary Supplements, and Nutraceuticals. *J. Spectr.* 2020, 1–2. doi:10.1155/2020/1397275

Dwyer, J. T., Coates, P. M., and Smith, M. J. (2018). Dietary Supplements: Regulatory Challenges and Research Resources. *Nutrients* 10 (1), 41. doi:10.3390/nu10010041

Dwyer, J. T., Saldanha, L., Bailen, R., Durazzo, A., Le Donne, C., Piccinelli, R., et al. (202110388). Commentary: An Impossible Dream? Integrating Dietary Supplement Label Databases Needs, Challenges, Next Steps. *103882 J. Food Comp. Anal.*

Eichner, A. K., Coyles, J., Fedoruk, M., Maxey, T. D., Lenaghan, R. A., Novitzky, J., et al. (2019). Essential Features of Third-Party Certification Programs for Dietary Supplements: a Consensus Statement. *Curr. Sport Med. Rep.* 18(5): 178–182. doi:10.1249/JSR.0000000000000595

Eliason, M., Eichner, A., Cancio, A., Bestervelt, L., Adams, B., and Deuster, P. (2012). Case Reports: Death of Active Duty Soldiers Following Ingestion of Dietary Supplements Containing 1,3-Dimethylamylamine (DMAA). *Mil. Med.* X (1). doi:10.7205/milmed-d-12-00265

FAO (2021). FAOLEX Database. Available at: http://www.fao.org/faolex/background/en/.

Fuller, D., Buote, R., and Stanley, K. (2017). A Glossary for Big Data in Population and Public Health: Discussion and Commentary on Terminology and Research Methods. *J. Epidemiol. Community Health* 71, 1113–1117. doi:10.1136/jech-2017-209608

Galimberti, A., de Mattia, F., Losa, A., Bruni, I., Federici, S., Casiraghi, M., et al. (2013). DNA Barcoding as a New Tool for Food Traceability. *Food Res. Int.* 50, 55–63. doi:10.1016/j.foodres.2012.09.036

Gee, P., Jackson, S., and Easton, J. (2010). Another Bitter Pill: a Case of Toxicity from DMAA Party Pills. *N. Z. Med. J.* 123, 124–127.

Gee, P., Tallon, C., Long, N., Moore, G., Boet, R., and Jackson, S. (2012). Use of Recreational Drug 1,3 Dimethylamylamine (DMAA) Associated with Cerebral Hemorrhage. *Ann. Emerg. Med.* 60, 431–434. doi:10.1016/j.annemergmed.2012.04.008

Gotti, R. (2011). Capillary Electrophoresis of Phytochemical Substances in Herbal Drugs and Medicinal Plants. *J. Pharm. Biomed. Anal.* 55 (4), 775–801. doi:10.1016/j.jpba.2010.11.041

Griffiths, P. R., and De Haseth, J. A. (2007). *Fourier Transform Infrared Spectrometry*. 2nd edition. Weinheim, Germany: Wiley.

Gurley, B. J., Steelman, S. C., and Thomas, S. L. (2015). Multi-ingredient, Caffeine-Containing Dietary Supplements: History, Safety, and Efficacy. *Clin. Ther.* 37 (2), 275–301. doi:10.1016/j.clinthera.2014.08.012

Gusev, P. A., Andrews, K. W., Savarala, S., Tey, P. T., Han, F., Oh, L., et al. (2020). Disintegration and Dissolution Testing of green tea Dietary Supplements: Application and Evaluation of United States Pharmacopeial Standards. *J. Pharm. Sci.* 109 (6), 1933–1942. doi:10.1016/j.xphs.2020.02.005

Handy, S. M., Pawar, R. S., Ottesen, A. R., Ramachandran, P., Sagi, S., Zhang, N., et al. (2021). HPLC-UV, Metabarcoding and Genome Skims of Botanical Dietary Supplements: a Case Study in Echinacea. *Planta Med.* 87 (4), 314–324. doi:10.1055/a-1336-1685

Harnly, J., Chen, P., Sun, J., Huang, H., Colson, K. L., Yuk, J., et al. (2016). Comparison of Flow Injection MS, NMR, and DNA Sequencing: Methods for Identification and Authentication of Black Cohosh (*Actaea Racemosa*). *Planta Med.* 82, 250–262. doi:10.1055/s-0035-1558113

Harnly, J., Lu, Y., Sun, J., and Chen, C. (2017). Botanical Supplements: Detecting the Transition from Ingredient to Product. *J. Food Compos. Anal.* 64, 85–92. doi:10.1016/j.jfca.2017.06.010

Haynes, E., Jimenez, E., Pardo, M. A., and Helyar, S. J. (2019). The Future of NGS (Next Generation Sequencing) Analysis in Testing Food Authenticity. *Food Control* 101, 134–143. doi:10.1016/j.foodcont.2019.02.010

Hibbert, D. B. (2016). Vocabulary of Concepts and Terms in Chemometrics (IUPAC Recommendations 2016). *Pure Appl. Chem.* 88, 4. doi:10.1515/pac-2015-0605

Hight, S. K., Kurita, K. L., McMillan, E. A., Bray, W., Clark, T. N., Shaikkh, A. F., et al. (2019). High-throughput Functional Annotation of Natural Products by Integrated Activity Profiling. *BioRxiv*. doi:10.1101/748129

Hurlbut, J. A., and Carr, J. R. (1998). Solid-phase Extraction Cleanup and Liquid Chromatography with Ultraviolet Detection of Ephedrine Alkaloids

in Herbal Products. *J. AOAC Int.* 81, 1121–1127. doi:10.1093/jaoac/81.6.1121

Ibragic, S., and Sofić, E. (2015). Chemical Composition of Various Ephedra Species. *Bosn. J. Basic Med. Sci.* 15 (3), 21–27. doi:10.17305/bjbms.2015.539

Institute of Medicine (2014). "Planning Committee for a Workshop on Potential Health Hazards Associated with Consumption of Caffeine in Food and Dietary Supplements; Food and Nutrition Board; Board on Health Sciences Policy; Institute of Medicine," in Caffeine in Food and Dietary Supplements: Examining Safety: Workshop Summary, Washington (DC) (National Academies Press (US)).

Ivanova, N. V., Kuzmina, M. L., Braukmann, T. W., Borisenko, A. V., and Zakharov, E. V. (2016). Authentication of Herbal Supplements Using Next-Generation Sequencing. *PLoS One* 11 (5), e0156426. doi:10.1371/journal.pone.0156426

Jadhav, N., Yan, M., Vetter, C. A., Kasisomayajula, S. V., and Gelling, V. J. (2013). "Infrared Spectroscopy (IR)," in *Encyclopedia of Tribology*. Editors Q. J. Wang and Y. W. Chung (Boston, MA: Springer). doi:10.1007/978-0-387-92897-5_1226

King, B. (1997). Metrology and Analytical Chemistry. *bridging Cult. gap Metrologia* 34 (1), 1–11. doi:10.1088/0026-1394/34/1/7

Kress, W. J., and Erickson, D. L. (2012). "DNA Barcodes: Methods and Protocols," in *Methods in Molecular Biology™ Series*. Editors I. Lopez and D. L. Erickson (SpringerHumana Press), XV, 470. 978-1-61779-591-6. doi:10.1007/978-1-61779-591-6_1

LaBudde, R. A., and Harnly, J. M. (2012). Probability of Identification: a Statistical Model for the Validation of Qualitative Botanical Identification methodsJ. *AOAC Int.* 95 (1), 273–285. doi:10.5740/jaoacint.11-266

Lee, I., Vo, J., Gao, Q., Purohit, P., Zarraga, V., Babajanian, S., et al. (2020). Single-laboratory Validation Study of a Proton NMR Method for the Determination of L-Arginine, L-Citrulline, and Taurine Contents in Dietary Supplements. *J. AOAC Int.* 103 (4), 1140–1147. doi:10.1093/jaoacint/qsaa002

Lo, Y. T., and Shaw, P. C. (2019). Application of Next-Generation Sequencing for the Identification of Herbal Products. *Biotechnol. Adv.* 37 (8), 107450. doi:10.1016/j.biotechadv.2019.107450

Mansouri, Y., Prokhorenko, V., and Ali Babar, M. (2020). An Automated Implementation of Hybrid Cloud. *J.Netw. Comp. Appl.* 167, 102740. doi:10.1016/j.jnca.2020.102740

Martínez-Sanz, J. M., Sospedra, I., Ortiz, C. M., Baladía, E., Gil-Izquierdo, A., and Ortiz-Moncada, R. (2017). Intended or Unintended Doping? A Review of the Presence of Doping Substances in Dietary Supplements Used in Sports. *Nutrients* 9, 1093. doi:10.3390/nu9101093

McCarthy, C., Canale, R., Alleman, R., Jr, Reed, J., and Bloomer, R. (2011). Biochemical and Anthropometric Effects of a Weight Loss Dietary Supplement in Healthy Men and Women. *Nutr. Metab. Insights* 6 (5), 13–22. doi:10.4137/NMI.S8566

Ministero della Salute (2020). Registro Degli Integratori Alimentari. 15 Giugno 2020Retrieved. Available at: http://www.salute.gov.it/portale/temi/p2_6.jsp?lingua=italiano&id=3668&area=Alimenti%20particolari%20e%20integratori&menu=registri.

Misra, B. B. (2021). New Software Tools, Databases, and Resources in Metabolomics: Updates from 2020. *Metabolomics : Official J. Metabolomic Soc.* 17 (5), 49. doi:10.1007/s11306-021-01796-1

Mok, D. K. W., and Chau, F. T. (2006). Chemical Information of Chinese Medicines: A challenge to Chemist. *Chemom. Intell. Lab. Syst.* 82, 210–217. doi:10.1016/j.chemolab.2005.05.006

Mudge, E. M., Brown, P. N., Rimmer, C. A., and Phillips, M. M. (2020). Determination of Curcuminoids in Turmeric Dietary Supplements by HPLC-DAD: Multi-Laboratory Study through the NIH-ODS/NIST Quality Assurance Program. *J. AOAC Int.* 103 (6), 1625–1632. doi:10.1093/jaoacint/qsaa069

Muela-Molina, C., Perelló-Oliver, S., and García-Arranz, A. (2021). False and Misleading Health-Related Claims in Food Supplements on Spanish Radio. An Analysis from a European Regulatory Framework. *Pub. Health Nutr.*, 1–24. doi:10.1017/S1368980021002007

Mullin, G. E., Limektkai, B., Wang, L., Hanaway, P., Marks, L., and Giovannucci, E. (2021). Dietary Supplements for COVID-19. *Adv. Exp. Med.Biol.* 1318, 499–515. doi:10.1007/978-3-030-63761-3_29

Nelson, K. M., Dahlin, J. L., Bisson, J., Graham, J., Pauli, G. F., and Walters, M. A. (2017). The Essential Medicinal Chemistry of Curcumin. *J. Med. Chem.* 60 (5), 1620–1637. doi:10.1021/acs.jmedchem.6b00975

Natural Medicines (2018). Natural Medicines Database. Available at https://naturalmedicines.therapeuticresearch.com/". (Accessed May 30, 2021).

Nguyen, T. D., Nguyen, M. H., Vu, M. T., Duong, H. A., Pham, H. V., and Mai, T. D. (2019). Dual-channeled Capillary Electrophoresis Coupled with Contactless Conductivity Detection for Rapid Determination of Choline and Taurine in Energy Drinks and Dietary Supplements. *Talanta* 193, 168–175. doi:10.1016/j.talanta.2018.10.002

O'Brien, S. K., Malacova, E., Sherriff, J. L., and Black, L. J. (2017). The Prevalence and Predictors of Dietary Supplement Use in the Australian Population. *Nutrients* 9, 1154. doi:10.3390/nu9101154

Otto, M. (2016). *Chemometrics: Statistics and Computer Application in Analytical Chemistry*. 3rd ed. New York, NY, USA: Wiley. 978-3-527-34097-2.

Palamar, J. (2010). How Ephedrine Escaped Regulation in the United States: a Historical Review of Misuse and Associated Policy. *Health Pol.* 99 (1), 1–9. doi:10.1016/j.healthpol.2010.07.007

Parnell, W. R., Wilso, N. C., and Smith, C. (2006). Dietary Supplements: Prevalence of Use in the New Zealand Population. *Nutr. Diet.* 63, 199–205. doi:10.1111/j.1747-0080.2006.00110.x

Parveen, I., Gafner, S., Techen, N., Murch, S. J., and Khan, I. A. (2016). DNA Barcoding for the Identification of Botanicals in Herbal Medicine and Dietary Supplements: Strengths and Limitations. *Planta Med.* 82 (14), 1225–1235. doi:10.1055/s-0042-111208

Pawar, R. S., Handy, S. M., Cheng, R., Shyong, N., and Grundel, E. (2017). Assessment of the Authenticity of Herbal Dietary Supplements: Comparison of Chemical and DNA Barcoding Methods. *Planta Med.* 83 (11), 921–936. doi:10.1055/s-0043-107881

Pehrsson, P. R., and Mitchell, D. C. (2020). Advancing Food and Nutrient Databases through Partnerships and Technology. *J. Food Comp. Anal.* 92, 103527. doi:10.1016/j.jfca.2020.103527

Pferschy-Wenzig, E. M., and Bauer, R. (2015). The Relevance of Pharmacognosy in Pharmacological Research on Herbal Medicinal Products. *Epil. Behav.* 52 (Part B), 344–362. doi:10.1016/j.yebeh.2015.05.037

Phillips, M. M., Rimmer, C. A., Wood, L. J., Lippa, K. A., Sharpless, K. E., Duewer, D. L., et al. (2011). Dietary Supplement Laboratory Quality Assurance Program: the First Five Exercises. *J. AOAC Int.* 94 (3), 803–814. doi:10.1093/jaoac/94.3.803

Porter, D. V. (2007). *Dietary Supplements: Ephedra. Report for Congress, Congressional Research Service*. Washington: US Congress.

Porter, D. V. (2002). *Dietary Supplements: Legislative and Regulatory Status. Report for Congress, Congressional Research Service*. Washington DC: US Congress.

Powers, M. (2015). Acute Stimulant Ingestion and Neurocognitive Performance in Healthy Participants. *J. Athl. Train.* 50 (5), 453–459. doi:10.4085/1062-6050-50.1.07

Raclariu, A. C., Heinrich, M., Ichim, M. C., and de Boer, H. (2018). Benefits and Limitations of DNA Barcoding and Metabarcoding in Herbal Product Authentication. *Phytochem. Anal.* 29 (2), 123–128. doi:10.1002/pca.2732

Restaino, O. F., De Rosa, M., and Schiraldi, C. (2020). High-performance Capillary Electrophoresis to Determine Intact Keratan Sulfate and Hyaluronic Acid in Animal Origin Chondroitin Sulfate Samples and Food Supplements. *Electrophoresis* 41 (20), 1740–1748. doi:10.1002/elps.202000028

Rimmer, C. A., Sharpless, K. E., Wise, S. A., Betz, J. M., and Coates, P. M. (2013). Standard Reference Materials for Dietary Supplement Analysis. *Anal. Bioanal. Chem.* 405 (13), 4337–4344. doi:10.1007/s00216-013-6942-x

Rizvi, R. F., Vasilakes, J., Adam, T. J., Melton, G. B., Bishop, J. R., Bian, J., et al. (2020). iDISK: the Integrated DIetary Supplements Knowledge Base. *J. Am. Med. Inf. Ass.* 27 (4), 539–548. doi:10.1093/jamia/ocz216

Rocco, A., Donati, E., Touloupakis, E., and Aturki, Z. (2018). Miniaturized Separation Techniques as Analytical Methods to Ensure Quality and Safety of Dietary Supplements. *Tr. Anal. Chem.* 103, 156–183. doi:10.1016/j.trac.2018.04.004

Roman, M. C. (2004). Determination of Ephedrine Alkaloids in Botanicals and Dietary Supplements by HPLCUV: Collaborative Study. *J. AOAC Int.* 87, 1–14. doi:10.1093/jaoac/87.1.1

Roytman, M. M., Poerzgen, P., and Navarro, V. (2018). Botanicals and Hepatotoxicity. *Clin. Pharmacol. Ther.* 104 (3), 458–469. doi:10.1002/cpt.1097

Rychlik, M., Zappa, G., Añorga, L., Belc, N., Castanheira, I., Donard, O., et al. (2018). Ensuring Food Integrity by Metrology and FAIR Data Principles. *Front. Chem.* 6, 49. doi:10.3389/fchem.2018.00049

Saldanha, L., Dwyer, J., Andrews, K., Betz, J., Harnly, J., Pehrsson, P., et al. (2015). Feasibility of Including green tea Products for an Analytically Verified Dietary Supplement Database. *J. Food Sci.* 80 (4), H883–H888. doi:10.1111/1750-3841.12838

Sander, L. C., Bedner, M., Duewer, D. L., Lippa, K. A., Phillips, M. M., Phinney, K. W., et al. (2013). The Development and Implementation of Quality Assurance Programs to Support Nutritional Measurements. *Anal. Bioanal. Chem.* 405 (13), 4437–4441. doi:10.1007/s00216-013-6864-7

Sander, L. C., Sharpless, K. E., Satterfield, M. B., Ihara, T., Phinney, K. W., Yen, J. H., et al. (2005). Determination of Ephedrine Alkaloids in Dietary Supplement Standard Reference Materials. *Anal. Chem.* 77 (10), 3101–3112. doi:10.1021/ac0484530

Sander, L. C., Sharpless, K. E., and Wise, S. A. (2006). Dietary Supplement Standard Reference Materials. *Life Sci.* 78 (18), 2044–2048. doi:10.1016/j.lfs.2005.12.037

Santana, J., Sharpless, K. E., and Nelson, B. C. (2008). Determination of Para-Synephrine and Meta-Synephrine Positional Isomers in Bitter orange-containing Dietary Supplements by LC/UV and LC/MS/MS. *Food Chem.* 109 (3), 675–682. doi:10.1016/j.foodchem.2007.12.076

Schilling, B., Hammond, K., Bloomer, R., Presley, C., and Yates, C. (2013). Physiological and Pharmacokinetic Effects of Oral 1,3-dimethylamylamine Administration in Men. *BMC Pharmacol. Toxicol.* 14 (52). doi:10.1186/2050-6511-14-52

Schrader, B. (1995). *Infrared and Raman Spectroscopy*. Weinheim, Germany: VCH Verlagsgesellschaft.

Sene, M., Gilmore, I., and Janssen, J. T. (2017). Metrology Is Key to Reproducing Results. *Nature* 547 (7664), 397–399. doi:10.1038/547397a

Sharpless, K., Anderson, D., Butler, T., Betz, J., Howell, D., Capar, S., et al. (2006). Preparation and Characterization of a Suite of Ephedra-Containing Standard Reference Materials. *J. AOAC Int.* 89 (6), 1483–1495. doi:10.1093/jaoac/89.6.1483

Shekelle, P. G., Hardy, M. L., Morton, S. C., Maglione, M., Mojica, W. A., Suttorp, M. J., et al. (2003b). Efficacy and Safety of Ephedra and Ephedrine for Weight Loss and Athletic Performance: a Meta-Analysis. *JAMA* 289 (12), 1537–1545. doi:10.1001/jama.289.12.1537

Shekelle, P. G., Morton, S. C., Hardy, M. L., Maglione, M. A., Booth, M., Roth, E., et al. (2003a). *Is it worth the Risk?* Santa Monica, CA: RAND Corporation. Available at: https://www.rand.org/pubs/research_briefs/RB4556.html.

Sherma, J. (2003). High-performance Liquid Chromatography/mass Spectrometry Analysis of Botanical Medicines and Dietary Supplements: a Review. *J. AOAC Int.* 86 (5), 873–881. doi:10.1093/jaoac/86.5.873

Sima, I. A., Andrási, M., and Sârbu, C. (2018). Chemometric Assessment of Chromatographic Methods for Herbal Medicines Authentication and Fingerprinting. *J. Chromatogr. Sci.* 56 (1), 49–55. doi:10.1093/chromsci/bmx080

Simmler, C., Graham, J. G., Chen, S. N., and Pauli, G. F. (2018). Integrated Analytical Assets Aid Botanical Authenticity and Adulteration Management. *Fitoterapia* 129, 401–414. doi:10.1016/j.fitote.2017.11.017

Small, E. (1996). Confusion of Common Names for Toxic and Edible "Star Anise" (*Illicium*) Species. *Econ. Bot.* 50, 337–339.

Smith, C., Wilson, N. C., and Parnell, W. R. (2005). Dietary Supplements: Characteristics of Supplement Users in New Zealand. *Nutr. Diet.* 62: 123–129. doi:10.1111/j.1747-0080.2005.00020.x

Sorkin, B., Betz, J. M., and Hopp, D. C. (2020). Toward FAIRness and a User-Friendly Repository for Supporting NMR Data. *Org. Lett.* 22, 2867. doi:10.1021/acs.orglett.0c01143

Sorkin, B. C., Kuszak, A. J., Bloss, G., Fukagawa, N. K., Hoffman, F. A., Jafari, M., et al. (2020). Improving Natural Product Research Translation: from Source to Clinical Trial. *FASEB J.* 34, 41–65. doi:10.1096/fj.201902143R

Spagnuolo, P. A., Ahmed, N., Buraczynski, M., Roma, A., Tait, K., and Tcheng, M. (2019), 4.44 - Analytical Methods – Functional Foods and Dietary Supplements. *Murray Moo-Young, Comprehensive Biotechnology*. Third Edition. Pergamon, 519–531. doi:10.1016/B978-0-444-64046-8.00238-X

Sun, M., Wang, S., Jing, Y., Li, L., He, M., Jia, Y., et al. (2019). Application of Delayed Luminescence Measurements for the Identification of Herbal Materials: a Step toward Rapid Quality Control. *Chin. Med.* 14, 47. doi:10.1186/s13020-019-0269-2

Urban, P. L. (2016). Quantitative Mass Spectrometry: an Overview. *Philos. Trans. A. Math. Phys. Eng. Sci.* 374, 20150382. doi:10.1098/rsta.2015.0382

U.S. Food and Drug Administration (2018). DMAA in Products Marketed as Dietary Supplements. Available at: https://www.fda.gov/food/dietary-supplement-products-ingredients/dmaa-products-marketed-dietary-supplements (Accessed May 30, 2020).

U.S. Food and Drug Administration (2004). Final Rule Declaring Dietary Supplements Containing Ephedrine Alkaloids Adulterated Because They Present an Unreasonable Risk. *Fed. Regist.* 69 (28), 6788–6854.

van Breemen, R. B. (2020). Role of Mass Spectrometry in Establishing Safety and Efficacy of Botanical Dietary Supplements. *Clin. Mass. Spectrom.* 15, 25–28. doi:10.1016/j.clinms.2019.12.001

Van Eck, N. J. (2011). *Methodological Advances in Bibliometric Mapping of Science*. PhD thesis. Rotterdam: Erasmus University.

Van Eck, N. J., and Waltman, L. (2009). Software Survey: VOSviewer, a Computer Program for Bibliometric Mapping. *Scientometrics* 84, 523–538. doi:10.1007/s11192-009-0146-3

Van Eck, N. J., and Waltman, L. (2010). Software Survey: VOSviewer, a Computer Program for Bibliometric Mapping. *Scientometrics* 84 (2), 523–538. doi:10.1007/s11192-009-0146-3

Van Eck, N. J., and Waltman, L. (2011). Text Mining and Visualization Using VOSviewer. *ISSI Newslett* 7, 50–54.

Vargas Medina, D. A., Vasconcelos Soares Maciel, E., and Lanças, F. M. (2020). Miniaturization of Liquid Chromatography Coupled to Mass Spectrometry. 3. Achievements on Chip-Based LC–MS Devices. *Trac Trend. Anal. Chem.* 131, 116003. doi:10.1016/j.trac.2020.116003

VOSviewer (2020). VOSviewer Software. version 1.6.16. Available at: www.vosviewer.com.

Vyawahare, H. R., Karde, P. P., and Thakare, V. M. (2018). "A Hybrid Database Approach Using Graph and Relational Database," in 2018 International Conference on Research in Intelligent and Computing in Engineering (San Salvador: RICE), 1–4. doi:10.1109/RICE.2018.8509057

Wallace, E. D., Todd, D. A., Harnly, J. M., Cech, N. B., and Kellogg, J. J. (2020). Identification of Adulteration in Botanical Samples with Untargeted Metabolomics. *Anal. Bioanal. Chem.* 412 (18), 4273–4286. doi:10.1007/s00216-020-02678-6

Waltman, L., Van Eck, N. J., and Noyons, E. C. (2010). A Unified Approach to Mapping and Clustering of Bibliometric Networks. *J. Informetrics* 4, 629–635. doi:10.1016/j.joi.2010.07.002

Wise, S. A., and Phillips, M. M. (2019). Evolution of Reference Materials for the Determination of Organic Nutrients in Food and Dietary Supplements-A Critical Review. *Anal. Bioanal. Chem.* 411 (1), 97–127. doi:10.1007/s00216-018-1473-0

Wu, A. H., Gerona, R., Armenian, P., French, D., Petrie, M., and Lynch, K. L. (2012). Role of Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HR/MS) in Clinical Toxicology. *Clin. Toxicol.* 50, 733–742. doi:10.3109/15563650.2012.713108

Wu, N., Balayssac, S., Danoun, S., Malet-Martino, M., and Gilard, V. (2020). Chemometric Analysis of Low-Field 1H NMR Spectra for Unveiling Adulteration of Slimming Dietary Supplements by Pharmaceutical Compounds. *Molecules* 25 (5), 1193. doi:10.3390/molecules25051193

Zeb, A., and Soininen, J.-P. (2021). Data Harmonisation as a Key to Enable Digitalisation of the Food Sector: A Review. *Food Bioprod. Proces.* doi:10.1016/j.fbp.202110.1016/j.fbp.2021.02.005

Zeng, Z., Chau, F. T., Chan, H. Y., Cheung, C. Y., Lau, T. Y., Wei, S., et al. (2008). Recent Advances in the Compound-Oriented and Pattern-Oriented Approaches to the Quality Control of Herbal Medicines. *Chin. Med.* 3, 9. doi:10.1186/1749-8546-3-9

Zhang, Y., Woods, R., Breitbach, Z., and Armstrong, D. (2012). 1,3-Dimethylamylamine (DMAA) in Supplements and geranium Products: Natural or Synthetic? *Drug Test. Anal.* 4 (12), 986–990. doi:10.1002/dta.1368

Zhang, L. X., Burdette, C. Q., Phillips, M. M., Rimmer, C. A., and Marcus, R. K. (2015). Determination of Isoflavone Content in SRM 3238 Using Liquid

Chromatography-Particle Beam/Electron Ionization Mass Spectrometry. *J. AOAC Int.* 98 (6), 1483–1490. doi:10.5740/jaoacint.15-112

Zovico, P., Curty, V., Leal, M., Meira, E. F., Dias, D. V., de Melo Rodrigues, L. C., et al. (2016). Effects of Controlled Doses of Oxyelite Pro on Physical Performance in Rats. *Nutr. Metab.* 13 (90). doi:10.1186/s12986-016-0152-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 17 January 2022
doi: 10.3389/fphar.2021.817885

Phytochemical Studies on Two Unexplored Endemic Medicinal Plants of India, *Barleria terminalis* and *Calacanthus grandiflorus*

Shreedhar S. Otari[1], *Suraj B. Patel*[1], *Manoj M. Lekhak*[2] *and Savaliram G. Ghane*[1]*

[1]*Plant Physiology Laboratory, Department of Botany, Shivaji University, Kolhapur, India,* [2]*Angiosperm Taxonomy Laboratory, Department of Botany, Shivaji University, Kolhapur, India*

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Vishwas Ananat Bapat, Shivaji University, India
Kalidas Shetty, North Dakota State University, United States
Rangappa Keri, Jain University, India
Adriano Mollica, University "G. d'Annunzio" of Chieti-Pescara, Italy

***Correspondence:**
Savaliram G. Ghane
sgg.botany@unishivaji.ac.in
ghaneram@gmail.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *18 November 2021*
Accepted: *27 December 2021*
Published: *17 January 2022*

Citation:
Otari SS, Patel SB, Lekhak MM and Ghane SG (2022) Phytochemical Studies on Two Unexplored Endemic Medicinal Plants of India, Barleria terminalis *and* Calacanthus grandiflorus. *Front. Pharmacol. 12:817885. doi: 10.3389/fphar.2021.817885*

Barleria terminalis Nees and *Calacanthus grandiflorus* (Dalzell) Radlk. are endemic medicinal plants of the Western Ghats of India. The aim of the present research work was to investigate phytochemical profile, potent bioactives using RP-HPLC, LC-MS and GC-MS and to evaluate their bioactivities. Acetone was found to be the best extraction medium for separating phytochemicals. Similarly, acetone and methanol extracts exhibited potential antioxidant properties. Ethanol extract of *B. terminalis* stem showed potent acetylcholinesterase (AChE) (89.10 ± 0.26%) inhibitory activity. Inhibition of α-amylase (36.96 ± 2.96%) activity was observed the best in ethanol extract of *B. terminalis* leaves and α-glucosidase inhibitory activity (94.33 ± 0.73%) in ethanol extract of *C. grandiflorus* stem. RP-HPLC analysis confirmed the presence of several phenolic compounds (gallic acid, hydroxybenzoic acid, vanillic acid, chlorogenic acid and coumaric acid) and phenylethanoid glycoside (verbascoside). The highest phenolics content were observed in *B. terminalis* (GA (4.17 ± 0.002), HBA (3.88 ± 0.001), VA (4.54 ± 0.001), CHLA (0.55 ± 0.004) mg/g DW, respectively). Similarly, LC-MS and GC-MS revealed the presence of phenolics, glycosides, terpenes, steroids, fatty acids, etc. Moreover, positive correlation between studied phytochemicals and antioxidants was observed in principal component analysis. Based on the present investigation, we conclude that *B. terminalis* and *C. grandiflorus* can be further explored for their active principles particularly, phenylethanoid glycosides and iridoids and their use in drug industry for pharmaceutical purposes.

Keywords: biological activities, endemic, HPLC, LC-MS, multivariate analysis, phytochemicals

Abbreviations: ABTS, (2, 2′-azinobis 3-ethylbenzthiazoline-6-sulfonic acid); AChE, acetylcholinesterase; CHLA, chlorogenic acid; COA, coumaric acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DNSA, dinitrosalicylic acid; DTNB, 5, 5-dithiobis-2-nitrobenzoic acid; DW, distilled water; FRAP, ferric reducing antioxidant property; GA, gallic acid; GC-MS, gas chromatography-mass spectrometry; HBA, hydroxybenzoic acid; HR-LC-MS, high-resolution liquid chromatography-mass spectrometry; MCA, metal chelating activity; PMA, phosphomolybdenum activity; PCA, principal component analysis; RP-HPLC, reversed phase high performance liquid chromatography; TIC, total iridoid content; TFC, total flavonoids content; TPC, total phenolic content; TPTZ-HCl, 2,4,6-Tripyridyl-S-triazine- hydrochloric acid; TTC, total tannin content; VA, vanillic acid.

1 INTRODUCTION

Acanthaceae Juss. is a large family comprising about 4,000 species and 220 genera over the globe. It comprises annual, perennial, herbs, shrubs or trees found in tropical, subtropical and a few in temperate regions (Kavitha et al., 2016). *Barleria terminalis* Nees and *Calacanthus grandiflorus* (Dalzell) Radlk. are the endemic plants of the Western Ghats of India (Tripp et al., 2013; Patil et al., 2019). The genus *Barleria* L. contains 285 species found in Asia and Africa, with the majority of its diversity found in tropical eastern and southern Africa (Darbyshire et al., 2019). In India, the genus is represented by 30 species (Patil et al., 2020). Extensive literature survey showed that *Barleria* is medicinally important genus and locally called as Vajradanti (Banerjee et al., 2012). The plant is widely used in the treatment of human diseases like anemia, toothache, cough, fever, asthma, bronchitis, diabetes, insect bites and inflammations (Banerjee et al., 2012; Sudheer and Praveen, 2021). Aerial parts and leaves of *Barleria* are known to be a vital source of iridoids (Amoo et al., 2011; Sudheer and Praveen, 2021). The leaves of the plant are known for flavonoids, saponins, sterols, tannins, and terpenoids. Similarly, flowers contain flavonoids and neohesperidoside. In the same way, aerial part contains balarenone, terpenoid, barlerinoside, saponins, flavonoids, phenolics, tannins, steroids, carbohydrates, phytosterols, acetyl-barlerin, β-sitosterol, iridoids and lupulinoside (Ata et al., 2009). This plant contains numerous medicinal properties (Sudheer and Praveen, 2021). On the other hand, there are no reports on the phytochemical or medicinal potential of *C. grandiflorus*.

So keeping in view the therapeutic potential of *Barleria* and *Calacanthus*, leaves and stem extracts of *B. terminalis* and *C. grandiflorus* were prepared for the first time utilizing a variety of solvents. The present study was designed to explore the phytochemicals and biological potential of both the species (antioxidant, anti-acetylcholine esterase and antidiabetic) as well as to identify and quantify phenolics and phenylethanoid glycoside (verbascoside) using RP-HPLC. Furthermore, LC-MS and GC-MS were used to screen potent secondary metabolites present in the plants. To the best of our knowledge, this is the first study to look into the potential benefits of the medicinal plants, *B. terminalis* and *C. grandiflorus*.

2 MATERIALS AND METHODS

2.1 Collection of Plant Materials and Extract Preparation

B. terminalis was collected from Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra (N 16°40.546', E 074°15.337'). *C. grandiflorus* were collected from Kumbhavade ghat, Sindhudurg district, Maharashtra (N 16°31.51.9′, E 73°50′01.4′). Both the specimens were deposited in SUK herbarium (Voucher No. SSO 001, SSO 030). Plant material was oven dried for 72 h at 60°C, ground into fine powder, and extraction was done by continuous shaking of finely ground powder (5 g) in respective solvents (30 ml each). Extracts were then centrifuged (5,000 rpm), concentrated and dissolved in 5 ml respective solvent. Prior to analysis, extracts were filtered through 0.2 μm nylon filter (HiMedia, India), used in experiment and stored at 4°C.

2.2 Phytochemical Analysis

2.2.1 Estimation of Total Phenolic Content, Total Flavonoid Content, Total Tannin Content

TPC was determined as per the method adopted by Patel and Ghane (2021) with some minor modifications. Results were expressed as mg tannic acid equivalent (TAE)/g extract. TFC was quantified according to method described by Sakanaka et al. (2005) and TTC was estimated as per Patel and Ghane (2021) with minor modifications. Catechin was used as standard (mg/ml) and results were expressed in mg catechin equivalent (CE)/g extract.

2.2.2 Estimation of Proanthocyanidins and Total Iridoid Content

Total proanthocyanidins were calculated according to the method described by Fawole et al. (2009) with minor modifications. Proanthocyanidins content was expressed as % per g DW and calculated by the following formula:

$$Proanthocyanidins\ (\%\ dry\ matter) = \frac{A550nm \times 78.26 \times Dilution\ factor}{\%\ Dry\ matter} \times 100$$

TIC was estimated according to method described by Levieille and Wilson (2002) with minor modifications. Results were expressed as mg harpagoside equivalent (HE)/g extract.

2.3 Determination of Antioxidant Potential

2.3.1 DPPH Scavenging Assay, FRAP Assay, ABTS Assay, Metal Chelating Assay, Phosphomolybdenum Assay

DPPH radical scavenging activity was determined as per earlier method (Attar and Ghane, 2021). The activity was expressed as mg ascorbic acid equivalent (AAE)/g extract. The FRAP method proposed by Benzie and Strain (1996) was adopted. FRAP values were indicated as mg Fe (II) equivalent/g extract. ABTS radical activity was evaluated according to Patel et al. (2018) with some minor modifications. Activity was expressed as mg trolox equivalent (TE)/g extract. Metal chelating activity was calculated as per Patel and Ghane (2021) and the activity expressed as mg EDTA equivalent (EE)/g extract. Phosphomolybdenum assay was performed according to method described by Prieto et al. (1999). Results were depicted as mg of ascorbic acid equivalent (AAE)/g extract.

2.3.2 Acetylcholinesterase Inhibitory and Anti–diabetic Activities

AChE inhibitory activity was examined according the method of Ghane et al. (2018) with some minor modifications. Galanthamine hydrobromide was used as standard, and results were expressed in percentage. Inhibition of α-amylase and α-glucosidase was evaluated as per our earlier protocol (Ghane et al.,

TABLE 1 | Extract yield, total phenolics content (TPC), total flavonoids content (TFC), total tannins content (TTC), proanthocyanidins and total iridoids content (TIC) of different solvent extracts of *B. terminalis* and *C. grandiflorus*.

Species	Plant part	Solvents	Yield (%)	TPC$^{\alpha}$	TFC$^{\beta}$	TTC$^{\beta}$	Proanthocyanidins$^{\lambda}$	TIC$^{\gamma}$
Barleria terminalis	Leaves	Acetone	2.62	158.7 ± 4.07^{a}	144.10 ± 3.42^{a}	200.60 ± 11.32^{c}	4.89 ± 0.61^{c}	3.28 ± 0.4^{b}
		Ethanol	4.14	94.36 ± 0.60^{c}	98.24 ± 1.85^{b}	102.42 ± 9.46^{e}	0.92 ± 0.16^{defg}	0.15 ± 0.0^{g}
		Methanol	2.84	105.63 ± 0.22^{b}	103.23 ± 6.94^{b}	32.72 ± 1.04^{h}	1.09 ± 0.23^{def}	5.77 ± 0.4^{a}
		water	5.48	40.07 ± 1.78^{f}	25.4 ± 0.06^{efg}	81.81 ± 4.81^{fg}	0.074 ± 0.013^{g}	2.68 ± 0.1^{bc}
	Stem	Acetone	0.70	60.60 ± 2.09^{e}	35.14 ± 3.60^{de}	74.54 ± 11.32^{g}	5.66 ± 0.26^{bc}	0.53 ± 0.0^{g}
		Ethanol	2.21	18.01 ± 0.28^{gh}	27.22 ± 1.44^{ef}	17.57 ± 9.46^{ij}	1.24 ± 0.30^{de}	0.18 ± 0.0^{g}
		Methanol	1.98	15.93 ± 0.61^{ghi}	20.76 ± 1.90^{fg}	6.66 ± 1.04^{j}	0.96 ± 0.23^{defg}	2.68 ± 0.2^{bc}
		Water	3.42	13.78 ± 0.24^{ih}	20.76 ± 3.66^{efg}	4.24 ± 4.81^{j}	0.16 ± 0.02^{fg}	3.34 ± 0.1^{b}
Calacanthus grandiflorus	Leaves	Acetone	2.36	81.74 ± 7.09^{d}	81.93 ± 1.38^{c}	290.30 ± 1.04^{a}	6.51 ± 0.08^{b}	0.42 ± 0.1^{g}
		Ethanol	2.74	24.7 ± 6.69^{g}	29.88 ± 1.79^{e}	124.24 ± 2.64^{dg}	1.78 ± 0.45^{d}	2.01 ± 0.1^{de}
		Methanol	2.46	16.11 ± 1.26^{ghi}	24.56 ± 0.52^{fg}	70.90 ± 1.21^{g}	1.10 ± 0.26d^{ef}	1.40 ± 0.0^{f}
		water	5.69	33.88 ± 0.38^{f}	14.77 ± 1.85^{efg}	90.30 ± 1.21^{ef}	0.07 ± 0.0019^{g}	2.26 ± 0.1^{cde}
	Stem	Acetone	1.02	105.33 ± 2.60^{b}	40.53 ± 6.12^{d}	248.48 ± 4.24^{b}	8.74 ± 0.67^{a}	2.92 ± 0.0^{bc}
		Ethanol	3.22	18.38 ± 0.28^{g}	22.43 ± 3.83^{fg}	52.12 ± 0.60^{h}	0.80 ± 0.18^{efg}	0.25 ± 0.00^{g}
		Methanol	3.54	9.98 ± 5.27^{hi}	19.70 ± 1.13^{fg}	35.75 ± 1.60^{h}	0.27 ± 0.05^{efg}	2.63 ± 0.1^{bcd}
		Water	3.89	7.10 ± 0.38^{i}	20.50 ± 3.52^{efg}	21.81 ± 4.19^{efhi}	0.063 ± 0.006^{g}	1.69 ± 0.6^{ef}

Values are means of three replicate determinations ±standard error. Mean values in the same column with different alphabets showed statistically significant differences ($p \leq 0.05$) according to Duncan's multiple range test. $^{\alpha}$(mg TAE/g extract), $^{\beta}$(mg CE/g extract), $^{\lambda}$(%/g DW), $^{\gamma}$(mg HE/g extract).

2018). Acarbose was used as a positive control and activity was evaluated on a percent basis.

2.4 Analysis of Phenolics, Verbascoside and Other Metabolites by RP-HPLC, HR-LC-MS and GC-MS

2.4.1 Preparation of Samples and Standard Solutions

Finely ground powder (500 mg) of *B. terminalis* and *C. grandiflorus* (leaves and stem) was extracted by using 10 ml methanol in an ultrasound bath. Homogenate was centrifuged, and supernatant collected, condensed and volume adjusted to 1 ml. Prior to analysis, the extract was filtered using 0.2 µm nylon filter (HiMedia, India). Different concentrations of standard solutions (20–100 µg/ml) were prepared and used to plot calibration curve using RP-HPLC.

2.4.2 RP-HPLC Analysis of Phenolics

HPLC apparatus consisted of quaternary pump, autosampler and UV detector (UV 2070) (Jasco, Japan, Model no. LC-2000 plus). Separation was performed using Hiber C18 column (5 µm, 250–4.6 mm). Mobile phase consisted of water: acetonitrile: glacial acetic acid (90:5:5), flow rate was 0.9 ml/min and 20 µl injection volume (Patel and Ghane, 2021). Sample peaks were monitored at 280 nm with 60 min as run time. Phenolic content was determined by comparing with standards and expressed as milligram per gram of dry weight (mg/g DW).

2.4.3 RP-HPLC Analysis of Verbascoside

RP-HPLC analysis of verbascoside was carried out using the same instrumentation as specified above. Separation of compound was achieved using methanol and water (90:10) as mobile phase with 1 ml/min flow rate and 20 µl injection volume (Dhakulkar et al., 2005). The peak was monitored at 320 nm with 30 min as run time. Experiments were performed in triplicates for assessing suitability of system and amount of verbascoside expressed as µg/g DW.

2.4.4 Identifications of Major Metabolites by LC-MS

LC-MS analysis was done by using HPLC-ESI-MS-NEG-PHENOMENEX in negative ionization mode. System was equipped with binary pump, auto sampler, thermostated column compartment and iFunnel quadrapole time-of-flight spectrometer (Q-TOF). Zorbax eclipse C18 column (4.6 × 250 mm, 5 µm particle size) was used for compounds separation at 25°C temperature. In the present study, 0.1% (v/v) formic acid (A) and acetonitrile (B) was used in gradient elution. Gradient was initiated at 80% A and 20% B to 30% B (after 10 min), followed by 40% B (40 min), 60% B (60 min) and 90% B (80 min) and finally returned to the initial conditions. Solvent system B was injected with flow rate of 0.8 ml/min. Mass spectrometer was operated in range of 100–1,000 m/z. N_2 gas was used as a nebulizer. Drying gas flow rate was 8 L/min at 325°C and nebulizer gas at 25 psi with fragmentor voltage 150 V (Patel and Ghane, 2021). For data analysis, mass hunter qualitative analysis software package (Agilent Technologies) was used. Detected compounds were validated on the basis of molecular formula, molecular mass, retention time and m/z ratio. For the authentication of compounds, details were compared with available literature and Metline personal metabolites database.

2.4.5 Identifications of Major Metabolites by GC-MS

GC-MS analysis was performed on Model QP 2010 series, Shimadzu, Tokyo, Japan, equipped with AOC-20i auto sampler and RTX-1 fused silica capillary column (30 m length, 0.25 mm id, and 0.25 µm thickness). Helium gas (purity 99.99%) was used as a carrier gas at a flow rate of 1.5 ml/min. The injector temperature was fixed at 280°C and samples were injected through split injection mode. The column oven program was set at 50°C for 2 min, then increased to 28°C with the rate of 10°C/min. Ion source temperature was applied to 230°C and interface temperature was set to 250°C. The mass range from 36

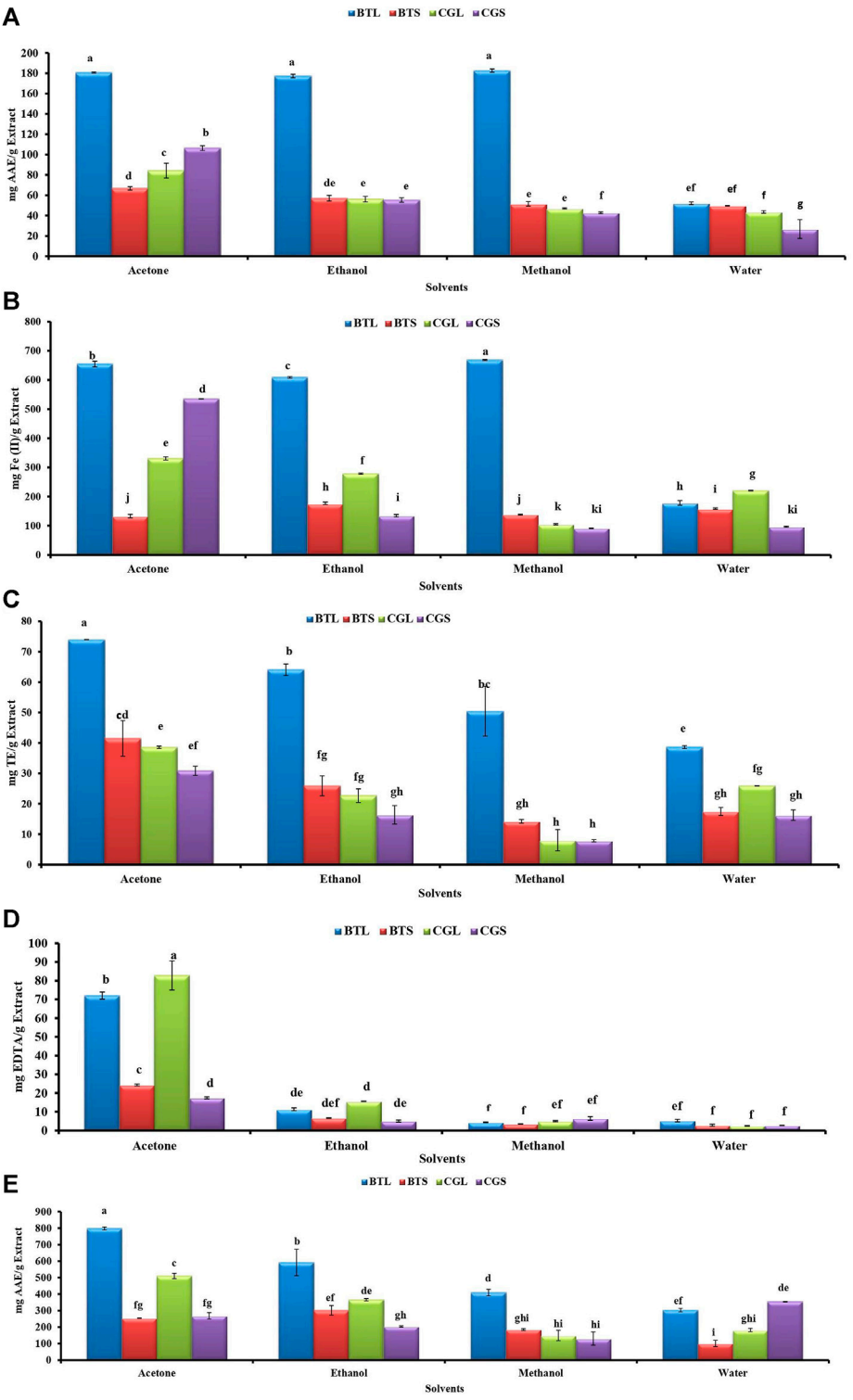


FIGURE 1 | Antioxidant activities from the different extracts of *B. terminalis* and *C. grandiflorus* **(A)** DPPH radical scavenging activity, **(B)** FRAP activity, **(C)** ABTS + radical scavenging activity, **(D)** Metal chelating activity, **(E)** Phosphopmolybdenum reduction activity. Note—All figures indicate different extracts from leaves and stems of BTL—*B. terminalis* leaves, BTS—*B. terminalis* stem and CGL—*C. grandiflorus* leaves and CGS—*C. grandiflorus* stem. According to DMRT, Bars having different alphabets showed statistically significant differences ($p \leq 0.05$).

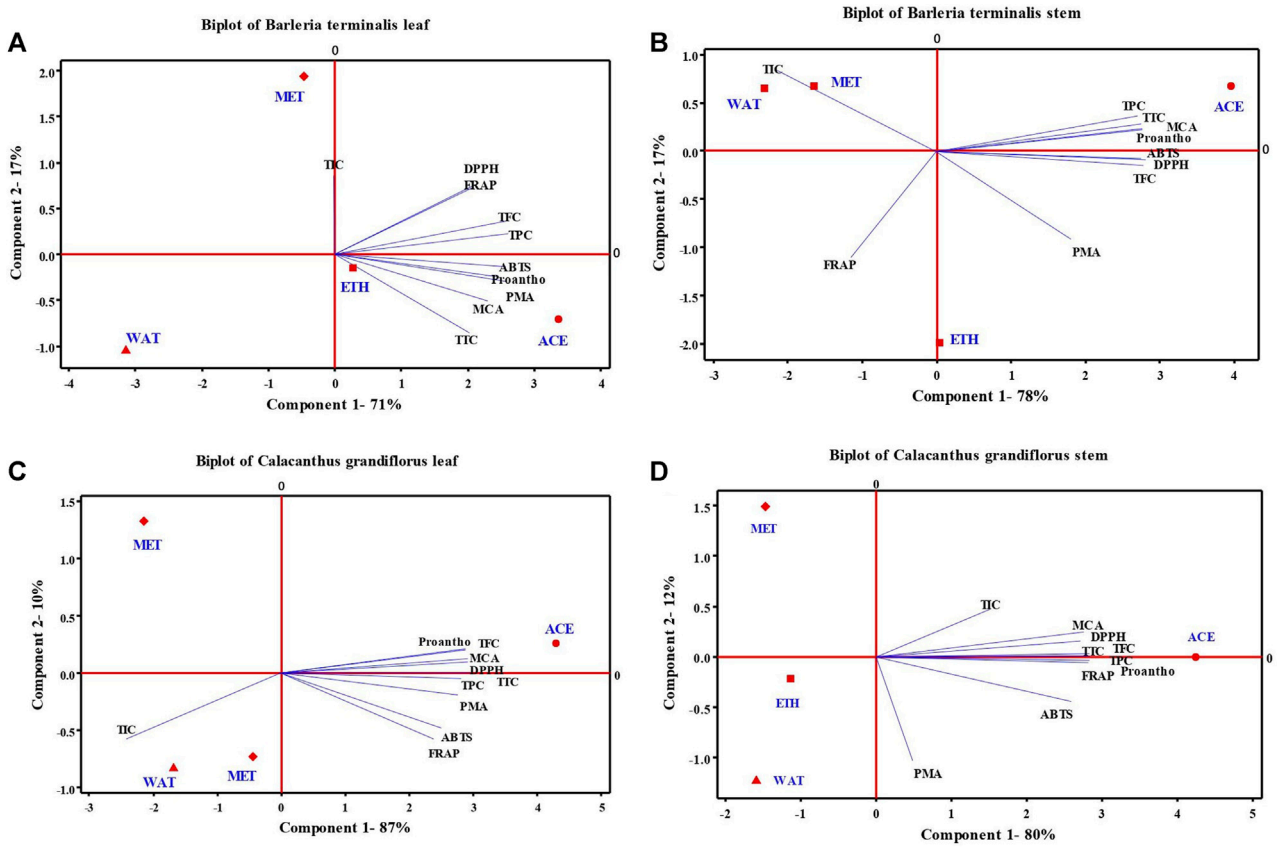


FIGURE 2 | Principal component analysis (scores and loading plots, biplot) depends on several phytochemical compounds analyzed in four different leaves and stem extract of *B. terminalis* **(A,B)** and C. *grandiflorus* **(C,D)** with their antioxidant activities (DPPH, ABTS, FRAP, PMA, and MCA). TPC, total phenolic content; TFC, total flavonoid content; TTC, total tannin content; Proantho, Proanthocyanidins content; TIC, total iridoid content.

TABLE 2 | α-Amylase, α-glucosidase, and acetyl cholinesterase inhibitory activities of leaves and stem extracts of *B. terminalis* and *C. grandiflorus*.

Species	Plant part	Solvent	α-Amylase$^{\alpha}$ inhibitory activity (%)	α-Glucosidase$^{\alpha}$ inhibitory activity (%)	Acetyl cholinesterase$^{\beta}$ inhibitory activity (%)
Barleria terminalis	Leaves	Acetone	20.02 ± 1.83^{cd}	3.94 ± 1.23^{ef}	12.34 ± 6.17^{gh}
		Ethanol	36.96 ± 2.96^{a}	26.82 ± 1.58^{d}	44.01 ± 10.46^{d}
		Methanol	31.27 ± 1.31^{ab}	28.44 ± 2.32^{d}	22.00 ± 7.78^{fg}
		Water	18.28 ± 3.38^{cd}	2.38 ± 2.08^{f}	ND
	Stem	Acetone	17.05 ± 3.67^{d}	50.91 ± 0.14^{c}	61.72 ± 0.80^{bc}
		Ethanol	34.37 ± 0.0^{a}	62.55 ± 0.85^{b}	89.10 ± 0.26^{a}
		Methanol	20.16 ± 2.54^{cd}	87.11 ± 0.47^{a}	26.03 ± 2.68^{efg}
		Water	18.56 ± 2.070^{cd}	4.94 ± 0.35^{e}	9.12 ± 0.26^{gh}
Calacanthus grandiflorus	Leaves	Acetone	26.14 ± 0.047^{bc}	4.05 ± 1.64^{ef}	19.05 ± 2.14^{fg}
		Ethanol	32.1 ± 1.27^{ab}	31.67 ± 2.85^{d}	77.83 ± 4.56^{ab}
		Methanol	14.8 ± 0.37^{d}	12.41 ± 3.35^{e}	32.20 ± 7.24^{ef}
		Water	23.83 ± 3.67^{c}	4.58 ± 0.94^{ef}	ND
	Stem	Acetone	17.43 ± 2.35^{d}	52.79 ± 3.02^{c}	87.76 ± 9.12^{a}
		Ethanol	25.85 ± 2.58^{bc}	94.33 ± 0.73^{a}	77.83 ± 2.95^{b}
		Methanol	19.08 ± 2.58^{cd}	64.11 ± 9.11^{b}	34.88 ± 10.46^{ef}
		Water	18.98 ± 3.62^{cd}	3.47 ± 1.64^{ef}	ND

$^{\alpha}$ % inhibition at standard acarbose at 100 µg – 36.84%, $^{\beta}$acetylcholine esterase inhibition at standard galanthamine (3 µg)—32.41%, ND- Not Detected. Values are means of three replicate determinations ±standard error. Mean values in the same column with different alphabets showed statistically significant differences ($p \leq 0.05$) according to Duncan's multiple range test (DMRT).

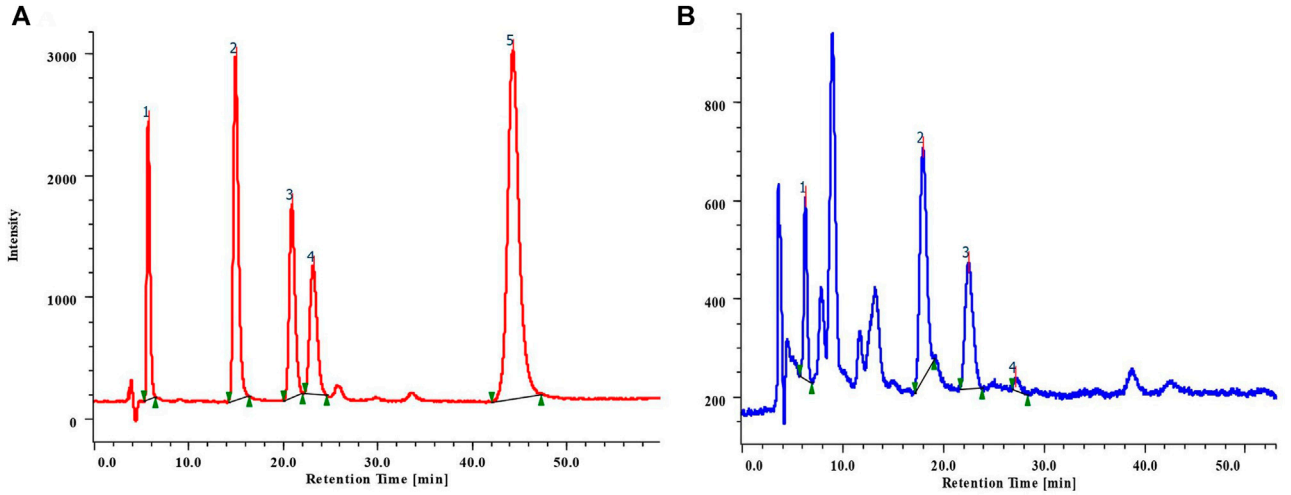


FIGURE 3 | (A) RP-HPLC chromatogram of mixture of standard phenolic compounds 1) GA; 2) HBA; 3) VA; 4) CHLA; 5) COA; **(B)** Phenolics detected from the leaves extract of *B. terminalis*.

TABLE 3 | HPLC analysis of phenolics from the leaves and stem extracts of *B. terminalis* and *C. grandiflorus*.

Plant samples	Phenolic compounds				
	GA$^{\alpha}$	HBA$^{\alpha}$	VA$^{\alpha}$	CHLA$^{\alpha}$	COA$^{\alpha}$
	(tR 6.0)	(tR 15.71)	(tR 21.62)	(tR 23.82)	(tR 44.87)
BTL	1.61 ± 0.003^{d}	3.88 ± 0.001^{a}	4.54 ± 0.001^{a}	0.39 ± 0.003^{b}	ND
BTS	4.17 ± 0.002^{a}	ND	0.45 ± 0.003^{b}	0.55 ± 0.004^{a}	1.73 ± 0.002^{b}
CGL	2.61 ± 0.002^{c}	0.003 ± 0.036^{b}	0.41 ± 0.001^{c}	ND	0.43 ± 0.002^{c}
CGS	3.26 ± 0.001^{b}	ND	0.52 ± 0.004^{d}	0.22 ± 0.002^{c}	52.82 ± 0.002^{a}

ND – Not detected, BTL – B. terminalis leaves, BTS – B. terminalis stem, CGL – C. grandiflorus leaves, CGS – C. grandiflorus stem. Values are means of three replicate determinations ±standard error. Mean values in the same column with different alphabets showed statistically significant differences (p < 0.05) according to DMRT. α (mg/g DW).

to 800 m/z was scanned at a rate of 3.0 scans/s. The total run time of GC-MS system was 36 min (Patel and Ghane, 2021). Compounds were identified by comparison with authentic spectra obtained from GC-MS library (NIST 11).

2.5 Statistical Analysis

All analyses were performed in triplicates and values are represented as average, with standard error. Data obtained from the experiments were subjected to one-way analysis of variance and significant differences between mean values were determined by Duncan's multiple range test ($p \leq 0.05$) using SPSS software ver. 16. Data derived from the studied phytochemicals and antioxidant activities from different extracts were subjected to Principal Component Analysis (PCA) (Minitab software ver. 19).

3 RESULTS

3.1 Phytochemical Analysis

In phytochemicals such as TPC, TFC, TTC, proanthocyanidins and TIC contents were determined and results are represented in **Table 1**. Among all the samples, *Calacanthus grandiflorus* leaves extract showed comparatively higher yield as compared to the stem extracts that ranged from 2.36 to 5.69%.

In the case of *B. terminalis,* the highest TPC and TFC content were found in acetone leaves extract (158.76 ± 4.07 mg TAE/g extract and 144.10 ± 3.42 mg CE/g extract, respectively). TTC ranged from 4 to 290 mg CE/g extract where aqueous stem extract possessed the lowest tannin content (4.24 ± 1.21 mg CE/g extract). Similarly, total iridoid content was recorded the highest in methanol extract of leaves (5.77 ± 0.4 mg HE/g extract) and the lowest was found in ethanol extract of stem (0.18 ± 0.0 mg HE/g extract) (**Table 1**).

In *C. grandiflorus*, aqueous extract of stem had the least content (7.10 ± 0.38 mg TAE/g extract) of TPC. In the same way, the lowest TFC was exhibited in aqueous leaves extract (14.77 ± 1.85 mg CE/g extract). Acetone extract of leaves revealed promising tannin content (290.30 ± 1.04 mg CE/g extract). Moreover, proanthocyanidins content ranged from 0.06 to 9%/g DW and acetone extract of stem had the highest proanthocyanidins (8.74 ± 0.67%/g DW) whereas water extracts exhibited the least (0.063 ± 0.006%/g DW) content (**Table 1**).

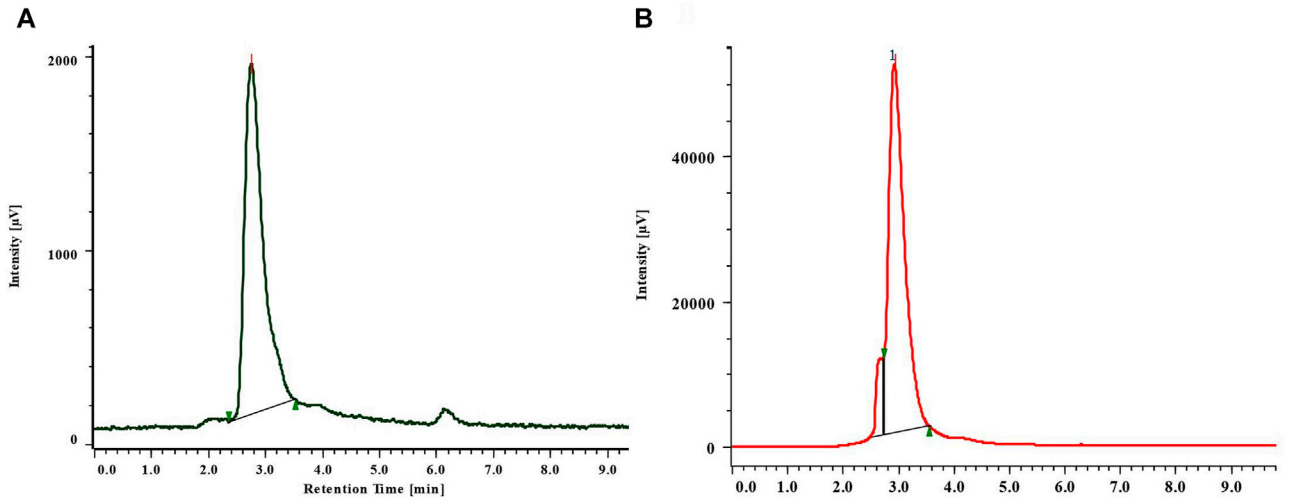


FIGURE 4 | RP-HPLC chromatogram of standard **(A)** and verbascoside from the leaves of *B. terminalis* **(B).**

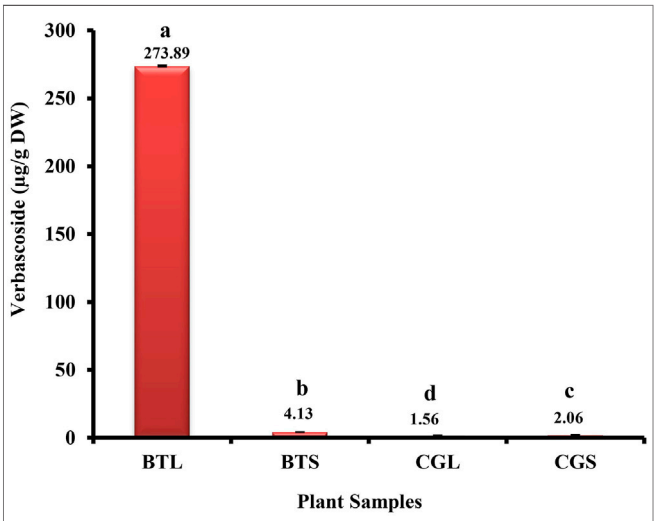


FIGURE 5 | HPLC analysis of verbascoside from *B. terminalis* and *C. grandiflorus*. BTL—*B. terminalis* leaves, BTS—*B. terminalis* stem, CGL—*C. grandiflorus* leaves, CGS—*C. grandiflorus* stem.

3.2 Antioxidant Analysis

Antioxidant activities like DPPH, FRAP, ABTS, MCA and PMA from different leaves and stem extracts of both the species are depicted in **Figure 1**. In *B. terminalis*, methanol extract of leaves showed promising DPPH scavenging and FRAP activity (182.59 ± 1.57 mg AAE/g extract and 668.24 ± 2.04 mg Fe(II)/g extract, respectively) (**Figures 1A,B**). Similarly, ABTS activity was noted the highest in the acetone extract of leaves (73.97 ± 0.04 mg TE/g extract) (**Figure 1C**). Acetone extract of leaves (797.61 ± 8.71 mg AAE/g extract) showed maximum PMA whereas the aqueous extract of stem exhibited the lowest activity (101.5 ± 18.95 mg AAE/g extract) (**Figure 1E**).

In *C. grandiflorus*, DPPH activity was observed the least in the water extract of stem (26.79 ± 9.30 AAE/g extract) (**Figure 1A**). Methanol extract of stem exhibited the lowest FRAP activity (91.33 ± 0.94 mg Fe (II)/g extract) (**Figure 1B**). The lowest ABTS activity (7.85 ± 0.37 mg TE/g extract) was registered with methanol extract of stem (**Figure 1C**). Antioxidant activity in terms of MC was found superior in *C. grandiflorus*. The acetone extract of leaves exhibited the highest MC activity (82.83 ± 7.79 mg EDTA/g extract) whereas the least activity was observed in the water extracts (2.42 ± 0.14 mg EDTA/g extract) (**Figure 1D**).

3.3 Chemometric Analysis

The PCA was performed to understand the relationship between the phytochemicals (TPC, TFC, TTC, proanthocyanidins and TIC) and antioxidant activities (DPPH, FRAP, ABTS, PMA, and MCA) studied from acetone, ethanol, methanol and water extracts of leaves and stem of *B. terminalis* and *C. grandiflorus* (**Figure 2**). From **Figures 2A,B**, it was confirmed that only acetone along with ABTS, proanthocyanidins, PMA, MCA and TTC occupied positive plane of component 1 in leaves; while in stem with TPC and TTC occupied positive plane of both components. PCA analysis of *C. grandiflorus* leaves and stem denoted 97 and 93% total variability respectively, out of which component 1 contributed for 87 and 80% variability. Similar to *B. terminalis* leaves, TPC, TFC, TTC, DPPH, ABTS, FRAP, PMA, MCA and proanthocyanidins enjoyed the positive plane of component 1 and exhibited largest distribution with the coefficients 0.323, 0.331, 0.339, 0.335, 0.288, 0.275, 0.317, 0.336, and 0.331, respectively (**Figure 2C**). PCA of stem extract of *C. grandiflorus* showed that all tested parameters enjoyed positive plane of component 1. Among the solvents studied, only acetone and methanol represented positive planes on component 1 and 2, respectively. Variables exhibited the largest distribution with the coefficients 0.353, 0.352, 0.353, 0.339, 0.323, 0.353, 0.061, 0.345, 0.353, and 0.189, respectively (**Figure 2D**).

TABLE 4 | Compounds detected from the leaf extracts of *B. terminalis* and *C. grandiflorus* by LC-MS.

Group	Name	Molecular formula	RT	m/z	Mass
Phenolics	Catechin-4beta-ol[a]	$C_{15}H_{14}O_7$	3.82	305.0677	306.075
	Hydroquinone[a]	$C_6H_6O_2$	10.91	109.0295	110.0369
	Salicylic acid[a,b]	$C_7H_6O_3$	12.77	137.024	138.0313
	Maritimetin[a]	$C_{15}H_{10}O_6$	20.16	285.0394	286.0467
	Sinapyl aldehyde[a]	$C_{11}H_{12}O_4$	21.31	207.0656	208.073
	4-Hydroxystyrene[b]	C_8H_8O	15.95	119.0501	120.0574
	Fraxin[a]	$C_{16}H_{18}O_{10}$	3.59	369.0829	370.0901
Glycoside	Antirrhinoside[a,b]	$C_{15}H_{22}O_{10}$	4.21	361.1125	362.1198
	Swertiamarin[b]	$C_{16}H_{22}O_{10}$	8.2	373.1133	374.1208
	Shanzhiside[b]	$C_{16}H_{24}O_{11}$	6.84	391.1237	392.131
Terpene glycoside	Leonuridine[b]	$C_{15}H_{24}O_9$	8.47	348.1409	348.1409
Steroid	Dexamethasone Acetate[a]	$C_{24}H_{31}FO_6$	13.61	433.2042	434.2115
	Hydroxyanthraquinone[a]	$C_{14}H_8O_3$	25.47	223.0397	224.0469
Anthraquinone	2-Hydroxymethylanthraquinonea[a]	$C_{15}H_{10}O_3$	27.98	237.0556	238.0628
Xanthonoid	Gambogic acid[a]	$C_{38}H_{44}O_8$	31.16	627.2974	628.3046
Ethyl ester	Trinexapac-ethyl[a]	$C_{13}H_{16}O_5$	25.36	251.0921	252.0994
Quinoline	Quinolin-2-ol[a]	C_9H_7NO	18.46	144.0452	145.0525
Ketone	Zingerone[a]	$C_{11}H_{14}O_3$	25.7	193.0869	194.0941
Tertiary alcohol	Ancymidol[b]	$C_{15}H_{16}N_2O_2$	18.53	255.1127	256.12
Iridoid monoterpenoid	Monotropein[b]	$C_{16}H_{22}O_{11}$	9.83	389.1077	390.1149

[a]*Compounds detected in B. terminalis.*
[b]*Compounds detected in C. grandiflorus.*

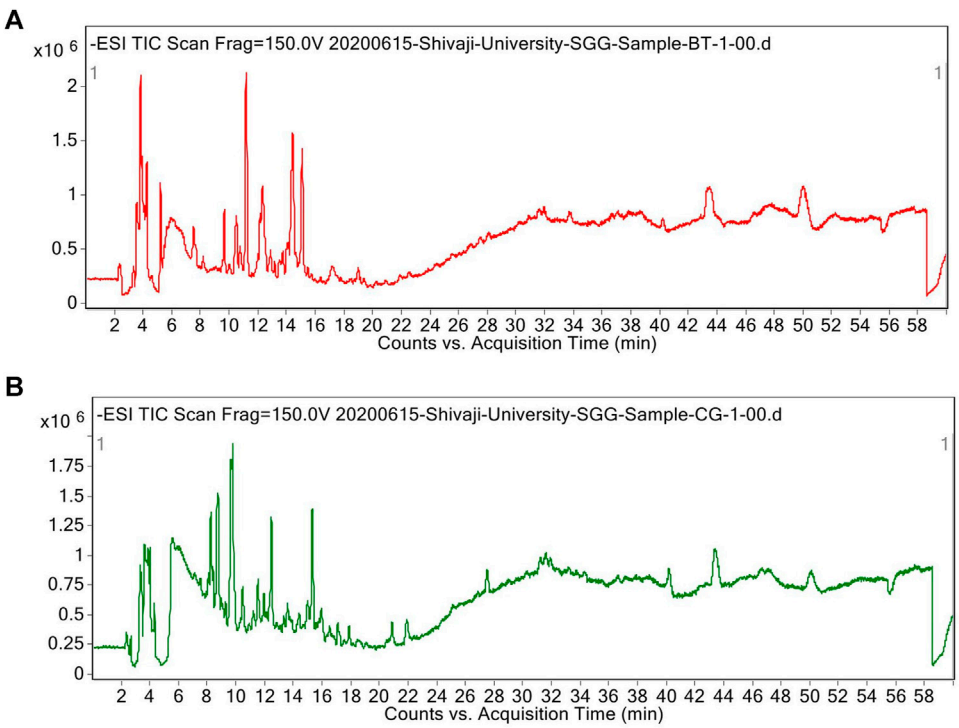


FIGURE 6 | Total ion chromatograms (TICs) of methanol extract of *B. terminalis* leaves **(A)** and *C. grandiflorus* leaves **(B)**.

3.4 Anti-Acetylcholinesterase and Anti-Diabetic Activities

Anti-acetylcholinesterase (AChE) and anti-diabetic activities of different solvent extracts of leaves and stem of *B. terminalis* and *C. grandiflorus* were performed and the results are depicted in **Table 2**. Anti-diabetic activity was analyzed by using α-amylase and α-glucosidase inhibition. In *B. terminalis*, maximum α-amylase inhibition was found in the ethanol extract of leaves (36.96 ± 2.96%) and the least α-glucosidase inhibitory (2.38 ± 2.08%) activity in the aqueous extract of leaves. The ethanol

TABLE 5 | Compounds detected from the leaves extracts of *B. terminalis* and *C. grandiflorus* by GC-MS.

Peak no	Group	Name	Molecular formula	RT	[a]Area (%)	[b]Area (%)
1	Phenol	Phenol, 2,4-bis(1,1-dimethylethyl)[a]	$C_{17}H_{30}OSi$	16.764	0.84	ND
2	Phthalate ester	Diethyl Phthalate[b]	$C_{12}H_{14}O_4$	17.76	ND	3.21
3	Fatty acid	Tetradecanoic acid[a,b]	$C_{14}H_{28}O_2$	19.559	5.54	0.91
4	Diterpene	Neophytadiene[a]	$C_{20}H_{38}$	20.328	0.91	ND
7	Fatty acid	n-Hexadecanoic acid[a,b]	$C_{16}H_{32}O_2$	21.597	20.45	19.31
8	Diterpene alcohol	Phytol[a]	$C_{20}H_{40}O$	23.026	4.40	ND
11	Fatty acid	Octadecanoic acid[a,b]	$C_{18}H_{36}O_2$	23.301	5.71	11.45
22	Triterpene	Squalene[b]	$C_{30}H_{50}$	28.74	ND	3.84
23	Fat soluble compound	Vitamin E[b]	$C_{29}H_{50}O_2$	32.22	ND	0.77
25	Phytosterol	Stigmasterol[b]	$C_{29}H_{48}O$	34.42	ND	2.85

[a]*Compounds detected in B. terminalis.*
[b]*Compounds detected in C. grandiflorus, ND- not detected.*

extract of stem showed the highest AChE inhibition (89.10 ± 0.26%) while the aqueous extract the least (9.12 ± 0.26%) (**Table 2**). Similarly, methanolic extract of leaves of *C. grandiflorus* exhibited the lowest α-amylase (14.8 ± 0.37%) inhibitory activity whereas the ethanol extract of the stem exhibited the highest *α*-glucosidase inhibitory (94.33 ± 0.73%) activity (**Table 2**).

3.5 Detection of Phenolics, Verbascoside and Other Bioactives

Results suggested that phenolics were present in the methanolic leaves and stem extracts of *B. terminalis* and *C. grandiflorus*. Five phenolic compounds, *viz*. gallic acid (GA) (tR 6.0), hydroxybenzoic acid (HBA) (tR 15.71), vanillic acid (VA) (tR 21.62), chlorogenic acid (CHLA) (tR 23.82), and coumaric acid (COA) (tR 44.87) were identified and quantified (**Figure 3A**; **Table 3**). The methanolic extract of stem of *B. terminalis* showed the highest GA content (4.17 ± 0.002 mg/g DW) whereas the lowest was observed in leaves (1.61 ± 0.003 mg/g DW). HBA content (3.88 ± 0.001 mg/g DW) was recorded maximum in leaves (**Figure 3B**). VA was found maximum in the leaves (4.54 ± 0.001 mg/g DW) and the minimum in the stem (0.45 ± 0.003 mg/g DW). CHLA was detected in very low quantity as compared to other phenolics. In all tested samples, the highest CHLA was recorded in the stem extract (0.55 ± 0.004 mg/g DW) (**Table 3**).

In the case of *C. grandiflorus*, significant amount of GA content was found in the stem (3.26 ± 0.001 mg/g DW) followed by the leaves (2.61 ± 0.002 mg/g DW) extract. VA was found maximum in the stem (0.52 ± 0.004 mg/g DW) and minimum in the leaves (0.41 ± 0.001 mg/g DW) extract. CHLA was observed to be the lowest in the stem (0.22 ± 0.002 mg/g DW) extract. The highest content of COA was reported in the stem (52.82 ± 0.002 mg/g DW) extract (**Table 3**).

Verbascoside was detected and quantified from the methanolic extracts of both the species (**Figure 4** and **Figure 5**). All the plant parts showed remarkable quantity of verbascoside, in which the highest content was found in *B. terminalis* leaves (273.89 ± 0.6 μg/g DW) followed by *B. terminalis* stem (4.13 ± 0.1 μg/g DW) (**Figure 5**). Similarly, remarkable quantity of verbascoside was observed in *C. grandiflorus* stem (2.06 ± 0.1 μg/g DW) and leaves (1.56 ± 0.1 μg/g DW) (**Figure 5**). Compounds detected in extracts of *B. terminalis* and *C. grandiflorus* leaves by LC-MS are shown in **Table 4** and **Figures 6A,B**. Likewise, using GC-MS several bioactive constituents were identified from the methanol extracts of both the species (**Table 5**). All the compounds from the both species were identified on the basis of chromatogram area, peak, molecular weight and molecular formula.

4 DISCUSSION

This investigation reported reliable amount of phenolics, flavonoids, tannins, proanthocyanidins and iriodoids from the leaves and stem of both the species. Our findings support the results of Amoo et al. (2011) who observed significant amount of phenolics, flavonoids, tannins, proanthocyanidins and iridoid content in methanolic extract leaves, stem and root of three *Barleria* species. Antioxidant potential of leaves and stem of *B. terminalis* and *C. grandiflorus* were comparable to the findings of Amoo et al. (2011) and Yadav et al. (2012). Extraction of phytochemicals depends on their solubility in different solvents and hence, the variability (quantitative and qualitative) in phytochemicals could be on account of different solvents used. Also, the extraction is dependent upon the extraction process, the genotype, and many intrinsic and extrinsic factors (Attar and Ghane, 2019).

Amoo et al. (2011) reported that methanol extract of leaves *B. greenii* and *B. albostellata* showed promising inhibition of AChE (68 and 22%, respectively). Investigations of *α*-amylase and *α*-glucosidase inhibitory activity are the key steps to discover the plants with antidiabetic potential as they play important role in the cure of diabetes (Ghane et al., 2018; Attar and Ghane, 2019; Patel et al., 2020; Taslimi et al., 2020). In the present investigation, correlation among phytochemicals and antioxidant activities from both the leaves and stem extracts of various solvents of both the species was found by PCA. Acetone was found to be the most superior for extracting phytochemicals. Acetone extracts showed higher antioxidant activity than ethanol, methanol and water extracts. This solvent can be used for the extraction of natural antioxidants from both the species.

HPLC analysis confirmed the presence of potent phenolics and phenylethanoid glycoside (verbascoside). Bioactive compounds found in plants also play important role in

pharmaceutics and food industry, where some secondary metabolites showed potential biological activities (Nescatelli et al., 2017; Stefanucci et al., 2018; Uysal et al., 2019; Lekhak et al., 2021; Mollica et al., 2021; Patel and Ghane, 2021). Ranade et al. (2016) concluded that among phenolics, GA and CA were found the most common and abundant in leaves and stem of *B. prionitis*. Verbascoside was firstly isolated from the Mullein plant (*Verbascum sinuatum* L.). It has potent biological activities such as antioxidant, anti-inflammatory, antineoplastic, wound healing, neuroprotective, etc. (Alipieva et al., 2014). Kanchanapoom et al. (2004) isolated verbascoside from the methanolic extract of entire plant of *B. strigosa*. Additionally, LC-MS and GC-MS analysis revealed several potent metabolites from the methanolic extract of both species. Studies on phytochemical investigations supported appreciable bioactivities from the studied plants coupled with the presence of bioactive compounds.

5 CONCLUSION

The presence of considerable amount of phenolics, flavonoids, tannins, terpenoids, proanthocyanidins and iridoids in *B. terminalis* and *C. grandiflorus* led to appreciable antioxidant, antidiabetic, and anti-acetyl cholinesterase potential. Acetone was discovered to be the best solvent for extracting phytochemicals that exhibited potential antioxidant properties in the current study. Ethanol extracts of leaves and stem revealed promising antidiabetic and acetyl cholinesterase inhibitory activities. The presence of phenolics, verbascoside, and other bioactive substances were confirmed using RP-HPLC, LC-MS, and GC-MS. The best source of phenolics and phenylethanoid glycoside was discovered to be *B. terminalis* leaves. Biological activities and secondary metabolites differ from one organ to the other as well as from one season to the next. As India is home to 30 species of *Barleria,* it would be worthwhile to undertake extensive research to look into the phytochemical diversity in the genus and identify potential bioactive compounds that might be used to treat diabetes, neurological disorders, and produce new medications. In addition, the pharmaceutical industry needs to pay more attention to the creation of important pharmaceuticals (phenolics, iridoids, and phenylethanoid glycosides) from *Barleria*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SG designed the experiment. SO and SP carried out experiment, SO drafted manuscript and ML, SP, and SG critically reviewed the manuscript. SG participated in statistical analysis and helped to improve the manuscript. All authors contributed to the article.

ACKNOWLEDGMENTS

Authors are thankful to the Head, Department of Botany, Shivaji University, Kolhapur, India for providing necessary facility. UGC-DRS-SAP and DST-FIST programs are duly acknowledged.

REFERENCES

Alipieva, K., Korkina, L., Orhan, I. E., and Georgiev, M. I. (2014). Verbascoside-a Review of its Occurrence, (Bio)synthesis and Pharmacological Significance. *Biotechnol. Adv.* 32, 1065–1076. doi:10.1016/j.biotechadv.2014.07.001

Amoo, S. O., Ndhlala, A. R., Finnie, J. F., and Van Staden, J. (2011). Antifungal, Acetylcholinesterase Inhibition, Antioxidant and Phytochemical Properties of Three *Barleria* Species. *South Afr. J. Bot.* 77, 435–445. doi:10.1016/j.sajb.2010.11.002

Ata, A., Kalhari, K. S., and Samarasekera, R. (2009). Chemical Constituents of *Barleria Prionitis* and Their Enzyme Inhibitory and Free Radical Scavenging Activities. *Phytochemistry Lett.* 2, 37–40. doi:10.1016/j.phytol.2008.11.005

Attar, U. A., and Ghane, S. G. (2019). *In Vitro* antioxidant, Antidiabetic, Antiacetylcholine Esterase, Anticancer Activities and RP-HPLC Analysis of Phenolics from the Wild Bottle Gourd (*Lagenaria siceraria* (Molina) Standl.). *South Afr. J. Bot.* 125, 360–370. doi:10.1016/j.sajb.2019.08.004

Attar, U. A., and Ghane, S. G. (2021). Proximate Composition, Ionomics, Phytochemical, Antioxidant, Anti-diabetic and Acetylcholinesterase Inhibitory Activity of *Cucumis* Species from Western Ghats of India. *Indian J. Pharm. Sci.* 83 (4), 679–694. doi:10.36468/pharmaceutical-sciences.819

Banerjee, D., Maji, A. K., Mahapatra, S., and Banerji, P. (2012). *Barleria prionitis* Linn.: A Review of its Traditional Uses, Phytochemistry, Pharmacology and Toxicity. *Res. J. Phytochem.* 6, 31–41. doi:10.3923/rjphyto.2012.31.41

Benzie, I. F., and Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": the FRAP Assay. *Anal. Biochem.* 239, 70–76. doi:10.1006/abio.1996.0292

Darbyshire, I., Tripp, E. A., and Chase, F. M. (2019). A Taxonomic Revision of Acanthaceae Tribe Barlerieae in Angola and Namibia. Part 1. *Kew Bull.* 74 (5), 1–85. doi:10.1007/s12225-018-9791-0

Dhakulkar, S., Ganapathi, T. R., Bhargava, S., and Bapat, V. A. (2005). Induction of Hairy Roots in *Gmelina arborea* Roxb. And Production of Verbascoside in Hairy Roots. *Plant Sci.* 169, 812–818. doi:10.1016/j.plantsci.2005.05.014

Fawole, O. A., Ndhlala, A. R., Amoo, S. O., Finnie, J. F., and Van Staden, J. (2009). Anti-inflammatory and Phytochemical Properties of Twelve Medicinal Plants Used for Treating Gastro-Intestinal Ailments in South Africa. *J. Ethnopharmacol.* 123, 237–243. doi:10.1016/j.jep.2009.03.012

Ghane, S. G., Attar, U. A., Yadav, P. B., and Lekhak, M. M. (2018). Antioxidant, Anti-diabetic, Acetylcholinesterase Inhibitory Potential and Estimation of Alkaloids (Lycorine and Galanthamine) from *Crinum* Species: An Important Source of Anticancer and Anti-alzheimer Drug. *Ind. Crops Prod.* 125, 168–177. doi:10.1016/j.indcrop.2018.08.087

Kanchanapoom, T., Noiarsa, P., Ruchirawat, S., Kasai, R., and Otsuka, H. (2004). Phenylethanoid and Iridoid Glycosides from the Thai Medicinal Plant, *Barleria strigosa*. *Chem. Pharm. Bull.* 52 (5), 612–614. doi:10.1248/cpb.52.612

Kavitha, K., Sujatha, K., and Manoharan, S. (2016). Antidiabetic Potential of Acanthaceae Family. *Int. J. Pharm. Sci. Rev. Res.* 36 (1), 30–37.

Lekhak, M. M., Patel, S. B., Otari, S. S., Lekhak, U. M., and Ghane, S. G. (2021). Bioactive Potential and RP-HPLC Detection of Phenolics and Alkaloids (Lycorine and Galanthamine) from Ultrasonic-Assisted Extracts of *Crinum* Roots. *South Afr. J. Bot.* doi:10.1016/j.sajb.2021.07.024

Levieille, G., and Wilson, G. (2002). *In Vitro* propagation and Iridoid Analysis of the Medicinal Species *Harpagophytum procumbens* and *H . zeyheri*. *Plant Cell Rep.* 21, 220–225. doi:10.1007/s00299-002-0520-6

Mollica, A., Scioli, G., Della Valle, A., Cichelli, A., Novellino, E., Bauer, M., et al. (2021). Phenolic Analysis and *In Vitro* Biological Activity of Red Wine, Pomace and Grape Seeds Oil Derived from *Vitis vinifera* L. Cv. Montepulciano d'Abruzzo. *Antioxidants* 10, 2–17. doi:10.3390/antiox10111704

Nescatelli, R., Carradori, S., Marini, F., Caponigro, V., Bucci, R., De Monte, C., et al. (2017). Geographical Characterization by MAE-HPLC and NIR Methodologies and Carbonic Anhydrase Inhibition of Saffron Components. *Food Chem.* 221, 855–863. doi:10.1016/j.foodchem.2016.11.086

Patel, S. B., and Ghane, S. G. (2021). Phyto-constituents Profiling of *Luffa echinata* and *In Vitro* Assessment of Antioxidant, Anti-diabetic, Anticancer and Anti-acetylcholine Esterase Activities. *Saudi J. Biol. Sci.* 28 (7), 3835–3846. doi:10.1016/j.sjbs.2021.03.050

Patel, S. B., Attar, U. A., and Ghane, S. G. (2018). Antioxidant Potential of Wild *Lagenaria siceraria* (Molina) Standl. *Thai J. Pharm. Sci.* 42, 90–96.

Patel, S. B., Attar, U. A., Sakate, D. M., and Ghane, S. G. (2020). Efficient Extraction of Cucurbitacins from *Diplocyclos Palmatus* (L.) C. Jeffrey: Optimization Using Response Surface Methodology, Extraction Methods and Study of Some Important Bioactivities. *Sci. Rep.* 10, 2109–2112. doi:10.1038/s41598-020-58924-5

Patil, S. S., Tamboli, A. S., Yadav, S. R., and Lekhak, M. M. (2019). A New Species of *Barleria* (Acanthaceae), its Morphotaxonomy, Cytogenetics and Phylogenetic Placement. *Plant Syst. Evol.* 305, 933–947. doi:10.1007/s00606-019-01613-2

Patil, S. S., Ganesan, R., Yadav, S. R., and Lekhak, M. M. (2020). Taxonomy of *Barleria morrisiana* E. Barnes & C. E. C. Fisch. (Acanthaceae), a Little-Known Species of the Biligiri Ranganathaswamy temple Tiger reserve, Karnataka, India. *Curr. Sci.* 119 (10), 1611–1612.

Prieto, P., Pineda, M., and Aguilar, M. (1999). Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E. *Anal. Biochem.* 269, 337–341. doi:10.1006/abio.1999.4019

Ranade, R., Jain, A., and Joshi, N. (2016). Estimation of Phenolic Compounds by RP-HPLC and Antioxidant Activity in Leaf and Stem Extracts of *Barleria prionitis* L. *Int. J. Pharm. Sci. Res.* 7 (6), 2445–2457. doi:10.13040/IJPSR.0975-8232.7(6).2445-57

Sakanaka, S., Tachibana, Y., and Okada, Y. (2005). Preparation and Antioxidant Properties of Extracts of Japanese Persimmon Leaf tea (Kakinoha-Cha). *Food Chem.* 89, 569–575. doi:10.1016/j.foodchem.2004.03.013

Stefanucci, A., Mollica, A., Macedonio, G., Zengin, G., Ahmed, A. A., and Novellino, E. (2018). Exogenous Opioid Peptides Derived from Food Proteins and Their Possible Uses as Dietary Supplements: A Critical Review. *Food Rev. Int.* 34 (1), 70–86. doi:10.1080/87559129.2016.1225220

Sudheer, W. N., and Praveen, N. (2021). Phytochemical, Pharmacological and Tissue Culture Studies of Some Important Species of the Genus *Barleria* L. (Acanthaceae) - a Review. *Plant Sci. Today* 8 (3), 491–500. doi:10.14719/pst.2021.8.3.1117

Taslimi, P., Köksal, E., Gören, A. C., Bursal, E., Aras, A., Kılıç, Ö., et al. (2020). Anti-alzheimer, Antidiabetic and Antioxidant Potential of *Satureja cuneifolia* and Analysis of its Phenolic Contents by LC-MS/MS. *Arabian J. Chem.* 13, 4528–4537. doi:10.1016/j.arabjc.2019.10.002

Tripp, E. A., Daniel, T. F., Fatimah, S., and McDade, L. A. (2013). Phylogenetic Relationships within Ruellieae (Acanthaceae) and a Revised Classification. *Int. J. Plant Sci.* 174 (1), 97–137. doi:10.1086/668248

Uysal, A., Ozer, O. Y., Zengin, G., Stefanucci, A., Mollica, A., Picot-Allain, C. M. N., et al. (2019). Multifunctional Approaches to Provide Potential Pharmacophores for the Pharmacy Shelf: *Heracleum sphondylium* L. subsp. *ternatum* (Velen.) Brummitt. *Comput. Biol. Chem.* 78, 64–73. doi:10.1016/j.compbiolchem.2018.11.018

Yadav, S. A., Raj, A. J., and Sathishkumar, R. (2012). *In Vitro* antioxidant Activity of *Barleria noctiflora* L. F. *Asian Pac. J. Trop. Biomed.* 2, 716–722. doi:10.1016/S2221-1691(12)60302-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer VAB declared a shared affiliation, with the authors to the handling editor at the time of the review.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 17 January 2022
doi: 10.3389/fphar.2021.796509

Phytochemical Analysis, Antioxidant and Bone Anabolic Effects of *Blainvillea acmella* (L.) Philipson

Rohanizah Abdul Rahim[1,2], Putri Ayu Jayusman[3], Vuanghao Lim[2], Nor Hazwani Ahmad[2], Zuratul Ain Abdul Hamid[4], Sharlina Mohamed[2], Norliza Muhammad[1], Fairus Ahmad[5], Norfilza Mokhtar[6], Norazlina Mohamed[1], Ahmad Nazrun Shuid[7]* and Isa Naina Mohamed[1]*

[1]Pharmacology Department, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, [2]Advanced Medical and Dental Institute, Universiti Sains Malaysia, Pulau Pinang, Malaysia, [3]Department of Craniofacial Diagnostics and Biosciences, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, [4]School of Materials and Mineral Resources Engineering, Universiti Sains Malaysia, Pulau Pinang, Malaysia, [5]Anatomy Department, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, [6]Physiology Department, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, [7]Faculty of Medicine UiTM, Sg Buloh, Malaysia

OPEN ACCESS

Edited by:
Massimo Lucarini,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Inês Mansinhos,
University of Algarve, Portugal
Fazleen Izzany Abu Bakar,
Universiti Tun Hussein Onn Malaysia, Malaysia
Clovis Macêdo Bezerra Filho,
Federal University of São Paulo, Brazil
Cemil Çolak,
İnönü University, Turkey
Nasreddine El Omari,
Mohammed V University, Morocco
Churdsak Jaikang,
Chiang Mai University, Thailand

***Correspondence:**
Ahmad Nazrun Shuid
anazrun@uitm.edu.my
Isa Naina Mohamed
isanaina@ppukm.ukm.edu.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 17 October 2021
Accepted: 09 December 2021
Published: 17 January 2022

Citation:
Abdul Rahim R, Jayusman PA, Lim V, Ahmad NH, Abdul Hamid ZA, Mohamed S, Muhammad N, Ahmad F, Mokhtar N, Mohamed N, Shuid AN and Naina Mohamed I (2022) Phytochemical Analysis, Antioxidant and Bone Anabolic Effects of Blainvillea acmella (L.) Philipson. Front. Pharmacol. 12:796509. doi: 10.3389/fphar.2021.796509

Blainvillea acmella (L.) Philipson [Asteraceae] (*B. acmella*) is an important medicinal plant native to Brazil, and it is widely known as a toothache plant. A plethora of studies have demonstrated the antioxidant activities of *B. acmella* and few studies on the stimulatory effects on alkaline phosphatase (ALP) secretion from bone cells; however, there is no study on its antioxidant and anabolic activity on bone cells. The study aimed to evaluate the phytochemical contents of aqueous and ethanol extracts of *B. acmella* using gas chromatography mass spectrometry (GCMS) and liquid chromatography time of flight mass spectrometry (LCTOFMS) along with the total phenolic (TPC) and flavonoid (TFC) contents using Folin-Ciocalteu and aluminum colorimetric methods. The extracts of *B. acmella* leaves were used to scavenge synthetic-free radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays. The bone anabolic effects of *B. acmella* extracts on MC3T3-E1 cells were measured with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoium bromide (MTT) at 1, 3, 5, and 7 days, Sirius-red and ALP at 7 and 14 days, and Alizarin Red S at 14 and 21 days. Comparatively, ethanol extract of *B. acmella* (*Ba*E) contributed higher antioxidant activities (IC_{50} of 476.71 µg/ml and 56.01 ± 6.46 mg L-ascorbic acid/g against DPPH and FRAP, respectively). Anabolic activities in bone proliferation, differentiation, and mineralization were also higher in *B. acmella* of ethanol (*Ba*E) than aqueous (*Ba*A) extracts. Positive correlations were observed between phenolic content (TPC and TFC) to antioxidant (ABTS and FRAP) and anabolic activities. Conversely, negative correlations were present between phenolic content to antioxidant (DPPH) activity. These potential antioxidant and bone anabolic activities in *Ba*E might be due to the phytochemicals confirmed through GCMS and LCTOFMS, revealed that terpenoids of α-cubebene, cryophyllene, cryophyllene oxide, phytol and flavonoids of pinostrobin and apigenin were the compounds contributing to both antioxidant and anabolic effects in *Ba*E. Thus, *B. acmella* may be a valuable antioxidant and anti-

osteoporosis agent. Further study is needed to isolate, characterize and elucidate the underlying mechanisms responsible for the antioxidant and bone anabolic effects.

Keywords: Phytochemical, GCMS, LCMS, *Blainvillea acmella*, *Spilanthes acmella*, bone anabolic, osteoblast, antioxidant

INTRODUCTION

According to the World Health Organization (WHO) Global Atlas of Traditional, Complementary, and Alternative Medicine (Bodeker et al., 2005), traditional medicine has been used widely by the world population to treat diseases. Approximately 70–95% of the population in the developing world uses traditional medicine as primary care (Robinson and Zhang, 2011). The use of medicinal plants or isolated active compounds from plants has gained attention due to their perceived lower toxicity.

Osteoporosis is a chronic skeletal condition that is characterized by deterioration in bone microarchitectural and reduction in bone mass, leading to decreased bone strength and increased risk of bone fracture (Clynes et al., 2020). Reactive oxygen species (ROS) may inhibit the function of osteoblast cells in bone formation. This would lead to imbalances between bone formation and bone resorption, contributing to osteoporosis (Domazetovic et al., 2017). Current treatments such as calcitonin, estrogen, and bisphosphonates are anti-bone resorptive drugs that inhibit osteoclast activity (Yang et al., 2018). However, long-term use of these treatments is associated with several side effects, such as cancer, femur fractures, osteonecrosis of the jaw, myocardial infarction, thromboembolic events, and skin reaction (DRESS syndrome) (Khosla and Hofbauer, 2017; Pherwani and Sathaye, 2020).

Given the contraindications of anti-bone resorptive drugs, it is important to develop plant-based therapeutic agents that will stimulate proliferation and differentiation of osteoblastic cells in the treatment of osteoporosis with minimal side effects than conventional treatments (Martiniakova et al., 2020). The osteoblast is one of the bone cells that play a major role in bone formation for new bone development (Kenkre and Bassett, 2018). Osteoblast has been used widely as a model system to study bone differentiation and mineralization based on the resemblance between the *in vitro* osteogenic differentiation and *in vivo* bone formation (Owen et al., 1990; Quarles et al., 1992; Stein and Lian, 1993; Hayatullina et al., 2018).

Blainvillea acmella (L.) Philipson (*B. acmella*) [Asteraceae], synonym as *Spilanthes acmella* (L.) L. (**Figure 1**) is a herbal plant belonging to the Asteraceae family (Abdul Rahim et al., 2021). The flowers and leaves of *B. acmella* have a pungent taste and cause tingling and numbness on the tongue. Other than being used as a spice for appetizers, it is also traditionally used to treat toothache, stomatitis (Nakatani and Nagashima, 1992), wound healing, snakebite remedy (Ong and Nordiana, 1999), and sore throat (Dubey et al., 2013). *B. acmella* plant was also reported to possess anti-malaria activities (Spelman et al., 2011). The extracts have also been used as nutritional supplements and cosmetics to reduce skin wrinkles *via* constriction of blood vessels (Sharma et al., 2011). In Malaysia, *B. acmella* is popularly known as "Subang Nenek or Pokok Getang" and it is consumed as a herbal treatment for tooth pain (Ong and Nordiana, 1999).

Previous studies reported that *B. acmella* extract exhibited antioxidant, antimicrobial, vasorelaxant (Prachayasittikul et al., 2009; Sharma and Arumugam, 2021), anti-inflammatory (Kim et al., 2018), and aphrodisiac (Sharma et al., 2011) activities. In terms of bone activities, it was able to raise alkaline phosphatase levels (Widyowati, 2011). These properties were contributed by the presence of phytochemicals in *B. acmella* extract. Phenolics, coumarin, triterpenoid, stigmasterol, and stigmasteryl glucoside contributed to the antioxidant activity, whereas vasorelaxant properties were enhanced by pentacyclic and 3-acetylaleuritolic acid (Wongsawatkul et al., 2008; Prachayasittikul et al., 2009). Spilanthol and other alkyl amides present in *B. acmella* extract also contributed significantly to its anti-inflammatory (Sharma et al., 2011; Bakondi et al., 2019) and antioxidant (Abeysiri et al., 2013; Sharma and Arumugam, 2021) activities. A recent study also reported that components in *B. acmella* extract such as 1,3-butanediol 3-pyroglutamate, 2-deoxy-d-ribono-1,4-lactone, methyl pyroglutamate, ampelopsisionoside, icariside B1, bezyl-α-1-arabinopyranosyl-(1,6)-β-d-glucopyranoside (Widyowati et al., 2020a), methyl threonolactones, and pyroglutamates contributed to its ALP activity and mineralization of osteoblast MC3T3-E1 cells (Widyowati et al., 2020b).

However, limited studies have been carried out on the effects of *B. acmella* extract on bone cells. Studies have shown that n-butanol and water fractions from ethanol extract (70%) of *B. acmella* leaves increased ALP activity in osteoblast cells *in-vitro* (Widyowati, 2011). In addition, the use of *B. acmella* extracts followed by exercise activity on mice increased the bone formation rate of mouse femur trabecular (Laswati et al., 2015). Methyl threonolactone glucopyranoside group, methyl threonolactonefructofuranoside, methyl pyroglutamates, and amine cell derivatives isolated from *B. acmella* methanol extracts were recently demonstrated to significantly increase the ALP and mineralization activities of osteoblast cells (Widyowati et al., 2020a; Widyowati et al., 2020b).

Osteoporosis is related to the phytochemicals and antioxidant activity in plants. Previous studies have shown that phenolic compounds are the major secondary metabolites in the plant (Swallah et al., 2020) that contribute to antioxidant activity lower the risk of osteoporosis. Flavonoid compounds are the major compounds in the phenolic group with antioxidant activity (Đudarić et al., 2015).

Nonetheless, no study has been conducted to determine the anabolic effects of the ethanol and aqueous extracts of *B. acmella* on osteoblast cells, as well as compounds that may contribute to the antioxidant and bone anabolic activities. In this study, the ethanol and aqueous extracts of *B. acmella* leaves were analyzed phytochemically for phenolic and flavonoid contents, and their

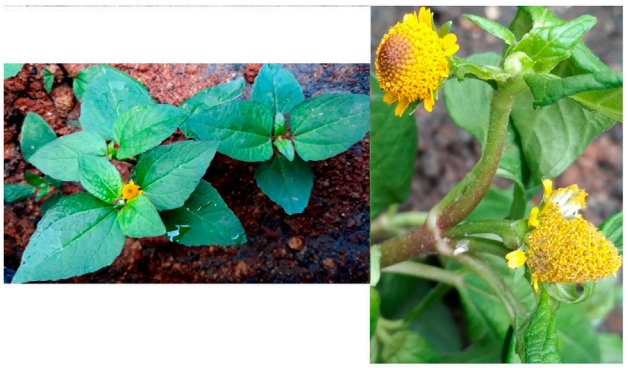


FIGURE 1 | Image of *Blainvillea acmella* (L.) Philipson [Asteraceae] plant with yellow flowers.

antioxidant activities were measured using DPPH, ABTS, and FRAP assays. Purified water and ethanol were chosen as the maceration technique to extract the biological active compound in *B. acmella* leaves. The relationship between phenolic compounds, antioxidant and anabolic activities were determined. The bone anabolic effects of the *B. acmella* extracts on osteoblast cells (MC3T3-E1) were evaluated by measuring osteoblast proliferation, collagen formation, ALP and mineralization activities. Finally, the possible compounds that contribute to both activities were identified using the GCMS and LCTOFMS systems.

The results from this study will provide the data on the anabolic effect of *B. acmella* extracts on the osteoblast cells, MC3T3-E1, and the correlation between phytochemical presence, antioxidant and bone anabolic activities. Compounds identified will contribute to the antioxidant and anabolic activities of *B. acmella*.

MATERIALS AND METHODS

The study was approved by the UKM ethical committee (Approval no: UKM PPI/111/8/JEP-2019-293).

Plant Identification

The plant *Blainvillea acmella* (L.) Philipson [Asteraceae] was collected from Pagoh, Muar, Johor (coordinates 2.136742; 102.765206) of South Malaysia. Botanical identification of *Blainvillea acmella* (L.) Philipson [Asteraceae] plant was conducted by the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia (USM). The voucher specimen: No. 11771 was identified by Dr Rahmad Zakaria and Dr. Farah Alia Nordin and deposited at the Herbarium Unit, School of Biological Sciences, USM.

Moisture Content Determination

The moisture content of *B. acmella* leaves was determined according to the previous method (Manimaran et al., 2019). *B. acmella* leaves were dried at 40°C in the dryer until the weight became constant. The moisture content of the leaves was determined based on the formulation given:

$$\text{Moisture content of } B.\ acmella\ (\%) = (\text{Original sample weight} - \text{Final sample weight}) \div (\text{Original sample weight}) \times 100$$

Preparation of *B. acmella* Leaves Ethanol and Aqueous Extracts

The *B. acmella* leaves extracts were prepared based on the modified methods (Gavamukulya et al., 2014; Abdul Rahim et al., 2018). Running water was used to wash the whole plant and it was separated into stems, flowers, and leaves. *B. acmella* leaves were consistently dried in the dryer at 40°C and the dried *B. acmella* leaves were grinded into a fine powder with the use of a herb Grinder. For ethanol extract, fine powdered *B. acmella* leaves were macerated in ethanol 95% (100%) while for aqueous extract, *B. acmella* leaves were macerated in purified water (100%). The *B. acmella* plant was extracted at 1 g: 10 ml (w/v) and placed in a 120 rpm shaker at room temperature for three days. Supernatants were collected daily for three days, centrifuged, filtered, lyophilized, and kept in an airtight container at −20°C for further analysis.

Yield Determination of *B. acmella* Leaves Extracts

The yield of *B. acmella* leaves extracts was calculated by comparing the weight of the lyophilized dried *B. acmella* extract with the weight of the original dried *B. acmella* as in the formulation given below (Nipornram et al., 2018):

$$\text{The yield of } B.\ acmella \text{ extracts } (\%) = \text{Weight of lyophilized extract} \div \text{Weight of original dried } B.\ acmella \times 100$$

Determination of Polyphenol Content in Ethanol (*Ba*E) and Aqueous (*Ba*A) Extracts of *B. acmella* Leaves

Total Phenolic Content (TPC) of *Ba*E and *Ba*A

The TPC of the extracts was determined using Folin-Ciocalteu (FC) method with modification (Luo et al., 2018). *Ba*E and *Ba*A (2 mg/ml) were dissolved in purified ethanol 95% and purified water, respectively and then filtered using a 0.2 µm filter (Polyethersulfone, FisherbrandTM). The extracts (20 µl) were then mixed with Folin-Ciocalteau reagent (80 µl), homogenized, and incubated at room temperature for 4 min. Disodium carbonate (Na_2CO_3) solution (100 µl, 7.5% w/v) was then added, followed by incubation at room temperature for 1 h, and the absorbance was measured at 750 nm using a microplate reader (Biotek, San Diego, United States). A standard solution of gallic acid was used for calibration curve preparation (0.3, 0.6, 1.2, 2.3, 4.7, 9.4, 18.8, 37.5, 75, 150, and 300 mg/l). The total phenolic content was expressed as mg gallic acid equivalents (mg GAE/g) of dry weight.

Total Flavonoid Content (TFC) of *Ba*E and *Ba*A

The TFC of *B. acmella* leaves extracts was determined using the aluminum chloride colorimetric method with modification from previous studies (Chandra et al., 2014; Luo et al., 2018). *Ba*E and *Ba*A (2 mg/ml) were dissolved in purified ethanol 95% and purified water, respectively, followed by filtration using a 0.2 µm filter (Polyethersulfone, FisherbrandTM). The extracts (50 µl) were then mixed with distilled water (220 µl) and aluminum chloride ($AlCl_3$) solution (15 µl, 10% w/v), followed by incubation at room temperature for 15 min, and the absorbance was measured at 405 nm using a microplate reader (Biotek, San Diego, United States). The flavonoid content of each extract was calculated using a standard calibration of quercetin (0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, and 50 mg/l), whereas the total flavonoid content was expressed as mg quercetin equivalent (mg QE/g) of dry weight.

Antioxidant Activity of *Ba*E and *Ba*A

2,2-Diphenyl-1-Picrylhydrazyl Radical Scavenging Assay

Antioxidant activity of extracts was measured using DPPH radical scavenging assay in 96-well plates with modified methods from previous studies (Barrientos et al., 2020). *B. acmella* leaves extract (0.1, 0.2, 0.3, 0.7, 1.3, 2.6, 5.3, 10.5, 21.1, 42.1 and 84.2 mg/l) were mixed with ethanolic DPPH and incubated for 30 min at room temperature. L-ascorbic acid was used as a positive control. The absorbance was measured at 517 nm using a microplate reader (Biotek, San Diego, United States). The result was expressed as the percentage of DPPH radical inhibition at 50% (IC_{50}). Antioxidant activity was calculated based on the formulation given:

$$\text{DPPH radical scavenging activity (\%)} = (\text{Absorbance of the control} - \text{Absorbance of the sample}) \div (\text{Absorbance of the control}) \times 100$$

2,2′-Azino-Bis-(3-Ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Assay

ABTS assay of the extracts was measured with modification as described by previous authors (Paranagama et al., 2020). The ABTS radical cation (ABTS +) was produced by reacting a 7 mM ABTS (5 ml) stock solution with 140 mM potassium persulfate (88 µl) and the mixture was incubated at room temperature in the dark for 16 h. The ABTS + solution was then diluted with ethanol to obtain an absorbance of 0.70 at 734 nm. The assay was carried out by mixing *B. acmella* extracts (40 µl) with ABTS + solution (100 µl) and incubated at room temperature for 6 min. L-ascorbic acid was used as a positive control. The result was expressed as the percentage of ABTS radical inhibition at 50% (IC_{50}). ABTS radical scavenging activity was calculated based on the formulation given:

$$\text{ABTS radical scavenging activity (\%)} = (\text{Absorbance of the control} - \text{Absorbance of the sample}) \div (\text{Absorbance of the control}) \times 100$$

Ferric Reducing Antioxidant Power Assay

FRAP assay of the extracts was measured with modification as described by previous researchers (Paranagama et al., 2020). The FRAP reagent was freshly prepared by mixing TPTZ (2,4,6-tri (2-pyridyl)-1,3,5-triazine) solution (10 mM) in hydrochloric acid (HCL) (40 mM), ferric chloride ($FeCl_3$) (20 mM), and acetate buffer (300 mM) (pH 3.6) at ratios of 1:1:10 (v/v/v). The extracts (40 µl) were then mixed with FRAP reagents (150 µl), incubated at room temperature for 15 min, and the absorbance was measured at 593 nm. L-ascorbic acid solutions (0.005, 0.01, 0.02, 0.04, 0.1, 0.1, 0.2, 0.3, 0.6, 1.2, 2.4, 4.9, 9.8, 19.5 and 39.1 mg/l) were used as the standard curve and expressed as mg L- ascorbic acid equivalents/g of dry weight extracts (mg AAE/g).

Effects of *Ba*E and *Ba*A on MC3T3-E1 Cells Proliferation and Differentiation

Cell Culture Conditions

Mouse osteoblast cells (MC3T3-E1) (ATCC, Rockville, MD, UDA) were grown in alpha Modified Eagle Medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic-antimycotic (Gibco Life Technologies, Ins., Grand Island, NY, United States). The cells were grown in a 75 cm^2 flask in a humidified atmosphere of 95% air with 5% CO_2 at 37°C and the growth medium was changed every 3 days. Once the cells reached 90% confluence, the cells were detached using 0.25% trypsin-EDTA. The cell counting procedure was performed using a hemocytometer (Hirschmann techcolor, Eberstadt, Germany) and viewed under an inverted microscope (CKX41, Olympus, Selangor, Malaysia).

Cytotoxicity Analysis and Effects of *B. acmella* Extracts on Cell Proliferation

MTT assay was used to assess cell metabolic activity according to the protocol kit (G4100, Promega). Briefly, the cells were seeded in 96-well plates (1×10^3 cells/cm^2) and treated with *B. acmella* extracts (1500, 750, 375, 187.5, 93.75, 46.88, 23.44, 11.72, 5.86 and 2.93 µg/ml) at 37°C with 5% CO_2. At designated time points (1, 3, 5, and 7 days), dye solution (10 µl) was added to the cell culture, followed by 3 h of incubation period in the incubator at 37°C to allow cellular tetrazolium conversion. Then, the cell-culture media was mixed with a stop solution. The absorbance was measured with a microplate reader (Biotek, San Diego, United States) at a wavelength of 570 nm. For each incubation period, the percentage of cell proliferation was calculated based on the formulation given:

$$\text{Cell proliferation (\%)} = (\text{Absorbance of treated cells} - \text{Absorbance of the blank}) \div (\text{Absorbance of the control} - \text{Absorbance of the blank}) \times 100$$

Sirius Red Assay

Collagen content was determined using the Sirius red-based colorimetric assay based on a method modified from previous

studies (Ahmad et al., 2018). MC3T3-E1 cells were seeded on a 24-well plate (1×10^5 cells/cm^2) and incubated at 37°C with 5% CO_2. The cells were treated at 80% confluence in the presence of osteogenic media and incubated with various concentrations of *Ba*E (2.92 and 23.44 μg/ml), and *Ba*A (5.85 and 11.72 μg/ml) for 7 and 14 days. The treatment media was changed every 3 days. After the incubation periods, the cell layer was washed with Dulbecco's phosphate-buffered saline (DPBS) and air-dried overnight in an incubator. The cells were then stained with Sirius red dye reagent (0.1% Sirius red in picric acid) for 1.5 h in the dark under mild shaking. Stained cells were washed with hydrochloric acid (HCL) (0.01 M) to remove all non-bounded dye until the solution becomes colorless. The stain cells were dissolved in a sodium hydroxide (NaOH) (0.01 M) for 30 min with mild shaking. The absorbance was measured at 540 nm using a multiskan spectrum (Thermo Scientific, Corston, Bath, United Kingdom). The percentage of collagen was calculated based on the formula given:

$$\text{Percentage of collagen (\%)} = \text{Absorbance of treated cells} \div \text{Absorbance of untreated (control) cells} \times 100$$

Alkaline Phosphatase (ALP) Activity

ALP activity was analyzed with the use of an Alkaline Phosphatase Assay Kit (ab83369, Abcam Inc., Cambridge, United Kingdom). Mouse osteoblast cell (MC3T3-E1) was seeded on a 24-well plate (1×10^5 cells/cm^2) and incubated at 37°C with 5% CO_2. The cells were treated at 80% confluence in the presence of osteogenic media and incubated with various concentrations of *Ba*E (2.92 and 23.44 μg/ml), and *Ba*A (5.85 and 11.72 μg/ml) for 7 and 14 days. The media was changed every 3 days. After the incubation periods, the cell layer was washed with cold DPBS and resuspended in ALP buffer (50 μL). The cells were then homogenized using an ultrasonic homogenizer (150/VT, Virginia, United States) for 2–3 s and kept on ice. Next, the cells were centrifuged at 4°C, 12,000 rpm for 15 min. The supernatants were collected for ALP activity and measured at 405 nm using a multiskan spectrum (Thermo Scientific, Corston, Bath, United Kingdom). The results were expressed as ALP activity of treated cells to untreated (control) cells:

$$\text{ALP activity}\left(\frac{\text{nmol}}{\text{well}}\text{pNPP}\right) = \text{Absorbance of treated cells} \div \text{Absorbance of untreated (control) cells} \times 100$$

Alizarin Red S Assay

Calcified calcium nodule formation was determined using Alizarin Red S staining based on a method modified from Ahmad et al. (2018). MC3T3-E1 cells were seeded on a 24-well plate (1×10^5 cells/cm^2) and incubated at 37°C with 5% CO_2. The cells were treated at 80% confluence in the presence of osteogenic media and incubated with various concentrations of *Ba*E (2.92 and 23.44 μg/ml) and *Ba*A (5.85 and 11.72 μg/ml) for 14 and 21 days. The treatment media was changed every 3 days. After the incubation periods, the cell layer was washed with DPBS and then fixed with paraformaldehyde (4%) for 15 min at room temperature. The cells were then washed with DPBS and stained with Alizarin Red S solution 40 mM (pH 4.2) for 30 min at room temperature. Stained cells were washed with water to remove all non-bounded dye until the solution becomes colorless. The stained cells were dissolved in cetyl peridium chloride (10%) in sodium dihydrogen phosphate (10 mM) for 30 min with mild shaking. A multiskan spectrum (Thermo Scientific, Corston, Bath, United Kingdom) was used to measure the absorbance at 570 nm. The percentage of mineralization was calculated based on the formula given:

$$\text{Percentage of mineralization (\%)} = \text{Absorbance of treated cells} \div \text{Absorbance of untreated (control) cells} \times 100$$

Identification of Compound in *Ba*E using Gas Chromatography Mass Spectrometry (GCMS) and Liquid Chromatography Time of Flight Mass Spectrometry (LCTOFMS) System

Sample Preparation for *Ba*E

*Ba*E was dissolved in ethanol (95%) at a concentration of 10 mg/ml and 25 mg/ml and filtered using Whatman PVDF Syringe filter, 0.2 μm (Polyethersulfone, FisherbrandTM). The extracts were then transferred into vials for GCMS and LCTOFMS analysis respectively.

Instrumentation and Chromatographic Condition of GCMS

Chemical compound identification was performed with some modifications (Anholeto et al., 2017). GCMS system consisting of an Agilent 6890 gas chromatography coupled with an Agilent 5973 mass spectrometer. Separation of compounds was carried out using HP-5 MS capillary column (30 m × 0.25 mm × 0.25 μm). The analysis was set at: injector at 220°C; split mode at 1:10 ratio; oven temperature at 40°C–250°C (2.5°C min^{-1}); carrier gas: helium at a flow rate of 1.0 ml min^{-1}; injection volume at 1 μl. MS condition was set with an ionizing voltage at 71 eV. The ionization source was set at 250°C. The scanning range was set between 30 amu for low mass and 1,000 amu for high mass with a solvent delay of 5.00 min. The total ion chromatograms (TICs) and mass spectra were recorded using MSD Chemstation Data Analysis software. Mass spectral identification of unknown chemical compounds in extracts was performed by comparing with NIST library with match quality above 85% (Samsurrijal et al., 2019).

Instrumentation and Chromatographic Condition of LCTOFMS

Phenolic compounds were determined using the liquid chromatography time of flight mass spectrometry (LCTOFMS) system. The detector used was a mass spectrometer with an ESI interface. Separation was carried out by injecting 10 μL aliquots into a C18 reversed-phase column (Zorbax, 150 mm, 4.6 μm) at room temperature. The elution was performed as reported in **Table 1**. The

TABLE 1 | Chromatographic gradient for LCTOFMS analysis.

Time (minutes)	A (Acetonitrile)	B (1% Formic acid in water)
0	10	90
15	11	89
18	15	85
20	20	80
23	30	70
25	35	65
28	40	60
30	45	55
50	60	40
60	10	90

mass spectrometer operated in full-scan MS mode, from m/z 100 to 1,200 in positive modes. The electrospray interface (ESI) provided nebulization by applying 3.0 kV ion spray voltage and capillary temperature of 200°C. Instrument control, data acquisition was provided by Masslynk 4.1 software. Data processing and chemical compound identification in extracts were performed by MS-DIAL software (RIKEN, version 4.7) (http://prime.psc.riken.jp/compms/msdial/main) with a score above 80%, peak detection at 5,000 amplitude, and MS identification using Vaniya Fiehn Natural Product Library.

Statistical Analysis

Results were presented as mean ± standard deviation (SD) and mean ± standard error of the mean (SEM). The slope of the calibration curve and the coefficient of determination (R^2) were obtained using MS Excel version 2110. Data obtained for cytotoxicity, proliferative, and antioxidant activity were normally distributed using Shapiro-Wilk and statistically processed with a one-way analysis of variance (ANOVA) test with Tukey HSD post-hoc tests to determine the significant differences among samples. ($p < 0.05$) was accepted as statistically significant. Control (untreated) and treatments groups were compared for cytotoxicity and proliferative activities. Statistical relationships with a linear model between TPC, TFC, DPPH, FRAP, ABTS, proliferation, ALP, collagen and mineralization were carried out using Pearson's correlation analysis. This analysis was performed to assess if two different variables are associated. All statistical analyses were conducted using the Statistical Package for Social Science (IBM SPSS statistical software version 26, IBM, New York, NY, United States).

RESULTS

Percentage of Moisture Content and Extraction Yield of Ethanol and Aqueous Extract of *B. acmella* Leaves

The residual moisture content in *B. acmella* leaves was 84.56% ± 1.45. Extraction yield of *Ba*E was 7.04% ± 1.30 and *Ba*A was 11.97% ± 0.31.

TABLE 2 | TPC and TFC of *B. acmella* extracts.

Extracts	TPC (mg GAE/g dry weight)	TFC (mg QE/g dry weight)
*Ba*E	30.25 ± 0.85[a]	30.06 ± 1.18[a]
*Ba*A	24.38 ± 2.15[b]	2.41 ± 0.12[b]
Calibration	Y = 0.004x + 0.001 (R^2 = 0.999)	Y = 0.035x + 0.032 (R^2 = 0.999)

BaE: B. acmella ethanol extract; BaA: B. acmella aqueous extract. Values represent mean ± standard deviation, n = 6. Different superscript letters show significant differences (p < 0.05) in the same analysis. Calibration curves: Y = mx+c; Y = absorbance, x = concentration of gallic acid (GAE) or quercetin (QE).

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

TPC and TFC of *Ba*E were 30.25 ± 0.85 mg GAE/g dry weight and 30.06 ± 1.18 mg QE/g dry weight, respectively. On the other hand, the TPC and TFC of *Ba*A were 24.38 ± 2.15 mg GAE/g dry weight and 2.41 ± 0.12 mg QE/g dry weight, respectively. **Table 2** shows the results of TPC and TFC of *B. acmella* leaves extracts. TPC and TFC of *Ba*E were significantly higher ($p < 0.01$) than *Ba*A.

Antioxidant Activity (DPPH, ABTS and FRAP Assays)

Antioxidant activity for DPPH and ABTS assays in *B. acmella* extracts was reported as the amount of antioxidants required to decrease the initial concentration at 50% (IC_{50}). **Table 3** shows the IC_{50} value of *B. acmella* extracts in DPPH and ABTS assays. DPPH assay revealed that *Ba*E contributed to significantly lower IC_{50} values than *Ba*A. Meanwhile, for the ABTS assay, no significant differences ($p > 0.05$) in the IC_{50} values were observed for *Ba*A and *Ba*E. For FRAP assay, *Ba*E contributed to significantly higher antioxidant activity ($p < 0.01$) than *Ba*A.

Cytotoxicity Analysis of *B. acmella* Extracts on Osteoblast Cells (MC3T3-E1)

The cytotoxicity analysis of MC3T3-E1 cells after treatment with *Ba*A (A) and *Ba*E (B) leaves, at concentrations of 2.93 μg/ml to 1,500 μg/ml at day 1, 3, 5, and 7 are shown in **Figure 2**.

Results showed that concentrations of extracts at 750 μg/ml to 1,500 μg/ml for all days and extracts at 46.88, 93.75 and 187.5 μg/ml on day 7 were toxic to cells, with less than 50% of viable cells compared to control for all days. Meanwhile, no cytotoxicity was found for both extracts at concentrations of 2.93 μg/ml to 187.5 μg/ml with more than 50% of viable cells at all days except at 46.88, 93.75 and 187.5 μg/ml on day 7. There were fluctuations in the percentage of cell proliferation from low to high doses at all treatment days. On day 1, *Ba*E induced a significantly higher cell proliferation than control ($p < 0.05$) at the treatment concentrations of 2.93 μg/ml (141.32%) and 23.44 μg/ml (118.99%). On day 5, *Ba*A caused a significantly higher cell proliferation than the control ($p < 0.05$) at treatment concentrations of 46.88 μg/ml (147.76%) and 93.75 μg/ml, which were about 134.53%, respectively. When compared, *Ba*E induced higher cell proliferation at lower concentrations and earlier days than *Ba*A.

Based on these findings, *Ba*E concentrations (2.93 μg/ml and 23.44 μg/ml) and *Ba*A concentrations (5.8 μg/ml and 11.72 μg/

TABLE 3 | DPPH, ABTS and FRAP activities of *Ba*E and *Ba*A.

Value	Samples/Antioxidant assays	*Ba*A	*Ba*E	L- ascorbic acid
Antioxidant activity, IC_{50} (μg/ml)	DPPH	860.67[a]	476.71[b]	20.25[c]
	ABTS	192.56[a]	201.49[a]	13.70[b]
Antioxidant activity (mg AAE/g)	FRAP	3.31 ± 0.53[a]	56.01 ± 6.46[b]	-

BaE: B. acmella leaves ethanol extract; BaA: B. acmella leaves aqueous extract. mg AAE/g: mg L-ascorbic acid equivalent/g. Value represents mean ± standard deviation, n = *6; Different superscript letters show significant differences between means in the same assays at* $p < 0.05$.

ml), which caused the highest significant cell proliferation on day 1 were chosen for further assessments on the differentiation of MC3T3-E1 cells.

Effects of *B. acmella* Extracts on Cell Proliferation Activity of Osteoblast Cells (MC3T3-E1)

Clodronate is the earliest group of non-nitrogen bisphosphonates that have been widely used in the clinical treatment for bone-related diseases by enhancing bone inhibition (Koch et al., 2011; Frediani and Bertoldi, 2015; Nardi et al., 2016; Pherwani and Sathaye, 2020). Bisphosphonates reacted with osteoblast cells and altered bone metabolism by stimulating bone formation (Corrado et al., 2017). Previous studies reported that clodronate affects the anabolic activity of bone cells by enhancing the differentiation and mineralization of MC3T3-E1 cells (Itoh et al., 2003; Koch et al., 2011; Maruotti et al., 2012). Therefore, clodronate has been used as a positive control to study the effects of *B. acmella* extracts on MC3T3-E1 cells.

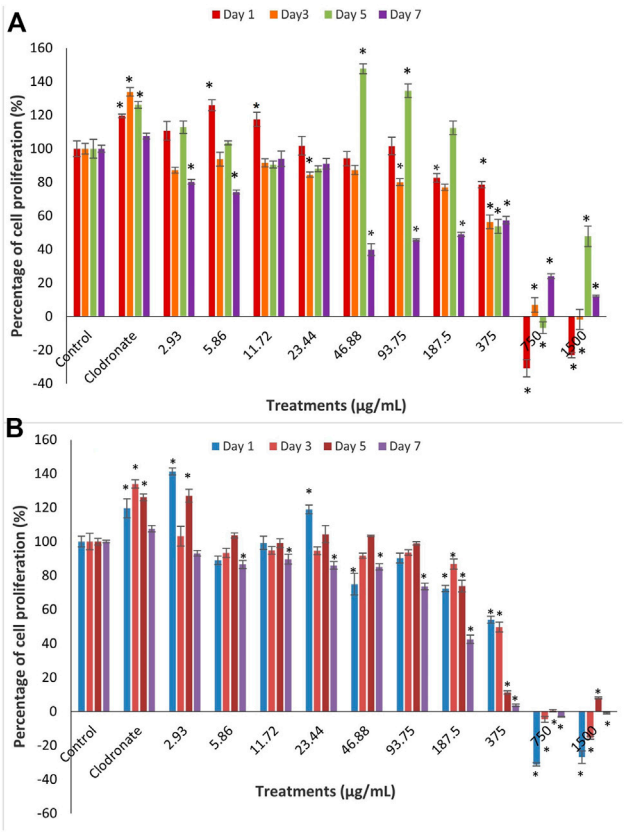


FIGURE 2 | Cell proliferation of MC3T3-E1 (osteoblast) cells after treatment with different concentrations of *B. acmella* **(A)** aqueous (*Ba*A) and **(B)** ethanol (*Ba*E) extracts at Day 1, Day 3, Day 5 and Day 7 of incubation. Results (optical densities) were calculated as the percentage of unexposed control cultures and represented by mean ± SEM. *p-value less than 0.05 ($p < 0.05$) indicated significant difference when compared to untreated cells (control) ($n = 6$) at same incubation times.

Results in **Figure 3** showed the cell proliferation activity of MC3T3-E1 cells after treatment at the determined concentration of *B. acmella* extracts on day 1. The highest cell proliferation was significantly observed at the *Ba*E concentration of 2.93 μg/ml, which was about 141.32% compared to clodronate and other treatment concentrations. As for *Ba*A, significantly ($p < 0.05$) higher cell proliferation than control was observed at the concentrations of 5.86 μg/ml, which was about 125.94%. However, no significant difference ($p > 0.05$) was found between BaA_5.86, BaA_11.72 and BaE_23.44.

The morphological features of MC3T3-E1 cells treated with the four selected concentrations of *B. acmella* extracts (BaA_5.86, BaA_11.72, BaE_2.93 and BaE_11.72), on day 1 were compared with controls using an inverted microscope and the micrographs were shown in **Figure 4**.

The micrographs showed that MC3T3-E1 cells have a flat, polygonal appearance and were uniformly thin with smooth extended cytoplasm. It also has a spindle-shaped morphology with a fibroblastic appearance. The number of cells increased on day 1 after treatment with *B. acmella* extracts compared to control and clodronate. The result obtained was also in agreement with the MTT proliferation assay, as shown in **Figure 3**. These findings indicated that the osteoprotective effect of *B. acmella* extracts were *via* amplification of osteoblast proliferation. These results also revealed that 2.93 μg/ml was the most compatible concentration of *B. acmella* followed by others *B. acmella* (BaE_23.44, BaA_5.86 and BaA_11.72) to provoke a significant proliferation of MC3T3-E1 cells compared to control (untreated cells).

Effects of *B. acmella* Extracts on Differentiation Activities of Osteoblast Cells (MC3T3-E1)

Collagen Determination and Alkaline Phosphatase Activity

The effects of *Ba*E and *Ba*A on early osteoblast differentiation were first assessed by measuring the percentage of collagen

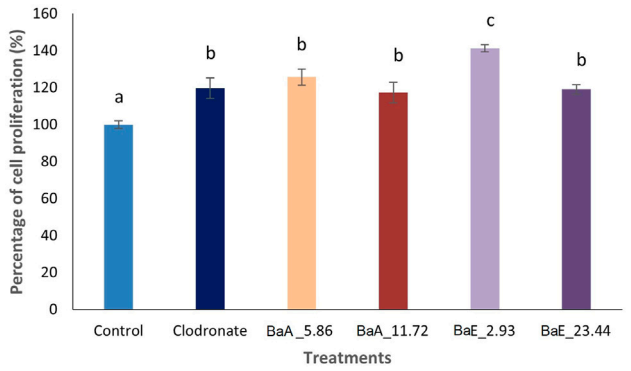


FIGURE 3 | Cell proliferation activity of MC3T3-E1 (osteoblast) cells after treatment with different concentrations of *B. acmella* aqueous (*Ba*A) and ethanol (*Ba*E) extracts at Day 1. BaE_2.93 (*Ba*E at 2.93 μg/ml), BaE_23.44 (*Ba*E at 23.44 μg/ml), BaA_5.86 (*Ba*A at 5.86 μg/ml) and BaA_11.72 (*Ba*A at 11.72 μg/ml). Results (optical densities) were calculated as mean ± SEM. Different alphabets indicated significance differences ($p \leq 0.05$) ($n = 6$) between treatments.

content and ALP activity of MC3T3-E1 cells at 7 and 14 days of treatment. The percentage of collagen secreted by osteoblast was estimated by Sirius red staining with red color formation by the reaction of sulfonic acid in Sirius red and the collagen fibers. **Figure 5** shows the photomicrographs of MC3T3-E1 cells with or without treatment with *B. acmella* leaves extracts and stained with Sirius red.

The red color staining of MC3T3-E1 cells receiving *B. acmella* leaves extracts treatment was more intense and widely distributed in the whole plate region on day 14 compared to the control cells. *Ba*E at a concentration of 23.44 μg/ml showed the highest red color intensity due to collagen depositions. The stained cells were further dissolved, and the absorbance of *B. acmella* leaves extracts was measured. Furthermore, the percentage of collagen content was calculated by comparing it with the untreated cells (control).

In line with the collagen staining results, *B. acmella* treatments produced an increase in collagen synthesis after 14 days of treatment as shown in **Figure 6**.

Results showed that the collagen percentage of cells increased significantly ($p < 0.05$) from day 7 to day 14 with clodronate and

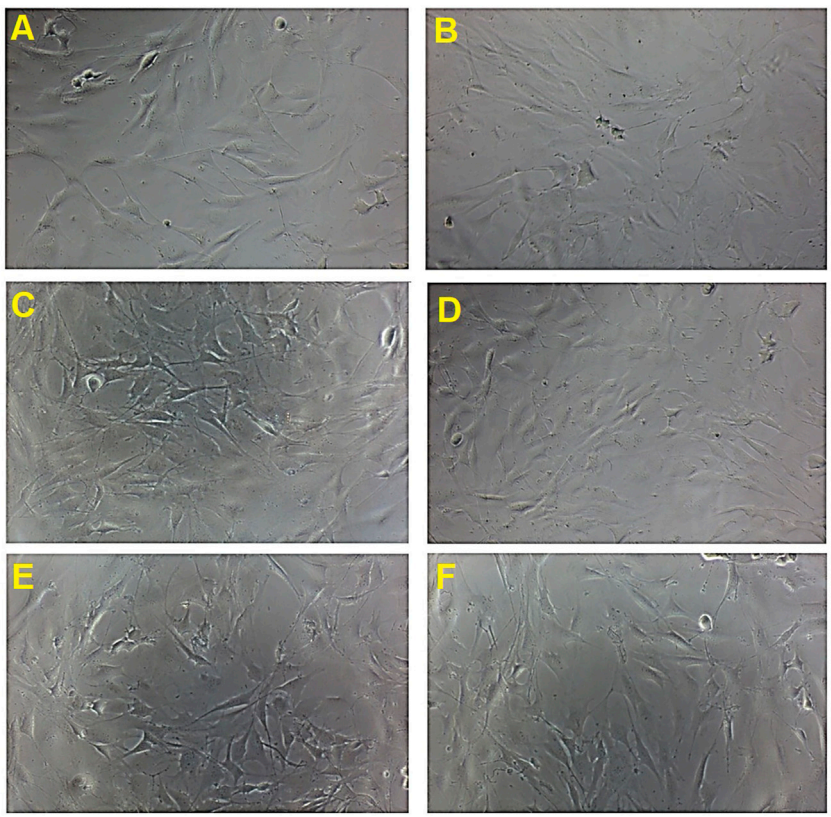


FIGURE 4 | Cell proliferation activity and morphological changes in MC3T3-E1 cells treated *with B. acmella* extracts. **(A)**: Control (untreated cells); **(B)**: Positive control (Clodronate); **(C)**: BaE_2.93 (*Ba*E at 2.93 μg/ml), **(D)**: BaE_23.44 (*Ba*E at 23.44 μg/ml), **(E)**: BaA_5.86 (*Ba*A at 5.86 μg/ml) and **(F)**: BaA_11.72 (*Ba*A at 11.72 μg/ml) at Day 1 compared to control (untreated cells) at 100x magnification.

FIGURE 5 | Photomicrographs of collagen-stained cells in *B. acmella* aqueous (*Ba*A) and ethanol (*Ba*E) extracts at Day 7 and Day 14. Control (untreated cells), Positive control (Clodronate), BaE_2.93 (BaE at 2.93 μg/ml), BaE_23.44 (BaE at 23.44 μg/ml), BaA_5.86 (BaA at 5.86 μg/ml) and BaA_11.72 (BaA at 11.72 μg/ml).

B. acmella treatments compared to control cells. On day 7, the collagen percentage of *Ba*E-treated cells (2.93 and 23.44 μg/ml) were significantly higher ($p < 0.05$) than control cells. However, no significant difference was found between the two concentrations of *Ba*E-treated cells. On day 14, the percentage of collagen was significantly increased from 2.93 μg/ml to 23.44 μg/ml.

With aqueous extract (*Ba*A) treatment, the collagen content was significantly increased ($p < 0.05$) compared to control at concentration of 5.86 μg/ml and 11.72 μg/ml on day 14. Meanwhile, no significant difference was found for both aqueous extracts of *B. acmella* on day 7 compared to the control. The highest collagen content was found on day 14 with *Ba*E at the concentration of 23.44 μg/ml, which was about 196.81% higher than the control. However, no significant difference was observed between *Ba*E at the concentration of 23.44 μg/ml and clodronate. Similar results were found on day 7 as the treatment with ethanol extracts (BaE_2.93 and BaE_23.44) showed higher collagen content compared to aqueous extracts (BaA_5.86 and BaA_11.72), which was not significantly different ($p > 0.05$) compared to clodronate.

Figure 7 shows the percentage of ALP activity of *B. acmella* leaves for 7 and 14 days. The ALP activity was increased from day 7 to day 14 for all treatments. Ethanol extracts showed higher ALP activity compared to aqueous extract. All treatment groups had significantly higher ALP activity than control for all days except 11.72 μg/ml of *Ba*A on day 7. The highest ALP activity was found on day 14 with *Ba*E at the concentration of 2.93 μg/ml, which was about 2.31 and 1.35 times more than control and clodronate, respectively. However, no significant difference was found between ethanol extract concentrations of 2.93 μg/ml and 23.44 μg/ml, when the same-day treatment was compared. Likewise, no significant difference was found between aqueous extracts (BaA_5.86 and BaA_11.72) on day 14.

Mineralization (Calcium Depositions)

The deposition of calcium noduls in MC3T3-E1 cells after treatment with *Ba*E (2.93 and 23.44 μg/ml) and *Ba*A (5.86 and 11.72 μg/ml) were determined using Alizarin Red staining on day 14 and day 21 as shown in **Figure 8**.

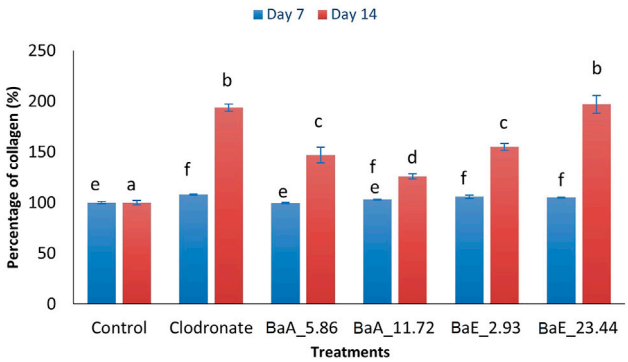


FIGURE 6 | Percentage of collagen in *B. acmella* aqueous (*Ba*A) and ethanol (*Ba*E) extracts. Control (untreated cells), Positive control (Clodronate), BaE_2.93 (*Ba*E at 2.93 μg/ml), BaE_23.44 (*Ba*E at 23.44 μg/ml), BaA_5.86 (*Ba*A at 5.86 μg/ml) and BaA_11.72 (*Ba*A at 11.72 μg/ml). Different alphabet shows significant different ($p < 0.05$) between treatments in a same day. The data are presented as mean ± SEM ($n = 6$).

Alizarin Red S staining revealed the calcium in the bone nodules by forming a bright-colored alizarin red s-complex. It shows evidence of matrix mineralization in the calcified region. No observable differences were noted between the group on day 14 except at BaE_2.93. On day 21, the red staining in MC3T3-E1 cells was intense with 2.93 and 23.44 μg/ml of ethanol extracts, followed by 11.72 μg/ml of aqueous extract and clodronate. Furthermore, the percentage of calcium depositions was determined as shown in **Figure 9**.

Generally, ethanol extracts contributed to a higher percentage of minerals compared to aqueous extracts. On day 14, only 2.93 μg/ml ethanol extract was found to be significantly higher than untreated cells (control). On day 21, both concentrations of ethanol extracts (2.93 μg/ml and 23.44 μg/ml) showed significantly higher mineral percentages ($p < 0.05$) compared to control and clodronate. No significant difference was found between the two concentration of ethanol extracts on day 21.

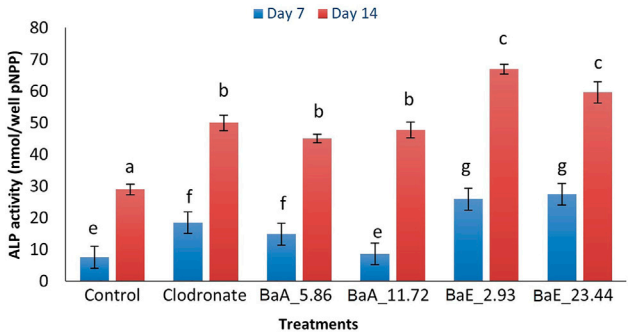


FIGURE 7 | ALP activity in *B. acmella* aqueous (*Ba*A) and ethanol (*Ba*E) extracts. Control (untreated cells), Positive control (Clodronate), BaE_2.93 (*Ba*E at 2.93 µg/ml), BaE_23.44 (*Ba*E at 23.44 µg/ml), BaA_5.86 (*Ba*A at 5.86 µg/ml) and BaA_11.72 (*Ba*A at 11.72 µg/ml). Different alphabets shows significant different ($p < 0.05$) between treatments in a same day. The data are presented as mean ± SEM ($n = 6$).

Correlation Analysis Between TPC, TFC, Antioxidant (DPPH, ABTS and FRAP) and Anabolic (Cells Proliferation, Collagen, ALP and Mineral) Activities

Table 4 show the Pearson correlation between TPC, TFC, antioxidant and anabolic activities of *B. acmella* using Pearson's correlation analysis. No significant correlation was found between ABTS and cell proliferation. A highly significant positive correlation was found between 1) TPC, TFC and FRAP assay 2) TFC, cell proliferation, collagen, ALP activity and mineralization 3) TPC and ALP activity and 4) FRAP, cell proliferation, collagen, ALP activity and mineralization 5) TFC and ABTS 6) ABTS, collagen and mineralization. A highly negative correlation was found between 1) TPC, TFC, DPPH and FRAP assay 2) DPPH assay, cell proliferation, collagen, ALP activity and mineralization. A moderate positive correlation was found between 1) TPC, cell proliferation and collagen activity and 2) ALP activity and ABTS assay. A moderate negative correlation was found between ABTS and DPPH assay.

GCMS and LCTOFMS Analysis

Based on the superior antioxidant and MC3T3-E1 cells proliferation and differentiation activities showed by the ethanol extract of *B. acmella* (*Ba*E), the extract was further analyzed using GCMS and LCTOFMS systems. The identified compounds might be responsible for these actions. **Figure 10A** and **Figure 10B** shows the GCMS and LCTOFMS total ion chromatograms (TIC) of compounds from *Ba*E.

Based on the matching quality at 85% (Samsurrijal et al., 2019) and above, GCMS analysis showed that *Ba*E contained 91 chemical compounds, including 30 known and 61 unknown compounds. Alkyl amides (30.93%) was the most abundant group present in *Ba*E extract, followed by terpenoid (16.56%), alkene (4.27%), fatty acid ester (2.95%), phenolic (2.22%), phthalic acid ester (0.69%), and alkana (0.31%) groups. Meanwhile, 19.89% of abundance compounds were contributed by the unknown compounds. Spilanthol (29.53%), the marker compound for *B. acmella* was the major compound in *Ba*E.

Supplementary Table S1 and **Supplementary Table S2** shows known biological active compounds in *Ba*E using GCMS and LCTOFMS respectively. Several biological active compounds identified in *Ba*E contribute to the stem cell and osteoblast proliferation, antioxidant and bone anabolic activities. GCMS analysis shows the highest antioxidant activity contributed by alkyl amides (35.09%) from the total abundance of 59.27% antioxidant activity. The highest anabolic activity contributed by diterpenoids (10.98%) from the total abundance (20.57%) of anabolic activity. Diterpenoids (10.98%) contributed to both antioxidant and anabolic activities from the total abundance of terpenoid (17.66%) activity.

LCTOFMS analysis combined with MS-DIAL software at a score of more than 80%, showed that *Ba*E contained 169 chemical compounds, including 32 known and 137 unknown compounds, which includes fatty acid (5.57%), flavonoids (4.95%), alkyl amides (4.00%), amino acids (2.58%), terpenoids (1.95%), guaianes (1.22%), alkaloids (1.18%), vitamin B (1.06%), purine nucleosides (0.57%), methoxy phenols (0.48%), coumarin (0.09%), phenolic acids (0.01%). A total of 5.44% phenolic compounds were detected.

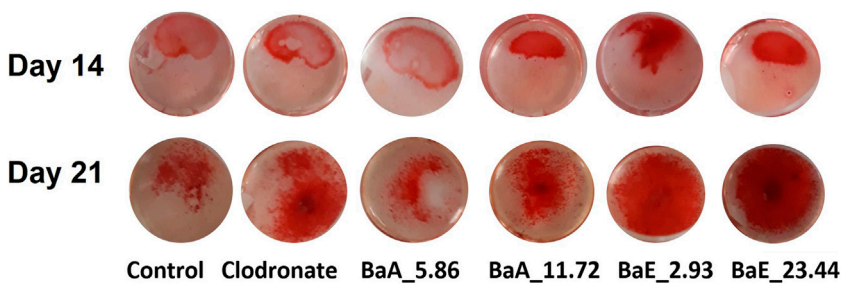


FIGURE 8 | Photomicrographs of mineral depositions stained with Alizarin Red staining in *B. acmella* aqueous (*Ba*A) and ethanol (*Ba*E) extracts on Day 14 and Day 21. Control (untreated cells), Positive control (Clodronate), BaE_2.93 (BaE at 2.93 µg/ml), BaE_23.44 (BaE at 23.44 µg/ml), BaA_5.86 (BaA at 5.86 µg/ml) and BaA_11.72 (BaA at 11.72 µg/ml).

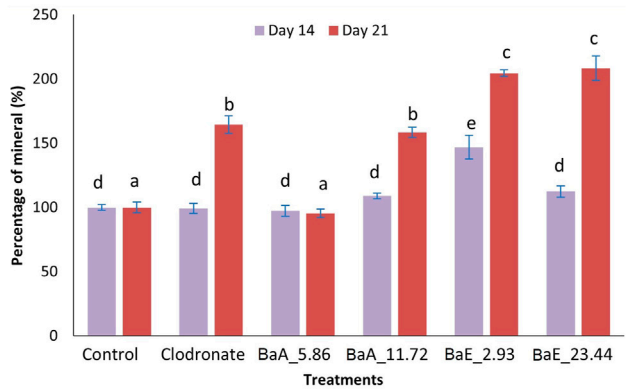


FIGURE 9 | Percentage of mineral in *B. acmella* aqueous (*Ba*A) and ethanol (*Ba*E) extracts. Control (untreated cells), Positive control (Clodronate), BaE_2.93 (*Ba*E 2.93 µg/ml), BaE_23.44 (*Ba*E 23.44 µg/ml), BaA_5.86 (*Ba*A 5.86 µg/ml) and BaA_11.72 (*Ba*A 11.72 µg/ml) different alphabets show significant different ($p < 0.05$) between treatments in a same day. The data are presented as mean ± SEM ($n = 6$).

DISCUSSIONS

Moisture Content, Extraction Yield, Phytochemical Content and Antioxidant Activity of *B. acmella* Leaves Extracts

Moisture content and extraction yield are parts of herbs characterization. It is one of the essential quality standards in the standardization process of herbal drugs. The percentage of moisture content of *B. acmella* in this study was 84.56%, higher than *B. acmella* cultivated in India which was 4.32% (Kumar and Ravindra, 2018). A study by Otles and Yalcin (Otles and Yalcin, 2012), showed that the percentage of moisture content in roots, stalks, and stems of netter plants that were planted in various locations ranged from 6.3 to 88.88%. Several factors, such as seasons, geographical origin, and environmental conditions contribute to the moisture content in plants (Azonwade et al., 2018). Therefore, the difference in the percentage of moisture content between the present and previous studies might be due to the variation in seasons, geographical origin, and environmental conditions, such as climate. Tee et al. (2014) reported that the low moisture content of *D. edulis* and *D. rostrata* fruits indicated that the fruits have a high dry matter content and highly nutrient-dense pulp. Thus, these properties might explain the disparities observed between the *B. acmella* leaves investigated in the present study compared to the research by Kumar and Ravindra (2018).

A secondary metabolite is a biologically active compound present in low amounts in plants. Therefore, the extraction method is critical in obtaining a high yield of phytochemicals with minimal changes to the functional properties of the biologically active compounds in the plant. The extraction method employed in this study involved a maceration technique using purified water and ethanol to extract the active compound in *B. acmella* leaves. This method was chosen due to its cost-effectiveness and close resemblance to the traditional extraction method of herbal medicine. Purified water (100%) and 95% ethanol (100%) were used in our extraction method instead of other solvents, such as methanol, hexane, and diethyl ether, as they are green solvents, suitable for human consumption, reusable and nontoxic (Ben Yakoub et al., 2018).

Several factors contributed to the extraction yields, such as the polarity of solvents (Do et al., 2014). The solvent polarity reacts based on the sample properties, analyte chemical properties, and interactions between the matrix and analyte (Dhanani et al., 2017). These factors contribute to the different groups of compounds being extracted (Chandra et al., 2014; Iloki-Assanga et al., 2015; Ghasemzadeh et al., 2018). In this study, water and ethanol were used as solvents for extraction to evaluate the phytochemical and biological activity of *B. acmella*. For standardization, the same cultivated area and batch of *B. acmella* were used, and the extraction parameters were fixed during the extraction process.

This was in agreement with the findings by previous study on the successive extraction with petroleum ether, benzene, chloroform, ethanol and water which reported that water extract (3.730%) of *B. acmella* roots, stems and leaves contributed to the highest extraction yield followed by ethanol (2.305%), chloroform (0.825%), petroleum ether (0.736%) and

TABLE 4 | Pearson correlation between TPC, TFC, antioxidant and anabolic activities.

		TFC	DPPH	ABTS	FRAP	% Cell proliferation	% Collagen	ALP activity	% Mineralization
TPC	R	0.887	−0.869	0.574	0.881	0.622	0.643	0.835	0.834
	p	0.000**	0.000**	0.050*	0.000**	0.031*	0.024*	0.001**	0.001**
TFC	R		−0.995	0.718	0.993	0.724	0.810	0.892	0.853
	p		0.000**	0.009**	0.000**	0.008**	0.001**	0.000**	0.000**
DPPH	R			−0.686	−0.985	−0.725	−0.800	−0.889	−0.835
	p			0.014*	0.000**	0.008**	0.002**	0.000**	0.001**
ABTS	R				0.698	0.380	0.888	0.692	0.724
	p				0.012*	0.223	0.000**	0.013*	0.008**
FRAP	R					0.738	0.816	0.887	0.882
	p					0.006**	0.001**	0.000**	0.000**

*TPC: total phenolic content; TFC: total flavonoid content; DPPH (2,2-diphenyl-1-picrylhydrazyl); ABTS (2,2'-azino-bis (3- ethylbenzothiazoline-6-sulfonate); FRAP (Ferric reducing antioxidant power); P= p-value (significant); R = Pearson's correlation; n = 12. **Correlation is significant at the p < 0.01 and *correlation is significant at the p < 0.05.*

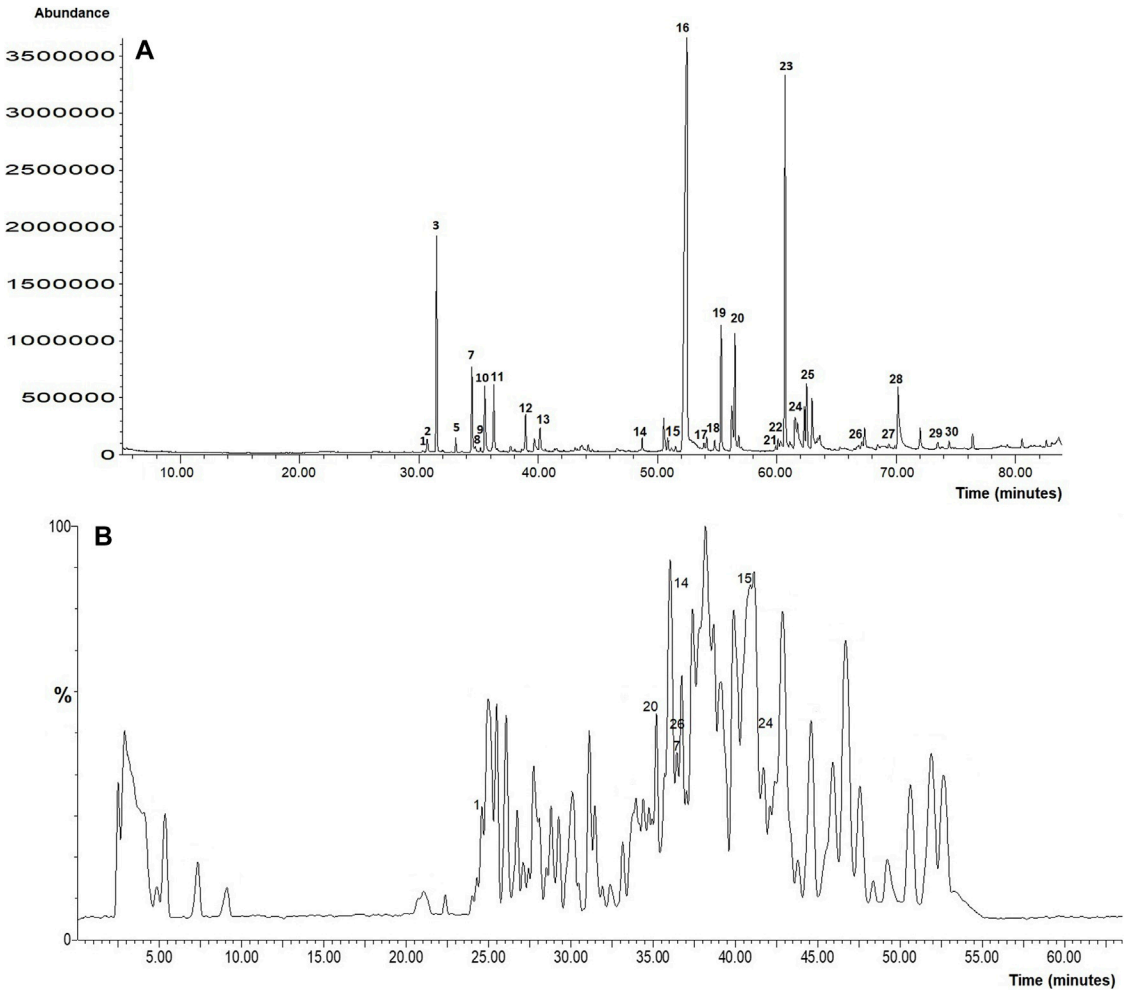


FIGURE 10 | (A) GCMS and **(B)** LCTOFMS total ion chromatograms of compounds from *Ba*E. Known compounds were labeled according to **Supplementary Table S1** and **Supplementary Table S2**. Each number labeled at chromatogram referred to the number in **Supplementary Table S1** and **Supplementary Table S2**.

benzene (0.735%) extracts (Tanwer and Vijayvergia, 2010). In a study on a different plant, Do et al. (Do et al., 2014) found that the extraction yield in *Limnophila aromatica* aqueous extract (25.58 ± 1.04%) was higher than ethanol extract (17.03 ± 2.66%). Do et al. (2014) concluded that the extraction yield increased with solvent polarity. The same pattern was found in another plant, *Adansonia digitata* (Ismail et al., 2019). Therefore, the extraction yield of *B. acmella* in the current study increased with solvent polarity, meaning that compared to ethanol, more phytochemicals were extracted as the polar solvent increased (i.e., more water content). This was attributed to the increased solubility of phytochemicals in water compared to ethanol. Protein and carbohydrates were found to be more soluble in water than ethanol (Hwang and Thi, 2014). Thus, the higher extraction yield in *Ba*A compared to *Ba*E, may be contributed by the higher amount of protein and carbohydrate in the *B. acmella* plant.

Phenolic compounds are important secondary metabolite groups present in plants and contribute significantly to the antioxidant activity in plants (Baba and Malik, 2015; Swallah et al., 2020). Thus, phenolic compounds can be used as a basis in screening antioxidant activity in plants. Flavonoids are the typical phenolics that contribute to antioxidant activity (Đudarić et al., 2015). They act by suppressing reactive oxygen formation, chelating trace elements involved in free-radical production, scavenging reactive species, and upregulating antioxidant defense (Ammar et al., 2009; El-Din and El-ahwany, 2016).

Folin-Ciocalteu (FC) and aluminum colorimetric methods were widely used to evaluate TPC and TFC. FC method is a fast, simple, inexpensive, robust, and reliable method to quantify phenolics in samples (Prior et al., 2005). Results from this study revealed that total phenolic and flavonoid contents were significantly influenced by the type of extraction solvent employed.

*Ba*E was found to have significantly higher TPC and TFC than *Ba*A. Similar findings were reported for *C. olitorius* (Ben Yakoub et al., 2018) and *Moringa oleifera* L. (Nobossé et al., 2018), indicating that ethanol extracts have higher TPC and TFC

compared to aqueous extract. In contrast, Ismail et al. (2019) reported that the aqueous extract of *Adansonia digitata* has higher TPC and TFC than ethanol extract. Likewise, Gavamukulya et al. (2014), showed that *Annona muricata* leaves aqueous extract has a higher TPC than ethanol extract. The highest TPC and TFC in *Ba*E compared to previous studies are due to the characteristics of the phenolic compounds in *B. acmella* that are less hydrophilic compared to *Adansonia digitata* and *Annona muricata*.

There are numerous assays to evaluate the antioxidant activity of plant extracts. DPPH, ABTS, FRAP, ORAC, and CUPRIC (Dudonne et al., 2009; Esmaeili and Sonboli, 2010; Ozyurek et al., 2011; Moreno et al., 2020) are chemical screening methods used to evaluate the potential of extracts as an antioxidant agent. There is no pharmacological relevance using these chemical assays and no evidence for therapeutic health benefits. Different antioxidant methods produced diverse antioxidant activities due to the various mechanisms and reactions to the group of compounds present in plants (Floegel et al., 2011). The most widely used method of evaluating the antioxidant activity of plant extracts as potential antioxidant agents were DPPH, ABTS, and FRAP assays (Sridhar and Charles, 2019; Ilyasov et al., 2020).

DPPH and ABTS assay can scavenge radicals and reduce the redox-active compound by accepting an electron or hydrogen radical from antioxidants present in an extract to become a stable diamagnetic molecule. Meanwhile, FRAP assay can reduce non-stable ferric iron (Fe^{3+}) to stable ferrous ion (Fe^{2+}) by accepting an electron from antioxidants present in an extract and terminate the oxidation chain reaction (Bibi Sadeer et al., 2020).

Further evaluation on the antioxidant activity of *B. acmella* as a potential antioxidant agent was carried out in this study using the chemical screening method of DPPH, ABTS, and FRAP assays. L-ascorbic acid was chosen as a positive control. L-ascorbic acid is one of the synthesis antioxidants and has been widely used by researchers to study the antioxidant activity in plant extracts (Manssouri et al., 2020; Ho et al., 2021). A low IC_{50} value indicates the better antioxidant activity of the extracts as a potential antioxidant agent. The reaction between antioxidant and radical reagent caused the purple color of DPPH radical to turn to pale yellow (Prior et al., 2005), whereas the ABTS radical became blue to green color (Opitz et al., 2014). The color changes in DPPH and ABTS assays indicated the scavenging effects of the tested plant extract. In the ABTS assay, the radical cation was directly generated in a stable form using potassium persulfate before the treatment process to prevent the interference of compounds that will affect radical formation. Hence, the assay will become less susceptible to artifacts and prevents the overestimation of antioxidant capacity (Mzid et al., 2017). In the FRAP assay, the intense blue color complex was formed when the ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to ferrous (Fe^{2+}) by the action of electron-donating antioxidants (Benzie and Devaki, 2017).

Furthermore, the current findings showed that ethanol extract of *Ba*E (476.71 μg/ml) contributed to significantly lower IC_{50} values of DPPH assay than the aqueous extract of *Ba*A (860.67 μg/ml) ($p < 0.05$). This indicated that *Ba*E has higher antioxidant activity, at about 1.8 times higher than *Ba*A. Meanwhile, the ABTS assay showed no significant difference between *Ba*A (192.562 μg/ml) and *Ba*E (201.489 μg/ml), and a lower IC_{50} value compared to the DPPH assay. These findings were in line with a previous study by Izabela and Anna (Grzegorczyk-Karolak and Kiss, 2018) on antioxidant properties (DPPH and ABTS assays) of *Salvia virdis* L. shoots. They reported that based on the DPPH assay, ethanol extract contributed to significantly higher antioxidant activities than aqueous extract. No significant difference in the antioxidant activities of ethanol and aqueous extracts was found with ABTS assay. Similar patterns were also reported in studies on *Urtica urens* (Mzid et al., 2017), *Harpagophytum procumbens* (Grabkowska et al., 2016) and *Rehmanniaglutinosa* (Piątczak et al., 2014).

ABTS assay is an aqueous-based assay that favors hydrophilic compounds, whereas DPPH assay is an organic (alcohol) and aqueous-based assay with an affinity for hydrophilic and hydrophobic compounds (Mzid et al., 2017; Grzegorczyk-Karolak and Kiss, 2018). Therefore, some highly water-soluble compounds in aqueous extracts may scavenge more ABTS radicals than DPPH radicals. This contributed to the lower IC_{50} value of *Ba*A in the ABTS assay (192.562 μg/ml) compared to the DPPH assay (476.71 μg/ml).

A further antioxidant test was carried out with the FRAP assay. The findings showed a similar pattern to the DPPH assay. Specifically, the antioxidant activity of *Ba*E (56.01 ± 6.46 mg L-ascorbic acid/g dry weight) was 17 times higher than *Ba*A (3.31 ± 0.53 mg L-ascorbic acid/g dry weight). The FRAP finding results corroborate a previous study which reported that non-oil seed legumes ethanol extracts had higher antioxidant activity than aqueous extracts (Diniyah et al., 2020).

Correlation Between TPC and TFC to Antioxidant Activities (DPPH, ABTS, and FRAP) for *B. acmella* Extracts

The relationship between phenolic content (TPC and TFC) in *B. acmella* leaves extracts, and antioxidant activities (DPPH, ABTS, and FRAP scavenging activities) were analyzed by determining the Pearson's correlations between them.

Results showed a significant positive correlation between TPC, TFC and antioxidant activities (FRAP, ABTS), while a significant negative correlation between TPC, TFC and DPPH activity of *B. acmella*.

A high negative correlation between TFC ($R = -0.82$) in *Prunus persica* L (Mokrani and Madani, 2016), TPC ($R = -0.791$) in *Dittrichia viscosa* L. (Mssillou et al., 2021) and DPPH scavenging activity revealed that the lower TFC and TPC in plant extracts contributed to the higher DPPH radical scavenging activity. The present findings are in line with previous research showing negative correlations between TPC, TFC and DPPH scavenging activity in *B. acmella* extracts. TPC and TFC contribute to the high negative correlation ($R = -0.869$, $R = -0.995$), which indicates that the lower the amount of TFC and TPC in *B. acmella* extracts, the lower the IC_{50} value, thus a better antioxidant potential. These results suggest that other compounds than phenolics and flavonoids play a key role in the inhibiting and trapping DPPH free radicals.

A high positive significant correlation was observed between TFC and antioxidant activity for ABTS (R = 0.718) and FRAP (R = 0.993) assays. This result corroborates the reports from previous studies. *Ginkgo biloba* extract showed positive correlations (R = 0.614) between TFC to FRAP activity (Sati et al., 2013) and *Morinda citrifolia* extract (R = 0.810) between TFC to ABTS activity (Thoo et al., 2010). Meanwhile, a high positive correlation was observed between TPC and FRAP (R = 0.881) and it was in line with a previous study in *Alphitonia philippinensis* extract (Ahmed et al., 2019). A high positive correlation indicated a high amount of TPC and TFC contributed to high FRAP value, thus a better antioxidant potential, suggesting that TPC and TFC plays a role in reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The significantly positive correlations between TPC, TFC, ABTS, and FRAP in the present study suggested that the antioxidant activity of *B. acmella* leaves extracts was proportional to the concentration of phenolic and flavonoid compounds. Thus, it can be postulated that phenolic and flavonoid compounds contributed to the antioxidant activity of *B. acmella* extracts in ABTS and FRAP assays. This is due to the involvement of a single electron transfer mechanism in these three methods (Contreras-Calderón et al., 2011).

Further analysis was carried out on the relationship between antioxidant assays and negative correlations were detected between DPPH and ABTS (R = −0.686) and DPPH and FRAP (R = −0.985) in *B. acmella* extracts. These results are consistent with previous work by Thoo et al. (Thoo et al., 2010) on the *Morinda citrifolia* extract, which showed that there was a high negative correlation (R = −0.531) between DPPH and ABTS assays. The high negative correlation (R = −0.85) between DPPH and FRAP is in line with the findings of a study on *Prunus persica* L. extract (Mokrani and Madani, 2016). The current study indicated that some bioactive compounds in *B. acmella* have radical scavenging activity with ABTS and FRAP but not with DPPH assays.

In conclusion, phytochemicals will dissolve in the solvents depending on the polarity of solvents. The types of phytochemicals present in *B. acmella* extracts contributed to the activity in different antioxidant assays. High phenolic and flavonoid content in *B. acmella* contributed to the high antioxidant activity of ABTS and FRAP assays. Other compounds than phenolics and flavonoids contributed to the high antioxidant activity of the DPPH assay. Therefore, these finding results showed, the combination of group in plant secondary metabolites were contributes to the antioxidant activity in *B. acmella* extracts.

Proliferation and Differentiation Activity of *B. acmella* Leaves Extracts on MC3T3-E1 Osteoblast Cells

Anabolic therapy is one of the approaches currently used to increase bone differentiation and bone formation in bone diseases such as osteoporosis. Therefore, a study was conducted on MC3T3-E1 osteoblast cells to determine if *B. acmella* leaves extracts have bone anabolic effects. Osteoblast cell growth is characterized by proliferation, differentiation, and mineralization of the matrix. MC3T3-E1 cell line was used in this study as a model to evaluate the effect of *B. acmella* leaves extracts on bone formation. These widely used cells differentiate into osteoblast-like cells and further develop into mature osteoblasts (Kim et al., 2014).

The MTT colorimetric assay is a widely used method to assess cellular metabolic activity in cell proliferation analysis. The principle is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by NAD(P)H-cellular oxidoreductase enzymes to insoluble formazan for the determination of viable cell number. Our results showed that *B. acmella* extracts could promote osteoblast proliferation, differentiation, and mineralization at appropriate concentrations of extracts. **Figure 2B** and **Figure 3** showed that *Ba*E induced significantly higher cell proliferation compared to the control ($p < 0.05$) at the concentrations of 2.93 and 23.44 μg/ml on day 1. As for *Ba*A in **Figure 2A**, there were significantly higher cell proliferation than the control ($p < 0.05$) at the concentrations of 46.88 and 93.75 μg/ml on day 5, and concentrations of 5.8 μg/ml and 11.72 μg/ml on day 1. When compared, *Ba*E induced higher cell proliferation at lower concentrations and earlier days compared to *Ba*A. Therefore, concentrations of 2.93 μg/ml and 23.44 μg/ml for *Ba*E and 5.86 μg/ml and 11.72 μg/ml for *Ba*A were selected for further evaluation of the differentiation and mineralization activities of MC3T3-E1 cells.

Alkaline phosphatase (ALP) and collagen (COL1) are early osteogenic markers in matrix maturation and essential enzymes for osteoblast differentiation (Wu et al., 2017). Bone ALP, a glycoprotein found on osteoblast surfaces, reflects the biosynthetic activity of bone formation (Hyun et al., 2014; Wang et al., 2015; Luo et al., 2018). ALP induces and promotes mineralization in MC3T3-E1 cells after the formation of confluent monolayers (Thu et al., 2017). It is released into the osteoid to initiate minerals deposition (Lee and Choi, 2006). Mineral depositions indicate bone formation activity by deposition of calcium in the bone nodules (Ahmad et al., 2018). As osteoblasts differentiation is severely compromised in osteoporosis (Zhang et al., 2018), the promotion of osteoblast differentiation is an effective strategy to prevent pathological bone loss.

The present findings showed that the proliferation (141.32%) and ALP activity (66.91 nmol/well pNPP) were highest at *Ba*E concentration of 2.93 μg/ml while collagen percentage (196.81%) was highest at *Ba*E concentration of 23.44 μg/ml. No significant difference ($p > 0.05$) was found between the ALP activity of the two concentrations of *Ba*E 2.93 μg/ml and 23.44 μg/ml. This indicated that both extracts showed the most significant contribute to the highest ALP activity and it was correlated with collagen production. Similar findings were reported in a previous study, showing ALP activity correlation with collagen production of MC3T3-E1 cells after treatment with *Eurycoma longifolia* (Thu et al., 2017). The relationship between collagen production and ALP activity was contributed by the interactions between collagens matrix and integrin receptors (Thu et al., 2017). Results from this study suggested that treatment with *Ba*E may promote interactions between collagen matrix and

integrin receptors in MC3T3-E1 cells. Calcium deposition was detectable in MC3T3-E1 cells during the first 14 days of culture cells. The highest mineralization was found in BaE_23.44 (208.39%) and BaE_2.93 (204.57%) with no significant different ($p > 0.05$) was found between them.

In the present finding, ALP activity in *Ba*E was more than 200% higher than control on day 14. This ALP activity was higher compared to that of *B. acmella* leaves of Widyowati (2011), which was 169% higher than control. The different solvent polarities used during extraction may have contributed to different phytochemicals being extracted. According to Lamien-Meda et al. (2010) (Lamien-Meda et al., 2010), different locations, climatic conditions, altitudes, and temperatures exposed by the plants may also influence the synthesis of phytochemicals. Thus, the different ALP activity in the present findings compared to Widyowati (2011) may be due to the different solvent extraction used and the different location or environment of the *B. acmella* plantation. These factors may contribute to phytochemical variation and abundance in *B. acmella* plant.

In summary, both *B. acmella* extracts contributed to the early and late differentiation activity of MC3T3-E1 cells. Ethanol extract (*Ba*E) produced higher osteoblast differentiation compared to aqueous extract (*Ba*A). The highest collagen content were observed at the *Ba*E concentrations of 23.44 μg/ml, respectively. The highest ALP activity and mineral depositions were observed at the *Ba*E concentrations of 2.93 μg/ml and 23.44 μg/ml, respectively with no significant different between both concentrations. Thus, the findings from this study indicated that both *Ba*E concentrations promoted higher cell proliferation, differentiation, and mineralization than *Ba*A. It can be deduced that *Ba*E could promote osteoblast cell proliferation, differentiation, and mineralization for new bone formation. Further study is needed to isolate the chemical compounds present in the most competent solvent extract (*Ba*E) that might have contributed to the antioxidant and bone anabolic activities.

Relationship Between TPC and TFC to Anabolic Activities (Collagen, ALP and Mineralization) of *B. acmella* Extracts

The relationship between phenolic content (TPC, TFC) and antioxidant activities (DPPH, FRAP, ABTS) in *B. acmella* leaves extracts and anabolic activities (Collagen, ALP and Mineralization) were analyzed by determining the Pearson's correlations between them.

A high significant positive correlation was found between TFC and FRAP assay to cell proliferation, collagen, ALP and mineralization activity, between TPC to ALP and mineralization activity, and ABTS assay to collagen and mineralization activity. A moderate significant positive correlation was found between TPC to cell proliferation and collagen activity and ABTS assay to cell proliferation and ALP activity. Meanwhile, a highly significant negative correlation was found between DPPH to cell proliferation, collagen, ALP and mineralization activity. The findings from this study were supported by previous studies. Flavonoid compounds of rutin isolated from *Chrozophora tinctoria* (Abdel-Naim et al., 2018) and phenolic compounds of paradol isolated from *Aframomum meleguta* enhances bone cells proliferation and ossification (Abdel-Naim et al., 2017). A review by Torre (2017) reported that bone anabolic activity is related to the phenolic compound due to their antioxidant properties.

Phytochemical Compounds Identified With Antioxidant and Bone Anabolic Activities of *B. acmella* Leaves Ethanol Extract

GCMS and LCMS are versatile tools for separation, quantification, quantitation, and identification of unknown compounds. It provides excellent separation of compounds, fast method and is capable of producing high-quality chemical fingerprints and determination of compounds, qualitatively, and quantitatively. These could be useful for elucidating the relationship between compounds present in plants and their pharmacological effects (Liang et al., 2004; Değirmenci and Erkurt, 2020). GCMS is suitable for non-targeted metabolite profiling of volatile and thermally stable non-polar or derivatized polar metabolites as well as targeted analysis of derivatized primary metabolites. Meanwhile LCMS is suitable for non-volatile, polar or thermally labile (Tugizimana et al., 2013). Therefore, GCMS and LCTOFMS were used to determine the compounds that may contribute to the antioxidant and bone anabolic activities of *Ba*E.

Supplementary Tables S1, S2 showed the compound present in the *B. acmella*. There are new compounds detected in the present study compared to the review in previous studies (Abdul Rahim et al., 2021) and it has been identified in other plants. The difference in the phytochemical present in the present study cultivated in Malaysia compared to the previous study cultivated over the world was due to the different environmental conditions. A review by Tohidi et al. (2019) reported that the number and compound presented in *Thymus* genus were different in different locations.

GCMS analysis shows a high abundance of terpenoids in *Ba*E which contributed to the antioxidant activity confirmed the higher antioxidant activity in the ABTS compared to the DPPH assay employed in this study. This is consistent with a previous study, where *P. ecuadorense*, which is rich in sesquiterpene and monoterpene, produced better antioxidant activity in the ABTS assay (Valarezo et al., 2020). Moreover, the ABTS method has been demonstrated to be suitable for assessing lipophilic antioxidants (Andrade et al., 2017). Hence, the lower antioxidant activity in the DPPH assay in this study could also be explained by the inability of terpenoids to donate a hydrogen atom. Terpenoids have low solubility in the reaction medium of the DPPH assay, which uses ethanol as the solvent (Mata et al., 2007).

Phenolics includes flavonoids are significant plant compounds that have prominent antioxidant activity due to their hydroxyl group. The total phenolic, flavonoid, and antioxidant activity (ABTS and FRAP) and anabolic activity (cell proliferation, collagen, ALP, mineralization) of *B. acmella* extracts were significantly and positively correlated. Therefore, the present of flavonoids using LCTOFMS in *Ba*E contributed to the antioxidant and anabolic activities. The high levels of

terpenoids in GCMS analysis, which have antioxidant activity close to that of flavonoid constituents, break free-radical chain reactions, and caused their irreversible oxidation into inert compounds (Değirmenci and Erkurt, 2020).

Meanwhile, other compounds including alkylamide, fatty acid ester, alkene, alkane, coumarine, alkaloid and purine nucleosides in *Ba*E demonstrated a significantly negative correlation between the antioxidant activity (DPPH) and anabolic activity and that of the total phenolic and flavonoid content.

Natural antioxidative molecules act synergistically against free radicals (Lu and Foo, 2001; Mokrani and Madani, 2016). Therefore, the antioxidant and anabolic potential of the studied *Ba*E may be attributed to the presence of terpenoids (α-cubebene, caryophyllene, caryophyllene oxide and phytol) and flavonoids (apigenin and pinostrobin). A synergism between compounds in *Ba*E may contribute to antioxidant and anabolic activities.

CONCLUSION

In summary, this study is the first attempt to demonstrate the bone anabolic effects of *B. acmella* aqueous and ethanol exracts by the proliferation, differentiation, and mineralization activities on osteoblasts. The ethanol extract was superior to aqueous extracts, as it was believed to contain an abundance of phytochemicals with antioxidant and bone anabolic activities. This was proven by the different antioxidant activities found in DPPH, ABTS, and FRAP antioxidant assays, with positive and negative correlations. The current results revealed that terpenoids, alkyl amides, fatty acid esters, alkene, alkane, coumarin, alkaloid and purine nucleosides in *Ba*E were considered to contribute to antioxidant activities in *Ba*E and bone anabolic effects of the *Ba*E extract. Among the identified compounds in *Ba*E, terpenoids of α-cubebene, caryophyllene, caryophyllene oxide, phytol and flavonoids of pinostrobin and apigenin are the compounds that may contribute to both antioxidant and anabolic activities.

Therefore, the current results showed that *Ba*E leaves have the potential to be developed further as antioxidant and anti-osteoporosis agents. Further studies need to be performed to isolate, characterized, and elucidate the specific compounds and underlying mechanisms responsible for these antioxidant and anabolic activities.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

RR performed the whole research. VL, NA, ZA, SM, FA, NMu, NMo, IN, AS provided their professional expertise. PJ, IN, AS provided their technical support. RR, IN, AS supervised the whole project. All authors have approved the content of the submitted manuscript.

FUNDING

This research was funded by Dana Impak Perdana UKM (DIP-2018-015) and Dana Fundamental PPUKM (FF-2019-236) from Universiti Kebangsaan Malaysia (UKM).

ACKNOWLEDGMENTS

The authors would like to thank the Pharmacology Department, Faculty of Medicine, Universiti Kebangsaan Malaysia and Advanced Medical and Dental Institute, Universiti Sains Malaysia by providing the facilities and chemicals and also staffs of the Pharmacology Department, Faculty of Medicine, Universiti Kebangsaan Malaysia and Advanced Medical and Dental Institute, Universiti Sains Malaysia for all their contributions in this study. I would like to thank to Universiti Sains Malaysia and Ministry of Higher Education, Malaysia for the scholarship given.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.796509/full#supplementary-material

REFERENCES

Abdel-Naim, A. B., Alghamdi, A. A., Algandaby, M. M., Al-Abbasi, F. A., Al-Abd, A. M., Abdallah, H. M., et al. (2017). Phenolics Isolated From Aframomum Meleguta Enhance Proliferation and Ossification Markers in Bone Cells. *Molecules.* 22 (9), 1467. doi:10.3390/molecules22091467

Abdel-Naim, A. B., Alghamdi, A. A., Algandaby, M. M., Al-Abbasi, F. A., Al-Abd, A. M., Eid, B. G., et al. (2018). Rutin Isolated From Chrozophora Tinctoria Enhances Bone Cell Proliferation and Ossification Markers. *Oxidative Med. Cell Longevity.* 2018, 1–10. doi:10.1155/2018/5106469

Abdul Rahim, R., Ahmad, N. H., Al Azzam, K. M., Mat, I., and Mat, I. (2018). Determination and Quantification of the Vinblastine Content in Purple, Red, and White Catharanthus Roseus Leaves Using RP-HPLC Method. *Adv. Pharm. Bull.* 8 (1), 157–161. doi:10.15171/apb.2018.201910.15171/apb.2018.019

Abdul Rahim, R., Jayusman, P. A., Muhammad, N., Mohamed, N., Lim, V., Ahmad, N. H., et al. (2021). Potential Antioxidant and Anti-Inflammatory Effects of Spilanthes Acmella and its Health Beneficial Effects: A Review. *Int. J. Environ. Res. Public Health.* 18 (7), 3532. doi:10.3390/ijerph18073532

Abeysiri, G. R. P. I., Dharmadasa, R. M., Abeysinghe, D. C., and Samarasinghe, K. (2013). Screening of Phytochemical, Physico-Chemical and Bioactivity of Different Parts of Acmella Oleraceae Murr. (Asteraceae), a Natural Remedy for Toothache. *Ind. Crops Prod.* 50, 852–856. doi:10.1016/j.indcrop.2013.08.043

Ahmad, H., Jamal, J. A., Aladdin, N. A., Husain, K., Mohd Sofi, N. S., Mohamed, N., et al. (2018). Demethylbelamcandaquinone B (Dmcq B) Is the Active Compound of *Marantodes Pumilum* Var. Alata (Blume) Kuntze With Osteoanabolic Activities. *Molecules.* 23 (7), 1–19. doi:10.3390/molecules23071686

Ahmed, J., Salim, K. A., Lim, L. B. L., and Jama, A. M. (2019). Evaluation of Antioxidant Activity and Phytochemical Screening of Leaves, Barks, Stems and Fruits of Alphitonia Philippinensis (Rhamnaceae) from Brunei Darussalam. *Pharmacognosy J.* 11 (5), 951–961. doi:10.5530/pj.2019.11.151

Ammar, R. B., Bhouri, W., Sghaier, M. B., Boubaker, J., Skandrani, I., Neffati, A., et al. (2009). Antioxidant and Free Radical-Scavenging Properties of Three

Flavonoids Isolated from the Leaves of *Rhamnus Alaternus* L. (Rhamnaceae) : A Structure-Activity Relationship Study. *Food Chem.* 116 (1), 258–264. doi:10.1016/j.foodchem.2009.02.043

Andrade, K. S., Poncelet, D., and Ferreira, S. R. S. (2017). Sustainable Extraction and Encapsulation of Pink Pepper Oil. *J. Food Eng.* 204, 38–45. doi:10.1016/j.jfoodeng.2017.02.020

Anholeto, L. A., Oliveira, P. R., Rodrigues, R. A. F., Spindolados, C. D. S. S., Labruna, M. B., Pizano, M. A., et al. (2017). Potential Action of Extract of *Acmella* Oleracea (L.) R.K. Jansen to Control Amblyomma Cajennense (Fabricius, 1787) (Acari: Ixodidae) Ticks. *Ticks Tick Borne Dis.* 8 (1), 65–72. doi:10.1016/j.ttbdis.2016.09.018

Azonwade, F. E., Paraïso, A., Agbangnan Dossa, C. P., Dougnon, V. T., N'tcha, C., Mousse, W., et al. (2018). Physicochemical Characteristics and Microbiological Quality of Honey Produced in Benin. *J. Food Qual.* 2018, 1–13. doi:10.1155/2018/1896057

Baba, S. A., and Malik, S. A. (2015). Determination of Total Phenolic and Flavonoid Content, Antimicrobial and Antioxidant Activity of a Root Extract of Arisaema Jacquemontii Blume. *J. Taibah Univ. Sci.* 9 (4), 449–454. doi:10.1016/j.jtusci.2014.11.001

Bakondi, E., Singh, S. B., Hajnády, Z., Nagy-Pénzes, M., Regdon, Z., Kovács, K., et al. (2019). Spilanthol Inhibits Inflammatory Transcription Factors and iNOS Expression in Macrophages and Exerts Anti-Inflammatory Effects in Dermatitis and Pancreatitis. *Int. J. Mol. Sci.* 20 (17), 1–18. doi:10.3390/ijms20174308

Barrientos, R., Fernández-Galleguillos, C., Pastene, E., Simirgiotis, M., Romero-Parra, J., Ahmed, S., et al. (2020). Metabolomic Analysis, Fast Isolation of Phenolic Compounds, and Evaluation of Biological Activities of the Bark From *Weinmannia Trichosperma* Cav. (Cunoniaceae). *Front. Pharmacol.* 11 (May), 780–813. doi:10.3389/fphar.2020.00780

Ben Yakoub, A. R., Abdehedi, O., Jridi, M., Elfalleh, W., Nasri, M., and Ferchichi, A. (2018). Flavonoids, Phenols, Antioxidant, and Antimicrobial Activities in Various Extracts from Tossa Jute Leave (*Corchorus Olitorus* L.). *Ind. Crops Prod.* 118, 206–213. doi:10.1016/j.indcrop.2018.03.047

Benzie, I. F. F., and Devaki, M. (2017). The Ferric Reducing/antioxidant Power (FRAP) Assay for Non-Enzymatic Antioxidant Capacity: Concepts, Procedures, Limitations and Applications. *Meas. Antioxidant Activity Capacity: Recent Trends Appl.*. John, Wiley and Sons, Inc., 77–106. doi:10.1002/9781119135388.ch5

Bibi Sadeer, N., Montesano, D., Albrizio, S., Zengin, G., and Mahomoodally, M. F. (2020). The Versatility of Antioxidant Assays in Food Science and Safety-Chemistry, Applications, Strengths, and Limitations. *Antioxidants.* 9 (8), 709–739. doi:10.3390/antiox9080709

Bodeker, G., Ong, C. K., Burford, G., and Shein, K. (2005). *WHO Global Atlas of Traditional, Complementary and Alternative Medicine*. Kobe, Japan: World Health Organization.

Chandra, S., Khan, S., Avula, B., Lata, H., Yang, M. H., Elsohly, M. A., et al. (2014). Assessment of Total Phenolic and Flavonoid Content, Antioxidant Properties, and Yield of Aeroponically and Conventionally Grown Leafy Vegetables and Fruit Crops: A Comparative Study. *Evid. Based Complement. Alternat Med.* 2014, 253875–253879. doi:10.1155/2014/253875

Clynes, M. A., Harvey, N. C., Curtis, E. M., Fuggle, N. R., Dennison, E. M., and Cooper, C. (2020). The Epidemiology of Osteoporosis. *Br. Med. Bull.* 133 (1), 105–117. doi:10.1093/bmb/ldaa005

Contreras-Calderón, J., Calderón-Jaimes, L., Guerra-Hernández, E., and García-Villanova, B. (2011). Antioxidant Capacity, Phenolic Content and Vitamin C in Pulp, Peel and Seed from 24 Exotic Fruits from Colombia. *Food Res. Int.* 44 (7), 2047–2053. doi:10.1016/j.foodres.2010.11.003

Corrado, A., Sanpaolo, E. R., Di Bello, S., and Cantatore, F. P. (2017). Osteoblast as a Target of Anti-Osteoporotic Treatment. *Postgrad. Med.* 129 (8), 858–865. doi:10.1080/00325481.2017.1362312

Değirmenci, H., and Erkurt, H. (2020). Relationship Between Volatile Components, Antimicrobial and Antioxidant Properties of the Essential Oil, Hydrosol and Extracts of Citrus Aurantium L. Flowers. *J. Infect. Public Health.* 13 (1), 58–67. doi:10.1016/j.jiph.2019.06.017

Dhanani, T., Shah, S., Gajbhiye, N. A., and Kumar, S. (2017). Effect of Extraction Methods on Yield, Phytochemical Constituents and Antioxidant Activity of *Withania Somnifera*. *Arabian J. Chem.* 10, S1193–S1199. doi:10.1016/j.arabjc.2013.02.015

Diniyah, N., Badrul Alam, M., and Lee, S.-H. (2020). Antioxidant Potential of Non-oil Seed Legumes of Indonesian's Ethnobotanical Extracts. *Arabian J. Chem.* 13 (5), 5208–5217. doi:10.1016/j.arabjc.2020.02.019

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., HuynhHuong, L. H., Soetaredjo, F. E., Ismadji, S., et al. (2014). Effect of Extraction Solvent on Total Phenol Content, Total Flavonoid Content, and Antioxidant Activity of Limnophila Aromatica. *J. Food Drug Anal.* 22 (3), 296–302. doi:10.1016/j.jfda.2013.11.001

Domazetovic, V., Marcucci, G., Iantomasi, T., Brandi, M. L., and Vincenzini, M. T. (2017). Oxidative Stress in Bone Remodeling: Role of Antioxidants. *Clin. Cases Miner Bone Metab.* 14 (2), 209–216. doi:10.11138/ccmbm/2017.14.2.20910.11138/ccmbm/2017.14.1.209

Dubey, S., Maity, S., Singh, M., Saraf, S. A., and Saha, S. (2013). Phytochemistry, Pharmacology and Toxicology of *Spilanthes Acmella*: A Review. *Adv. Pharmacol. Sci.* 2013, 423750–423759. doi:10.1155/2013/423750

Đudarić, L., Fužinac-Smojver, A., Muhvić, D., and Giacometti, J. (2015). The Role of Polyphenols on Bone Metabolism in Osteoporosis. *Food Res. Int.* 77, 290–298. doi:10.1016/j.foodres.2015.10.017

Dudonné, S., Vitrac, X., Coutière, P., Woillez, M., and Mérillon, J. M. (2009). Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *J. Agric. Food Chem.* 57, 1768–1774. doi:10.1021/jf803011r

Esmaeili, M. A., and Sonboli, A. (2010). Antioxidant, Free Radical Scavenging Activities of *Salvia Brachyantha* and its Protective Effect Against Oxidative Cardiac Cell Injury. *Food Chem. Toxicol.* 48 (3), 846–853. doi:10.1016/j.fct.2009.12.020

El-Din, S. M., and El-ahwany, A. M. D. (2016). Bioactivity and Phytochemical Constituents of marine Red Seaweeds (Jania rubens,Corallina mediterraneaandPterocladia Capillacea). *J. Taibah Univ. Sci.* 10 (4), 471–484. doi:10.1016/j.jtusci.2015.06.004

Floegel, A., Kim, D.-O., Chung, S.-J., Koo, S. I., and Chun, O. K. (2011). Comparison of ABTS/DPPH Assays to Measure Antioxidant Capacity in Popular Antioxidant-Rich US Foods. *J. Food Compost. Anal.* 24 (7), 1043–1048. doi:10.1016/j.jfca.2011.01.008

Frediani, B., and Bertoldi, I. (2015). Clodronate: New Directions of Use. *Clin. Cases Miner Bone Metab.* 12 (2), 97–108. doi:10.11138/ccmbm/2015.12.2.097

Gavamukulya, Y., Abou-Elella, F., Wamunyokoli, F., and AEl-Shemy, H. (2014). Phytochemical Screening, Anti-oxidant Activity and *In Vitro* Anticancer Potential of Ethanolic and Water Leaves Extracts of Annona Muricata (Graviola). *Asian Pac. J. Trop. Med.* 7S1 (S1), S355–S363. doi:10.1016/S1995-7645(14)60258-3

Ghasemzadeh, A., Jaafar, H. Z. E., Bukhori, M. F. M., Rahmat, M. H., and Rahmat, A. (2018). Assessment and Comparison of Phytochemical Constituents and Biological Activities of Bitter Bean (*Parkia Speciosa* Hassk.) Collected from Different Locations in Malaysia. *Chem. Cent. J.* 12 (1), 12–19. doi:10.1186/s13065-018-0377-6

Grąbkowska, R., Matkowski, A., Grzegorczyk-Karolak, I., and Wysokińska, H. (2016). Callus Cultures of *Harpagophytum Procumbens* (Burch.) DC. Ex Meisn.; Production of Secondary Metabolites and Antioxidant Activity. *South Afr. J. Bot.* 103, 41–48. doi:10.1016/j.sajb.2015.08.012

Grzegorczyk-Karolak, I., and Kiss, A. K. (2018). Determination of the Phenolic Profile and Antioxidant Properties of *Salvia Viridis* L. Shoots: A Comparison of Aqueous and Hydroethanolic Extracts. *Molecules.* 23 (6), 1468. doi:10.3390/molecules23061468

Hayatullina, Z., Mohamed, N., Soelaiman, I.-N., and Muhammad, N. (2018). Kesan Polifenol Minyak Kelapa Dara Ke Atas Aktiviti Enzim Antioksidan Dan Lipid Peroksida Pada Sel Osteoblas. *Sains Malaysiana.* 47 (10), 2401–2409. doi:10.17576/jsm-2018-4710-16

Ho, D. V., Nguyen, H. T., Vu, T. Y., Pham, T. V., and Nguyen, H. M. (2021). Antioxidant Activity of a New Xanthone Derivative from Aspidistra Letreae: *In Vitro* and In Silico Studies. *Chem. Biodivers.* 18 (4), e2001008. doi:10.1002/cbdv.202001008

Hwang, E. S., and Thi, N. D. (2014). Effects of Extraction and Processing Methods on Antioxidant Compound Contents and Radical Scavenging Activities of Laver (*Porphyra Tenera*). *Prev. Nutr. Food Sci.* 19 (1), 40–48. doi:10.3746/pnf.2014.19.1.040

Hyun, H., Park, H., Jeong, J., Kim, J., Kim, H., Oh, H. I., et al. (2014). Effects of Watercress Containing Rutin and Rutin Alone on the Proliferation and Osteogenic Differentiation of Human Osteoblast-Like Mg-63 Cells. *Korean J. Physiol. Pharmacol.* 18 (4), 347–352. doi:10.4196/kjpp.2014.18.4.347

Iloki-Assanga, S. B., Lewis-Luján, L. M., Lara-Espinoza, C. L., Gil-Salido, A. A., Fernandez-Angulo, D., Rubio-Pino, J. L., et al. (2015). Solvent Effects on Phytochemical Constituent Profiles and Antioxidant Activities, Using Four Different Extraction Formulations for Analysis of *Bucida Buceras* L. And *Phoradendron Californicum*. *BMC Res. Notes.* 8 (1), 396–414. doi:10.1186/s13104-015-1388-1

Ilyasov, I. R., Beloborodov, V. L., Selivanova, I. A., and Terekhov, R. P. (2020). ABTS/PP Decolorization Assay of Antioxidant Capacity Reaction Pathways. *Int. J. Mol. Sci.* 21 (3), 1131. doi:10.3390/ijms21031131

Ismail, B. B., Pu, Y., Guo, M., Ma, X., and Liu, D. (2019). LC-MS/QTOF Identification of Phytochemicals and the Effects of Solvents on Phenolic Constituents and Antioxidant Activity of Baobab (*Adansonia Digitata*) Fruit Pulp. *Food Chem.* 277 (April 2018), 279–288. doi:10.1016/j.foodchem.2018.10.056

Itoh, F., Aoyagi, S., Furihata-Komatsu, H., Aoki, M., Kusama, H., Kojima, M., et al. (2003). Clodronate Stimulates Osteoblast Differentiation in ST2 and MC3T3-E1 Cells and Rat Organ Cultures. *Eur. J. Pharmacol.* 477 (1), 9–16. doi:10.1016/j.ejphar.2003.08.011

Kenkre, J. S., and Bassett, J. (2018). The Bone Remodelling Cycle. *Ann. Clin. Biochem.* 55 (3), 308–327. doi:10.1177/0004563218759371

Khosla, S., and Hofbauer, L. C. (2017). Osteoporosis Treatment: Recent Developments and Ongoing Challenges. *Lancet Diabetes Endocrinol.* 5 (11), 898–907. doi:10.1016/S2213-8587(17)30188-2

Kim, M., Song, Y., and Hwang, J.-K. (2014). Kirenol Stimulates Osteoblast Differentiation Through Activation of the BMP and Wnt/β-Catenin Signaling Pathways in MC3T3-E1 Cells. *Fitoterapia.* 98, 59–65. doi:10.1016/j.fitote.2014.07.013

Kim, S. H., Kim, K. J., Kang, H. J., Son, Y. J., Choi, S. W., and Lee, M. J. (2018). The Dual Role of Oat Bran Water Extract in Bone Homeostasis Through the Regulation of Osteoclastogenesis and Osteoblast Differentiation. *Molecules.* 23 (12),23. doi:10.3390/molecules23123119

Koch, F. P., Merkel, C., Al-Nawas, B., Smeets, R., Ziebart, T., Walter, C., et al. (2011). Zoledronate, Ibandronate and Clodronate Enhance Osteoblast Differentiation in a Dose Dependent Manner-Aa Quantitative *In Vitro* Gene Expression Analysis of Dlx5, Runx2, OCN, MSX1 and MSX2. *J. Craniomaxillofac. Surg.* 39 (8), 562–569. doi:10.1016/j.jcms.2010.10.007

Kumar, K. S., and Ravindra, N. (2018). *Spilanthes Acmella*: Pharmacognostic and Phytochemical Studies. *J. Pharm. Adv. Res.* 1 (7), 329–334.

Lamien-Meda, A., Nell, M., Lohwasser, U., Börner, A., Franz, C., Novak, J., et al. (2010). Investigation of Antioxidant and Rosmarinic Acid Variation in the Sage Collection of the Genebank in Gatersleben. *J. Agric. Food Chem.* 58 (6), 3813–3819. doi:10.1021/jf903993f

Laswati, H., Agik, M., and Widyowati, R. (2015). Effect of *Spilanthes Acmella* and Exercise on Osteoblast Cells Femur in Mice Dexamethasone Induced. *Medit Litbangkes.* 25 (1), 43–50. doi:10.22435/mpk.v25i1.4095.43-50

Lee, K. H., and Choi, E. M. (2006). *Rubus Coreanus* Miq. Extract Promotes Osteoblast Differentiation and Inhibits Bone-Resorbing Mediators in MC3T3-E1 Cells. *Am. J. Chin. Med.* 34 (4), 643–654. doi:10.1142/S0192415X0600417X

Liang, Y. Z., Xie, P., and Chan, K. (2004). Quality Control of Herbal Medicines. *J. Chromatogr. B Analyt Technol. Biomed. Life Sci.* 812, 53–70. doi:10.1016/j.jchromb.2004.08.041

Lu, Y., and Yeap Foo, L. (2001). Antioxidant Activities of Polyphenols from Sage (Salvia officinalis)fficinalis).Pdf. *Food Chem.* 75, 197–202. doi:10.1016/s0308-8146(01)00198-4

Luo, X., Cui, J., Zhang, H., Duan, Y., Zhang, D., Cai, M., et al. (2018). Ultrasound Assisted Extraction of Polyphenolic Compounds from Red Sorghum (Sorghum Bicolor L.) Bran and Their Biological Activities and Polyphenolic Compositions. *Ind. Crops Prod.* 112, 296–304. doi:10.1016/j.indcrop.2017.12.019

Manimaran, P., Sanjay, M. R., Senthamaraikannan, P., Saravanakumar, S. S., Siengchin, S., Pitchayyapillai, G., et al. (2019). Physico-Chemical Properties of Fiber Extracted from the Flower of Celosia Argentea Plant. *J. Nat. Fibers.* 18 (00), 464–473. doi:10.1080/15440478.2019.1629149

Manssouri, M., Znini, M., and Majidi, L. (2020). Studies on the Antioxidant Activity of Essential Oil and Various Extracts of Ammodaucus Leucotrichus Coss. & Dur. Fruits from Morocco. *J. Taibah Univ. Sci.* 14 (1), 124–130. doi:10.1080/16583655.2019.1710394

Martiniakova, M., Babikova, M., and Omelka, R. (2020). Pharmacological Agents and Natural Compounds: Available Treatments for Osteoporosis. *J. Physiol. Pharmacol.* 71 (3), 1–14. doi:10.26402/jpp.2020.3.01

Maruotti, N., Corrado, A., Neve, A., and Cantatore, F. P. (2012). Bisphosphonates: Effects on Osteoblast. *Eur. J. Clin. Pharmacol.* 68 (7), 1013–1018. doi:10.1007/s00228-012-1216-7

Mata, A. T., Proença, C., Ferreira, A. R., Serralheiro, M. L. M., Nogueira, J. M. F., and Araújo, M. E. M. (2007). Antioxidant and Antiacetylcholinesterase Activities of Five Plants Used as Portuguese Food Spices. *Food Chem.* 103 (3), 778–786. doi:10.1016/j.foodchem.2006.09.017

Mokrani, A., and Madani, K. (2016). Effect of Solvent, Time and Temperature on the Extraction of Phenolic Compounds and Antioxidant Capacity of Peach (Prunus Persica L.) Fruit. *Separation Purif. Technology.* 162, 68–76. doi:10.1016/j.seppur.2016.01.043

Moreno, M. A., Zampini, I. C., and Isla, M. I. (2020). Antifungal, Anti-inflammatory and Antioxidant Activity of Bi-Herbal Mixtures With Medicinal Plants From Argentinean highlands. *J. Ethnopharmacol.* 253, 112642. doi:10.1016/j.jep.2020.112642

Mssillou, I., Agour, A., Slighoua, M., Tourabi, M., Nouioura, G., Lyoussi, B., et al. (2021). Phytochemical Characterization, Antioxidant Activity, and *In Vitro* Investigation of Antimicrobial Potential of Dittrichia Viscosa L. Leaf Extracts against Nosocomial Infections. *Acta Ecologica Sinica.* (August). doi:10.1016/j.chnaes.2021.09.021

Mzid, M., Regaieg, W., and Rebai, T. (2017). Antioxidant and Antimicrobial Activities of Ethanol and Aqueous Extracts from *Urtica Urens*. *Pharm. Biol.* 55 (1), 775–781. doi:10.1080/13880209.2016.1275025

Nakatani, N., and Nagashima, M. (1992). Pungent Alkamides from *Spilanthes Acmella* L. Var. Oleracea Clarke. *Biosci. Biotechnol. Biochem.* 56 (5), 759–762. doi:10.1271/bbb.56.759

Nardi, A., Ventura, L., Cozzi, L., and Tonini, G. (2016). Clodronate News of Efficacy in Osteoporosis. *Clin. Cases Miner Bone Metab.* 13 (1), 33–35. doi:10.11138/ccmbm/2016.13.1.033

Nipornram, S., Tochampa, W., Rattanatraiwong, P., and Singanusong, R. (2018). Optimization of Low Power Ultrasound-Assisted Extraction of Phenolic Compounds from Mandarin (*Citrus Reticulata* Blanco Cv. Sainampueng) Peel. *Food Chem.* 241, 338–345. doi:10.1016/j.foodchem.2017.08.114

Nobossé, P., Fombang, E. N., and Mbofung, C. M. F. (2018). Effects of Age and Extraction Solvent on Phytochemical Content and Antioxidant Activity of Fresh *Moringa Oleifera* L. Leaves. *Food Sci. Nutr.* 6 (8), 2188–2198. doi:10.1002/fsn3.783

Ong, H. C., and Nordiana, M. (1999). Malay Ethno-Medico Botany in Machang, Kelantan, Malaysia. *Fitoterapia* 70 (5), 502–513. doi:10.1016/S0367-326X(99)00077-5

Opitz, S. E. W., Smrke, S., Goodman, B. A., and Yeretzian, C. (2014). Methodology for the Measurement of Antioxidant Capacity of Coffee: A Validated Platform Composed of Three Complementary Antioxidant Assays. In*Processing and Impact on Antioxidants in Beverages* (Elsevier). doi:10.1016/B978-0-12-404738-9.00026-X

Otles, S., and Yalcin, B. (2012). Phenolic Compounds Analysis of Root, Stalk, and Leaves of Nettle. *Scientific World J.* 2012, 1–12. doi:10.1100/2012/564367

Owen, T. A., Aronow, M., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M. S., et al. (1990). Progressive Development of the Rat Osteoblast Phenotype *In Vitro*: Reciprocal Relationships in Expression of Genes Associated with Osteoblast Proliferation and Differentiation During Formation of the Bone Extracellular Matrix. *J. Cell Physiol.* 143 (3), 420–430. doi:10.1002/jcp.1041430304

Özyürek, M., Güçlü, K., and Apak, R. (2011). The Main and Modified CUPRAC Methods of Antioxidant Measurement. *Trac Trends Anal. Chem.* 30 (4), 652–664. doi:10.1016/j.trac.2010.11.016

Paranagama, M. P., Piyarathne, N. S., Nandasena, T. L., Jayatilake, S., Navaratne, A., Galhena, B. P., et al. (2020). The Porphyromonas Gingivalis Inhibitory Effects, Antioxidant Effects and the Safety of a Sri Lankan Traditional Betel Quid - an *In Vitro* Study. *BMC Complement. Med. Ther.* 20 (1), 259–312. doi:10.1186/s12906-020-03048-6

Pherwani, P. U., and Sathaye, S. (2019). Treatment of Osteoporosis: Current Scenario from a Research Perspective. *Indian J. Pharm. Educ. Res.* 54 (1), 8–16. doi:10.5530/ijper.54.1.2

Piątczak, E., Grzegorczyk-Karolak, I., and Wysokińska, H. (2014). Micropropagation of *Rehmannia Glutinosa* Libosch.: Production of Phenolics and Flavonoids and Evaluation of Antioxidant Activity. *Acta Physiol. Plant.* 36 (7), 1693–1702. doi:10.1007/s11738-014-1544-6

Prachayasittikul, S., Suphapong, S., Worachartcheewan, A., Lawung, R., Ruchirawat, S., Prachayasittikul, V., et al. (2009). Bioactive Metabolites from *Spilanthes Acmella* Murr. *Molecules.* 14 (2), 850–867. doi:10.3390/molecules14020850

Prior, R. L., Wu, X., and Schaich, K. (2005). Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *J. Agric. Food Chem.* 53, 4290–4302. doi:10.1021/jf0502698

Quarles, L. D., Yohay, D. A., Lever, L. W., Caton, R., and Wenstrup, R. J. (1992). Distinct Proliferative and Differentiated Stages of Murine MC3T3-E1 Cells in Culture: an *In Vitro* Model of Osteoblast Development. *J. Bone Miner Res.* 7 (6), 683–692. doi:10.1002/jbmr.5650070613

Robinson, M. M., and Zhang, X. (2011). *The World Medicines Situation 2011. Traditional Medicines: Global Situation, Issues and Challenges*. Geneva, Switzerland: World Health Organization.

Samsurrijal, S. F., Rahim, R. A., Azizan, N. S., Noor, S. N. F. M., and Vuanghao, L. (2019). Enhancement of Dental Pulp Stem Cell Viability Using *Acmella Oleraceae* Extract Combined with Bioactive Glass. *J. Biomed. Clin. Sci. (Jbcs).* 4 (1), 34–43.

Sati, P., Pandey, A., Rawat, S., and Rani, A. (2013). Phytochemicals and Antioxidants in Leaf Extracts of *Ginkgo Biloba* with Reference to Location, Seasonal Variation and Solvent System. *J. Pharm. Res.* 7 (9), 804–809. doi:10.1016/j.jopr.2013.09.001

Sharma, R., and Arumugam, N. (2021). N-alkylamides of Spilanthes (Syn: Acmella): Structure, Purification, Characterization, Biological Activities and Applications - a Review. *Future Foods.* 3 (February), 100022. doi:10.1016/j.fufo.2021.100022

Sharma, V., Boonen, J., Chauhan, N. S., Thakur, M., De Spiegeleer, B., and Dixit, V. K. (2011). Spilanthes Acmella Ethanolic Flower Extract: LC-MS Alkylamide Profiling and its Effects on Sexual Behavior in Male Rats. *Phytomedicine.* 18 (13), 1161–1169. doi:10.1016/j.phymed.2011.06.001

Spelman, K., Depoix, D., McCray, M., and Grellier, P. (2011). The Traditional Medicine Spilanthes Acmella, and the Alkylamides Spilanthol and Undeca-2e-Ene-8,10-Diynoic Acid Isobutylamide, Demonstrate *In Vitro* and *In Vivo* Antimalarial Activity. *Phytother Res.* 25 (7), 1098–1101. doi:10.1002/ptr.3395

Sridhar, K., and Charles, A. L. (2019). *In Vitro* antioxidant Activity of Kyoho Grape Extracts in DPPH and ABTS Assays: Estimation Methods for EC50 Using Advanced Statistical Programs. *Food Chem.* 275, 41–49. doi:10.1016/j.foodchem.2018.09.040

Stein, G. S., and Lian, J. B. (1993). Molecular Mechanisms Mediating Developmental and Hormone-Regulated Expression of Genes in Osteoblasts. *Cell Mol. Biol. Bone*. Academic Press, Inc., 47–95. doi:10.1016/b978-0-08-092500-4.50006-1

Swallah, M. S., Sun, H., Affoh, R., Fu, H., and Yu, H. (2020). Antioxidant Potential Overviews of Secondary Metabolites (Polyphenols) in Fruits. *Int. J. Food Sci.* 2020, 1–8. doi:10.1155/2020/9081686

Tanwer, B. S., and Vijayvergia, R. (2010). Molluscicidal Activity of *Spilanthus Acemella* Murr. On the Snail *Lymnaea Acuminate*. *Pharmacognosy J.* 2 (15), 31–33. doi:10.1016/S0975-3575(10)80075-6

Tee, L. H., Yang, B., Nagendra, K. P., Ramanan, R. N., Sun, J., Chan, E. S., et al. (2014). Nutritional Compositions and Bioactivities of *Dacryodes* Species: A Review. *Food Chem.* 165, 247–255. doi:10.1016/j.foodchem.2014.05.084

Thoo, Y. Y., Ho, S. K., Liang, J. Y., Ho, C. W., and Tan, C. P. (2010). Effects of Binary Solvent Extraction System, Extraction Time and Extraction Temperature on Phenolic Antioxidants and Antioxidant Capacity from Mengkudu (*Morinda citrifolia*). *Food Chem.* 120 (1), 290–295. doi:10.1016/j.foodchem.2009.09.064

Thu, H. E., Mohamed, I. N., HussainNaina, Z., Shuid, A. N., and ShuidNazrun, A. (2017). Eurycoma Longifolia as a Potential Alternative to Testosterone for the Treatment of Osteoporosis: Exploring Time-Mannered Proliferative, Differentiative and Morphogenic Modulation in Osteoblasts. *J. Ethnopharmacol.* 195, 143–158. doi:10.1016/j.jaim.2017.04.005

Tohidi, B., Rahimmalek, M., and Trindade, H. (2019). Review on Essential Oil, Extracts Composition, Molecular and Phytochemical Properties of Thymus Species in Iran. *Ind. Crops Prod.* 134 (April), 89–99. doi:10.1016/j.indcrop.2019.02.038

Torre, E. (2017). Molecular Signaling Mechanisms behind Polyphenol-Induced Bone Anabolism. *Phytochem. Rev.* 16 (6), 1183–1226. doi:10.1007/s11101-017-9529-x

Tugizimana, F., Piater, L., and Dubery, I. (2013). Plant Metabolomics: A New Frontier in Phytochemical Analysis. *S. Afr. J. Sci.* 109 (5–6), 1–11. doi:10.1590/sajs.2013/20120005

Valarezo, E., Flores-Maza, P., Cartuche, L., Ojeda-Riascos, S., and Ramírez, J. (2020). Phytochemical Profile, Antimicrobial and Antioxidant Activities of Essential Oil Extracted from *Ecuadorian* Species *Piper Ecuadorense* Sodiro. *Nat. Prod. Res.* 0 (0), 1–6. doi:10.1080/14786419.2020.1813138

Wang, X., He, Y., Guo, B., Tsang, M. C., Tu, F., Dai, Y., et al. (2015). *In Vivo* Screening for Anti-Osteoporotic Fraction from Extract of Herbal Formula Xianlinggubao in Ovariectomized Mice. *PLoS ONE.* 10 (2), e0118184–17. doi:10.1371/journal.pone.0118184

Widyowati, R. (2011). Alkaline Phosphatase Activity of *Graptophyllum Pictum* and *Sphilanthes Acmella* Fractions Against Mc3T3 E1 Cells As Marker of Osteoblast Differentiation Cells. *Int. J. Pharm. Pharm. Sci.* 3, 1–4.

Widyowati, R., Ekasari, W., and Purwitasari, N. (2020a). Amine Derivative from the Aerial Part of Spilanthes Acmella Murr. And Their Alkaline Phosphatase Activity. *Nat. Prod. J.* 10 (5), 571–577. doi:10.2174/2210315509666190807161413

Widyowati, R., Sulistyowaty, M. I., Uyen, N. H., Sugimoto, S., Yamano, Y., Otsuka, H., et al. (2020b). New Methyl Threonolactones and Pyroglutamates of *Spilanthes Acmella* (L.) L. And Their Bone Formation Activities. *Molecules.* 25 (11), 2500. doi:10.3390/molecules25112500

Wongsawatkul, O., Prachayasittikul, S., Isarankura-Na-Ayudhya, C., Satayavivad, J., Ruchirawat, S., and Prachayasittikul, V. (2008). Vasorelaxant and Antioxidant Activities of *Spilanthes Acmella* Murr. *Int. J. Mol. Sci.* 9 (12), 2724–2744. doi:10.3390/ijms9122724

Wu, C. F., Lin, Y. S., Lee, S. C., Chen, C. Y., Wu, M. C., and Lin, J. S. (2017). Effects of *Davallia Formosana* Hayata Water and Alcohol Extracts on Osteoblastic MC3T3-E1 Cells. *Phytother Res.* 31 (9), 1349–1356. doi:10.1002/ptr.5860

Yang, D. U., Siddiqi, M. H., Ahn, S., Kang, S., Noh, H. Y., and Yang, D. C. (2018). *In Vitro* Evaluation of the Potential Therapeutic Role of Dendropanax Morbifera Extract in Ameliorating Osteoporosis and Resultant Bone Impairment Using MC3T3-E1 Cells. In Vitro *Cell Dev Biol Anim.* 54 (5), 346–354. doi:10.1007/s11626-018-0242-x

Zhang, H., Zhang, G.-P., Jiang, H., and Gong, Z.-F. (2018). (2 R ,3 S)-Pinobanksin-3-Cinnamate Promotes Osteoblast Differentiation Through cAMP and cGMP Pathways. *Revista Brasileira de Farmacognosia.* 28 (4), 468–473. doi:10.1016/j.bjp.2018.05.006

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 17 January 2022
doi: 10.3389/fphar.2021.793200

Culinary Spices in Food and Medicine: An Overview of *Syzygium aromaticum* (L.) Merr. and L. M. Perry [Myrtaceae]

Gloria Aderonke Otunola *

Medicinal Plants and Economic Development (MPED) Research Center, Department of Botany, University of Fort Hare, Alice, South Africa

Spices-dried aromatic parts of plants (leaves, seeds, bark, roots, rhizomes, buds, etc) used to enhance flavour, taste and colour (sensory quality) of foods, are increasingly finding other useful roles in healthcare beyond their primary use as culinary organoleptic enhancers. Several spices are currently being investigated for their potential health benefits, because of the failing efficacy, toxicity and high cost associated with conventional drugs. One such spice: *Syzygium aromaticum* (L.) Merr. and L.M.Perry [Myrtaceae] (Clove), has a multi-dimensional role in diet, medicine, functional foods and nutraceuticals, agriculture, among other industries. Peer-reviewed articles, mostly from PubMed and Google Scholar, were consulted for the purpose of this review. The nutritional and phytochemical contents, selected biological activities as well as some functional foods and beverages of clove and their uses for human health are presented. Although these observations are largely empirical, the efficacious attributes have led to their pharmacological applications in the indigenous system of medicine all over the world and bridge between food, diet and medicine. Considering the GRAS status of clove, more studies on bioavailability, accumulation, toxicity, dosage and efficacy of clove as a spice drug or functional foods in biological systems especially in humans are required. Meanwhile, clove and its products can be used as co-adjuvants in the prevention, treatment and management of chronic diseases. Further, many applications of clove in food, health, cosmetics, pharmaceutics, nanoparticles and agricultural industries are still open for investigations.

Keywords: culinary spices (food additives for taste), clove, pharmacological, nutritional content, functional foods and nutraceuticals

OPEN ACCESS

Edited by:
Michael Heinrich,
UCL School of Pharmacy,
United Kingdom

Reviewed by:
Mohamed Fawzy Ramadan Hassanien,
Umm al-Qura University, Saudi Arabia
Nilufer Orhan,
Gazi University, Turkey
Amit Baran Sharangi,
Bidhan Chandra Krishi Viswavidyalaya, India
Pudji Widodo,
Jenderal Soedirman University, Indonesia

***Correspondence:**
Gloria Aderonke Otunola
gotunola@ufh.ac.za

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *11 October 2021*
Accepted: *07 December 2021*
Published: *17 January 2022*

Citation:
Otunola GA (2021) Culinary Spices in Food and Medicine: An Overview of Syzygium aromaticum *(L.) Merr. and L. M. Perry [Myrtaceae]. Front. Pharmacol. 12:793200. doi: 10.3389/fphar.2021.793200*

1 INTRODUCTION

The interest of consumers and researchers into how food and diet prevent and treat many disease conditions and promote human health has increased in recent years. The concept of food has gone beyond the supply of basic nutrients, to optimal nutrition-the capacity of food to over and above the supply of macro- and micro-nutrients, to promote health and well-being of individuals, reduce or prevent the risk of developing diseases and sometimes reverse them (Viuda-Martos et al., 2011).

A complex relationship exists between food, health, and humans. Naturally, foods provide normal growth and development, but recent trends have demonstrated that foods can also provide health benefits that co-exist with traditional medical approaches to disease treatment when fortified with phytonutrients. Foods beyond the basic nutritional functions have potential benefits to promote

TABLE 1 | Uses of spices.

Dietary	Medicinal	Cosmetic	Religious
Flavouring	Antioxidants, Anticancer	Perfumes	Anointing oils
Colouring	Anti-obesity, Antidiabetic	Deodorants	Incense
Pungency	Antihelmintic, Cardioprotective	Antiseptic soaps	Purification rites
Deodorizing/masking	Antimicrobial, Insect repellent	Hair health	Rituals
Sugar replacement	Aromatherapy		
Salt replacement			
Fat reduction			

health, longevity and reduce the risk of diseases. A rapidly increasing area of research is that of functional foods and nutraceuticals also referred to as foods with physiological or health benefits (Myrie and Jones, 2011). The terms fortified or functional foods are used to describe extracts and whole foods that provide protective, preventive and possibly curative effects in cancer and other chronic disease progression (Litwin et al., 2018). Nutraceuticals on the other hand refer to components/extracts of food and non-food commodities taken in the medicinal forms such as tablets, capsules, powders, liquids and potions. Functional or fortified foods resemble traditional foods in their form, but provide health benefits beyond what is rendered by their nutrient components (Shahidi, 2009).

Plant foods contain many phytomedicines-bioactive compounds known as "phytochemicals." Some groups of phytochemicals which have, or appear to have significant health potentials are carotenoids, phenolic compounds (flavonoids, phytoestrogens, and phenolic acids), phytosterols and phytostanols, tocotrienols, organosulfur compounds, and non-digestible carbohydrates (dietary fiber and prebiotics). Bioactive plant components from medicinal plants, foods, herbs, and spices are being widely examined for their ability to provide health benefits against coronary heart disease, hypercholesterolemia, high blood pressure, diabetes, inflammation, cancers, microbial, viral, and parasitic infections and play a role in supporting treatment with modern medicine (Quilez et al., 2003; Izuegbuna et al., 2019; Ozdal et al., 2021).

Spices are aromatic or pungent vegetable substances, often dried seeds, buds, fruits, roots, or barks, primarily used for flavouring, colouring or preserving food, or to mask other flavours (Dubey, 2017). In the culinary arts, it is any dried part of a plant, other than the leaves, used for seasoning and flavouring a recipe, but not used as the main ingredient. The green leafy parts of plants used in this way are considered herbs. Every other part of the plant, including dried bark, roots, berries, seeds, twigs, or anything else that is not the green leafy part, is considered a spice. Several reports of their flavour enhancement, reduction of the need for salt, sugar and fat, preservative, digestive improvement, bioactive component and health promoting uses abound in the literature.

For ages, spices have been used as seasonings, colourants and flavourings, as well as for medicinal purposes by a huge population of the world to treat several health problems such as cancer, diabetes, obesity, hepatic, renal, and cardiovascular diseases (Baselga-Escudero et al., 2017; Otunola and Afolayan, 2018; Jiang, 2019; Batiha et al., 2020). Several spices are currently being investigated for their potential health benefits, because of the failing efficacy, toxicity and high cost associated with conventional drugs.

1.2 The Role of Spices in Food/Diet

The use of spices and herbs (**Table 1**) dates back to time immemorial and transcends early civilization. In most cuisines, spices are used as adjuncts to flavour, colour, or enhance the taste of foods. Because of their strong flavours, spices are used in small quantities and therefore do not add high amount of extra calories to the diet. However, some spices have considerably high protein, fats, carbohydrates, mineral elements, vitamins, and phytonutrients/phytochemicals contents; thus making them excellent sources of bioactive compounds which contribute to the total biological activity of the whole meal, thus providing means of managing degenerative disorders and metabolic diseases (Bhathal et al., 2020).

Addition of spices to foods have resulted in improved flavour, value addition, preservative effects and longer shelf-life. For example, garlic and red chilli added to butterfat improved the flavour, red chilli, fennel or clove are used for pickles, while improved storage stability of groundnut oil was effected with red chilli and cinnamon leaves (Madsen and Bertelsen, 1995). Spices are also known to enhance digestion through the stimulation of digestive (pancreatic, terminal and small intestine) enzymes and secretion of bile, thus aiding the digestion and absorption of dietary fats (Platel and Srinivasan, 2000a; Platel and Srinivasan, 2000b; Srinivasan, 2005).

The nutritional content of spices especially with regards to macro- and micro-nutrients are important and vary from spice to spice and is dependent on several factors which include the part of the plant, harvesting technique, processing method, vegetative state, and environmental conditions, amongst others (Ereifej et al., 2015). These authors reported that a study of selected spices from Jordan revealed that dry matter of spices could range from 83.6 to 92.4%; ash 4.5–10.4%; carbohydrates 4.5–31%; protein 2.9–21.2%; fat 1.7–19.7; and fibre 25.7–59.2.

Another study reported that on dry weight basis, the crude protein of spices and herbs could range from 4.6 to 22.1%; fat (ether extract) 7.5–36.0%, total carbohydrate 34.6–71.9% and free fatty acids (as percent oleic acid) were generally low indicating good storage stability, while the flavour imparting essential oils (as percent oleoresin) were fairly high and ranged from 0.1 to 5.2% (Achinewhu et al., 1995). These data indicate that spices can contribute nutrients to the diet.

TABLE 2 | Some spices and their major bioactive components.

Spice	Bioactive components
Rosemary	Carnosic acid, carnosol, rosemarinic acid, and rosmanol
Sage	Carnosol, carnosic acid, rosmanol, and rosmarinic acid
Oregano	Derivatives of phenolic acids, flavonoids, and tocopherols
Thyme	Carvacrol, thymol, *cymene,* caryophyllene, carvone, and borneol
Marjoram	Flavonoids
Garlic	Allicin, alliin
Ginger	Zingiberol, gingerol, zingiberene, and shogaol
Pepper	Capsaicin, vitamin C, D, and carotenoids

Spices are also very important in food preservation and safety. They help to eliminate the risk of food spoilage caused by lipid oxidation and spoilage microbes. Polyphenolic compounds in spices confer antioxidant properties which scavenge free radicals, chelate transition metals, quench singlet oxygen, and thus prevent oxidation in foods (Hyldgaard et al., 2012). Again, spices can prevent the growth of spoilage microorganisms (food preservation) and inhibit or regulate the growth of pathogenic organisms leading to food safety (Tajkarimi et al., 2010).

1.3 Medicinal Uses of Spices

Most spices along with their culinary uses, also have health-enhancing properties and have been used traditionally and culturally for the prevention, treatment and management of several diseases and ailments. The health benefits of spices are many and diverse, and range from strengthening the immune system, as nutritional supplements, control of blood sugar and cholesterol levels. In addition to these, spices have anti-inflammatory, antioxidant, anti-obesity properties and can help in preventing other diseases like mental conditions, cancer and other chronic conditions (Gottardi et al., 2016). The therapeutic effects of several spices including *Zingiber officinale* Roscoe (ginger), *Capsicum frutescens* L. (cayenne pepper), *Cinnamomum verum* J. Presl (cinnamon), *Piper nigrum* L. (black pepper), *Curcuma longa* L. (turmeric), *Trigonella foenum-graecum* L.(fenugreek), *Salvia rosmarinus* Spenn. (rosemary) and *Allium sativum* L. (garlic) against several communicable, non-communicable, and chronic diseases have been reported (Tapsell et al., 2006; Dearlove et al., 2008; Kaefer and Milner, 2008; Panickar, 2013; Opara and Chohan, 2014: Jiang, 2019; Otunola and Afolayan, 2020). The specific biological activities that support human health in spices are attributed to the presence of phytonutrients/phytochemicals or bioactive compounds which have high antioxidant effects. Several spices and herbs and their bioactive compounds (**Table 2**) have and are currently undergoing extensive studies to evaluate and validate their beneficial therapeutic effects (Tapsell et al., 2006; Kochhar, 2008; Islam et al., 2013; Srinivasan, 2013; Otunola and Afolayan, 2015; Baselga-Escudero et al., 2017; Jiang, 2019).

Syzygium aromaticum (L.) Merr. and L. M. Perry (clove) is as a common aromatic spice with wide global usage in cuisines and alternative medicine. In traditional and folkloric medicine, the oil is use in dentistry because of its strong analgesic and antiseptic properties (Pulikottil and Nath, 2015; Devkota and Adhikari-Devkota, 2020). Extracts and essential oil of clove has wide applications medicinally as anti-cancer, antimicrobial, anti-halitosis, anti-diabetic, anti-obesity, anti-inflammatory; antioxidant, antiviral, aphrodisiac, amongst other uses (Hussain et al., 2017; Salama et al., 2018; Kaur and Kaushal, 2019; Batiha et al., 2020; Saeed et al., 2021).

This review aimed at collating the nutritional and pharmacological importance of clove an important culinary spice as a typical example of spices (foods) at the interface of diet and medicine specifically, to show that food can be medicine. The specific objective was to collate information on the main uses and functional properties of clove, culinary and medicinal (traditional and modern) and to highlight studies reporting these functionalities. It is a summary of some of the major culinary, medicinal and functional properties of *Syzygium aromaticum* (L.) Merr. and L. M. Perry (clove) and includes a section on functional foods and nutraceuticals containing clove.

2 METHODS

Information for this review was retrieved from repositories and search engines including PubMed, Google Scholar and Science Direct. Studies and reviews related to the culinary, nutritional and health benefits of clove, with emphasis on antioxidant, antimicrobial, anticancer, anti-diabetes, anti-inflammatory and anti-obesity potentials as well as functional foods from clove, published from 1995 to 2021, in peer-reviewed journals and reviews were retrieved. The search terms included *Syzygium aromaticum*, clove, phytochemical, culinary, pharmacological, anti-cancer, antimicrobial, anti-obesity, anti-diabetes, functional foods from clove.

2.1 *Syzygium aromaticum* (L.) Merr. and L. M. Perry [Myrtaceae]

Clove are the unopened flower bud of the clove tree of the family Myrtaceae. It is a species native to Indonesia and is used as a culinary spice globally (Kunnumakkara et al., 2009; Sachan et al., 2018). It is native to the Molucanna Islands (Indonesia), but now widely cultivated in many tropical regions like Zanzibar, Madagascar, Pakistan, India and Sri Lanka for commerce. As far back as 300 BC, Indonesia was the focus of Arab, Chinese, Indian and European traders for the major Indonesian spices of the time especially cloves (*Syzygium aromaticum* (L). Merr. and L. M. Perry) and nutmeg (*Myristica fragrans* Houtt.) (Sihotang et al., 2018). Historically, these spices were the cause for many exploration and expansion during the 15th century because they were highly sought after, caused great competition on finding the most efficient navigational route and conflict among those who wanted to monopolize the spice trade (https://mrlambertchemistry.wordpress.com/; Sihotang et al., 2018). A recent review (Haro-González et al., 2021) of Clove (*Syzygium aromaticum* L. Myrtaceae) essential oil revealed that the essential oil has numerous biological activities and wide application relevant to human and animal health, cosmetic, medical, flavouring, pesticide and food industries.

2.2 Scientific Classification

Clove belongs to the Kingdom: Plantae; Division: Magnoliophyta; Class: Magnoliopsida; Order: Myrtales; Family: Myrtaceae; Genus: Syzygium; Species: S. aromaticum (Clove); Name: Syzygium aromaticum (L.) Merr. and L. M. Perry (Bhowmik et al., 2012; Rahajaan, 2018; Saeed et al., 2021).

2.3 Description

Sujanapal and Kunhikannan (2017) described *Syzygium aromaticum* as a small evergreen tree, 6 m tall, conical crown, leaves 7–12 × 3–5 cm, elliptic or oblanceolate, base attenuate or cuneate, apex acuminate, margin entire, glabrous, coriaceous, punctate beneath; lateral nerves many, parallel, obscure, with intramarginal nerve; petiole 10–20 mm long, slender, glabrous. The flowers are pinkish-white, fragrant, in 4 cm long cymes; calyx 1.5 × 6 mm, tubular, verrucose with four hook-like involute ascending segments; petals up to 10 × 5 mm, elliptic, calyptrate; stamens many, inflexed in the bud; ovary inferior. Berry globoid or ellipsoid, dark purple with persistent calyx ring on the top. The flower buds are of commercial value as spice.

2.4 Nutritional Content and Culinary/Dietary Uses of Clove

Clove belongs to the generally regarded as safe (GRAS) foods list. It is used to flavour foods and beverages especially rice, masala and soups. Nutritionally, 100 g of clove contain 66 g carbohydrates, 6 g protein, 13 g fat and has 274 calories, as well as rich in vitamins A and B6; iron, calcium, magnesium and phosphorus (https://www.wildturmeric.net/clove-health-benefits-medicinal-uses/). Clove spice is pungent and astringent, enhance circulation, digestion and metabolism as well as alleviate stomach disorders (Dey and Mukherjee, 2021). Nutritionally, 100 g of ground clove is reported to contain Water 5.40–6.86 g; Food energy 323 (Kcal); Protein 5.98 g; Fat 20.06 g; Carbohydrate 61.22 g; Ash 5.88 g; Ca 0.646 g; P 105 mg; Na 243 mg; K 1102 mg; Fe 8.68 mg; Thiamin 0. 115 mg; Riboflavin 0.267 mg; Niacin 1.458 mg; Ascorbic acid 80.81 mg; Vitamin A 53 RE (Tainter and Grenis, 1993; Dey and Mukherjee, 2021). Clove has been used in some food formulations to enhance the nutritional, preservative and biological activities of such products. Some of these are highlighted.

A study on the sensory, antioxidant, and maillard reaction profiles of rye-buckwheat cakes enhanced with selected spices, which included clove revealed that the phenolic content, antioxidant capacity, browning properties, and overall acceptability of cakes enriched with clove, allspice, and spice were elevated and best (Przygodzka et al., 2015). According to Schlieck et al. (2021), addition of a blend of essential oils of clove, rosemary and *Origanum vulgare* L. (oregano) and vitamin E to replace conventional chemical antioxidants in dog feed improved the food quality and health of beagles considerably. Another study used and compared clove, ginger and *Cymbopogon citratus* (DC.) Stapf (lemon grass) as flavourants in cakes. The outcome revealed that clove spiced cake had a very nice texture, taste and nutrients and was best in terms of acceptability by consumers (Forlemu and Amadou, 2021). Other culinary uses of clove are as studding for *Allium cepa* L. (onions), *Solanum lycopersicum* L. (tomatoes), salads, herbal teas, and soups, to flavour meat products, cookies, pastries, sandwiches, pickles, puddings, chewing gums, spiced fruits, chocolates, soft drinks and candies (Hussain et al., 2017).

2.5 Metabolites of Clove

Several studies have investigated and reported on the metabolites present in the essential oil and various extracts of clove.

Alma et al. (2007) reported the presence of 18 components in clove bud essential oil, and the most abundant was eugenol (87%), chavibetal (19.7%), β-caryophyllene (13%). A GC-MS analysis of the essential oil of clove by El-Ghallab et al. (2020) revealed a total of 13 bioactive compounds of which Eugenol, β-Caryophyllene, eugenyl acetate and α-Humulene were the most abundant. Several other authors have reported varying abundance of phytochemicals for clove essential oil, but all pointed to the fact that eugenol was the major bioactive component (Viuda-Martos et al., 2007; Hussain et al., 2017; Shahidi and Hossain, 2018; Sgorbini et al., 2015). Other bioactives present in clove are kaempferol, quercetin and its derivates, caffeic, ferulic, elagic, and salicylic acids, as well as other minor constituents like α-humulene, β-humulene, methyl salicylate, crategolic acid, and benzaldehyde, that account for the characteristic pleasant fragrance of clove (Hussain et al., 2017). Six sesquiterpenes, α-cubebene (1.3%), α-copaene (0.4%), β-humulene (9.1%), β-caryophyllene (64.5%), γ-cadinene (2.6%) and δ-cadinene (2.6%) have been characterized from the hydrocarbon fraction of the freshly distilled Indian clove bud oil (Gopalakrishnan et al., 1984).

Nassar et al. (2007) evaluated the chemical constituents of clove (*Syzygium aromaticum*, Fam. Myrtaceae) using GC-MS, chemical and spectroscopic methods including 1D and 2D NMR for identifications of the compounds. They reported sixteen volatile compounds in the n-hexane extract with eugenol (71.56%) and eugenol acetate (8.99%) as the major components; limonin, ferulic aldehyde, and eugenol in the dichloromethane extract; tamarixetin 3-O-b-D-glucopyranoside, ombuin 3-O-b-D-glucopyranoside and quercetin from the ethanol extract. They also reported the hepatoprotective activity of the ethanol extract against paracetamol-induced liver injury in female rats.

Hemalatha et al. (2016) subjected the methanol, acetone and chloroform extracts of clove to phytochemical analysis and confirmed the presence of phenols, flavonoids, alkaloids, tannins, and saponins. The dichloromethane extract of clove bud oil showed the presence of carbohydrates, terpenoids, glycosides, steroids, sterols, tannins, and phenolic compounds (Kumar et al., 2010); some other studies of methanol extract reported saponins, alkaloids, flavanoids, cardiac glycosides, tannins, and steroids and forty-six phenolic compounds in methanol extract of clove (Jimoh et al., 2017; Kaur and Kaushal, 2019). Clove ethanolic extract contained fixed oil, tannins, phenolic compounds, terpenoids, cardiac glycosides but no saponin (Rosarior et al., 2021).

2.6 Medicinal and Pharmacological Importance of Clove

Historically, clove have been used in Ayurveda, Chinese and Western traditional medicine as a painkiller for dental emergencies (Daniel et al., 2009; Sachan et al., 2018). The antimicrobial, antioxidant, antiviral, anaesthetic, antiparasitic, antioxidant action, antiperspirant action, antiseptic property, carminative action, deodorant, digestive disorders, rubefacient, immune-boosting, stomachic actions Alzheimer's disease, among many others have been reported (Sujanapal and Kunhikannan, 2017; Sachan et al., 2018). Most of the medicinal and pharmacological properties attributed to clove are premised on its various phytochemical and bioactive constituents especially eugenol. The medicinal and pharmacological importance of clove essential oil and extracts are numerous and while transcending its dietary role, acts/points to the important link between food and medicine.

2.7 Antioxidant Activity

Clove oil has been accredited with high antioxidant activity, perhaps one of the best known oil for food or supplement, which is attributed to the phenolic compounds especially eugenol, thymol and eugenol acetate (Yadav and Bhatnagar, 2007; Dai et al., 2013; Nam and Kim, 2013; Mittal et al., 2014; Uddin et al., 2017; Kaur and Kaushal, 2019). The high antioxidant capacity of eugenol has been compared to that of pyrogallol and BHT. According to Fankem et al. (2017) clove essential oil exhibited ten times higher antiradical activity than BHT; and seven times greater than cocoa butter and clove essential oil mixture. When a linoleic acid emulsion was treated with 15 μg/ml of clove oil, 97.3% inhibition of lipid peroxidation was observed compared to inhibition by standard antioxidants like trollox, BHA, α-tocopherol and BHT which showed 95.6, 95.4, 84.6, and 99.7% inhibition respectively (Gulcin et al., 2012). Clove ethanolic extract exhibited dose-responsive antioxidant effect and better reducing potential, against DPPH, ABTS, and Ferric chloride respectively (Rosarior et al., 2021). Clove and lavender (*Lavandula angustifolia* Mill.) ethanol and aqueous extracts at 20, 40, and 60 μg/ml showed inhibitions up to 95% when tested as superoxide radical capture, scavenging of DPPH radical and as metal quelants (Cortés-Rojas et al., 2014). The powerful antioxidant activity of both extracts were attributed to strong hydrogen donating, metal chelating and free radical scavenging capacity.

Clove extract was also reported to exhibit antioxidant properties when tested using ferric reducing antioxidant power, oxygen radical absorbance capacity, DPPH, hydroxyl and superoxide radicals scavenging activities (Suantawee et al., 2015). According to Chatterjee and Bhattacharjee (2015) Mayonnaise formulated with eugenol-lean clove extract showed distinctly higher antioxidant activity and reducing power than mustard-formulated mayonnaise and the market sample.

2.8 Assessment

Although clove extracts and oil have been credited with very high antioxidant capacity, the antioxidant activities reported here are mostly "*in vitro*", which according to Gafner (2018) is present in all plants and as such not useful without additional data. Moreover, the tests (ABTS, ORAC, DPPH, TEAC, NO, and FRAP† assays) used for measuring total antioxidant activity, are non-specific, prone to interferences and the results are often unreliable; also the assurance that *in vitro* antioxidant activities will be observed in animal or human studies is uncertain.

2.9 Antimicrobial Activity

2.9.1 Antibacterial

Yassin et al. (2020a) evaluated the antibacterial efficiency of acetone, dichloromethane, ethanol and petroleum ether extracts of clove against *Escherichia coli, Salmonella typhi, Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA). The researchers reported that the dichloromethane extract exhibited the highest antibacterial potency against the pathogenic isolates.

A study by Radünz et al. (2019) showed that unencapsulated and encapsulated clove essential oil at 0.304 mg/ml inhibited *S. aureus*, *E. coli*, *L. monocytogenes*, and *S. Typhimurium* bacteria, independent of the microorganism membranes and *in situ* inhibition against *S. aureus* in meat products similar to burgers. The bactericidal action of clove essential oil is attributed to eugenol, which ruptures the bacterial cytoplasmic membrane, allowing leakage of ions and intracellular proteins, leading to cell death.

The high antibacterial effect of ethanol and methanol extracts of clove against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates have been reported (Garba et al., 2019). Ethanolic extract of clove was shown to have broad spectrum (Gram-positive and Gram-negative) inhibition on five pathogens causing urinary tract infections, this inhibition was stronger than that shown by pure eugenol at the same concentration (Rosarior et al., 2021). Antibacterial activity of methanol extract was reported for *Klebsiella pneumonia* (Hemalatha et al., 2015).

Eugenol, the major component of clove oil was shown to have bactericidal action against *Proteus mirabilis* at the minimum inhibitory concentration of 0.125% (v/v) and at 0.25% (v/v) it reduced viability and completely inhibited *P. mirabilis* population within 30 min of exposure (Devi et al., 2013).

Another study showed that hexane extract of clove (*Eugenia caryophylata*), at 0.2% exhibits the highest inhibition against the pathogenic potato black stem and soft rot bacteria, *Erwinia carotovora subsp. atroseptica* when compared to ethanol extracts of cinnamon (*Cinnamomum zelanicum*) and datura (*Datura metel*) at the same concentration (Al-Jeboory et al., 2012).

2.9.2 Antifungal

Yassin et al. (2020b) showed that *Syzygium aromaticum* (clove) ethyl acetate extract exhibited the highest antifungal activity against *C. tropicalis*, *C. albicans, C. glabrata,* and with minimum inhibitory concentration of 250 μg/disc and 500 μg/disc respectively, while the minimum fungicidal concentration was 0.5 mg/disc against *C. tropicalis* and 1 mg/disc against the *C.*

albicans and *C. glabrata.* The main bioactive compounds in the extract were eugenol (58.88%), eugenyl acetate (23.86%), *trans*-caryophyllene (14.44%) and α-humulene (1.88%).

Clove oil was reported to show maximum antifungal activity against *C. tropicalis, C. albicans,* and *C. guilliermondii* (Kumar et al., 2012). Encapsulated clove oil also exhibited strong antifungal action against *Fusarium oxysporum* (Estrada-Cano et al., 2017) and antiseptic effects on meat products at a concentration above 0.070% (Wang et al., 2018; Kaur and Kaushal, 2019).

2.9.3 Antiviral Activity

Aqueous extract of clove was reported to exhibit a dose-dependent antiviral effect against Feline Calicivirus, used as a surrogate for Human Norovirus, a food-borne virus. The effect was attributed to the main bioactive component-eugenol which showed similar antiviral activity to clove extract, although at lower level, an indication that other components in clove worked synergistically with eugenol to inactivate Feline Calcivirus (Aboubakr et al., 2016).

Eugenol and Eugenin isolated from clove bud essential oil was also shown to have potent inhibitory effect against herpes simplex virus at a dose of 10 μg/ml (Milind and Deepa, 2011). The antiviral efficacy of aqueous extracts of clove against herpes simplex virus type 1 (HSV-1) and influenza A virus when combined with acyclovir has also been reported (Batiha et al., 2020).

2.9.4 Assessment

According to the above mentioned studies, both the extracts and essential oil of clove possess antimicrobial activity. Most of these activities were dose dependent, broad spectrum and based on disruption of the microbial cytoplasmic membrane resulting in leakage of cytoplasmic content and subsequent cell death. However, most of the studies did not give the mechanism or type of inhibition, but all reported that clove can be used as a natural antibacterial, antifungal and antiviral agent in human/animal health, as well as the inhibition of pathogenic and spoilage microorganisms in foods and plants.

2.10 Anti-Inflammatory Properties of Clove

2.10.1 *In vitro*

Anti-inflammatory activity of clove (*Eugenia caryophyllata*) essential oil was evaluated by simulating inflamed human skin cells in a dermal fibroblast system (HDF3CGF). Clove essential oil at a concentration of 0.001% showed strong anti-inflammatory action, demonstrated through resistance to the production of pro-inflammatory biomarkers like interferon-inducible T-cell α-chemoattractant (I-TAC), vascular cell adhesion molecule-1 (VCAM-1) and collagen III expression at gene and protein levels (Han and Parker, 2017).

Bachiega et al. (2012) reported that aqueous extract of clove and eugenol in non-cytotoxic concentrations exerted immunomodulatory or anti-inflammatory action on cytokine production by murine macrophages incubated with clove or eugenol (5, 10, 25, 50 or 100 μg/well) for 24 h. They showed that 100 μg/well of clove inhibited the production of IL-1β, IL-6, and IL-10, eugenol did not affect IL-1β production but inhibited IL-6 and IL-10. They concluded that the immunomodulatory/anti-inflammatory effects of clove was by inhibiting LPS action and the possible mechanism of action may be through suppression of nuclear factor-κB pathway by eugenol, because it was the major bioactive of clove extract.

2.10.2 *In vivo*

Nikoui et al. (2017) investigated the anti-inflammatory and antipyretic effects of clove oil in healthy dogs after surgery. Animals surgically operated in the abdomen were grouped into four and administered 25 mg/kg of clove oil, 20 mg/kg betamethasone (anti-inflammatory), 15 mg/kg phenylbutazone (anti-pyretic), or nothing as control for 5 days consecutively. The researchers reported that clove oil-treated animals had significantly reduced edema, white blood cells, neutrophils, band neutrophils and rectal temperature compared to control. Also, histopathology assay showed that clove oil reduced inflammation significantly in dogs.

Also, the use of pure clove oil in the aromatherapy treatment of arthritis and rheumatism, in combination with honey for dermatitis and the paste (water and clove powder) for bites and cuts have been reported (Hussain et al., 2017).

Further, Naji et al. (2017) evaluated the anti-inflammatory capacity of *Syzygium aromaticum* extract (SAE) using formalin test and showed that mice fed with different doses of SAE exhibited visible increase in analgesia time and decrease in licking number ($p < 0.05$) compared to untreated animals.

Bachiega et al. (2012) showed that clove and eugenol exhibited immunomodulatory and anti-inflammatory action on cytokine production by murine macrophages when administered in non-toxic doses. In addition, clove oil at 0.05 and 0.20 ml/kg showed anti-inflammatory effect equal to that of anti-inflammatory drugs like etodolac and indomethacin at 0.025 and 0.1, and 0.05 and 0.2 ml/kg doses, respectively (Vicidomini et al., 2021).

2.10.3 Assessment

The anti-inflammatory actions of clove extracts and essential oils though documented, were mostly performed using *in vitro* and *in vivo* assays using cell lines and animals respectively. No clinical study involving human subjects were reported. Many studies evaluated the anti-inflammatory potentials using eugenol the major bioactive component of clove. The studies report the potential of clove extracts and oil and provide support for its use in folk medicine. However, well planned studies across all levels (*in vitro*, *in vivo* and clinical/human) including dosages are urgently needed to validate the anti-inflammatory use of clove.

2.11 Anti-Cancer Activity

2.11.1 *In vitro* Studies

Toxicity

Cytotoxicity of acetone, dichloromethane, ethanol and petroleum ether extracts of clove against human colon cancer (HCT) cell lines using MTT assay were investigated (Yassin et al., 2020a). The test revealed that ethanol extract of clove gave maximum cytotoxic potency against HCT cell line, dichloromethane extract

was least effective, whereas the petroleum ether and acetone clove extracts showed only moderate cytotoxicity against HCT cells.

Another study by Kello et al. (2020) revealed that treatment of MCF-7 cells with ethanol extract of clove (CBE) induced intrinsic caspase-dependent cell death associated with increased oxidative stress mediated by oxygen and nitrogen radicals, release of mitochondrial pro-apoptotic factors, signalling of oxidative stress-mediated DNA damage with modulation of cell antioxidant SOD (superoxide dismutase) system, and modulation activity of the Akt, p38 MAPK, JNK, and Erk 1/2 pathways.

Nirmala et al. (2019) evaluated the anticancer (cytotoxicity) potential of a clove bud essential oil-based nanoscale emulsion system (CB-4) against thyroid cancer cell line (HTh-7) using MTT, colony formation and Annexin V-FITC assays. The authors reported that 0.7 µL/ml of CB-4 was cytotoxic against thyroid cancer cell lines (HTh-7) but not to non-cancerous cells (Hek-293); also significant reduction was observed in the number of cancer cell colonies when treated with CB-4 for 7–10 days, while Annexin V-FITC confirmed that the clove bud oil-based emulsion has antiproliferative action on growth of HTh-7 cell line, an indication that apoptosis may be responsible for cell death though necrosis was also observed when stained with propidium iodide.

Khan et al. (2018) tested fluorescent magnetic submicronic polymer nanoparticles (FMSP-nanoparticles) alone (1.25, 12.5, 50, 75, and 100 μg/ml) and combined with crude clove extracts (50 μg/ml) on human breast cancer cells (MCF-7) for 24 and 48 h, using Trypan Blue, 4′,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. They showed that when treated with FMSP-nanoparticles alone, cancer cell viability was decreased to 55.40%, but decreased drastically to 8.50% when treated with combination of FMSP-nanoparticles and crude clove extracts.

Another study reported that clove essential oil at concentration of 0.011% was potent against proliferation of human dermal fibroblasts by significantly inhibiting the production of pro-inflammatory biomarkers (vascular cell adhesion molecule-1 (VCAM-1), interferon γ-induced protein 10 (IP-10), interferon-inducible T-cell α chemoattractant (I-TAC), and monokine induced by γ interferon (MIG) and tissue remodelling protein molecules, namely, collagen-I, collagen-III, macrophage colony-stimulating factor (M-CSF), and tissue inhibitor of metalloproteinase 2 (TIMP-2); as well as modulation of gene expression and altering signalling pathways critical for inflammation, tissue remodelling and cancer signalling processes (Han and Parker, 2017).

The *in vitro* antitumor effects of clove ethyl acetate extract and the bioactive component (oleanoic acid) responsible for the activity was investigated (Liu et al., 2014). The ethyl acetate extract and oleanoic acid were reported to be cytotoxic, inhibit tumor growth, promote G_0/G_1 cell cycle arrest and apoptosis in several human cancer cell lines in a dose-dependent manner, selectively increased protein expression of $p21^{WAF1/Cip1}$ and γ-H2AX and downregulated expression of cell cycle-regulated proteins.

Anti-proliferative activity of aqueous, ethanol and oil extracts of clove against HeLa (cervical cancer), MCF-7 (ER + ve) and MDA-MB-231 (ER—ve) breast cancer, DU-145 prostate cancer, TE-13 esophageal cancer cell lines and normal human peripheral blood lymphocytes was investigated. The results showed that clove oil at 300 µl/ml caused maximum (80%) and apoptotic cell death in TE-13 cells within 24 h, but minimal cell death in DU-145 cells with no significant cytotoxicity in human PBMC's at the same dose (Dwivedi et al., 2011). β-caryophyllene had no cytotoxic effect, but clove oil and eugenol were cytotoxic. They proposed the possibility that natural compounds found in clove could be used for the development of new treatment for esophageal cancer.

2.11.2 *In silico* Studies

Azadi et al. (2020) using *in silico* mathematical modelling and 1H Nuclear Magnetic Resonance spectroscopy on clove oil treated Raji cells, used the Metaboanalyst software to predict that 50% inhibitory concentration of clove oil was 50 µg/ml and 74 genes with differentiating metabolites consisting of amino acids, cholesterol and fucose. They also predicted that clove oil mechanism of anti-cancer action was against novel enzymes, like 24-dehydrocholesterol reductase and 7-dehydrocholesterol reductase in cholesterol biosynthesis, dehydrofolate reductase in one carbon metabolism and serine palmitoyl-transferase long chain in sphingolipid biosynthesis.

2.11.2 *In vivo* Studies

Kubatka et al. (2017) showed that dietary administration (0.1 and 1%) of clove was effective against induced mammary carcinoma in rat model through significant decrease in tumor frequency but not latency. The antitumor effect was mediated through caspase three activation, reduction of protein (Bcl-2, Ki67, vascular endothelial growth factors A, MDA, CD24, CD44) expressions and increased methylation of RASSF1A promoter, as well as expressions of ALDH1A1, H4K16ac, and H4K20me3. Colon cancer was induced in mice by injecting them with naked HT-29 tumor cells, the mice were then treated with ethyl acetate extract of *S. aromaticum* once daily for 5 days. The authors reported that tumor growth was significantly reduced in the extract-treated group (Kubatka et al., 2017).

In vivo, the antitumor activity of ethyl acetate extract and oleanoic acid from clove were investigated using the HT-29 tumor xenograft model (Liu et al., 2014). The extract showed higher activity than oleanoic acid (one of its bioactive components) and 5-fluorouracil (a chemotherapeutic agent) at suppressing the growth of colon tumor xenografts. Most of the changes reported were at the mRNA level, suggesting transcriptional regulation by ethyl acetate extract. The authors concluded that clove extract and oleanoic acid, could be novel therapeutic agents for the treatment of colorectal cancer.

2.11.3 Assessment

From the studies reviewed, clove extracts, essential oils and bioactive components exerted anti-cancer properties *in vitro* through cytotoxicity, apoptosis, chemopreventive, tumor formation, antiproliferative, modulation of gene expression

and cancer signalling processes actions. *In silico* evaluation have also been used to predict anti-cancer potential of clove. *In vivo*, clove extracts inhibited tumor growth through caspase three activation, gene and protein expressions. Unfortunately, as with anti-inflammatory action of clove, no human studies have been documented so far. Thus it is pertinent that future studies be initiated to investigate the anticancer effect of clove in humans including the dosage. Presently though, since clove is a GRAS food, it can be used as an adjuvant in cancer treatment.

2.12 Anti-Diabetic Activities

The anti-diabetic effects of clove essential oil and extracts are well documented. It is assumed that clove extract mimics the action of insulin on gene expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase; and that expression of several genes are regulated by clove and insulin in the same pattern (Milind and Deepa, 2011).

2.12.1 *In vitro*

Adefegha and Oboh (2012) reported that polyphenol-rich extracts from *Syzygium aromaticum* (L.) Merr. and Perry (Clove) buds inhibited the activity of carbohydrate hydrolyzing enzymes linked to type 2 diabetes and Fe2+-induced lipid peroxidation in rat pancreas in a dose-dependent manner *in vitro*. The authors reported that alpha-glucosidase inhibition was significantly higher than that of alpha-amylase and could be the mechanism by which clove elicits exerts its ameliorative effect on type 2 diabetes.

2.12.2 *In vivo*

Dietary intake of clove by streptozotocin-induced diabetic male Sprague-Dawley rats led to reduced tissue and cardiac muscle damage, reduced blood sugar and lipid peroxidation, decrease in hyperglycaemia-induced oxidative tissue damage and cataract formation in the eye lens (Lakshmi and Manasa, 2021).

Hydroalcoholic extracts of clove reportedly improved serum biomarkers, antioxidant status, and histopathological changes in kidneys of streptozotocin-induced diabetic rats by ameliorating glycaemic control, lipid profile and preventing kidney damage (Abtahi-Eivari et al., 2021).

Sammy et al. (2020) reported that when streptozotocin induced diabetic rats were administered aqueous extract of clove at 250 mg/kg body weight with metformin, glucose, ALT, AST, and other biomarkers were significantly downregulated compared to the untreated or no clove streptozotocin treated diabetic rats. Also, Abdulrazak et al. (2018) showed that high fat diet-induced type 2 diabetic rabbits treated with 12.5% dietary clove and 12.5% fermented ginger respectively for 6 weeks exhibited significant ($p < 0.05$) decrease in blood glucose levels and that the clove supplement effectively sustained anti-hyperglycemic activity, lower effect on leptin levels, with significant increase in insulin levels compared to diabetic control group.

According to Abd El-Rahman (2015) dietary inclusion of *Syzygium aromaticum* at 3, 5, 7 and10% for 4 weeks in the diet of alloxan-induced diabetic rats, caused significant decrease in serum glucose, total cholesterol, LDL-C, VLDL-C, triglyceride levels, AST, ALT, and ALP, as well as liver function and lipid profile.

Adefegha et al. (2014) reported that induced type 2 diabetes rat model fed on diets containing clove bud powder at 20–40 g kg−1 exhibited reduced blood glucose level compared to control diabetic rats without clove bud powder supplementation; in addition, reduced α-glucosidase and liver enzymes activities along with elevated levels of antioxidant status were observed.

Aqueous extracts of cinnamon and clove at 10 μg/ml showed very strong and potent inhibition of protein glycation in zebra fish (Jin and Cho, 2011).

2.12.3 Clinical

Bi et al. (2017) reported a study in which 36 people with T2D were given capsules containing 0, 1, 2, or 3 g of cloves per day for 30 days and a 10-days washout period. Results from the study showed that although no significance differences in responses to the dose was observed, diabetic patients who took clove supplement exhibited decrease in serum glucose, total cholesterol, triglycerides and LDL, compared to diabetic patients who did not.

2.13 Anti-Obesity Activities

2.13.1 *In vitro*

An alcoholic extract of clove (containing majorly-eugenol (42.27%), acetyl eugenol (29.12%), caryophyllene (15.40%), and humulene (3.22%) was reported to inhibit S-phase DNA replication of HepG2 cells and adipocyte differentiation of OP9 cells (Ding et al., 2017). *In vitro* treatment using clove ethanol extract on 3T3-L1 cells showed that the extract efficiently inhibited the conversion of cells into adipocytes in a dose-dependent manner (Jung et al., 2012).

2.13.2 *In vivo*

Ding et al. (2017) reported that an alcohol extract of clove [containing majorly-eugenol (42.27%), acetyl eugenol (29.12%), caryophyllene (15.40%), and humulene (3.22%)] prevents obesity in a mouse model by acting as a natural fatty acid inhibitor. The extract also reduced the development of high fat diet-induced obesity, body and abdominal adipose tissue weight, regulate total triglyceride, low-density lipoprotein cholesterol and lower lipid accumulation in the liver and epididymal adipose tissues.

In another study, Jin and Cho, (2011) showed that an aqueous extract of cinnamon and clove at 10 μg/ml administered to hyper-cholesterolaemic zebrafish exerted a potent dose-dependent cholesteryl ester transfer protein (CETP) inhibitory and hypolipidaemic activities; and that clove extract-fed group had the smallest increase in body weight and height and the strongest antioxidant activity after a 5-weeks high cholesterol diet.

Another study reported that clove ethanol extract reduced diet-induced obesity in mice fed high fat diet supplemented with 0.5% (w/w) for 9 weeks through down-regulation of adipogenic and lipogenic gene expression (Jung et al., 2012). Another study reported that simultaneous administration of clove and curcumin (*Curcuma longa* L.) extract on high fat diet-induced mice for 5 weeks led to reduced food intake, weight gain, adipose tissue,

liver weight, and regulation of lipid profiles (Pérez Gutiérrez and Arrioja, 2021).

2.13.2 Assessment

Regarding antidiabetic and anti-obesity potential of clove, most of the studies were on extracts and essential oils of clove and were mostly *in vivo* using mice or rat models and *in vitro* using cell lines and enzyme based assays. The activities were marked with reduction in fasting blood glucose, protein glycation, increased insulin production, improving glucose tolerance, and glucose and lipid metabolism-related enzyme activities and conversion of cells into adipocytes among other physiological actions. Only one clinical study in humans was reported for antidiabetic property of clove. These studies however, are not detailed enough, neither are they supported with human studies considering the GRAS status of clove.

Therefore, future studies should focus on the effect of clove on humans through randomized clinical trials, targeting specific biomarkers to confirm and validate the folkloric uses of clove as therapy for diabetes and obesity.

2.13.3 Functional foods and Nutraceuticals prepared with clove.

Paschoalinotto et al. (2021) showed that a tisane (T5) composed of lemon thyme, tutsan, cloves and cinnamon, exhibited health-promoting effects (lipid peroxidation inhibition, anti-inflammatory, and anti-diabetic activities) and potential for application in the food and nutraceutical industries.

In another study, Günes-Bayir et al. (2020) investigated the effect of adding clove (*Syzygium aromaticum*) in three different concentrations (0.1, 0.3, and 1%) and propolis (0.03%) to probiotic yoghurt as follows: 1) yoghurt without propolis or clove (control group); 2) yoghurt with propolis (0.03%); 3) yoghurt with propolis (0.03%) and clove (0.1%); 4) yoghurt with propolis (0.03%) and clove (0.3%); 5) yoghurt with propolis (0.03%) and clove (1.0%) respectively on the microbiological, chemical and sensory properties of probiotic yoghurts. They reported that whereas propolis exerted antibacterial effect on bacteria colonies except *Streptococcus thermophiles*, clove reduced the suppressive effect of propolis on bacteria colonies by supporting the number of *B. animalis subsp. lactis* growth, improving the microbial qualities and sensory acceptability of the probiotic yoghurts positively.

Nirmala et al. (2019) prepared nanoscale-based emulsions from essential oil of clove buds (*Syzygium aromaticum*) and varying concentrations of Tween 20 and Tween 80 surfactants. Cytotoxicity of the stable oil-based emulsion was evaluated using MTT colony formation, and Annexin V-FITC assays against thyroid cancer cell line (HTh-7). The authors reported that the product has potential as alternative cancer candidate drug because it showed apoptotic and reductive effect on cancer cell proliferation.

Cinnamon (*Cinnamomum zeylanicum*), fenugreek (*Trigonella foenum-graecum*), shallot (*Allium hirtifolium Boiss*) and clove (*Syzygium aromaticum*) were evaluated for their antidiabetic efficacy in rats, singly and as a polyherbal formulation (Tetraherbs) in streptozotocin (STZ)-induced diabetic rats with fasting blood sugar above 350 mg/dl. The rats were treated orally with 75 mg/kg dose ethanolic extracts of each specie separately, or in equal combination formulation (Tetraherbs) at 100–300 mg/kg once daily for 28 days. Tetraherbs gave a higher blood glucose lowering activity and pancreatic β cell regeneration than for each plant (Kiani et al., 2018).

Another study by Ramos et al. (2017) evaluated an herbal extract containing green mate (*Ilex paraguariensis*), clove (*Syzygium aromaticum*), and lemongrass (*Cymbopogon citratus*) optimized in fermented milk with or without sweet potato pulp. Addition of a lyophilized extract (1 g 100 g–1) of 87.5% clove and 12.5% green mate increased the antioxidant and total phenolic content, though fermented milk with added sweet potato pulp had the best sensory acceptance. Twenty-one-day storage revealed slight decrease in total phenolic acid and total reducing capacity. The authors proposed the addition and use of this extract for the development of new dairy foods with potential functional properties.

The safety and anti-ulcerogenic activity of a Clovinol, a novel polyphenol-rich extract of clove buds (*Syzygium aromaticum* L) was reported by Issac et al. (2015). Clovinol was derived as a water soluble free flowing powder from clove buds, without the characteristic pungency and aroma. The extract was characterized by electrospray ionization-time of flight mass spectrometry (ESI-TOF-MS) and evaluated for antioxidant, anti-inflammatory and anti-ulcerogenic activities in mice and rat models. The product exhibited significant antioxidant and anti-inflammatory effects measured by cellular antioxidant levels and inhibition of carrageenan-induced paw swelling in mice; while its anti-ulcerogenic activity was confirmed by greater than 97% inhibition of ethanol-induced stomach ulcers in Wistar rats administered at 100 mg per kg b w. orally and up regulation of *in vivo* antioxidants. Lipid peroxidation, oxidative stress and gastrointestinal health of ulcer induced rats were also improved by Clovinol, which was shown to be safe at 5 and 2.5 g per kg body weight for acute and 28 days of repeated dose toxicity studies respectively.

Babajide et al. (2013) blended equal portions of cucumber (50%) and pineapple (50%) juices with clove and ginger powder at 0.25% (CPCLG1), 0.5 (CPCLG2), 0.75% (CPCLG3), and 1% (CPCLG4) (w/v) respectively to develop a new fruit drink with health benefits. They reported the presence of alkaloids, flavonoids, saponins, steroids, tannins, terpenoids and phlobatannins. Various Quantities of isoquinoline, octanoic acid, metroprolol, fumaric acid, benzoquinone, and betaxolol among others.

Balaji et al. (2012) investigated the antidiabetic potentials of a novel polyherbal preparation containing *Asparagus racemosus, Emblica officinalis, Salacia oblonga, Syzygium aromaticum,* and *Tinospora cordifolia* in equal ratios. The authors showed that the product reverted the activities of glycolytic and gluconeogenic enzymes in diabetic rats and that lipid profile, antioxidant status and glycogen content were improved, with decrease in lipid peroxidation.

2.13.4 Assessment

The studies reported here are some examples of the use of clove in preparation of functional foods and beverages, tailored towards the management of some chronic diseases. Some of these show that the therapeutic property of clove can withstand food processing/thermal changes and are carried through to the final products. However, more studies are needed to determine appropriate vehicles, doses, shelf-life and toxicity/adverse consequences in human trials to validate these therapeutic claims. These functional foods however, can be used co-adjuvantly in the prevention, treatments and management of chronic diseases.

3 CONCLUSION, LIMITATIONS, AND FUTURE RESEARCH NEEDS

The traditional, historical and cultural use of spices in diet and medicine cannot be overemphasized, because along with their culinary uses, most spices also have health-enhancing properties as demonstrated by clove (*Syzygium aromaticum* (L.) Merr. and L. M. Perry [Myrtaceae]).

Traditionally and pharmacologically, the medicinal uses of clove spice are diverse and seemingly unending. This review collated available information on nutritional and phytochemical constituents, forms (powders, extracts, infusions, and combination with other spices, etc.), concentrations/dosages, selected biological/health activities, as well as functional foods derived from clove spice were considered.

Some of these include use of clove oil for toothache, dental caries and pyorrhea; headache, sore throat, respiratory disorders (coughs, colds, bronchitis, asthma, sinusitis, and tuberculosis) and digestive system ailments. In traditional medicines of Australia and Asian countries, clove is used for ear ache, anti-inflammatory, analgesic, antipyretic, antifungal, antibacterial and peptic ulcer treatments. Other uses include as an anaesthetic, hepatoprotective, anthelmintic, memory recall, anti-stress, analgesic, anticonvulsant, antimycotic, insecticidal, antimutagenic, and antiulcerogenic amongst other uses (Milind and Deepa, 2011; Agrawal et al., 2018; El-Ghallab et al., 2020; Yadav et al., 2020).

This overview has revealed documented scientific evidence that clove spice possesses antioxidant, anti-microbial, anti-viral, anti-inflammatory, anti-cancer, anti-diabetic and anti-obesity functions amongst other reported properties. However, this could be further buttressed through clinical studies.

Thus, the medicinal and pharmacological importance of spices are an excellent validation of the common statement "Let food be thy medicine and medicine thy food; " and the link between food and medicine.

The review was limited by the fact that so many of the studies reported were *in vitro* with very few clinical studies. Most of the studies also did not report the percentage of effectiveness of the extract or oil and the mechanism of action.

Future research needs and focus should be on well-planned and executed studies on the medicinal properties of clove using different models including *in vitro, in vivo, ex vivo,* and clinical/human studies. Considering the fact that clove is a GRAS food, it is important that human studies be performed and documented. More studies on bioavailability, accumulation, toxicity, dosage and efficacy of clove as a spice drug or functional foods in biological systems especially humans are required. Further, many applications of clove in the food, health, cosmetics, pharmaceutics, nanoparticles, and agricultural industries are still open for investigations.

AUTHOR CONTRIBUTIONS

GAO is responsible for all aspects of this review.

FUNDING

This study was supported by the South Africa National Research Foundation, Research and Innovation Support and Advancement (NRF: RISA) CSURG: Grant No: 121264.

REFERENCES

Abd El-Rahman, A. N. (2015). Anti-Diabetic and Hepatoprotective Effect of Clove (*Syzygium Aromaticum* Linn) on Rats Induced by Alloxan. *J. Home Econ.* 25 (3), 133–150.

Abdulrazak, A., Tanko, Y., Mohammed, A., Mohammed, K. A., Sada, N. M., and Dikko, A. A. (2018). Effects of Clove and Fermented Ginger on Blood Glucose, Leptin, Insulin and Insulin Receptor Levels in High Fat DietInduced Type 2 Diabetic Rabbits. *Niger. J. Physiol. Sci.* 33 (1), 89–93.

Aboubakr, H. A., Nauertz, A., Luong, N. T., Agrawal, S., El-Sohaimy, S. A., Youssef, M. M., et al. (2016). *In Vitro* antiviral Activity of Clove and Ginger Aqueous Extracts against Feline Calicivirus, a Surrogate for Human Norovirus. *J. Food Prot.* 79 (6), 1001–1012. doi:10.4315/0362-028X.JFP-15-593

Abtahi-Eivari, S. H., Shokoohi, M., Ghorbani, M., Kalarestaghi, H., Shoorei, H., Bahrami, J., et al. (2021). Effects of Hydroalcoholic Extracts of Clove (*Syzygium Aromaticum*) on Serum Biomarkers, Antioxidant Status and Histopathological Changes of Kidneys in Diabetic Rats. *Crescent J. Med. Biol. Sci.* 8 (4), 269–275.

Achinewhu, S. C., Ogbonna, C. C., and Hart, A. D. (1995). Chemical Composition of Indigenous Wild Herbs, Spices, Fruits, Nuts and Leafy Vegetables Used as Food. *Plant Foods Hum. Nutr.* 48, 341–348. doi:10.1007/BF01088493

Adefegha, S. A., Oboh, G., Adefegha, O. M., Boligon, A. A., and Athayde, M. L. (2014). Antihyperglycemic, Hypolipidemic, Hepatoprotective and Antioxidative Effects of Dietary Clove (Szyzgium Aromaticum) Bud Powder in a High-Fat Diet/streptozotocin-Induced Diabetes Rat Model. *J. Sci. Food Agric.* 94 (13), 2726–2737. doi:10.1002/jsfa.6617

Adefegha, S. A., and Oboh, G. (2012). *In Vitro* inhibition Activity of Polyphenol-Rich Extracts from Syzygium Aromaticum (L.) Merr. & Perry (Clove) Buds against Carbohydrate Hydrolyzing Enzymes Linked to Type 2 Diabetes and Fe(2^+)-Induced Lipid Peroxidation in Rat Pancreas. *Asian Pac. J. Trop. Biomed.* 2 (10), 774–781. doi:10.1016/S2221-1691(12)60228-7

Agrawal, P., Kotagiri, D., and Kolluru, V. C. (2018). Comparative Analysis of Antimicrobial Activity of Herbal Extracts against Pathogenic Microbes. *Adv. Biochem. Biotechnol.* 10, 2574–2578. doi:10.29011/2574-7258.000063

Alma, M. H., Ertaş, M., Nitz, S., and Kollmannsberger, H. (2007). Chemical Composition and Content of Essential Oil from the Bud of Cultivated Turkish

Clove (*Syzygium Aromaticum* L.). *BioRes* 2, 265–269. doi:10.15376/biores.2.2.265-269

Al–Jeboory, H. H., Al–Fadhial, F. A., Al–Ani, R. A., and Aish, A. A. (2012). Inhibition Activity of Clove Hexane Extract, Cinnamon and Datura Ethanol Extract against *Erwina Carotovora Sub Sp. Atroseptica* Causative Agent of Soft Rot and Black Stem Disease. *Iraqi J. Agric. Sci.* 43 (4), 41–48.

Azadi, M., Alemi, F., Sadeghi, S., Mohammadi, M., Rahimi, N. A., Mirzaie, S., et al. (2020). An Integrative In Silico Mathematical Modelling Study of the Anti-cancer Effect of Clove Extract (*Syzygium Aromaticum*) Combined with *In Vitro* Metabolomics Study Using 1HNMR Spectroscopy. *Iranian J. Biotechnol.* 18 (3), e2336. doi:10.30498/IJB.2020.141102.2336

Babajide, J. M., Olaluwoye, A. A., Taofik Shittu, T. A., and Adebisi, M. A. (2013). Physicochemical Properties and Phytochemical Components of Spiced Cucumber-Pineapple Fruit Drink. *Niger. Food J.* 31, 40–52. doi:10.1016/s0189-7241(15)30055-2

Bachiega, T. F., de Sousa, J. P., Bastos, J. K., and Sforcin, J. M. (2012). Clove and Eugenol in Noncytotoxic Concentrations Exert Immunomodulatory/anti-Inflammatory Action on Cytokine Production by Murine Macrophages. *J. Pharm. Pharmacol.* 64, 610–616. doi:10.1111/j.2042-7158.2011.01440.x

Balaji, V., Williams, A. A., Sathish, S., Mayilvanan, C., Narasimhan, A., and Balasubramanian, K. (2012). Antidiabetic Potentials of a Novel Polyherbal Preparation Formulated According to Principles of Siddha System of Medicine. *J. Evid. Based Complement. Altern Med* 17, 180–190. doi:10.1177/2156587212451027

Baselga-Escudero, L., Souza-Mello, V., Pascual-Serrano, A., Rachid, T., Voci, A., Demori, I., et al. (2017). Beneficial Effects of the Mediterranean Spices and aromas on Non-alcoholic Fatty Liver Disease. *Trends Food Sci. Tech.* 61, 14. doi:10.1016/j.tifs.2016.11.019

Batiha, G. E., Alkazmi, L. M., Wasef, L. G., Beshbishy, A. M., Nadwa, E. H., and Rashwan, E. K. (2020). *Syzygium Aromaticum* L. (Myrtaceae): Traditional Uses, Bioactive Chemical Constituents, Pharmacological and Toxicological Activities. *Biomolecules* 10 (2), 202. doi:10.3390/biom10020202

Bhathal, S. K., Kaur, H., Bains, K., and Mahal, A. K. (2020). Assessing Intake and Consumption Level of Spices Among Urban and Rural Households of Ludhiana District of Punjab, India. *Nutr. J.* 19 (1), 121–122. doi:10.1186/s12937-020-00639-4

Bhowmik, D., Kumar, K. S., Yadav, A., Srivastava, S., Paswan, S., and Dutta, A. S. (2012). Recent Trends in Indian Traditional Herbs *Syzygium Aromaticum* and its Health Benefits. *J. Pharmacognosy Phytochemistry* 1 (1), 13–22.

Bi, X., Lim, J., and Henry, C. J. (2017). Spices in the Management of Diabetes Mellitus. *Food Chem.* 217, 281–293. doi:10.1016/j.foodchem.2016.08.111

Chatterjee, D., and Bhattacharjee, P. (2015). Use of Eugenol-Lean Clove Extract as a Flavoring Agent and Natural Antioxidant in Mayonnaise: Product Characterization and Storage Study. *J. Food Sci. Technol.* 52, 4945–4954. doi:10.1007/s13197-014-1573-6

Cortés-Rojas, D. F., Fernandes de Souza, C. R., and Oliveira, W. P. (2014). Clove (*Syzygium Aromaticum*): a Precious Spice. *Asian Pac. J. Trop. Biomed.* 4 (2), 90–96. doi:10.1016/S2221-1691(14)60215-X

Dai, J. P., Zhao, X. F., Zeng, J., Wan, Q. Y., Yang, J. C., Li, W. Z., et al. (2013). Drug Screening for Autophagy Inhibitors Based on the Dissociation of Beclin1-Bcl2 Complex Using BiFC Technique and Mechanism of Eugenol on Anti-influenza A Virus Activity. *Plos One* 8, e61026. doi:10.1371/journal.pone.0061026

Daniel, A. N., Sartoretto, S. M., Schmidt, G., Caparroz-Assef, S. M., Bersani-Amado, C. A., and Cuman, R. K. N. (2009). Anti-inflammatory and Antinociceptive Activities A of Eugenol Essential Oil in Experimental Animal Models. *Rev. Bras. Farmacogn.* 19, 212–217. doi:10.1590/s0102-695x2009000200006

Dearlove, R. P., Greenspan, P., Hartle, D. K., Swanson, R. B., and Hargrove, J. L. (2008). Inhibition of Protein Glycation by Extracts of Culinary Herbs and Spices. *J. Med. Food* 11 (2), 275–281. doi:10.1089/jmf.2007.536

Devi, K. P., Sakthivel, R., Nisha, S. A., Suganthy, N., and Pandian, S. K. (2013). Eugenol Alters the Integrity of Cell Membrane and Acts against the Nosocomial Pathogen *Proteus mirabilis*. *Arch. Pharm. Res.* 36 (3), 282–292. doi:10.1007/s12272-013-0028-3

Devkota, H. P., and Adhikari-Devkota, A. (2020). "Cold Pressed Clove (*Syzygium Aromaticum*) Oil," in *Cold Pressed Oils 2020 Jan 1* (London, UK: Academic Press), 273–276.

Dey, B. K., and Mukherjee, S. S. (2021). Potential of Clove and its Nutritional Benefits in Physiological Perspective: A Review. *Int. J. Physiol. Nutr. Phys. Educ.* 6 (1), 103–106.

Ding, Y., Gu, Z., Wang, Y., Wang, S., Chen, H., Zhang, H., et al. (2017). Clove Extract Functions as a Natural Fatty Acid Synthesis Inhibitor and Prevents Obesity in a Mouse Model. *Food Funct.* 8 (8), 2847–2856. doi:10.1039/c7fo00096k

Dubey, S. (2017). Indian Spices and Their Medicinal Value. *Ijper* 51, s330–s332. doi:10.5530/ijper.51.3s.41

Dwivedi, V., Shrivastava, R., Hussain, S., Ganguly, C., and Bharadwaj, M. (2011). Comparative Anticancer Potential of Clove (Syzygium Aromaticum)-an Indian Spice-Aagainst Cancer Cell Lines of Various Anatomical Origin. *Asian Pac. J. Cancer Prev.* 12 (8), 1989–1993.

El Ghallab, Y., Al Jahid, A., Jamal Eddine, J., Ait Haj Said, A., Zarayby, L., and Derfoufi, S. (2020). *Syzygium Aromaticum* L.: Phytochemical Investigation and Comparison of the Scavenging Activity of Essential Oil, Extracts and Eugenol. *Adv. Tradit Med. (Adtm)* 20 (2), 153–158. doi:10.1007/s13596-019-00416-7

Ereifej, K. I., Feng, H., Rababah, T. M., Tashtoush, S. H., Al-U'datt, M. H., Al-Rabadi, G. J., et al. (2015). Microbiological Status and Nutritional Composition of Spices Used in Food Preparation. *Fns* 06, 1134–1140. doi:10.4236/fns.2015.612118

Estrada-Cano, C., Anaya-Castro, M. A., Munoz-Castellanos, L., Amaya-Olivas, N., Garcia-Triana, A., and Hernandez-Ochoa, L. (2017). Antifungal Activity of Microencapsulated Clove (*Eugenia Caryophyllata*) and Mexican Oregano (*Lippia Berlandieri*) Essential Oils against *Fusarium Oxysporum*. *J. Microbiol. Biochem. Tech.* 9, 567–571. doi:10.4172/1948-5948.1000342

Fankem, P. M., Kwanga, S. N., Sameza, M. L., Tchoumbougnang, F., Tchabong, R., and Ngoune, T. (2017). Antioxidant and Antifungal Activities of cocoa Butter (*Theobroma Cacao*), Essential Oil of *Syzygium Aromaticum* and a Combination of Both Extracts against Three Dermatophytes. *Am. Scientific Res. J. Eng. Tech. Sci.* 37, 255–272.

Forlemu, A. L., and Amadou, B. A. (2021). Production of Cake Using Herbs and Spices as Flavourants (Clove, Ginger, Lemongrass). *Int. J. Innovative Analyses Emerging Tech.* 1 (3), 1–7. Available at: http://openaccessjournals.eu/index.php/ijiaet/article/view/112.

Gafner, S. (2018). Scientific Journals Increasingly Skeptical of Antioxidant Research. *HerbalEGram* 117, 35.

Garba, L., Lawan, H. S., Puma, H. U., Abdullahi, M. M., Yusuf, I., and Mukhtar, M. D. (2019). Phytochemical Screening and *In Vitro* Bacteriostatic Effects of *Syzigium Aromaticum* (Clove) Extracts on Clinical Bacterial Isolates. *J. Biochem. Microbiol. Biotechnol.* 7 (1), 5–9.

Gopalakrishnan, M., Narayanan, C. S., and Mathew, A. G. (1984). Sesquiterpene Hydrocarbons from Clove Oil. *J. Lebensmittel-Wissenschaft Technologie B* 17, 42–43.

Gottardi, D., Bukvicki, D., Prasad, S., and Tyagi, A. K. (2016). Beneficial Effects of Spices in Food Preservation and Safety. *Front. Microbiol.* 7, 1394. doi:10.3389/fmicb.2016.01394

Gülçin, İ., Elmastaş, M., and Aboul-Enein, H. Y. (2012). Antioxidant Activity of Clove Oil - A Powerful Antioxidant Source. *Arabian J. Chem.* 5, 489–499. doi:10.1016/j.arabjc.2010.09.016

Güne-Bayir, A., Bilgin, M. G., Kutlu, S. S., Demirci, D., and Gölgeci, F. N. (2020). Microbiological, Chemical and Sensory Analyzes of Produced Probiotic Yoghurts Added Clove and Propolis: Probiotic Yoghurt Added Propolis and Clove. *Icontech Int. J.* 4 (2), 1–14. doi:10.46291/ICONTECHvol4iss2pp1-14

Han, X., and Parker, T. L. (2017). Anti-inflammatory Activity of Clove (*Eugenia Caryophyllata*) Essential Oil in Human Dermal Fibroblasts. *Pharm. Biol.* 55 (1), 1619–1622. doi:10.1080/13880209.2017.1314513

Haro-González, J. N., Castillo-Herrera, G. A., Martínez-Velázquez, M., and Espinosa-Andrews, H. (2021). Clove Essential Oil (Syzygium Aromaticum L. Myrtaceae): Extraction, Chemical Composition, Food Applications, and Essential Bioactivity for Human Health. *Molecules* 26, 6387. doi:10.3390/molecules26216387

Hemalatha, R., Nivetha, P., Mohanapriya, C., Sharmila, G., Muthukumaran, C., and Gopinath, M. (2016). Phytochemical Composition, GC-MS Analysis, *In Vitro* Antioxidant and Antibacterial Potential of Clove Flower Bud (*Eugenia Caryophyllus*) Methanolic Extract. *J. Food Sci. Technol.* 53 (2), 1189–1198. doi:10.1007/s13197-015-2108-5

Hussain, S., Rahman, R., Mushtaq, A., and Zerey-Belaskri, A. E. (2017). Clove: A Review of a Precious Species with Multiple Uses. *Int. J. Chem. Biochem. Sci. (Ijcbs)* 11, 129–133.

Hyldgaard, M., Mygind, T., and Meyer, R. L. (2012). Essential Oils in Food Preservation: Mode of Action, Synergies, and Interactions with Food Matrix Components. *Front. Microbiol.* 3, 12. doi:10.3389/fmicb.2012.00012

Islam, S., Nasrin, S., Khan, M. A., Hossain, A. S., Islam, F., Khandokhar, P., et al. (2013). Evaluation of Antioxidant and Anticancer Properties of the Seed Extracts of *Syzygium Fruticosum* Roxb. Growing in Rajshahi, Bangladesh. *BMC Complement. Altern. Med.* 13 (1), 142. doi:10.1186/1472-6882-13-142

Issac, A., Gopakumar, G., Kuttan, R., Maliakel, B., and Krishnakumar, I. M. (2015). Safety and Anti-ulcerogenic Activity of a Novel Polyphenol-Rich Extract of Clove Buds (*Syzygium Aromaticum* L). *Food Funct.* 6 (3), 842–852. doi:10.1039/c4fo00711e

Izuegbuna, O., Otunola, G., and Bradley, G. (2019). Estimation of Phytochemical, Vitamins Composition and Antioxidant Activity of *Pelargonium Inquinans* Leaves. *Pharmacognosy J.* 11 (2), 237. doi:10.5530/pj.2019.11.37

Jiang, T. A. (2019). Health Benefits of Culinary Herbs and Spices. *J. AOAC Int.* 102 (2), 395–411. doi:10.5740/jaoacint.18-0418

Jimoh, S. O., Arowolo, L. A., and Alabi, K. A. (2017). Phytochemical Screening and Antimicrobial Evaluation of *Syzygium Aromaticum* Extract and Essential Oil. *Int.J.Curr.Microbiol.App.Sci* 6, 4557–4567. doi:10.20546/ijcmas.2017.607.476

Jin, S., and Cho, K. H. (2011). Water Extracts of Cinnamon and Clove Exhibits Potent Inhibition of Protein Glycation and Anti-atherosclerotic Activity *In Vitro* and *In Vivo* Hypolipidemic Activity in Zebrafish. *Food Chem. Toxicol.* 49 (7), 1521–1529. doi:10.1016/j.fct.2011.03.043

Jung, C. H., Ahn, J., Jeon, T. I., Kim, T. W., and Ha, T. Y. (2012). *Syzygium Aromaticum* Ethanol Extract Reduces High-Fat Diet-Induced Obesity in Mice through Downregulation of Adipogenic and Lipogenic Gene Expression. *Exp. Ther. Med.* 4, 409–414. doi:10.3892/etm.2012.609

Kaefer, C. M., and Milner, J. A. (2008). The Role of Herbs and Spices in Cancer Prevention. *J. Nutr. Biochem.* 19 (6), 347–361. doi:10.1016/j.jnutbio.2007.11.003

Kaur, K., and Kaushal, S. (2019). Phytochemistry and Pharmacological Aspects of *Syzygium Aromaticum*: A Review. *J. Pharmacognosy Phytochemistry* 8 (1), 398–406.

Kello, M., Takac, P., Kubatka, P., Kuruc, T., Petrova, K., and Mojzis, J. (2020). Oxidative Stress-Induced DNA Damage and Apoptosis in Clove Buds-Treated MCF-7 Cells. *Biomolecules* 10 (1), 139. doi:10.3390/biom10010139

Khan, F. A., Akhtar, S., Almohazey, D., Alomari, M., and Almofty, S. A. (2018). Extracts of Clove (*Syzygium Aromaticum*) Potentiate FMSP-Nanoparticles Induced Cell Death in MCF-7 Cells. *Int. J. Biomater.* 2018, 8479439. doi:10.1155/2018/8479439

Kiani, Z., Hassanpour-Fard, M., Asghari, Z., and Hosseini, M. (2018). Experimental Evaluation of a Polyherbal Formulation (Tetraherbs): Antidiabetic Efficacy in Rats. *Comp. Clin. Pathol.* 27 (6), 1437–1445. doi:10.1007/s00580-018-2755-9

Kochhar, K. P. (2008). Dietary Spices in Health and Diseases (II). *Indian J. Physiol. Pharmacol.* 52 (4), 327–354.

Kubatka, P., Uramova, S., Kello, M., Kajo, K., Kruzliak, P., Mojzis, J., et al. (2017). Antineoplastic Effects of Clove Buds (*Syzygium Aromaticum* L.) in the Model of Breast Carcinoma. *J. Cel Mol Med* 21, 2837–2851. doi:10.1111/jcmm.13197

Kumar, A., Thakur, S., Thakur, V. C., Kumar, A., Patil, S., and Vohra, M. P. (2012). Antifungal Activity of Some Natural Essential Oils against Candida Species Isolated from Blood Stream Infection. *J. Krishna Inst. Med. Sci. Univ.* 1, 61–66.

Kumar, U., Kumar, B., Bhandhari, A., and Kumar, Y. (2010). Phytochemical Investigation and Comparison of Antimicrobial Screening of Clove and Cardamom. *Int. J. Pharm. Sci. Res.* 1, 138–147.

Kunnumakkara, A. B., Koca, C., Dey, S., Gehlot, P., Yodkeeree, S., Danda, D., et al. (2009). "Traditional Uses of Spices: an Overview," in *Molecular Targets and Therapeutic Uses of Spices: Modern Uses for Ancient Medicine* Singapore: World Scientific Publishing Co. Pte.Ltd, 1–24. doi:10.1142/9789812837912_0001

Lakshmi, V. J., and Manasa, K. (2021). Various Phytochemical Constituents and Their Potential Pharmacological Activities of Plants of the Genus Syzygium. *Ajptr* 11 (2), 68–85. doi:10.46624/ajptr.2021.v11.i2.006

Litwin, N., Clifford, J., and Johnson, S. (2018). Functional Foods for Health. *Hum. Nutr.* 4, 18.

Liu, H., Schmitz, J. C., Wei, J., Cao, S., Beumer, J. H., Strychor, S., et al. (2014). Clove Extract Inhibits Tumor Growth and Promotes Cell Cycle Arrest and Apoptosis. *Oncol. Res.* 21, 247–259. doi:10.3727/096504014X13946388748910

Madsen, H. L., and Bertelsen, G. (1995). Spices as Antioxidants. *Trends Food Sci. Tech.* 6 (8), 271–277. doi:10.1016/s0924-2244(00)89112-8

Milind, P., and Deepa, K. (2011). Clove: a champion Spice. *Int. J. Res. Ayurveda Pharm.* 2 (1), 47–54.

Mittal, M., Gupta, N., Parashar, P., Mehra, V., and Khatri, M. (2014). Phytochemical Evaluation and Pharmacological Activity of *Syzygium Aromaticum*: a Comprehensive Review. *Int. J. Pharm. Pharm. Sci.* 6 (8), 67–72.

Myrie, S. B., and Jones, P. J. H. (2011). "Functional Foods and Obesity," in *Functional Foods* (Cambridge, UK: Woodhead Publishing Limited), 234–260. doi:10.1533/9780857092557.2.234

Naji, H. H., Amer, J. O., and Walaa, F. O. (2017). Analgesic and Anti-inflammatory Effects of Hydro Alcoholic Extract of (*Syzygium Aromaticum*) in Albino Mice. *Kufa J. Vet. Med. Sci.* 8 (2), 56–62.

Nam, H., and Kim, M. M. (2013). Eugenol with Antioxidant Activity Inhibits MMP-9 Related to Metastasis in Human Fibrosarcoma Cells. *Food Chem. Toxicol.* 55, 106–112.

Nassar, M. I., Gaara, A. H., El-Ghorab, A. H., Farrag, A., Shen, H., Huq, E., et al. (2007). Chemical Constituents of Clove (*Syzygium Aromaticum*, Fam. Myrtaceae) and Their Antioxidant Activity. *Revista Latinoamericana de Química.* 35 (3), 47.

Nikoui, V., Ostadhadi, S., Bakhtiarian, A., Abbasi-Goujani, E., Habibian-Dehkordi, S., Rezaei-Roshan, M., et al. (2017). The Anti-inflammatory and Antipyretic Effects of Clove Oil in Healthy Dogs after Surgery. *PharmaNutrition* 5, 52–57. doi:10.1016/j.phanu.2017.02.003

Nirmala, M. J., Durai, L., Gopakumar, V., and Nagarajan, R. (2019). Anticancer and Antibacterial Effects of a Clove Bud Essential Oil-Based Nanoscale Emulsion System. *Int. J. Nanomedicine* 14, 6439–6450. doi:10.2147/IJN.S211047

Opara, E. I., and Chohan, M. (2014). Culinary Herbs and Spices: Their Bioactive Properties, the Contribution of Polyphenols and the Challenges in Deducing Their True Health Benefits. *Int. J. Mol. Sci.* 15 (10), 19183–19202. doi:10.3390/ijms151019183

Otunola, G. A., and Afolayan, A. J. (2020). "A Review of the Anti-diabetic Activities of Ginger," in *Ginger Cultivation and its Antimicrobial and Pharmacological Potentials*. Editor H. Wang (London, UK: IntechOpen Ltd). ISBN 978-1-83880-030-7. doi:10.5772/intechopen.88899

Otunola, G. A., and Afolayan, A. J. (2015). Antidiabetic Effect of Combined Spices ofAllium sativum,Zingiber officinaleandCapsicum Frutescensin Alloxan-Induced Diabetic Rats. *Front. Life Sci.* 8 (4), 314–323. doi:10.1080/21553769.2015.1053628

Otunola, G. A., and Afolayan, A. J. (2018). *In Vitro* antibacterial, Antioxidant and Toxicity Profile of Silver Nanoparticles green-synthesized and Characterized from Aqueous Extract of a Spice Blend Formulation. *Biotechnol. Biotechnological Equipment* 32 (3), 724–733. doi:10.1080/13102818.2018.1448301

Ozdal, T., Tomas, M., Toydemir, G., Kamiloglu, S., and Capanoglu, E. (2021). "Introduction to Nutraceuticals, Medicinal Foods, and Herbs," in *Aromatic Herbs in Food 2021 Jan 1* (London, UK: Academic Press), 1–34.

Panickar, K. S. (2013). Beneficial Effects of Herbs, Spices and Medicinal Plants on the Metabolic Syndrome, Brain and Cognitive Function. *Cent. Nerv Syst. Agents Med. Chem.* 13 (1), 13–29. doi:10.2174/1871524911313010004

Paschoalinotto, B. H., Dias, M. I., Pinela, J., Pires, T. C. S. P., Alves, M. J., Mocan, A., et al. (2021). Phytochemical Characterization and Evaluation of Bioactive Properties of Tisanes Prepared from Promising Medicinal and Aromatic Plants. *Foods* 10 (2), 475. doi:10.3390/foods10020475

Pérez Gutiérrez, R. M., and Arrioja, M. W. (2021). Rapid Model to Evaluate the Anti-obesity Potential of a Combination of *Syzygium Aromaticum* (Clove) and *Cuminun Cyminum* (Cumin) on C57BL6/j Mice Fed High-Fat Diet. *J. Visualized Experiments (Jove)* 31, 173. doi:10.3791/62087

Platel, K., and Srinivasan, K. (2000a). Influence of Dietary Spices and Their Active Principles on Pancreatic Digestive Enzymes in Albino Rats. *Nahrung* 44 (1), 42–46. doi:10.1002/(SICI)1521-3803(20000101)44:1<42:AID-FOOD42>3.0.CO;2-D

Platel, K., and Srinivasan, K. (2000b). Stimulatory Influence of Select Spices on Bile Secretion in Rats. *Nutr. Res.* 20 (10), 1493–1503. doi:10.1016/s0271-5317(00)80030-5

Przygodzka, M., Zieliński, H., Ciesarová, Z., Kukurová, K., and Lamparski, G. (2015). Study on Sensory Quality, Antioxidant Properties, and Maillard Reaction Products Formation in rye-buckwheat Cakes Enhanced with Selected Spices. *J. Chem.* 2015, 1–9. Article ID 418639. doi:10.1155/2015/418639

Pulikottil, S. J., and Nath, S. (2015). Potential of Clove of *Syzygium Aromaticum* in Development of a Therapeutic Agent for Periodontal Disease: A Review. *South Afr. Dental J.* 70 (3), 108–115.

Quilez, J., Garcia-Lorda, P., and Salas-Salvadó, J. (2003). Potential Uses and Benefits of Phytosterols in Diet: Present Situation and Future Directions. *Clin. Nutr.* 22 (4), 343–351. doi:10.1016/s0261-5614(03)00060-8

Radünz, M., da Trindade, M. L., Camargo, T. M., Radünz, A. L., Borges, C. D., Gandra, E. A., et al. (2019). Antimicrobial and Antioxidant Activity of Unencapsulated and Encapsulated Clove (*Syzygium Aromaticum*, L.) Essential Oil. *Food Chem.* 276, 180–186. doi:10.1016/j.foodchem.2018.09.173

Rahajaan, J. D. (2018). The Meaning of Nutmeg and Clove north Maluku in Popular Culture. *Balong Int. J. Des.* 1 (1), 897–906. doi:10.25134/balong.v1i1.1198

Ramos, L. R., Santos, J. S., Daguer, H., Valese, A. C., Cruz, A. G., and Granato, D. (2017). Analytical Optimization of a Phenolic-Rich Herbal Extract and Supplementation in Fermented Milk Containing Sweet Potato Pulp. *Food Chem.* 221, 950–958. doi:10.1016/j.foodchem.2016.11.069

Rosarior, V. L., Lim, P. S., Wong, W. K., Yue, C. S., Yam, H. C., and Tan, S.-A. (2021). Antioxidant-rich Clove Extract, a strong Antimicrobial Agent against Urinary Tract Infections-Causing Bacteria *In Vitro*. *Tlsr* 32 (2), 45–63. doi:10.21315/tlsr2021.32.2.4

Sachan, A. K., Kumar, S., Kumari, K., and Singh, D. (2018). Medicinal Uses of Spices Used in Our Traditional Culture: Worldwide. *J. Med. Plants Stud.* 6 (3), 116–122.

Saeed, M., Khan, M. S., Alagawany, M., Farag, M. R., Alqaisi, O., Aqib, A. I., et al. (2021). Clove (Syzygium Aromaticum) and its Phytochemicals in Ruminant Feed: an Updated Review. *Rendiconti Lincei. Scienze Fisiche e Naturali* 32, 273–285. doi:10.1007/s12210-021-00985-3

Salama, M., El-Ahwany, A. M., Elkenany, E. T., and Abdeldaim, A. A. (2018). An *In Vitro* Study on Antimicrobial and Anticancer Potentiality of Thyme and Clove Oils. *Rendiconti Lincei. Scienze Fisiche e Naturali.* 29 (1), 131–139. doi:10.1007/s12210-018-0672-0

Sammy, J. F., Emine, D. G. T., and Nwachuku, E. O. (2020). Evaluation of Anti-diabetic, Hepatoprotective and Antilipidemic Potentials of *Syzygium Aromaticum* (Clove) on Albino Rats. *Jocamr* 11 (1), 38–50. doi:10.9734/jocamr/2020/v11i130178

Schlieck, T. M., Petrolli, T. G., Bissacotti, B. F., Copetti, P. M., Bottari, N. B., Morsch, V. M., et al. (2021). Addition of a Blend of Essential Oils (Clove, Rosemary and Oregano) and Vitamin E to Replace Conventional Chemical Antioxidants in Dog Feed: Effects on Food Quality and Health of Beagles. *Arch. Anim. Nutr.* 75 (5), 389–403. doi:10.1080/1745039X.2021.1960091

Sgorbini, B., Bicchi, C., Cagliero, C., Cordero, C., Liberto, E., and Rubiolo, P. (2015). Herbs and Spices: Characterization and Quantitation of Biologically-Active Markers for Routine Quality Control by Multiple Headspace Solid-phase Microextraction Combined with Separative or Non-separative Analysis. *J. Chromatogr. A.* 1376, 9–17. doi:10.1016/j.chroma.2014.12.007

Shahidi, F., and Hossain, A. (2018). Bioactives in Spices, and Spice Oleoresins: Phytochemicals and Their Beneficial Effects in Food Preservation and Health Promotion. *Jfb* 3, 8–75. doi:10.31665/jfb.2018.3149

Shahidi, F. (2009). Nutraceuticals and Functional Foods: Whole versus Processed Foods. *Trends Food Sci. Tech.* 20 (9), 376–387. doi:10.1016/j.tifs.2008.08.004

Sihotang, V. B., Yang, G., Chi, X., and Huang, L. (2018). The Ethnobotanical Survey of Clove, Pepper, and Nutmeg and Their Utilization by Chinese and Indonesian People. *J. Trop. Biol. Conservation (Jtbc)* 15, 15â–27.

Srinivasan, K. (2013). Dietary Spices as Beneficial Modulators of Lipid Profile in Conditions of Metabolic Disorders and Diseases. *Food Funct.* 4 (4), 503–521. doi:10.1039/c2fo30249g

Srinivasan, K. (2005). Role of Spices beyond Food Flavoring: Nutraceuticals with Multiple Health Effects. *Food Rev. Int.* 21 (2), 167–188. doi:10.1081/fri-200051872

Suantawee, T., Wesarachanon, K., Anantsuphasak, K., Daenphetploy, T., Thien-Ngern, S., Thilavech, T., et al. (2015). Protein Glycation Inhibitory Activity and Antioxidant Capacity of Clove Extract. *J. Food Sci. Technol.* 52 (6), 3843–3850. doi:10.1007/s13197-014-1452-1

Sujanapal, P., and Kunhikannan, C. (2017). "The Genus Syzygium in Western Ghats. "*The Genus Syzygium 2017 April 7*. Boca Raton, FL: CRC Press, 15–56. doi:10.1201/9781315118772-3

Tainter, R. D., and Grenis, T. A. (1993). *Spice and Seasoning Food Science and Technology*. New York: VCH Publishers.

Tajkarimi, M. M., Ibrahim, S. A., and Cliver, D. O. (2010). Antimicrobial Herb and Spice Compounds in Food. *Food control* 21 (9), 1199–1218. doi:10.1016/j.foodcont.2010.02.003

Tapsell, L. C., Hemphill, I., Cobiac, L., Patch, C. S., Sullivan, D. R., Fenech, M., et al. (2006). Health Benefits of Herbs and Spices: the Past, the Present, the Future. *Med. J. Aust.* 185 (S4), S1–S24. doi:10.5694/j.1326-5377.2006.tb00548.x

Uddin, M. A., Shahinuzzaman, M., Rana, M. S., and Yaakob, Z. (2017). Study of Chemical Composition and Medicinal Properties of Volatile Oil from Clove Buds (*Eugenia Caryophyllus*). *Int. J. Pharm. Sci. Res.* 8 (2), 895–899. doi:10.13040/IJPSR.0975-8232

Vicidomini, C., Roviello, V., and Roviello, G. N. (2021). Molecular Basis of the Therapeutical Potential of Clove (*Syzygium Aromaticum* L.) and Clues to its Anti-COVID-19 Utility. *Molecules* 26 (7), 1880. doi:10.3390/molecules26071880

Viuda-Martos, M., Ruíz-Navajas, Y., Fernández-López, J., and Pérez-Álvarez, J. A. (2007). Chemical Composition of the Essential Oils Obtained From Some Spices Widely used in Mediterranean Region. *Acta Chim. Slov.* 54 (4), 921

Viuda-Martos, M., Sendra, E., Pérez-Alvarez, J. A., Fernández-López, J., Amensour, M., and Abrini, J. (2011). Identification of Flavonoid Content and Chemical Composition of the Essential Oils of Moroccan Herbs: Myrtle (*Myrtus Communis* L.), Rockrose (*Cistus Ladanifer* L.) and Montpellier Cistus (*Cistus Monspeliensis* L.). *J. Essent. Oil Res.* 23 (2), 1–9. doi:10.1080/10412905.2011.9700439

Wang, Y.-F., Jia, J.-X., Tian, Y.-Q., Shu, X., Ren, X.-J., Guan, Y., et al. (2018). Antifungal Effects of Clove Oil Microcapsule on Meat Products. *LWT* 89, 604–609. doi:10.1016/j.lwt.2017.11.042

Yadav, A. S., and Bhatnagar, D. (2007). Free Radical Scavenging Activity, Metal Chelation and Antioxidant Power of Some of the Indian Spices. *Biofactors* 31 (3-4), 219–227. doi:10.1002/biof.5520310309

Yadav, S., Gupta, S. K., Bharti, D., and Yogi, B. (2020). *Syzygium Aromaticum* (Clove): A Review on Various Phytochemicals and Pharmacological Activities in Medicinal Plant. *World J. Pharm. Res.* 9 (11), 349–363. doi:10.20959/wjpr202011-18663

Yassin, M. T., Al-Askar, A. A., Mostafa, A. A. F., and El-Sheikh, M. A. (2020a). Bioactivity of Syzygium Aromaticum (L.) Merr. & L.M.Perry Extracts as Potential Antimicrobial and Anticancer Agents. *J. King Saud Univ. - Sci.* 32 (8), 3273–3278. doi:10.1016/j.jksus.2020.09.009

Yassin, M. T., Mostafa, A. A., and Al-Askar, A. A. (2020b). *In Vitro* anticandidal Potency of *Syzygium Aromaticum* (Clove) Extracts against Vaginal Candidiasis. *BMC Complement. Med. Ther.* 20 (1), 25–29. doi:10.1186/s12906-020-2818-8

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

SYSTEMATIC REVIEW
published: 20 January 2022
doi: 10.3389/fphar.2021.809866

Neglected and Underutilised Crops: A Systematic Review of Their Potential as Food and Herbal Medicinal Crops in South Africa

Fhatuwani Nixwell Mudau[1]*, *Vimbayi Grace Petrova Chimonyo*[2,3], *Albert Thembinkosi Modi*[2] *and Tafadzwanashe Mabhaudhi*[2,4]*

[1]School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa, [2]Centre for Transformative Agricultural and Food Systems, School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa, [3]International Maize and Wheat Improvement Center (CIMMYT), Harare, Zimbabwe, [4]International Water Management Institute (IWMI-GH), West Africa Office, Kumasi, Ghana

The African continent harbours many native species with nutraceutical and pharmaceutical potential. This study reviewed underutilised crops in South Africa to determine their potential as food and herbal medicinal crops. Over 5,000 species have been identified and earmarked for their medical attributes in formal and informal setups. Researchers, plant breeders and policymakers have mostly ignored the development potential of these crops. Consequently, their value chains are poorly developed. In South Africa, there is a wide range of neglected and underutilised crops, which were historically popular and used by communities; however, over the years, they have lost their status within farming systems and been relegated to the status of neglected and underutilised. Recently, driven by the need to transition to more sustainable and resilient food systems, there has been renewed interest in their potential as food and herbal medicinal crops to establish new value chains that include vulnerable groups. They are now gaining global attention, and their conservation and sustainable utilisation are now being prioritized. The review confirmed that several of these crops possess nutraceutical and pharmaceutical properties, highlighting their potential for development as food and herbal medicines. However, current production levels are too low to meet the requirements for industrial development; research and development should focus on all aspects of their value chain, from crop improvement to utilisation. A transdisciplinary approach involving a wide range of actors is needed to develop the identified neglected and underutilised crops' potential as food and herbal medicinal crops and support the development of new and inclusive value chains.

Keywords: food and nutrition security (FNS), nutraceutical, orphan crops, pharmaceutical (PhC), sustainable diets

OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Jen-Tsung Chen, National University of Kaohsiung, Taiwan
Nqobile Masondo, Agricultural Research Council of South Africa (ARC-SA), South Africa
Sheikh Mansoor, Sher-I-Kashmir Institute of Medical Sciences, India
Bhakti Tanna, Gujarat Biotechnology Research Centre (GBRC), India
Nokwanda Makunga, Stellenbosch University, South Africa

***Correspondence:**
Fhatuwani Nixwell Mudau
mudauf@ukzn.ac.za
Tafadzwanashe Mabhaudhi
mabhaudhi@ukzn.ac.za

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *05 November 2021*
Accepted: *23 December 2021*
Published: *20 January 2022*

Citation:
Mudau FN, Chimonyo VGP, Modi AT and Mabhaudhi T (2022) Neglected and Underutilised Crops: A Systematic Review of Their Potential as Food and Herbal Medicinal Crops in South Africa. Front. Pharmacol. 12:809866. doi: 10.3389/fphar.2021.809866

1 INTRODUCTION

Rural communities within South Africa (SA) practise rainfed subsistence agriculture and generally derive low economic returns from farming activities (Beddington et al., 2011). Poverty, unemployment and food and nutrition insecurity are concentrated within these communities (Shackleton et al., 2008). Also, these communities are plagued by the coexistence of

undernutrition (i.e., thinness, stunting and underweight) and overnutrition (i.e., overweight and obesity) or diet-related non-communicable diseases (Tsegay et al., 2014; Govender et al., 2016; Thow et al., 2018; Dakora and Belane, 2019). This phenomenon is known as the triple burden of malnutrition (Modjadji and Madiba, 2019). Increasing agricultural activities within these communities has often been viewed as improving food and nutrition security, reducing household poverty, increasing youth employment, and promoting rural development (RSA, 2010). However, due to water scarcity and farmers being located in marginal production environments, and in part changing climate and low capacity to adapt, productivity remains low. It is argued that current agricultural activities within these communities are too mainstream and lack the necessary innovation to allow rural economic development. Given these challenges, there is a need to embrace a new paradigm that promotes context-specific best-bet agricultural technologies that can perform under climate change and meet market demands for sustainable and healthy diets. One strategy could be to diversify cropping systems to include multipurpose food and herbal medicinal plants with nutraceutical, pharmaceutical and economic potential.

Since recorded history, food and herbal medicinal plants have been an integral part of human existence and the traditional African healthcare system. Africa is endowed with huge agrobiodiversity, which herbalists and traditional healers have histrorically used in prescribing medicines for common ailments. Van Wyk (2011) reported that the "African Plant Checklist and Database Project" identified 50,136 angiosperm taxa that occur in sub-Sharan Africa (32,424 taxa in tropical Africa and 22,755 taxa in southern Africa). Africa is estimated to contain between 40 and 45,000 plants with development potential, with more than 5,000 species already used in the formal and informal market as herbal medicinal plants (Van Wyk, 2011; Tasheva and Kosturkova, 2012). Statistics from southern Africa estimates 3,000 species or 13.8% of the flora (Van Wyk and Gericke, 2000), representing 13.5% of the flora is used for herbal medicines. With increasing health awareness and dietary shifts towards healthier foods, there has been an increase in the popularity, production and marketing of functional food crops such as amaranth (*Amaranthus tricolor* L.), bush tea (*Sutherlandia frutescens* L.), honeybush tea (*Cyclopia* Vent.), ginger [*Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt] and mint (*Mentha spp* L.). These crops serve as dual purpose as functional herbal medicinal and food crops or plant-based dietary compounds for therapeutic, nutraceutical and pharmaceutical benefits.

In response, there has been an increase in food and nutrition, health, ethnobiological and ethnopharmacological research along the food-medicine continuum. These research interests have been reinforced by the overwhelming evidence linking diets and health and disease occurrences. However, most studies have primarily addressed popular therapeutic, nutraceutical and pharmaceutical remedies. They have often sidelined traditional and indigenous food crops [hereafter referred to as Neglected and underutilised functional medicinal crop species (NUFMS)]. Furthermore, several attempts to promote these crops have been met with numerous constraints, including but not limited to knowledge about their true medicinal value, poorly developed value chains and conservation practices. It is important to generate information to support the mainstreaming of NUFMS because of their potential to contribute to sustainable rural development, employment creation, food and nutrition security and improved human health and livelihoods. Research, development and innovation have a role to play in commercialising NUFMS through developing high-value products for the food and pharmaceutical industries. The study aimed to identify the range of selected functional neglected and underutilised crops found in South Africa and their nutraceutical and phytochemical properties. In addition, the study outlined a possible strategy for developing the neglected and underutilised functional medicinal crops value chain.

2 MATERIALS AND METHODS

To fulfil the objectives of this research, the methodology of the current study was structured into four phases, namely 1) a general review of key terms and definitions to be used in the review 2) a mixed-methods review of the literature to establish the current status of food and herbal medicinal plants and identify gaps to their mainstreaming, 3) systematic review to quantify the amount of knowledge on a) diversity of functional food and herbal medicinal NUS b), pharmaceutical and nutraceuticals properties, and c) priority NUFMS and 4) proposed production strategy for priority underutilised functional food and herbal medicinal crops. Details of these stages are outlined below.

Phase 1: Definition of terms

Various definitions have been used to describe NUFMS, each with a different meaning and context, causing an incoherent body of literature on these crops. This study identified correct and/or contextualised definitions of key terms in this review. Each definition of a term includes, as a rule, a short formal definition, some additional characteristics and references if available. The terms to be defined include "medicinal plants", "functional food crops", "functional medicinal plants", "Neglected and underutilised crops", "dietary supplements", "pharmaceutical", and "nutraceuticals". We used official guidelines, position papers, statements and reports of international societies, and original papers and review articles in scientific international journals as references.

Phase 2: Identifying priority functional medicinal crops

This phase of the study was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. The main databases used were SCOPUS and Web of Science. Google Scholar was also used for identifying keywords during the planning of the systematic review.

Phase 3: Status of underutilised functional medicinal crops in South Africa

For this phase, a mixed-method review approach, which included combining quantitative and qualitative research or outcomes with process studies, was used to compile the literature on the status of food and herbal medicinal crops. Where applicable, the emphasis was placed on the use of

TABLE 1 | Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
Honeybush	*Cyclopia* (Vent.) *spp*	5	South Africa	L	S	Lv	Reduce digestive problems, gives relief for arthritis and to treat diabetes, relaxation and stress relief, colic, hypertension and hypotension, chest ailments, diarrhoea, immune-boosting, blood circulation and blood cleanser, kidney ailments, diabetes, eczema (internally), stomach ailments, constipation, appetite stimulant, breastfeed (stimulate milk in the mother), provide nutrition for the baby and animals when mother's milk dries up, colds and flu, cosmetics, Contains flavonoids and polyphenols. Has anti-tumour, anti-inflammatory, anti-obesity, anti-oxidant, cardioprotective and anti-diabetic properties	Joubert et al. (2011), Joubert et al. (2008), Okole and Odhav (2004), van Wyk, (2008), Van Wyk and Viljoen (2011)
Tigernut	*Cyperus esculentus* (L.)	2	Benin	L	H	Sd	To treat colon cancer, heart disease, diabetics, obesity, gastrointestinal, Aphrodisiac, Carminative, diuretic, emmenagogue, flatulence, indigestion, diarrhoea, dysentery, excessive thirst, vein expansion, and it is gluten-free. Used as a source of crude fibre, calcium and iron	Dansi et al. (2012)
Ground Bean	*Macrotyloma geocarpum* (Harms) Maréchal and Baudet	2	Benin and Togo	L	H	Sd	Source of crude fat, arginine, amino acids, protein, calcium, potassium, Phosphorus Iron, Zinc, Lysine, Methionine, thiamine, Riboflavin, niacin, Phenylalanine, Histidine, Tryptophane	Bedi and Peter-ikechukwu (2015)
Winged bean	*Psophocarpus tetragonolobus* (L.) D.C.	1	India	L	Cl	Sd	Rich in proteins, oils, vitamins and carbohydrates. Has anti-oxidant, anti-inflammatory, anti-nociceptive, anti-bacterial, anti-fungal, anti-proliferative and cytotoxic activity	Chay et al. (2018)
Sword bean	*Canavalia gladiate* (Jacq.) DC.	1	India	L	Cl	Sd, Pd	treatment of vomiting, abdominal dropsy, kidney-related lumbago, asthma, obesity, stomach-ache, dysentery, coughs, headache, intercostal neuralgia, epilepsy, schizophrenia, inflammatory diseases and swellings. It is rich in carbohydrates, proteins, oils and minerals (K, Mg, Ca, P and S)	Vadivel et al. (2010)
Sunn hemp	*Crotalaria juncea* (L.)	1	India	L	S	F, Sh	The plant is used to purify the blood and is used to treat impetigo and psoriasis	Sangakkara et al. (2003)
Lablab	*Dolichos lablab* (L.) *or Lablab purpureus* (L.) Sweet	1	Kenya	L	Cl	Sd	—	Sennhenn et al. (2017)
Pigeon pea	*Cajanus cajan* (L.) Millsp.	3	Canada, Benin, India	L	S	Lv, St, Sd	It is a source of proteins; fibre, calcium, potassium, magnesium, phosphate	Bedi and Peter-ikechukwu, (2015), Chalwe et al. (2002), Ouma and Jeruto (2010)
Winged bean	*Psophocarpus tetragonolobus* (L.) D.C.	1	Malaysia	L	Cl	Sd	Source of peptides and treatment of ailments caused by microbes	Bhattacharjee et al. (2019)
Bambara groundnut	*Vigna subterranea* (L.) Verdc.	2	Benin, Southern Africa	L	H	Sd	Source of moisture, protein, carbohydrate, energy, crude fibre, calcium, potassium, magnesium, sodium, phosphate, iron, zinc, copper, ascorbic acid, B carotene, Lysine, methionine, thiamine,	Chibarabada et al. (2017), Chibarabada et al. (2017), Olayinka Atoyebi et al. (2017)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
							riboflavin, phenylalanine, histidine, niacin, tryptophan, valine, threonine, leucine, isoleucine, fat, ash, phenolics and flavonoids	
Velvet bean	*Mucuna pruriens* (L.) DC. var utilis	5	India	L	Cl	Sd	Treatment of cancer and microbial diseases	Ceballos et al. (2012), Janardhanan (2000), Kaizzi et al. (2004), Ojiem et al. (2007), Pugalenthi et al. (2005)
Grass pea	*Lathyrus sativus* (L.)	1	—	L	Cl	Sd	It possesses many pharmacological effects included anti-oxidant, nervous, anti-diabetic, analgesic, anti-pyretic and cardioprotective effects. The current review discussed the chemical constituents and pharmacological effects of Lathyrus sativus. The oil from the seeds is a powerful and dangerous cathartic (stimulating bowel evacuation). It contains starch, cane sugar, leguminvicilin, legumelin, fixed oil, gum resin, oleoresin, alkaloids, carbohydrates, flavonoids, terpenes, phenols, tannins, vitamin C, riboflavin, carotenoids, beta-carotene, proteins and amino acid	Lambein et al. (2019)
Clusterbean	*Cyamopsis tetragonoloba* (L.) Taub	1	India	L	Cl	Sd	Source of amino acids, ascorbic acid, and Lysine used to treat heart diseases and colon cancer	Rani and Punia (2015)
Broad bean	*Vicia faba* (L.)	1	Spain	L	Cl	Sd	protein, carbohydrates, B group vitamins, and minerals, volute vitamins, folic acid, niacin, and vitamin C, dietary fibre and macro and micronutrient	Manners and van Etten (2018)
African yam bean	*Sphenostylis stenocarpa* (Hochst. ex A.Rich.) Harms	1	Benin	L	—	Sd	Source of B carotene and phenolics. Treatment of cancer	Obidiegwu et al. (2020)
Black gram	*Vigna mungo* (L.) Hepper	2	India	L	H	Sd	The seedpods are diuretic and lithotripic, and the inside of the green pods is rubbed on warts to remove them. Source for lipids, proteins, carbohydrates, amino acids, energy, fibre, ash, lysine, phenolics and leucine. Used for the treatment of cancer and heart diseases	Rathore et al. (2012)
Drumstick	*Moringa oleifera* (L.)	7	Nigeria, India, Mauritius, Spain, South Africa	T	T	Sd, Pd, Lv, R	Almost all tree parts are eaten or used as ingredients in traditional herbal medicines. This especially applies to the leaves and pods, commonly eaten in parts of India and Africa. To date, Moringa oleifera may lead to modest reductions in blood sugar and cholesterol. It may also have antioxidant and anti-inflammatory effects and protect against arsenic toxicity. In addition, it has anti-microbial, anti-oxidant, anti-cancer, cardiovascular, hepatoprotective, anti-ulcer, diuretic, anti-urolithiatic, and anti-helminthic. Its multiple pharmaceutical effects are capitalized as therapeutic remedies for various diseases in the traditional medicinal system. Moringa leaves are an excellent source of calcium, potassium Iron, magnesium, phosphorus, zinc, vitamin A, vitamin B1 (thiamine), B2	Gangadhar and Praseetha (2019), Abd El-Razik et al. (2019), Neergheen-Bhujun et al. (2020), Palanisamy et al. (2019), Pande et al. (2018), Singh et al. (2016), Tesfay et al. (2011)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
							(riboflavin), B3 (niacin), B-6 folate and ascorbic acid (vitamin C), oils, fatty acids, micro-macro minerals elements and various phenolics	
Baobab/African Baobab	*Adansonia digitate* (L.)	3	Benin, Ghana	T	T	Fr, Sd, R, B, St, Lv, F, Sh	To treat diabetes, cancer, diuretics, inflammatory, hypolipidemic, fever and flavonoids. It is a source of Arginine, minerals, calcium, sodium, potassium, magnesium, manganese, iron, zinc, phenolics, Vitamin A, B, C; proteins and carbohydrates	Dansi et al. (2012), De Caluwé et al. (2009), Nyadanu and Lowor (2015)
Breadfruit	*Artocarpus altilis* (Parkinson) Fosberg	2	Benin, india	T	T	—	The sources are fats, carbohydrates, proteins, crude fibre, calcium, sodium, phosphate, magnesium, zinc, manganese, potassium, copper, molybdenum, and vitamins. Assists in treating indigestion, diarrhoea, malaria, vomiting and fever. It can be used externally for wound cleaning	Gulla et al. (2020), Singh et al. (2016)
African fan palm	*Borassus aethiopum* (Mart.)	1	Benin	T	T	Fr, R	The roots may be used to treat stomach parasites, bronchitis, sore throats, and asthma. The leaves are said to be an aphrodisiac, and the sap is reported to have many uses. The African fan plant is a good source of protein, fat, ash, fibre, amino acids, aspartic acid, threonine, serine, glutamic acid, proline glycine, methionine, isoleucine, leucine, minerals, sulphur, potassium, magnesium, calcium, sodium, zinc, iron, manganese, and copper	Bolade and Bello (2006)
Blackberry	*Rubus fruticosus* (L.)	1	Benin	T	T	—	Useful in treating cancer, diarrhoea, dysentery, whooping cough, anaemia, toothache, mouth ulcer, sore throat, haemorrhoids, and minor bleeding. Virtually all parts of the plants are used traditionally to treat skin-related diseases, diabetes, diarrhoea, hypertension, cough and bronchitis. Source of protein, crude fibre, ash, carbohydrates, carotenoids, vitamin C, and minerals. Blackberry is also a good source of nutrients; manganese, copper, dietary fibre carbohydrates, zinc, magnesium, minerals, and vitamins	Dansi et al. (2012), Odebunmi and Oluwaniyi (2010)
Mulberry	*Morus alba* (L.)	2	India	T	T	Fr, Lv	Used for treating dizziness, insomnia, premature ageing, skin irritation and DM2. Contains vitamin C, zinc, calcium, iron, potassium, phosphorus, and magnesium	Zhang et al. (2018)
Physic nut	*Jatropha curcas* (L.)	1	India	T	T	Fr, Sd, R, B, St, Lv, F, Sh	Source of Ascorbic acid, phenolics, anthocyanins and flavonoids. Treatment of cancer, inflammation, and diabetes	Achten et al. (2010)
Jackfruit	*Artocarpus heterophyllus* (Lam.)	1	India	T	S	—	Presence of many secondary metabolites, including diterpenoids, sesquiterpenoids, alkaloids, flavonoids, phenols, lignans, coumarins and cyclic peptides. pharmacological activities, such as anti-inflammatory, anti-oxidant, anti-microbial, anti-viral, anti-cancer,	Kushwaha et al. (2021)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
							anti-diabetic, anti-coagulant, hepatoprotective, analgesic and abortifacient effects	
Velvet tamarind	*Dialium guineense* (Willd.)	1	Benin	T	H	—	Sources of proteins, carbohydrates and amino acids	Fandohan et al. (2010)
Marvel of Peru or four o'clock flower	*Mirabilis jalapa* (L.)	1	India	S	—	Lv, Sd, Sh, St	Treating ulcers, diarrhoea, boils, stomach-ache, boils, skin diseases and asthma. It is an anti-syphilitic, anti-bacterial and a vermifuge	Bhattacharjee et al. (2019)
Chocolate weed	*Melochia corchorifolia* (L.)	1	India	S	H	Sd, Lv, St	diuretic, purgative, and for vulnerary, aphrodisiac, reduce inflammation, For candida, chagas disease, colic, constipation, contusions, diarrhoea, dysentery, earache, oedema, eczema, freckles, herpes, hives, itch, intestinal parasites, liver problems, pain, skin problems, skin infections, syphilis, vaginal discharge, urinary insufficiency, wounds, worms	Bhattacharjee et al. (2019)
Cannabis	*Cannabis sativa* (L.)	2	Canada	S	S	Sd, L	Cannabis is commonly used for long-term or severe pain, nausea caused by chemotherapy and painful muscle spasms. Cannabis is a good source of essential fatty acids, amino acids, dietary fibre, enzymes, vitamins, proteins, carbohydrates, water, fat, trace amounts of calcium, potassium, sodium, minerals, flavonoids, carotenoids, terpenes and phytocannabinoid acids	Aderinola et al. (2019), Caplan et al. (2019)
Hibiscus or Roselle	*Hibiscus sabdariffa* (L.)	3	Egypt, India, Japan	S	H	Lv, F, Sd	Reduces inflammation treatment of pain, spasms, asthma, insomnia, depression, and loss of appetite	Al-Sayed et al. (2020), Bhattacharjee et al. (2019), Abd El-Razik et al. (2019)
Cape periwinkle; graveyard plant	*Catharanthus roseus* (L.) G.Don	1	Israel	S	H	Lv, Sh	Source of K, Mg, Mn, Fe, CHO, Zn, P and vitamin C. Used for the treatment of diabetes, indigestion, blood pressure. It also has biotic functions	Levy et al. (1983)
Cassava	*Manihot esculenta* (Crantz)	1	Philippines	S	S	R, Tu, Lv, Sh	Treatment of high blood pressure, liver diseases and fevers, mild laxative, anti-bacterial. Rich in phytochemicals like polyphenols, especially anthocyanins, polysaccharides and organic acids. Source of manganese, copper, molybdenum and ascorbic acid	Publishers (2018)
Donkey berry	*Grewia flavescens* (Juss)	2	Niger, India	S	S	B, Lv, Fr	Can be used for wound cleaning, menstrual, stomach problems. Good source of potassium, calcium, manganese, iron, copper, zinc, crude fibre, ash, and carbohydrates	Gebauer et al. (2013), Kumar et al. (2017)
Carob	*Ceratonia silique* (L.)	2	South Africa	S	S	St, Lv, F, Pd, R	Treatment of Aphrodisiac, diarrhoea, cancer, heart disease, inflammation, microbial. Provide flavonoids, tannins, and steroids	Batlle and Tous (1997), Kleynhans et al. (2018)
Cancer bush	*Sutherlandia frutescens* (L.) R.Br. *or Lessertia frutescens* (L.) Goldblatt and J.C.Manning	3	South Africa	S	S	St, Lv, F, Pd, R	Used to treat chickenpox, diabetes, cancer, menopausal symptoms, influenza, rheumatoid arthritis, peptic ulcers, anxiety, clinical depression, HIV infection and external wounds. Caner bush is amino acids, proline, and alanine	Kleynhans et al. (2018), Masenya et al. (2020), Van Wyk (2011)
Ethiopian eggplant	*Solanum aethiopicum* (L.)	1	Ghana	S	S	Fr	Treatment of cancer and diabetics	Nyadanu and Lowor (2015)
African eggplant	*Solanum macrocarpon* (L.)	2	Ghana	S	S	—	Source of fats, carbohydrates, proteins, crude fibre, Ca, Na, P, Mg, Mn, K, Fe, Zn, Cu, Mo, vitamin	Nyadanu and Lowor (2015)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
							A, Vitamin C. Treatment of diabetes, dysentery haemorrhoids, bowel movement and Blood pressure	
Bitter eggplant	*Solatium insanum,* (L.)	1	Sri Lanka	S	S	Sd, Lv, R	Source of fats, CHO, proteins, C-fibre, Ca, Na, P, Mg, Mn, K, Fe, Zn, Cu, Mo and vitamin c	Samarakoon et al. (2018)
Miracle fruit	*Synsepalum dulcificum* (Schumach. and Thonn.) William Freeman Danielferl	1	Africa	S	H	Fr	Source of carbohydrates, vitamin A, vitamin C and phenolics	Tchokponhoué et al. (2017)
Cactus pear	*Opuntia robusta* J.C.Wendl. ex Pfeiff	2	South Africa	S	S	Fr	Used for type 2 diabetes, high cholesterol, obesity, alcohol hangover, colitis, diarrhoea, and benign prostatic hypertrophy (BPH). Good source of minerals, amino acids, vitamin C, E, K, and beta-carotene, flavonoids, and antioxidants	Venter et al. (2019)
Pea eggplant	*Solanum torvum* (Sw.)	2	India	S	S	R, Fr	Source of vitamin C, Leucine, flavonoids and anthocyanin. Treatment of diabetes and gastrointestinal conditions	Bhattacharjee et al. (2019), Nyadanu and Lowor (2015)
Quinoa	*Chenopodium quinoa* C.L. Willdenow (Willd.)	1	China	P	H	Sd	Rich in fibre, minerals, antioxidants, and all nine essential amino acids. Quinoa is a good source of protein, lipids, ash, crude fibre, carbohydrate, and energy	Amjad et al. (2015)
Buck wheat	*Fagopyrum esculentum* (Moench)	3		P	H	Sd, F	Treatment of bacterial diseases	Bashir et al. (2021), Horbowicz and Obendorf (2005), Kumari and Chaudhary (2020)
Amaranth	*Amaranthus spinosus* (L.)	3	India	P	H	Lv, Sh	Amaranth is used to treat diarrhoea, ulcers, swollen mouth and throat. The leafy vegetable is rich in fibre, protein, calories, protein, carbohydrates, fat, manganese, magnesium, phosphorus, iron, selenium, copper	Bhattacharjee et al. (2019), Rastogi and Shukla (2013), Verma et al. (2017)
Amaranth	*Amaranthus tricolor* (L.)	3	India	P	H	Lv, Sh	Treatment of eczema. It is a source of flavonoids, steroids, lipids, carbohydrates, crude fibre, amino acids, minerals, protein, ash, B carotene and phenolics	Bhattacharjee et al. (2019), Rastogi and Shukla (2013), Verma et al. (2017)
Elephant foot yam	*Amorphophalus campanulatus* (Dennst.) Nicolson	1	India	RT	H	Tu	Source of crude fat, crude protein, crude fibre, Calcium, Manganese, iron, zinc, copper, Vitamin A, B-carotene, foliate, lysine and methionine. Used as a diuretic	Bhattacharjee et al. (2019)
Up yam	*Dioscorea bulbiferaa* (L.)	1	India	RT	H	Tu	Source of calcium, manganese, phosphate, iron, zinc, copper, Vitamin A, B-carotene and foliate. Used for the treatment of eczema and inflammation	Bhattacharjee et al. (2019)
Lesser yam	*Dioscorea esculenta* (Lour.) Burkill	2	India, Philippines	RT	H	Tu, St	Lesser yam is used to treat piles, dysentery, syphilis, ulcers, leprosy, diabetes, asthma, cough, and cancer. Nutritional composition includes protein, crude fibre, ash, and fat	Bhattacharjee et al. (2019)
Taro	*Colocasia esculenta* (L.) Schott	5	India, Benin, Philippines, Tanzania	RT	H	Cm, Lv, Sh, St	Treatment of diarrhoea, help control blood sugar, reduce risk of heart disease, weight loss, anti-cancer properties. Good source of protein, carbohydrates, fat, fibre, vitamins, potassium, folate, calcium, magnesium, phosphorus, and iron	Bhattacharjee et al. (2019), Grimaldi et al. (2018), Gulla et al. (2020), Okole and Odhav (2004), Singh et al. (2016), Son et al. (2021)
Greater yam	*Dioscorea alata* (L.)	2		RT	H	Tu	Treatment of ulcers, boost brain health, reduce inflammation, and	Bhattacharjee et al. (2019), Crop and Society (2019), Obidiegwu et al.

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
			India, Philippines, Sub Saharan Africa				improve blood sugar control. Source of carbohydrate, vitamin B6, copper, manganese, potassium	(2020), Osei et al. (2019), Singh et al. (2016)
Yam	*Dioscorea dumetorum* (Kunth) Pax	4	Benin, India	RT	Cl	Tu	Rich in Vitamin C as well as starch. It contains calcium, phosphorous, thiamine, riboflavin, niacin, oxalic acid, calcium oxalate, sapotoxin and flavones, apigenin and luteolin. Has Anti-microbial, Anti-hepatotoxic, Anti-cancer, Anti-Lipidperoxidative, Anti-bacterial and Anti-fungal, Anti-diabetic, Anti-melanogenic, Anthelmintic, Anti-microbial, Anti-hepatotoxic, Anti-cancer, Anti-Lipidperoxidative, anti-bacterial and Anti-fungal, Anti-diabetic, Anti-melanogenic, Anthelmintic properties	Dansi et al. (2012), Obidiegwu et al. (2020), Paul et al. (2020)
Sweet potato	*Ipomea batatas* (L.) Lam.	3	Benin, Pakistan	RT	Cr	—	Sweet potato may be used to promote gut health, treat cancer, vision, support immune system and brain function. Great source of fibre, minerals and vitamins	Dansi et al. (2012), Galvao et al. (2021), Singh et al. (2016)
Giant taro	*Alocasia macrorrhiza* (L.) G.Don	1	Philippines	RT	H	St, Lv	Provides moisture, crude fat, protein, ash, crude fibre, minerals, calcium, sodium, potassium, magnesium, manganese, phosphate, iron, zinc, copper and phenolics. It treats cancer, heart disease, diabetes, dysentery, inflammation, gonorrhoea, haemorrhages, hypertension, helminths. It is a source of tannins and flavonoids	Publishers (2018)
Ethiopian potato	*Plectranthus edulis* (Vatke) A.J.Paton	1	Ethiopia	RT	H	Tu	Used to treat urinary disorders, including bladder infection (cystitis), prostatic hyperplasia, prostate cancer, lung diseases, and other cancer. Contains fat, fibre, sodium, potassium, carbohydrates, vitamins, minerals and other essential nutrients	Gulla et al. (2020)
Wild ginger	*Siphonochilus aethiopicus* (Schweinf.) B.L.Burtt	3	South Africa	RT	H	—	Used to treat intestinal ailments, relieve stomach aches and cramps. Reduces stress, pain, anxiety. Contains fat, sodium, carbohydrates, sugars, protein, and calories	Geldenhuys, (2007), Okole and Odhav (2004), Xego et al. (2016)
Tannia	*Xanthosoma sagittifolium* (L.) Schott	4	India, Ghana, Philippines	RT	H	Cm, St, Lv, Tu, R	Source of moisture, fats, crude protein, protein, crude fibre, minerals, sodium, potassium, manganese, phosphates, zinc, iron, copper, lysine, methionine, histidine, and Isoleucine	Bhattacharjee et al. (2019), Grimaldi et al. (2018), Nyadanu and Lowor (2015), Singh et al. (2016)
White seed melon	*Cucumeropsis manni* (Naudin)	1	Benin	Cu	Cr	Sd, Fr	Juice from the fruit is mixed with other ingredients to treat the cord-relic of newborn babies until it drops off. Source of carbohydrates, proteins, essential amino acids, fatty acids, minerals, and vitamins	Onawola and Asagbra (2012)
Watermelon	*Citrullus lanatus* (Thunb.) Matsum. and Nakai	1	Benin	Cu	Cr	Sd, Fr	Used to treat urinary tract infection, alcohol poisoning, hypertension, diabetes, gonorrhoea, and diarrhoea. Good source of copper, vitamin B5, lycopene, and vitamin C	Dansi et al. (2012), Gullino et al. (2020)
Bottle gourd	*Lagenaria siceraria* (Molina) Standl.	1	China	Cu	Cr	—	It is a source of crude fats, moisture, proteins, carbohydrates,	Zhang et al. (2020)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
							energy, amino acids, minerals and Vitamin A	
Bitter gourd	*Momordica charantia* (L.)	2	India, South Africa	Cu	Cr	Fr	Provides proteins, potassium, iron and fibre. It is used in the treatment of cancer and as an aphrodisiac	Bhattacharjee et al. (2019), Kutu and Magongwa (2017)
Wax gourd	*Benincasa hispida* ((Thunb.) Cogn.)	1	India	Cu	Cr	—	Source of proteins, fibre, amino acids, ascorbic acid and B carotene	Bhattacharjee et al. (2019)
Bitter melon	*Momordica charantia* (L.)	1	Korea	Cu	Cr	Fr	Used to fight cancer, diabetes, and many infectious diseases and treat eye-related diseases. Contains calories, fat, sodium, carbohydrates, fibre, sugar, and protein	Chung et al. (2016)
Pumpkin	*Cucurbita pepo var. styriaca* (L.)	1	Iran	Cu	Cr	Sd, R, F	Source of vitamin A, B carotene, Fat, Protein, Crude fibre, Minerals, Calcium, sodium, potassium, magnesium, phosphates, iron, copper. Assists with bowel movement and treatment of helminths, liver complaints and intestinal diseases	Soltani et al. (2016)
Napier grass	*Pennisetum purpureum* (Schumach)	1	Africa	C	H	—	Treatment of cancer and viral diseases. Reduces inflammation and	Khan et al. (2010)
Pearl millet	*Cenchrus americanus* (L.) Morrone	6	Benin, Kenya, India	C	H	Sd	Treat iron deficiency anaemia, reduce blood sugar levels, aids in weight loss and microbial actions. Good source of energy, moisture, protein, fat, mineral, fibre, carbohydrate, calcium, phosphorus and iron. Control blood sugar, improve digestive health. Good source of vitamins, phosphorus, potassium, antioxidants, niacin, calcium, and iron	Avashthi et al. (2018), Dansi et al. (2012), Gulla et al. (2020), Kamble et al. (2019), Ndiku et al. (2016), Srivastava et al. (2021)
Barley	*Hordeum vulgare* (L.)	1	Himalaya	C	H	Sd	Sources of Na, P, Mg, Mn, K, Fe, Zn, Cu, Mo, Ca, and oleic, palmitic and stearic acids	Bungla et al. (2017)
Proso millet	*Panicum miliaceum* (L.)	1	Himalaya	C	H	Sd	Source of moisture, fat, protein, ash, carbohydrate, amino acid and Vitamin A	Bungla et al. (2017)
Fonio millet	*Digitaria exilis* (Kippist) Stapf	1	Benin	C	H	—	The amino acid methionine is important for the body's cartilage production. Helps strengthen nails and hair. Fonio millet is a good source of thiamine niacin, riboflavin carbohydrates, protein, fat, fibre, and iron	Dansi et al. (2012)
Foxtail millet	*Setaria italica* (L.) P. Beauvois	1	Himalaya	C	H	Sd	Good for cardiac health, regulates blood sugar level, lower blood cholesterol, improves digestion and immunity	Bungla et al. (2017)
Finger millet	*Eleusine coracana* (L.)	4	India, United States, Canada	C	H	Sd, Lv	Source of protein, carbohydrates, crude fibre, energy, thiamine, riboflavin, niacin, phenylalanine, threonine, valine, leucine and isoleucine	Bungla et al. (2017); Hall et al. (2021); Kamble et al. (2019); Singh et al. (2010)
Sorghum	*Sorghum bicolor* (L.)	2	Benin	C	H	—	Source of moisture, fat, protein, ash, carbohydrate, amino acid and Vitamin A, Source of phenolics	—
Maize	*Zea mays* (L.)	4	Philippines, Iran, Vietnam	C	H	Sh, Sd	Source of moisture, fat, protein, ash, carbohydrate, amino	Ndiku et al. (2016), Sales et al. (2018), Sarkar et al. (2020), Sharifi-Rad et al. (2016)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
Scarlet pimpernel	*Chenopodium album* (L.)	1	Egypt	H	H	—	Source of phenolics, Treat iron deficiency anaemia, reduce blood sugar levels, and microbial actions	Abd El-Razik et al. (2019)
Bush tea	*Athrixia phylicoides* DC.	5	South Africa	H	S	Lv	cleansing or purifying the blood, treating boils, headaches, infected wounds, cuts, and the solution may also be used as a foam bath. Treatment of various ailments such as boils, acne, colds, loss of voice, and throat infection as a gargle. significantly high polyphenols, tannins, antioxidants, quercetin, flavonoids, alkaloids, polysaccharides, amino acids, lipids, vitamins, and inorganic elements	De Caluwé et al. (2009), Okole and Odhav, (2004), Slabbert et al. (2019), Zonyane et al. (2019)
Sweet clover	*Melilotus officinalis* (L.) Pall	1	Egypt	H	H	—	Used to treat microbial infections and diabetes. It is a source of carbohydrates, sodium, potassium, magnesium, manganese, phosphates, iron, zinc, Vitamin A	Abd El-Razik et al. (2019)
Lemongrass	*Cymbopogon flexuosus* (Nees ex Steud.) W.Watson	2	India, Saudi Arabia	H	H	Fr	Used to treat stomach and intestinal spasms, ache, high blood pressure, convulsion, pain, vomiting, cough, achy joint, and fever. Rich in minerals, various essential nutrients and vitamins	Dagar et al. (2013), Sujatha et al. (2011)
Rapeseed/Sarson	*Brassica napus* (L.)	2	United States, India	H	H	—	Source of moisture, fat, CHO, protein, fibre, Fe, Zn, B carotene, lysine, phenolics and flavonoids	Saroop and Kaul, (2015), Velasco et al. (2007)
Water hyssop	*Bacopa monnieri* (L.) Pennell	2	India	H	H	Lv	Contains powerful antioxidants, may reduce inflammation, boost brain function, reduce ADHD symptoms, may prevent anxiety and stress. Source of carbohydrates, fat, proteins, and minerals	Bhattacharjee et al. (2019), Verma et al. (2017)
Safflower	*Carthamus tinctorius* (L.)	2	China, Pakistan	H	H	Sd, F	Used to traditionally treat painful joints, trauma, dysmenorrhea, amenorrhea, postpartum, and abdominal pain. The nutritional value includes copper, tryptophan, fat, vitamin B1, and phosphorus	Koley et al. (2020), Liu et al. (2016)
Fennel flower	*Nigella sativa* (L.)	1	Iran, Egypt	H	H	R, Sh	Treatment of diabetes and gastro-intestinal bowel movement. It is used as an aphrodisiac, diuretic and assists with indigestion	Al-Sayed et al. (2020), Abd El-Razik et al. (2019), Srivastava et al. (2021)
Ladies' fingers or Okra	*Abelmoschus esculentus* (L.) Moench	1	Africa, India, Iran	H	H	Sd, R, St, Fr	An infusion of the root is used to treat syphilis, the juice of the roots is used to treat cuts, wounds, and boils. Good source of calories fats, sodium, potassium, carbohydrates, protein, dietary fibre, proteins, vitamins, and iron	Okole and Odhav (2004), Singh et al. (2016), Xavier et al. (2019)
Plantain	*Plantago major* (L.)	3	Sweden, Egypt	H	H	Lv	Helps with memory. Sources of carbohydrates. fibre, Ca, K, P, Fe, Cu, Vitamin A	Abd El-Razik et al. (2019), Paul et al. (2020), Zubair et al. (2011)
Toothache plant	*Acmella oleracea* (L.) R.K.Jansen	1	Benin	H	H	—	Used to treat toothache, throat and gum infections, stomach, diuretic, dry mouth, and gastric ulcers. Good source of fat, carbohydrates, nitric oxide, and hydroxytoluene	Dansi et al. (2012)
Creeping woodsorrel	*Oxalis corniculata* (L.)	1	India	H	H	Lv	Source of proteins, moisture, carbohydrates, minerals, Ca, Mg, Na, Mn, P, Fe, vitamin A, Vitamin C, B carotene, oleic and palmitic acid. Treatment of heart disease, cancer, inflammation, blood pressure,	Bhattacharjee et al. (2019)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
							hypertension, ulcers, tannins and flavonoids	
Chinese water chestnut	*Eleocharis dulcis* (Burm.f.) Trin. ex Hensch	1	India	H	H	Tu	Source of fats and lipids, crude proteins, energy, crude fibre, calcium, manganese, sodium, potassium, iron, zinc, copper and vitamin A and C. It is used for the treatment of gastrointestinal problems, including dysentery. It is used as a diuretic and treatment of gonorrhoea and fever	Bhattacharjee et al. (2019)
Lamb's quarters	*Chenopodium album* (L.)	3	India, Egypt	LV	H	Lv, Sh	Source of flavonoids. Treatment of inflammation, cardiovascular diseases, diarrhoea and helminths	Bhattacharjee et al. (2019), Abd El-Razik et al. (2019), Verma et al. (2017)
False sesame	*Ceratotheca sesamoides* (Endl.)	1	Benin	LV	H	Lv	Source of proteins, carbohydrates and amino acids	—
Black sesame	*Sesamum radiatum* (Schumach. and Thonn)	1	Benin	LV	H	Sd, Lv	Sources of carbohydrates. fibre, Ca, K, P, Fe, Cu, Vitamin A, Vitamin C, ascorbic acid and B carotene	Dansi et al. (2012)
—	*Crassocephalum rubens* (Juss. and Jacq.) S	2	Benin	LV	H	Lv	Used to treat indigestion, upset stomach, headaches, epilepsy, fresh wounds, to stop nose bleeding, swollen lips and sleeping sickness. Good source of crude protein, lipid, ash, fibre, carbohydrates, and energy	Dansi et al. (2012), Nyadanu and Lowor (2015)
Spiderflower	*Cleome gynandra* (L.)	3	Benin, India	LV	H	Lv	Source of phenolics. Treatment of cancer, viral diseases, diabetes, hypertension, inflammatory conditions, ulcers and wound cleaning	Dansi et al. (2012), Okole and Odhav (2004), Saroop and Kaul (2015)
Jute mallow	*Corchorus olitorius* (L.)	2	Benin, Ghana	LV	H	Lv, Sd	A good remedy for aches, pains, dysentery, enteritis, fever, pectoral pains, ascites, piles, and tumours. It is also a rich source of potassium, iron copper, manganese and zinc	Dansi et al. (2012), Nyadanu and Lowor (2015)
Thickhead, redflower ragleaf	*Crassocephalum crepidioides* (Benth.) S.Moore	2	Benin, Ghana	LV	H	Lv	Source of proteins, iron and potassium	Dansi et al. (2012), Nyadanu and Lowor (2015)
Wild mustard	*Brassica juncea* (L.)	1	India	LV	H	—	Source of moisture, fats, proteins, carbohydrates, fibre, minerals, Ca, Na, K, Mg, Mn, P, linoleic, oleic, Palmitic and stearic acid	Gangadhar and Praseetha (2019)
Kales	*Brassica oleracea* (L.)	4	Spain, Netherlands	LV	H	Lv	Source of proteins, lipids Cu, Fe, Zn, Na, Zn, Mg, Mn and vitamin c. Used for treating diabetes, inflammation, hypertension, Malaria, liver complaints, helminths, hepatic insufficiency and has biotic functions	Giambanelli et al. (2016), Nyadanu and Lowor, (2015), Sharifi-Rad et al. (2016), Velasco et al. (2007)
Common dandelion	*Taraxacum officinale* F. H. Wigg	1	Iran	LV	H	R, Sh	Source of moisture, fat, protein, carbohydrates, crude fibre, energy, calcium, potassium, iron, ascorbic acid, phosphorus, copper. Used in treating inflammation, blood pressure, lactogenic cancer, viral diseases and haemorrhoids. It is used as an aphrodisiac	Sharifi-Rad et al. (2016)
Purslane	*Portulaca oleracea* (L.)	1	NA	LV	H	Sd	Can be used as a febrifuge, anti-septic, vermifuge. Has a good	Srivastava et al. (2021)

(Continued on following page)

TABLE 1 | (*Continued*) Results of neglected functional medicinal crop species from the systematic review. [1]Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P) [2]Growth behaviour - Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S), [3]Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).

Common name	Scientific name	Citation frequency	Country of use	Crop type[a]	Growth behaviour[b]	Parts used[c]	Pharmaceutical and nutraceutical properties	References[d]
Hyacinthus	*Hyacinthaceae*	2	South Africa	LV	Cr	R, Fr, Sh	content in sodium, potassium, carbohydrates, protein, vitamin C Used to treat rheumatism, cardiac, urinary infection, dermatological problems, stomach, haemorrhoid, and prostate disease. Hyacinthus is a good source of crude lipids, ash, fibre, proteins and minerals; potassium, and sodium	Masondo et al. (2014), McCartan and Van Staden (1999)

[a]*Crop type -Legume (L), Herb (H), Cereal (C), Cucurbit (Cu), Root and tuber (RT), Tree (T), Shrub (S), Leafy vegetable (LV), Pseudocereal (P).*
[b]*Growth behavior—Herb (H), Creeper (Cr), Climber (Cl), Tree (T), Shrub (S).*
[c]*Plant part—Root (R), Shoot (Sh), Seed (Sd), Stem (St), Flower (Fr), Pod (Pd), Leaves (Lv), Bark (B), Corm (Cm), Tuber (Tu).*
[d]*Reference cites can be found in Supplementary information document 1*

literature from SA, with some comparisons to regional literature. Narrowing the search to SA allowed for an assessment of local knowledge relative to international knowledge on functional food and herbal medicinal crops.

Phase 4: Identify a production strategy for priority underutilised functional medicinal crops

The NUFMS industry is considered an "uncharted" economy dominated by unsustainable informal production and market systems. Since the development of NUFMS plants in South Africa is at its infancy, a value chain approach was used in developing a guideline for the commercialisation of NUFMS plants.

2.1 Search Strategy

A two-step approach was used to focus the review and provide an in-depth assessment of NUFMS nutraceutical and pharmaceutical properties and priorities.

The first stage: planning the review

In this stage, research questions were identified, a protocol was developed, and eventually, the protocol was validated to see if the approach was feasible. The research questions, publication venues, initial search strings, and publication selection criteria were also defined. When all this information was defined, the protocol was revised to see if it represented a proper review protocol.

Research questions:

1) The current review aimed to obtain insight into NUS's nutraceutical and pharmaceutical properties. Following four research questions (RQs) were defined.
2) What is the body of knowledge around the nutraceutical and pharmaceutical properties of NUFMS?
3) What are the Nutraceutical and pharmaceutical characteristics of NUFMS?
4) Which phytochemicals are associated with the Nutraceutical and Pharmaceutical characteristic of NUFMS?

Identifying keywords

The second step of the planning stage was an internal process to identify keywords, terms, and phrases used in the actual search strings. The review's objective was copied and pasted into Google scholar, and the top ten articles that appeared were downloaded and reviewed for keywords, terms and phrases. After identifying the articles, we verified the relevance and reliability of each article by searching for them in indexed databases (Scopus and Web of Science).

The ten articles were (Rigat et al., 2009; Dansi et al., 2012; Donno et al., 2015; Bhartiya et al., 2015; Campanaro et al., 2019; Deb et al., 2019; Lambein et al., 2019; Donno and Turrini, 2020; Kumari and Chaudhary, 2020; Udeh et al., 2020)

The second stage: a systematic review

We reviewed the ten articles, and a total of 34 terms (**Table 1**) were identified for use as keywords. From the review of the ten articles, it was observed that, in many instances, research articles on NUS often insinuate nutraceutical and pharmaceutical benefits without specifically mentioning the actual attribute(s). However, research articles expanded on the phytochemical characteristics that allow nutraceutical and pharmaceutical benefits later in the main text. Therefore, it was important to include *nutraceutical, pharmacological, phytochemical, pharmaceutical and medicinal*. Overall, the main objective was to articulate the nutraceutical and pharmaceutical attributes of NUFMS. Within the ten articles, we identified the following attributes: *antifungal, anti-bacterial, anti-viral, anti-mutagenic, antihepatotoxic, antiinflammatory, antihistaminic, anti-immuno-modulatory, anti-hypolipidemic, anti-diabetic, anti-convulsant, anti-carcinogenic, anti-hypolipidemic, anti-acetylcholinesterase, anti-neuropathic, anti-hypertensive, anti-analgesic, anti-helminths, anti-malaria, lactogenic, aphrodisiaque, diuretic, hepatoprotective, hypotensive, and carminative*. We also observed that these terms had variations, e.g., anti-fungal could be written as "antifungal" or "anti-fungal". These variants were also included in the search string. In the selected ten articles, it was also observed that several terms had been used to refer to neglected and underutilised crops. Using some of the identified terms and incorporating those obtained from expert knowledge, we came up with the following terms *indigenous, neglected, traditional, orphan, native, underutilis(z)ed, future, medicinal crops*, which were also used as keywords.

To address RQ 1) and 2), searches were conducted using above stated keywords and search combinations outlined in **Table 1**.

2.2 Data Mining, Analysis and Presentation

The second stage was conducting the review to identify priority NUFMS. When conducting the literature search, the cluster of key terms was systematically used in each database, and these were later combined to come up with a list of articles. The lists from the different databases were combined and duplicates removed (**Supplementary Table S1** and **Figure 1**). The list of articles was exported to Excel, where data on authors, year of publication, type of publication, crop species and type, nutraceutical and pharmacological characteristics, phytochemical properties and any other information to help answer the research questions were extracted and stored. Articles excluded from the final list included those discussing crop species not found in Africa, whose focus was very broad and not on nutraceutical and pharmacological properties of neglected and underutilised crop species and were not available in English. After that, the relevant articles were left and necessary data was extracted, the data were then subjected to bibliometric analysis. Bibliometric analysis is a quantitative method used to assess published articles and has become helpful to evaluate peer-reviewed studies in a specific field of research (Small, 1973; Lozano et al., 2019). The evolutionary trends were inferred from statistically assessing the occurrence and co-occurrence of key terms used to map trends in NUFMS using VOSviewer software. The titles and abstracts of articles in the final database (with 105 articles in **Table 2**) were used in the VOSviewer to investigate how concepts and topics have evolved over the years.

3 RESULTS AND DISCUSSION

3.1 Conceptual Definitions of Key Terms

According to Blum (2016), "correct definitions and use of terms are not just a matter of formality but is essential for emerging research." Consensus in the use of terms and definitions determine the success or failure of research, and the lack of it undermines attempts to reproduce the specific outputs, hence outcomes (Blum, 2016). Currently, there is an array of terms used in defining the medicinal properties of crops, each with a different meaning and context, and this has tended to work against NUFMS. In this section, we define and explain key terms that have been used to describe

3.1.1 Medicinal Plants

A medicinal plant contains active compounds or therapeutic properties in one or more of its organs, which can be pharmacologically beneficial to the human body (Rasool, 2012; Namdeo, 2018). Medicinal plants, also called medicinal herbs, have been discovered and used in traditional medicine practices since prehistoric times. Medicinal plants are widely used in non-industrialised societies, mainly because they are cheaper than modern medicines. Currently, the science of medicinal plants has been recognised as "alternative medicine" where synergetic effects and preventative properties have been studied (Rasool, 2012).

3.1.2 Functional Food Crops

Functional foods are defined as "a food category in which the products are either modified or fortified with substances that have a preventive or therapeutic effect beyond their original nutritional value" (Jonas and Beckmann, 1998). Functional foods can also be defined as "foods that are similar in appearance to a conventional food and possess physiological benefits or reduce the risk of chronic disease beyond basic nutritional functions" (Sawahla, 2014). Functional foods should be consumed at sufficient levels to positively impact the body (Vattem and Maitin, 2015).

3.1.3 Neglected and Underutilised Crops

Neglected and underutilised crop species (NUS) refer to crop species that were primarily grown in their native communities but are currently losing their popularity (Padulosi et al., 2002). These crops have significant potential as food and industrial crops but are marginalised, if not entirely sidelined, by researchers, breeders, policymakers, producers and traders (Mabhaudhi et al., 2017). Consequently, they have poorly developed and understood value chains. They are non-commodity crops and belong to a large, biodiverse group of domesticated, semi-domesticated or wild species and, in most instances, are locally adapted (Padulosi et al., 2013). They are cultivated in traditional systems, mostly under subsistence level, while using informal seed systems (Dansi et al., 2012). Due to the need to increase agricultural productivity, NUS have been receiving attention because of their potential in promoting food and nutrition security in marginal areas.

3.1.4 Dietary Supplement

Dietary supplements are another major nutraceutical class that includes concentrated food-derived nutrients (Phillips and Rimmer, 2013). Dietary supplements are not intended to replace food but are designed to provide added nutrients or perceived health benefits to daily food consumption.

3.1.5 Pharmaceuticals

Pharmaceuticals are therapeutic and biologically active substances used to treat diseases (Taylor, 2015). They are structures that makeup drugs that can be complex or simple aromatic molecules (Taylor, 2015). Pharmaceuticals can be packaged as capsules, tablets, liquid or gel, and the quantity taken should be within a prescribed limit; otherwise, effectiveness will be diminished.

3.1.6 Nutraceuticals

Nutraceuticals refers to the science dealing with the bioactive plant-based compounds that are isolated, packaged to be distributed in medicinal form and consumed to alter or maintain normal body functions (Das et al., 2012; Verma et al., 2017). The term also includes whole plants providing nutritional supplementation, such as honeybush tea, which provides antioxidants. The term "nutraceutical" was derived from "nutrition" and "pharmaceutical". Nutraceuticals can be used as part of the diet to prevent or treat diseases. Some countries have not adopted the term "nutraceutical"; hence other terminologies are used to describe them in that regard. For

example, in the United States of America, they are labelled as "dietary supplements" while in India, they are referred to as "foods for special dietary use". In South Africa, the term "nutraceuticals" is not recognised in its drug-control law; they are referred to as "complementary medicine" and regulated by the South African Health Products Regulatory Authority (SAHPRA).

3.2 Characteristics of Searched Literature

3.2.1 Diversity of Functional Medicinal NUCs

The NUFMS in this study were of nutritional or medicinal significance being used to treat specific ailments within communities. The study listed 226 plants belonging to 105 medicinal species sampled worldwide (**Table 1**) but found in South Africa. The medicinal plants pooled were of use in 29 countries. The regions represented by the sampled articles included West Africa (32%), North Africa (4%), East Africa (2%), Southern Africa (6%), Asia (47%), North America (3%), South America (1%), Europe (4%) and the Middle East (1%). The plants included legumes (16), pseudocereals (4), cereals (9), cucurbits (7), herbs (9), leafy vegetables (10), roots and tubers (10), shrubs (14) and trees (9). The plants presented a variety of growth behaviours, and the parts utilised also varied.

From the literature review results, South African research has focused on seven of the 105 neglected and underutilised functional medicinal crops. These include honeybush (*Cyclopia* Vent.), drumstick (*Moringa oleifera* L.), carob (*Ceratonia siliqua* L.), cancer bush (*Sutherlandia frutescens* L.), wild ginger (*Siphonochilus aethiopicus* (Schweinf.) B.L.Burtt), bitter gourd (*Momordica charantia* L.) and cactus pear (*Opuntia robusta* H.L.Wendl. ex Pfeiff.). Among these, the honey bush was the most frequently cited plant, appearing in five of the 105 articles of the sampled pool. Collectively, the plants had medicinal uses as antioxidants, anti-inflammatory and anti-cancer properties. Other ailments and conditions treated included arthritis, diabetes, helminthiasis, liver problems and fever. Nutritionally, the plants were utilised as appetite stimulants and a source of minerals. The plants include legumes (honeybush), shrubs (carob, cancer bush, cactus pear), root and tuber (wild ginger), tree (drumstick) and cucurbit (bitter gourd) (**Table 1**).

3.2.2 Main Research Themes Coming out of the Literature

In evaluating the sourced literature on NUMFS, results (**Figures 2**, **3**) showed that research into nutraceutical and pharmaceutical properties started trending globally in 2012, mostly in China and south-east Asia. During this period, the emphasis was placed on conservation. From 2014 to 2015, the period witnessed a shift from conservation to outlining the potential benefits of these crops (**Figure 3**). More recently, studies have focused on understanding the underlying mechanisms for the purported benefits. A closer look at the keywords showed three main thematic areas coming out of the source literature (**Figures 2**, **3**).

The first thematic area, denoted by the red cluster, comprised terms that highlighted the biochemical properties of NUFMS (**Supplementary Table S1**). The thematic area represented the largest cluster; the keyword antioxidant activity had the highest citation (**Figure 3**). The compounds in this cluster associated with antioxidant properties of NUFMS included phenolic compounds, minerals, carotenoids, glucosinolates and proteins. Glucosinolates and proteins are located on the cluster's periphery, indicating their role as indirect antioxidants. Glucosinolates stimulate natural antioxidant systems in the body while proteins work through inhibiting lipid oxidation. Purslane and amaranth were the only NUFMS frequently cited in the pool of research sampled for the network mapping.

The second thematic area denoted by the green cluster showed that research was still focusing on the sustainable use and production of NUFMS. The keyword "conservation" was mostly cited in articles that fell into this cluster, followed by tissue culture (**Figure 3** and **Supplementary Table S1**). Tissue culture has been used to clone desirable NUFMS genomes, including buckwheat. Buckwheat is one of the most genetically diverse NUFMS, with 10,000 accessions currently stored in various gene banks globally (Chauhan et al., 2010). In addition, its popularity has increased, based on its diverse properties, which has enabled it to be used for nutritional security, medicinal purposes, industrial and as a cash crop (Suvorova, 2016). Similar research efforts are being made to document the NUFMS in different world regions; this is one of the crucial steps in their developmental journey. For instance, tissue culture technology has opened extensive research areas for micro-propagation, secondary metabolite production and biodiversity conservation for medicinal and endangered crop species (Tasheva and Kosturkova, 2012). Other biotechnological tools such as cryopreservation and genetic transformation are important to select, multiply and conserve the critical genotypes of medicinal plants. Cryopreservation is a long-term conservation method in liquid nitrogen and allows for the conservation of endangered medicinal plants (Wang et al., 2021). Genetic transformation may be a powerful tool for enhancing the productivity of novel secondary metabolites (Narayani and Srivastava, 2017). Genetic improvement of NUFMS is also currently underway (Jayaramachandran et al., 2020). Efforts are being made to genetically engineer sesame (*Sesamum indicum* L.) to improve its productivity through mutagenesis (Jayaramachandran et al., 2020). Sesame production has been affected by inherently low productivity; the genetically modified mutant has managed to produce larger seed weight and higher seed yield. DNA technology has also been used to ascertain, confirm and trace plant origins and phylogenetic associations. Barcoding using DNA has been used to ascertain the genealogy of some legumes such as lablab [*Lablab purpureus* (L.) Sweet], marama bean [*Tylosema esculentum* (Burch.) Schreiber] and cowpea [*Vigna unguiculata* (L.) Walp] (Popoola et al., 2019).

The blue cluster, which represents the third thematic area, is associated with the role of NUFMS in food and nutritional security. The keywords in this cluster included nutrition, food security, climate change and intercropping (**Figure 3** and **Supplementary Table S1**). The research in this cluster indicates a response to climate change impacts on food production systems to ensure food and nutritional security. Intercropping, therefore, has been suggested as an intervention. According to Mabhaudhi et al. (2017), a key

strategy to adapt to a changing climate is developing and promoting underutilised crop species (in our case NUFMS). Exploiting the large reservoir of underutilised crops would provide a more diversified agricultural system and food sources necessary to address food and nutrition security concerns under climate change (Mabhaudhi et al., 2019). Furthermore, there is vast potential for these crops to increase agricultural diversification and minimise environmental degradation; this has a direct impact on climate adaptation and mitigation (Beddington et al., 2011; Mabhaudhi et al., 2019).

The rest of the clustering was species dependant e.g. *Dioscorea* and bioactive compounds; safflower (*Carthamus tinctorius* L.) and gene sequencing. The studies falling into these clusters assessed (quantitatively and qualitatively) the properties of NUFMS using biotechniques (**Figure 3** and **Supplementary Table S1**).

3.2.3 Identified Crop Types and Their Distribution

3.2.3.1 Legumes

The most cited leguminous plant included honeybush tea, velvet bean [*Mucuna pruriens* (L) DC.] and pigeon pea [*Cajanus cajan* (L.) Millsp.]. They are mostly utilised in India and Africa, growing as climbers or shrubs. Seeds are the most used parts in 60% of the legumes listed in this study. The legumes were used as dual-purpose plants to provide nutritional elements and medicinal use. Legumes such as tiger nut (*Cyperus esculentus* L.), bambara groundnut, grass pea (*Lathyrus sativus* L.), broad bean (*Vicia faba* L.) have been cited in the treatment of cardiovascular diseases, cancer, and diabetes in addition to being sources of nutritional compounds (Fasoyiro et al., 2006). Additional properties were reported in honeybush, where an increase in appetite, reduction in digestive problems, and relief for arthritis have been noticed in patients taking the tea. The elements provided for by legumes included crude fibre, crude fat, various amino acids, and mineral elements such as iron, calcium, magnesium, and phosphorus. Only ground bean [*Macrotyloma geocarpum* (Harms) Maréchal and Baudet] and pigeon pea were singularly used to provide nutrients (**Table 1**).

3.2.3.2 Cucurbit

Cucurbits were popular in Asia (Korea, India, Iran, and China) and Africa (South Africa and Benin). Most plants in this category are creepers and have a dual function of providing nutrients and treating diseases except for *Cucumeropsis mannii*, bottle gourd [*Lagenaria siceraria* (Molina) Standl.], pumpkins (*Cucurbita pepo* L.) which provide nutritional compounds only. Their seeds and fruits provide nutrients such as vitamins, carbohydrates, fibre and minerals. The ailments treated by the listed cucurbits include bowel movements, helminthes, cancer and liver disease (**Table 1**).

3.2.3.3 Leafy Vegetables

Leafy vegetables are mostly utilised in Africa (Benin, Ghana), Europe (Spain and Netherlands), Asia (India and Iran). Lamb's quarters (*Chenopodium album* L.) and kale (*Brassica oleracea* L.) were the most frequently cited leafy vegetables. The leaves were primarily consumed, while in the case of common dandelion [*Taraxacum officinale* (L.) Weber ex F.H.Wigg.], jute mallow (*Corchorus olitorius* L.), black sesame (*Sesamum radiatum* Schumach. and Thonn.), lamb's quarters' seeds, roots and shoots were utilized (**Table 1**). They have been listed as sources of beta-carotene, phenolics, carbohydrates, fatty acids and minerals (Uusiku et al., 2010). The legumes were collectively used to treat diabetes, inflammation, hypertension, malaria, liver problems, helminths, cancer, haemorrhoids and indigestion. The spider flower (*Cleome gynandra* L.) is particularly used as an aphrodisiac.

3.2.3.4 Cereals

This group is mostly associated with grasses, and their seeds are consumed primarily to provide carbohydrates. In addition, the seeds have been explored for containing lysine; minerals such as sodium, potassium, magnesium, manganese, phosphates, iron, zinc; vitamin A and B; phenolics and flavonoids (**Table 1**). The listed cereals were rarely used for medicinal purposes except for finger millet [*Eleusine coracana* (L.) Gaertn.], which treated some microbial infections and diabetes. In maize (*Zea mays* L.) and finger millet, shoots and leaves are consumed and their seeds. Cereals were reported in Africa (Benin and Kenya), Asia (India, Philippines, Iran, Vietnam) and America (Canada and United States). Maize and finger millet were the most frequently cited plants in the group.

3.2.3.5 Pseudocereals

Six articles mentioned the use of pseudocereals as NUFMS in India (n = 2), China (n = 1) and unspecified geographical locations (n = 3). Buckwheat (*Fagopyrum esculentum* Moench.) and amaranth were used medicinally to treat eczema, while *Amaranthus spinosus* (L.) was a diuretic. Nutritionally, pseudocereals were sources of flavonoids, steroids, lipids, carbohydrates, crude fibre, amino acids, minerals, protein, ash, beta-carotene and phenolics. Parts utilised included seeds, leaves, flowers and shoots.

3.2.3.6 Roots and Tubers

The geographical distribution of roots and tubers cited in articles selected for the study was confined to Asia (India and Philippines) and Africa (Ethiopia, Benin, South Africa, Tanzania). The most popular plant in the group was taro [*Colocasia esculenta* (L.) Schott] (n = 5; **Table 1**). Tubers were mostly consumed while the stem, leaves, shoots and corm were used in crops such as taro (Tattiyakul et al., 2006). Medicinal functions included use in the treatment of diarrhoea (*Dioscorea bulbifera* L.), ulcers [*Dioscorea esculenta* (Lour.) Burkill], kidney stones and appendicitis (taro). The yam [*Dioscorea dumetorum* (Kunth) Pax] has been used to treat or relieve many ailments associated with cancer, heart disease, diabetes, dysentery, inflammation, gonorrhoea, haemorrhages, and hypertension helminths. Nutritional contributions of roots and tubers included carbohydrates, crude fat, protein, crude fibre, minerals and phenolics (**Table 1**).

3.2.3.7 Shrubs

Another popular class of NUFMS comprised plants classified as shrubs (n = 25). The plants were identified in Africa (South Africa, Ghana, Sri Lanka, Niger) and Asia (India, China, Philippines). Dual-purpose species used for both medicinal and nutritive purposes included donkey berry (*Grewia flavescens* Juss), Ethiopian eggplant (*Solanum aethiopicum* L.) and miracle fruit [*Synsepalum dulcificum* (Schumach. and Thonn.) Daniell] (Hirakawa et al., 2014 and **Table 1**). Shrubs used for the sole purpose of treating ailments included cannabis (*Cannabis sativa* L.) for inflammation; cancer bush for cancer and diabetes; pea eggplant (*Solanum torvum* Schltdl.) for curbing bacterial diseases, and cassava (*Manihot esculenta* Crantz.) to clean wounds. Shrubs used to provide nutrition only included hibiscus or roselle (*Hibiscus sabdariffa* L.), which is a source of manganese, copper, molybdenum and ascorbic acid; African eggplant (*Solanum macrocarpon* L.) for provision of fats, carbohydrates, proteins, crude fibre, minerals and vitamin c; bitter eggplant (*Solatium insanum* L.) for provision of carbohydrates, vitamin A, vitamin C and phenolics (**Table 1**).

3.2.3.8 Trees

Tree species considered were from many regions, particularly West Africa (n = 7), Southern Africa (n = 2), Asia (n = 4) and Southern Europe (n = 1) (**Table 1**). The group is home to drumstick, cited most frequently (n = 7) among all the plant species considered in this study. A wide variety of plant parts were used and of note was the baobab (*Adansonia digitate* L.), which had eight parts being utilised for medicinal and nutritional purposes. These parts included fruits, seeds, roots, bark, stem, leaves, flowers and shoots (**Table 1**). Trees were used to treat diseases and conditions such as cancer, blood pressure, ulcers, inflammation, diabetes, diarrhoea, malaria and fever. Nutritional contribution by this group of plants included the provision of fats, carbohydrates, proteins, crude fibre, minerals and vitamins.

3.2.3.9 Botanical Drugs/Herbs

Plants classified as herbs were mentioned in articles mainly from Asia. Fruits, seeds, leaves, flowers and roots were used to utilise their medicinal and nutritional properties. Most herbs listed worked exclusively as either medicinal or nutritional. Medicinal herbs included lemongrass [*Cymbopogon flexuosus* (Nees ex Steud.) Will. Watson] and bush tea (*Athrixia phylicoides* DC.) for diabetes, improving bowel movements, enhancing sexual potency and as a diuretic substance and water hyssop [*Bacopa monnieri* (L.) Wettst.] to enhance memory. Herbs used for nutritional purposes were fennel flower (*Nigella sativa* L.) and ladies' fingers or okra [*Abelmoschus esculentus* (L.) Moench]. Dual-purpose herbs included safflower (*Carthamus tinctorius* L.) used as a source of flavonoids and for treatment of diarrhoea, cardiovascular diseases, helminths and diarrhoea and plantain (*Plantago major* L.) used as a source of phenolics and for treatment of cancer, viral diseases, diabetes, hypertension, inflammatory conditions, ulcers and cleaning wounds (**Table 1**).

3.3 Chemical Constituents of Functional Medicinal Crops

Plants have been used for thousands of years to flavour and conserve food, treat health disorders, and prevent diseases. Active compounds produced during secondary vegetal metabolism are usually responsible for the biological properties of some plant species used for various nutraceutical and pharmaceutical purposes. These active compounds also enhance their survival. These constituents have been identified as alkaloids (Varsha et al., 2013), glycosides (Firn, 2010), flavonoids (Varsha et al., 2013), phenolics (Puupponen-Pimiä et al., 2001), saponins (Vashist and Sharma, 2013), tannins (Varsha et al., 2013), essential oils (Canales-Martinez et al., 2008), and steroids (Madziga et al., 2010). In this section, we provide an overview of these constituents

Alkaloids contain highly active nitrogenous molecules and have anti-cancer and immune-stimulant properties (Shakkarpude et al., 2020). Examples of alkaloids found in plants include caffeine, theobromine and theophylline, classified as purine alkaloids and act as stimulants that increase heart function, vasodilation, and metabolism (Anaya et al., 2006). Alkaloids are being used to synthesise drugs, including vincristine, a common drug for leukaemia, made from Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) (Barrales-Cureño et al., 2019; Shakkarpude et al., 2020).

Bitters: The plants in this group have a characteristic bitter taste that stimulates the digestive system, including the salivary glands (Shakkarpude et al., 2020). As a result, bitters are considered effective as appetite-stimulants and ensure a well-functioning digestive system (Sahu et al., 2013)—examples of bitters are aloes, wormwood and hops.

Cardiac Glycosides: The group comprises steroids that work directly on the cardiac muscles. They have been used traditionally as poison and placed on arrows' tips for hunting (Morsy, 2017). With advances in medical technology, drugs have been synthesised with the correct levels to treat heart diseases. In addition to cardiac muscle stimulating properties, cardiac glycosides are diuretics that assist in draining fluids out of the body (Shakkarpude et al., 2020). Examples of cardiac glycosides include digitoxin, digoxin and convallotaxin. Cardiac glycosides are found in the families Apocynaceae and Asclepiadaceae (Shakkarpude et al., 2020).

Cyanogenic Glycosides: Some plants, such as the wild cherry and the elderberry, produce hydrogen cyanide as a defence mechanism that relaxes the muscle, and in high amounts, it can be highly poisonous to humans (Yulvianti and Zidorn, 2021). Some examples of cyanogenic glycoside include amygdalin, which can be isolated from bitter almonds [*Prunus dulcis* (Mill.)]. In wild cherry and elderberry, the compounds are used to pacify coughs (Shakkarpude et al., 2020).

Flavonoids: Flavonoids are broadly found in nature and comprise secondary polyphenol metabolites with a wide range of medicinal properties (Wang et al., 2018). More than 9,000 types of flavonoids have been isolated from plants so far, and these are known to have anti-viral, anti-inflammatory, cardioprotective, anti-cancer, anti-ageing properties (Wang

et al., 2018; Shakkarpude et al., 2020). Flavonoids can be found in various plants, including vegetables as quercetin and kaempferol (Ngameni et al., 2013). Chalcones, flavones, and isoflavones can be found in the *Dorstenia angusticornis* (L.) and cashew plant (*Anacardium occidentale* L.). Lemons also contain flavonoids and assist in strengthening the capillaries. They are responsible for colouring some plants characterised by red, blue, and purple pigments or yellow and white pigmentation on fruits and flowers (Ngameni et al., 2013; Shakkarpude et al., 2020).

Minerals: Minerals are acquired by plants from the soil hence are found in many plants. Minerals are responsible for ensuring general health, including disease prevention. Intake of sodium, potassium, magnesium, calcium, manganese, copper, zinc and iodine in the diet reduces the risk of cardiovascular disease (Sanchez-Castillo et al., 1998). Trace elements are significant in ensuring human metabolic processes as components of proteins such as haemoprotein and haemoglobin (Imelouane et al., 2011). Calcium is responsible for healthy teeth and bones, while sodium and potassium act as regulators. The horsetail plant [*Equisetum arvense* (L.)] is known to assist in repairing connective tissue.

Phenols: Phenolic compounds have chemopreventative properties, which enable them to be anti-carcinogenic (Huang et al., 2009). Examples of phenolic compounds found in herbal medicinal plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans and quinones. Apart from anti-cancer properties, phenolic compounds have anti-microbial properties that prevent infection (Shakkarpude et al., 2020). More than 8,000 phenolic compounds have been isolated from herbal medicinal plants, including wintergreen [*Gaultheria procumbens* (L.)], mint [*Mentha spicata* (L.)] and willow [*Salix alba* (L.)] (Tungmunnithum et al., 2018).

Polysaccharides: Polyssachaccharides are macromolecules that exist as structural molecules in plants. They exist in plants as monosaccharides in the form of starch and cellulose. Cellulose and hemicellulose are insoluble in water and bulky, and hence they assist in bowel movements. Other bioactivities of polysaccharides in herbal medicinal plants include anti-tumour activity, antioxidant activity, anti-coagulant activity, anti-diabetic activity, radioprotection effect, anti-viral activity, hypolipidemic immunomodulatory (Wang et al., 2008; Xie et al., 2016).

Proanthocyanins are compounds closely related to tannins and flavonoids, which give plants their colouring. The compounds have anti-inflammatory, antioxidant and anti-cancer properties hence are important in heart, eyes and feet health (Shakkarpude et al., 2020). Plants that contain large quantities of proanthocyanins include blackberries ad hawthorn berries. The bioavailability of proanthocyanidins is primarily influenced by polymerization, which is rendered insignificant in the gastric tract (Golovinskaia and Wang 2021).

Saponins: Saponins are secondary metabolites, surface-active glycosides with a characteristic foaming ability (Shakkarpude et al., 2020). Steroidal and triterpenoid are the two types of naturally occurring saponins. Their benefits include lowering cholesterol levels, treating diarrhoea and having anti-microbial properties (Desai et al., 2009). Plants containing high levels of saponins include agave, wild yam, and several lily family members.

Tannins: Tannins are polyphenols of a high molecular weight converted to quinones when oxidised (Pereira et al., 2015). Their bitter taste is part of the protective mechanisms in plants to protect them from herbivores. They are, however, utilised in processing leather during the tanning stage. They have a binding effect on proteins, thereby creating a protective layer that prevents microbes' action (Pereira et al., 2015). They are highly concentrated in the bark, sap, fruits and leaves of plants such as oak bark [*Quercus* (L.)] and black catechu [*Senegalia catechu* (L.f.) P.J.H.Hurter and Mabb].

Vitamins: Vitamins are compounds with many uses and are required in small amounts to maintain a constant body environment. Antioxidant vitamins, particularly Vitamin A, C and E, scavenge for free radicals in the body. Fruits and vegetables are sources of vitamins, while plants such as rose hips [*Rosa canina* (L.)] and sea buckthorn [*Hippophae rhamnoides* (L.)] are rich in vitamins B, C and E. Ascorbate or vitamin is also a cofactor for ascorbate peroxidase (Smith et al., 2007) and phylloquinone or vitamin K is involved in the electron transport chain.

Volatile oils: Volatile oils are highly complex oils extracted from plants and are used to produce essential oils. They contain more than 100 compounds (Shakkarpude et al., 2020). Essential oils have anti-microbial, anti-carcinogenic, anti-diabetic and antioxidant properties (Reddy, 2019). *Apiaceae, Lamiaceae, Myrtaceae, Poaceae*, and *Rutaceae* families are significant sources of essential oils.

Antioxidants: are molecules that inhibit the production of free radicals in the body through oxidation. Compounds that act as antioxidants include flavonoids, phenolics, sterols, alkaloids, carotenoids and glucosinolates (Saranya et al., 2017). Medicinal plants such as turmeric (*Curcuma longa* L.), cinnamon (*Cinnamomum verum* L.), onion [*Allium sativum* (L.)], ginger [*Zingiber officinale* (Roscoe.)], safflon (*Crocus sativus* (L.)], hopbush [*Dodonaea viscosa* Jacq. subsp. *angustifolia* (L.f.) J.G. West], Barleria noctiflora [*Barleria noctiflora* (L.)], cashew tree [*Anacardium occidentale* (L.)], Indian Thornapple [*Datura fastuosa* (L.)] and fever nut [*Caesalpinia bonducella* (L.) Fleming] are among many other plants that have antioxidant properties.

3.4 Pharmacological Properties

Studies to determine herbal medicinal plants' chemical profile and composition reveal the complexity and variety of compounds contributing to plants' various uses in treating numerous aliments, including life-threatening diseases such as HIV/AIDS, cancer, cardiovascular, and diabetes. Sexually transmitted infections (STIs) are among the most common reasons people use herbal medicines and visit traditional healers in South Africa. NUFMS plays an important role in providing nutrition and treating chronic diseases, especially in many indigenous communities and developing regions. Neglected and underutilised functional medicinal crop species exhibit various pharmaceutical properties that include, but are not limited to, anti-inflammatory, anti-spasmodic, antioxidative, anti-bacterial, anti-fungal, anti-cancer, anti-allergic,

hypoglycemic, analgesic, immunomodulatory, anti-stress, anti-ulcerogenic, anti-hypertensive, hepatoprotective, chemopreventive, radioprotective, anti-tumour, and anti-pyretic (**Figure 4**). The majority of the plant species identified (91.3%) had multiple pharmacological uses, with 45 species (42.4%) used in the treatment of five or more ailments, 18 species (16.5%) treating between five and three ailments, and 15 species (11.5%) treating three or fewer ailments (**Table 1**). Gastro-intestinal disorders, STIs, cold, cough and sore throat and gynaecological problems were treated with using most of the identified herbal medicinal plant species (**Table 1**). Gastro-intestinal disorders, particularly cholera, diarrhoea, and dysentery, are a major concern in South Africa and the whole region (Ribeiro et al., 2010) due to poor access to clean water, sanitation and hygiene. Sexually transmitted infections are a major public health concern in developing countries, with their transmission rate regarded as one of the highest in the world (Van Vuuren and Naidoo, 2010).

4 PRODUCTION STRATEGY FOR NEGLECTED AND UNDERUTILISED FUNCTIONAL MEDICINAL CROPS

The NUFMS industry is considered an "uncharted" economy dominated by local production and market systems, which are less organised. In response to high population growth rates, rapid urbanisation and the important cultural value placed on traditional medicines and health remedies, the demand for these plants is expected to grow (Mander et al., 2007). Currently, the demand for NUFMS plants out-strips supplies. This is evidenced by the high level of over-harvesting and extinction from the wild and resultant high prices in the market. Most crop species harvested from the wild have been considered endangered herbal medicinal plant species. On the other hand, low yielding potential has meant low productivity due to low genetic and breeding focus and poor agronomic practices. The depletion of wild stocks and low productivity have been attributed to the unsustainable access and availability of NUFMS. Concurrently, it has now been recognised that their conservation and production should be coupled with poverty alleviation initiatives for rural communities. There is a growing need to increase research and development on NUMFS since they are 1) an important plant genetic resource, 2) a resource for the synthesis of natural medicines and nutrients, and 3) support the health and nutrition of poor South Africans. The motivation to conserve and cultivate these plants should involve a socio-economic and technical approach in which attention is given to the synergistic interlinkages between economic, technical, cultural and institutional dimensions of domestication. Key activities will thus be used to formulate a strategic business plan. These will include conservation and propagation, research and development, marketing and distribution and policy.

Since the development of NUFMS plants in South Africa is at its infancy, understanding their current status using a value chain approach will be considered in developing a guideline for the commercialisation of NUFMS plants.

4.1 Understanding the Status of Functional Medicinal Crops

There is a need to improve information, the supply, marketing and production of functional medicinal plant material (Akinola et al., 2020). This is because wild plants are under extreme pressure due to increased demand from local and export markets, and the productivity of many of these crop species remains far too low for meaningful economic returns (Sivakumar et al., 2004; Tasheva and Kosturkova, 2012; Mabhaudhi et al., 2017; Akinola et al., 2020). In addition, climate variability and change have resulted in reduced availability and potency of these crop species owing to unfavourable growing conditions (Mabhaudhi et al., 2017). Therefore, the status of functional medicinal crops in SA was reviewed to identify existing gaps, opportunities, and challenges for developing future research capacity. It is hoped that a framework to transform these crops into a commercially viable enterprise can be developed through understanding the status. Also, within the context of SA, we can identify priority herbal medicinal crops. This can be achieved through the research value chain, i.e. breeding/crop improvement—production—agro-processing—marketing (**Figure 5**).

A value chain refers to the "full range of activities which are required to bring a product or service from its commencement, through the different phases of product delivery to final consumers, and final disposal after use" (Padulosi et al., 2002). Such an approach was considered ideal for the development of herbal medicinal plants. It would allow for a holistic strategy that gave equal focus on all aspects required to commercialise herbal medicinal plants (Gómez and Ricketts, 2013; Manyise and Dentoni, 2021). In the context of rural agricultural development, value-chain approaches have since become a popular strategy to encourage greater participation in national and international markets (Sharma and Chen, 2020). Such a strategy has also been proposed to promote lesser products such as NUFMS (Padulosi et al., 2002); however, little has been done to develop sustainable value chains. Value chains of NUFMS remain poorly developed, with few activities and actors (Tran et al., 2013). There is a need to identify actors and their key roles to successfully transform NUFMS into commodity crops. **Figure 5** proposes a simple food value chain. It outlines the "who", "when", and "how" considerations to guide scaling NUFMS value chains. Support activities play a pivotal role in identifying and enhancing the "value" that must be added, thus incentivising a broader spectrum of actors (Tran et al., 2013). This set of activities has been coined the "research value chain" (**Figure 5**).

The value chain approach is also accepted for developing and promoting emerging technologies across different sectors (Sharma and Chen, 2020). The lack of an explicit focus on the supportive role of research, development and innovation (RDI) may partly explain the slow progress in developing NUFMS value chains. Research, development and innovation are needed to generate evidence, and integrate indigenous knowledge; much information on NUFMS currently resides in indigenous knowledge systems. A clear and targeted RDI roadmap could unlock the potential of NUFMS. As an entry point, there is a need

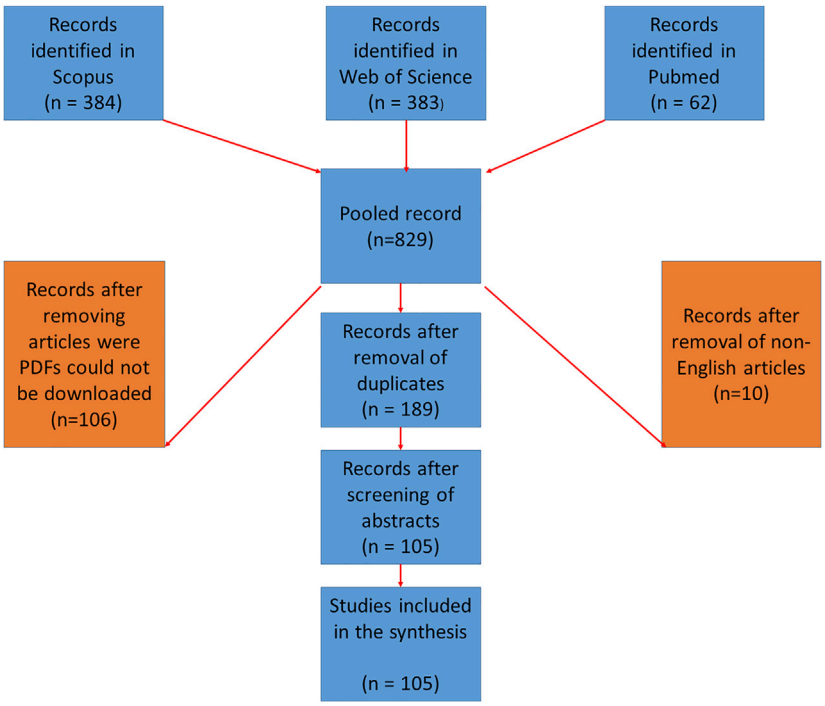


FIGURE 1 | PRISMA diagram of the selected articles for the literature synthesis.

to consider key activities for sustainable production and use of these crop species (**Figure 6**).

4.2 Conservation and Production

The initial point should be at the start-up point of the value chain, i.e., for successful and sustainable commercialisation of NUFMS to occur, the supply side has to be developed. This entails shifting from current practices of harvesting wild stocks, which are already showing signs of depletion and focussing on conservation, domestication and sustainable cultivation of herbal medicinal plants. In this regard, the following is therefore proposed:

Short-term: in the short term, there is a need to introduce conservation strategies for herbal medicinal plants, especially those flagged as vulnerable or near endangered. These strategies will ensure consistent plant genetic resources to expand the medicinal plant's industry. Therefore, collectors of NUFMS should be educated on sustainable harvesting practices (Box 1). As part of the strategy, *in situ* conservation, which entails on-site conservation through improved forms of controlled use of naturally growing plants, should be explored.

Medium-term: ex-situ conservation, i.e., off-site conservation, where part of the population is removed from a threatened habitat and placed in a new location, should be explored. This will allow for the regeneration of declining wild stocks and provide new material to meet demand while relieving wild stocks. This stage also involves the setting up and/or establishing dedicated medicinal plants nurseries in areas where targeted farmers are located.

Long-term: as a long-term strategy to conservation and meet demand, *in-domo* conservation should be explored. This would involve conservation by developing cultivation practices to conserve herbal medicinal plant species. *In-domo* conservation will form the basis for promoting the cultivation of herbal medicinal plants within smallholder farming communities across the country. This stage will require support from RDI, policymakers, civil society and community engagement to co-develop and co-implement sustainable agricultural intensification practices.

4.3 Value Addition

Value addition is often the mid-point of the value chain and includes activities from the farm gate up to distribution. It includes activities such as post-harvest handling and storage as well as agro-processing. The objective of this stage is to add to the value of the raw material and improve its utilisation. For developing countries and rural economic development, this is a key point of the value chain, which remains under-developed and may explain why farmers continue to earn less from their production. It also provides the most opportunities for setting up new agro-processing industries in rural areas, product development and supporting inclusive rural economic development. The following should be noted:

- Within the context of medicinal plants, agro-processing refers to the set of techno-economic activities carried out for conservation, production and handling of plants to make

TABLE 2 | Identified key experts and their role in the commercialisation process of medicinal plants.

Expertise	Role
Agronomist	To improve techniques for the cultivation of medicinal plants
Conservation campaigners	To persuade the public of the need to conserve medicinal plants
Ecologists	To understand the ecosystem in which medicinal plants grow
Ethnobotanists	To identify the use of plants as medicines in traditional science
Health policy-makers	To include conservation and utilisation of medicinal plants in their policy and planning
Horticulturists	To cultivate medicinal plants
Legal experts	To develop effective legal mechanisms that ensure that the collection of medicinal plants is at sustainable levels
Manufacturers	To develop processed products and by-products such as pills, lotions ointments
Nurseries	To produce and supply propagules and seedlings of medicinal plants
Pharmacognosists	To study the application of medicinal plants
Plant breeders	To breed improved strains of medicinal plants for cultivation
Plant genetic resources specialist	To assess and map the genetic variation in medicinal plants and maintain seed banks of medicinal plants
Plant pathologist	To protect the cultivated medicinal plants from pests and diseases without using dangerous chemicals
Seed biologists	To understand the germination and storage requirements of the seed of different medicinal plants
Taxonomist	To identify medicinal plants accurately
Traditional health practitioners	To provide information on the use and availability of medicinal pants

them usable as herbal medicines, food supplements and/or industrial raw material;

- Agro-processing of these plants includes all operations from the stages of cultivation, harvesting and post-harvest handling until the material reaches the end-users in the desired quality;
- Developing agro-processing capacity for NUFMS will certainly have a significant impact on the sustainable supply and marketing of a wide range of plant products;
- There is a lack of local information describing the pharmacology, post-harvest handling and storage information for the range of plants currently utilised in South Africa;
- As a starting point, this review recommends the adoption of the World Health Organisation (Organización Mundial de la Salud, and World Health Organization, 2003) guidelines on Good Agricultural and Collection Practices (GACP) and post-harvest processing for NUFMS;
- Due to the lack of robust and empirical information describing the pharmacology and post-harvest handling and storage of NUFMS, there is a need for RDI investments in this area; and
- While some knowledge already exists in indigenous knowledge (IK) mostly held by traditional healers and herbalists, this IK remains poorly documented. Research and development should initially seek to document this IK and then integrate it with scientific knowledge. The integration of IK and scientific knowledge will open new and inclusive pathways for allowing traditional healers and herbalists to participate in the knowledge economy.

An Agri-hub should be set up to address the agro-processing aspect of the value chain. However, it is critical to note that the success of the Agri-hub depends largely on RDI investments into the entry point of the value chain (i.e., conservation, propagation and propagation) as well as components of the mid-point value chain that have been discussed above.

4.4 Logistics

Logistics management is a cross-cutting aspect of value chains involved in organising, controlling and implementing operations along the value chain. It also manages the forward and backward interactions of the value chain. In this regard, logistics planning plays a critical role in the viability and sustainability of any value chain. Since value chains for herbal medicinal plants are still under-developed, the following logistical considerations should form part of the strategy:

Pre-production: there is a need to establish nurseries for priority NUFMS plants within suitable sites. These will be used as a source of plant material for supplying identified farmers and encouraging cultivation;

Production: there is a need to invest in infrastructure such as tunnels/greenhouses to support all-year round production of NUFMS plants and thus guarantee the stability of supply;

Technology: there is a need to invest in sustainable and efficient irrigation technologies as most environments in which NUFMS grow are water-scarce areas. To mitigate increasing demand for water and energy, complementary technologies such as solar pumps, rainwater harvesting and conservation and use of grey water, could be considered. This will contribute to the overall resilience of production;

Input support: in the initial phase, there is a need to support identified farmers with inputs, i.e., planting material, fertilisers, herbicides and pesticides. This will ensure yield potential is realised and that quality material is also produced. Input support should also be complemented with farmer training so that over time farmers are capable procuring own inputs and managing the enterprise; and

Post-harvest: integrated pre-and post-harvest management practices should be developed for identified priority NUFMS. Like nurseries, at least adequate packhouses should be established. Low-cost water and energy efffcient technologies such as air and solar drying and biogas digesters should be prioritised as these can easily be set up within rural communities. This will ensure that quality established during field production is maintained throughout the value chain.

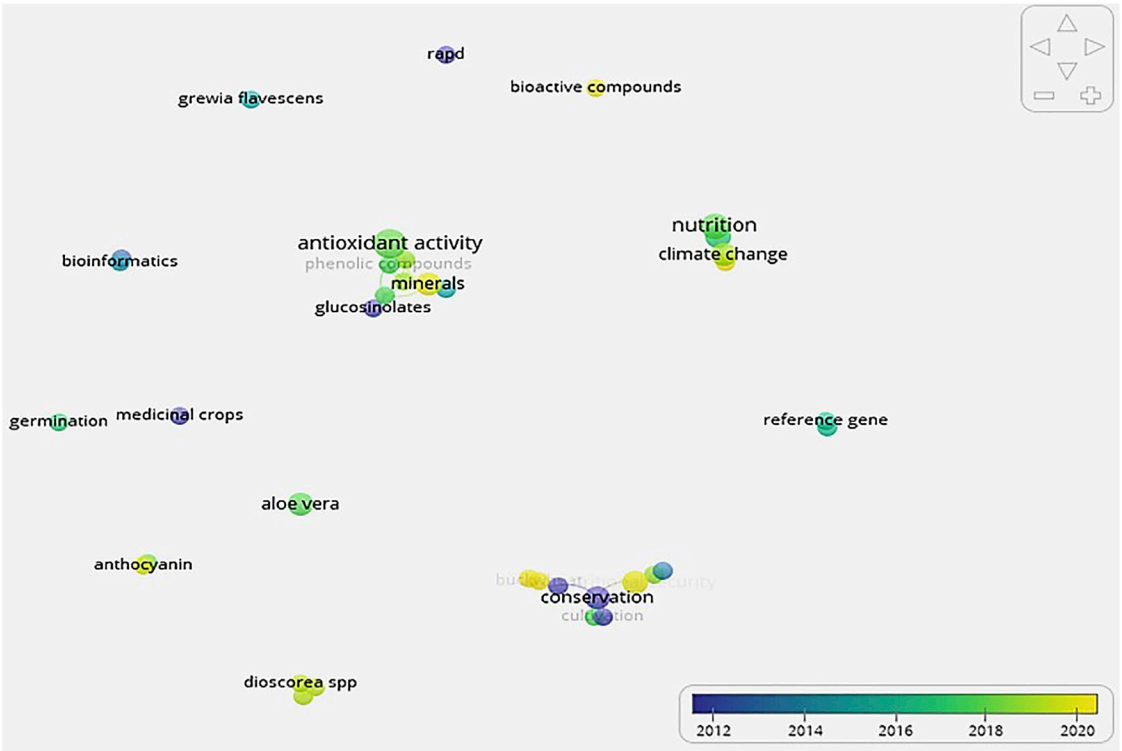


FIGURE 2 | Direction and revolution of topical concepts on neglected and underutilised functional medicinal crops (NUFMS) derived using data from titles and abstracts in VosViewer.

4.5 Setting up Cooperatives

The logistical considerations also give rise to opportunities or setting up cooperatives to address various aspects of logistics as follows:

Primary co-ops: these will be related to managing logistics linked to the start-up/entry point of the value chain. These activities include the production of herbal medicinal plants. As such, smaller cooperatives that offer geographic and economies of scale advantages, in terms of land area, could be explored as a starting point.

Secondary co-ops: could target common/shared interest areas within the appropriate location. Given that part of the strategy recommends setting up nurseries in target areas, this could be a point of formation combining the various primary co-ops in each of the areas to form a secondary co-op for managing the nurseries, and

Tertiary co-ops: this is high level and specialised co-op which will possibly manage the procurement of inputs, aggregation and distribution logistics (refrigerated trucks and packhouses).

4.6 Research, Development and Innovation for Herbal Medicinal Plants

Research, development and innovation on herbal medicinal plants is still limited due to the historical neglect of RDI investments and prioritisation that favoured a few major crops. Successful commercialisation of medicinal plants requires that RDI on NUFMS be focused on unlocking and supporting points in the value chain. In this regard, RDI on the chemical properties and value of plants and their extracts and the development of commercial, pharmaceutical, and nutritional products that may arise from the chemical analysis is required. In addition, indigenous knowledge systems linked to plants and their uses and the ecological and economic considerations relating to production should be documents and integrated with scientific knowledge. Activities that can lead to achieving these goals include:

- Continuous research into the agronomic considerations and conservation of priority medicinal plants.
- Research on traditional knowledge and medicine systems.
- Research op agro-technique, biodiversity, biotechnology and genetic improvement medicinal plants. This includes biosynthesis and metabolic assays, tissue culture and propagation, phytochemical research; plant-derived agents for cancer, immune-related diseases and hepatotoxicity
- Quality control assessment and research on active ingredients and substances of NUFMS.

4.7 Agribusiness Accelerators

There is a strong case to provide technical and agribusiness support for the farmers along with the various points of the NUFMS value chain through establishing dedicated Agribusiness Accelerators (AAs) consisting of transdisciplinary stakeholders (researchers/academics, government, private sector, civil society,

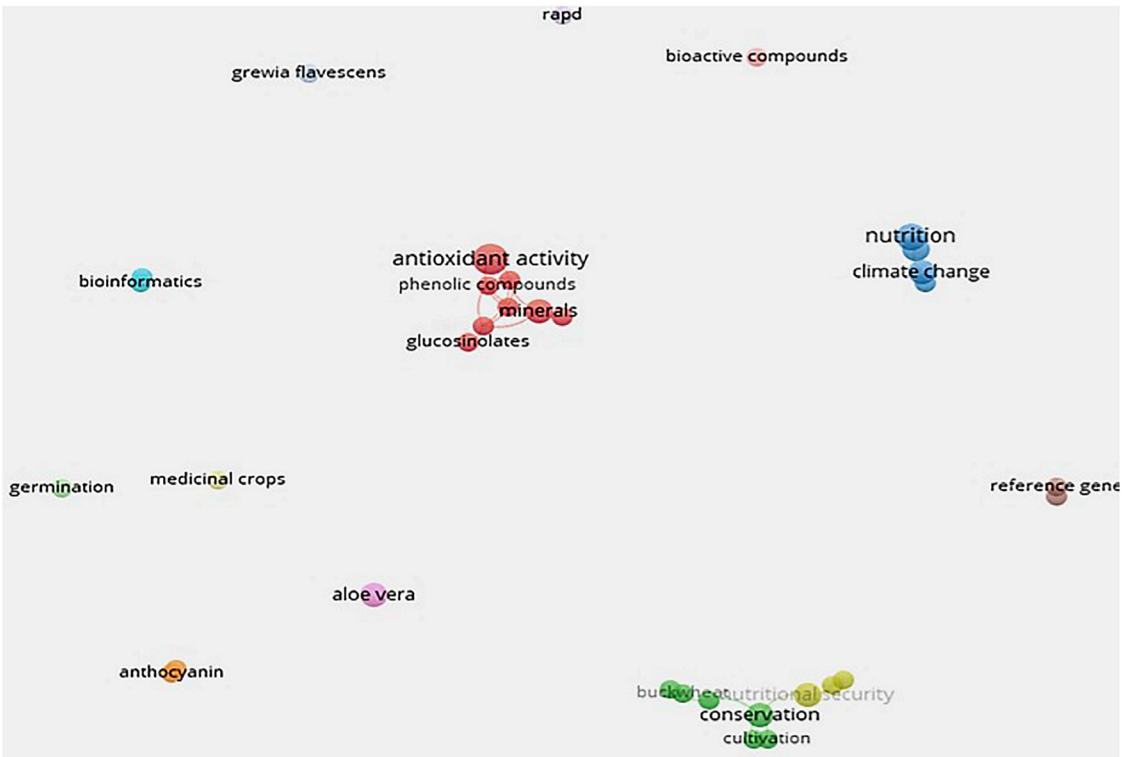


FIGURE 3 | Topical concepts on neglected and underutilised functional medicinal crops (NUFMS) derived using data from titles and abstracts in VosViewer.

communities/farmers, and traditional healers/herbalists). The role of the AAs will be as follows:

- To co-develop and co-implement an RDI roadmap for supporting the NUFMS value chain;
- To provide monitoring and evaluation support and capacity for interventions and investments made in targeted areas;
- To provide technical support geared at propagation, production, post-harvest handling and storage;
- To support the establishment of production enterprises by providing information on best management practices related to sustainable conservation and production of NUFMS, and
- To oversee capacity development of farmers by offering regular training and field visits and farmer field days. The AAs will initiate knowledge development concerning the production of NUFMS.

The role of the AA is both strategic and critical to the unpacking of any strategy, and guiding the sustainable commercialisation of NUFMS. As such, the formation of the AAs should be prioritised as it is catalytic to the overall success of the NUFMS RDI Roadmap.

4.8 Capacity Development

The lack of knowledge transfer between researchers and farmers, extension services policymakers and lobbyists is widely recognised. This is also indicative of a lack of transdisciplinarity and knowledge co-creation and co-implementation. This knowledge bottleneck may affect the successful commercialisation of herbal medicinal plants. Researchers need to translate, package, and transmit their findings for information regarding medicinal plants to reach intended beneficiaries. Research—training—deployment constitutes a 3-dimensional capacity development approach that can increase knowledge skills and experiences in the herbal medicinal plant industry. Capacity development is how individuals and organisations obtain, strengthen and maintain the capabilities to set and achieve their development objectives over time. In this regard, capacity is about the growth of individuals and/or institutes in herbal medicinal plants' knowledge, skills, and experience. For this to happen, the following is proposed

Research: Database containing medicinal plants should be created and accessible to herbal medicinal plant stakeholders. The development of such a database should involve all stakeholders to allow for knowledge co-creation, and integration of IK. After that, research findings on medicinal plants should be translated into user-friendly formats so that all stakeholders can easily locate and understand the findings. This would entail producing training guidelines, brochures, newsletters, policy briefs, seminars, media coverage and active community engagement.

Training: Stakeholders will need to be trained on effective and efficient ways to execute their roles within the value chain. Using available information, regular training workshops should be done

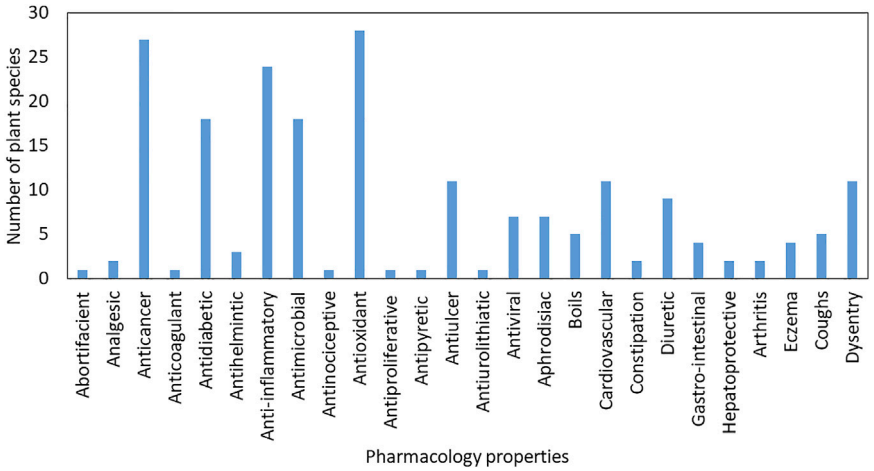


FIGURE 4 | Total frequency of articles citing pharmacological properties possessed by neglected and underutilised functional medical crops found in South Africa.

to build and strengthen capacity. The focus should be on extension officers and community heads who are centrally positioned to support other farmers. Evaluations should be done based on changes in performance, based around the four main issues: institutional arrangements, leadership, knowledge, and accountability.

4.9 Key Stakeholders

To fully support the commercialisation of medicinal plants, actors or key stakeholders who directly and indirectly participate in the value chain need to be identified and their roles clearly stated. **Table 2** identifies key experts and institutes that may be required for the successful commercialisation of medicinal plants and outlines their role.

5 PRIORITISATION OF FUNCTIONAL MEDICINAL PLANTS

The report's initial sections highlighted a wide range of NUFMS plants with potential within the nutraceutical and pharmaceutical industry. However, for allocating resources and developing targeted interventions, there is a need to streamline these and develop a priority list of NUFMS. The following key points should be noted:

1) The initial part of the report provided a list of NUFMS crops based on literature and was not exhaustive but provided a basis for prioritisation, and
2) Priority NUFMS should possess exceptional and desribale qualities (**Supplementary Table S1**) defined in terms of:
   a) *Nutritional value*: this is regarded as part of food quality and is a measure of a well-balanced ratio of the essential nutrients' carbohydrates, fat, protein, minerals, and vitamins in items of food or diet concerning the nutrient requirements of their consumer,
   b) *Pharmaceutical value*: therapeutic indications, pharmacological effects, pharmacokinetic properties, physicochemical properties, and molecular mechanisms underlying the therapeutic benefits,
   c) *Cultural value:* forms part of the core principles and ideals upon which an entire community exists and protects and relies upon for existence and harmonious relationship,
   d) *Environmental importance*—provides several regulatory and support services to ecosystems which include, but are not limited to, photosynthesis, nutrient cycling, the creation of soils, and the water cycle, pollination, decomposition, water purification, erosion and flood control, and carbon storage and climate regulation, and
   e) *Economic potential:* the crop's potential for economic development and growth and creation of surplus-value.

The list of priority NUFMS crops provided below is provisional. It still needs to be verified by relevant stakeholders such as traditional healers/herbalists, pharmaceuticals and the targeted industry, as part of a broader transdisciplinary approach.

Cyclopia spp (Honeybush tea) Currently, approximately 200 ha, mostly of *C. genistoides* and *C. subternata* shrubs, are under cultivation but cater to demand, wild harvesting, especially of *C. intermedia*, still contributes the major part of the annual production. It is estimated that 75% of honeybush tea is still harvested from the wild. Traditionally, leafy shoots and flowers were fermented and dried to prepare tea (Joubert et al., 2011). The increase in demand has placed natural populations not well-protected in jeopardy through unsustainable. Researchers have played a vital role in the "rediscovery" of this product and the development of the industry. Commercial cultivation and factory-based production have increased the access and value of honeybush tea. Extracts of the tea are gaining more scientific attention due to their phenolic composition (Agapouda et al., 2020).

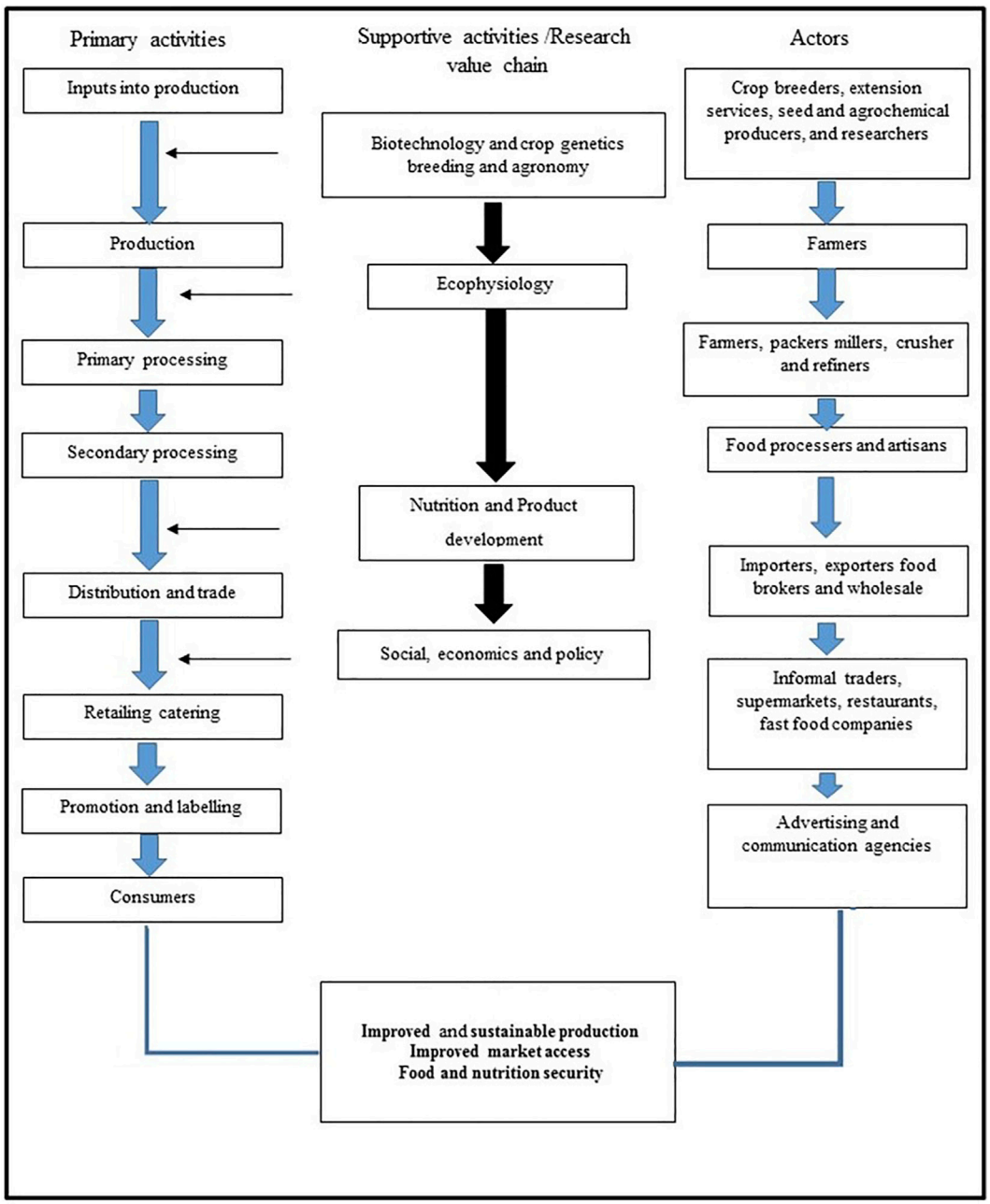


FIGURE 5 | Value chain of neglected and underutilised crops indicating primary and support activities and actors involved during the primary activities (Source: Mabhaudhi et al., 2017).

Athrixia phylicoides DC. (Bush tea) is an indigenous South Africa shrub commonly used as an anti-depressant and aphrodisiac (Ajao et al., 2019). This shrub naturally grows in semi-arid regions with limited canopy coverage, such as grassland and forest biomes of South Africa (Limpopo, Free State, KwaZulu Natal and Eastern Cape Provinces) and Swaziland (Herman et al., 2000). Traditionally, the herb is greatly treasured as a traditional herbal medicine to treat diabetes, heart disease and hypertension, acne, boils, colds, cuts, headache, infected wounds, loss of voice and throat infection (Nchabeleng et al., 2013; Mathivha et al., 2019). It is also recommended as a potent blood cleanser (Mudau, 2012) and is further substantiated by its high levels of total polyphenol content (Mudau et al., 2005). The commercialisation of bush tea is a potential prospect in developing high-value products for the beverage and pharmaceutical industries (Tshivhandekano et al., 2018). There is, however, a need for an alternative supply of plant material as wild plants are under extreme pressure due to increased demands

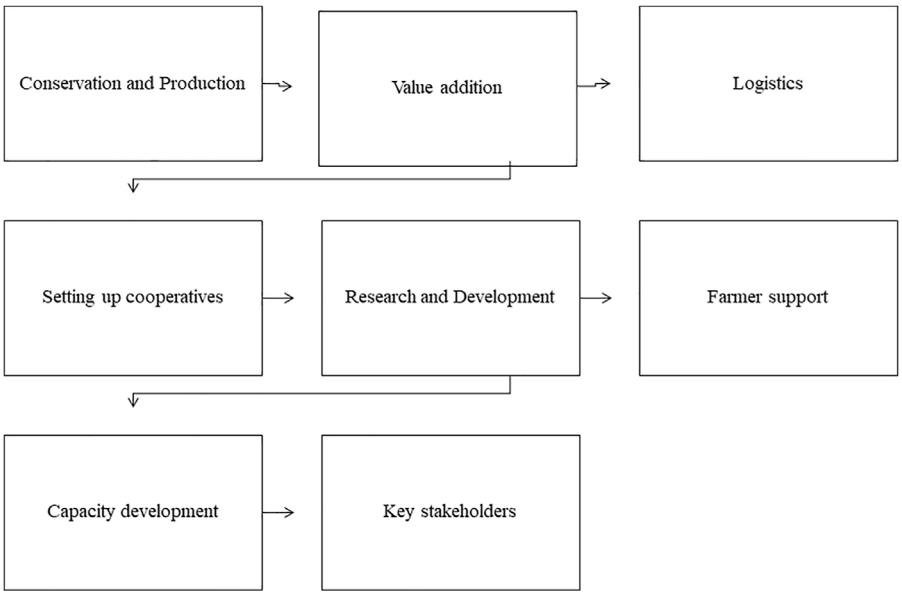


FIGURE 6 | Production strategy for neglected and underutilised functional medicinal crops.

from local and export markets. Lerotholi et al. (2017) reported that the intensive harvesting of bush tea due to the increasing demand has, in many places, resulted in overexploitation and is a serious threat to biodiversity in the region. The only option for the crop species is sustainable conservation and cultivation to mitigate the impacts of unsustainable harvesting from the wild (Cunningham, 1993).

Brassica oleracea var. acephala (Kale) is a cruciferous vegetable characterised by leaves along the stem, which, in recent years, have gained great popularity as a "superfood". Consequently, it is listed in many lists of the healthiest vegetables in popular culture. Although kale has been cultivated for several centuries and has been included in many traditional meals, especially in the Mediterranean, it has become very popular in South Africa. However, its popularity is as dichotomous as its households' current food and nutrition security status. Kale is a superfood among the health-conscious upper-class, who primarily use it as an antioxidant booster. In the poor communities of SA, kale and its timely indigenisation and increased use support the notion of food crop globalisation (Traill, 1997; Ukonu, 2016; Liu et al., 2019). This notion has been supported by the southward migration of people from Africa's central and western regions, where these leafy vegetables are considered a staple, to SA in search of employment opportunities (NEPAD (New Partnership for Africa's Development, 2018). These crops have become indigenised and remain underutilised, creating opportunities to develop new value and transformation of those already existing, supporting rural agricultural development and food and nutrition security (Mabhaudhi et al., 2019). Kale has become a very popular crop among organic farmers due to its good tolerance for wide and extreme temperature fluctuations.

Colocasia esculenta (Taro) is a herbaceous perennial herb that grows to a height of 1–2 m. The main stem is an edible starch-rich underground structure (Chivenge et al., 2015). It is called the corm, from which leaves grow upwards, roots grow downwards while corms, cormels and runners grow laterally (Son et al., 2021). The root system is fibrous and confined mainly to the top layer of the soil (Sunitha et al., 2013). Corms in the dasheen type of taro are cylindrical and large. They are up to 30 cm long and 15 cm in diameter and constitute the main edible part of the plant (Modi and Mabhaudhi, 2013). In the eddboe types, the corms are small, globoid and surrounded by several corms and cormels. The corm and cormels constitute a significant portion of the edible harvest of eddoe taro. Since taro is gluten free and has low protein, high-calorie content, and low-fat levels, taro consumption can benefit individuals with dietary restrictions such as those presenting allergies, especially in children and gluten-intolerant individuals, contributing to reducing the risk of obesity and type II diabetes.

Moringa oleifera is a common tree native to India and cultivated throughout subtropical areas from West Africa to Fiji and is a source of food and medicine (Sreelatha and Padma, 2011). In South Africa, moringa grows in the Limpopo, Free State, Mpumalanga, KwaZulu-Natal and Gauteng provinces. According to traditional African and Indian (Ayurvedic) medicine, moringa has almost 540 compounds that can treat or prevent 300 health issues (Freiberger et al., 1998; Rivas et al., 2013; Aderinola et al., 2019). In developing countries, the moringa leaf powder is commonly used as a medicinal herb rather than food, as in Asian populations. It is often taken as a supplement by HIV-infected people to enhance immunity and manage opportunistic infections. In SA, several flagship projects have been initiated due to growing interest. One example includes the Agricultural Research Council's Vegetable and Ornamental Plants group in Roodeplaat researching moringa propagation, cultivation

practices, processing, storage (shelf-life) and analysis on biological activities, safety and phytochemistry (Mashamaite et al., 2021).

6 CONCLUSION

Several neglected and underutilised crops have nutraceutical and pharmaceutical properties that make them ideal for commercialisation. Also, they can contribute to sustainable and healthy diets and sustainable agricultural intensification, making them an important resource for food systems transformation. Thus, they can deliver on multiple objectives. Currently, unsustainable harvesting and utilisation of wild populations threatens several species, which are now endangered and facing extinction. Sustainable conservation and cultivation practices are urgently needed to mitigate further population declines in the wild. Their nutraceutical and pharmaceutical properties provide a clear starting point for developing commercial food and herbal medicinal products. To effectively meet the requirements for nutraceutical and pharmaceutical development, there is a need to develop their value chains; however, limited research and development done so far limit their potential. Therefore, RDI should focus on all aspects of their value chain to unlock their commercial potential and develop new inclusive value chains. Transdisciplinary approaches are needed to ensure that these new value chains are well-supported by public and private sectors, inclusive and equitable.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

FM, VC, and AM had the original idea for the article and conceptualised the article; VC did the initial data collection and analysis. VC then led the write-up of the manuscript, and all co-authors read and revised the manuscript; AM did the critical review and redrafting; TM and FM were responsible for the funding acquisition.

FUNDING

This study forms part of the Sustainable and Healthy Food Systems (SHEFS) programme supported by the Wellcome Trust's Our Planet, Our Health programme (Grant Number: 205200/Z/16/Z), TM and AM are supported by the SHEFS programme.

ACKNOWLEDGMENTS

The research team would like to acknowledge Evelyn Tatenda Kamba for assisting with the initial manuscript draft and revisions. The Water Research Commission of South Africa is acknowledged for funding through WRC Project No. K5/2717//4 "Developing a guideline for rainfed production of underutilised indigenous crops and estimating green water use of indigenous crops based on available models within selected bio-climatic regions of South Africa' and WRC CON 2020/2021-00420 on "Water use and nutritional water productivity of bush tea". The uMngeni Resilience Project (URP), funded by the Adaptation Fund, is acknowledged for supporting AM and TM.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.809866/full#supplementary-material

REFERENCES

Abd El-Razik, T., Hendawy, S., Abouziena, H., Amer, H., and Hussein, M. (2019). Winter Weeds and its Control in the Medicinal Plants in Egypt: a Survey Study. *Egypt. Pharmaceut J.* 18, 16. doi:10.4103/epj.epj_13_18

Achten, W. M. J., Maes, W. H., Reubens, B., Mathijs, E., Singh, V. P., Verchot, L., et al. (2010). Biomass Production and Allocation in Jatropha Curcas L. Seedlings under Different Levels of Drought Stress. *Biomass and Bioenergy* 34, 667–676. doi:10.1016/j.biombioe.2010.01.010

Aderinola, T. A., Fagbemi, T. N., Enujiugha, V. N., Alashi, A. M., and Aluko, R. E. (2019). *In Vitro* antihypertensive and Antioxidative Properties of Alcalase-Derived Moringa Oleifera Seed Globulin Hydrolysate and its Membrane Fractions. *J. Food Process. Preserv.* 43, e13862. doi:10.1111/jfpp.13862

Agapouda, A., Butterweck, V., Hamburger, M., de Beer, D., Joubert, E., and Eckert, A. (2020). Honeybush Extracts (*Cyclopia Spp.*) Rescue Mitochondrial Functions and Bioenergetics against Oxidative Injury. *Oxid. Med. Cel. Longev.* 2020, 1948602–1948614. doi:10.1155/2020/1948602

Ajao, A. A., Sibiya, N. P., and Moteetee, A. N. (2019). Sexual Prowess from Nature: A Systematic Review of Medicinal Plants Used as Aphrodisiacs and Sexual Dysfunction in Sub-saharan Africa. *South Afr. J. Bot.* 122, 342–359. doi:10.1016/j.sajb.2018.08.011

Akinola, R., Pereira, L. M., Mabhaudhi, T., de Bruin, F.-M., and Rusch, L. (2020). A Review of Indigenous Food Crops in Africa and the Implications for More Sustainable and Healthy Food Systems. *Sustainability* 12, 3493. doi:10.3390/SU12083493

Al-Sayed, H. M., Hegab, S. A., Youssef, M. A., Khalafalla, M. Y., Almaroai, Y. A., Ding, Z., et al. (2020). Evaluation of Quality and Growth of roselle (Hibiscus sabdariffa L.) as Affected by Bio-Fertilizers. *J. Plant Nutr.* 43, 1025–1035. doi:10.1080/01904167.2020.1711938

Amjad, M., Akhtar, S. S., Yang, A., Akhtar, J., and Jacobsen, S.-E. (2015). Antioxidative Response of Quinoa Exposed to Iso-Osmotic, Ionic and Non-ionic Salt Stress. *J. Agro Crop Sci.* 201, 452–460. doi:10.1111/jac.12140

Anaya, A. L., Cruz-Ortega, R., and Waller, G. R. (2006). Metabolism and Ecology of Purine Alkaloids. *Front. Biosci.* 11 (1), 2354–2370. doi:10.2741/1975

Avashthi, H., Pathak, R. K., Pandey, N., Arora, S., Mishra, A. K., Gupta, V. K., et al. (2018). Transcriptome-wide Identification of Genes Involved in Ascorbate-Glutathione Cycle (Halliwell-Asada Pathway) and Related Pathway for Elucidating its Role in Antioxidative Potential in finger Millet (Eleusine Coracana (L.)). *3 Biotech.* 8, 499. doi:10.1007/s13205-018-1511-9

Barrales-Cureño, H. J., Reyes, C. R., García, I. V., Valdez, L. G. L., De Jesús, A. G., Cortés Ruíz, J. A., et al. (2019). *Alkaloids of Pharmacological Importance in* Catharanthus Roseus. London: Intech Open Ltd, 18. Vol. 1.

Bashir, E., Mahajan, R., Mir, R. A., Dar, W. A., and Zargar, S. M. (2021). Unravelling the Genetic Variability and Population Structure of Buckwheat (Fagopyrum spp.): a Collection of north Western Himalayas. *Nucleus* 64, 93–101. doi:10.1007/s13237-020-00319-y

Batlle, I., and Tous, J. (1997). *Carob Tree. Ceratonia Siliqua L. Promoting the Conservation and Use of Underutilized and Neglected Crops*. Rome. Italy: Institute of Plant Genetics and Crop Plant Research, Gatersleben/ International Plant Genetic Resources Institute.

Beddington, J., Asaduzzaman, M., and Fernandez, A. (2011). *Achieving Food Security in the Face of Climate Change: Summary for Policymakers from the Commission on Sustainable Agriculture and Climate Change*.

Bedi, E. N., and Peter-ikechukwu, A. I. (2015). Comparison of Proximate Composition of Some Cultivars of Chickpea (Cicer Arietinum L.) Cultivated in Owerri , Imo State. *Nigeria* 37, 103–110.

Bhattacharjee, T., Sen, D., Irenaeus, T. K. S., Das, S. P., and Chakraborty, A. (2019). Diversity of Underutilized Vegetable Crops in Tripura. *Acta Hortic.* 1241, 121–134. doi:10.17660/ActaHortic.2019.1241.19

Bhartiya, A., Aditya, J. P., and Kant, L. (2015). Nutritional and Remedial Potential of an Underutilised Food Legume Horse Gram (*Macrotyloma Uniflorum* (Lam.) Verdc): A Review. *J. Anim. Plant Sci.* 25 (4), 908–920.

Blum, A. (2016). Stress, Strain, Signaling, and Adaptation--Not Just a Matter of Definition. *J. Exp. Bot.* 67, 562–565. doi:10.1093/jxb/erv497

Bolade, M. K., and Bello, S. B. (2006). Selected Physicochemical Properties of Flour from the Root of African Fan Palm(Borassus Aethiopum). *Int. J. Food Properties* 9, 701–713. doi:10.1080/10942910600575641

Bungla, P. S., Tewari, G., Rawal, R. S., Bhatt, I. D., Tewari, L. M., Jugran, A. K. R., et al. (2017). Comparative *In Vitro* Anti-oxidant Activity, Morphological and Phytochemical Profiling of Traditional Crops from Kumaim Himalaya. *J. Indian Chem. Soc.* 94, 181–189.

Campanaro, A., Tommasi, N., Guzzetti, L., Galimberti, A., Bruni, I., and Labra, M. (2019). DNA Barcoding to Promote Social Awareness and Identity of Neglected, Underutilized Plant Species Having Valuable Nutritional Properties. *Food Res. Int.* 115, 1–9. doi:10.1016/j.foodres.2018.07.031

Canales-Martinez, M., Rivera-Yañez, C. R., Salas-Oropeza, J., Lopez, H. R., Jimenez-Estrada, M., Rosas-Lopez, R., et al. (2017). Antimicrobial Activity Of Bursera Morelensis Ramed;rez Essential oil. *African J. Tradit. Complement. Altern. Med.* 14, 74–82. doi:10.21010/ajtcam.v14i3.8

Caplan, D., Dixon, M., and Zheng, Y. (2019). Increasing Inflorescence Dry Weight and Cannabinoid Content in Medical Cannabis Using Controlled Drought Stress. *HortScience* 54, 964–969. doi:10.21273/HORTSCI13510-18

Ceballos, A., Valdivia, C., Rivera, J., and Arce, M. (2012). *Velvet Bean (Mucuna Pruriens Var. Utilis) a Cover Crop as Bioherbicide to Preserve the Environmental Services of Soil*.

Chalwe, A., Chiona, M., and Simwambana, M. S. C. (2002). Improving Household Food Security by Using Planted Fallows in Zambia. *Discov. Innov.* 14, 76–81.

Chauhan, R. S., Gupta, N., Sharma, S. K., Rana, J. C., Sharma, T. R., and Jana, S. (2010). Genetic and Genome Resources in Buckwheat Present Status and Future Perspectives. *Eur. J. Plant Sci. Biotechnol.* 4 (Special Issue 1), 33–44.

Chay, S. Y., Salleh, A., Sulaiman, N. F., Zainal Abidin, N., Hanafi, M. A., Zarei, M., et al. (2018). Blood-pressure Lowering Efficacy of Winged Bean Seed Hydrolysate in Spontaneously Hypertensive Rats, Peptide Characterization and a Toxicity Study in Sprague-Dawley Rats. *Food Funct.* 9, 1657–1671. doi:10.1039/c7fo01769c

Chibarabada, T., Modi, A., and Mabhaudhi, T. (2017). Expounding the Value of Grain Legumes in the Semi- and Arid Tropics. *Sustainability* 9, 60. doi:10.3390/su9010060

Chibarabada, T. P., Modi, A. T., and Mabhaudhi, T. (2017). Nutrient Content and Nutritional Water Productivity of Selected Grain Legumes in Response to Production Environment. *Int. J. Environ. Res. Public Health* 14, 1300. doi:10.3390/ijerph14111300

Chivenge, P., Mabhaudhi, T., Modi, A. T., and Mafongoya, P. (2015). The Potential Role of Neglected and Underutilised Crop Species as Future Crops under Water Scarce Conditions in Sub-saharan Africa. *Int. J. Environ. Res. Public Health* 12, 5685–5711. doi:10.3390/ijerph120605685

Chung, I.-M., Thiruvengadam, M., Rekha, K., and Rajakumar, G. (2016). Elicitation Enhanced the Production of Phenolic Compounds and Biological Activities in Hairy Root Cultures of Bitter Melon (Momordica Charantia L.). *Braz. Arch. Biol. Technol.* 59, 1–10. doi:10.1590/1678-4324-2016160393

Crop, A., and Society, S. (2019). Positive Selection and Neem Leaf. *Powder Factors* 27, 631–640.

Cunningham, A. B. (1993). African Medicinal Plants: Setting Priorities at the Interface between Conservation and Primary Health Care. People and Plant Initiative Working Paper 1. Nairobi. Available at: uuw.rbgkeworg.uk/peopleplant/wp/wp.

Dagar, J. C., Tomar, O. S., Minhas, P. S., and Kumar, M. (2013). Lemongrass (Cymbopogon Flexuosus) Productivity as Affected by Salinity of Irrigation Water, Planting Method and Fertilizer Doses on Degraded Calcareous Soil in a Semi-arid Region of Northwest India. *Indian J. Agric. Sci.* 83, 734–738.

Dakora, F. D., and Belane, A. K. (2019). Evaluation of Protein and Micronutrient Levels in Edible Cowpea (*Vigna Unguiculata L. Walp*.) Leaves and Seeds. *Front. Sustain. Food Syst.* 3, 70. doi:10.3389/fsufs.2019.00070

Dansi, A., Vodouhè, R., Azokpota, P., Yedomonhan, H., Assogba, P., Adjatin, A., et al. (2012). Diversity of the Neglected and Underutilized Crop Species of Importance in benin. *ScientificWorld Journal* 2012, 932947. doi:10.1100/2012/932947

Das, L., Bhaumik, E., Raychaudhuri, U., and Chakraborty, R. (2012). Role of Nutraceuticals in Human Health. *J. Food Sci. Technol.* 49 (2), 173–183. doi:10.1007/s13197-011-0269-4

De Caluwé, E., De Smedt, S., Assogbadjo, A. E., Samson, R., Sinsin, B., and Van Damme, P. (2009). Ethnic Differences in Use Value and Use Patterns of Baobab (Adansonia digitataL.) in Northern Benin. *Afr. J. Ecol.* 47, 433–440. doi:10.1111/j.1365-2028.2008.01023.x

Deb, C. R., Khruomo, N., and Paul, A. (2019). Underutilized Edible Plants of Nagaland: A Survey and Documentation from Kohima, Phek and Tuensang District of Nagaland, India. *Am. J. Plant Sci.* 10 (01), 162–178. doi:10.4236/ajps.2019.101014

Desai, S. D., Desai, D. G., and Kaur, H. (2009). Saponins and Their Biological Activities. *Pharm. Times* 41 (3), 13–16.

Donno, D., and Turrini, F. (2020). Plant Foods and Underutilized Fruits as Source of Functional Food Ingredients: Chemical Composition, Quality Traits, and Biological Properties. *Foods* 9 (10), 7–10. doi:10.3390/foods9101474

Donno, D., Cerutti, A. K., Prgomet, I., Mellano, M. G., and Beccaro, G. L. (2015). Foodomics for mulberry Fruit (Morus spp.): Analytical Fingerprint as Antioxidants' and Health Properties' Determination Tool. *Food Res. Int.* 69, 179–188. doi:10.1016/j.foodres.2014.12.020

Fandohan, B., Assogbadjo, A. E., Kakaï, R. G., Kyndt, T., Caluwé, E. D., Codjia, J. T. C., et al. (2010). Women's Traditional Knowledge, Use Value, and the Contribution of Tamarind (*Tamarindus indica* L.) to Rural Households' Cash Income in Benin. *Econ. Bot.* 64, 248–259. doi:10.1007/s12231-010-9123-2

Fasoyiro, S. B., Ajibade, S. R., Omole, A. J., Adeniyan, O. N., and Farinde, E. O. (2006). Proximate, Minerals and Antinutritional Factors of Some Underutilized Grain Legumes in South-western Nigeria. *Nutr. Food Sci.* 36, 18–23. doi:10.1108/00346650610642151

Firn, R. (2010). *Nature's Chemicals: The NaturalProducts that Shaped Our World*. Oxford: Oxford University Press.

Freiberger, C. E., Vanderjagt, D. J., Pastuszyn, A., Glew, R. S., Mounkaila, G., Millson, M., et al. (1998). Nutrient Content of the Edible Leaves of Seven Wild Plants from Niger. *Plant Foods Hum. Nutr.* 53, 57–69. doi:10.1023/A:1008080508028

Galvao, A. C., Nicoletto, C., Zanin, G., Vargas, P. F., and Sambo, P. (2021). Nutraceutical Content and Daily Value Contribution of Sweet Potato Accessions for the European Market. *Horticulturae* 7, 23–14. doi:10.3390/horticulturae7020023

Gangadhar, L., and Praseetha, P. K. (2019). Identification and Molecular Phylogenetic Relationship of Selected Medicinal Plants-Ethano Medicinal Importance. *Ind. Jour. Publ. Health Rese. Develop.* 10, 1248–1252. doi:10.5958/0976-5506.2019.01842.4

Gebauer, J., Bernholt, H., and Hammer, K. (2013). Grewia Flavescens: A Potential Horticultural Crop? *Genet. Resour. Crop Evol.* 60, 1915–1919. doi:10.1007/s10722-013-0003-3

Geldenhuys, C. J. (2007). Weeds or Useful Medicinal Plants in the Rural home Garden? *Food Nutr. Bull.* 28, S392–S397. doi:10.1177/15648265070282s219

Giambanelli, E., Verkerk, R., D'Antuono, L. F., and Oliviero, T. (2016). The Kinetic of Key Phytochemical Compounds of Non-heading and Heading Leafy *Brassica oleracea* Landraces as Affected by Traditional Cooking Methods. *J. Sci. Food Agric.* 96, 4772–4784. doi:10.1002/jsfa.7844

Golovinskaia, O., and Wang, C. K. (2021). Review of Functional and Pharmacological Activities of Berries. *Molecules* 26, 3904. doi:10.3390/molecules26133904

Gómez, M. I., and Ricketts, K. D. (2013). Food Value Chain Transformations in Developing Countries: Selected Hypotheses on Nutritional Implications. *Food Policy* 42, 139–150. doi:10.1016/j.foodpol.2013.06.010

Govender, L., Pillay, K., Siwela, M., Modi, A., and Mabhaudhi, T. (2016). Food and Nutrition Insecurity in Selected Rural Communities of KwaZulu-Natal, South Africa-Linking Human Nutrition and Agriculture. *Int. J. Environ. Res. Public Health* 14, 17. doi:10.3390/ijerph14010017

Grimaldi, I. M., Leke, W. N., Borokini, I., Wanjama, D., and Van Andel, T. (2018). From Landraces to Modern Cultivars: Field Observations on Taro Colocasia Esculenta (L.) Schott in Sub-saharan Africa. *Genet. Resour. Crop Evol.* 65, 1809–1828. doi:10.1007/s10722-018-0651-4

Gulla, A., Getachew, A., Haile, T. G., and Molla, F. (2020). Evaluation of Acid-Modified Ethiopian Potato (Plectranthus Edulis) Starch as Directly Compressible Tablet Excipient. *Biomed. Res. Int.*, 1–15. doi:10.1155/2020/9325173

Gullino, M. L., Gilardi, G., Bertetti, D., and Garibaldi, A. (2020). Emerging Soilborne Pathogens and Trends in Their Management. *Acta Hortic.* 1270, 9–22. doi:10.17660/ActaHortic.2020.1270.2

Hall, N. D., Patel, J. D., McElroy, J. S., and Goertzen, L. R. (2021). Detection of Subgenome Bias Using an Anchored Syntenic Approach in Eleusine Coracana (finger Millet). *BMC Genomics* 22, 175. doi:10.1186/s12864-021-07447-y

Herman, P. P., Retief, J., Koekemoer, E., and Welman, W. G. (2000). *Seed Plants of Southern Africa*. Editors O. A. Leister Pretoria, South Africa: National Botanical Institute.

Hirakawa, H., Shirasawa, K., Miyatake, K., Nunome, T., Negoro, S., Ohyama, A., et al. (2014). Draft Genome Sequence of Eggplant (*Solanum Melongena L.*): the Representative Solanum Species Indigenous to the Old World. *DNA Res.* 21, 649–660. doi:10.1093/dnares/dsu027

Horbowicz, M., and Obendorf, R. L. (2005). Fagopyritol Accumulation and Germination of Buckwheat Seeds Matured at 15, 22, and 30°C. *Crop Sci.* 45, 1264–1270. doi:10.2135/cropsci2004.0431

Huang, W. Y., Cai, Y. Z., and Zhang, Y. (2009). Natural Phenolic Compounds from Medicinal Herbs and Dietary Plants: Potential Use for Cancer Prevention. *Nutr. Cancer* 62 (1), 1–20. doi:10.1080/01635580903191585

Imelouane, B., Tahri, M., Elbastrioui, M., Aouinti, F., and Elbachiri, A. (2011). Mineral Contents of Some Medicinal and Aromatic Plants Growing in Eastern Morocco. *J. Mater. Environ. Sci.* 2 (2), 104–111.

Janardhanan, V. (2000). Nutritional and Anti-nutritional Composition of Velvet Bean: an Under-utilized Food Legume in South India. *J. Food Sci. Nutr.* 51 (4), 279. doi:10.1080/09637480050077167

Jayaramachandran, M., Saravanan, S., Motilal, A., Prabhu, P. C., Hepziba, S. J., Swain, H., et al. (2020). Genetic Improvement of a Neglected and Underutilised Oilseed Crop: Sesame (*Sesamum indicum L.*) through Mutation Breeding. *Nucleus* 63 (3), 293–302. doi:10.1007/s13237-020-00329-w

Jonas, M. S., and Beckmann, S. C. (1998). Functional Foods: Consumer Perceptions in Denmark and England. Centre for Market Surveillance, Research and Strategy for the Food Sector Working Paper Number 55. Available at: https://pure.au.dk/portal/files/91/wp55.pdf (Accessed July 26, 2021).

Joubert, E., Gelderblom, W. C., Louw, A., and de Beer, D. (2008). South African Herbal Teas: Aspalathus Linearis, Cyclopia Spp. And Athrixia Phylicoides-Aa Review. *J. Ethnopharmacol.* 119, 376–412. doi:10.1016/j.jep.2008.06.014

Joubert, E., Joubert, M. E., Bester, C., de Beer, D., and De Lange, J. H. (2011). Honeybush (Cyclopia spp.): From Local Cottage Industry to Global Markets - the Catalytic and Supporting Role of Research. *South Afr. J. Bot.* 77, 887–907. doi:10.1016/j.sajb.2011.05.014

Kaizzi, C. K., Ssali, H., and Vlek, P. L. G. (2004). The Potential of Velvet Bean (Mucuna Pruriens) and N Fertilizers in maize Production on Contrasting Soils and Agro-Ecological Zones of East Uganda. *Nutrient Cycling in Agroecosystems* 68, 59–72. doi:10.1023/B:FRES.0000012233.27360.60

Kamble, D. B., Singh, R., Rani, S., Kaur, B. P., Upadhyay, A., and Kumar, N. (2019). Optimization and Characterization of Antioxidant Potential, *In Vitro* Protein Digestion and Structural Attributes of Microwave Processed Multigrain Pasta. *J. Food Process. Preserv.* 43, 14125. doi:10.1111/jfpp.14125

Khan, Z. R., Midega, C. A., Bruce, T. J., Hooper, A. M., and Pickett, J. A. (2010). Exploiting Phytochemicals for Developing a 'push-Pull' Crop protection Strategy for Cereal Farmers in Africa. *J. Exp. Bot.* 61, 4185–4196. doi:10.1093/jxb/erq229

Kleynhans, R., Bulannga, M., Nenungwi, L., Lehlaleroa, M. T., Matsiliza-Mlathi, B., and Slabbert, M. M. (2018). Preliminary Investigations on Germination of Sutherlandia Frutescens and Emergence of Ceratonia Siliqua Seed. *Acta Hortic.* 1204, 153–160. doi:10.17660/ActaHortic.2018.1204.20

Koley, T. K., Khan, Z., Oulkar, D., Singh, B., Bhatt, B. P., and Banerjee, K. (2020). Profiling of Polyphenols in Phalsa (Grewia Asiatica L) Fruits Based on Liquid Chromatography High Resolution Mass Spectrometry. *J. Food Sci. Technol.* 57, 606–616. doi:10.1007/s13197-019-04092-y

Kumar, V., Uthappa, A. R., Srivastava, M., Vijay, D., Kumaranag, K. M., Manjunatha, N., et al. (2017). Floral Biology of Grewia Flavescens Juss.: an Underutilized Crop. *Genet. Resour. Crop Evol.* 64, 1789–1795. doi:10.1007/s10722-017-0536-y

Kumari, A., and Chaudhary, H. K. (2020). Nutraceutical Crop Buckwheat: a Concealed Wealth in the Lap of Himalayas. *Crit. Rev. Biotechnol.* 40, 539–554. doi:10.1080/07388551.2020.1747387

Kushwaha, R., Fatima, N. T., Singh, M., Singh, V., Kaur, S., Puranik, V., et al. (2021). Effect of Cultivar and Maturity on Functional Properties, Low Molecular Weight Carbohydrate, and Antioxidant Activity of Jackfruit Seed Flour. *J. Food Process. Preserv.* 45, 15146. doi:10.1111/jfpp.15146

Kutu, F. R., and Magongwa, S. M. (2017). Effect of Pre-treatment and Preservation Methods on the Nutritional Quality of Bitter Gourd Leaves from Two Different Sources. *Rese. Crop* 18, 675–682. doi:10.5958/2348-7542.2017.00115.2

Lambein, F., Travella, S., Kuo, Y. H., Van Montagu, M., and Heijde, M. (2019). Grass Pea (Lathyrus Sativus L.): Orphan Crop, Nutraceutical or Just plain Food? *Planta* 250, 821–838. doi:10.1007/s00425-018-03084-0

Lerotholi, L., Chaudhary, S. K., Combrinck, S., and Viljoen, A. (2017). Bush tea (*Athrixia Phylicoides*): A Review of the Traditional Uses, Bioactivity and Phytochemistry. *South Afr. J. Bot.* 110, 4–17. doi:10.1016/j.sajb.2016.06.005

Levy, A., Milo, J., Ashri, A., and Palevitch, D. (1983). Heterosis and Correlation Analysis of the Vegetative Components and Ajmalicine Content in the Roots of the Medicinal Plant-Catharanthus Roseus (L.) G. Don. *Euphytica* 32, 557–564. doi:10.1007/BF00021468

Liu, F., Guo, D. D., Tu, Y. H., Xue, Y. R., Gao, Y., and Guo, M. L. (2016). Identification of Reference Genes for Gene Expression Normalization in Safflower (Carthamus tinctorius). *Revista Brasileira de Farmacognosia* 26, 564–570. doi:10.1016/j.bjp.2016.05.006

Liu, X., Jones, P. J., Motuzaite Matuzeviciute, G., Hunt, H. V., Lister, D. L., An, T., et al. (2019). From Ecological Opportunism to Multi-Cropping: Mapping Food Globalisation in Prehistory. *Quat. Sci. Rev.* 206, 21–28. doi:10.1016/j.quascirev.2018.12.017

Lozano, S., Calzada-Infante, L., Adenso-Díaz, B., and García, S. (2019). Complex Network Analysis of Keywords Co-occurrence in the Recent Efficiency Analysis Literature. *Scientometrics* 120, 609–629. doi:10.1007/S11192-019-03132-W

Mabhaudhi, T., Chimonyo, V. G. P., Hlahla, S., Massawe, F., Mayes, S., Nhamo, L., et al. (2019). Prospects of Orphan Crops in Climate Change. *Planta* 250, 695–708. doi:10.1007/s00425-019-03129-y

Mabhaudhi, T., Chimonyo, V., and Modi, A. (2017). Status of Underutilised Crops in South Africa: Opportunities for Developing Research Capacity. *Sustainability* 9 (9), 1569. doi:10.3390/su9091569

Madziga, H. A., Sanni, S., and Sandabe, U. K. (2010). Phytochemical and Elemental Analysis of Acalypha Wilkesiana Leaf. *J. Amer. Sci.* 6 (11), 510–514.

Mander, M., Ntuli, L., Diederichs, N., and Mavundla, K. (2007). "Economics Of The Traditional Medicine Trade In South Africa," in *South African Health Review*. Editors S. Harrison, R. Bhana, and A. Ntuli (Pretoria: Health Systems Trust).

Manners, R., and van Etten, J. (2018). Are Agricultural Researchers Working on the Right Crops to Enable Food and Nutrition Security under Future Climates? *Glob. Environ. Change* 53, 182–194. doi:10.1016/j.gloenvcha.2018.09.010

Manyise, T., and Dentoni, D. (2021). Value Chain Partnerships and Farmer Entrepreneurship as Balancing Ecosystem Services: Implications for Agri-Food Systems Resilience. *Ecosyst. Serv.* 49. doi:10.1016/j.ecoser.2021.101279

Masenya, T. A., Pofu, K. M., and Mashela, P. W. (2020). Responses of Cancer bush (Sutherlandia Frutescens) and meloidogyne Javanica to Increasing Concentration of Nemafric-Bl Phytonematicide. *Res. Crops* 21, 615–620. doi:10.31830/2348-7542.2020.096

Mashamaite, C. V., Pieterse, P. J., Mothapo, P. N., and Phiri, E. E. (2021). *Moringa Oleifera* in South Africa: A Review on its Production, Growing Conditions and Consumption as a Food Source. *S. Afr. J. Sci.* 117. doi:10.17159/sajs.2021/8689

Masondo, N. A., Finnie, J. F., and Van Staden, J. (2014). Pharmacological Potential and Conservation prospect of the Genus Eucomis (Hyacinthaceae) Endemic to Southern Africa. *J. Ethnopharmacol.* 151, 44–53. doi:10.1016/j.jep.2013.11.002

Mathivha, L. P., Thibane, V. S., and Mudau, F. N. (2019). Anti-diabetic and Anti-proliferative Activities of Herbal Teas, Athrixia Phylicoides DC and Monsonia Burkeana Planch. Ex Harv, Indigenous to South Africa. *Br. Food J.* 121 (4), 964–974. doi:10.1108/bfj-10-2018-0697

McCartan, S. A., and Van Staden, J. (1999). Micropropagation of Members of the Hyacinthaceae with Medicinal and Ornamental Potential - A Review. *South Afr. J. Bot.* 65, 361–369. doi:10.1016/S0254-6299(15)31023-1

Modi, A. T., and Mabhaudhi, T. (2013). *Water Use and Drought Tolerance of Selected Traditional and Indigenous Crops. Final Report of Water Research Commission Project K5/1771//4. ISBN 978-1-4312-0434-2*. Pretoria, South Africa: Water Research Commission.

Modi, M., Modi, A., and Hendriks, S. (2006). Potential Role for Wild Vegetables in Household Food Security: a Preliminary Case Study in Kwazulu-Natal, South Africa. *Afr. J. Food Agric. Nutr. Dev.* 6, 1–13. doi:10.4314/ajfand.v6i1.19167

Modjadji, P., and Madiba, S. (2019). The Double burden of Malnutrition in a Rural Health and Demographic Surveillance System Site in South Africa: a Study of Primary Schoolchildren and Their Mothers. *BMC Public Health* 19, 1087. doi:10.1186/s12889-019-7412-y

Morsy, N. (2017). *Cardiac Glycosides in Medicinal Plants. Aromatic and Medicinal Plants–Back to Nature*. London: Intechopen, 29–45.

Mudau, F., and Ik, M. (2012). "Bush tea as a Herbal Beverage and Medicinal Plant," in *Tea in Health and Disease Prevention*. 1st ed. (Oxford, UK: Elsevier), 182–192.

Mudau, N. F., Soundy, P., and du Toit, E. S. (2005). Plant Growth and Development of Bush Tea as Affected by Nitrogen, Phosphorus, and Potassium Nutrition. *HortSci* 40, 1898–1901. doi:10.21273/HORTSCI.40.6.1898

Namdeo, A. G. (2018). "Cultivation of Medicinal and Aromatic Plants," in *Natural Products and Drug Discovery* (Amsterdam, Netherlands: Elsevier), 525–553. doi:10.1016/b978-0-08-102081-4.00020-4

Narayani, M., and Srivastava, S. (2017). Elicitation: a Stimulation of Stress in *In Vitro* Plant Cell/tissue Cultures for Enhancement of Secondary Metabolite Production. *Phytochem. Rev.* 16 (6), 1227–1252. doi:10.1007/s11101-017-9534-0

NEPAD (New Partnership for Africa's Development) (2018). Intra-Continental Migration, a windfall for Africa [WWW Document]. *CEO-BLOG*.Available at: https://www.nepad.org/content/intra-continental-migration-windfall-africa

Nchabeleng, L., Mudau, F. N., and Mariga, I. K. (2012). Effects Of Chemical Composition Of Wild Bush Tea (Athrixia Phylicoides DC.) Growing At Locations Differing In Altitude, Climate And Edaphic Factors. *J. Med. Plants Res.* 6 (9), 1662–1666. doi:10.5897/JMPR11.1453

Ndiku, H., Mwitari, P., Irungu, B., and Ngule, C. (2016). Antibacterial Activity of Pennisetum Glaucum Crude Extracts and Pre-purified Fractions against Selected Pathogenic Microorganisms. *Bjpr* 14, 1–9. doi:10.9734/bjpr/2016/28434

Neergheen-Bhujun, V. S., Ruhomally, Z. B., Dunneram, Y., Boojhawon, R., and Chan Sun, M. (2020). Consumption Patterns, Determinants and Barriers of the Underutilised Moringa Oleifera Lam in Mauritius. *South Afr. J. Bot.* 129, 91–99. doi:10.1016/j.sajb.2019.01.027

Ngameni, B., Fotso, G. W., Kamga, J., Ambassa, P., Abdou, T., Fankam, A. G., et al. (2013). "Flavonoids and Related Compounds from the Medicinal Plants of Africa," in *Medicinal Plant Research in Africa* (Amsterdam, Netherlands: Elsevier), 301–350. doi:10.1016/b978-0-12-405927-6.00009-6

Nyadanu, D., and Lowor, S. T. (2015). Promoting Competitiveness of Neglected and Underutilized Crop Species: Comparative Analysis of Nutritional Composition of Indigenous and Exotic Leafy and Fruit Vegetables in Ghana. *Genet. Resour. Crop Evol.* 62, 131–140. doi:10.1007/s10722-014-0162-x

Obidiegwu, J. E., Lyons, J. B., and Chilaka, C. A. (2020). The Dioscorea Genus (Yam)-An Appraisal of Nutritional and Therapeutic Potentials. *Foods* 9, 1–45. doi:10.3390/foods9091304

Odebunmi, E., and Oluwaniyi, O. O. (2010). Comparative Proximate Analysis of Some Food Condiments Heavy Metal Adsorption onto Nanoparticles View Project Heterogeneous Catalytic Hydrodeoxygenation K View Project Comparative Proximate Analysis of Some Food Condiments. *J. Appl. Sci. Res.* 6, 272.

Ojiem, J. O., Vanlauwe, B., De Ridder, N., and Giller, K. E. (2007). Niche-based Assessment of Contributions of Legumes to the Nitrogen Economy of Western Kenya Smallholder Farms. *Plant Soil* 292, 119–135. doi:10.1007/s11104-007-9207-7

Okole, B. N., Odhav, B., and Bornman, C. H. (2004). Commercialisation of Plants in Africa. *South Afr. J. Bot.* 70, 109–115. doi:10.1016/S0254-6299(15)30270-2

Olayinka Atoyebi, J., Osilesi, O., Adebawo, O., and Abberton, M. (2017). Evaluation of Nutrient Parameters of Selected African Accessions of Bambara Groundnut (Vigna Subterranea (L.) Verdc.). *Ajfn* 5, 83–89. doi:10.12691/ajfn-5-3-1

Onawola, O., and Asagbra, A. (2012). Comparative Soluble Nutrient Value of Ogiri Obtained from Dehulled and Undehulled Boiled Melon Seeds (Cucumeropsis Mannii). *Food Sci. Qual. Manage.* 4, 10–16.

Organización Mundial de la Salud, and World Health Organization (2003). *WHO Guidelines On Good Agricultural And Collection Practices [GACP] For Medicinal Plants* Geneva: World Health Organization.

Osei, K., Ennin, S. A., Aighewi, B., Aidoo, A. K., Lamptey, J. N. L., Mochiah, M. B., et al. (2019). Enhancing Productivity of Farmer-Saved Seed Yam in Ghana: Positive Selection and Neem Leaf Powder Factors. *Afr. Crop Sci. J.* 27, 631. doi:10.4314/acsj.v27i4.6

Ouma, G., and Jeruto, P. (2010). Sustainable Horticultural Crop Production through Intercropping: The Case of Fruits and Vegetable Crops: A Review. *Abjna* 1, 1098–1105. doi:10.5251/abjna.2010.1.5.1098.1105

Padulosi, S., Hodgkin, T., Williams, J. T., and Haq, N. (2002). *Underutilized Crops: Trends, Challenges and Opportunities in the 21st Century, Managing Plant Genetic Diversity*. Wallingford: CAB International.

Padulosi, S., Thompson, J., and Rudebjer, P. (2013). *Fighting Poverty, Hunger and Malnutrition with Neglected and Underutilized Species (NUS): Needs, Challenges and the Way Forward*. Rome: Bioversity International.

Palanisamy, A., Kannan, D., Kavitha, M., Ravindran, C., and Rajangam, J. (2019). Potential Vegetable Crops - Need for Exploration and Conservation for Nutritional Security for Rural Livelihoods. *Acta Hortic.* 1241, 69–74. doi:10.17660/ActaHortic.2019.1241.11

Pande, V. C., Kurothe, R. S., Kumar, G., Singh, H. B., and Tiwari, S. P. (2018). Economic Assessment of Agri-Horticulture Production Systems on Reclaimed Ravine Lands in Western India. *Agroforest Syst.* 92, 195–211. doi:10.1007/s10457-016-0025-x

Paul, C., Debnath, A., Chakraborty, K., Ghosh, S., Bhattacharjee, A., and Debnath, B. (2020). Sex-specific Variations in Phytochemicals and Antimicrobial Potentiality of Dioscorea. *Futur. J. Pharm. Sci.* 6, 4. doi:10.1186/s43094-020-00076-4

Pereira, A. V., Santana, G. M., Góis, M. B., and Sant'Ana, D. G. (2015). Tannins Obtained from Medicinal Plants Extracts against Pathogens: Anti-microbial Potential. *Battle Against Microb. Pathog. Basic Sci. Technol. Adv. Educ. Programs*, 228–235.

Phillips, M. M., and Rimmer, C. A. (2013). Functional Foods and Dietary Supplements. *Anal. Bioanal. Chem.* 405, 4323–4324. doi:10.1007/s00216-013-6846-9

Popoola, J., Ojuederie, O., Omonhinmin, C., and Adegbite, A. (2019). "Neglected and Underutilized Legume Crops: Improvement and Future Prospects," in *Recent Advances in Grain Crops Research* (London, UK: IntechOpen).

Publishers, W. A. (2018). Diversity and Change in Food Wellbeing. *Divers. Chang. Food Wellbeing* 2018, 43–69. doi:10.3920/978-90-8686-864-3

Pugalenthi, M., Vadivel, V., and Siddhuraju, P. (2005). Alternative Food/feed Perspectives of an Underutilized Legume Mucuna Pruriens Var. Utilis-Aa Review. *Plant Foods Hum. Nutr.* 60, 201–218. doi:10.1007/s11130-005-8620-4

Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A., et al. (2001). Anti-microbial Properties of Phenolic Compounds from Berries. *J. Appl. Microbiol.* 90, 494–507.

Rani, M., and Punia, D. (2015). Nutritional Evaluation of Matar Prepared Incorporating green Beans Powder. *Ann. Biol.* 31, 161–163.

Rasool, H. B. A. (2012). Medicinal Plants (Importance and Uses). *Pharm. Anal. Acta* 3 (10), 2153–2435. doi:10.4172/2153-2435.1000e139

Rastogi, A., and Shukla, S. (2013). Amaranth: A New Millennium Crop of Nutraceutical Values. *Crit. Rev. Food Sci. Nutr.* 53, 109–125. doi:10.1080/10408398.2010.517876

Rathore, R. S., Nawange, D. D., Solanki, R. S., and Singh, R. P. (2012). Identification of Suitable Ideotypes of Blackgram (Vigna Mungo L. Hepper) for Intercropping with Sorghum (Sorghum Bicolar L. Moench). *Legum. Res.* 35, 72–74.

Reddy, D. N. (2019). "Essential Oils Extracted From Medicinal Plants And Their Applications," in *Natural Bio-Active Compounds* (Singapore: Springer), 237–283.

Ribeiro, A., Romeiras, M. M., Tavares, J., and Faria, M. T. (2010). Ethnobotanical Survey in Canhane Village, District of Massingir, Mozambique: Medicinal Plants and Traditional Knowledge. *J. Ethnobiol. Ethnomed* 6, 33. doi:10.1186/1746-4269-6-33

Rigat, M., Bonet, M. À., Garcia, S., Garnatje, T., and Vallès, J. (2009). Ethnobotany of Food Plants in the High River Ter valley (Pyrenees, Catalonia, Iberian Peninsula): Non-crop Food Vascular Plants and Crop Food Plants with Medicinal Properties. *Ecol. Food Nutr.* 48 (4), 303–326. doi:10.1080/03670240903022320

Rivas, R., Oliveira, M. T., and Santos, M. G. (2013). Three Cycles of Water Deficit from Seed to Young Plants of Moringa Oleifera Woody Species Improves Stress Tolerance. *Plant Physiol. Biochem.* 63, 200–208. doi:10.1016/j.plaphy.2012.11.026

RSA (2010). *The New Growth Plan*. Pretoria, Republic of South Africa: RSA.

Sahu, P. K., Giri, D. D., Singh, R., Pandey, P., Gupta, S., Shrivastava, A. K., et al. (2013). Therapeutic and Medicinal Uses of *Aloe Vera*: a Review. *J. Pharm. Pharmacol.* 4 (8), 599. doi:10.4236/pp.2013.48086

Sales, Z. G., Juanico, C. B., Dizon, E. I., and Hurtada, W. A. (2018). Indigenous Pigmented Corn (Zea mays L.) Flour as Substitute for All-Purpose Flour to Improve the Sensory Characteristics and Nutrient Content of Crackers. *Malays. J. Nutr.* 24, 617–625.

Samarakoon, S. M. P. H., Ranil, R. H. G., Fonseka, R. M., Sivarnanthawerl, T., Fonseka, H., Senarathna, S. M. A. C., et al. (2018). Morphological Characterization and Assessment of Selected Biochemical Properties of Solanum Insanum L.: Screening of Local Plant Genetic Resources for Crop Improvement. *J. Agric. Sci.* 13, 45–54. doi:10.4038/jas.v13i1.8299

Sanchez-Castillo, C. P., Dewey, P. J. S., Aguirre, A., Lara, J. J., Vaca, R., Leon de la Barra, P., et al. (1998). The Mineral Content of Mexican Fruits and Vegetables. *J. Food Compost. Anal.* 11, 340–356. doi:10.1006/jfca.1998.0598

Sangakkara, U. R., Richner, W., Schneider, M. K., and Stamp, P. (2003). Impact of Intercropping Beans (Phaseolus vulgaris L.) and Sunhemp (Crotalaria Juncea L.) on Growth, Yields and Nitrogen Uptake of maize (Zea mays L.) Grown in the Humid Tropics during the Minor Rainy Season. *Maydica* 48, 233–238.

Saranya, B., Sulfikarali, T., Chindhu, S., Muneeb, A. M., Leela, N. K., and Zachariah, T. J. (2017). Turmeric and Cinnamon Dominate in Anti-oxidant Potential Among Four Major Spices. *J. Spices. Aromat. Crops* 26 (1), 803. doi:10.25081/josac.2017.v26.i1.803

Sarkar, D., Walker-Swaney, J., and Shetty, K. (2020). Food Diversity and Indigenous Food Systems to Combat Diet-Linked Chronic Diseases. *Curr. Dev. Nutr.* 4, 3–11. doi:10.1093/CDN/NZZ099

Saroop, S., and Kaul, V. (2015). *Cleome viscosa*: a Promising Underutilized Minor Crop. *Genet. Resour. Crop Evol.* 62, 1121–1126. doi:10.1007/s10722-015-0305-8

Sawahla, S. (2014). Functional Food. *J. Nutri.Food. Sci.* 2 (4), 1022.

Sennhenn, A., Njarui, D. M. G., Maass, B. L., and Whitbread, A. M. (2017). Exploring Niches for Short-Season Grain Legumes in Semi-arid Eastern Kenya - Coping with the Impacts of Climate Variability. *Front. Plant Sci.* 8, 699. doi:10.3389/fpls.2017.00699

Shackleton, S., Campbell, B., Lotz-Sisitka, H., and Shackleton, C. (2008). Links between the Local Trade in Natural Products, Livelihoods and Poverty Alleviation in a Semi-arid Region of South Africa. *World Develop.* 36, 505–526. doi:10.1016/j.worlddev.2007.03.003

Shakkarpude, J., Jain, A., and Bhimte, A. (2020). Active Ingredients of Herbal Drugs. *J. Pharm. Biol. Sci.* 8 (2), 92–94.

Sharifi-Rad, J., Sharifi-Rad, M., and Teixeira da Silva, J. A. (2016). Morphological, Physiological and Biochemical Responses of Crops (Zea mays L., Phaseolus vulgaris L.), Medicinal Plants (Hyssopus Officinalis L., Nigella Sativa L.), and Weeds (Amaranthus Retroflexus L., *Taraxacum officinale* F. H. Wigg) Exposed to SiO2 Nan. *J. Agric. Sci. Technol.* 18, 1027–1040.

Sharma, T., and Chen, J. (2020). Barriers in Cultivation and Marketing of Medicinal and Aromatic Plants in Uttarakhand, India: A Stakeholder Perspective. *Int. J. Ecol. Dev.* 35, 1–24.

Singh, B., Bhatt, S., Uniyal, P., Pal, V., and Chauhan, D. S. (2010). *In Vitro* bioassay to Study Phytotoxicity Effects of Stevia rebaudiana on Food Crops. *J. Trop. For. Sci.* 22, 364–369.

Singh, S., Waman, A. A., Bohra, P., Gautam, R. K., and Roy, S. D. (2016). Conservation and Sustainable Utilization of Horticultural Biodiversity in Tropical Andaman and Nicobar Islands, India. *Genet. Resour. Crop Evol.* 63, 1431–1445. doi:10.1007/s10722-016-0445-5

Sivakumar, G., Krishnamurthy, K. V., Hahn, E. J., and Paek, K. Y. (2004). Enhancedin Vitroproduction of Colchicine inGloriosa superbaL. - an Emerging Industrial Medicinal Crop in South India. *J. Hortic. Sci. Biotechnol.* 79, 602–605. doi:10.1080/14620316.2004.11511813

Slabbert, E. L., Malgas, R. R., Veldtman, R., and Addison, P. (2019). Honeybush (Cyclopia spp.) Phenology and Associated Arthropod Diversity in the Overberg Region, South Africa. *Bothalia* 49, 1–13. doi:10.4102/abc.v49i1.2430

Small, H. (1973). Co-citation in the Scientific Literature: A New Measure of the Relationship between Two Documents. *J. Am. Soc. Inf. Sci.* 24, 265–269. doi:10.1002/asi.4630240406

Smith, A. G., Croft, M. T., Moulin, M., and Webb, M. E. (2007). Plants Need Their Vitamins Too. *Curr. Opin. Plant Biol.* 10, 266–275. doi:10.1016/j.pbi.2007.04.009

Soltani, F., Karimi, R., and Kashi, A. (2016). Essential Oil Composition and Total Phenolic Compounds of Naked and normal Seed Types of Different Accessions of Cucurbita Pepo L. In Iran. Iran. *J. Plant Physiol.* 7, 1889–1897. doi:10.22034/ijpp.2016.532428

Son, H. N., Kingsbury, A., and Hoa, H. T. (2021). Indigenous Knowledge and the Enhancement of Community Resilience to Climate Change in the Northern Mountainous Region of Vietnam. *Agroecology Sustain. Food Syst.* 45, 499–522. doi:10.1080/21683565.2020.1829777

Sreelatha, S., and Padma, P. R. (2011). Modulatory Effects of Moringa Oleifera Extracts against Hydrogen Peroxide-Induced Cytotoxicity and Oxidative Damage. *Hum. Exp. Toxicol.* 30, 1359–1368. doi:10.1177/0960327110391385

Srivastava, R., Srivastava, V., and Singh, A. (2021). Multipurpose Benefits of an Underexplored Species Purslane (Portulaca Oleracea L.): A Critical Review. *Environ. Manage.*. doi:10.1007/s00267-021-01456-z

Sujatha, S., Bhat, R., Kannan, C., and Balasimha, D. (2011). Impact of Intercropping of Medicinal and Aromatic Plants with Organic Farming Approach on Resource Use Efficiency in Arecanut (Areca Catechu L.) Plantation in India. *Ind. Crops Prod.* 33, 78–83. doi:10.1016/j.indcrop.2010.09.001

Sunitha, S., Ravi, V., George, J., and Suja, G. (2013). Aroids and Water Relations: An Overview. *J. Root Crop* 39, 1–10.

Suvorova, G. (2016). "Buckwheat Tissue Cultures and Genetic Transformation," in *Molecular Breeding and Nutritional Aspects of Buckwheat* (Cambridge, MA, USA: Academic Press), 365–375. doi:10.1016/b978-0-12-803692-1.00029-8

Tasheva, K., and Kosturkova, G. (2012). The Role of Biotechnology for Conservation and Biologically Active Substances Production of Rhodiola Rosea: Endangered Medicinal Species. *ScientificWorldJournal* 2012, 274942. doi:10.1100/2012/274942

Tattiyakul, J., Asavasaksakul, S., and Pradipasena, P. (2006). Chemical and Physical Properties of Flour Extracted from Taro *Colocasia Esculenta* (L.) Schott Grown in Different Regions of Thailand. *ScienceAsia* 32, 279. doi:10.2306/scienceasia1513-1874.2006.32.279

Taylor, D. (2015). The Pharmaceutical Industry and the Future of Drug Development. *Issues Environ. Sci. Tech.* 41. doi:10.1039/9781782622345-00001

Tchokponhoué, D. A., N'Danikou, S., Hale, I., Van Deynze, A., and Achigan-Dako, E. G. (2017). Early Fruiting in Synsepalum Dulcificum (Schumach. & Thonn.) Daniell Juveniles Induced by Water and Inorganic Nutrient Management. *F1000Res* 6, 399. doi:10.12688/f1000research.11091.1

Tesfay, S. Z., Bertling, I., Odindo, A. O., Seyoum Workneh, T., and Mathaba, N. (2011). Levels of Anti-oxidants in Different Parts of Moringa (Moringa Oleifera) Seedling. *Afr. J. Agric. Res.* 6, 5123–5132. doi:10.5897/AJAR11.1101

Thow, A. M., Greenberg, S., Hara, M., Friel, S., duToit, A., and Sanders, D. (2018). Improving Policy Coherence for Food Security and Nutrition in South Africa: a

Qualitative Policy Analysis. *Food Sec.* 10, 1105–1130. doi:10.1007/s12571-018-0813-4

Traill, B. (1997). Globalisation in the Food Industries? *Eur. Rev. Agric. Econ.* 24, 390–410. doi:10.1093/erae/24.3-4.390

Tran, N., Bailey, C., Wilson, N., and Phillips, M. (2013). Governance of Global Value Chains in Response to Food Safety and Certification Standards: The Case of Shrimp from Vietnam. *World Develop.* 45, 325–336. doi:10.1016/j.worlddev.2013.01.025

Tsegay, Y. T., Rusare, M., Mistry, R., Teka Tsegay, Y., Rusare, M., and Mistry, R. (2014). *Hidden Hunger in South Africa: The Faces of Hunger and Malnutrition in a Food-Secure Nation*. Oxford, Crowley: Oxfam International.

Tshivhandekano, I., Ngezimana, W., Tshikalange, T. E., Makunga, N. P., and Mudau, F. N. (2018). Nitrogen Application Influences Quality, Pharmacological Activities and Metabolite Profiles ofAthrixia phylicoidesDC. (Bush tea) Cultivated under Greenhouse and Field Conditions. *Acta Agriculturae Scand. Section B - Soil Plant Sci.* 68, 388–400. doi:10.1080/09064710.2017.1412496

Tungmunnithum, D., Thongboonyou, A., Pholboon, A., and Yangsabai, A. (2018). Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. *Medicines (Basel)* 5 (3), 93. doi:10.3390/medicines5030093

Udeh, E. L., Nyila, M. A., and Kanu, S. A. (2020). Nutraceutical and Antimicrobial Potentials of Bambara Groundnut (Vigna Subterranean): A Review. *Heliyon* 6 (10), e05205. doi:10.1016/j.heliyon.2020.e05205

Ukonu, I. C. (2016). The Effect of Fast Food Globalisation on Students' Food Choice. *World Acad. Sci. Eng. Technol. J. Soc. Behav. Educ. Econ. Bus. Ind. Eng.* 10.

Uusiku, N. P., Oelofse, A., Duodu, K. G., Bester, M. J., and Faber, M. (2010). Nutritional Value of Leafy Vegetables of Sub-saharan Africa and Their Potential Contribution to Human Health: A Review. *J. Food Compost. Anal.* 23, 499–509. doi:10.1016/j.jfca.2010.05.002

Vadivel, V., Doss, A., and Pugalenthi, M. (2010). Evaluation of Nutritional Value and Protein Quality of Raw and Differentially Processed Sword Bean [Canavalia Gladiata (Jacq.) DC.] Seeds. *Afr. J. Food Agric. Nutr. Dev.* 10, 34. doi:10.4314/ajfand.v10i7.59034

D. A. Vattem and V. Maitin (Editors) (2015). *Functional Foods, Nutraceuticals and Natural Products: Concepts and Applications* (Lancaster, PA, USA: DEStech Publications, Inc).

Van Vuuren, S. F., and Naidoo, D. (2010). An Antimicrobial Investigation of Plants Used Traditionally in Southern Africa to Treat Sexually Transmitted Infections. *J. Ethnopharmacol* 130, 552–558. doi:10.1016/j.jep.2010.05.045

Van Wyk, B.-E., and Gericke, N. (2000). *People's Plants: A Guide to Useful Plants of Southern Africa*. Pretoria South Africa: Briza publications. 1875093192.

Van Wyk, B.-E. (2011). The Potential of South African Plants in the Development of New Medicinal Products. *South Afr. J. Bot.* 77, 812–829. doi:10.1016/j.sajb.2011.08.011

Van Wyk, B.-E., and Viljoen, A. (2011). Special Issue on Economic Botany. *South Afr. J. Bot.* 77, 809–811. doi:10.1016/j.sajb.2011.09.008

van Wyk, B. E. (2008). A Broad Review of Commercially Important Southern African Medicinal Plants. *J. Ethnopharmacol.* 119, 342–355. doi:10.1016/j.jep.2008.05.029

Varsha, S., Agrawal, R. C., and Sonam, P. (2013). Phytochemical Screening and Determination of Anti-bacterial and Anti-oxidant Potential of Glycyrrhiza Glabra Root Extracts. *J. Environ. Res. Develop.* 7 (4), 1552–1558.

Vashist, H., and Sharma, D. (2013). Pharmacognostical Aspects of Glycyrrhiza Glabra. *Asian J. Pharm. Clin. Res.* 6 (4), 55–59.

Velasco, P., Cartea, M. E., Gonzalez, C., Vilar, M., and Ordas, A. (2007). Factors Affecting the Glucosinolate Content of Kale (*Brassica oleracea* Acephala Group). *J. Agric. Food Chem.* 55, 955–962. doi:10.1021/jf0624897

Venter, S. L., Fouché, H. J., De Wit, M., Mavengahama, S., Coetzer, G. M., Swart, W. J., et al. (2019). The Effect of Fostering Partnerships on Broadening the Food Base: the Role of Cactus Pear, an Underutilised Crop with Unlimited Potential - the South African Perspective. *Acta Hortic.* 1247, 237–244. doi:10.17660/ActaHortic.2019.1247.32

Verma, A., Srivastava, K., Singh, S., and Singh, H. (2017). *Nutraceuticals and Innovative Food Products for Healthy Living and Preventive Care I*, 304–322.

Wang, J., Zhang, Q., Zhang, Z., and Li, Z. (2008). Antioxidant Activity of Sulfated Polysaccharide Fractions Extracted from Laminaria Japonica. *Int. J. Biol. Macromol* 42 (2), 127–132. doi:10.1016/j.ijbiomac.2007.10.003

Wang, M.-R., Lambardi, M., Engelmann, F., Pathirana, R., Panis, B., Volk, G. M., et al. (2021). Advances in Cryopreservation of In Vitro-derived Propagules: Technologies and Explant Sources. *Plant Cel Tiss Organ. Cult* 144 (1), 7–20. doi:10.1007/s11240-020-01770-0

Wang, T. Y., Li, Q., and Bi, K. S. (2018). Bioactive Flavonoids in Medicinal Plants: Structure, Activity and Biological Fate. *Asian J. Pharm. Sci.* 13 (1), 12–23. doi:10.1016/j.ajps.2017.08.004

Xavier, F., Kumar, R., Yadav, R. K., Behera, T. K., and Khade, Y. P. (2019). Studies on Combining Ability of Okra Genotypes for Protein, Total Dietary Fibre and mineral Content. *Ind. Jour. Hort.* 76, 672–677. doi:10.5958/0974-0112.2019.00107.5

Xego, S., Kambizi, L., and Nchu, F. (2016). Threatened Medicinal Plants of South Africa: Case of the Family Hyacinthaceae. *Afr. J. Trad. Compl. Alt. Med.* 13, 169–180. doi:10.4314/ajtcam.v13i3.20

Xie, J.-H., Jin, M.-L., Morris, G. A., Zha, X.-Q., Chen, H.-Q., Yi, Y., et al. (2016). Advances on Bioactive Polysaccharides from Medicinal Plants. *Crit. Rev. Food Sci. Nutr.* 56 (Suppl. 1), S60–S84. doi:10.1080/10408398.2015.1069255

Yulvianti-, M., and Zidorn, C. (2021). *Chemical Diversity of Plant Cyanogenic Glycosides: An Overview of Reported Natural*.

Zhang, D.-Y., Wan, Y., Hao, J.-Y., Hu, R.-Z., Chen, C., Yao, X.-H., et al. (2018). Evaluation of the Alkaloid, Polyphenols, and Antioxidant Contents of Various mulberry Cultivars from Different Planting Areas in Eastern China. *Ind. Crops Prod.* 122, 298–307. doi:10.1016/j.indcrop.2018.05.065

Zhang, H., Tan, J., Zhang, M., Huang, S., and Chen, X. (2020). Comparative Transcriptomic Analysis of Two Bottle Gourd Accessions Differing in Fruit Size. *Genes (Basel)* 11, 359. doi:10.3390/genes11040359

Zonyane, S., Chen, L., Xu, M.-J., Gong, Z.-N., Xu, S., and Makunga, N. P. (2019). Geographic-based Metabolomic Variation and Toxicity Analysis of Sutherlandia Frutescens L. R.Br. - an Emerging Medicinal Crop in South Africa. *Ind. Crops Prod.* 133, 414–423. doi:10.1016/j.indcrop.2019.03.010

Zubair, M., Nybom, H., Lindholm, C., and Rumpunen, K. (2011). Major Polyphenols in Aerial Organs of Greater Plantain (Plantago Major L.), and Effects of Drying Temperature on Polyphenol Contents in the Leaves. *Scientia Horticulturae* 128, 523–529. doi:10.1016/j.scienta.2011.03.001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 25 January 2022
doi: 10.3389/fphar.2021.791049

Citrullus colocynthis (L.) Schrad (Bitter Apple Fruit): Promising Traditional Uses, Pharmacological Effects, Aspects, and Potential Applications

Qin-Yuan Li[1†], Mahzaib Munawar[2†], Muhammad Saeed[2†], Ji-Qiang Shen[1†], Muhammad Sajjad Khan[2], Sobia Noreen[3], Mahmoud Alagawany[4], Muhammad Naveed[5], Asadullah Madni[3] and Chang-Xing Li[1*]*

[1]Department of Human Anatomy, Medical Institute of Qinghai University, Xining, China, [2]Department of Poultry and Animal Breeding, Faculty of Animal Production and Technology, The Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan, [3]Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan, [4]Department of Poultry, Faculty of Agriculture, Zagazig University, Zagazig, Egypt, [5]School of Pharmacy, Nanjing Medical University, Nanjing, China

OPEN ACCESS

Edited by:
Massimo Lucarini,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Syed Nasir Abbas Bukhari,
Al Jouf University, Saudi Arabia
Zohara Yaniv Bachrach,
Agricultural Research Organization, Volcani Center, Israel

***Correspondence:**
Muhammad Sajjad Khan
drsajjad2@yahoo.com
Chang-Xing Li
lcx1535@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to "Ethnopharmacology", a section of the journal Frontiers in Pharmacology

Received: 07 October 2021
Accepted: 09 December 2021
Published: 25 January 2022

Citation:
Li Q-Y, Munawar M, Saeed M, Shen J-Q, Khan MS, Noreen S, Alagawany M, Naveed M, Madni A and Li C-X (2022) Citrullus colocynthis (L.) Schrad (Bitter Apple Fruit): Promising Traditional Uses, Pharmacological Effects, Aspects, and Potential Applications. Front. Pharmacol. 12:791049. doi: 10.3389/fphar.2021.791049

Health consciousness and increased knowledge about the side effects of synthetic drugs have enhanced interest in traditional medicines. Medicinal plants offer cures for various diseases, leading to improved living standards. This has brought ethnomedicinal studies into the spotlight and increased demand for herb-based medicines. *Citrullus colocynthis* is an herbaceous plant containing an abundance of nutrients that play a key role in the improvement of wellbeing. *C. colocynthis* has many biological properties, such as antioxidative, hypoglycemic, antibacterial, anti-cancerous, anti-inflammatory, analgesic, gastrointestinal tract, reproduction, protection, anti-microbial, antidiabetic, hypolipidemic, antineoplastic, profibrinolytic, anti-allergic, pesticidal, and immune-stimulatory. There are numerous bioactive compounds like cucurbitacin, flavonoids, and polyphenols in *C. colocynthis* that give it medicinal properties. Herein, we have extensively compiled, reviewed, and analyzed significant information on *C. colocynthhis* from the best published available evidence in PubMed, Scopus (Embase), Web of Science (Web of Knowledge), Cochrane Library, and Google Scholar, etc. Scientific literature evidenced that owing to the bioactive constituents, including cucurbitacin, polyphenols, flavonoids, and other potent molecules, *C. colocynthis* has many pharmacological and physiological functions. It possesses multi-beneficial applications in treating various disorders of humans and animals. So, the primary purpose of this comprehensive review is to provide an overview of the findings of positive impacts and risks of *C. colocynthis* consumption on human health, especially in poultry and veterinary fields. In the future, this narrative article will be aware of discoveries about the potential of this promising natural fruit and its bioactive compounds as the best nutraceuticals and therapeutic drugs in veterinary and human medicine.

Keywords: *Citrullus colocynthis*, traditional uses, health aspects, human, poultry

1 INTRODUCTION

Plants have supplied many essential human needs, including a variety of therapeutic medications (Alagawany et al., 2020, 2021a, b; Dhama et al., 2021). Therefore, deliberate efforts towards cultivation are crucial for the continuous availability of those plant species. Medicinal plants have been used in healthcare for a long time, and their use to prevent and treat illness is expanding worldwide (Dhama et al., 2018; Bilal et al., 2021; Reda et al., 2021; Saeed et al., 2021). The medicinal properties of plants are due to the natural chemicals/compounds they contain (Saeed et al., 2019; Alagawany et al., 2021c; Garg et al., 2021; Zhang et al., 2021). Plants are a source of food and act as raw materials from which a variety of drugs are synthesized (Hassan, 2012). *Citrullus colocynthis* is a desert plant and a source of several bioactive compounds such as essential oils, glycosides, flavonoids, alkaloids, and fatty acids. Medicinal plants improve the immune system. The dried fruit pulp of *C. colocynthis* has been used to treat gastrointestinal disorders like indigestion, gastroenteritis, and intestinal parasites. *C. colocynthis* also has excellent pharmacological properties, such as being a laxative and purgative; it is anti-diabetic, anti-inflammatory, anthelmintic, and anti-cancerous. The fruit has been studied extensively for its antimicrobial, antioxidant, and anti-inflammatory activities (Hussain et al., 2014). *C. colocynthis* seed powder (CCSP) has been used as an emulsifier, fat binder, and flavoring (De Smet., 1997). *C. colocynthis* has also long been utilized in popular cuisine. Some of its medicinal characteristics include antioxidant, anti-inflammatory, anti-diabetic, and antibacterial activities (Kamran et al., 2018). Its pharmacological properties include antioxidative, hypoglycemic, antibacterial, anti-cancerous, anti-inflammatory, analgesic (Sanafi et al., 2006). *C. colocynthis* has antidiabetic, hypolipidemic, antineoplastic, profibrinolytic, antiallergic, antimicrobial, pesticidal, and immune-stimulatory effects. It also affects the reproductive system and fertility (Meybodi, 2020). *C. colocynthis* acts as an antioxidant and anesthetic in humans (Hyderi et al., 2015); its oil can be used to treat constipation (Qureshi et al., 2010), while an extract showed anti-tumor activity on cancerous cells (Abdulridha et al., 2020) and its leaves are anti-cancerous and anti-adipogenic (Perveen et al., 2020). Phytochemical screening of *C. colocynthis* fruit extract revealed anti-diarrheal properties (Dhakad, 2017). The irregular use of antimicrobials results in drug resistance in animals and humans, adversely affecting their health. Therefore, in 2006, the European Union prohibited antibiotics as growth promoters (Milanov et al., 2016). Due to this restriction, many alternative antimicrobials are being used, and preferences trend towards photogenic products extracted from herbs and spices with known antimicrobial properties (Bajagai et al., 2020). Many other products have been selected as alternatives to antibiotic growth promoters; these include probiotics, prebiotics, enzymes, organic acids, acidifiers, antioxidants, and phytogenic additives (Perić et al., 2009).

In Pakistan, the poultry industry is a key sub-sector of the livestock industry, with current investment of >750 billion and a growth rate of 7.5% per annum. Pakistan is the 11th largest poultry producer globally, with an estimated population of 64.01 million layers, 1,407.73 million broilers, and 14.34 million breeders (Pakistan economic survey, 2020). This indicates the strong growth and importance of, as well as prospects for broiler farming in Pakistan. The antimicrobial growth promoters boost feed conversion and body weight gain as they change the composition and activity of gut microflora (Al Dobaib and Mousa, 2009). The focus of broiler production is growth and performance, and the latter and health depend on the microflora present in the lower gastrointestinal tract (GIT) of broiler chicken (Rinttilä and Apajalahti, 2013). Change or imbalance in gut microbiota can adversely affect nutrient utilization and gut health (Choct., 2009). Phytobiotics are natural, less toxic, and residue-free. Growth promoters improve digestive capacity and growth, increase nutrient availability, and reduce potential pathogens in the GIT (Yitbarek, 2015). These additives also improve feed intake, thus improving the feed conversion and weight gain of broiler chickens (Ertas et al., 2005). Phytobiotics are added to poultry feed and are considered an antimicrobial substitute. These compounds can be used as replacements for antibiotic growth promoters because of their antibacterial, antifungal, antiparasitic, and immune stimulatory attributes, resulting in improved product performance of chickens (Abd El Ghany and Yazar Soyadı, 2020). Ten bioactive components were isolated from *C. colocynthis* seeds (CCS). CCS are anti-microbial, immune-stimulating, and enhance growth. CCSP improves production performance and alleviates immune suppression (Alzarah et al., 2021). CCS contains 13.5% protein, is rich in methionine and cysteine, and is limited in lysine. The *in vitro* digestibility of seed protein is 75.9% (Sawaya et al., 1986).

Previous research has reported multiple benefits of *C. colocynthis* for humans, livestock, and poultry. This literature, from various sources, has been reviewed. As the literature on the use of *C. colocynthis* in poultry and its importance in humans is limited, we recommend further research on it and its extract in manufacturing poultry and human medicine. We aim to broaden the scope of *C. colocynthis* use and increase the awareness of scientists and veterinarians regarding the benefits of this plant for human and poultry health.

2 BOTANICAL DESCRIPTION

C. colocynthis is a perennial plant with perennial roots and angular, tough, rough, and vine-like stems that spread on the ground and may climb up from there. They produce a single yellow flower at leaf axils. They are monoecious and have long peduncles and tuberous rootstock sprouting long trailing or climbing stems (Pravin et al., 2013) (**Table 1**).

3 GEOGRAPHIC DISTRIBUTION

The plant is native to the arid sandy areas of West Asia, Arabia, tropical Africa, and the Mediterranean (Pravin et al., 2013). It is also widely distributed in the desert area of Pakistan (Kamran et al., 2018). *C. colocynthis* originated in Asia and the Mediterranean Basin, particularly Turkey and Nubia, to the western coastal regions of Africa, the Sahara, and Egypt in the east. It is also found in India and

TABLE 1 | Botanical description of *C. colocynthis* (Pravin et al., 2013).

Botanical description	
Roots and stem	Perennial roots, stems are angular, tough, and rough vine-like that spread on the ground and may climb up
Seeds	Yellow to brown in color, smooth in texture, and oval in shape
Flowers	A single yellow color flower at leaf axils. They are monoecious and have long peduncles
Leaves	Angular and about 5–10 cm long. They are triangular, rough, and green
Fruit	15-30 fruits that are about 7–10 cm in diameter. Color may be yellow or green with yellow stripes. Fruit pulp contains oval seeds

C. colocynthis *is a desert plant, as shown in* ***Figure 1***

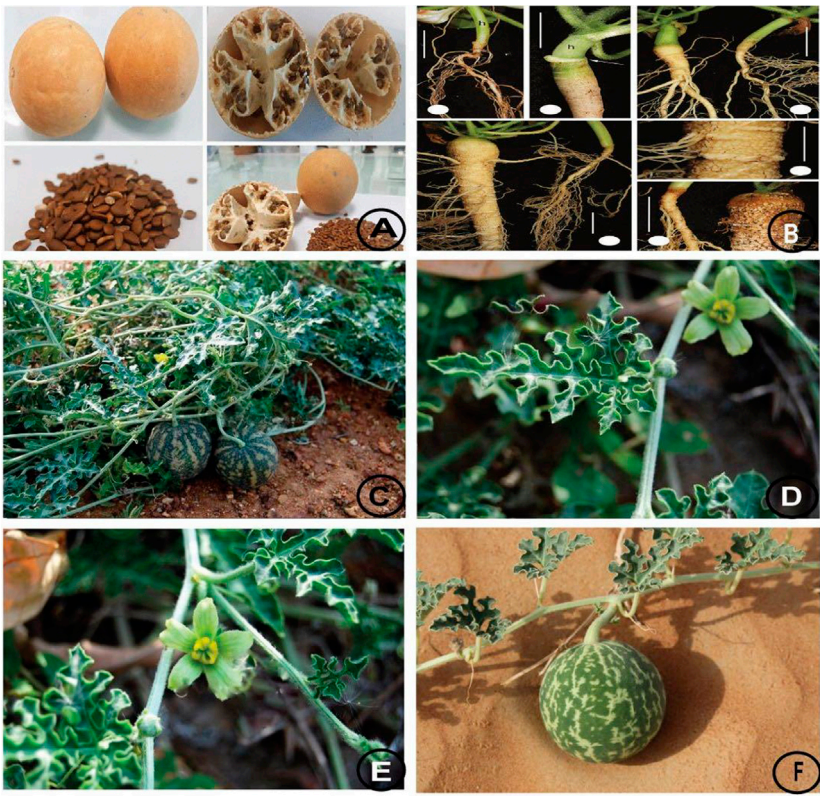


FIGURE 1 | (A) Seeds **(B)** roots **(C)** plant **(D)** leaf **(E)** flower and **(F)** fruit of C. colocynthis.

the northern coastal regions of the Caspian and Mediterranean seas. *C. colocynthis* belongs to the Cucurbitaceae family, and its common names are shown in **Table 2**.

3.1 Proximate Composition

The proximate composition of *C. colocynthis* is given in **Table 3**. The proximate analysis of *C. colocynthis* revealed 24.37% protein, 1.91% fiber, 10.88% carbohydrate, 56.61% fat, 3.15% ash, and 3.08% moisture (Ogundele et al., 2012).

4 TRADITIONAL USES

C. colocynthis can be used to treat gastrointestinal conditions and pulmonary, skin, and bacterial infections (Hameed et al., 2020); constipation; edema, cancer, and diabetes (Kumar et al., 2008). The dried pulp of the fruit of *C. colocynthis* is used as a remedy for gastrointestinal disorders like indigestion, gastroenteritis, and intestinal parasites (Hussain et al., 2014). The plant is also used to treat diabetes, liver problems, weak bowel movements, and obstruction or paralysis of the intestine (Rahimi et al., 2012). The fruit extract is used as an analgesic (Heydari et al., 2015). The vital pharmacological effects of *C. colocynthis* are shown in **Figure 2**.

5 PHYTOCHEMISTRY

C. colocynthis contains several bioactive compounds like cucurbitacin, flavonoids, and polyphenols, which impart

TABLE 2 | Common names used for *C. colocynthis* (De Smet., 1997; Elltayeib et al., 2020; Pravin et al., 2013).

Common names	Languages
Colocynth	English
Bitter gourd	
Bitter apple	
Bitter cucumber	
Koloquinthe	German
Coloquinte	French
Indravaruni	Sanskrit
Handhal	Arabic
Ghurunba/Kortuma	Punjabi
Makhal	Bengali
Paedikari Attutummatti	Tamil
Kadu indravani	Marathi
Indrayan	Gujarati
Paikummatti	Malyalam
Indrayan	Hindi
Maraghonae	Pashto

medicinal properties (Bhasin et al., 2020). The phytochemical constituents of *C. colocynthis* are shown in **Table 4**.

Three flavone glucosides–isovitexin, isosaponarin, isoorientin, and the two cucurbitacin glucosides 2-glucopyranosyl-cucurbitacin L and glucopyranosyl cucurbitacin were extracted from the fruits of the locally growing *C. colocynthis* and identified. The flavonoids were shown to have considerable antioxidant effects, which is a key characteristic for treating various disorders because reactive oxygen species play an important role in inflammation, cancer, tissue damage, and a variety of diseases (Delazar et al., 2006). Phytochemical screening also revealed the presence of tannins, flavonoids, alkaloids, saponins, and glycosides in *C. colocynthis*. The chemical components of the ethanolic extract of *C. colocynthis*, including alkaloids, glycosides, and flavonoids, could have a strong antibacterial effect (Najafi et al., 2010). Terpenoids, steroids, alkaloids, flavonoids, glycosides, phenols, tannins, flavones, and saponins were found in crude extracts of *C. colocynthis* (Ahmed et al., 2019). Carbohydrates, proteins, tannins, distinct amino acids, steroids, phenolic compounds, alkaloids, glycosides, terpenoids, and cucurbitacins A, B, C, D, E, J, and L were also all found in various preparations of *C. colocynthis* (Mazher et al., 2020).

5.1 Bioactive Compounds and their Structure-Activity Relationship

5.1.1. Cucurbitacin

Colocynthosides A, cucurbitacin L, and cucurbitacin B were isolated from the fruit of *C. colocynthis*. The main cucurbitane-type triterpene glycoside and its aglycon, Cucurbitacin E 2-O—D-glucopyranoside, and cucurbitacin E, showed anti-allergic properties (Yoshikawa et al., 2007). Natural cucurbitacins are triterpenoid chemicals famous for their bitter taste and toxicity. Due to their cytotoxic activities, cucurbitacins play an important role in drug discovery, particularly in anticancer drug development (Chen et al., 2005). **Figure 3** shows the structure of various Cucurbitacin.

The structure-activity relationship of the anti-cancerous effects of cucurbitacin and their derivatives, which are capable of electrophilic attack on cellular structures or genetic material, have been studied. This could be used to derive new anti-cancerous agents (Lang et al., 2014).

5.2 Glycosides, Flavonoids, and Phenolic Acids

C. colocynthis fruit contained 2-O—D-glucopyranosyl-Cucurbitacin L, 2-O—D-glucopyranosyl-Cucurbitacin I and isosaponarin. Kaempferol, quercetin, myricetin, catechin, gallic acid, vanillic acid, p-hydroxybenzoic acid, p-coumaric acid, caffeic acid, sinapic acid, chlorogenic acid, and ferulic acid were also found in *C. colocynthis* (Delazar et al., 2006; Hussain et al., 2014). Flavonoid C-glycosides show considerable anticancer and antitumor action and antibacterial, antifungal, antioxidative, anti-diabetic, anti-inflammatory, antiviral, and hepatoprotective activities, among other biological benefits (Xiao et al., 2016). The structure-activity relationship for quercetin and structurally similar flavonoids has a strong tumor necrosis factor-alpha inhibitory effect and a positive chemical potential and negative electrophilicity index that was considered beneficial (Geoffrey et al., 2020).

5.3 Fatty Acids

Stearic, linolenic, oleic, linoleic, myristic, and palmitic acids were present in CCS (Gurudeeban et al., 2010). **Figure 4** shows various phytochemicals along with their mechanism of action.

6 MEDICINAL PROPERTIES

The bitter and spicy *C. colocynthis* fruit is used to treat colds, diarrhea, parasitic worms, the expulsion of wind, tumors, ascites, leukoplakia, ulcers, asthma, bronchitis, diabetes insipidus, jaundice, splenomegaly, neck tuberculosis, constipation, anemia, throat diseases, elephantiasis, and joint pain; it is also used as an antipyretic. The root can be used to treat jaundice, ascites, urinary disorders, rheumatism; in children, and it can be used against enlarged abdomens, coughs, and asthma attacks. A root plaster can also be used to treat breast inflammation. An application of the fruit or root with a mixture of water and/or Nux vomica can treat papules and acne (Pravin et al., 2013). Different studies on *C. colocynthis* have been summarized in **Table 5**.

TABLE 3 | Proximate composition of *C. colocynthis* (Akpambang et al., 2008).

Parameter	*Citrullus colocynthis*
Protein	25.73b ± 0.06
Fat	46.24c ±0.02
Moisture	4.85a±0.04
Ash	4.48b ± 0.02
Fiber	5.00b ± 0.07
Carbohydrate	13.70b ± 0.02

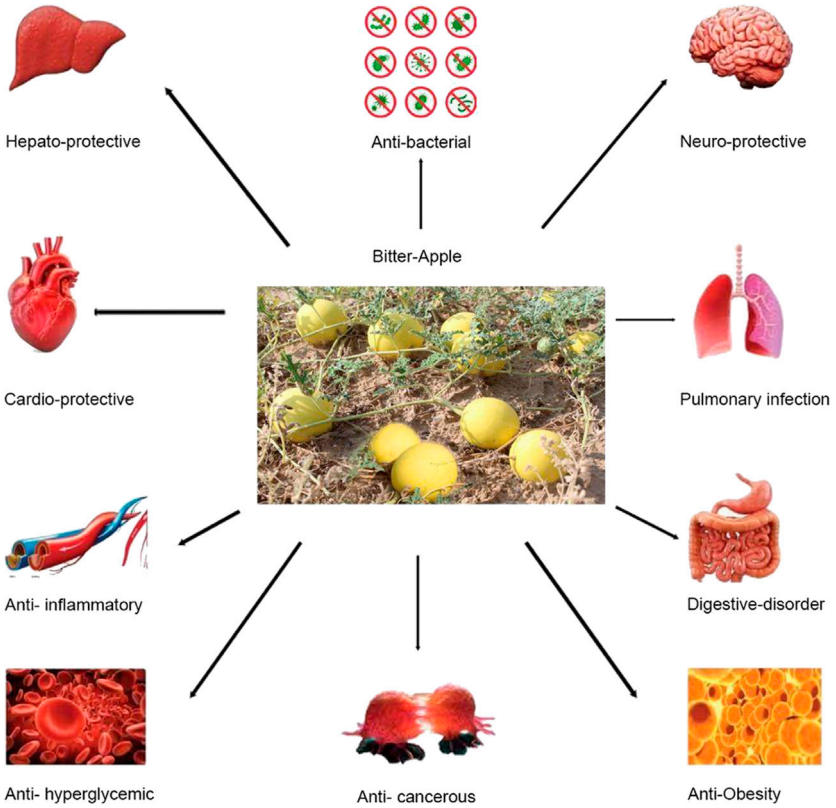


FIGURE 2 | Promising pharmacological effects of *C. colocynthis*.

TABLE 4 | Chemical constituents of *C. colocynthis* (Al-Snafi, 2016).

- ❖Carbohydrates
- ❖Alkaloids
- ❖Proteins
- ❖Glucosides
- ❖Phenolics, flavonoids
- ❖Tannins
- ❖Saponins
- ❖Cardiac glycolipids
- ❖Flavone, terpenoids
- ❖Cucurbitacins
- ❖Anthranol
- ❖Saponarin
- ❖Steroids
- ❖Trace elements

7 PHARMACOLOGICAL EFFECTS OF *C. COLOCYNTHIS*

C. colocynthis has many therapeutic uses and has also been studied for its various pharmacological effects. It is considered an excellent therapeutic agent for the trachea, gut, and cardiovascular system (Hussain et al., 2014).

7.1 Antimicrobial Properties

Previous studies report that aqueous and diluted acetone extracts (from the plant's roots, stems, leaves), and three maturation stages of its fruit and seeds of *C. colocynthis* plant are active against Gram-positive and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Enterococcus faecalis*), but have a more substantial effect on newer bacteria. The broth dilution method measured the minimal inhibitory concentration (MIC) preventing visible bacterial growth. MIC was tested for concentrations ranging from 0.10 to 6.50 mg/ml. For aqueous extracts of immature fruits, the MIC was 0.20 mg/ml for *Escherichia coli, Pseudomonas aeruginosa.* The activity depends on the strains, plant organs, stage of maturity, and the nature of the extraction (Marzouk et al., 2009).

The effect of the ethanolic extract of the *C. colocynthis* fruit was studied by the well diffusion method and disc diffusion method, and results showed that it has a standard antibacterial effect on both Gram-positive bacteria (*S. aureus* and *Bacillus subtilis*) and Gram-negative bacteria like *Klebsiella pneumoniae*. However, the ethanolic extract from the pulp was more active against Gram-positive bacteria, while the seed extract was slightly less effective against both types of bacteria (Hameed et al., 2020). Both aqueous and diluted acetone extracts showed antibacterial effects against both Gram-positive and Gram-negative bacteria as evaluated by *in vitro* study; the best results came from an aqueous fruit extract and poorest from a root extract. Immature seeds and fruits showed the highest antibacterial activity. The highest

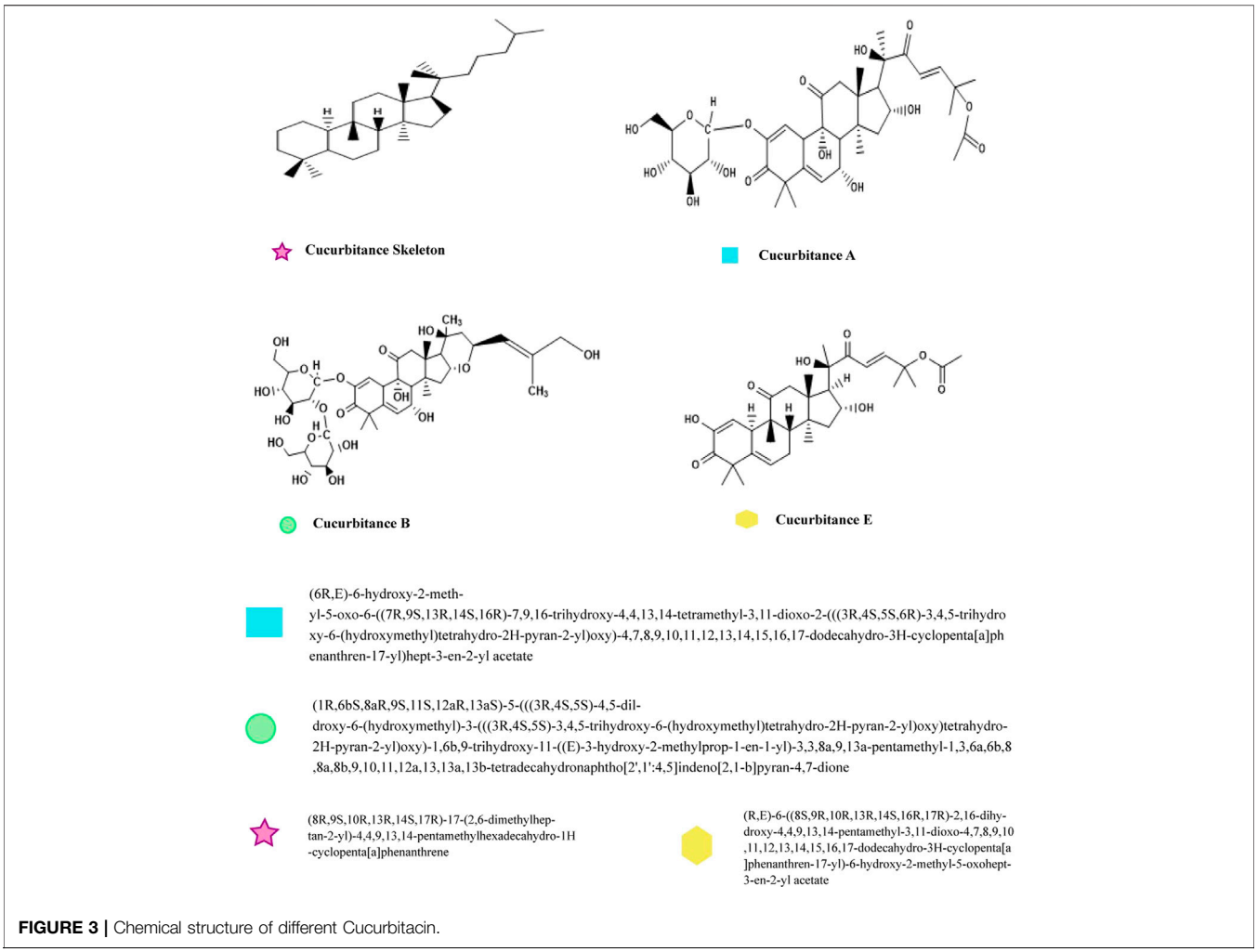


FIGURE 3 | Chemical structure of different Cucurbitacin.

MICs were obtained from the fruit aqueous extracts 0.20 mg/ml against *E. coli* and *P. aeruginosa* (Khatibi and Teymorri, 2011). The ethyl acetate extract of the leaves showed promising results against Gram-positive and Gram-negative bacteria (*Salmonella enteritidis, B. cereus, Escherichia coli, S. aureus, Enterococcus faecalis,* and *P. aeruginosa*) using the agar disc well-diffusion method. MIC values were obtained for the ethyl acetate extract 0.625 mg/ml against *Bacillus cereus* (Chawech et al.,. 2015).

7.2 Anti-Fungal Properties

The aqueous plant extract and a diluted acetone extract of *C. colocynthis* (roots, stems, leaves, and fruit and seeds at various stages of maturity) were selected for testing against several strains of *Candida* (*Candida glabrata, C. albicans, C. parapsilosis,* and *C. kreusei*). In a water extract, the mature fruit had the greatest anti-*Candida* effect among all strains (MIC 0.20 mg/ml); the immature fruit was the most active in an acetone extract against all strains (Marzouk et al., 2009). Using an ethanol extract of *C. colocynthis* fruit on various fungal species (*Fusarium oxysporum, C. albicans, Aspergillus fumigatus,* and *A. niger*) were assessed *in vitro* and produced good results against all strains, particularly *C. albicans*. The efficiency of the extracts was enhanced by increasing the concentration thereof. The results showed that all fungal strains were sensitive against the extracts of the fruit pulp, seeds, and roots of *C. colocynthis* (Hameed et al., 2020).

The anti-mycotic activity of the ethanol extract of *C. colocynthis* fruit was tested against pathogenic plant fungi using the agar dilution method and showed promising results. An organic extract of *C. colocynthis* fruit can thus be used as to alternative synthetic fungicide in agro-industries (Hadizadeh et al., 2009).

7.3 Antioxidant Effects

The methanolic fruit extract of *C. colocynthis* was found to be a good antioxidant. It exhibited good free radical scavenging activity due to the presence of gallic acid, a phenolic compound. The highest antioxidant and free radical scavenging ability of the fruit extract was observed at a concentration of 2,500 mg ml^{-1} (Kumar et al., 2008). Cucurbitacin is also an effective antioxidant. that can eliminate free radicals like hydroxyl radicals, superoxide anions, and singlet oxygen. It can also completely inhibit lipid peroxidation and

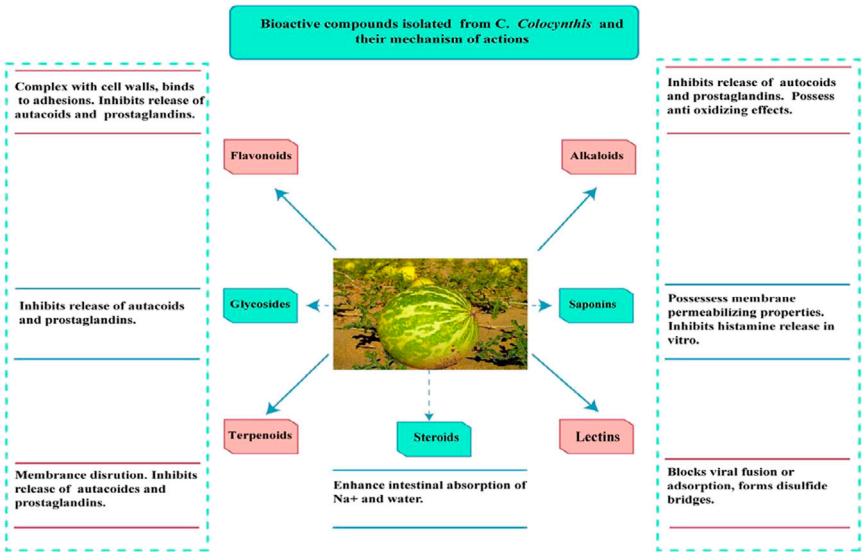


FIGURE 4 | Phytoconstituents of *C. Colocynthis* and their mechanism of action.

oxidation (Bernard and Olayinka, 2010). Phytochemical screening of *C. colocynthis* extracts revealed that the natural compounds present therein make it an excellent antioxidant (Benariba et al., 2013). *C. colocynthis* oil can boost the function of antioxidant enzymes and protect the liver from injury (Amamou et al., 2015). An *in-vitro* study states that *C. colocynthis* can prevent the damage caused by free radicals to the body. Various biochemicals in *C. colocynthis* make it a good antioxidant (Rizvi et al., 2018).

7.4 Anti-Inflammatory and Analgesic Properties

C. colocynthis water extracts were found to possess anti-inflammatory and analgesic activities. All extracts displayed palliative and anti-inflammatory potential at unique doses despite causing acute toxicity. The outcomes of the problem were acquired from unripe fruits and seeds. Stem and root extracts reduced big inhibitory endeavors in analgesic and anti-inflammatory models (Marzouk et al., 2009). The main bioactive chemical components in the chloroform part of CCS extracts came from the separation and characterization of glycoside 11-deoxycucurbitacinI2ObD at the doses of 0.5 and 1 mg/kg body weight in two animal models. The compounds studied demonstrated strong analgesic and anti-inflammatory effects in two animal models (Marzouk et al., 2013). The *in vivo* analgesic and anti-inflammatory actions of organic extracts of unripe fruits and seeds of Tunisian melon were studied. All extracts showed marked analgesic and anti-inflammatory effects at different doses. *C. colocynthis* Schrad appeared to interfere with histamine and serotonin pathways and strongly interfered with prostaglandin and kinin-like pathways (Marzouk et al., 2011). The methanolic extract of *C. colocynthis* leaves was evaluated for anti-inflammatory activity using different *in vivo* screening models. It had an inhibitory effect on the edema of the paw caused by different inflammatory drugs at the doses of 250 and 500 mg/kg, the infiltration of leukocytes, and the formation of exudate caused by carrageenan, thus presenting an anti-inflammatory effect on the acute and subacute phases of inflammation (Rajamanikam et al., 2010).

7.5 Anti-Hyperglycemic Activity

Various extracts of *C. colocynthis* peel–aqueous, alkaloidal, saponin, and glycosidic–were examined for their effects on plasma glucose levels in rabbits. The activity of the saponin extract on fasting blood sugar levels of alloxan-induced diabetic rabbits was examined. Normal rabbits orally (300 mg/kg) administered with an aqueous extract of *C. colocynthis* showed noticeably low plasma glucose levels after 1 h; this increased to high levels after 2, 3, and 6 h. The saponin extract lowers the fasting glucose levels after 1 and 2 h and considerably after 3 and 6 h (Abdel Hassan et al., 2000). The ethanol extract of *C. colocynthis* at the dose rate of 300 mg/kg on the blood glucose attention within the alloxan brought about diabetes in rats. The results showed that *C. colocynthis* could lower blood glucose markedly in contrast to manipulating the diabetic team. CCS were also shown to have a marked anti-hyperglycemic effect, supporting the everyday use of *C. colocynthis* to treat diabetes mellitus (Oryan et al., 2014). Wistar rats and streptozotocin-diabetic rats were injected with various extracts of CCS (total alkaloids, aqueous, saponin, and glycosidic) intraperitoneally to examine their anti-hyperglycemic activity. The results showed that these extracts had a good anti-hyperglycemic effect on the diabetic rats and stabilized the blood glucose of the control rats to within a normal range. The aqueous extract 2.5 g/kg (BW) showed the highest activity by decreasing the blood glucose

TABLE 5 | Biological effects and health benefits of *C. colocynthis*.

Plant part	Traditional use	Specie	References
Dried fruit pulp	Gastrointestinal disorders	Human	Hussain et al. (2014)
Fruit	Antioxidant, antimicrobial, and anti-inflammatory	Human	Hussain et al. (2014)
Fruit	Cold, diarrhea, deworming, antipyretic, expulsion wind, treatment of tumors, ascites, leukoplakia, ulcers, asthma, bronchitis, diabetes insipidus, jaundice, splenomegaly, neck tuberculosis, constipation, anemia, diseases of the throat, elephantiasis, and joint pain	Human	Pravin et al. (2013)
Root	Jaundice, ascites, diseases of the urinary system, rheumatism, and to treat an enlarged abdomen, and cough	Human	Pravin et al. (2013)
Ethanolic extract of the *C. colocynthis*/aqueous and diluted acetone extracts/ethyl acetate extract from leaves	Anti-bacterial	Bacteria (Gram-positive and Gram-negative)	Hameed et al.(2020), Khatibi and Teymorri. (2011), Chawech et al. (2015)
Ethanol extraction of *C. colocynthis*	Anti-hyperglycemic	Rat	Oryan et al. (2014)
Seed	Anti- Heat stress	Poultry	Alzarah et al. (2021)
Hydroalcoholic peel extract	Cardioprotective	Rabbit	Tabani et al. (2018)
Hydro-alcoholic leaf extract	Anti-hyperglycemic and anti-hyperlipidemic	Rat	Ebrahimi et al. (2016)
Colocynth oil	Anti-obesity	Rat	Meziane et al. (2012)
The ethanol extract of *C. colocynthis* fruit	Anti-mycotic	Plant pathogenic fungi	Hadizadeh et al. (2009), Hameed et al. (2020)
Ethanol extract of *C. colocynthis*	Antifertility	Rat	Chaturvedi et al. (2003)
C. colocynthis plant	Diabetes, liver problems. weak bowel movements, Obstruction, or paralysis of the intestine		Rahimi et al. (2012)
Fruit extract	Analgesic	Human	Hyderi et al. (2015)
Leaves	Anticancerous, Anti-adipogenic, and hypolipidemic	Human	Perveen et al. (2020)
C. colocynthis	Anti-fertility	Human	Amal et al. (2016)
C. colocynthis fruit extract	Anti-tumor	Human cell lines	Saeed et al. (2019)
C. colocynthis fruit	Anthelmintic	Animals	Qureshi et al. (2010)
C. colocynthis oil	Constipation	Human	Qureshi et al. (2010)
C. colocynthis extract	Anti-cancer	Human	Abdulridha et al. (2020)
C. colocynthis pulp and seed	Anti-diabetic	Rabbit	Shafaei et al. (2014)
C. colocynthis fruit	Anti-diabetic	Human	Huseini et al. (2009)
Hydro-alcoholic *C. colocynthis* fruit extract	Anti-diarrheal	Rat	Dhakad. (2017)
Hydro-ethanolic pulpy flesh of *C. colocynthis* with its seeds	Anti-hyperglycemic	Rat	Ghauri et al. (2020)
C. colocynthis extract	Anti-oxidant		Benariba et al. (2013)
C. colocynthis fruit extract	Anti-convulsant	Rat	Mehrzadi et al. (2016)
Leaf and root extract	Skin disorders	Humans	Upadhyay et al. (2007)
Roots paste	Joint problems	Humans	Upadhyay et al. (2007)
C. colocynthis	Anti-obesity	Rat	Sari et al. (2019)

level (Lahfa et al., 2017). The hydro-ethanolic pulpy flesh of *C. colocynthis* also demonstrated an exceptional anti-hyperglycemic effect in a diabetic rat at the dose rate of 300 mg/kg by decreasing its blood glucose and triglyceride, and cholesterol levels. *In vitro* testing also showed that *C. colocynthis* inhibited glucosidase, which is responsible for postprandial hyperglycemia, strongly indicating that it is a potential candidate for a hyperglycemia treatment (Ghauri et al., 2020).

The *C. colocynthis* fruit possesses insulin-enhancing activity. This activity may explain in part its antidiabetic effects in traditional medicine. It also identifies the *C. colocynthis* as a source of a potential novel insulin enhancer that may prove to be useful to reduce hyperglycemia in type 2 diabetes. The ethyl acetate fractions of aqueous non-defatted seed and pulp extracts were used. Two extracts enhanced the insulin-induced translocation of glucose transporter (GLUT4) from intracellular storage sites towards the plasma membrane and accordingly increased insulin-induced glucose uptake. Several of our findings suggested that pulp extract, which increased glucose uptake more than its seed homolog, increased GLUT4 translocation and glucose uptake by acting on the same intracellular signaling cascade as the one employed by insulin (Drissi et al., 2021).

7.6 Anti-Obesity Activity

Results from the administration of 4%colocynth oil to the offspring of overweight rats suggest that it can aid in weight reduction, maintenance of a healthy lipid profile, and controlling glucose levels. This suggests that the oil has a remedial and regulating effect on obesity (Meziane et al., 2012). The effect of glycoside and alkaloid extracts of colocynth were studied on 26 adult male Wistar rats. Animals administered with alkaloids showed weight regression, while those given glycosides were appropriately sized, starting from the 6th week. It became a widespread give-up of treatment (Tabani et al., 2018). These

results suggest that *C. colocynthis* seed oil has good potential for treating obesity and related problems (Sari et al., 2019).

7.7 Anti-Tumor Activity

The anti-tumor activity of *C. colocynthis* can be attributed to different pathways and properties, including apoptotic pathways, antioxidant and anti-inflammatory effects, inhibition of the Wnt/ß-catenin signaling pathway, and anti-metastatic effects. The cucurbic acid in *C. colocynthis* gives the plant its anti-cancerous properties (Abdulridha et al., 2020). The methanolic extract of *C. colocynthis* leaves and its two fractions, ethyl acetate and chloroform, possess notable anti-cancerous effects on the human breast cancer cell line. Bioassays showed a marked reduction in the multiplication and growth of cells treated with these extracts compared to untreated cells. The presence of cyclin-CDK inhibitors means that *C. colocynthis* extract can arrest human breast cancer cells (Perveen et al., 2021). Colocynth fruit pulp extracts can also block the proliferation and metastatic activity of breast cancer cells and prevent cell migration, the induction of cell apoptosis and cell proliferation, and inhibit cancer stemness properties in breast cancer cells (Chowdury et al., 2017). By modulating the metabolism of lipids, *C. colocynthis* leaves showed excellent potential as anti-cancerous agents in treating human breast cancer (Perveen et al., 2020). The extract of *C. colocynthis* fruit also showed anti-tumor activity on cancerous cell lines (Saeed et al., 2019).

7.8 Hepatoprotective Activity

The glycoside and alkaloid extract of colocynth (70 mg/kg single intraperitoneal injection) were analyzed for their effect on metabolic and histological liver disorders in Wistar rats. Treatments therewith showed hypoglycemic, lipid-lowering, and hepatoprotective effects. There was a marked increase in the levels of the liver function markers aspartate aminotransferase, ALT, and alkaline phosphatase (Tabani et al., 2018). The administration of ethanolic extracts of *C. colocynthis* (200 mg/kg BW), as opposed to paracetamol, resulted in hepatotoxicity in albino rats. The 90% ethanolic extract of *C. colocynthis* leaves exhibited *in-vivo* hepatoprotective effects that can be attributed to cell membrane stabilization and liver cell regeneration (Dar et al., 2012).

The hydro-alcoholic extract of *C. colocynthis* leaves (75 mg/kg body weight orally for 3 weeks) showed good anti-hyperglycemic and anti-hyperlipidemic effects. In addition, *C. colocynthis* leaf extract might also have a protective effect on the liver, as demonstrated by the markedly lower fasting blood sugar, low-density lipoprotein, cholesterol, alanine aminotransferase, creatinine, aspartate aminotransferase, urea, triglycerides, and bilirubin levels in diabetic rats to which it was administered (Ebrahimi et al., 2016).

7.9 Cardioprotective Activity

Experiments on male rabbits suggest that the administration of adrenaline prompted myocardial damage, as shown by the increased ranges of histomorphological adjustments in the myocardium associated with free radical manufacturing in cardiac tissue. *C. colocynthis* provided cardiac protection by decreasing oxidative stress caused by the experimental myocardial infarction, preventing the free radical-arbitrated damage of a catecholamine attack. The hydro-alcoholic extract of *C. colocynthis* peel also showed cardioprotective potential in experimentally induced myocardial infarction in rabbits, as shown by improvements in histological variations and the estimation of different biochemical and inflammatory markers in injured cardiac tissue. Rabbits pretreated with extract 300 mg/kg for 14 successive days significantly prevented the effect of adrenaline and maintained the biochemical parameters at a normal level (Manzoor et al., 2020).

7.10 Neuroprotective Activity

The neuroprotective efficacy of *C. colocynthis* was observed by estimating its effect on endogenous antioxidant molecules in brain samples of a rat with rotenone-induced Parkinson's disease (Ahmed et al., 2019). The therapeutic impact of *C. colocynthis* and its protective mechanisms confirmed that it showed an excellent neuroprotective impact, lessening oxidative stress and inhibiting apoptotic cell death in both *in-vitro* and *in-vivo* model (Chen et al., 2019). Treatment with hydro-alcoholic *C. colocynthis* pulp extract also showed an anticonvulsant effect in rats. Injection of the *C. colocynthis* extract (25 and 50 mg/kg) exhibited protection against seizure, prolonged the onset of a seizure significantly, and decreased the duration of seizures (Mehrzadi et al., 2016).

All these studies, either *in-vitro* or *in-vivo*, are suggestive of promising effects of *C. colocynthis* and validate its use in traditional medicine as a treatment of gastrointestinal, pulmonary infection and skin infections, constipation, edema, bacterial infections, cancer, diabetes, gastrointestinal disorders, liver problems and as an analgesic.

7.11 Toxicity Assessment

The effect of methanolic extract of *C. colocynthis* fruit was evaluated on male albino Wister rats to assess its toxicity. The bone marrow, liver, and kidney functions of the animals were measured using preferred techniques. The acute median deadly dose of the extract was calculated to be 1,311, 45 mg/kg. Plasma AST, urea, ALT, and creatinine titers were affected to a notable extent, indicating that the extract was hepato-nephrotoxic. These findings confirmed that the consumption of the extract of ripe *C. colocynthis* fruit has some undesirable effects on the bone marrow, liver, and kidneys of rats (Soufane et al., 2013).

The membranolytic effect of some *C. colocynthis* components can cause intestinal damage (Javadzadeh et al., 2013). In a study of the subchronic hemotoxicity and cytotoxicity of *C. colocynthis* on albino rats, the oral LD50 for extraction of *C. colocynthis* flowers was found to be 162.4 mg/kg of bodyweight. Pathological adjustments to the lung, liver, kidney, spleen, stomach and intestine of the treated rats were also recorded (Elgerwi et al., 2013). The noxiousness of ingesting an extract with 10% *C. colocynthis* fruits was checked in the rats. The outcomes of

C. colocynthis treatment were depression, ruffled hair, low body weight, feeding efficiency, and entero-hepato-nephropathy. Diarrhea is a clear sign of *C. colocynthis* poisoning. Lesions were observed on the organs in addition to leukopenia, anemia, modifications in serum enzyme (AST, ALT ALP, and ALT) levels, and concentrations of whole protein, urea, bilirubin, albumin, and one of a kind serum constituent (Al-Yahya et al., 2000). *C. colocynthis* is a strong laxative, with one case report suggesting that ingestion of the former causes inflammation of the colon with bloody diarrhea (Goldfain et al., 1989). High doses of *C. colocynthis* have detrimental effects on liver cells (Dehghan and Panjeh, 2006). High doses of its pulp extract, in particular, were deadly in rabbits, causing dehydration owing to severe diarrhea, heart failure due to cardio-stimulatory action, hepatorenal insults, or hypoglycemia; seed extract caused mild intestinal lesions (Shafaei et al., 2012). Hepatic damage, watery diarrhea, hypoglycemia, and hypotension were observed in a man who received high doses of a *C. colocynthis* fruit decoction to treat constipation (Rezvani et al., 2011). Chickens fed a diet of 10% *Citrullus* developed reversible lesions in their livers, small intestines, and kidneys (Bakhiet and Adam, 1995). Ten sheep fed fresh *C. colocynthis* fruits and leaves developed poisoning symptoms and died between 4 and 25 days of being dosed. Diarrhea, dyspnea, anorexia, and loss of condition are clinical symptoms (Elawad et al., 1984). Oral administration of *C. colocynthis* fruit fruits 0.25 g/kg/day with *Rhazya stricta* leaves resulted in dehydration, loss of condition, profuse diarrhea, ataxia, and recumbency prior to death within 26 days (Adam et al., 2000).

8 APPLICATIONS IN POULTRY

CCS was fed to 144-day-old straight-run chicks as a potential source of protein in feed, in place of soybean meal. The feeding experiment revealed that including up to 15% of the whole seed in the feed resulted in the normal growth of the chicks. However, the inclusion of 15% unprocessed meals depressed growth and showed a poor feed conversion ratio (FCR) (Sawaya et al., 1986).

CCSP was fed to 360-day-old Ross strain broiler chickens as a 0, 2, 4, and 6% supplement in feed. The result of the study showed that the 6% supplement in feed improved live body weight and dressing percentage while decreasing feed intake and FCR (Ali et al., 2012).

C. colocynthis fruit powder was fed to 100 broiler chickens, among which 100 chicks were given this on the sixth day after inoculation with *Eimeria tenella*. The power was supplemented in feed at 0.05, 0.01, 0.15, and 0.00%. The result showed that the 0.15% *C. colocynthis* fruit powder supplement was the most efficient at preventing coccidiosis (ALamery and ALsaeq, 2011). The effects of CCS meal (CCSM) on 270-day-old male Cobb broiler chickens were studied. CCSM was supplemented through feed at 0, 2, and 4%, and results showed that supplementation at 4% improved carcass weight, dressing percentage, and live body weight. As the dietary level of CCSM increased, feed intake decreased, and FCR was impaired (Ali et al., 2012). In another study, *C. colocynthis* was fed to 240-day-old Ross broiler chicks to check the effects of the former on growth performance and intestinal morphology. Here, *C. colocynthis* was supplemented at 0, 0.2, 0.4, and 0.6% of bitter cucumber feed with 0 and 0.01% protein. The results showed that supplementation at 0.6% improved feed intake, body weight gain, breast meat, and carcass yield while reducing FCR. Villus height, crypt depth, and intestinal mucosal muscle also increased (Hashemi et al., 2016).

In a different study, *C. colocynthis* fruit pulp was fed to replace antibiotic growth promoters with 400-day-old Ross broiler chicken chicks. Here, *C. colocynthis* fruit pulp was supplemented at 1 g/kg feed and 1.5 g/kg. The result showed that supplementation of *C. colocynthis* fruit pulp at the latter rate could replace antibiotic growth promoters (Kamran et al., 2018). In a separate study, CCS was fed *via* feed to 300 Cobb 21-day-old broiler chickens subjected to chronic heat stress at the rate of 0.1%. The result showed that this supplementation rate improved immune response and production performance in the heat-stressed group but had no effect on a control (thermo-neutral) group (Alzarah et al., 2021). The promising effects of *C. colocynthis* for poultry nutrition are shown in **Figure 5**.

9 VETERINARY USES

At 4 g/day, a polyherbal combination including *C. colocynthis* modified gene expression to promote growth and health from the pre-ruminant to weaning phase. Some gene expression research indicates that polyherbal therapy enhanced lipid, protein, carbohydrate, and immune response metabolism. These findings support using plant chemicals in animal feed (Díaz Galván et al._, 2021). Supplementation with *C. colocynthis* fruit showed potential to minimize methanogenesis and improve rumen fermentation. However, *in vivo* testing on ruminants is required to evaluate the persistence of benefits as well as health issues (Hundal et al., 2020). De-oiled CCS cake was added to dairy cow feed and showed no effect on milk yield (Khatri et al., 1993).

Ten sheep were fed fresh *C. colocynthis* fruits and leaves. The sheep developed poisoning symptoms and died within 4–25 days after being dosed. They showed symptoms of dyspnea, diarrhea, loss of condition, and anorexia (Elawad et al., 1984). *C. colocynthis* fruits oral dosing with *Rhazya stricta* leaves proved deadly within 26 days, resulting in ataxia, profuse diarrhea, loss of condition, dehydration, and recumbency prior to death (Adam et al., 2000). A trial of the ingestion and metabolism of *C. colocynthis* was undertaken in 12 yearlings Najdi sheep to investigate the consumption of crude protein in CCS meal as this was shown to be a good partial substitute for soybean meal in sheep diets (Bhattacharya, 1990). After reviewing the literature, we found that there is currently a lack of research-based data on the use of *C. colocynthis* in veterinary science. More research is needed to determine its importance and note effective inclusion levels in the diet of animals.

10 APPLICATIONS IN HUMANS

The methanolic extract of *C. colocynthis* leaves and its two fractions, ethyl acetate, and chloroform possess notable anti-cancerous effects. Bioassays showed a significant reduction in the multiplication and growth of treated cells compared to untreated cells. Owing to the expression of cyclin-CDK inhibitors, *C. colocynthis* arrests the cell cycle in human breast tumor cells (Perveen et al., 2021). *C. colocynthis* is used for treating colorectal cancer in humans. Cucurbic acid present in *C. colocynthis* extract is believed to stop the multiplication of cancerous cells. The anti-tumor activity of *C. colocynthis* can be attributed to different pathways and effects, such as apoptotic pathways, antioxidant and anti-inflammatory effects, inhibition of Wnt/ß-catenin signaling pathway, and anti-metastatic effects (Abdulridha et al., 2020). CCSP lowers the cholesterol level in non-diabetic patients (Rahbar and Nabipour, 2010). The *C. colocynthis* plant acts as a good anti-diabetic agent in humans with type II diabetes as it reduces glucose and cholesterol levels (Youshan et al., 2015; Chenghe et al., 2014). *C. colocynthis* fruit pulp of mature seed can also be used to treat tuberculosis, and it was found to have active anti-bacterial properties against various strains of normal and drug-resistant *mycobacterium* (Archana et al., 2013). The methanolic extract of *C. colocynthis* fruit is also active against several food-borne bacteria hazardous to human health (Kim et al., 2014). *C. colocynthis* also shows excellent potential as an anti-cancerous agent for treating human breast cancer *via* the modulation of lipid metabolism (Perveen et al., 2020). Consumption of *C. colocynthis* for a long time could lead to anti-fertility issues in both males and females (Chaturvedi et al., 2003; Amal et al., 2016). *C. colocynthis* fruit extract shows anti-tumor activity on drug-resistant cancerous cell lines (Saeed et al., 2019). *C. colocynthis* oil can be used for treating constipation in humans (Qureshi et al., 2010). *C. colocynthis* fruit has shown promising results in treating diabetic patients. A dose of 300 mg/day given to patients showed no adverse effects on their health (Huseini et al., 2009). An extract of *C. colocynthis* leaves can be used to treat skin infections, and a paste of *C. colocynthis* roots can be applied to treat joint problems (Upadhyay et al., 2007). *C. colocynthis* has also been used to treat edema, cancer, constipation, bacterial infections, and diabetes and has been used as an abortifacient (Delazar et al., 2006). *C. colocynthis* is a desert shrub with a long history as a valuable oil source and medicinal plant. During the first 4 weeks of ripening, yield and size of fruit, seed output, and overall oil yield were all at their peak. *C. colocynthis* has the potential to be grown as a source of edible oil. The oil has a laxative effect and contains between 80 and 85 percent unsaturated oleic and linoleic fatty acids, making it a high-quality oil for human consumption (Schafferman et al., 1998).

The health benefits of *C. colocynthis* are shown in **Figure 6**.

11 LIMITATION AND FUTURE RESEARCH

The chemical cucurbitacin, found in colocynth, irritates mucous membranes, especially those in the stomach and intestines. Colocynth is extremely dangerous to use. The Food and Drug Administration (FDA) prohibited it in 1991. Even tiny doses of colocynth can induce severe stomach and gut lining irritation, bloody diarrhea, kidney damage, bloody urine, and inability to pee. Convulsions, paralysis, and death are some of the other adverse effects. Colocynth dosage is determined by a number of factors, including the user's age, health, and other circumstances. There is insufficient scientific evidence to define a suitable dosing range for colocynth. Keep in mind that natural products aren't always safe and that doses are crucial. Before using, be sure to read the product label and consult your pharmacist, physician, or another healthcare expert. According to reports, ingestion of merely 1-1/2 tablespoons of the powder has resulted in death. Colocynth is unsuitable for use during pregnancy and breastfeeding. Ingestion of less than 2 gm of the powder has been reported to result in death. In clinical studies, as little as 300 mg of colocynth powder was found to cause moderate diarrhea.

Oral administration of *C. colocynthis* fruits 0.25 g/kg/day with Rhazya stricta leaves proved deadly within 26 days, resulting in dehydration, loss of condition, profuse diarrhea, ataxia, and recumbency before death (Adam et al., 2000). The treatment regimen having 10% of *C. colocynthis* fruits was once checked for rats. The outcomes characterized by *C. colocynthis* treatment were depression, ruffled hair, low physique weight, low feed efficiency, and entero-hepato-nephropathy (Al-Yahya et al., 2000). Chickens fed a 10% Citrullus diet developed reversible lesions in the liver, small intestine, and kidney (Bakhiet and Adam, 1995). 60 ml of decoction of the plant fruit taken by a 48-year old man to treat constipation resulted in watery diarrhea, hypotension and hypoglycemia, and hepatic injury (Rezvani et al., 2011). *C. colocynthis* being stimulant laxatives can cause the body's potassium levels to drop. Low potassium levels might exacerbate digoxin side effects. Lanoxin: Colocynth can induce diarrhea in some patients who are taking Warfarin. Diarrhea can make Warfarin less effective and raise the risk of bleeding. Taking colocynth with water pills may cause the body's potassium levels to drop too low.

The biological activity of the extracts and isolated compounds have been discovered, particularly in antidiabetic, anticancer, anti-inflammatory, antioxidant, insecticidal, and antibacterial areas. Interestingly, the plant has been demonstrated to have a high nutritional value since it is a strong source of protein, has edible seed oil, and contains certain vital minerals such as calcium, potassium, and magnesium, all of which are known to have medical benefits. Despite the fact that growing interest has driven greater research on *C. colocynthis*' phytochemistry and pharmacology, there are still many areas where existing understanding might be improved. Furthermore, there is a scarcity of information concerning its mode of action and dosing rate. In recent pharmacological investigations, various traditional applications of the *C. colocynthis* fruit have been verified; however, some of these studies were only examined *in vitro*. As a result, *in vivo* experiments should be used to evaluate further the efficacy and safety of *C. colocynthis* fruit extracts and isolated chemicals. *In previous studies, C. colocynthis* has been shown to have many roles in people, cattle, and fowl. The aforementioned system's literature has

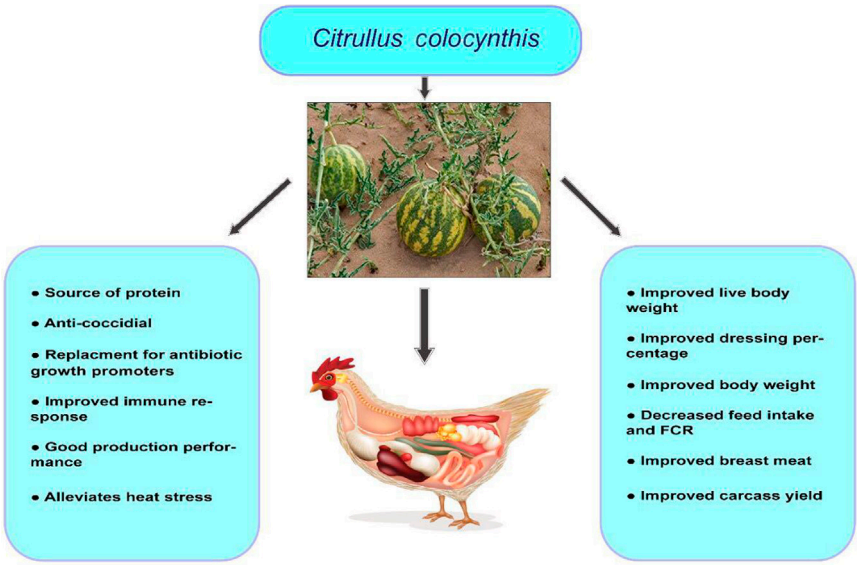


FIGURE 5 | Application of *C. colocynthis* in avian nutrition.

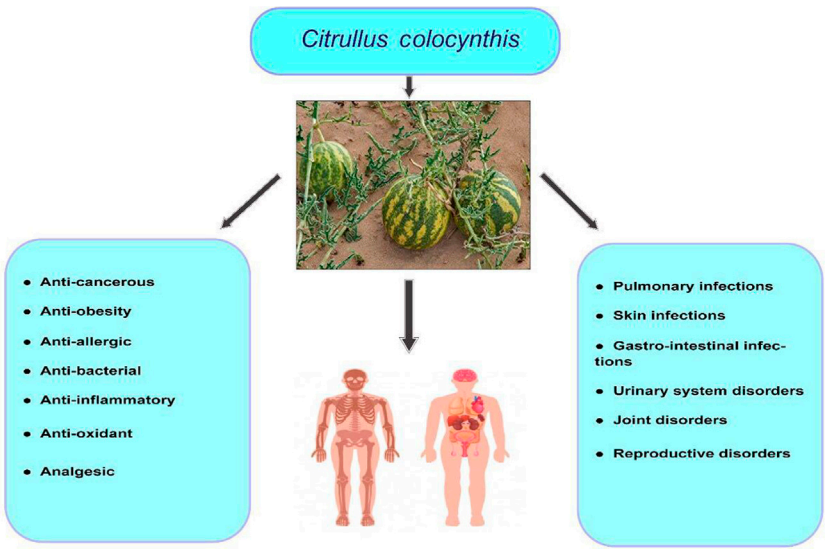


FIGURE 6 | Health benefits of *C. colocynthis* in humans.

been evaluated from a variety of sources. Because there is limited research on the use of *C. colocynthis* in poultry, veterinary medicine, and human medicine, it is the forward reassessment to advocate *C. colocynthis* plant and extract for use in poultry and human medicine manufacturing.

12 CONCLUSION AND PERSPECTIVES

In the present assessment, the nutrient composition and medicinal qualities of *C. colocynthis* have been evaluated based on various previous studies. This review strongly indicates that *C. colocynthis* is a fruit crop that could benefit the treatment of a range of diseases. Although *C. colocynthis* has high dietary value, it is not widely known. More investigations are required to spotlight the utility of such fruit crops as a dietary supplement that can enhance fitness. This review demonstrates that *C. colocynthis* is a medicinal plant with a wide variety of pharmacological properties that might make it useful and effective in numerous medical applications. To date, no review article has published comprehensive literature about its uses in

poultry, veterinary and human areas. This review gives a thorough insight into its phytochemistry along with the structure-activity relationship of some bioactive compounds, pharmacology, beneficial effects, limitations, and drug interaction. The study compiles the most recent data present on *C. colocynthis*. So, the objective of this review is to provide comprehensive data about the benefits and limitations of *C. colocynthis*, as the data about inclusion levels and its use and possible side effect are still not precise and need to be validated by pharmacological investigations against various disorders *in vivo*. *C. colocynthis* has many vital health-promoting effects like neurological, physiological as well as biological functions, but still, their mechanism of action behind these properties in different species is not known and needs to be exploited. The future avenues for the veterinary and pharmaceutical researchers would be to identify more of these demanding areas and document reliable markers (bio and molecular) which are responsible for a vast array of *C. colocynthis's* benefits.

AUTHOR CONTRIBUTIONS

Q-YL, MM, and MS: Conceptualization. MK and J-QS: Review and editing. MA, MN, SN, and AM: Original draft, writing–review and editing and C-XL: Supervision.

FUNDING

This work was financed by the Qinghai University School of Medicine Young and Middle-aged Scientific Research Fund Project (Natural Science) (#. 2020-kyy-8) and the Qinghai Fundamental Scientific and Technological Research Plan (#. 2018-ZJ-730, 2019-SF-134) and CUVAS.

ACKNOWLEDGMENTS

All the authors of the manuscript also thank and acknowledge their respective Universities and Institutes.

REFERENCES

Abd El-Ghany, W. A., and Yazar Soyadı, Y. A. (2020). Phytobiotics in Poultry Industry as Growth Promoters, Antimicrobials and Immunomodulators - A Review. *jwpr* 10, 571–579. doi:10.36380/jwpr.2020.65

Abdel-Hassan, I. A., Abdel-Barry, J. A., and Tariq Mohammeda, S. (2000). The Hypoglycaemic and Antihyperglycaemic Effect of *Citrullus colocynthis* Fruit Aqueous Extract in normal and Alloxan Diabetic Rabbits. *J. Ethnopharmacol* 71 (1-2), 325–330. doi:10.1016/s0378-8741(99)00215-9

Abdulridha, M. K., Al-Marzoqi, A. H., and Ghasemian, A. (2020). The Anticancer Efficiency of *Citrullus colocynthis* toward the Colorectal Cancer Therapy. *J. Gastrointest. Cancer* 51 (2), 439–444. doi:10.1007/s12029-019-00299-6

Adam Sei, S. E. I., Al-Farhan, A. H., and Al-Yahya, A. (2000). Effect of Combined *Citrullus colocynthis* and Rhazya Stricta Use in Najdi Sheep. *Am. J. Chin. Med.* 28 (03n04), 385–390. doi:10.1142/S0192415X00000453

Ahmed, M., Ji, M., Qin, P., Gu, Z., Liu, Y., Sikandar, A., et al. (2019). Phytochemical Screening, Total Phenolic and Flavonoids Contents and Antioxidant Activities of *Citrullus colocynthis* L. And Cannabis Sativa L. *Appl. Ecol. Environ. Res.* 17, 6961–6979. doi:10.15666/aeer/1703_69616979

Akpambang, V. O. E., Amoo, I. A., and Izuagie, A. A. (2008). Comparative Compositional Analysis on Two Varieties of Melon (Colocynthis citrullus and Cucumeropsis Edulis) and a Variety of almond (Prunus Amygdalus). *Res. J. Agric. Biol. Sci.* 4 (6), 639–642.

Al-Dobaib, S. N., and Mousa, H. M. (2009). Benefits and Risks of Growth Promoters in Animal Production. *J. Food Agric. Environ.* 7 (2), 202–208.

Al-Snafi, P. D. A. E. (2016). Chemical Constituents and Pharmacological Effects of Cynodon Dactylon- A Review. *Iosrphr* 06 (7), 17–31. doi:10.9790/3013-06721731

Al-Yahya, M. A., Al-Farhan, A. H., and Adam, S. E. (2000). Preliminary Toxicity Study on the Individual and Combined Effects of *Citrullus colocynthis* and Nerium Oleander in Rats. *Fitoterapia* 71 (4), 385–391. doi:10.1016/s0367-326x(00)00135-0

Alagawany, M., Abd El-Hack, M. E., Saeed, M., Naveed, M., Arain, M. A., Arif, M., et al. (2020). Nutritional Applications and Beneficial Health Applications of green tea and L-Theanine in Some Animal Species: A Review. *J. Anim. Physiol. Anim. Nutr. (Berl)* 104, 245–256. doi:10.1111/jpn.13219

Alagawany, M., El-Saadony, M. T., Elnesr, S. S., Farahat, M., Attia, G., Madkour, M., et al. (2021c). Use of Lemongrass Essential Oil as a Feed Additive in Quail's Nutrition: its Effect on Growth, Carcass, Blood Biochemistry, Antioxidant and Immunological Indices, Digestive Enzymes and Intestinal Microbiota. *Poult. Sci.* 100 (6), 101172. doi:10.1016/j.psj.2021.101172

Alagawany, M., Elnesr, S. S., Farag, M. R., Tiwari, R., Iqbal Yatoo, M., Karthik, K., et al. (2021a). Nutritional Significance of Amino Acids, Vitamins and Minerals as Nutraceuticals in Poultry Production and Health - A Comprehensive Review. *Vet. Q.* 41 (1), 1–45. doi:10.1080/01652176.2020.1857887

Alagawany, M., Farag, M. R., Abdelnour, S. A., Dawood, M. A. O., Elnesr, S. S., and Dhama, K. (2021b). Curcumin and its Different Forms: A Review on Fish Nutrition. *Aquaculture* 532 (2021), 736030. doi:10.1016/j.aquaculture.2020.736030

ALamery, M. M., and ALsaeq, A. A. (2011). Addition Powder Citrullus Colocynthis Fruits to the Broiler Ratio for Treat's Coccidiosis Type E. Tenella. *AL-Qadisiyah J. Vet. Med. Sci.* 10 (2), 131–137.

Ali, S. A., Abdalla, H. O., and Elamin, M. A. (2012). *Citrullus colocynthis* (Handal) Seed Meal as a Natural Feed Supplementation in Broiler Chickens' Diets. *Egypt. Poult. Sci. J.* 32 (2), 237–246.

Alzarah, M. I., Althobiati, F., Abbas, A. O., Mehaisen, G. M. K., and Kamel, N. N. (2021). *Citrullus colocynthis* Seeds: A Potential Natural Immune Modulator Source for Broiler Reared under Chronic Heat Stress. *Animals (Basel)* 11 (7), 1951. doi:10.3390/ani11071951

Amamou, F., Nemmiche, S., Meziane, R. K., Didi, A., Yazit, S. M., and Chabane-Sari, D. (2015). Protective Effect of Olive Oil and Colocynth Oil against Cadmium-Induced Oxidative Stress in the Liver of Wistar Rats. *Food Chem. Toxicol.* 78, 177–184. doi:10.1016/j.fct.2015.01.001

Bajagai, Y. S., Alsemgeest, J., Moore, R. J., Van, T. T. H., and Stanley, D. (2020). Phytogenic Products, Used as Alternatives to Antibiotic Growth Promoters, Modify the Intestinal Microbiota Derived from a Range of Production Systems: an *In Vitro* Model. *Appl. Microbiol. Biotechnol.* 104 (24), 10631–10640. doi:10.1007/s00253-020-10998-x

Bakhiet, A. O., and Adam, S. E. (1995). An Estimation of *Citrullus colocynthis* Toxicity for Chicks. *Vet. Hum. Toxicol.* 37 (4), 356–358.

Ben Geoffrey, A. S., Christian Prasana, J., and Muthu, S. (2020). Structure-activity Relationship of Quercetin and its Tumor Necrosis Factor Alpha Inhibition Activity by Computational and Machine Learning Methods. *Mater. Today Proc.* doi:10.1016/j.matpr.2020.07.464

Benariba, N., Djaziri, R., Bellakhdar, W., Belkacem, N., Kadiata, M., Malaisse, W. J., et al. (2013). Phytochemical Screening and Free Radical Scavenging Activity of *Citrullus colocynthis* Seeds Extracts. *Asian Pac. J. Trop. Biomed.* 3 (1), 35–40. doi:10.1016/S2221-1691(13)60020-9

Bernard, S. A., and Olayinka, O. A. (2010). Search for a Novel Antioxidant, Anti-inflammatory/analgesic or Anti-proliferative Drug: Cucurbitacins Hold the Ace. *J. Med. Plants Res.* 4 (25), 2821–2826. doi:10.5897/JMPR.9001120

Bhasin, A., Singh, S., and Garg, R. (2020). Nutritional and Medical Importance of Citrullus Colocynthis-A Review. *Plant Arch.* 20 (2), 3400–3406.

Bhattacharya, A. N. (1990). *Citrullus colocynthis* Seed Meal as a Protein Supplement for Najdi Sheep in Northern Saudi Arabia. *Anim. feed Sci. Technol.* 29 (1-2), 57–62. doi:10.1016/0377-8401(90)90093-N

Bilal, R. M., Liu, C., Zhao, H., Wang, Y., Farag, M. R., Alagawany, M., et al. (2021). Olive Oil: Nutritional Applications, Beneficial Health Aspects and its Prospective Application in Poultry Production. *Front. Pharmacol.* 12, 723040. doi:10.3389/fphar.2021.723040

Chabane Sari, M., Nemmiche, S., Benmehdi, H., Amrouche, A., Lazouni Hamadi, A., and Chabane Sari, D. (2019). Hypolipidemic and Antioxidant Effects ofCitrullus colocynthisSeeds Oil in High-Fat Diets Induced Obese Rats. *Phytothérapie* 17 (6), 310–320. doi:10.3166/phyto-2018-0066

Chaturvedi, M., Mali, P. C., and Ansari, A. S. (2003). Induction of Reversible Antifertility with a Crude Ethanol Extract of *Citrullus colocynthis* Schrad Fruit in Male Rats. *Pharmacology* 68 (1), 38–48. doi:10.1159/000068727

Chawech, R., Jarraya, R., Girardi, C., Vansteelandt, M., Marti, G., Nasri, I., et al. (2015). Cucurbitacins from the Leaves of *Citrullus colocynthis* (L.) Schrad. *Molecules* 20 (10), 18001–18015. doi:10.3390/molecules201018001

Chen, J. C., Chiu, M. H., Nie, R. L., Cordell, G. A., and Qiu, S. X. (2005). Cucurbitacins and Cucurbitane Glycosides: Structures and Biological Activities. *Nat. Prod. Rep.* 22 (3), 386–399. doi:10.1039/b418841c

Chen, Y., Sa, Y., Wang, G., Pan, X., Zhen, Y., Cheng, X., et al. (2019). The Protective Effects of citrullus Colocynthis on Inhibiting Oxidative Damage and Autophagy-Associated Cell Death in Parkinson's Disease. *J. Taiwan Inst. Chem. Eng.* 100, 18–25. doi:10.1016/j.jtice.2019.04.003

Chowdhury, K., Sharma, A., Kumar, S., Gunjan, G. K., Nag, A., and Mandal, C. C. (2017). Colocynth Extracts Prevent Epithelial to Mesenchymal Transition and Stemness of Breast Cancer Cells. *Front. Pharmacol.* 8, 593. doi:10.3389/fphar.2017.00593

Dar, A. I., Saxena, R. C., and Bansal, S. K. (20122012). Hepatoprotection: A Hallmark of *Citrullus colocynthis* L. Against Paracetamol Induced Hepatotoxicity in Swiss Albino Rats. *Ajps* 03, 1022–1027. doi:10.4236/ajps.2012.327121

De Smet, P. A. G. M. (1997). "Citrullus Colocynthis," in *Adverse Effects of Herbal Drugs* (Berlin, Heidelberg: Springer), 29–36. doi:10.1007/978-3-642-60367-9_4

Dehghan, F., and Panjeh, S. M. (2006). *The Toxic Effect of Alcoholic Extract of* Citrullus colocynthis *on Rat Liver*.

Delazar, A., Gibbons, S., Kosari, A. R., Nazemiyeh, H., Modarresi, M. A. S. O. U. D., Nahar, L. U. T. F. U. N., et al. (2006). Flavone C-Glycosides and Cucurbitacin Glycosides from *Citrullus colocynthis*. *Daru* 14 (3), 109–114.

Dhakad, Pk. (2017). Phytochemical Investigation and Anti-diarrheal Activity of Hydroalcoholic Extract of Fruits of *Citrullus colocynthis* (L.) Schrad. (Cucurbitaceae). *J. Mol. Genet. Med.* 11 (305), 1747–0862. doi:10.4172/1747-0862.1000305

Dhama, K., Karthik, K., Khandia, R., Munjal, A., Tiwari, R., Rana, R., et al. (2018). Medicinal and Therapeutic Potential of Herbs and Plant Metabolites/Extracts Countering Viral Pathogens - Current Knowledge and Future Prospects. *Curr. Drug Metab.* 19 (3), 236–263. doi:10.2174/1389200219666180129145252

Dhama, K., Sharun, K., Gugjoo, M. B., Tiwari, R., Alagawany, M., Iqbal Yatoo, Yatoo., et al. (2021). A Comprehensive Review on Chemical Profile and Pharmacological Activities of Ocimum Basilicum. *Food Rev. Int.*, 1–29. doi:10.1080/87559129.2021.1900230

Díaz Galván, C., Méndez Olvera, E. T., Martínez Gómez, D., Gloria Trujillo, A., Hernández García, P. A., Espinosa Ayala, E., et al. (2021). Influence of a Polyherbal Mixture in Dairy Calves: Growth Performance and Gene Expression. *Front. Vet. Sci.* 7. doi:10.3389/fvets.2020.623710

Ebrahimi, E., Mohammadzadeh, G., Mansouri, E., and Aberomand, M. (2016). Effects of Hydro-Alcoholic Leaf Extract of citrullus Colocynthis on Biochemical Factors and Histopathological Changes in Streptozotocin-Induced Diabetic Rats. *Jundishapur J. Nat. Pharm. Prod.* 11 (3). doi:10.17795/jjnpp-33214

Elawad, A. A., Abdel Bari, E. M., Mahmoud, O. M., and Adam, S. E. (1984). The Effect of *Citrullus colocynthis* on Sheep. *Vet. Hum. Toxicol.* 26 (6), 481–485.

Elgerwi, A., Benzekri, Z., Awaidat, S., El-Magdoub, A., Abusnina, A., and El-Mahmoudy, A. (2013). Subchronic Haemotoxicity and Histotoxicity of *Citrullus colocynthis*. *J. Am. Sci.* 9 (5).

Elltayeib, A. A. E., Mohamed, B. K., and Elshami, R. M. (2020). Chemical Properties and Antibacterial Effect of the Oil from Seeds of *Citrullus colocynthis*. *Insi Chem. Biochem*. doi:10.33552/icbc.2020.01.000512

Ertas, O. N., Guler, T., Çiftçi, M., DalkIlIç, B., and Simsek, U. G. (2005). The Effect of an Essential Oil Mix Derived from Oregano, Clove and Anise on Broiler Performance. *Int. J. Poult. Sci.* 4 (11), 879–884. doi:10.3923/ijps.2005.879.884

Garg, A. K., Faheem, M., and Singh, S. (2021). Role of Medicinal Plant in Human Health Disease. *Asian J. Plant Sci. Res.* 11 (1), 19–21.

Ghauri, A. O., Ahmad, S., and Rehman, T. (2020). *In Vitro* and *In Vivo* Anti-diabetic Activity of *Citrullus colocynthis* Pulpy Flesh with Seeds Hydro-Ethanolic Extract. *J. Complement. Integr. Med.* 17 (2). doi:10.1515/jcim-2018-0228

Goldfain, D., Lavergne, A., Galian, A., Chauveinc, L., and Prudhomme, F. (1989). Peculiar Acute Toxic Colitis after Ingestion of Colocynth: a Clinicopathological Study of Three Cases. *Gut* 30 (10), 1412–1418. doi:10.24941/ijcr.40170.11.202010.1136/gut.30.10.1412

Gurudeeban, S., Satyavani, K., and Ramanathan, T. (2010). Bitter Apple (*Citrullus colocynthis*): An Overview of Chemical Composition and Biomedical Potentials. *Asian J. Plant Sci.* 9 (7), 394–401. doi:10.3923/ajps.2010.394.401

Hadizadeh, I., Peivastegan, B., and Kolahi, M. (2009). Antifungal Activity of Nettle (Urtica Dioica L.), Colocynth (*Citrullus colocynthis* L. Schrad), Oleander (Nerium Oleander L.) and Konar (Ziziphus Spina-Christi L.) Extracts on Plants Pathogenic Fungi. *Pak J. Biol. Sci.* 12 (1), 58–63. doi:10.3923/pjbs.2009.58.63

Hameed, B., Ali, Q., Hafeez, M. M., and Malik, A. (2020). Antibacterial and Antifungal Activity of Fruit, Seed and Root Extracts of *Citrullus colocynthis* Plant. *Biol. Clin. Sci. Res. J.* 2020 (1). doi:10.47264/bcsrj010103310.54112/bcsrj.v2020i1.33

Hashemi, M., Jalali, S. M. A., and Kheiri, F. (2016). The Effect of Using of Bitter Cucumber Fruit Powder, *Citrullus colocynthis*, and Probiotic on Growth Performance and Intestinal Morphology of Broiler Chicks. *J. Med. Herbs* 7 (3), 193–200.

Heydari, M., Homayouni, K., Hashempur, M. H., and Shams, M. (2015). Topical *Citrullus colocynthis* (Bitter Apple) Extract Oil in Painful Diabetic Neuropathy: A Double-Blind Randomized Placebo-Controlled Clinical Trial. *J. Diabetes* 8, 246–252. doi:10.1111/1753-0407.12287

Hundal, J. S., Wadhwa, M., Singh, J., Dhanoa, J. K., and Kaur, H. (2020). Potential of *Citrullus colocynthis* as Herbal Feed Additive for Ruminants. *Indian J. Anim. Sci.* 90 (2), 244–248.

Huseini, H. F., Darvishzadeh, F., Heshmat, R., Jafariazar, Z., Raza, M., and Larijani, B. (2009). The Clinical Investigation of *Citrullus colocynthis* (L.) Schrad Fruit in Treatment of Type II Diabetic Patients: a Randomized, Double Blind, Placebo-Controlled Clinical Trial. *Phytother Res.* 23 (8), 1186–1189. doi:10.1002/ptr.2754

Hussain, A. I., Rathore, H. A., Sattar, M. Z., Chatha, S. A., Sarker, S. D., and Gilani, A. H. (2014). *Citrullus colocynthis* (L.) Schrad (Bitter Apple Fruit): A Review of its Phytochemistry, Pharmacology, Traditional Uses and Nutritional Potential. *J. Ethnopharmacol* 155 (1), 54–66. doi:10.1016/j.jep.2014.06.011

Javadzadeh, H. R., Davoudi, A., Davoudi, F., Valizadegan, G., Goodarzi, H., Mahmoodi, S., et al. (20132013). *Citrullus colocynthis* as the Cause of Acute Rectorrhagia. *Case Rep. Emerg. Med.* 2013, 1–5. doi:10.1155/2013/652192

Kamran, Z., Ruby, T., Hussain, M., Ali, S., Ahmad, S., Koutoulis, K. C., et al. (2018). *Comparative Efficacy of* Citrullus colocynthis *Fruit Powder and Popular Antibiotic Growth Promoters in Broilers. 6th Mediterranean Poultry Summit.*

Khatibi, R., and Teymorri, J. (2011). Anticandidal Screening and Antibacterial of *Citrullus colocynthis* in South East of Iran. *J. Hortic. For.* 3 (13), 392–398. doi:10.5897/JHF11.030

Khatri, L. M., Nasir, M. K. A., Saleem, Rubina., and Valhari, M. U. (1993). *Pakistan J. Scientific Ind. Res.* 36, 383. BCI:BCI199497409740.

Kim, M. G., Lee, S. E., Yang, J. Y., and Lee, H. S. (2014). Antimicrobial Potentials of Active Component Isolated from *Citrullus colocynthis* Fruits and Structure-Activity Relationships of its Analogues against Foodborne Bacteria. *J. Sci. Food Agric.* 94 (12), 2529–2533. doi:10.1002/jsfa.6590

Kumar, S., Kumar, D., Manjusha, K., Saroha, K., Singh, N., and Vashishta, B. (2008). Antioxidant and Free Radical Scavenging Potential of *Citrullus colocynthis* (L.) Schrad. Methanolic Fruit Extract. *Acta Pharm.* 58 (2), 215–220. doi:10.2478/v10007-008-0008-1

Lahfa, F. B., Azzi, R., Mezouar, D., and Djaziri, R. (2017). Hypoglycemic Effect of *Citrullus colocynthis* Extracts. *Phytothérapie* 15 (2), 50–56. doi:10.1007/s10298-015-0997-4

Lang, K. L., Silva, I. T., Machado, V. R., Zimmermann, L. A., Caro, M. S., Simões, C. M., et al. (2014). Multivariate SAR and QSAR of Cucurbitacin Derivatives as Cytotoxic Compounds in a Human Lung Adenocarcinoma Cell Line. *J. Mol. Graph Model.* 48, 70–79. doi:10.1016/j.jmgm.2013.12.004

Manzoor, A., Khan, I. A., Iqbal, M. O., Kousar, S., Munawar, s. H., Manzoor, Z., et al. (2020). *Evaluation of Cardioprotective Potential of Hydroalcoholic Peel Extract of Citrullus Colocynthis*. doi:10.21203/rs.3.rs-124440/v1

Marzouk, B., Marzouk, Z., Décor, R., Edziri, H., Haloui, E., Fenina, N., et al. (2009). Antibacterial and Anticandidal Screening of Tunisian *Citrullus colocynthis* Schrad. From Medenine. *J. Ethnopharmacol* 125 (2), 344–349. doi:10.1016/j.jep.2009.04.025

Marzouk, B., Marzouk, Z., Fenina, N., Bouraoui, A., and Aouni, M. (2011). Anti-inflammatory and Analgesic Activities of Tunisian *Citrullus colocynthis* Schrad. Immature Fruit and Seed Organic Extracts. *Eur. Rev. Med. Pharmacol. Sci.* 15 (6), 665–672. doi:10.1016/j.jep.2009.11.027

Marzouk, B., Mahjoub, M. A., Bouraoui, A., Fenina, N., Aouni, M., and Marzouk, Z. (2013). Anti-inflammatory and Analgesic Activities of a New Cucurbitacin Isolated from *Citrullus colocynthis* Seeds. *Med. Chem. Res.* 22 (8), 3984–3990. doi:10.1007/s00044-012-0406-2

Mazher, M., Ishtiaq, M., Mushtaq, W., Maqbool, M., Zahid, N., Husain, T., et al. (2020). Comprehensive Review of Phytochemistry and Bioactivities of Citrullus Colocynthis (L.) Schrad. *Pharm. Res.* 4 (4), 486. doi:10.33215/sjom.v4i1.486

Mehrzadi, S., Shojaii, A., Pur, S. A., and Motevalian, M. (2016). Anticonvulsant Activity of Hydroalcoholic Extract of *Citrullus colocynthis* Fruit: Involvement of Benzodiazepine and Opioid Receptors. *J. Evid. Based Complement. Altern Med* 21 (4), NP31–5. doi:10.1177/2156587215615455

Meybodi, M. S. K. (2020). A Review on Pharmacological Activities of *Citrullus colocynthis* (L.) Schrad. *Asian J. Res. Rep. Endocrinol.*, 25–34.

Meziane, R. K., Khemmar, L., Amamou, F., Yazit, M., and Didi, A. (2012). Anti-obesity and Anti-hyperlipidemic Effect of *Citrullus colocynthis* Oil in the Offspring of Obese Rats. *Ann. Biol. Res.* 3 (5), 2486–2490. www.scholarsresearchlibrary.com.

Milanov, D., Ljubojevic, D., Cabarkapa, I., and Aleksic, N. (2016). Impact of Antibiotics Used as Growth Promoters on Bacterial Resistance. *Food Feed Res.* 43 (2), 83–92. doi:10.5937/FFR1602083M

Najafi, S., Sanadgol, N., Nejad, B. S., Beiragi, M. A., and Sanadgol, E. (2010). Phytochemical Screening and Antibacterial Activity of *Citrullus colocynthis* (Linn.) Schrad against *Staphylococcus aureus*. *J. Med. Plants Res.* 4 (22), 2321–2325. doi:10.5897/JMPR10.192

Ogundele, J. O., Oshodi, A. A., and Amoo, I. A. (2012). Comparative Study of Amino Acid and Proximate Composition of Citurllus Colocynthis and Citrullus vulgaris Seeds. *Pakistan J. Nutr.* 11 (3), 247.

Oryan, A., Hashemnia, M., Hamidi, A. R., and Mohammadalipour, A. (2014). Effects of Hydro-Ethanol Extract of *Citrullus colocynthis* on Blood Glucose Levels and Pathology of Organs in Alloxan-Induced Diabetic Rats. *Asian Pac. J. Trop. Dis.* 4 (2), 125–251. doi:10.1016/S2222-1808(14)60328-510.3923/pjn.2012.247.251

Pakistan economic survey (2020-2021). Accessed on https://www.finance.gov.pk/survey_2021.html.

Parveen, B., Upadhyay, B., Roy, S., and Kumar, A. (2007). Traditional Uses of Medicinal Plants Among the Rural Communities of Churu District in the Thar Desert, India. *J. Ethnopharmacol* 113 (3), 387–399. doi:10.1016/j.jep.2007.06.010

Peric, L., Zikic, D., and Lukic, M. (2009). Application of Alternative Growth Promoters in Broiler Production. *Biotec Anim. Husb* 25 (5-6-1), 387–397. doi:10.2298/BAH0906387P

Perveen, S., Ashfaq, H., Ambreen, S., Ashfaq, I., Kanwal, Z., and Tayyeb, A. (2021). Methanolic Extract of *Citrullus colocynthis* Suppresses Growth and Proliferation of Breast Cancer Cells through Regulation of Cell Cycle. *Saudi J. Biol. Sci.* 28 (1), 879–886. doi:10.1016/j.sjbs.2020.11.029

Perveen, S., Ashfaq, H., Shahjahan, M., Manzoor, A., and Tayyeb, A. (2020). *Citrullus colocynthis* Regulates De Novo Lipid Biosynthesis in Human Breast Cancer Cells. *J. Cancer Res. Ther.* 16 (6), 1294–1301. doi:10.4103/jcrt.JCRT_206_20

Pravin, B., Tushar, D., Vijay, P., and Kishanchnad, K. (2013). Review on *Citrullus colocynthis*. *Int. J. Res. Pharm. Chem.* 3 (1), 46–53. doi:10.1089/acm.2011.0297

Qureshi, R., Bhatti, G. R., and Memon, R. A. (2010). Ethnomedicinal Uses of Herbs from Northern Part of Nara Desert, Pakistan. *Pak J. Bot.* 42 (2), 839–851.

Rahbar, A. R., and Nabipour, I. (2010). The Hypolipidemic Effect of *Citrullus colocynthis* on Patients with Hyperlipidemia. *Pak J. Biol. Sci.* 13 (24), 1202–1207. doi:10.3923/pjbs.2010.1202.1207

Rahimi, R., Amin, G., and Ardekani, M. R. (2012). A Review on *Citrullus colocynthis* Schrad.: from Traditional Iranian Medicine to Modern Phytotherapy. *J. Altern. Complement. Med.* 18 (6), 551–554. doi:10.1089/acm.2011.0297

Rasool Hassan, B. A. (2012). Medicinal Plants (Importance and Uses). *Pharm. Anal. Acta* 03, 32153–32435. doi:10.4172/2153-2435.1000e139

Reda, F. M., El-Saadony, M. T., El-Rayes, T. K., Farahat, M., Attia, G., and Alagawany, M. (2021). Dietary Effect of Licorice (Glycyrrhiza Glabra) on Quail Performance, Carcass, Blood Metabolites and Intestinal Microbiota. *Poult. Sci.*, 100, 101266, doi:10.1016/j.psj.2021.101266

Rezvani, M., Hassanpour, M., Khodashenas, M., Naseh, G., Abdollahi, M., and Mehrpour, O. (2011). Citrullus Colocynthis (Bitter Apple) Poisoning; A Case Report. *Indian J. Forensic Med. Toxicol.* 5 (2). http://bsid.bums.ac.ir/dspace/handle/bums/7901.

Rinttilä, T., and Apajalahti, J. (2013). Intestinal Microbiota and Metabolites-Implications for Broiler Chicken Health and Performance. *J. Appl. Poult. Res.* 22 (3), 647–658. doi:10.3382/japr.2013-00742

Rizvi, T. S., Khan, A. L., Ali, L., Al-Mawali, N., Mabood, F., Hussain, J., et al. (2018). *In Vitro* oxidative Stress Regulatory Potential of *Citrullus colocynthis* and Tephrosia Apollinea. *Acta Pharm.* 68 (2), 235–242. doi:10.2478/acph-2018-0012

Saeed, M., Boulos, J. C., Elhaboub, G., Rigano, D., Saab, A., Loizzo, M. R., et al. (2019). Cytotoxicity of Cucurbitacin E from *Citrullus colocynthis* against Multidrug-Resistant Cancer Cells. *Phytomedicine* 62, 152945. doi:10.1016/j.phymed.2019.152945

Saeed, M., Naveed, M., Arif, M., Kakar, M. U., Manzoor, R., Abd El-Hack, M. E., et al. (2021). *Biomed. Pharmacother.* 270, 113772. doi:10.1016/j.jep.2020.113772

Saeed, M., Naveed, M., BiBi, J., Ali Kamboh, A., Phil, L., and Chao, S. (2019). Potential Nutraceutical and Food Additive Properties and Risks of Coffee: a Comprehensive Overview. *Crit. Rev. Food Sci. Nutr.* 59 (20), 3293–3319. doi:10.1080/10408398.2018.1489368

Sawaya, W. N., Daghir, N. J., and Khalil, J. K. (1986). *Citrullus colocynthis* Seeds as a Potential Source of Protein for Food and Feed. *J. Agric. Food Chem.* 34 (2), 285–288. doi:10.1021/jf00068a035

Schafferman, D., Beharav, A., Shabelsky, E., and Yaniv, Z. (1998). Evaluation ofCitrullus Colocynthis, a Desert Plant Native in Israel, as a Potential Source of Edible Oil. *J. Arid Environments* 40 (4), 431–439. doi:10.1006/jare.1998.0454

Shafaei, H., Rad, J. S., Delazar, A., and Behjati, M. (2014). The Effect of Pulp and Seed Extract of Citrullus Colocynthis, as an Antidaibetic Medicinal Herb, on Hepatocytes Glycogen Stores in Diabetic Rabbits. *Adv. Biomed. Res.* 3, 258. doi:10.4103/2277-9175.148230

Shafaei, H., Esmaeili, A., Rad, J. S., Delazar, A., and Behjati, M. (2012). *Citrullus colocynthis* as a Medicinal or Poisonous Plant: a Revised Fact. *J. Med. Plants Res.* 6 (35), 4922–4927. doi:10.5897/JMPR11.264

Soufane, S., Bedda, A., Mahdeb, N., and Bouzidi, A. (2013). Acute Toxicity Study on *Citrullus colocynthis* Fruit Methanol Extract in Albino Rats. *J. Appl. Pharm. Sci.* 3 (6), 88. doi:10.7324/JAPS.2013.3614

Tabani, K., Birem, Z., Halzoune, H., Saiah, W., Lahfa, F., Koceir, E. A., et al. (2018). Therapeutic Effect of Alkaloids and Glycosides of Colocynth Seeds on Liver Injury, Associated with Metabolic Syndrome in Wistar Rats, Subject to Nutritional Stress. *Pak J. Pharm. Sci.* 31, 277–290.

Xiao, J., Capanoglu, E., Jassbi, A. R., and Miron, A. (2016). Advance on the Flavonoid C-Glycosides and Health Benefits. *Crit. Rev. Food Sci. Nutr.* 56 Suppl 1 (Suppl. 1), S29–S45. doi:10.1080/10408398.2015.1067595

Yitbarek, M. B. (2015). Phytogenics as Feed Additives in Poultry Production: a Review. *Int. J. Extensive Res.* 3, 49–60.

Yoshikawa, M., Morikawa, T., Kobayashi, H., Nakamura, A., Matsuhira, K., Nakamura, S., et al. (2007). Bioactive Saponins and Glycosides. XXVII. Structures of New Cucurbitane-type Triterpene Glycosides and Antiallergic

Constituents from *Citrullus colocynthis*. *Chem. Pharm. Bull. (Tokyo)* 55 (3), 428–434. doi:10.1248/cpb.55.428

Zhang, L.-X., Li, C.-X., Kakar, M. U., Khan, M. S., Wu, P.-F., Amir, R. M., et al. (2021). Resveratrol (RV): A Pharmacological Review and Call for Further Research. *Biomed. Pharmacother.* 143, 112164. doi:10.1016/j.biopha.2021.112164

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 07 February 2022
doi: 10.3389/fphar.2022.813142

Buchu (*Agathosma betulina* and *A. crenulata*): Rightfully Forgotten or Underutilized?

Thomas Brendler[1,2,3] *and Mona Abdel-Tawab*[*,4,5]

[1]*Department of Botany and Plant Biotechnology, University of Johannesburg, Johannesburg, South Africa,* [2]*Plantaphile, Collingswood, NJ, United States,* [3]*Traditional Medicinals Inc., Rohnert Park, CA, United States,* [4]*Central Laboratory of German Pharmacists, Eschborn, Germany,* [5]*Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University, Frankfurt am Main, Germany*

Today, the term *buchu* refers to the two species in commerce, *Agathosma betulina* (P.J.Bergius) Pillans and *Agathosma crenulata* (L.) Pillans (Rutaceae). Its traditional use in urinary tract infections and related ailments made it a popular remedy, specifically in the US, in 19th century, but with the advent of antibiotics it became largely obsolete. Recent focus is on technological use and on the essential oil for use in the perfume and food-flavouring industry. A review of the scarce pharmacological research revealed moderate antimicrobial activity for a leaf extract but not the essential oil of both species in the MIC assay. In the 5-lipoxygenase (5-LO) assay the essential oil of both species revealed IC_{50} values of 50.37 ± 1.87 µg/ml and 59.15 ± 7.44 µg/ml, respectively. In another study 98% inhibitory activity was determined for 250 µg/ml of an ethanolic extract of *A. betulina* on cyclooxygenase (COX)-1 and a 25% inhibitory activity on COX-2. Analgesic activity of an ethanolic extract of *A. betulina* was shown in mice. Moderate antioxidant activity was determined for methanol:dichlormethane extracts of *A. betulina* and *A. crenulata* and an aqueous extract of *A. betulina* showed a Trolox equivalent antioxidant capacity (TEAC) of 11.8 µM Trolox. Recent *in vitro* studies with a commercial aqueous extract of *buchu* revealed increased uptake of glucose added to 3T3-L1 cell line, significant inhibition of the respiratory burst of neutrophils and monocytes, reduction in the expression of adhesion molecules and inhibition of the release of IL-6 and TNF-α. In diabetic rats the ingestion of aqueous *buchu* extract completely normalized the glucose level and in rats receiving a high fat diet the consumption of aqueous *buchu* extract resulted in less weight gain and less intraperitoneal fat gain as well as reduction of elevated blood pressure to normal associated with cardioprotective effects. Limitations in the hitherto conducted research lie in the undisclosed composition of the *buchu* extracts used and the difficulty in extrapolating data from animal studies to humans. Health claims for *buchu* products need to be substantiated by randomized, double-blind and placebo-controlled studies. Only then can they be promoted for their true therapeutic potential.

Keywords: buchu, ethnobotany, commercialization, pharmacological activity, phytochemical composition

OPEN ACCESS

Edited by:
Massimo Lucarini, Council for Agricultural Research and Economics, Italy

Reviewed by:
Ian Edwin Cock, Griffith University, Australia
E. Manikandan, Thiruvalluvar University, India

***Correspondence:**
Mona Abdel-Tawab
tawab@em.uni-frankfurt.de

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *11 November 2021*
Accepted: *11 January 2022*
Published: *07 February 2022*

Citation:
Brendler T and Abdel-Tawab M (2022) Buchu (Agathosma betulina and A. crenulata): Rightfully Forgotten or Underutilized? Front. Pharmacol. 13:813142. doi: 10.3389/fphar.2022.813142

INTRODUCTION

Agathosma is a genus of 150 species of flowering plants in the family Rutaceae indigenous to South Africa. The two species in commerce are now known as *A. betulina* (P.J.Bergius) Pillans and *A. crenulata* (L.) Pillans. Their common name *buchu*, however, was historically applied to multiple aromatic species of this and other genera. Traditionally, *buchu* has been used by the Khoisan for spiritual and medicinal purposes (Smith, 1966). Initially noted by the early settlers, knowledge and use of *buchu* spread to Europe and later to the United States (US). *Buchu* belongs to a handful of Southern African medicinal plants which reached international markets through colonial interests and entrepreneurship, more or less unaffected by barriers of entry which were more recently introduced by health product regulations in the target markets as well as bioprospecting legislation in the countries of origin (Brendler et al., 2008; Brendler, 2009; Stander et al., 2019; Brendler, 2020; Brendler, 2021; Brendler and Cock, 2021; Brendler et al., 2021). It has been compendial since 1826 for its diuretic effects and use in the treatment of genito-urinary tract infections, however, became obsolete in the 20th century due to the sparsity of scientific evidence for its efficacy and the advent of antibiotics. Today's interest is focused on technological use and the essential oil for use in perfumes and as a flavouring agent. Nevertheless, *buchu* is still found in numerous herbal preparations, sold over-the-counter (OTC) or on the internet, for promoting health and treatment of urinary tract disorders. Despite its unique and exciting history, many questions regarding *buchu's* pharmacological properties and potential therapeutic effects remain to be answered.

HISTORY OF BUCHU

Taxonomy

Nomenclature of *buchu* is complicated and species identification hampered by historical references omitting authorities. Linné first recorded the genus as *Diosma* in 1756 (Linné, 1756), specifically *D. crenulata* and *D. crenata*, followed by Thunberg in his *Prodromus* (Thunberg, 1794) and his botanical thesis dedicated to *Diosma* (Thunberg, 1797) (**Figure 1**). Ecklon and Zeyher introduced the genus as *Barosma* and provided detailed botanical and geographical data (Ecklon and Zeyher, 1835). Until the authoritative revision by Pillans, multiple authorities named and renamed species, therefore, it seems appropriate to include here the full synonymy for the two species of *Agathosma* in commerce, while botanical descriptions of the species can be found in the revision (Pillans, 1950):

A. crenulata (L.) Pillans = *Diosma crenulata* L.; *D. crenata* L.; *D. latifolia* Andrews; *D. serratifolia* Curt.; *Parapetalifera odorata* Wendl.; *Barapelutiflora serrata* Wendl.; *Barosma odorata* Willd.; *Baryosma odorata*, *B. serratifolia* Roem. & Schultes; *Bucco crenata* Roem. & Schultes; *Adenandra cordata*, *A. serratifolia* Link; *Barosma serratifolia* Willd.; *Diosma odorata* DC.; *Barosma crenata* Sweet; *Agathosma latifolia* Loud.; *Barosma crenulata* Hook.; *B. eckloniana* O. Berg.

A. betulina (P.J.Bergius) Pillans = *Hartogia betulina* P.J.Bergius; *Diosma betulina* Thunb.; *Bucco betulina* Roem. & Schultes; *Diosma crenata* Lodd.; *Barosma betulina* Bartl. & Wendl.


DISSERTATIO BOTANICA
DE
DIOSMA,

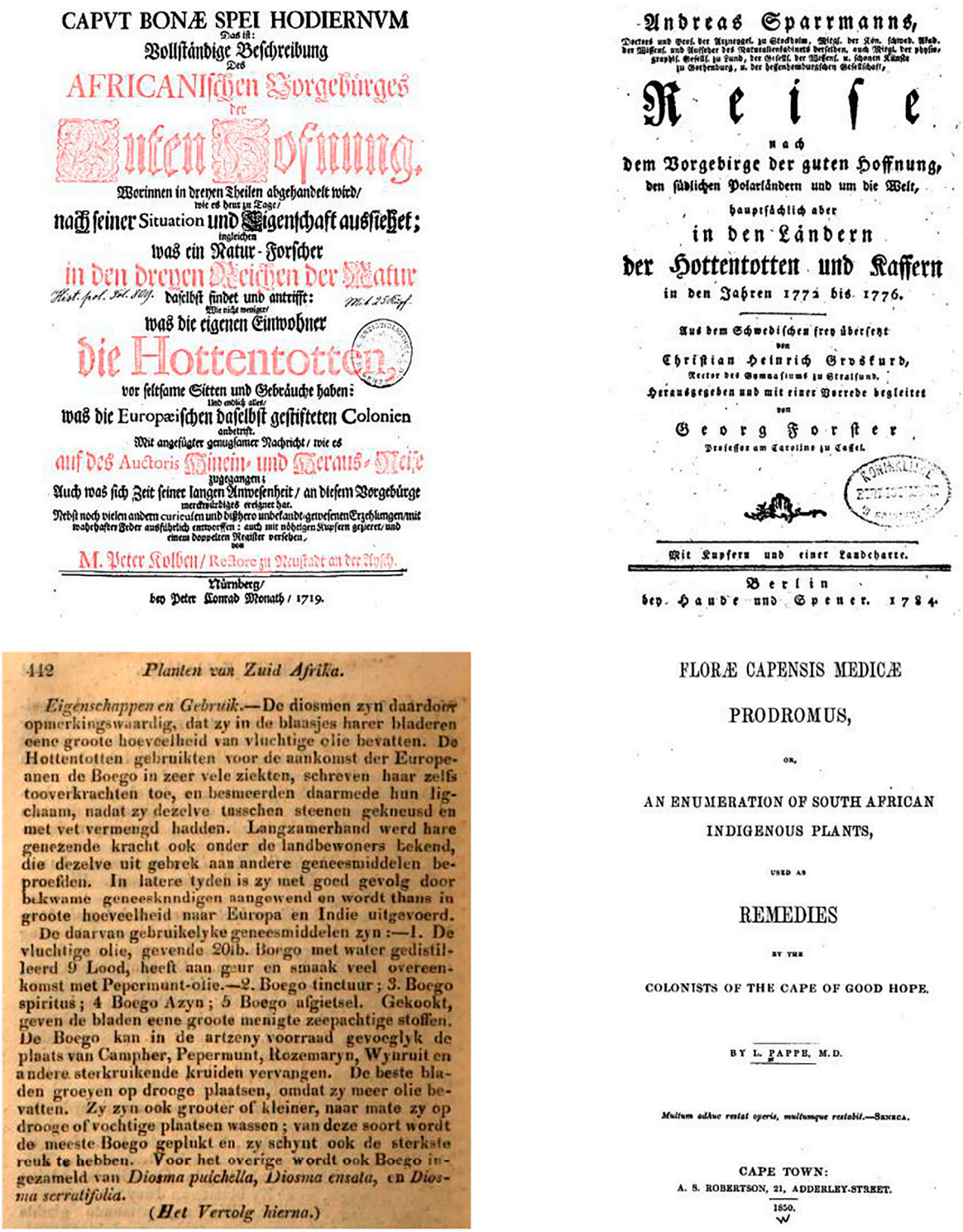
QUAM
VENIA EXP. FACULT. MED. UPSAL.
PRÆSIDE
CAROL. PET.
THUNBERG,

EQUITE AURAT. REG. ORD. DE VASA,
MEDIC. DOCT. PROFESS. MED. ET BOTAN. REG. ET ORD. ACAD. CÆSAR. NAT. CURIOS. REG. SCIENT. LONDIN. HOLM. SOCIET. SCIENT. UPSAL. PHILAD. PATR. HOLM. BEROL. NAT. SCRUT. PARIS. ET HAFNIENS. HIST. NAT. LUND. HARLEM. AMSTELD. ZELAND. NIDROSIENS. TICINENS. IENENS. HALENS. NAT. SCRUT. LINN. LONDIN. PHYS. GÖTTINGENS. MEDIC. EDIMBURG. ET NAT. STUDIOS. IBID. MEMBRO; NEC NON ACAD. SCIENT. PARIS. MONSPELIENS. AGRICULT. PARIS. MEDIC. LONDIN. FLORENTIN. ET BATAVIN. IND. OR. CORRESPOND.

PUBLICO SUBJICIT EXAMINI.
CAROL. JOH. PENTZ,
WESTHOGOTHUS.
IN AUDIT. BOTAN. D. 17 MAII 1797.
H. C.

UPSALIÆ,
LITTERIS JOH. FR. EDMAN, REG. ACAD. TYPOGR.

FIGURE 1 | Title page of Thunberg's Dissertatio Botanica de Diosma (1799), *Spiraea Africana* odorata in Commelin (1706).



CAPVT BONÆ SPEI HODIERNVM
Das ist:
Vollständige Beschreibung
Des
AFRICANischen Vorgebürges
der
Guten Hofnung.
Worinnen in dreyen Theilen abgehandelt wird/
wie es heut zu Tage/
nach seiner Situation und Eigenschaft aussiehet;
ingleichen
was ein Natur-Forscher
in den dreyen Reichen der Natur
daselbst findet und antrifft:
Wie nicht weniger/
was die eigenen Einwohner
die Hottentotten,
vor seltsame Sitten und Gebräuche haben:
Und endlich alles/
was die Europæischen daselbst gestifteten Colonien
anbetrift.
Mit angefügter genugsamer Nachricht/ wie es
auf des Auctoris Hinein- und Heraus-Reise
zugegangen;
Auch was sich Zeit seiner langen Anwesenheit/ an diesem Vorgebürge
merckwürdiges ereignet hat.
Nebst noch vielen andern curieusen und bißhero unbekandt-gewesenen Erzehlungen/mit
wahrhafter Feder ausführlich entworffen: auch mit nöhtigen Kupfern gezieret/ und
einem doppelten Register versehen,
von
M. Peter Kolben/ Rectore zu Neustadt an der Aysch.
Nürnberg/
bey Peter Conrad Monath/ 1719.

Andreas Sparrmanns,
Doctors und Prof. der Arzneygel. zu Stockholm, Mitgl. der Kön. schwed. Akad. der Wissensch. und Vorsteher des Naturalienkabinets derselben, auch Mitgl. der physiographisch. Gesellsch. zu Lund, der Gesellsch. der Wissensch. u. schönen Künste zu Gothenburg, u. der hessenhomburgischen Gesellschaft,
Reise
nach
dem Vorgebirge der guten Hoffnung,
den südlichen Polarländern und um die Welt,
hauptsächlich aber
in den Ländern
der Hottentotten und Kaffern
in den Jahren 1772 bis 1776.
Aus dem Schwedischen frey übersetzt
von
Christian Heinrich Groskurd,
Rector des Gymnasiums zu Stralsund.
Herausgegeben und mit einer Vorrede begleitet
von
Georg Forster
Professor am Carolino zu Cassel.
Mit Kupfern und einer Landcharte.
Berlin
bey Haude und Spener. 1784.

442 *Planten van Zuid Afrika.*

Eigenschappen en Gebruik.—De diosmen zyn daardoor opmerkingswaardig, dat zy in de blaasjes harer bladeren eene groote hoeveelheid van vluchtige olie bevatten. De Hottentotten gebruikten voor de aankomst der Europeanen de Boego in zeer vele ziekten, schreven haar zelfs tooverkrachten toe, en besmeerden daarmede hun ligchaam, nadat zy dezelve tusschen steenen gekneusd en met vet vermengd hadden. Langzamerhand werd hare genezende kracht ook onder de landbewoners bekend, die dezelve uit gebrek aan andere geneesmiddelen beproefden. In latere tyden is zy met goed gevolg door bekwame geneeskundigen aangewend en wordt thans in groote hoeveelheid naar Europa en Indie uitgevoerd.

De daarvan gebruikelyke geneesmiddelen zyn:—1. De vluchtige olie, gevende 20lb. Boego met water gedistilleerd 9 Lood, heeft aan geur en smaak veel overeenkomst met Pepermunt-olie.—2. Boego tinctuur; 3. Boego spiritus; 4 Boego Azyn; 5 Boego afgietsel. Gekookt, geven de bladen eene groote menigte zeepachtige stoffen. De Boego kan in de artzeny voorraad gevoeglyk de plaats van Campher, Pepermunt, Rozemaryn, Wynruit en andere sterkruikende kruiden vervangen. De beste bladen groeyen op droege plaatsen, omdat zy meer olie bevatten. Zy zyn ook grooter of kleiner, naar mate zy op droege of vochtige plaatsen wassen; van deze soort wordt de meeste Boego geplukt en zy schynt ook de sterkste reuk te hebben. Voor het overige wordt ook Boego ingezameld van *Diosma pulchella*, *Diosma ensata*, en *Diosma serratifolia*.

(*Het Vervolg hierna.*)

FLORÆ CAPENSIS MEDICÆ
PRODROMUS,
OR,
AN ENUMERATION OF SOUTH AFRICAN
INDIGENOUS PLANTS,
USED AS
REMEDIES
BY THE
COLONISTS OF THE CAPE OF GOOD HOPE.
BY L. PAPPE, M.D.
Multum adhuc restat operis, multumque restabit.—SENECA.
CAPE TOWN:
A. S. ROBERTSON, 21, ADDERLEY-STREET.
1850.

FIGURE 2 | Title page of Kolb (1719), title page of Sparrmann (1784), excerpt from Ecklon (1826), title page of Pappe (1850).

Common names include *boegoe, boechoe, boekoe, boggoa, bookoo, bouchou, bugu, buccho, bucchuu, bucco, buchu, bucku and buku* (Smith, 1966).

Ethnobotany and Ethnobiology

Before moving on to discuss the records of traditional use of *buchu* made by early settlers, colonists, and explorers, it must be

stressed that the knowledge of *buchu* and its medicinal properties by the Khoisan precedes written records, probably by centuries. The Digital Bleek and Lloyd[1], a digital archive of the ethnographical exploration of the Khoisan people, lists the use of *buchu* in multiple every-day, spiritual and medicinal contexts. The Khoisan and other indigenous peoples considered multiple aromatic species as *buchu* and used them in dance rituals, for anointment, beautification, perfume, and also as medicine (Smith, 1966; Low, 2007). Within the realm of those recorded uses, traditional knowledge is owned by the Khoisan and should be attributed and respected as such (Low, 2007).

The first published record of *boggoa* leaves being used in tribal dance practices was made by an early settler in 1668 and later reiterated in other settlers' records (Dapper et al., 1933; Smith, 1966). Interestingly, and contrary to secondary sources, neither van der Stel's travel journal from 1685 (van der Stel, 1979) nor the *Codex Witsenii* of 1692 (Wilson, 2002) included *buchu* species. Commelin, in 1706, described three species in his *Horti Medici Amstelaedamensis* as *Spiraea Africana* (Commelin, 1706) and noted the great importance to the local "hottentots" (**Figure 1**). Kolb first mentioned the common name *buchu*, not *boegoe* as claimed by Smith (1966), and described the use of the pleasant smelling, dried and powdered leaves for aches and anointments (Kolb, 1719) (**Figure 2**). This constitutes the first record for medicinal use, again contrary to Smith, who ascribed the first medicinal use record to Sparrman et al. (1784) (**Figure 2**), who indeed reported on the use of *bucku* for anointment and as a strong medicine, but without providing further detail. He also alluded to different species being plentiful, while one of them is particularly valued: "*Some species are common in the Cape, one, however, to be found somewhere near the gold river, is so precious that one thimble of the powder is paid for with a lamb*" (author's transl.) (Sparrman et al., 1784). Burchell, in his *Travels in the interior of southern Africa* recorded names and uses (Burchell, 1822). Latrobe followed shortly after and for the first time noted the use of *buchu* brandy: "*. . . we found the larger species of bukku, one of the most aromatic, medicinal, plants in the country, and justly esteemed for its healing properties. Its leaves steeped in brandy or vinegar, and the bottle placed in the heat of the sun, emit an unctuous juice, by which the fluid is rendered as thick as honey, and applied particularly for the healing of contusions, sores, and all external complaints. The Hottentots also use it for inward hurts, by mixing a spoonful of it with warm water . . .* " (Latrobe, 1818). In the same year, de Candolle praised the pleasant smell of *buchu* essential oil and its spasmolytic properties (De Candolle, 1818).

Between 1826 and 1831, Ecklon published a series of articles in *Het Nederduitsch Zuid-Afrikaansch Tydschrift* on the identity, habitat and virtues of local medicinal plants, and specifically on *boego* (*Diosma crenata*) (Ecklon, 1826). Therein he gave a detailed description and a contemporary summary of uses: "*. . .Hottentots used the boego in many diseases, even ascribing magical powers to it, and smearing their bodies with it, after they had bruised it between stones and mixed it with fat. . . . In recent times it has been successfully used by skilled physicians and is now exported in great quantity to Europe and India. . . . The boego can conveniently replace camphor, peppermint, rosemary, rue, and other strong-smelling herbs in the medical supply." (author's transl.)* (**Figure 2**).

Given that Pappe was associated with Ecklon, and undoubtedly acquainted with Mackrill, Liesching and other contemporaries (see below), his statement that "*I am not aware of any publication on South African Materia Medica, except that of a small dissertation by Thunberg, which appeared in the year 1785*" (Pappe, 1847; Pappe, 1850) seems bizarre; he did, however, add a few details to his description reflecting overseas research, e.g., the identification of diosmin, attribution of medicinal uses to the essential oil, and new uses: as a diuretic, for gout and rheumatism, urinary ailments, as an appetite stimulant, and even cholera (Pappe, 1850) (**Figure 2**).

Later accounts (Watt and Breyer-Brandwijk, 1962; Smith, 1966) drew from these early sources.

From an ethnobiological point of view *buchu* was a highly prized Khoisan traditional remedy and remained one of the most popular herbal medicines in South-Africa. The traditional use of *buchu* encompasses the treatment of kidney and urinary tract infections, cold, stomach ailments, rheumatism, gout and fever. Externally it was applied as an antiseptic wash to infected wounds and as a compress to relieve swelling, bruising and sprains. In traditional practice *A. betulina* is most commonly taken orally in form of an aqueous infusion, sometimes sweetened with brown sugar, or as tincture in brandy. Other dosage forms include a vinegar infusion for external application as an antiseptic wash or embrocation (South African National Biodiversity Institute, 2005).

Commercialization

Buchu arrived in Europe via two distinct and chronologically almost parallel paths, only one of which is documented in the literature. Generally, Joseph Mackrill (1762–1820) is credited with the introduction of *buchu* into the United Kingdom (UK) around 1815 (Theal, 1908; Gunn and Codd, 1981; Low, 2007), and from there to Ireland and the United States. Since Mackrill had spent time in the US prior to his arrival in the Cape, there may very well also have been a direct (trade) connection, but this could not be confirmed.

Richard Reece (1775–1831), a London pharmacist and wholesaler became the proprietor of *buchu* in the Anglo-Saxon world (Reece, 1822; Reece, 1824; Reece, 1827; Reece, 1836) and on a small scale also into Germany, where Friedrich Jobst, pharmacist in Bavaria became his "broker" around 1825 (Jobst, 1825). But Theal (Theal, 1908) and all citing authors in this singular accreditation underestimate the vibrancy of the Cape colony population and its vast and intricate network into Central Europe: next to the UK primarily to the Netherlands and Germany. Indeed, around the same time, *buchu* was introduced into Central Europe via Amsterdam. A German pharmacist and quinine fabricant in Nordhorn (today in Lower Saxony, Germany), Ernst Firnhaber, who procured chinchona bark from Amsterdam and supplied quinine to the Dutch colonies (Kühle, 1970), in 1826 published a note by

[1]http://lloydbleekcollection.cs.uct.ac.za/.

Friedrich Ludwig Liesching (1757–1841) on the virtues of *buchu* (Firnhaber, 1826) which also mentioned its already established use in the Netherlands. Firnhaber's friendship with the editor of the journal, notable scientist, and co-founder of what became the German pharmacists association, Rudolph Brandes, helped spread the word throughout the association, which by 1821 already had over 100 members, and resulted in further publications and scientific investigation (Brandes, 1826; Brandes, 1827).

Mackrill and Liesching were contemporaries in the Cape. The medical community was small, it can safely be assumed that they were acquainted. Mackrill, an Englishman, arrived in the Cape from Maryland (US) around 1806 and was admitted to practice as a surgeon in August 1807 (Gunn and Codd, 1981; Glen and Germishuizen, 2010).

At that point in time, Liesching was already well established. He had landed at the Cape at the end of 1787 as surgeon-major of the 1st battalion of the *Württembergische Kapregiment*, which served the Dutch East India Company as mercenaries. The regiment moved on in 1791, but Liesching stayed behind and established himself among the medical community (Deacon and Van Heyningen, 2004). In 1800, he started *Dr Liesching and Company, Apothecaries and Retail Shop* in partnership with Jean Jacques von Ziegler (1766-?) at 61 Loop Street, Cape Town, which was to become one of the largest apothecary shops in the colony (Burrows, 1958). There they were joined by Carl Ferdinand Heinrich von Ludwig (1784–1847), at the time pharmacist in Amsterdam, who applied for a position as a pharmacy assistant to Liesching in 1805, and was approved as an apothecary by the *Supreme Medical Committee*—a body set up to control practice of medicine and pharmacy—in 1807 (Price, 1974). *Buchu*—the universal panacea—featured prominently in the pharmacy's portfolio for decades, even after *Liesching & Co.* was sold and renamed *De Engel Apotheek* in 1836 (McMagh, 1992). In 1808, Liesching and von Ziegler established a botanical garden at The Knoll (**Figure 2**), on Kloof Road, above Botany (now Bantry) Bay. The main building of the estate was demolished in the early 2000s to make room for real-estate development (Hart, 2001). The garden held specimens of useful indigenous and exotic flora (Spohr, 1968; McMagh, 1992). Unfortunately, no exact records of its holdings survive, thus, albeit highly likely, cultivation of *buchu* can only be assumed.

As of 1810 Mackrill practiced at 10 Burg Street, Cape Town, where he was visited by Burchell (1822). By 1814, Mackrill had sold his residence and set up an experimental farm for the cultivation of tobacco in Somerset West (Skead, 2009). During this period, he was collecting (and probably also propagating) specimens of the useful indigenous flora and knowledge about its use (Kannemeyer, 1951; Gunn and Codd, 1981; Glen and Germishuizen, 2010). Purportedly, his notes provided input to Pappe's *Florae Capensis Medicae Prodromus* (Pappe, 1850).

In 1816, Christian Ignatius Latrobe (1758–1836), clergyman of the Moravian church, visited both Mackrill on his Somerset farm and Liesching at Botany Bay (Latrobe, 1818). Latrobe's account also mentioned a Reverend Hesse, who was in his company on the visit to Liesching, and, according to Smith (1966), sent (or took) seeds and specimens of *buchu* to Germany. According to Smith, Latrobe and/or Hesse were likely also responsible for *buchu* being sent to Moravian missionaries in Calcutta and Madras to treat intestinal colic.

In 1815, Ludwig married into money to evolve as Baron von Ludwig of the Ludwigsburg Gardens, three acres of land in Kloof Street (now Tamboerskloof), Cape Town, which he started to develop in 1829. The gardens, however, were more focused on naturalizing exotic species. To which extent, if at all, he utilized them for the propagation of native species and whether these were part of his business operation is unclear. One fact stands out, however: throughout the years he was collecting native plants with fellow countryman Ludwig Beil near Swellendam, in the Cape Flats, Somerset East, Worcester, Tulbagh, Karsrivier, and Potberg, and in 1838 both accompanied German scientist C.F.F. Krauss on a collection trip to Natal, affording plenty of opportunity to pass on knowledge about *buchu* (Bradlow, 1965).

Thus, not only both Mackrill and Liesching, but also their respective associates (**Figure 3**) should all be considered to have had a hand in the introduction of *buchu* to Europe and the US.

Buchu products quickly gained popularity, however, less so in Central Europe, but rather in the UK and the US. Unfortunately, there are no epidemiological records for urinary tract ailments in the 19th century for either region. Therefore, no correlations with the popularity of *buchu* can be made. The immense success of *buchu* preparations in the US can thus only be attributed to the marketing practices of manufacturers of patent formulas at the time. One of the most (in)famous protagonists was Henry T. Helmbold (1826–1892), who started his patent medicine business in 1846 as a retail druggist with "*Helmbold's Extract Buchu—cures diabetes, gravel, brick-dust deposits, irritations of the bladder and diseases arising from exposure or imprudence, etc.*" and other medicines. He opened his first store in Philadelphia in 1850, the largest and best-known in New York in 1862. By 1865 Helmbold's *buchu* was the bestselling patent medicine on the US market. For this, he spent enormous amounts of money on advertising, mostly in newspapers: ~US$ 500,000 (about 10 million US$ today) each for the years 1869–71. For the distribution of his products, Helmbold had his own 4c postage stamp (**Figure 4**) (The Historian, 1912; Young, 1961).

In 1863, Bedford provided some insight in the wholesale value of *buchu*: long-leaved (*B. serratifolia*) and short-leaved (*B. crenulata*) were traded at 80 and 40 cents per pound, respectively (Bedford, 1863). With the Titanic was lost a shipment of eight bales of *buchu* in its cargo (Anonymous, 1912a). For the variety of products in the US market at the beginning of the 20th century, see e.g., the *Drug Department* of the *Druggist Circular* (Anonymous, 1912b).

Buchu remained to be a popular remedy way into the 20th century, interest only began to wane with the discovery of antibiotics and synthetic diuretics. Compton (1922), Lawson and Clark (1932) provided some insight into trade volumes of *buchu* during the 1920s (**Table 1**). Exports had peaked in 1873 at 400,000 pounds, with the bulk going to the US, some via the UK, but also to Europe (mostly UK and Germany). Meanwhile, in the UK, popularity held steady, but around the turn of the century supply was notoriously short. Random checks in *The Chemist and Druggist* confirm steady sales both for the national market and for

FIGURE 3 | Friedrich Ludwig Liesching, Liesching's cottages at Botany Bay (1832), Baron von Ludwig (~1840), Joseph Mackrill's gravestone at Maitland cemetery, Cape Town.

re-export (Anonymous, 1897; Anonymous, 1908). Only, in the early 1920s, demand appears to decline (Anonymous, 1922).

Lawson and Clark (1932) also reported on first cultivation attempts, and the profitability thereof. Cultivation, specifically climatic and soil conditions, preparation, seeding, transplanting, culture, harvesting and drying were detailed by Werner (1949), implying a sufficiently steady interest in the crop.

When axenic cultures of *A. betulina* were inoculated with the soil yeast *Cryptococcus laurentii* and cultivated for 5 months under glasshouse conditions, the root growth increased by 51% (Cloete et al., 2009).

Scientific Investigation and Compendial Representation

Over the 100 years following its introduction to Europe and the US, *buchu* became the subject of multiple scientific investigations and publications. Reece first published on *buchu* in the *Monthly Gazette of Health* (his own "journal") in 1822 (Reece, 1822). Case reports by McDowell on the efficacy of *buchu* leaf infusions and tinctures in urinary ailments followed (McDowell, 1824), laying the foundation for the inclusion in the Dublin pharmacopoeia (Anonymous, 1826). Another account by Reece followed in 1824 (Reece, 1824) and in 1825, Jackson reported on the use of *buchu* from Calcutta (see above) (Jackson, 1825).

Following the introduction to Germany (Jobst, 1825; Brandes, 1826; Firnhaber, 1826), Jorritsma reported from Amsterdam, on 1) the connection of Liesching to a wholesale pharmacy in Amsterdam (Gebroeders Rouffaer) and 2) several case reports on the successful treatment of urinary tract ailments in the Netherlands (Jorritsma, 1826). *Richard's Medizinische Botanik* (Kunze, 1826) contained a detailed botanical description of *fol. Diosmae crenatae*, followed by a reiteration of all previously mentioned sources. Noteworthy is a footnote to the account that mentioned a supplier of *buchu* leaves, Brückner, Lampe & Co., a wholesale pharmacy founded in 1750 in Merseburg (Germany), at the time headquartered in Leipzig with subsidiaries in Berlin and Hamburg and business relations reaching as far as Russia and the Unites States (Dufour von Féronce, 1900). The fact that they had 40 pounds of *buchu* readily available suggests a lively trade in Germany at the time.

Cadet de Gassicourt (1827) and Brandes (1827) conducted the first investigations into the chemical composition of *buchu* leaves. Brandes first isolated a substance which he called diosmin. Nees von Esenbeck questioned identity and synonymy of *D. crenata* and *D. serratifolia*, an early indication for admixture and adulteration (Nees von Esenbeck, 1827). Nourij, in his 1827 dissertation, apparently unaware of Brandes' experiments, reiterated the already known history and proceeded to report his own investigation of various *buchu* preparations confirming the results of Cadet de Gassicourt. He also added a number of case reports to those already presented by Jorritsma (Nourij, 1827). Meanwhile, Reece developed a portfolio of preparations "*as a remedy for morbid irritability of the bladder, prostate gland, spasmodic stricture, irritative gleet, fluor albus, and morbid irritation of the rectum, &c.*" which were advertised in his 1827 *Catalogue Of Drugs* (Reece, 1827). In 1828, Nees von Esenbeck included *D. crenata* and *D. serratifolia* in his *Plantae*

FIGURE 4 | Helmbold ~1871, one of his storefronts, Helmbold's extract of *buchu,* his proprietary postage stamp.

TABLE 1 | Exports of *buchu* 1920–1931[a].

Year	Exports (in pounds)	Value (in US$)
1920	139,149	246,109
1921	124,842	93,309
1922	124,046	76,608
1923	204,297	129,213
1924	152,657	87,310
1925	198,691	79,966
1926	186,589	42,967
1927	139,444	29,359
1928	203,350	39,648
1929	220,669	38,684
1930	157,919	24,879
1931	197,426	26,622

[a]*the inverse trend of volumes and values is noteworthy, however, impacted by currency fluctuations.*

Officinalis (Nees von Esenbeck, 1828). Autenrieth's and Möckel's dissertations of 1830 were the first treaties with focus on medical applications (Autenrieth, 1830; Möckel, 1830). Long (1831) recommended the use of *buchu* over an extract of belladonna in cases of urethral strictures. By 1836, Reece's *Popular Catalogue of Drugs* was littered with formulas containing *buchu* and its essential oil (Reece, 1836). Another dissertation (Bruinsma, 1838) summarized advancements in research hitherto and promoted the use of *buchu* as a diuretic, diaphoretic, and stimulant. Two publications of 1847 and 1848 suggested *buchu* for oedema (anasarca, dropsy) (Anonymous, 1847; Hoskins, 1848). Meanwhile, *buchu* had become largely established in European pharmacopoeias (**Figures 5**, **6**, **Table 2**).

In 1861, Strumpf in his *Allgemeine Pharmakopöe*, condensed all hitherto available data into his entry on Folia Bucco (Strumpf, 1861). Flückiger further investigated the microscopic structure of *buchu* leaves and reported a layer of mucilage on their upper side between the epidermis and the mesophyll [Flückiger 1873, cited in Flückiger and Hanbury (1874)]. This description was part of a detailed monograph, which once again summarized the current knowledge.

Jones, with reference to Bedford's analyses (Bedford, 1863), provided data on ash and soluble matter—relevant for quality control—for the three compendial species at the time (*B. betulina*, *B. crenulata* and *B. serratifolia*) (Jones, 1879). Maisch (1881) and

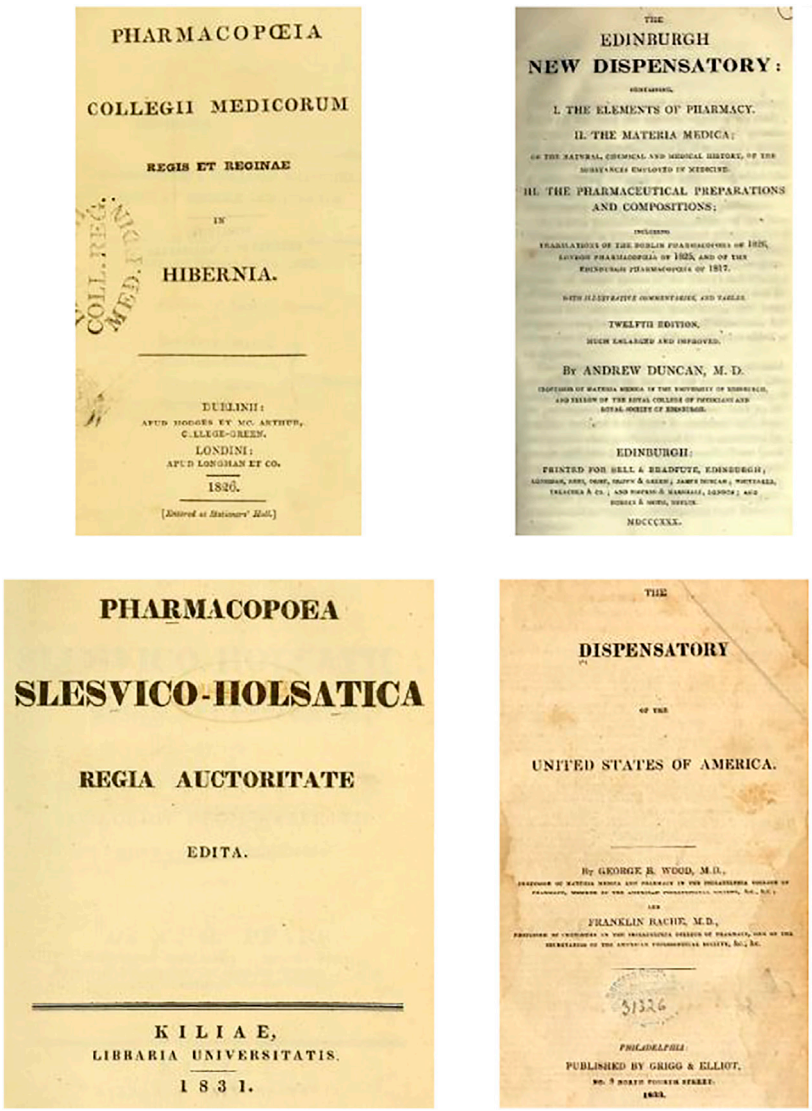


FIGURE 5 | Title pages of the earliest pharmacopeia entries for *buchu* in chronological order: Dublin pharmacopoeia 1826, Edinburgh dispensatory 1830, Schleswig-Holstein pharmacopoeia 1831, US dispensatory 1833.

Spica (1885) continued the investigations into the composition of *buchu* essential oil, the former reported on the stearoptene (diosphenol) from *B. betulina* essential oil, the latter disproved the presence of salicylic acid. Shimoyama repeated Flückiger's experiment and confirmed his results; he also noted the presence of hesperidin crystals (Shimoyama, 1888), the latter being further investigated in terms of distribution and amount by Zenetti (1895). It is noteworthy that both Shimoyama and Zenetti conducted their investigations at least partially on *Diosma alba* Thunb. [synonym of *Coleonema album* (Thunb.) Bartl. & H.L.Wendl.], a related South African Rutaceae species, and then extrapolated their results to compendial *buchu*. Kondakov and Bachtschiew conducted the first comparative elucidation of the essential oil compounds from different sources. Their two oil samples differed significantly in their diosphenol content, leading to the suspicion that they were not derived from the same species. They further referenced and extended an earlier investigation in their laboratory which found three compounds, rather than the previously reported two, a hydrocarbon compound of limonene and dipentene, a ketone (menthone) and diosphenol (Kondakow and Bachtschiew, 1901). McKenzie, (1906) focused on the elucidation of disophenol, or *bucco* camphor, and described its first synthesis via oxidation of oxymethylenmenthone with ozone. Wander revisited the hesperidin in *buchu*, confirmed diosmin, barosmin and hesperidin to be identical, and summarized all there was known on the presence of hesperidin in Rutaceae (Wander, 1925). In 1944, Feldman and Youngken published a pharmacognostic review of *buchu* with many sources cited therein that are not included here (Feldman and Youngken, 1944). This account constituted the most

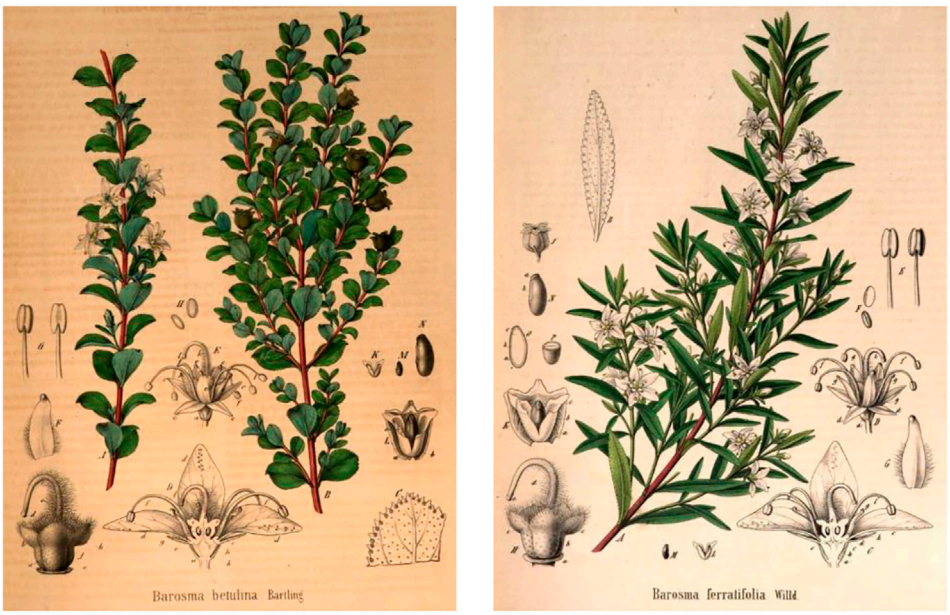


FIGURE 6 | *Agathosma* spp. compendial in the *Pharmacopoeia Borussica* (1846ff) (Berg and Schmidt, 1858).

TABLE 2 | History of compendial representation of *buchu*, compiled from (Anonymous, 1826; Duncan, 1830; Pfaff, 1831; Wood and Bache, 1833; Anonymous, 1835; Anonymous, 1836; Anonymous, 1837; Royal College of Physicians of Edinburgh, 1839; Anonymous, 1842; Anonymous, 1846; Anonymous, 1860; Anonymous, 1916; Bruntz and Jaloux, 1918).

Country/region	***Barosma betulina* (P.J.Bergius) Bartl. & H.L.Wendl., *Diosma betulina* Thunb**	***Barosma crenata* (L.) Sweet., *Diosma crenata* L.**	***Barosma serratifolia* (Curtis) Willd., *Barosma crenulata* (L.) Hook., *Diosma crenulata* L.**
Belgium		(1)—1854	(1)—1854
Denmark	(6)—1893	(5)—1868	(3)—1840
France	(4)—1884	(3)—1866	(4)—1884
Germany	(DAB Erg.-B. 4)—1916	(6)—1846	(6)—1846
Prussia			
Hesse		(2)—1860	(1)—1835
Schlsw.-Holstein		(1)—1831	
Hamburg		(1)—1835	1852[a] (2)—1837
Lower Saxony		1852[a] (2)—1837	
Saxony			
Greece		(1)—1837	
Ireland		(2)—1826	
Dublin			
Netherlands	(2)—1871	(1)—1851	(2)—1871
Norway	(2)—1870	(2)—1870	(2)—1870
Portugal	(3)—1876		(3)—1876
Spain	(6)—1884		(6)—1884
Sweden	(7)—1869	(6)—1846	(7)—1869
United Kingdom	(1)—1864	(1)—1864	(1)—1864
London		1836	
Edinburgh		1830, (11) 1839	
USA	(7)—1882[b]	(2)—1833[b]	(3)—1842[b]

Numbers in parentheses refer to the respective editions.

[a]*noted as relevant but not in Pharmacopoea hannoverana nova (Stromeyer, 1852).*

[b]*Dispensatory of the US of America (1833), not in the US Pharmacopoeia (USP) until 1842.*

detailed summary of all aspects of identity and quality control at the time.

Buchu was monographed in the first edition of the *Dispensatory of the US of America* (Wood and Bache, 1833)—referring to the Dublin pharmacopoeia because the article was not yet listed in the USP. *Buchu* entered the *Primary List of Materia Medica* in the second decennial revision of the USP (Anonymous, 1842), listed as: "*Diosma*. *Buchu*. The leaves of *Diosma crenata*." A monograph for one preparation was included, *Infusum Diosmae* USP. In the fifth decennial revision (1870), the name of the article changed from *Diosma* to "*Buchu*. The leaves of *Barosma crenata*, and of other species of *Barosma*." Preparations included *Extractum Buchu Fluidum* and *Infusum Buchu*. *Infusum Buchu* was dismissed from the sixth decennial revision (1880). *Elixir Buchu*, *Elixir Buchu Compositum*, *Elixir Buchu et Potassii Acetatis*, and *Extractum Buchu Fluidum Compositum* appeared in the first edition of the *National Formulary* (NF) (American Pharmaceutical Association, 1888). Squire summarized compendial monographs from British, US and other pharmacopoeias and noted for the *British Pharmacopoeia* (BP) 1898 an increase of strength of *Tinctura Buchu* from 1:8 to 1:5, and further *Infusum Buchu*, which at that point was unique to the BP (Squire, 1899). Culbreth, in his 1906 edition of *Manual of Materia Medica and Pharmacology*, provided a lengthy monograph for *Barosma betulina*, including the mention of common adulterants; at that point *B. crenulata* was no longer compendial (Culbreth, 1906).

Buchu and *Fluidextractum Buchu* were dismissed from the eleventh decennial revision (USP XI, 1930). *Buchu* (raw material) entered the sixth edition of the NF (NF VI, 1936). Monographs for preparations also remained compendial in the NF after dismissal from the USP: *Elixir Buchu*; *Elixir Buchu Compositum*; *Elixir Buchu et Potassii Acetatis*; *Elixir Buchu, Juniperi et Potassii Acetatis*; *Fluidextractum Buchu*; *Fluidextractum Buchu Compositum*. *Buchu* and preparations made from it last appeared in the tenth edition of the NF (NF X, 1955), in force from 1955 until 1960. Monographs for *buchu* and preparations made from it were dismissed from the eleventh edition of the NF (NF XI, 1960).

By 1899, *buchu* was still not compendial in Austria, Germany, Hungary, Italy, Russia or Switzerland (Squire, 1899). Nonetheless, the inaugural edition of *Hager's Handbuch der Pharmaceutischen Praxis* (1880), a monumental reference for the pharmacist, which has been in print and updated regularly to this day, contained a detailed monograph for *Barosma* (Hager, 1880) noting its reluctant use in Central Europe despite it purportedly being more efficacious than bearberry [*Arctostaphylos uva-ursi* (L.) Spreng]. According to Schneider (1974), *Folia Bucco* appeared in the supplements to the German pharmacopeia (DAB) around 1900. Madaus (1938), in another seminal compendium, the *Lehrbuch der biologischen Heilmittel*, provided a detailed account for *bucco*, including a summary of its etymology, botany, history, uses, and directions of use, based on US and UK compendial monographs. He mentioned its presence in the supplement to DAB VI, which, however, was only published in 1941. In fact, the first compendial monograph for Germany *Extractum Bucco fluidum* was published in the supplement to DAB IV (Anonymous, 1916).

The 1949 *British Pharmaceutical Codex* (BPC) listed buchu, specifically *Infusum Buchu Concentratum* and *Infusum Buchu Recens* as per BP 1932, and *Tinctura Buchu*. Noteworthy is the entry on action and uses: "[It] is now superseded by sulfonamides and penicillin [. . .]" (Anonymous, 1949). It was still present in the BPC 1963 (Wade, 1977). While it is unclear exactly when it was omitted from the BP—it was no longer compendial by 1992—*buchu* retained its spot in the *British Herbal Pharmacopoeia* throughout all three editions (1971ff) (Scientific Committee, 1983). A 1997 query of the UK Medicines Control Agency's (now Medicines and Healthcare Products Regulatory Agency) database of registered products yielded 17 products (liquid and solid dosage forms) of 10 manufacturers containing *buchu* as a sole ingredient or in combinations. All products had been licensed as GSL (General Sales List) between 1988 and 1996 (unpublished data). Only two products transitioned into the Traditional Herbal Medicinal Product category post 2004, however, neither are actively marketed. Nonetheless, the UK compendium *Herbal Medicines* (Barnes et al., 2013) includes a monograph for *buchu* to this day. Other European national pharmacopoeias also retained *buchu*, e.g., the *Pharmacopée Française* (ANSM, 1983) only earmarked it for omission in 2015, but keeps it currently listed in an addendum.

To inform the German *Kommission E* monograph (Kommission, 1990), an unpublished report was compiled (Franz and Hoth, 1987) in 1987. It contained a summary of botanical, biochemical and pharmacological data. It can be assumed that the decision of *Kommission E* to publish a "negative" monograph for *buchu:* "*Since the claimed effectiveness has not been documented, the application of buchu leaf cannot be recommended. The use of buchu leaf as an aroma or flavor corrigent in tea mixture is acceptable,*" was based on the absence of toxicological and clinical data. At the time, six combination products containing *buchu* leaf were on the German market. The *Kommission E* verdict effectively dealt a 'death sentence' to *buchu* products in Germany, as health claims could no longer be assigned to products and by 1994 medicinal products containing *buchu* had largely disappeared (Anonymous, 1994). The German drug database *AMIce* lists a total of 129 products containing *buchu* which are no longer licensed. The only remaining are homoeopathic products as per the first German homoeopathic pharmacopoeia (HAB 34) (Schwabe, 1934), albeit *buchu* having become obsolete and no longer listed in HAB 1 (Anonymous, 1978). It does, however, remain compendial in the *Homeopathic Pharmacopoeia of US* to this day.

BUCHU TODAY

The 1990s saw some revival of interest in the medicinal properties of *buchu*, but primarily in its country of origin, meanwhile it was still traded as a flavour in both Europe and the US (Simpson, 1998; Cohen et al., 2020). In 1999, the Agricultural Research Council (ARC) of South Africa initiated a project to cultivate

buchu commercially to prevent it from becoming extinct (Coetzee, 1999). Demand for *buchu* oil increased significantly from the beginning of 1990, and with cultivation proving difficult, price hikes led to unsustainable harvesting practices. To remedy this situation, local manufacturers like Afriplex (Pty) Ltd. and Puris Natural Aroma Chemicals (Pty) Ltd. initiated numerous cultivation projects, e.g., by 2008, Afriplex had 82 ha with 3.5 million *buchu* plants under cultivation (Afriplex, 2008). In 2009, Bhat and Moskowitz identified eight commercial tea products containing *buchu* in the South African market (Bhat and Moskovitz, 2009). In 2011, Lubbe and Verpoorte noted the production volume for essential oil of *buchu* to be in the 1–50 tons per annum range, in 2010 the price for raw material was given at US$ 56/kg (Lubbe and Verpoorte, 2011). A few recent reviews point out the potential as an herbal medicinal product (Moolla and Viljoen, 2008; Street and Prinsloo, 2013; Skosana et al., 2014; Huisamen et al., 2019), however, from an international perspective the sleeping beauty is still waiting for its prince.

Phytochemical Composition

Taxonomically, the two species *A. betulina* and *A. crenulata* can be mainly distinguished by their leaf form. In addition, the cultivation of both species outside their natural habitat resulted in the formation of hybrid plants (Collins et al., 1996). The phytochemical composition of the leaf oil of *A. betulina* and *A. crenulata* was extensively investigated by several research groups applying gas chromatography (GC) coupled to a flame ionisation detector or mass spectrometer including also cluster analysis. Fluck et al. identified pulegone and diosphenol as constituents of *buchu* oil (Fluck et al., 1961). Lamparsky and Schudel isolated 8-mercapto-*p*-menthan-3-one, a sulphur-containing terpene responsible for the flavour and aroma of the oil (Lamparsky and Schudel, 1971). Kaiser et al. identified more than 120 components including the already known pulegone, diosphenol and 8-mercapto-*p*-menthan-3-one (Kaiser et al., 1975). Collins et al. detected a total of 56 compounds including 14 new and found pulegone to be the key identification marker. They described two chemotypes for *A. betulina* depending on the diosphenol and isomenthone content. The diosphenol chemotype is characterized by high (ψ)-diosphenol (>10%) and diosphenol (>12%) and low isomenthone concentrations (<28%), the isomenthone chemotype by high isomenthone (>31%) and low (ψ)-diosphenol (<0.16%) and diosphenol (<0.14%). No chemotypes were found for *A. crenulata* oil (Collins et al., 1996). In their study of the chemical composition of *A. betulina*, *A. crenulata* and their hybrid, Posthumus et al. (1996) identified several rare bi- and tri-functionalized monoterpenes besides the commonly known monoterpenes. These included hydroxylated diosphenols, several hydroxymenthones and some acetates thereof. 8-hydroxy-4-menthen-3-one and 8-hydroxy-menthone were suspected to be decomposition products of pulegone (Posthumus et al., 1996). Finally, Viljoen et al. were able to confirm the data obtained from the previous phytochemical studies (Viljoen et al., 2006). Relevant findings are summarized in **Table 3**.

Whereas most of the monoterpenes identified in *buchu* oil may be also found in many other plants, *buchu* is the only genus that produces diosphenol, responsible for the distinctive blackcurrant flavor; hence its use in the food industry to enhance fruit flavors in sweets and beverages (Moolla and Viljoen, 2008).

The presence of diosphenol as a distinctive constituent of *A. betulina* essential oil in addition to pseudo-diosphenol, limonene, 1,8-cineole, menthone, isomenthone and *trans*-8-mercapto-*p*-menthan-3-one was also confirmed in a recent quality control protocol published as part of a monograph by Viljoen and colleagues. Chemical profiling of a methanol extract of *A. betulina* revealed the presence of hesperidin, rutin, and diosmin, all serving as non-volatile marker compounds (Viljoen et al., 2021).

Pharmacological Activity

Pharmacological experiments in the early 2000s were mostly carried out with the hydro-distilled essential oils and/or methanol-dichloromethane (1:1) extracts of both *Agathosma* species.

Antimicrobial Activity

Based on the traditional use of *buchu* in urinary tract infections, several assays have been utilized to study the antimicrobial activity of the hydro-distilled essential oils and methanol-dichloromethane (1:1) extracts of *A. betulina* and *A. crenulata*. It is noteworthy, however, that these extracts do not correspond with traditional extraction methods as an infusion in water or a tincture in ethanol.

Applying the micro-titre plate dilution method in the minimum inhibitory concentration (MIC) assay, the methanol-dichloromethane (1:1) extracts of both species revealed moderate antimicrobial activity with a MIC in the range of 2 mg/ml–4 mg/ml against the tested pathogens *Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans*. Much higher MIC values in the range of 3 mg/ml–32 mg/ml were determined for the essential oils of both species. Unfortunately these MIC values are too high and indicate low activity. By comparison the MIC of the respective controls ranged between 2.5×10^{-3} and 6.3×10^{-3} mg/ml (Viljoen et al., 2006; Moolla et al., 2007).

In another study by Lis-Balchin et al. utilizing the agar disc diffusion assay the essential oil of both species (10 µL undiluted) did not demonstrate antimicrobial activity against *Enterococcus hirae* and *Pseudomonas aeruginosa*, and very low activity against *Escherichia coli*, *Saccharomyces cerevisiae* and *S. aureus* (Lis-Balchin et al., 2001). This is not surprising, since the selected assay is not suitable for essential oils, because of the lack of solubility in aqueous environments, which consequently results in low diffusion.

Steenkamp et al. investigated the antimicrobial activity of aqueous and ethanolic extracts of *A. betulina* on *E. coli* using the micro-well dilution method revealing no effect of either extract on the growth of *E. coli* (Steenkamp et al., 2006).

The methanol:dichlormethane extract (1:1) of *A. betulina* was re-investigated by Sandasi revealing MIC values ranging between

TABLE 3 | Comparable overview of the phytochemical composition of the essential oil determined for *A. betulina* and *A. crenulata* and the hybrid of both.

Study	*A. betulina*	*A. crenulata*	Hybrid
Fluck et al. (1961)	Limonene, menthone, diosphenol, *l*-pulegone, (ψ)-diosphenol (an isomer of diosphenol)	Limonene, menthone, traces of diosphenol, *l*-pulegone, (ψ)-diosphenol	n.a
Kaiser et al. (1975)	Limonene 17% Menthone 17% Isomenthone 43% Isopulegone 4% Pulegone 3% ψ-diosphenol 8% Diosphenol 9% 8-mercapto-*p*-menthan-3-one ++ 8-acetylthio-*p*-menthan-3-one +	Limonene 9% Menthone 6% Isomenthone 22% Isopulegone 10% Pulegone 50% ψ-diosphenol 1% Diosphenol 1% 8-mercapto-*p*-menthan-3-one + 8-acetylthio-*p*-menthan-3-one ++	n.a
Collins et al. (1996)	Pulegone 2.4–4.5% 8-mercapto-*p*-menthan-3-one > 8-acetylthio-*p*-menthan-3-one *cis*-8-mercapto-*p*-menthan-3-one > *trans*-8-mercapto-*p*-menthan-3-one	Pulegone 31.6%–73.2% 8-acetylthio-*p*-menthan-3-one > 8-mercapto-*p*-menthan-3-one	Simultaneous presence of relatively high concentrations of both pulegone and diosphenol 8-mercapto-*p*-menthan-3-one > 8-acetylthio-*p*-menthan-3-one *cis*-8- mercapto-*p*-menthan-3-one > *trans*-8- mercapto-*p*-menthan-3-one
Posthumus et al. (1996)	(Iso)menthone 31% ψ-diosphenol 41% *cis*- and *trans*-8-mercapto-*p*-menthan-3-one 3% + two decomposition products of pulegone 8-hydroxy-4-menthen-3-one 8-hydroxy-menthone	Pulegone 54% *trans*-acetylthio-*p*-menthan-3-one 7 + two decomposition products of pulegone 8-hydroxy-4-menthen-3-one 8-hydroxy-menthone	Intermediate composition including (iso)menthone 55%
Viljoen et al. (2006)	Limonene 23.7% Menthone 29.2% Isomenthone 14.2% Pulegone 8.4% Diosphenol 2.5% *cis*-8-mercapto-*p*-menthan-3-one 0.1 *trans*-8-mercapto-*p*-menthan-3-one 0.1%	Limonene 13.4% Menthone 16.6% Isomenthone 7.3% Diosphenol 0.1% *cis*-8-mercapto-*p*-menthan-3-one *trans*-8-mercapto-*p*-menthan-3-one 0.1%	n.a

n.a., not analyzed.

3 and 6 mg/ml against *Listeria monocytogenes, Pseudomonas aeruginosa*, *Candida albicans*, *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Enterococcus faecalis* in the MIC microplate assay. It also prevented the growth and development of biofilms by preventing the attachment of bacteria to the polyvinyl chloride surface in the crystal violet (CV) assay, except for *C. albicans* (Sandasi, 2008).

Unfortunately, the phytochemical composition of the tested methanol-dichloromethane extract has not been investigated, so it can only be postulated that the antimicrobial effects observed may likely be attributed to inherent coumarins and flavonoids. But although antimicrobial acting flavonoids may be also expected to be present in the ethanolic extracts of *A. betulina* the latter revealed no effect. Obviously, the methanol-dichloromethane extract also contains other components that are not found in the aqueous and ethanolic extracts, which are responsible for the moderate antimicrobial activity. Also, the monoterpenes identified in the essential oils should have revealed more potent antimicrobial effects. The poor activity of the essential oils compared to the extracts was attributed by Moolla to the insolubility of the essential oil in the growth medium, a negative influence of the pH of the medium, or a possible inactivation of the essential oil components by constituents of the growth medium (Moolla, 2005).

For the time being it can thus be concluded that only the methanol-dichloromethane (1:1) extracts of both *Agathosma* species exert low to moderate activity against micro-organisms responsible for urinary tract infections like *E. coli* and *K. pneumoniae*. Aqueous or ethanolic extracts as well as the essential oils were shown to be inactive. The missing phytochemical characterization of the applied extracts prevents interrogating the causes for the observed differences.

Anti-Inflammatory Activity

In the 5-lipoxygenase (5-LO) assay the essential oils of *A. betulina* and *A. crenulata* revealed IC_{50} values of 50.37 ± 1.87 μg/ml and 59.15 ± 7.44 μg/ml, respectively, indicating low 5-LO inhibitory activity (Viljoen et al., 2006). Investigations on individual oil components that might contribute to the anti-inflammatory activity have not been carried out, but Moola and Viljoen

assumed that the monoterpene limonene may be responsible for the observed inhibition of 5-LO *in vitro* as it is present in both species and is known for its anti-inflammatory effects (Moolla and Viljoen, 2008). It is worth mentioning that other *Agathosma* species like *A. collina* Ecklon & Zeyher and *A. namaquensis* Pillans revealed better 5-LO inhibitory activity, reflected in IC_{50} values corresponding to 25.98 ± 1.83 μg/ml and 31.54 μg/ml, respectively.

Aqueous and ethanolic extracts of *A. betulina* revealed a higher activity for the ethanolic extract (250 μg/ml) on cyclooxygenase (COX)-1 (98% inhibition) and COX-2 (25% inhibition). Both extracts were obtained by extracting 1 g of dried plant material with either 10 ml of water or ethanol in an ultrasound bath for 30 min, after which the extracts were filtered and evaporated to dryness (Steenkamp et al., 2006).

Other pharmacological targets playing a role in inflammation, e.g., microsomal prostaglandin E synthase-1 have not been investigated.

Apart from the limited *in vitro* investigations, one double-blind placebo-controlled study in 30 male participants reported reduction of swelling and pain in exercise-induced muscle damage following topical application of *A. betulina* oil containing gel three times a day (Lambert et al., 2002).

Taken together, the current data situation with regard to anti-inflammatory effects of the essential oil or aqueous and ethanolic extracts is very poor, thus more investigations including detailed phytochemical characterization of the tested oils or extracts are needed to come to a final conclusion with regard to the anti-inflammatory efficacy.

Analgesic Activity

Analgesic activity has been investigated by Chiguvare et al. in Swiss albino mice receiving 200 mg/kg of an ethanolic extract of *A. betulina* compared to 200 mg/kg silver nanoparticles prepared from the ethanolic extract, and 100 mg/kg of aspirin as positive control. The silver nanoparticles showed better analgesic properties than aspirin, reflected in a lower number of paw licks in the formalin test. The inhibition values for the silver nanoparticles synthesized at different temperatures ranged between 73% and 98% for the neurogenic phase and between 55% and 80% in the inflammatory phase. The inhibition values for the crude ethanolic extract were 55% and 45% for the neurogenic and inflammatory phase, respectively, compared to 84% and 81% for aspirin (Chiguvare et al., 2016).

Apart from a general screening on the presence of glycosides, flavonoids, alkaloids, terpenes, steroids, tannins, saponins and proteins in the ethanolic extracts no detailed phytochemical characterization of the individual components has been carried out. Thus, the significance of this single test for evaluating the analgesic activity of ethanolic *A. betulina* extracts is very limited.

Antioxidant Activity

The radical scavenging activity of methanol-dichloromethane (1:1) extracts of several *Agathosma* species has been tested in the 2,2-diphenyl-β-picrylhydrazyl (DPPH) and 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays. In the DPPH assay, both *A. betulina* and *A. crenulata* extracts showed poor antioxidant activity with IC_{50} values >100 μg/ml. In contrast, moderate activity with IC_{50} values corresponding to 37.75 ± 0.54 μg/ml and 33.32 ± 0.33 μg/ml was determined for both extracts in the ABTS assay. These seemingly contradictory results, however, point at the fact that mechanisms of both reagents are different and suggest interactions at different stages of the oxidative process (Moolla et al., 2007). The outcomes confirmed results of an earlier study (O'Brien, 2005), in which acetone, 80% methanol and aqueous extracts were tested for their antioxidant activities in the DPPH assay. The only extract that suppressed the oxidation of linoleic acid was the acetone extract containing the four lipophilic flavonoids 3 and 3,3′-dimethyl ethers of quercetin and the 3 and 3,4′ dimethyl ethers of kaempferol. The 80% methanol extract contained hyperoside, rutin and a novel compound designated agathosin, which was shown to be a quercetin glucoside esterified with oleuropeic acid. The main antioxidative compound in the aqueous extract was rutin. But despite the presence of quercetin, kaempferol and rutin, all of which have known radical scavenging properties, only a poor correlation could be found between the total phenolic content of the different extracts and the radical scavenging activity.

On the other hand, Steenkamp et al. determined a scavenging activity for hydroxyl radicals generated by a Fenton-type reaction for very high concentrations of the aqueous and ethanolic extract of *A. betulina* (4 mg/ml) corresponding to 80% using electron spin resonance spectrophotometry (Steenkamp et al., 2006). However, these concentrations are far away from being of relevance for practical application.

Thring et al. reported a TEAC of 11.8 μM Trolox when applying 25 μg aliquots of an aqueous extract of *A. betulina* (Berg) Pill. (10 mg/ml). The aqueous extract was obtained by extracting 500 mg dried ground herb in 10 ml boiling water, followed by sonication for 15 min, filtration on the following day, and drying with a fan. Trolox refers to the standard applied in the ABTS+ diammonium salt free radical assay. Here the correlation analysis showed a significant correlation ($p = 0.001$) between total phenolic content (0.246 mg/ml, as equivalents of gallic acid determined by the Folin-Ciocalteu assay) and TEAC. In the superoxide dismutase (SOD) assay *buchu* aqueous extract inhibition activity was reported at 20.49% compared to 85.02% of the positive control (SOD at 3.33 units final volume). SOD is a naturally occurring enzyme that protects the cell from the reactive and damaging superoxide anion (O_2^-) by dismuting it into O_2 and H_2O_2 (Thring et al., 2009).

All experiments indicate that different *buchu* extracts may exert antioxidant effects in different experimental settings. Unfortunately, they were carried out without detailed analysis of the respective extract compositions, and applying too high concentrations, so no conclusions can be drawn regarding effective extract components and effective concentrations in practical applications. Further research is therefore needed.

Effects of Buchu Water on Metabolic Syndrome

A recent set of investigations carried out on the quest of Cape Kingdom Nutraceuticals addressed the efficacy of aqueous *buchu* extract (presumably from *A. betulina* and *A. crenulata*) on

diseases related to metabolic syndrome in *in vitro* and *in vivo* test systems. The extract, marketed commercially as *buchu* water, is a water condensate recovered during the steam distillation of commercial *buchu* oil, back-extracted using ethyl alcohol followed by drying under reduced pressure and re-suspension in methanol. As the oil is distilled at low temperatures under vacuum, the water contains not only the water-soluble hydrophilic compounds but also some of the more volatile oil-soluble compounds. Targeted analysis using LC-MS revealed the presence of hesperidin, rutin, diosmin, quercetin and pulegone besides other molecular species with unknown structures (Huisamen et al., 2019). Unfortunately, no quantitative analysis was conducted.

In vitro Effects on Glucose Metabolism

The effect of the aqueous extract in comparison to pure *buchu* oil on excessive glucose utilization was tested on Chang Liver cell line of hepatocyte origin and 3T3-L1 cell line of adipose tissue origin. In the Chang Liver cell line only *buchu* oil showed a dose-dependent increase in the uptake of the additional glucose (1 mg/ml) added to the medium by 12–21% in the concentration range from 0.15–0.6 ppm. In comparison, the positive control metformin at 1 μM exhibited 23%–25% increase in uptake. In the 3T3-L1 cell line only the aqueous extract caused a 35%–40% increase in the glucose uptake, comparable to 35% observed increase obtained with 1 μM insulin serving as positive control. The observed difference in sensitivity of the tested cell lines to oil or aqueous extract is not surprising, as they also respond differently to well-established anti-glycaemic drugs like metformin or insulin depending on their origin (Huisamen et al., 2019).

Anti-Inflammatory Activity

In human peripheral blood cells, the extract caused a significant inhibition of the respiratory burst of neutrophils and monocytes and of the expression of adhesion molecules (CD11b/CD18) in the range of 1:400 to 1:3,200 dilution of a 600 μg/ml stock solution. The latter effect was more pronounced in neutrophils than in monocytes. Furthermore, the extract inhibited the release of potent cytokines like interleukin (IL)-6 and tumour necrosis factor (TNF)-α. In general the round leafed *buchu* extract was more effective than the oval leafed *buchu* extract in three of the total extract fractions tested, only in one fraction the oval leafed *buchu* extract showed a greater inhibitory activity (Huisamen et al., 2019). However, these results are very difficult to comprehend since detailed information on the preparation and phytochemical composition of the different fractions of the total aqueous extract are missing. Moreover, these results have not been verified by any other research group.

Surprisingly, the above results were considered sufficient to verify the observations made *in vitro* in animal models, although no mechanism of action can be deduced from the *in vitro* experiments and no identification of the effective compound(s) was carried out.

Anti-Diabetic, Anti-Obesity, Anti-Hypertensive Effects of Buchu Water in Animals

To investigate anti-diabetic effects, type 1 diabetes (T1D) was induced in adult male Wistar rats by injecting streptozotocin leading to the chemical destruction of 50% the pancreatic β-cells. Free access to diluted *buchu* water was given 3 weeks after the streptozotocin injection for a duration of 14 weeks. For inducing type 2 diabetes (T2D) the diet-induced obesity (DIO) model was applied in young rats by administering rat chow diet supplemented with sugar and condensed milk for 16 weeks. *Buchu* water treatment started 8 weeks after receiving the DIO diet and continued for the remaining 8 weeks.

In T1D rats with glucose levels <20 mmol/L the aqueous *buchu* extract completely normalized glucose levels and in T1D rats with glucose levels >20 mmol/L, they were significantly reduced compared to the untreated T1D group. The T2D rats ingesting *buchu* water did not accumulate additional intraperitoneal fat as happened in the DIO group not receiving *buchu* water. The whole-body glucose tolerance revealed no significant differences between control animals and DIO animals that ingested *buchu* water. The insulin sensitivity at organ level measured in isolated ventricular cardiomyocytes was significantly enhanced in the control and DIO group receiving *buchu* water. The insulin secretion was enhanced in the DIO group receiving *buchu* water, accompanied by a significant increase in the C-peptide levels and a significant upregulation of the pancreatic transcription factors musculoaponeurotic fibrosarcoma homolog A (Maf A) and pancreatic duodenal homeobox 1 (Pdx-1). The latter is an indication for the regeneration of pancreatic β-cells.

Since both animal models represent so-called pre-stages of type 1 and type 2 diabetes, it was concluded that *buchu* water can be used as treatment option in newly diagnosed pre-type 1 diabetic patients. It must, however, be emphasized that human T1D, in contrast to the chemically induced TD1 in rats, is an autoimmune disease where the regeneration of pancreatic β-cells may be counteracted by autoantibodies. Because of the generally known low bioavailability of the very low concentrated polyphenols in the ingested aqueous *buchu* extract, it was hypothesized that *buchu* extract may exert influences on the gut microbiome, which in turn could produce substances like γ-aminobutyric acid (GABA), known to act on the transcription factors involved in pancreatic redifferentiation (Huisamen et al., 2019). Nonetheless, much more work is required to verify this hypothesis.

Based on the observed effects of aqueous *buchu* extract on intraperitoneal fat, the anti-obesity effect was studied in rats receiving a high-fat diet (HFD) for 16 weeks that was composed of normal rat chow supplemented with 10% saturated fat, 10% fructose, 10% casein and 1% cholesterol, rendering animals insulin-resistant and hypertensive. The HFD significantly increased the rats' body weight from 381.3 ± 9.5 g (control group) to 451.8 ± 15.1 g (HFD group) and the intraperitoneal fat from 8.6 ± 0.4 g (control group) to 24.5 ± 0.7 g (HFD group). The consumption of *buchu* water resulted in less weight gain corresponding to 394.1 ± 14.0 g and less intraperitoneal fat gain

corresponding to 15.9 ± 1.3 g in the HFD group. The consumption of *buchu* water resulted in a significant reduction in fat cell size ($p < 0.0001$), as well as significantly lower leptin levels ($p = 0.0003$) compared to the HFD group without intake of *buchu* water. No significant differences were observed in the adiponectin, TNF-α and IL-6 levels. *Buchu* water specifically lowered the elevated mRNA levels of peroxisome proliferator-activated γ receptor (PPARγ) to control values but not that of PPARα in the HFD group. The total cholesterol levels in both the control and HFD group ($p = 0.03$) and the plasma triglycerides in the HFD group were reduced ($p = 0.03$).

Again, ingredients responsible for the observed effects could not be identified since the *buchu* water intake corresponded to a mean of 30 ml per day and the concentrations of the included flavonoids were very low at 0.0005 mg/L diosmin, 0.007 mg/L quercetin, 0.001 mg/L hesperidin, and 0.0035 mg/L rutin (Huisamen et al., 2019).

When HFD rats ingested *buchu* water, the initial rise in blood pressure declined to control values at week 14 and infarct development induced in the isolated perfused hearts by regional ischemia was significantly smaller compared to the HFD group with no *buchu* ingestion. Serum aldosterone levels, as an indicator of the renin-angiotensin-aldosterone system, could be significantly lowered to 259.7 ± 65.5 pg/ml compared to 619.9 ± 136.1 pg/ml in the HFD group without *buchu* ingestion (Huisamen et al., 2019).

These findings suggest positive effects of *buchu* water on glucose homeostasis, weight gain, intraperitoneal fat gain, blood pressure and cardio-protection. It should be noted that dosing in the animal studies was equivalent to a human weighing approximately 70 kg drinking 250 ml/day of the extract. However, in the absence of clinical trials, no findings exist that verify these observations that were made in rats in humans. Clinical studies are urgently needed, especially in view of the different pancreatic biochemistry and the difficulties in extrapolating the data obtained from rats to humans. Unless these clinical trials are conducted, the reported positive anti-diabetic, anti-obesity, and anti-hypertensive effects of buchu water in rats remain questionable in humans. Even in rats the described effects remain inconclusive because of the missing information on extract composition. They also remain questionable as long they are not further verified by independent research studies supporting these observations.

Toxicity

The toxicity of methanol-dichloromethane (1:1) extracts of *Agathosma* species was evaluated by Moolla et al. using the MTT [3-(4,5-dimethyl-2-thiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide] cellular viability assay. In this assay several dilutions of methanol-dichloromethane (1:1) extracts displayed different degrees of cellular inhibition, but extracts of *A. betulina* and *A. crenulata* were not toxic at concentrations up to 100 μg/ml (Moolla et al., 2007).

The essential oils of both species proved to exhibit higher toxicity at the concentration tested, both having IC_{50} values of <0.0001 μg/ml (Viljoen et al., 2006). Essential oils at high doses were found to be hepatotoxic in rats, affecting liver and uterine functions. This was attributed to *R*-(+)-pulegone which is known to be a hepatotoxic compound causing depletion of glutathione at high doses. This depletion along with excess pulegone leads to centrilobular hepatocellular necrosis (Moolla, 2005). Caution should be exercised with essential oil from *A. crenulata* that contains higher amounts of pulegone. Pulegone is not water soluble, thus this caution does not apply to water extracts. Pulegone has been approved by the US FDA for use in the food industry (with a FEMA GRAS status) and is listed among the authorized synthetic flavouring substances (CFR 21–172.515). The no effect level of pulegone in beverages as stated in the *Opinion of the Scientific Committee on Food on the risks to human health of Polycyclic Aromatic Hydrocarbons in Food* is 100 mg/kg (European Commission, 2002). Consequently, the aqueous *buchu* extract applied in above animal experiments containing 74.22 mg/L ± 1.1 mg/L pulegone is well below the acceptable level indicated by the FDA and the LC_{50} value of 25.91 mg/ml determined by Raza et al. (2016).

Market Presence of *Buchu* Products

As mentioned before, *buchu* is currently primarily used in the fragrance and flavour industry due to its sulphur-containing compounds and sensory properties.

Despite preliminary research suggesting pharmacological potential regarding its antimicrobial, anti-inflammatory, and antioxidant properties, *buchu* has not retained its place in the mainstream market. This is unsurprising as many of the effects have only been observed at exorbitantly high doses in the respective animal models. Effects reported for aqueous *buchu* extracts from animal studies cannot be related to putative active ingredients either, as doses were too low for explaining any direct actions on pharmacological targets. At the same time, urgently needed clinical trials have not been conducted.

Nevertheless, *buchu* enjoys a reputation as a general health tonic and is promoted to possess anti-inflammatory, antioxidant and antibacterial properties. It is marketed as a dietary supplement in various forms including teas, dried, whole, and powdered leaves, liquid and powder extracts, oils, tinctures, waters, capsules, tablets, gels and creams, that are moreover often adulterated (Raman et al., 2015). Online *buchu* features among ten plants commonly claiming benefit in kidney diseases (Vamenta-Morris et al., 2014). It is found in herbal preparations sold OTC or online associated with following claims: “blood pressure support,” “support cardiovascular health,” “digestive support” and in combination with cranberry for “traditional urinary support,” “supports body’s health against bacteria in the urinary tract” as well as in combination with other herbal ingredients to “support kidney health” and “support urinary bladder health.” This is alarming because such claims have neither been widely investigated nor substantiated by peer review (Vamenta-Morris et al., 2014). At the same time scientific literature has to date failed to prove any benefit in humans.

Other than in medicinal applications, *buchu* has recently found use in the green synthesis of nanoparticles (Thema et al., 2015; Pal et al., 2019). This opens up novel dimensions in the field of biotechnology and nanomedicine. Whereas physical and chemical routes are associated with high energy consumption,

low yield, high cost, and environmental damage, biological pathways using plants or plant-based extracts as chelating agents represent a cost effective, simpler and eco-friendly approach. In fact the biosynthesis approach has been demonstrated to be effective in the synthesis of metal and oxide nanoparticles as e.g., the green synthesis of cadmium oxide nanoparticles based on *Hibiscus subdariffa* flower extract (Thovhogi et al., 2016), the green synthesis of silver nanoparticles of the crude extract of *Syzgium aromaticum* (Venugopal et al., 2017a), the green synthesis of SnO_2 nanoparticles via *Aspalathus linearis* (Diallo et al., 2016), the green synthesis of samarium oxide nanoparticles via *Callistemon viminalis* extract (Sone et al., 2015), the biosynthesis of *Beta vulgaris* extract mediated silver nanoparticles with enhanced anticancer activity (Venugopal et al., 2017b), and the synthesis of single-phase α-Cr_2O_3 nanoparticles using *Callistemon viminalis*' red flower extract (Sone et al., 2016), just to mention a view.

This is of great importance as it opens up new advantageous applications in biomedical, drug delivery, and food industries as well as in agriculture, and textile industries.

SUMMARY AND DISCUSSION

The popularity of *buchu,* i.e., *A. betulina* and *A. crenulata* in medicinal use may stem from its traditional use by the indigenous peoples of South Africa. This medicinal application was successfully disseminated by settlers, colonists, and explorers, which led to the tremendous popularity in Europe and the United States in the 19th century. However, because of the sparsity of scientific evidence and the advent of antibiotics the interest in buchu began to wane in the 20th century.

Whereas the phytochemical differences between the two *Agathosma* species have been well illustrated, recent pharmacological studies unfortunately could not alleviate the justified doubts regarding the traditional use of buchu for the treatment of urinary tract infection. Hence the overall modest number of assays addressing the antimicrobial activity revealed only low to moderate effects against micro-organisms responsible for urinary tract infection for a methanol-dichloromethane (1:1) extract that was not used traditionally and no effects for the essential oil or aqueous or ethanolic extracts. Taken together, the results of the antimicrobial tests remain inconclusive because the tentative active coumarins and flavonoids inherent in the different extracts have not been identified, preventing thus a direct comparison. Surprisingly also the monoterpenes in the essential oils failed to exert any antimicrobial effects they are known for. This was explained by the insolubility or reciprocal inactivation of the inherent ingredients in buchu by the ingredients in the growth medium. However, no further investigations were carried out to verify or deny these hypotheses.

ther pharmacological assays devoted to the study of anti-inflammatory, analgesic and antioxidant effects must be considered to be of limited scientific value, which is attributed to the very small number of potential pharmacological targets that have been tested and the extremely high doses applied being far away from doses applied in daily practice.

Recent studies on the effect of aqueous *buchu* extract on the metabolic syndrome should also be treated with caution as the promising results observed in animal studies may not be readily extrapolated to humans because of different pathophysiologies and the general absence of clinical trials.

Therefore, to answer the question whether *buchu* has been rightfully forgotten or has been just underutilized more studies of high scientific value are needed. Thus, extracts of *buchu* should be subjected to well-designed experiments *in vitro* and *in vivo* taking into consideration at first instance a detailed phytochemical characterization of the extract components and the application of clinically relevant doses. Based on the above future research activities should focus on the methanol-dichlormethane (1:1) extract of *A. betulina*, as it showed the most promising results in the antimicrobial studies carried out. In case of verified positive effects *in vitro*, the results obtained should be further tested for efficacy in urinary tract infections in randomized, double-blind, and placebo-controlled clinical trials. Also various promising antioxidant pharmacological activities and health benefits reported for citrus flavonoids (Mahmoud et al., 2019) like hesperidin (Li and Schluesener, 2017) and diosmin (Zheng et al., 2020) should be considered for *A. betulina*, as it also contains these components. In this context the effects of *A. betulina* on lowering the risks for degenerative diseases like cancer, cardiovascular diseases, Alzheimer's disease and Parkinson disease should be further investigated. In case of verified pharmacological activity *in vitro* and in human *A. betulina* extracts may be utilized as food supplements for its preventive effects on degenerative diseases. In general, *A. betulina* should be preferred over *A. crenulata*, because of the lower amounts of the hepatotoxic pulegone, inherent to both species. Moreover also other species like *A. collina* or *A. namaquensis* should receive an increasing focus of attention. Results obtained should be verified in randomized, double-blind, and placebo-controlled clinical trials. But until these studies are available the answer to the question "rightfully forgotten or underutilized" remains open.

CONCLUSION

Taken together, pharmacological studies carried out to date failed to confirm the traditional use or historical popularity of *buchu*. Hence, products containing *buchu* should be treated with caution in that the deduced effects of *buchu* may not occur. Only on the basis of well characterized extracts and realistic doses applied in pharmacological assays and clinical trials a final verdict on the therapeutic potential of *buchu* can be made, resulting in an appropriate classification as a dietary supplement or medicinal product with proven pharmacological activity. In this context future studies should focus on the antimicrobial and antioxidant effects of *A. betulina* extracts providing the base for more evident use of buchu in the treatment of urinary tract infections or as food supplement in degenerative diseases. Also the application of *A. betulina* in the green synthesis of nanoparticles should receive more attention in the future.

AUTHOR CONTRIBUTIONS

TB: Introduction, History of *buchu*; MA-T: *Buchu* today; TB and MA-T: Summary, Conclusions.

FUNDING

We acknowledge support by the Open Access Publication Fund of Goethe University and the German Research Foundation.

REFERENCES

Afriplex (2008). *Afriplex Buchu Cultivation Project*. Paarl: Afriplex Pty Ltd.

American Pharmaceutical Association (1888). *The National Formulary of Unofficinal Preparations*. Washington, DC: American Pharmaceutical Association.

Anonymous (1847). Buchu in Anasarca. *Prov Med. Surg. J.* 11, 615.

Anonymous (1912a). Cargo of Titanic Valued at $420,000 *The New York Times*. April 21, 1912.

Anonymous (1835). *Codex Medicamentarius Hamburgensis*. Hamburg: Perthes.

Anonymous (1912b). Drug Department. *The Druggist Circular* 56, 1236.

Anonymous (1916). *Ergänzungsbuch Zum Deutschen Arzneibuch, Vierte Ausgabe (Erg-B. 4)*. Stuttgart: Deutscher Apotheker-Verein.

Anonymous (1978). *Homöopathisches Arzneibuch, 1. Ausgabe*. Stuttgart: Deutscher Apotheker Verlag.

Anonymous (1846). *Pharmacopoea Borussica. Editio Sexta*. Berlin: Decker.

Anonymous (1860). *Pharmacopoea Hassiae Electoralis, Editio Altera Emendatior*. Kassel.

Anonymous (1837). *Pharmacopoea Saxonica, jussu regio et auctoritate publica*. Dresden: In Bibliopolio Aulico Waltheriano.

Anonymous (1826). *Pharmacopoeia Collegii Medicorum Regis et Reginae in Hibernia*. Dublin: Hodges & McArthur.

Anonymous (1836). *Pharmacopoeia Collegii Regalis Medicorum Londinensis*. London: Woodfall.

Anonymous (1994). *Rote Liste. Editio Cantor*. Aulendorf: Bundesverband der Pharmazeutischen Industrie e.V.

Anonymous (1949). *The British Pharmaceutical Codex*. London: The Pharmaceutical Press.

Anonymous (1897). *The Chemist and Druggist: A Weekly Journal of Pharmacy and the Drug Trade*. London: Benn Brothers.

Anonymous (1908). *The Chemist and Druggist: A Weekly Journal of Pharmacy and the Drug Trade*. London: Benn Brothers.

Anonymous (1922). *The Chemist and Druggist: A Weekly Journal of Pharmacy and the Drug Trade*. London: Benn Brothers.

Anonymous (1842). *The Pharmacopoeia of the United States of America [2nd Ed. 1840]*. Philadelphia: Grigg & Elliot.

ANSM (1983). "Buchu (Barosma)," in *Pharmacopée Française, 10ème Édition*, 1–4.

Autenrieth, H. F. (1830). *Dissertatio inauguralis de diosma crenata eiusque in morbis efficacia adiectis quibusdam thesibus chirurgicis. MD*. Tübingen.

Barnes, J., Anderson, L., Phillipson, J. D., Smith, M., and Veitch, N. C. (2013). *Herbal Medicines*. 4th ed. London: The Pharmaceutical Press.

Bedford, P. W. (1863). "On the Comparative Values of the Commercial Varieties of Buchu Leaves," in *Proceedings of the American Pharmaceutical Association* (Baltimore: Merrihew & Thompson), 211–212.

Berg, O. C., and Schmidt, C. F. (1858). *Darstellung und Beschreibung sämmtlicher in der Pharmacopoea borussica aufgeführten offizinellen Gewächse oder der Theile und Rohstoffe, welche von ihnen in Anwendung kommen, nach natürlichen Familien*. Leipzig: Förstner.

Bhat, R. B., and Moskovitz, G. (2009). Herbal Medicinal Teas from South Africa. *Phyton* 78, 67–73. doi:10.32604/phyton.2009.78.067

Bradlow, F. R. (1965). *Baron von Ludwig and the Ludwig's-Burg Garden*. Cape Town: Balkema.

Brandes, R. (1827). Chemische Untersuchung der Buccoblätter (Fol. Diosmae crenatae). *Arch. Pharm.* 22, 229–251. doi:10.1002/ardp.18270220302

Brandes, R. (1826). Ueber die Bucco-Blätter. *Arch. Pharm.* 17, 207–213. doi:10.1002/ardp.18260170102

Brendler, T. (2021). From Bush Medicine to Modern Phytopharmaceutical: A Bibliographic Review of Devil's Claw (*Harpagophytum* spp.). *Pharmaceuticals* 14, 726. doi:10.3390/ph14080726

Brendler, T. (2009). "Umckaloabo: from a Patent Remedy to a Modern Herbal Pharmaceutical Based on Pelargonium Sidoides with Clinically Proven Efficacy," in *African Natural Plant Products, Volume I: Discoveries and Innovations in Chemistry, Bioactivity, and Applications* (Washington, DC: ACS Publications), 295–319. doi:10.1021/bk-2009-1021.ch017

Brendler, T., and van Wyk, B. E. (2008). A Historical, Scientific and Commercial Perspective on the Medicinal Use of Pelargonium Sidoides (Geraniaceae). *J. Ethnopharmacol* 119, 420–433. doi:10.1016/j.jep.2008.07.037

Brendler, T., Brinckmann, J. A., Feiter, U., Gericke, N., Lang, L., Pozharitskaya, O. N., et al. (2021). Sceletium for Managing Anxiety, Depression and Cognitive Impairment: A Traditional Herbal Medicine in Modern-Day Regulatory Systems. *Curr. Neuropharmacology* 19, 1384–1400. doi:10.2174/1570159x19666210215124737

Brendler, T., and Cock, I. E. (2021). *Cape Aloe Bitters – Past and Present*. Washington, DC: South African Journal of Botany In press. doi:10.1016/j.sajb.2021.11.054

Brendler, T. (2020). "The Rise and Fall of Hoodia: A Lesson on the Art and Science of Natural Product Commercialization," in *African Natural Plant Products, Volume III: Discoveries and Innovations in Chemistry, Bioactivity, and Applications* (Cape Town: ACS Publications), 313–324. doi:10.1021/bk-2020-1361.ch017

Bruinsma, A. F. A. (1838). *Dissertatio medica inauguralis de diosma crenata. MD*. Leiden.

Bruntz, L., and Jaloux, M. (1918). *Plantes Officinales et Plantes a Drogues Médicamenteuses*. Paris: Vigot.

Burchell, W. J. (1822). *"Travels in the interior of Southern Africa."*. London: Printed for Longman, Hurst, Rees, Orme, and Brown, 529–537.

Burrows, E. H. (1958). *A History of Medicne in South Africa up to the End of the Nineteenth Century*. Cape Town: Balkema.

Cadet de Gassicourt, F. (1827). Sur le Diosma crenata. *Bull. des travaux de la Société de pharmacie* 13, 106–112.

Chiguvare, H., Oyedeji, O. O., Matewu, R., Aremu, O., Oyemitan, I. A., Oyedeji, A. O., et al. (2016). Synthesis of Silver Nanoparticles Using Buchu Plant Extracts and Their Analgesic Properties. *Molecules* 21, 774. doi:10.3390/molecules21060774

Cloete, K. J., Valentine, A. J., Stander, M. A., Blomerus, L. M., and Botha, A. (2009). Evidence of Symbiosis between the Soil Yeast Cryptococcus Laurentii and a Sclerophyllous Medicinal Shrub, Agathosma Betulina (Berg.) Pillans. *Microb. Ecol.* 57 (4), 624–632. doi:10.1007/s00248-008-9457-9

Coetzee, C. (1999). *Buchu Commercialization: South Africa*. Elsenburg: Agricultural Research Council.

Cohen, S. M., Eisenbrand, G., Fukushima, S., Gooderham, N. J., Guengerich, F. P., Hecht, S. S., et al. (2020). FEMA GRAS Assessment of Natural Flavor Complexes: Mint, Buchu, Dill and Caraway Derived Flavoring Ingredients. *Food Chem. Toxicol.* 135, 110870. doi:10.1016/j.fct.2019.110870

Collins, N. F., Graven, E. H., Van Beek, T. A., and Lelyveld, G. P. (1996). Chemotaxonomy of Commercial Buchu Species (Agathosma betulinaandA. Crenulata). *J. Essent. Oil Res.* 8, 229–235. doi:10.1080/10412905.1996.9700607

Commelin, C. (1706). *Horti medici amstelæ damensis plantæ rariores et exoticæ ad vivum æri incisæ*. Lugduni Batavorum: Apud Fredericum Haringh.

Compton, R. H. (1922). Medicinal and Aromatic Plants in South Africa. *Veld & Flora* 8, 9.

Culbreth, D. M. R. (1906). *A Manual of Materia Medica and Pharmacology*. 4th edition. Philadelphia: Lea Brothers & Co.

Dapper, O., Ten Rhijne, W., and Grevenbroek, J. G. (1933). *The Early Cape Hottentots: Described in the Writings of Olfert Dapper (1668), Willem Ten Rhyne (1686) and Johannes Gulielmus de Grevenbroek (1695)*. Cape Town: The Van Riebeeck Society.

De Candolle, A. P. (1818). *Versuch über die Arzneikräfte der Pflanzen verglichen mit den äussern Formen und der natürlichen Klasseneintheilung derselben*. Aarau: Heinrich Remigius Sauerländer.

Deacon, H., and Van Heyningen, E. (2004). "Opportunities outside Private Practice before 1860," in *The Cape Doctor in the Nineteenth Century: A Social History*. Editors H. Deacon, H. Phillips, and E. Van Heyningen (Amsterdam - New York: Ed. Rodopi B. V), 133–168.

Diallo, A., Manikandan, E., Rajendran, V., and Maaza, M. (2016). Physical & Enhanced Photocatalytic Properties of green Synthesized SnO2 Nanoparticles via Aspalathus Linearis. *J. Alloys Comp.* 681, 561–570. doi:10.1016/j.jallcom.2016.04.200

Dufour von Féronce, A. (1900). *Hundertfünfzig Jahre einer deutschen Drogenhandlung 1750–1900. Ein Beitrag zur Geschichte ihrer Firma herausgegeben am 7. Februar 1900 von Brückner, Lampe & Co. Leipzig Berlin Hamburg*. Leipzig: Meisenbach, Riffahrt & Co.

Duncan, A. (1830). *The Edinburgh New Dispensatory*. 12th ed. Edinburgh: Bell & Bradfute.

Ecklon, C. F. (1826). Kruidkundige beschrijving en aanmerkingen betreffende eenige planten van Zuid-Afrika. *Het Nederduitsch zuid-afrikaansch tydschrift* 3, 439–442.

Ecklon, C. F., and Zeyher, C. L. P. (1835). *Enumeratio plantarum Africae australis extratropicae quae collectae, determinatae et expositae, pars 1*. Hamburg: Perthes & Besser.

European Commission (2002). *Opinion of the Scientific Committee on Food on the Risks to Human Health of Polycyclic Aromatic Hydrocarbons in Food. SCF/CS/CNTM/PAH/29 Final*. Brussels.

Feldman, H. S., and Youngken, H. W. (1944). A Pharmacognostical Study of Buchu. *J. Am. Pharm. Assoc. (Scientific ed.)* 33, 277–288. doi:10.1002/jps.3030330806

Firnhaber, E. (1826). Kurze Beschreibung der Arzneykräfte der Bucco, welche auf dem Borgebirge der guten Hoffnung zu finden, verfaßt vom Dr. Liesching, daselbst wohnhaft. *Arch. Pharm.* 16, 246–247. doi:10.1002/ardp.18260160319

Fluck, A. A. J., Mitchell, W., and Perry, H. M. (1961). Composition of Buchu Leaf Oil. *J. Sci. Food Agric.* 12, 290–292. doi:10.1002/jsfa.2740120405

Flückiger, F. A., and Hanbury, D. (1874). *Pharmacographia: A History of the Principal Drugs of Vegetable Origin, Met with in Great Britain and British India*. London: Macmillan.

Franz, G., and Hoth, A. (1987). *Monographie-Entwurf Barosma Betulina Bartl. Folium*. Regensburg: Institut für Pharmazeutische Biologie der Universität Regensburg.

Glen, H. F., and Germishuizen, G. (2010). "Mackrill, Joseph (1762–1820)," in *Botanical Exporation of Southern Africa*. 2nd edition (Pretoria: SANBI), 280–281.

Gunn, M., and Codd, L. E. (1981). *Botanical Exploration of Southern Africa*. Cape Town: A. A.: Balkema.

Hager, H. (1880). *Handbuch der Pharmaceutischen Praxis, erster Theil*. Berlin: Springer.

Hart, T. (2001). *A Report on the Demolition of "The Knoll" (Known Historically as Botany House), Kloof Road, Bantry Bay*. Rondebosch: Visser and Visser Consulting Quantity Surveyors.

Hoskins, E. (1848). Buchu in Dropsy. *BMJ* s1-12, 120–121. doi:10.1136/bmj.s1-12.5.120

Huisamen, B., Bouic, P. D. J., Pheiffer, R. H., and Van Vuuren, M. (2019). *Medicinal Effects of Agathosma (Buchu) Extracts*. Berlin: Cape Town: AOSIS (Pty) Ltd.

Jackson, A. R. (1825). Notice Respecting the Employment of the Leaves of the Diosma Crenata, at the Cape of Good Hope. *Trans. Med. Phys. Soc. Calcutta* 1, 371.

Jobst, F. (1825). Ueber Bukku-Blätter. *Repertorium für die Pharmacie* 22, 51–56. doi:10.24355/dbbs.084-201007190902-0

Jones, H. W. (1879). The Amount of Ash and Soluble Matter in the Three Sorts of Buchu. *Am. J. Pharm.* 1879, 198–199. (1835-1907) April.

Jorritsma, T. A. (1826). An Den Redacteur. *Algemeene Konst- en Letter-Bode* 1, 114–119.

Kaiser, R., Lamparsky, D., and Schudel, P. (1975). Analysis of Buchu Leaf Oil. *J. Agric. Food Chem.* 23, 943–950. doi:10.1021/jf60201a021

Kannemeyer, M. (1951). Dr. Mackrill's Notebook. *Africana notes and news* 8, 55–59.

Kolb, P. (1719). *Caput Bonae Spei hodiernum, das ist: vollständige Beschreibung des africanischen Vorgebürges der Guten Hofnung: worinnen in dreyen Theilen abgehandelt wird, wie es heut zu Tage, nach seiner Situation und Eigenschaft aussiehet*. Nürnberg: Monath.

Kommission, E. (1990). *Barosmae Folium/Buccoblätter*. Bundesanzeiger *22a*, 04853.

Kondakow, I., and Bachtschiew, N. (1901). Ueber das ätherische Oel der Buccoblätter und über den Bau der Bestantheile desselben. *J. Prakt. Chem.* 63, 49–76. doi:10.1002/prac.19010630104

Kühle, E. (1970). "Apotheker Firnhaber, Bürgermeister von Nordhorn 1843–72," in *Jahrbuch des Heimatvereins der Grafschaft Bentheim*, 97–105.

Kunze, G. (1826). *A. Richards Medizinische Botanik*. Berlin: Enslin.

Lambert, M. I., Burgess, T., and Noakes, T. D. (2002). *The Efficacy of Buchu (Agathosma Betulina) in Treating Symptoms of Pain and Swelling from Exercise-Induced Muscle Damage*. Newlands, South Africa: MRC/UCT Research Unit of Exercise Science and Sports Medicine, Sports Science Institute of South Africa.

Lamparsky, D., and Schudel, P. (1971). p-Menthane-8-thiol-3-one, a New Component of Buchu Leaf Oil. *Tetrahedron Lett.* 12, 3323–3326. doi:10.1016/s0040-4039(01)97168-0

Latrobe, C. I. (1818). *Journal of a Visit to South Africa, in 1815, and 1816*. London: Ackerman.

Lawson, E. B., and Clark, W. G. (1932). Essential Oils in South Africa. *Oil Soap* 9, 162–165. doi:10.1007/bf02543567

Li, C., and Schluesener, H. (2017). Health-promoting Effects of the Citrus Flavanone Hesperidin. *Crit. Rev. Food Sci. Nutr.* 57, 613–631. doi:10.1080/10408398.2014.906382

Linné, C. V. (1756). *Centuria Plantarum [I.-] II*. Upsaliae: exc. L. M. Höjer.

Lis-Balchin, M., Hart, S., and Simpson, E. (2001). Buchu (Agathosma Betulina and A. Crenulata, Rutaceae) Essential Oils: Their Pharmacological Action on guinea-pig Ileum and Antimicrobial Activity on Microorganisms. *J. Pharm. Pharmacol.* 53, 579–582. doi:10.1211/0022357011775703

Long, E. (1831). On the Use of the Extract of Belladonna, and Buchu Leaves, in Spasmodic Strictures of the Urethra. *Lond. Med. Phys. J.* 10, 283–285.

Low, C. H. (2007). Different Histories of Buchu: Euro-American Appropriation of San and Khoekhoe Knowledge of Buchu Plants. *Environ. Hist. Camb* 13, 333–361. doi:10.3197/096734007x228309

Lubbe, A., and Verpoorte, R. (2011). Cultivation of Medicinal and Aromatic Plants for Specialty Industrial Materials. *Ind. Crops Prod.* 34, 785–801. doi:10.1016/j.indcrop.2011.01.019

Madaus, G. (1938). "Bucco, von Barosma betulina, Rutaceae," in *Lehrbuch der Biologischen Heilmittel* (Hildesheim: Olms), 741–744. (Reprint 1979).

Mahmoud, A. M., Hernandez Bautista, R. J., Sandhu, M. A., and Hussein, O. E. (2019). Beneficial Effects of Citrus Flavonoids on Cardiovascular and Metabolic Health. *Oxidative Med. Cell. longevity* 2019, 1–19. doi:10.1155/2019/5484138

Maisch, J. M. (1881). The Stearopten of Buchu Leaves. *Am. J. Pharm.* 53, 331–333. (1835-1907).

McDowell, E. (1824). "Cases of Disease of the Bladder Treated with Buchu Leaves,". *Transactions of the Association of Fellows and Licentiates of the King and Queen's College of Physicians in Ireland*, 4, 131–141.

McKenzie, C. E. (1906). *Abbau und Synthese des Buccokamphers (Diosphenol)*. PhD, Friedrich-Wilhelms-Universität.

McMagh, P. (1992). *The Three Lieschings: Their Times and Contribution to Cape Medicine 1800-1843*. Cape Town: The Society for the History of Pharmacy in South Africa.

Möckel, H. A. (1830). *De diosma crenata oleo crotonis tiglii et carbone animali. Dissertatio pharmacologico-medica*. Leipzig: Staritz.

Moolla, A. (2005). A Phytochemical and Pharmacological Investigation of Indigenous Agathosma Species. *MSc*. University of the Witwatersrand.

Moolla, A., and Viljoen, A. M. (2008). 'Buchu' -Agathosma Betulina and Agathosma Crenulata (Rutaceae): a Review. *J. Ethnopharmacol* 119, 413–419. doi:10.1016/j.jep.2008.07.036

Moolla, A., Van Vuuren, S. F., Van Zyl, R. L., and Viljoen, A. M. (2007). Biological Activity and Toxicity Profile of 17 Agathosma (Rutaceae) Species. *South Afr. J. Bot.* 73, 588–592. doi:10.1016/j.sajb.2007.05.007

Nees von Esenbeck, T. F. L. (1828). *Plantae Officinalis Oder Sammlung Officineller Pflanzen*. Düsseldorf: Arnz & Comp.

Nees von Esenbeck, T. F. L. (1827). Ueber die Buccoblätter. *Arch. Pharm.* 21, 48. doi:10.1002/ardp.18270210111

Nourij, F. G. (1827). *Dissertatio Medica Inauguralis, Exhibens Hlstoriam Botanicam, Chemico-Pharmaceuticam Et Medicam Follorum Diosmae Serratifoliae (Vulgo Foliorum Buchu). MD*. Groningen: Academia Groningana.

O'Brien, D. (2005). A Phytochemical-Bioactivity Investigation of the South African Traditional Herbal Medicine, Agathosma Betulina (Buchu). *PhD*. Reading: University of Reading.

Pal, G., Rai, P., and Pandey, A. (2019). in *Green Synthesis of Nanoparticles: A Greener Approach for a Cleaner Future*. Editors A. K. Shukla and S. Iravani (Elsevier), 1–26. doi:10.1016/b978-0-08-102579-6.00001-0

Pappe, L. (1847). *A List South African Indigenous Plants Used as Remedies by the Colonists of the Cape of Good Hope*. Cape Town: Pike.

Pappe, L. (1850). *Florae Capensis Medicae Prodromus*. Cape Town: Robertson.

Pfaff, C. H. (1831). *Pharmacopoea Slesvico-Holsatica/regia auctoritate et sub auspiciis Collegii Regii Sanitatis Slesvico-Holsatici*. Kiel: Libraria Universitatis.

Pillans, N. S. (1950). A Revision of Agathosma. *J. South Afr. Bot.* 16, 55–117.

Posthumus, M. A., Van Beek, T. A., Collins, N. F., and Graven, E. H. (1996). Chemical Composition of the Essential Oils of *Agathosma betulina, A. crenulata* and an *A. betulina* x crenulata hybrid (*buchu*). *J. Essent. Oil Res.* 8, 223–228. doi:10.1080/10412905.1996.9700606

Price, C. H. (1974). Origins of Medicine and Pharmacy at the Cape of Good Hope. *S Afr. Med. J.* 48, 1128–1130. doi:10.10520/AJA20785135_27034

Raman, V., Sagi, S., Galal, A. M., Avula, B., Viljoen, A., and Khan, I. A. (2015). Adulteration in Commercial Buchu Dietary Supplements: Analyses of Commercial and Authentic Buchu Samples and Comparative Studies of Agathosma Betulina and Agathosma Crenulata by Microscopy and HPTLC. *South Afr. J. Bot.* 100, 122–131. doi:10.1016/j.sajb.2015.05.012

Raza, A., Muhammad, F., De Sousa, D. P., Khaliq, T., Aslam, B., Andrade, L., et al. (2016). *In Vitro* and *In Vivo* Toxicological Evaluations of Methyl Ferulate, Methyl P-Coumarate, and Pulegone 1,2-epoxide. *Pharm. Biol.* 54, 523–529. doi:10.3109/13880209.2015.1052148

Reece, R. (1827). *An Alphabetical Catalogue of Drugs; with a Specification of Their doses, The Diseases for the Cure or Palliation of Which They Are Administered. Twelfth Edition, with Considerable Additions*. London: Reece & Co.

Reece, R. (1822). *On Buchu Leaves. The Monthly Gazette of Health Vol. 6 for the Year 1821, 799, 812, 864–865, 909-910, 1075-1076, 1080, 1175*. London: Reece & Co.

Reece, R. (1824). *On Buchu Leaves. The Monthly Gazette of Health Vol. 9 for the Year 1824, 1010, 1083–1084*. London: Reece & Co.

Reece, R. (1836). *The Popular Catalogue of Drugs, Specifying Their Properties, the Doses to Adults and Children of Different Ages, the Retail Prices &c. Forming a Complete Modern Dispesatory*. Fifteenth edition. London: Reece & Co.

Royal College of Physicians of Edinburgh (1839). *The Pharmacopoeia of the Royal College of Physicians of Edinburgh*. Edinburgh: A. & C. Black.

Sandasi, M. (2008). *The Effect of Plant Extracts on Biofilm Formation and Development. M Tech*. Pretoria: Tshwane University of Technology.

Schneider, W. (1974). *Lexikon zur Arzneimittelgeschichte, Pflanzliche Drogen, Band V/1*. Frankfurt: Govi.

Schwabe, W. (1934). *Homöopathisches Arzbeibuch*. Leipzig: Verlag Dr. Willmar Schwabe.

Scientific Committee (1983). "Agathosma - Buchu," in *British Herbal Pharmacopoeia*. 2nd edition (Bournemouth: The British Herbal Medicine Association), 15–16.

Shimoyama, Y. (1888). Beiträge zur Kenntnis der Bukublätter. *Arch. Pharm.* 226, 64–70. doi:10.1002/ardp.18882260203

Simpson, D. (1998). Buchu--South Africa's Amazing Herbal Remedy. *Scott Med. J.* 43, 189–191. doi:10.1177/003693309804300610

Skead, C. J. (2009). *Mackrill, Joseph (1762-1820)*. Pretoria: SANBI.

Skosana, B., Aboua, G., and Du Plessis, S. S. (2014). "Buchu – The Multi-Purpose Ethnomedicinally Important Specie and Its Benefits in the Reproductive System," in *Antioxidant-Antidiabetic Agents And Human Health*. Editor O. Oguntibeju (London: IntechOpen).

Smith, C. A. (1966). *Common Names of South African Plants*. Pretoria, RSA: Department of Agricultural Technical Services.

Sone, B. T., Manikandan, E., Gurib-Fakim, A., and Maaza, M. (2016). Single-phase α-Cr2O3 Nanoparticles' green Synthesis Using Callistemon Viminalis' Red Flower Extract. *Green. Chem. Lett. Rev.* 9, 85–90. doi:10.1080/17518253.2016.1151083

Sone, B. T., Manikandan, E., Gurib-Fakim, A., and Maaza, M. (2015). Sm2O3 Nanoparticles green Synthesis via Callistemon Viminalis' Extract. *J. Alloys Comp.* 650, 357–362. doi:10.1016/j.jallcom.2015.07.272

South African National Biodiversity Institute (2005). *Agathosma Betulina Monograph*.

Sparrman, A. E., Groskurd, C. H., and Forster, J. G. A. (1784). *Andr. Sparrmanns Reise nach dem Vorgebirge der Guten Hoffnung, den südlichen Polarländern und um die Welt, hauptsächlich aber in den Ländern der Hottentotten und Kaffern. 1772 bis 1776*. Berlin: Haude & Spener.

Spica, P. (1885). *Ricerche Sulla Diosma Crenata*. Padova: Istituto chimico-farmaceutico della R. Università.

Spohr, O. H. (1968). Dr. Med. Friedrich Ludwig Liesching. *Q. Bull. S Afr. Libr.* 23, 45–55.

Squire, P. (1899). *Companion to the Latest Edition of the British Pharmacopceia, Comparing the Strength of its Various Preparations with Those of the United States, and Other Foreign Pharmacopceias, to Which Are Added Not Official Preparations, and Practical Hints on Prescribing*. 17th edition. London: Churchill.

Stander, M. A., Brendler, T., Redelinghuys, H., and Van Wyk, B.-E. (2019). The Commercial History of Cape Herbal Teas and the Analysis of Phenolic Compounds in Historic Teas from a Depository of 1933. *J. Food Compost. Anal.* 76, 66–73. doi:10.1016/j.jfca.2018.11.001

Steenkamp, V., Gouws, M. C., Gulumian, M., Elgorashi, E. E., and Van Staden, J. (2006). Studies on Antibacterial, Anti-inflammatory and Antioxidant Activity of Herbal Remedies Used in the Treatment of Benign Prostatic Hyperplasia and Prostatitis. *J. Ethnopharmacol* 103, 71–75. doi:10.1016/j.jep.2005.07.007

Street, R. A., and Prinsloo, G. (2013). Commercially Important Medicinal Plants of South Africa: A Review. *J. Chem.* 2013, 1–16. doi:10.1155/2013/205048

Stromeyer, F. a. W. (1852). *Pharmacopoea Medicaminum, Quae in Pharmacopoea Hannoverana Non Sunt Recepta*. Hannover: Hahn.

Strumpf, F. L. (1861). *Allgemeine Pharmakopöe nach den neuesten Bestimmungen oder die offizinellen Arzneien nach ihrer Erkennung, Bereitung, Wirkung und Verordnung*. Leipzig & Heidelberg: Winter'sche Verlagshandlung.

The Historian (1912). Who Helmbold Really Was. *The Druggist Circular* 56, 654–657.

Theal, G. M. (1908). *History of South Africa since September 1795*, 1. London: Swan Sonnenschein & Co.

Thema, F. T., Manikandan, E., Dhlamini, M. S., and Maaza, M. (2015). Green Synthesis of ZnO Nanoparticles via Agathosma Betulina Natural Extract. *Mater. Lett.* 161, 124–127. doi:10.1016/j.matlet.2015.08.052

Thovhogi, N., Park, E., Manikandan, E., Maaza, M., and Gurib-Fakim, A. (2016). Physical Properties of CdO Nanoparticles Synthesized by green Chemistry via Hibiscus Sabdariffa Flower Extract. *J. Alloys Comp.* 655, 314–320. doi:10.1016/j.jallcom.2015.09.063

Thring, T. S., Hili, P., and Naughton, D. P. (2009). Anti-collagenase, Anti-elastase and Anti-oxidant Activities of Extracts from 21 Plants. *BMC Complement. Altern. Med.* 9, 27–11. doi:10.1186/1472-6882-9-27

Thunberg, C. P. (1797). *Dissertatio botanica de Diosma. PhD.* Uppsala.

Thunberg, C. P. (1794). *Prodromus Plantarum Capensium, Quas in Promontorio Bonæ Spei Africes, Annis 1772-1775*. Pars Prior. Upsaliae: J. Edman.

Vamenta-Morris, H., Dreisbach, A., Shoemaker-Moyle, M., and Abdel-Rahman, E. M. (2014). Internet Claims on Dietary and Herbal Supplements in Advanced Nephropathy: Truth or Myth. *Am. J. Nephrol.* 40, 393–398. doi:10.1159/000368724

Van der Stel, S. (1979). *Journey to Namaqualand in 1685*. Cape Town: Human and Rossouw.

Venugopal, K., Ahmad, H., Manikandan, E., Thanigai Arul, K., Kavitha, K., Moodley, M. K., et al. (2017b). The Impact of Anticancer Activity upon Beta Vulgaris Extract Mediated Biosynthesized Silver Nanoparticles (Ag-NPs) against Human Breast (MCF-7), Lung (A549) and Pharynx (Hep-2) Cancer Cell Lines. *J. Photochem. Photobiol. B.* 173, 99–107. doi:10.1016/j.jphotobiol.2017.05.031

Venugopal, K., Rather, H. A., Rajagopal, K., Shanthi, M. P., Sheriff, K., Illiyas, M., et al. (2017a). Synthesis of Silver Nanoparticles (Ag NPs) for Anticancer Activities (MCF 7 Breast and A549 Lung Cell Lines) of the Crude Extract

of Syzygium Aromaticum. *J. Photochem. Photobiol. B.* 167, 282–289. doi:10.1016/j.jphotobiol.2016.12.013

Viljoen, A., Chen, W., Mulaudzi, N., Kamatou, G., and Sandasi, M. (2021). *Phytochemical Profiling of Commercially Important South African Plants*. Amsterdam: Academic Press.

Viljoen, A. M., Moolia, A., Van Vuuren, S. F., Van Zyl, R. L., Başer, K. H. C., Demirci, B., et al. (2006). The Biological Activity and Essential Oil Composition of 17 Agathosma (Rutaceae) Species. *J. Essent. Oil Res.* 18, 2–16. doi:10.1080/10412905.2006.12067112

Wade, A. E. (1977). *Martindale, the Extra Pharmacopoeia*. 27th edition. London: The Pharmaceutical Press.

Wander, G. (1925). *Über das" Hesperidin" einiger Pflanzen* PhD. Zurich: Eidgenössische Technische Hochschule.

Watt, J. M., and Breyer-Brandwijk, M. G. (1962). *The Medicinal and Poisonous Plants of Southern and Eastern Africa*. London, UK: Livingstone.

Werner, H. F. (1949). The Cultivation of Buchu. *Veld & Flora* 35, 13–14.

Wilson, M. L. (2002). *Codex Witsenii: annotated watercolours of landscapes, flora and fauna observed on the expedition to the Copper Mountains in the country of the Namaqua undertaken in 1685-6 by Simon van der Stel, Commander at the Cape of Good Hope; copied at the Cape in 1692 for Nicolaas Witsen, mayor of Amsterdam, member of the Amsterdam Chamber of the Dutch East India Company, ambassador to Great Britain & c, & c*. Cape Town: Iziko Museums.

Wood, G. B., and Bache, F. (1833). *The Dispensatory of the United States Of America*. Philadelphia: Grigg & Elliot.

Young, J. H. (1961). *The Toadstool Millionaires*. Princeton: Princeton University Press.

Zenetti, P. (1895). Das Vorkommen von Hesperidin in Folia Bucco und seine Krystallformen. *Arch. Pharm.* 233, 104–110. doi:10.1002/ardp.18952330204

Zheng, Y., Zhang, W., Shi, H., Li, Z., Liu, H., Chen, Z., et al. (2020). Metabolism and Pharmacological Activities of the Natural Health-Benefiting Compound Diosmin. *Food Funct.* 11, 8472–8492. doi:10.1039/d0fo01598a

Conflict of Interest: TB is an industry consultant DBA PlantaPhile and is part-time employed by Traditional Medicinals Inc.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 18 February 2022
doi: 10.3389/fphar.2022.816029

Ethnobotanical Survey on Bitter Tea in Taiwan

Jung Chao[1†], Ting-Yang Chen[2†], Li-Heng Pao[3,4], Jeng-Shyan Deng[5], Yung-Chi Cheng[6], Shan-Yu Su[7] and Shyh-Shyun Huang[5,8*]*

[1]Chinese Medicine Research Center, Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, Master Program for Food and Drug Safety, China Medical University, Taichung, Taiwan, [2]Chinese Medicine Research Center, Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan, [3]Graduate Institute of Health Industry Technology, Research Center for Food and Cosmetic Safety, and Research Center for Chinese Herbal Medicine, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan, Taiwan, [4]Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital, Taoyuan, Taiwan, [5]Department of Food Nutrition and Health Biotechnology, Asia University, Taichung, Taiwan, [6]Department of Pharmacology, Yale University School of Medicine, New Haven, CT, United States, [7]Department of Chinese Medicine, China Medical University Hospital, School of Post-Baccalaureate Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan, [8]School of Pharmacy, China Medical University, Taichung, Taiwan

OPEN ACCESS

Edited by:
Michael Heinrich,
University College London,
United Kingdom

Reviewed by:
Manuel Pardo de Santayana,
Autonomous University of Madrid,
Spain
Diego Rivera,
University of Murcia, Spain
Lukasz Luczaj,
University of Rzeszow, Poland

***Correspondence:**
Shan-Yu Su
shanyusu@gmail.com
Shyh-Shyun Huang
sshuang@mail.cmu.edu.tw

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 16 November 2021
Accepted: 13 January 2022
Published: 18 February 2022

Citation:
Chao J, Chen T-Y, Pao L-H, Deng J-S, Cheng Y-C, Su S-Y and Huang S-S (2022) Ethnobotanical Survey on Bitter Tea in Taiwan. Front. Pharmacol. 13:816029. doi: 10.3389/fphar.2022.816029

Ethnopharmacological evidence: In Taiwan, herbal tea is considered a traditional medicine and has been consumed for hundreds of years. In contrast to regular tea, herbal teas are prepared using plants other than the regular tea plant, Camellia sinensis (L.) Kuntze. Bitter tea (kǔ-chá), a series of herbal teas prepared in response to common diseases in Taiwan, is often made from local Taiwanese plants. However, the raw materials and formulations have been kept secret and verbally passed down by store owners across generations without a fixed recipe, and the constituent plant materials have not been disclosed.

Aim of the study: The aim was to determine the herbal composition of bitter tea sold in Taiwan, which can facilitate further studies on pharmacological applications and conserve cultural resources.

Materials and methods: Interviews were conducted through a semi-structured questionnaire. The surveyed respondents were traditional sellers of traditional herbal tea. The relevant literature was collated for a systematic analysis of the composition, characteristics, and traditional and modern applications of the plant materials used in bitter tea. We also conducted an association analysis of the composition of Taiwanese bitter tea with green herb tea (qing-cao-cha tea), another commonly consumed herbal tea in Taiwan, as well as herbal teas in neighboring areas outside Taiwan.

Results: After visiting a total of 59 stores, we identified 32 bitter tea formulations and 73 plant materials. Asteraceae was the most commonly used family, and most stores used whole plants. According to a network analysis of nine plant materials used in high frequency as drug pairs, Tithonia diversifolia and Ajuga nipponensis were found to be the core plant materials used in Taiwanese bitter tea.

Conclusion: Plant materials used in Taiwanese bitter tea were distinct, with multiple therapeutic functions. Further research is required to clarify their efficacy and mechanisms.

Keywords: Taiwanese bitter tea, field survey, Taiwanese traditional medicine, ethnobotany, health geography

INTRODUCTION

Herbal tea is a drink composed of plants other than *Camellia sinensis* (L.) Kuntze of the Theaceae family—in contrast to regular tea—and is prepared by decocting or brewing with hot water. Herbal tea is commonly prepared with local plants that can be easily obtained (Fu et al., 2018). The custom of brewing tea with herbs is found all over the world, including Europe (Soukand and Kalle, 2013; Soukand et al., 2013), America (Joubert et al., 2008), Africa (Roulette et al., 2018), and Asia (Hu, 2005). The herbs used could be a single herb or a mixture of multiple plants. In many areas, herbal teas are used as therapeutic vehicles to treat associated health conditions (Poswal et al., 2019).

In China, herbal tea has been consumed for more than 2000 years (Liu et al., 2013). Famous herbal teas in pan-China include those are drunk in Yao area (Jin et al., 2018), Lingnan (Liu Y. et al., 2013), Chaoshan (Li et al., 2017), and Fujian (Lin, 2014), and Taiwan (Chang, 2005). Furthermore, this beverage has been closely associated with the prevention and treatment of local common ailments (Li et al., 2017; Tan et al., 2017). The herbal teas in Lingnan (Liu Y. et al., 2013), Chaoshan (Li et al., 2017), and Fujian (Lin, 2014) are called the"cool tea," implying that the herbal teas are used against the hot weather in southern China. The type of herbal tea is also influenced by the traditional culture of various regions, thereby reflecting local characteristics (Hu, 2005).

Although the folk plants in Taiwan are widely used in religious rites, bathing, cuisine, and herbal tea, international literature on Taiwanese folk plants is limited. According to previous surveys, there are 1,217 wild or cultivated folk plants with medicinal purposes have been documented in Taiwan (Ministry of Health and Welfare, 2021). The two main herbal teas in Taiwan, bitter tea (kǔ-chá) and green herb tea (qīng-cǎo-chá), are the two most complicated applications of folk plants in Taiwan. Both of them are usually made by cooking a mixture of medicinal plants (Chang, 2004). The mixtures of medicinal plants, in which there are the mixtures of bioactive compounds, exert synergistic therapeutic effects (Gertsch, 2011; Gras et al., 2018). Herbal teas in Taiwan are believed to have originated in southern China. After arriving in Taiwan, the ingredients of original herbal teas gradually turned into readily available Taiwanese native plants, and then, herbal teas that are suitable for local people were gradually developed (Chen and Lin, 2012).

Although the formulations of bitter tea and green herb tea vary across Taiwanese stores, they are all primarily advertised as having the capacity to "clear heat" (Huang et al., 2020). In the principle of traditional Chinese medicine formulation, one to three medicinal materials play the role of core medicinal materials in an herbal mixture. Other medicinal materials are added to enhance the therapeutic effects, improve the taste, or reduce the side effects of the core plant medicinal materials (Zhou et al., 2016).

According to previous research and surveys, the main components of green herb tea in Taiwan are *Platostoma palustre* (Blume) A.J.Paton, *Bidens pilosa* L., *Pteris multifida* Poir., *Mentha arvensis* L., *Sphagneticola calendulacea* (L.) Pruski, and *Rhinacanthus nasutus* (L.) Kurz (Huang et al., 2020). These tea beverages prepared by mixing and decocting these plants have a refreshing, half bitter/half sweet taste, and are used for quenching thirst and relieving summer heat (Huang et al., 2020). The other Taiwanese main herbal tea, the Taiwanese bitter tea, is also known as liver nourishing tea (yǎng-gān-chá), and thick green herb tea (nóng-hóu-qīng-cǎo-chá). It is characterized by a bitter-dominated taste that is stronger than that of regular green herb tea (Chang, 2005). Although bitter tea has been consumed for hundreds of years in Taiwan, its components and core medicinal materials have not been comprehensively studied, which has impeded the investigation of its efficacy against various health conditions.

Traditional Chinese medicine categorizes medicinal materials according to their property and flavor. The property includes hot, warm, neutral, cool, cold. Hot and warm materials are usually taken to supply energy to the body, while cool and cold materials are taken to drain away the heat from the body (Liu et al., 2020). The flavors are divided into sour, bitter, sweet, spicy, salty, and plain. Flavor is the taste of herbs in the mouth. Each of the flavors has distinct medicinal therapeutic functions. Sour herbs astringe the leakage of fluids and energy; sweet herbs tonify and harmonize; spicy herbs disperse and move; salty herbs soften and purge; plain herbs leech out dampness; and finally, the traditional therapeutic function associated with bitter herbs is heat clearing and removing dampmess (Bensky et al., 2004; Lu et al., 2018; Liu et al.,. 2020). The heat-clearing and dampess-draining effects provided by the bitter taste are used to combat the discomfort caused by the summer rainy season (Wu, 2005). Moreover, according to a previous study, the administration of bitter drugs can help treat liver disease (Tsai et al., 2020). For example, *Gentiana scabra* Bunge, *Artemisia capillaris* Thunb., and *Scutellaria baicalensis* Georgi are the most classical and commonly used traditional Chinese medicines for liver disease (Tsai et al., 2020).

Only one of the two main types of herbal tea in Taiwan, the composition and pharmacology of green herb tea constituents have been studied and reported (Huang et al., 2020), whereas those of the raw materials of bitter tea have not yet been elucidated. According to local reference books, most raw materials used in such herbal teas are either cultivated locally or wildly harvested. They require simple process after collection and are decocted after drying (Chang, 2005). However, the formula of Taiwanese bitter tea is not in the public domain, considering that every store keeps their recipe a secret. In the present study, we surveyed sellers of folk herbal medicine in Taiwan. The survey was conducted via interviews using semi-structured questionnaires, and information recorded included the current status of the bitter tea market in Taiwan as well as the ingredients and formulations used. The results of the present study can help reveal the ethnopharmacological aspects of bitter tea consumed in Taiwan and facilitate the conservation of such unique cultural resources.

MATERIALS AND METHODS

Survey Area and Period

Taiwan, an island in East Asia, is located at 21°45′-25°56′N and 119°18′-124°34′E. It spans the Tropic of Cancer, covers an area of

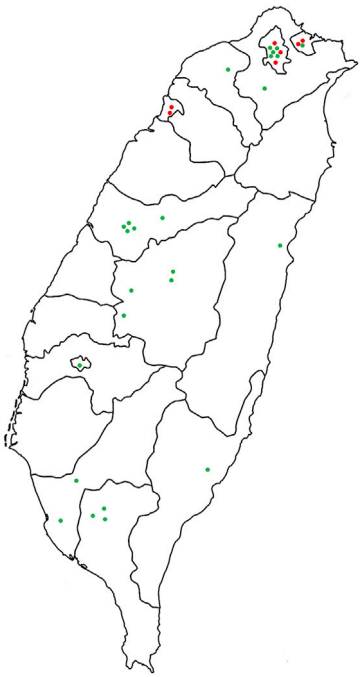


FIGURE 1 | Geographical distribution of 32 stores with available bitter tea formulations. Red dots indicate that the store provided bitter tea formulation but did not receive interviews (seven stores), and green dots indicate that the store provided complete bitter tea formulation and accepted interviews (25 stores).

36,197 km^2, and mostly has a subtropical climate with monsoons, warm and humid summers, and heavy rainfall. In the present study, 59 stores were visited, 25 of which provided information on bitter tea formulations and participated in the interviews. Seven stores only provided information on the bitter tea formula used in their stores but did not give interviews. The 32 sampled stores were distributed all over Taiwan (**Figure 1**). The survey period was from December 2018 to April 2019, and the entire research activity was reviewed and approved by Taiwan's Central Regional Research Ethics Center (CRREC-107-019) (**Supplementary Figure S1**).

Interviews and Data Collection

A semi-structured questionnaire was used for interviews, and the subjects were store owners selling bitter tea. The stores were found online or introduced to us by organizations involved with local medicinal plants. The store owners were invited to participate in the survey, and interviews were conducted on a voluntary basis. With the permission of the informants, written records, audio recordings, and photographs were obtained, and herbal medicines were acquired by the authors. The medicinal materials were then identified using the five senses identification method, with which the expert identify the origin of medicinal materials by touch, taste, smell, hearing, and visual observation, without any equipments (Hsieh et al., 2017). The specimens of the medicinal materials have been deposited in the herbarium at School of Pharmacy of China Medical University (CMU) in Taichung City, Taiwan.

The information collected by the interviews was divided into two parts. The first part included basic information on the store, including age and sex of the store owner, store location, and how long the store has been in operation. The second part was a semi-structured questionnaire consisting of the following questions: 1) what is the composition of bitter tea; 2) what is the traditional therapeutic indication of the bitter tea sold in your store; 3) how was the traditional knowledge of the bitter tea obtained; and 4) does the store sell dry or fresh raw materials needed to make the tea?

Data Collection of Bitter Tea Plant Materials in Taiwan

All data on the surveyed plant materials were collated, which included the following:

(1) Name, including scientific name, family name, and local name. The scientific and family names of plants are presented according to the Plant List nomenclature (The Royal Botanic Gardens, 2013) and the Plants of the World Online (Royal Botanic Garden, 2021).
(2) Part of the plant used. This was determined by identifying the raw plant materials.
(3) Frequency and use value (UV). Frequency refers to the number of stores using the plant material in the survey, and UV refers to the number of stores using the plant material/total number of stores in the survey as follows (Fu et al., 2018):

$$UV = \frac{\sum Ui}{N}$$

Where Ui represents the individual number of the *i*th medicinal material, and N represents the total number of bitter tea formulations.

(4) Traditional therapeutic functions. This information was obtained from the Committee on Chinese Medicine and Pharmacy (2003) and the second edition of The Committee on Chinese Medicine and Pharmacy (2011).
(5) The diversity of the medicinal plants was evaluated using Shannon diversity index (Gras et al., 2018), which was calculated as follows:

$$\text{Shannon diversity index} = -\sum_{i=1}^{n} Pi * log_2 Pi$$

Where *Pi* represents the proportion of the individual number of the *i*th medicinal material to the individual number of the total medicinal material. For example, *Andrographis paniculata* was used in 10 of the formulations, and the total number of individual of all species in this survey is 183. Then the *Pi* for *Andrographis paniculata* was obtained by dividing 10 by 183. Then the

"$Pi*log_2Pi$" for all the medicinal materials in the survey were summed up to get the Shannon diversity index.

(6) Modern pharmacology. Information was obtained by searching for the plant materials in the literature using the PubMed database[1]. Those cited in studies published before April 2021 and at a frequency greater than nine were included.
(7) Comparison between Taiwanese bitter tea and Taiwanese green herb tea. Based on the findings of Huang et al. (2020), we analyzed the similarities and differences between the plant materials used for each tea and further compared differences in flavor, UV, and application in modern pharmacology.
(8) Comparison of herbal tea composition across three regions in southern China. By referring to relevant published articles (Liu et al., 2013; Lin, 2014; Li et al., 2017), a Venn diagram was plotted using an online tool[2] to analyze the relationships between Taiwanese bitter tea and herbal tea from Lingnan, Chaoshan, and Fujian.

Core Network Analysis of Bitter Tea Use in Taiwan

Core network analysis was carried out using the Traditional Chinese Medicine Inheritance Support System (TCMISS) v2.5 (Wu et al., 2019; Chao et al., 2020; Wu Z. et al., 2020). Several matching frequency conditions were input into the software to find a suitable network diagram. Establishing connections when combinations of plant materials had a matching frequency greater than four was found the most proper to generate the network that clearly present the core medicinal materials. The length of the connection indicates the degree of matching frequency, and the size of the circles represent the relative UV.

RESULTS

Respondents' Data and Store Information

A total of 59 stores selling bitter tea were visited for this study. Among them, 25 participated in interviews and provided the formulations for bitter tea; 7 were unwilling to participate in the interviews but provided bitter tea formulations; and 27 declined to participate in interviews and did not provide formulations. Of the 25 traditional stores that participated in interviews, 50% have been operating for more than 50 years. The store owners were mainly male (76%), with ages ranging from 31 to 70 years. Knowledge on bitter tea formulations was mostly passed down through family members (76%) or apprentices (20%). All stores sold prepared bitter tea drinks (100%), 36% sold dry raw materials, and 48% sold both dry and fresh raw materials (**Supplementary Figure S2**). In addition, all bitter tea sellers sold green herb tea.

Plant Materials Used in Bitter Tea and Their Territories in Taiwan

In the present study, 73 plant materials from 72 plants belonging to 33 families and 67 genera were identified. Among the 73 medicinal materials, 60% of them were cultivated, 63% of them can be collected in the wild, and 37% were both cultivated and wild (**Supplementary Table S1**). The average number of plants materials in a formulation is 5.7 (**Supplementary Table S2**). The top five ranking species include *Andrographis paniculata* (Burm. f.) Nees, *Tithonia diversifolia* (Hemsl.) A. Gray (both UV = 0.312), *Ajuga nipponensis* Makino, *Ixeris chinensis* (Thunb. ex Thunb.) Nakai (both UV = 0.281), and *Ilex asprella* (Hook. & Arn.) Champ. ex Benth (UV = 0.25). Regarding the plant families, Asteraceae was the most frequently used, comprising 16.7% among 72 plants and 75.0% among 32 formulations, followed by Lamiaceae, comprising 15.3% among total plants and 65% among formulations (**Figure 2A**). The most used plant parts were the whole plant (41.1%), followed by the root and rhizome (20.6%) (**Figure 2B**).

The Shannon diversity index for all the 73 medicinal materials in Taiwan was 5.73. Among the northern, central, and southern Taiwan, the medicinal materials used in northern Taiwan were the most diverse, with a Shannon diversity index of 5.27. While the Shannon diversity indices of central and southern Taiwan were 4.92 and 4.11, respectively. Moreover, the more the north, the more species of medicinal materials were used. A total of 49 medicinal materials were used in bitter tea in northern Taiwan, while there were only 34 medicinal materials used in Central Taiwan, and even fewer in southern Taiwan, with only 20 medicinal materials (**Supplementary Table S3**). In these three areas, only nine medicinal materials were commonly used (**Figure 2C**). They were *Tithonia diversifolia* (stem), *Ixeris chinensis*, *Rhinacanthus nasutus* (L.) Kurz, *Orthosiphon aristatus* (Blume) Miq., *Andrographis paniculata* (Burm. f.) Nees, *Solanum incanum*, *Mallotus repandus* (Willd.) Muell.-Arg., *Sphagneticola calendulacea* (L.) Pruski, and *Pteris multifida* Poir.

Ethnomedicinal Functions of the Bitter Tea

When asked about the traditional applications of the bitter tea sold at a store, all sellers answered based on traditional concepts in Taiwan folk therapy; the bitter flavor is said to protect the liver, and the main ethnomedicinal function of bitter tea is "clearing heat, protecting the liver, and lowering liver fire." The sellers also stated that bitter tea is a type of folk therapy product for the liver.

The traditional applications of the 73 identified plant materials were revealed, excluding *T. diversifolia* (stem) and *A. keiskei* since there were no records on their properties and flavors (**Supplementary Table S1**). The cold, hot, warm, and cool properties were then analyzed for 71 plant materials; cold properties were the most common (46.5%) followed by cool (26.8%), jointly accounting for 73.3% of the plant material properties (**Figure 3A**). Moreover, 69% of the plant materials had a bitter flavor (**Figure 3B**). According to a comprehensive analysis of the four properties

[1]https://www.ncbi.nlm.nih.gov/pubmed
[2]http://bioinformatics.psb.ugent.be/webtools/Venn/

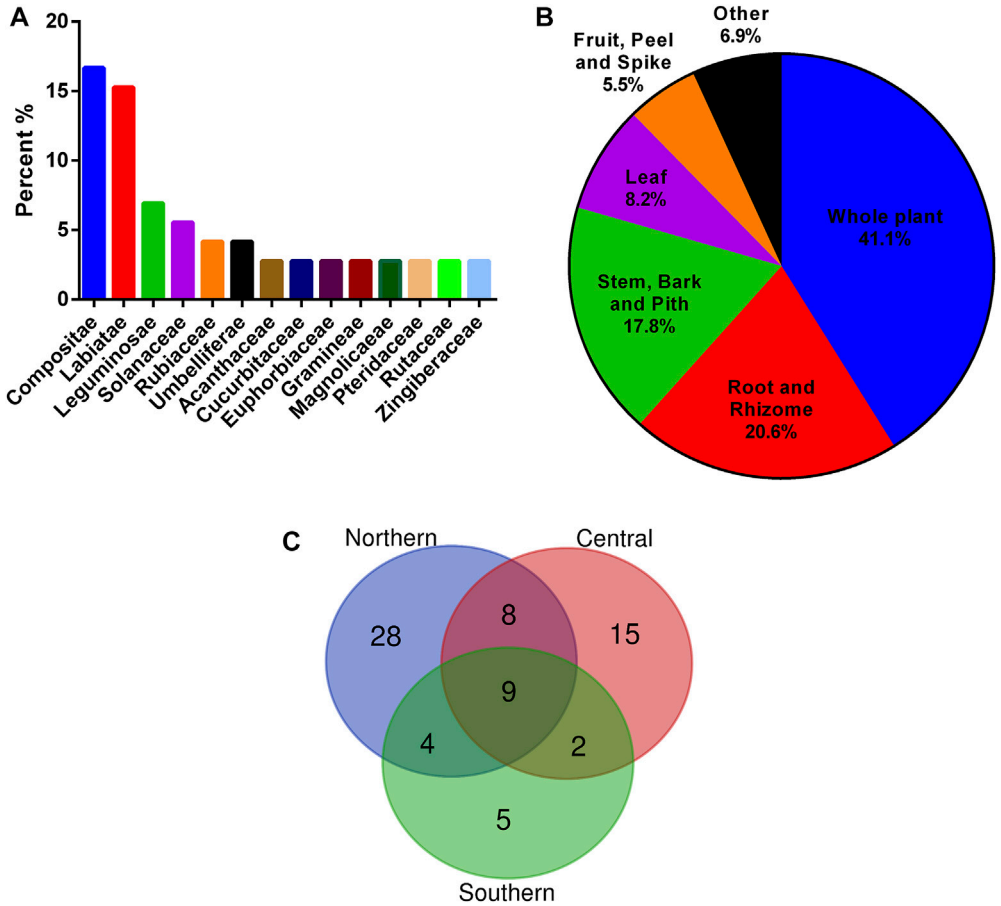


FIGURE 2 | Origin analysis of 73 plant materials used in bitter tea. **(A)** Families; **(B)** plant parts; **(C)** the number of shared plant materials in areas of Taiwan.

and five flavors, cold and bitter plants are the most commonly used for preparing bitter tea in Taiwan (**Figure 3C**). Analysis of the ethnomedicinal function showed that 63.9, 54.8, 25.0, and 25.0% of the 73 plant materials had heat-clearing, detoxification, detumescence, and diuresis effects, respectively (**Figure 3D**).

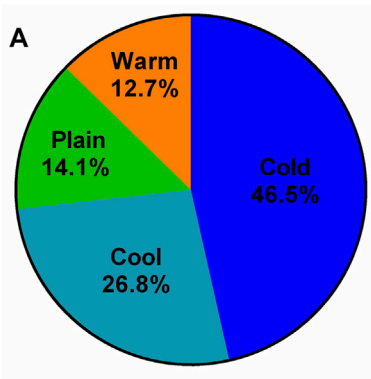
Among the 24 commonly used plant materials in Taiwanese bitter tea, those with a frequency of use greater than nine were collected to analyze their modern pharmacological applications (**Table 1**). Anti-inflammation was the most common pharmacological action (**Figure 3E**), followed by anticancer, antioxidant, antimicrobial, hepatoprotection, and antidiabetic effects.

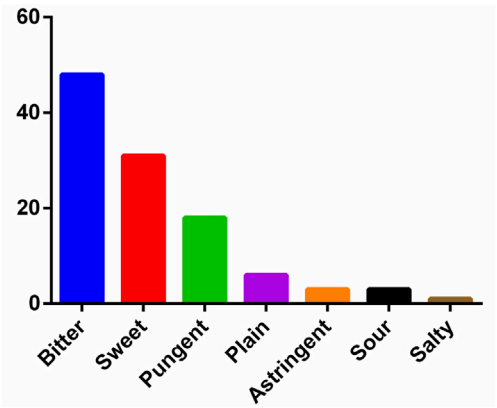
Comparison Between Taiwanese Bitter Tea and Green Herb Tea

We next compared the top 15 most commonly used plant materials between Taiwanese bitter tea and green herb tea (**Figure 4A**) and found an overlap of only four plant materials: *Glycyrrhiza uralensis* Fisch., *P. palustre*, *I. asprella*, and *Rhinacanthus nasutus* (L.) Kurz. Therefore, the plant materials used vary between these two tea types.

Plant materials with a bitter flavor were then divided into bitter cold and sweet cold; bitter cold plant materials accounted for 37.1 and 21.4% of the bitter tea and green herb tea plant materials, respectively, whereas sweet cold plants were used in the same proportion of approximately 19% (**Figure 4B**). The UV values of the top five most commonly used plant materials were higher for green herb tea than for bitter tea (**Figure 4C**). Moreover, the composition of green herb tea sold in different stores was shown to have minimal differences and high consistency, whereas the constituents of bitter tea differed among businesses in Taiwan. Therefore, the selection of plant materials for bitter tea is not consistent.

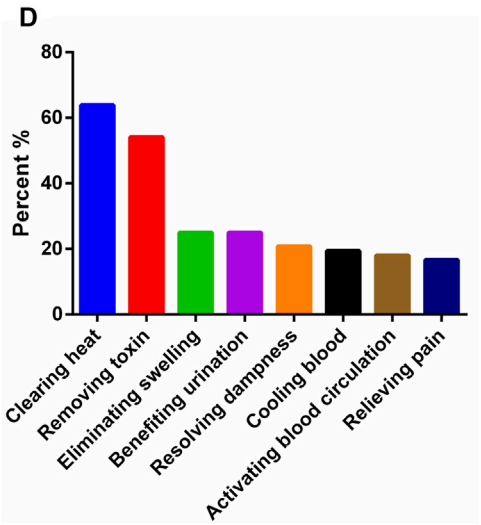
Anti-inflammation is the most published aspect in plant pharmacology research associated with the top 15 most commonly used plant materials in bitter tea and green herb tea (**Figure 4D**). For green herb tea, this is followed by antioxidant, antimicrobial, and anticancer effects, which is slightly different than that for bitter tea (**Figure 4D**).





C

	Sour	Bitter	Sweet	Pungent	Salty	Plain	Astringent
Cold	1.43	37.14	18.57	7.14	0.00	4.29	1.43
Cool	0.00	18.57	14.29	7.14	1.43	2.86	0.00
Neutral	0.00	7.14	8.57	4.29	0.00	1.43	1.43
Warm	2.86	5.71	2.86	7.14	0.00	0.00	1.43
Hot	0.00	0.00	0.00	0.00	0.00	0.00	0.00



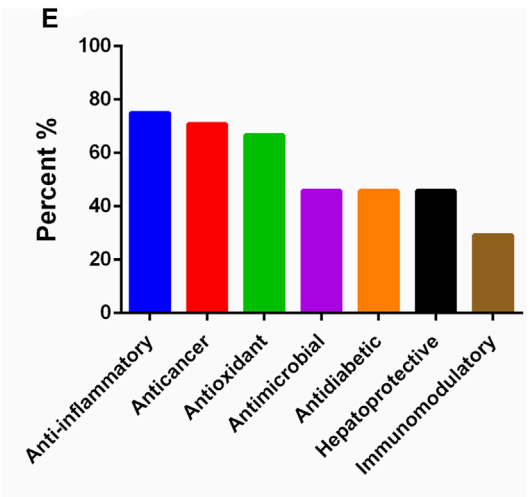


FIGURE 3 | Traditional and modern pharmacological analysis of plant materials used in Taiwanese bitter tea. **(A)** Nature; **(B)** flavor; **(C)** comprehensive analysis of nature and flavor (%); **(D)** ethnomedicinal effects; **(E)** modern pharmacological applications of plant materials with a frequency greater than nine.

Comparison of Taiwanese Bitter Tea With That of Herbal Tea From Lingnan, Chaoshan, and Fujian

In the south of China near Taiwan, there are other local herbal teas (liang-cha; cool tea) that are commonly consumed. Comparisons of Taiwanese bitter tea with herbal teas from the Lingnan area (Liu Y. et al., 2013), Chaoshan area (Li et al., 2017), and Fujian (Lin, 2014) revealed that the plant materials used in herbal teas vary across the four locations. Notably, 35 (48.0%) of the plant materials used in bitter tea, according to the survey, were limited to Taiwan (**Figure 5**).

Analysis of High-Frequency Drug Pairs and Core Network Analysis of Taiwanese Bitter Tea

To identify the drug pairs used in high frequencies, we performed core network analysis. Eight drug pairs appeared more than four times in 32 formulations and included nine medicinal plant materials

TABLE 1 | Modern pharmacological effects and traditional use of highly cited plant materials used in Taiwanese bitter tea (UV > 0.09).

Voucher specimen numberCMUBT-Xa	Scientific name	Family	Local name	Use value (%)	Related research on PubMed	Traditional use
1	*Andrographis paniculata* (Burm. f.) Nees	*Acanthaceae*	Chuān xīn lián (穿心蓮)	0.31	Antioxidant Mussard et al. (2020), anti-inflammatory Burgos et al. (2020), anti-hyperglycemic Wediasari et al. (2020), hepatoprotective Toppo et al. (2021), antimicrobial Hossain et al. (2021), anticancer Malik et al. (2021), immunomodulatory Liu et al. (2020), cardioprotective Lin et al. (2020), anti-hyperlipidemic, sexual functions and contraceptive Hossain et al. (2014), antiviral Jadhav and Karuppayil, (2021), Sa-Ngiamsuntorn et al. (2021), anti-snake venom Nayak et al. (2021), antimalarial Hassan et al. (2019), anti-Alzheimer Lu et al. (2019), and anti-obesity Chen et al. (2020)	Clears heat, resolves toxins, disperses swelling, and relieves pain
2	*Tithonia diversifolia (Hemsl.)* A. Gray (stem)	*Asteraceae (Compositae)*	Wǔ zhǎo jīn yīng (五爪金英)	0.31	Anti-inflammatory Broering et al. (2019), antimicrobial, antioxidant Ferreira Farias et al. (2019), anticancer Lu et al. (2017), analgesic Owoyele et al. (2004), anti-hyperlipidemic, antidiabetic Miura et al. (2005), antiviral Maregesi et al. (2010), gastroprotective Sanchez-Mendoza et al. (2011), immunomodulatory Ejelonu et al. (2017), antimalarial Afolayan et al. (2016), hepatoprotective Nguepi et al. (2021), anti-obesity, antiemetic, anti-diarrheal, anti-leishmania, and anti-trypanosoma Mabou Tagne et al. (2018)	-b
3	*Ajuga nipponensis* Makino	*Lamiaceae*	Bái mǎ wú gōng (白馬蜈蚣)	0.28	Antioxidant, hepatoprotective Hsieh et al. (2016), and anti-osteoclastogenic Wang, H. et al. (2021)	Disperses inflammation, cools the blood, and joins bone (Jiēgǔ)
4	*Ixeris chinensis* (Thunb.) Nakai	*Asteraceae (Compositae)*	Xiǎo jīn yīng (小金英)	0.28	Anticancer Xu et al. (2016), antiviral, hepatoprotective Shih et al. (2014), immunomodulatory Li et al. (2020), and antileukemic Chiang et al. (2004)	Clears heat, promotes urination, and calms the spirit
5	*Ilex asprella* (Hook. & Arn.) Champ. ex Benth	*Aquifoliaceae*	Wàn diǎn jīn (萬點金)	0.25	Anti-inflammatory Yang et al. (2018), anticancer Li et al. (2018), immunoregulatory Meng et al. (2018), lung-protective Dai et al. (2014), anti-hyperlipidemic Hu et al. (2012), and antiviral Zhang et al. (2018)	Clears heat, promotes urination, and calms the spirit

(Continued on following page)

TABLE 1 | (*Continued*) Modern pharmacological effects and traditional use of highly cited plant materials used in Taiwanese bitter tea (UV > 0.09).

Voucher specimen numberCMUBT-Xa	Scientific name	Family	Local name	Use value (%)	Related research on PubMed	Traditional use
6	*Bombax ceiba* L.[*Bombax malabaricum* DC.]	*Malvaceae [Bombacaceae]*	Mù mián gēn (木棉根)	0.22	Antidiabetic Bhargava and Shah (2020), antioxidant Komati et al. (2020), anti-inflammatory, antisteatotic Arafa et al. (2019), promotes osteoblast proliferation Chauhan et al. (2018), antimicrobial Shah et al. (2018), hepatoprotective Lee et al. (2017), anticancer Tundis et al. (2014), anti-obesity Gupta et al. (2013), promotes male sexual function, and antiviral Wang et al. (2013)	Dispels wind, stops itchiness, dispels wind-dampness, clears heat, resolves toxins, removes stasis, and relives pain
7	*Solanum incanum* L	*Solanaceae*	Huáng shuǐ qié(黃水茄)	0.19	Anticancer Wu, Y.H. et al. (2015), Yu et al. (2017), Al-Emam et al. (2018) and antimicrobial Lashin et al. (2021)	Disperses inflammation, resolves toxins, dispels wind, relieves pain, clears heat, and disperses inflammation
8	*Mallotus repandus* (Willd.) Muell.-Arg	*Euphorbiaceae*	Tǒng jiāo téng (桶交藤)	0.16	Analgesic Hasan et al. (2014), anti-inflammatory Hasan et al. (2014), antioxidant Lin et al. (1995), and hepatoprotective Mondal et al. (2020)	Clears heat, resolves the exterior, benefits the throat, prevents rashes, improves digestion, disperses swelling, and stops itch
9	*Physalis angulata* L	*Solanaceae*	Dēng long cǎo (燈籠草)	0.16	Immunomodulatory Vieceli et al. (2021), anti-inflammatory Sun, C.P. et al. (2017a), Sun, C.P. et al. (2017b), Wang, L. et al. (2021), antiproliferative Sun, C.P. et al. (2017b), Chairissy et al. (2019), antioxidant Adewoye et al. (2016), reno-restorative Adewoye et al. (2016), antiparasitic Meira et al. (2015), antileishmanial Da Silva et al. (2018), and anticancer Ma et al. (2017)	Clears heat, resolves toxins, disperses swelling, and removes stasis
10	*Rhinacanthus nasutus* (L.) Kurz	*Acanthaceae*	Bái hè líng zhī(白鶴靈芝)	0.16	Anticancer Siripong et al. (2006), Kupradinun et al. (2009), Siripong et al. (2009), Horii et al. (2012), Siripong et al. (2012), Boueroy et al. (2018), anti-obesity Ngoc et al. (2019), anti-glycation Shah et al. (2017), neuroprotective Chuang et al. (2017), acetylcholinesterase inhibitor Brimson and Tencomnao, (2011), Brimson et al. (2012), Chang, C.Z. et al. (2016), Boonyaketgoson et al. (2018), antioxidant Brimson et al. (2012), Shah et al. (2017), Zhao et al. (2019), antidiabetic Shah et al. (2017), Visweswara Rao et al. (2013a), neuraminidase inhibitor Kwak et al. (2018), hepatoprotective Visweswara Rao et al. (2013b), anti-inflammatory Zhao et al. (2019), antimicrobial Kernan et al. (1997), Puttarak et al. (2010), Ngoc et al. (2019), anti-allergic Tewtrakul et al. (2009), immunomodulatory Punturee et al. (2005), anti-	Moistens the lung and stops coughing, calms the liver and reduces fire, disperses swelling, resolves toxins, kills worms, and stops itchiness

(Continued on following page)

TABLE 1 | (*Continued*) Modern pharmacological effects and traditional use of highly cited plant materials used in Taiwanese bitter tea (UV > 0.09).

Voucher specimen numberCMUBT-Xa	Scientific name	Family	Local name	Use value (%)	Related research on PubMed	Traditional use
					Parkinson's Saleem et al. (2021), and antifungal Jeenkeawpieam et al. (2020)	
11	*Sigesbeckia orientalis* L	*Asteraceae (Compositae)*	Kǔ cǎo (苦草)Xī liàn cǎo (豨薟草)	0.16	Antimicrobial, anti-allergic, antithrombotic Wang, Q. et al. (2021), anti-inflammatory Nguyen et al. (2017), Chu et al. (2018), Engels et al. (2020), antihyperuricemic Nguyen et al. (2017), analgesic Nguyen et al. (2017), and anticancer Sun and Wang, (2006), Chang, C.C. et al. (2016)	Dispels wind-dampness and benefits sinew and bone
12	*Boehmeria nivea* (L.) Gaudich.[*Boehmeria nivea* (L.) Gaudich. var. *tenacissima* (Gaudich.) Miq.]	*Urticaceae*	Shān zhù má(山苧麻)	0.13	Anti-inflammatory Lim et al. (2020), laxative, antioxidant Lee et al. (2020), and antiproliferative Wang et al. (2019)	
13	*Glycyrrhiza uralensis* Fisch	*Leguminosae*	Gān cǎo (甘草)	0.13	Anticancer, antiulcer, spasmolytic, hepatoprotective, anti-inflammatory, antimicrobial Jiang et al. (2020), and anti-allergic Fouladi et al. (2019)	Supplements the spleen, boosts qi, relaxes tension, relieves pain, moistens lungs, stops coughing, drains fire, resolves toxins, and harmonizes the activity of other medicines
14	*Orthosiphon aristatus* (Blume) Miq	*Lamiaceae*	Huà shí cǎo (化石草)	0.13	Genoprotective Al-Dualimi et al. (2018), antibacterial Al-Dualimi et al. (2018), antidiabetic Damsud et al. (2014), antioxidant Hsu et al. (2010), anti-inflammatory Hsu et al. (2010), and antihypertensive Matsubara et al. (1999), Ohashi et al. (2000)	Clears heat, promotes urination, and clears kidney stones
15	*Platostoma palustre* (Blume) A.J.Paton [*Mesona chinensis* Benth.]	*Lamiaceae*	Xiān cǎo (仙草)	0.13	Antidiabetic Adisakwattana et al. (2014), Chusak et al. (2014), Liu S. et al. (2018), Yuris et al. (2019) and antioxidant Huang et al. (2019), Huang, L. et al. (2020)	Clears heat and resolves toxins
16	*Bidens pilosa* L.[*Bidens pilosa* L. var. *radiata* Sch. Bip.]	*Asteraceae (Compositae)*	Xián fēng cǎo (咸豐草)	0.09	Anticancer Arantes et al. (2021), gastroprotective Alvarez et al. (1999), Horiuchi et al. (2010), Anti-diabetic Chien et al. (2009), Ubillas et al. (2000) Anti-allergy Matsumoto et al. (2009), anti-inflammatory Hong et al. (2021), Xin et al. (2021) Anti-malaria Nadia et al. (2020) Anti-microbial Chiavari-Frederico et al. (2020) anti-coccidial, Yang et al. (2019), hepatoprotective Pegoraro et al. (2021), and anti-hypertensive Bilanda et al. (2017)	Clears heat, resolves toxins, promotes urination, and reduces jaundice
17	*Elephantopus scaber* L	*Asteraceae (Compositae)*	Ding shù wū(丁豎杇)	0.09	Anticancer Bai et al. (2020), Pandey et al. (2020), hepatoprotective Sulistyani and Nurkhasanah, (2020), anti-inflammatory Fu et al. (2020), Qi et al. (2020), antioxidant Aslam et al. (2016), antidiabetic, antimicrobial,	Clears heat, resolves toxins, promotes urination, and disperses swelling

(Continued on following page)

TABLE 1 | (*Continued*) Modern pharmacological effects and traditional use of highly cited plant materials used in Taiwanese bitter tea (UV > 0.09).

Voucher specimen numberCMUBT-Xa	Scientific name	Family	Local name	Use value (%)	Related research on PubMed	Traditional use
					and analgesic Hiradeve and Rangari, (2014)	
18	*Momordica charantia* L.[*Momordica charantia* L. var. *abbreviata* Ser.]	*Cucurbitaceae*	Shān kǔ guā(山苦瓜)	0.09	Anti-obesity Fan et al. (2021), anti-fatigue Hsiao et al. (2017), anti-inflammatory Perera et al. (2021), Tsai et al. (2016), antioxidant Akyuz et al. (2020), cell-protective Tsai et al. (2014), antimelanogenic Tsai et al. (2014), antidiabetic Kulkarni et al. (2021), and anticancer Ehigie et al. (2021)	Dispels wind, clears heat, clears the liver, and brightens the eyes
19	*Mucuna macrocarpa* Wall	*Leguminosae*	Xiě téng (血藤)	0.09	Antileukemic Lu et al. (2010)	Cures rheumatic pain (backache)
20	*Oldenlandia diffusa* (Willd.) Roxb.[*Hedyotis diffusa* Willd.]	*Rubiaceae*	Bái huā shé shé cǎo (白花蛇舌草)	0.09	Anticancer Chung et al. (2017), immunomodulatory, antioxidant Chen et al. (2016), and anti-inflammatory Zhu et al. (2018)	Clears heat, resolves toxins, engenders fluid, relieves thirst, and invigorates the blood
21	*Pteris multifida* Poir	*Pteridaceae*	Fèng wěi cǎo (鳳尾草)	0.09	Anti-cancer Kim et al. (2017), anti-neuroinflammatory Kim et al. (2016), anti-hyperlipidemic Wang et al. (2010), free radical-scavenging activity Wang et al. (2007), and anti-inflammatory Yin et al. (2018)	Clears heat, promotes urination, cools the blood, and resolves toxins
22	*Scutellaria barbata* D. Don	*Lamiaceae*	Bàn zhī lián (半枝蓮)	0.09	Anti-inflammatoryAkyuz et al. (2020), Liu H.L. et al. (2018), anticancerChen et al. (2017), Gao et al. (2014), Jin et al. (2017), Kan et al. (2017), Lin et al. (2017), Marconett et al. (2010), Ozmen et al. (2010), Sun, P. et al. (2017), Zhang et al. (2017), Zhang et al. (2021), antiproliferative Kim et al. (2008), Wu and Chen, (2009), attenuates diabetic retinopathy Mei et al. (2017), neuroprotective Wu et al. (2016), improves cognition Zhang and Li, (2016), antimicrobial Sato et al. (2000), Wu, T. et al. (2015), Yu et al. (2004), acaricidal Yang et al. (2013), and antioxidant Ye and Huang, (2012)	Clears heat, resolves toxins, invigorates the blood, eliminates stasis, disperses swelling, relieves pain, and cures cancer
23	*Sphagneticola calendulacea* (L.) Pruski [*Wedelia chinensis* (Osbeck) Merr.]	*Asteraceae (Compositae)*	Huáng huā mì cài (黃花蜜菜)	0.09	Antidiabetic Chen et al. (2021), Thao et al. (2018), anticancer Huang et al. (2016), Lin et al. (2007), Liu, M. et al. (2013), Tsai et al. (2009), Tsai et al. (2015), Tsai et al. (2017a), Tsai et al. (2017b), neuroprotective Lin et al. (2014), antibacterial Darah et al. (2013), anti-inflammatory Darah et al. (2013), and antioxidant Manjamalai and Berlin Grace, (2012)	Clears heat, resolves toxins, eliminates stasis, and disperses swelling
24	*Tithonia diversifolia* (Hemsl.) A.Gray (leaf)	*Asteraceae (Compositae)*	Wǔ zhǎo jīn yīng yè(五爪金英葉)	0.09	Hepatoprotective Nguepi et al. (2021), cardioprotective Ide et al. (2020), antioxidant, antimicrobial Ferreira Farias et al. (2019), antidiabetic, immunomodulatory, analgesic, antimalarial, anti-obesity,	Clears heat, resolves toxins, disperses swelling, and relieves pain

(Continued on following page)

TABLE 1 | (*Continued*) Modern pharmacological effects and traditional use of highly cited plant materials used in Taiwanese bitter tea (UV > 0.09).

Voucher specimen numberCMUBT-Xa	Scientific name	Family	Local name	Use value (%)	Related research on PubMed	Traditional use
					anti-hyperlipidemic, gastroprotective, antiemetic, antidiarrheal, antileishmanial, anti-trypanosomal, antivenin, and antiviral Mabou Tagne et al. (2018)	

[a]*CMUBT, china medical university bitter tea.*
[b]*No records for the stem of* T. diversifolia *in the Committee on Chinese Medicine and Pharmacy (2003) and the second edition of The Committee on Chinese Medicine and Pharmacy (2011).*

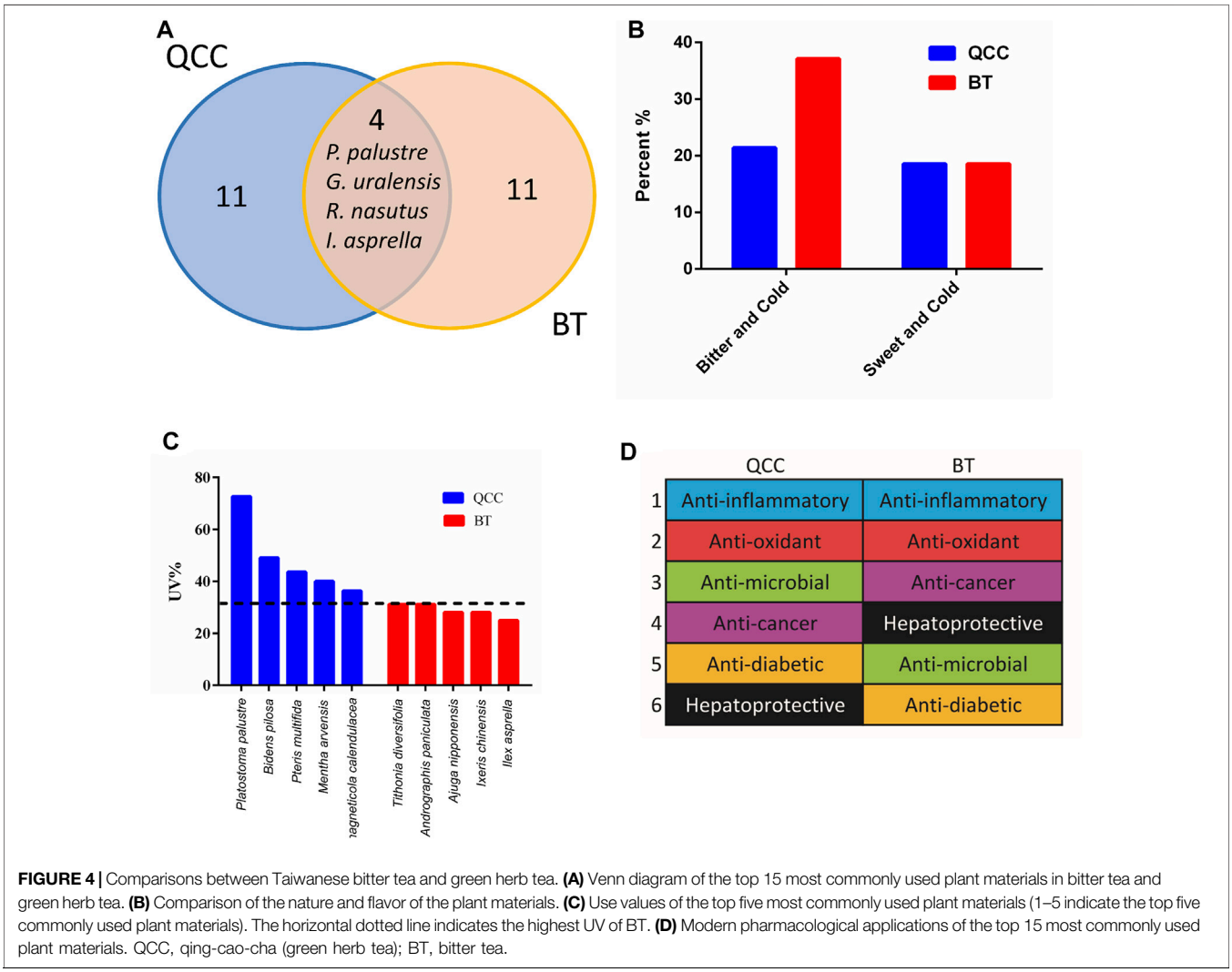


FIGURE 4 | Comparisons between Taiwanese bitter tea and green herb tea. **(A)** Venn diagram of the top 15 most commonly used plant materials in bitter tea and green herb tea. **(B)** Comparison of the nature and flavor of the plant materials. **(C)** Use values of the top five most commonly used plant materials (1–5 indicate the top five commonly used plant materials). The horizontal dotted line indicates the highest UV of BT. **(D)** Modern pharmacological applications of the top 15 most commonly used plant materials. QCC, qing-cao-cha (green herb tea); BT, bitter tea.

from *A. paniculata*, *I. asprella*, *T. diversifolia*, *A. nipponensis*, *Mallotus repandus* (Willd.) Muell.-Arg., *Bombax ceiba* L., *Solanum incanum* L., and *I. chinensis*. The most commonly used drug pairs were *A. paniculata–A. nipponensis* and *A. paniculata–I. asprella*, with a frequency of occurrence of 5 in 32 formulations.

We constructed a network diagram (**Figure 6**) of the core components of Taiwanese bitter tea using TCMISS v2.5, and found that the core medicinal materials were from *A. nipponensis* and *T. diversifolia*. The medicinal plants often matched with *A. nipponensis* were *I. chinensis*, *A. paniculata*, and *T. diversifolia*,

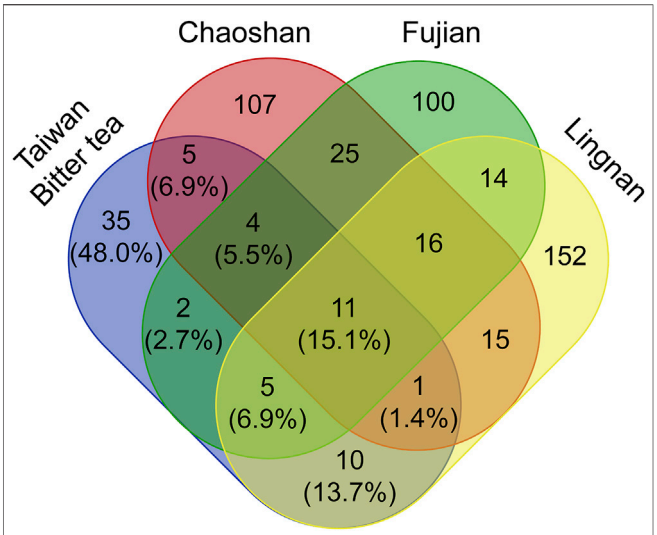


FIGURE 5 | Venn diagram showing the number of shared plant materials in herbal tea from different regions.

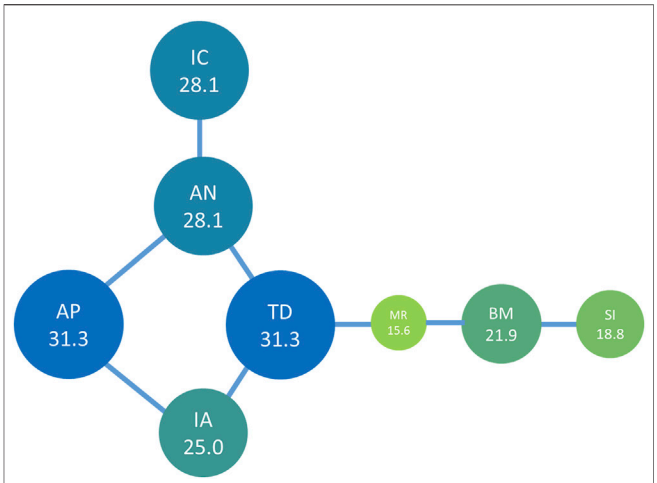


FIGURE 6 | Network analysis of the high frequency drug pairs found in Taiwanese bitter tea. The number is the UV of the plant material, and the length of the connecting line indicates co-occurrence frequency. The circle sizes represent the relative ratio of UV. AN, *Ajuga nipponensis* Makino; IC, *Ixeris chinensis* (Thunb.) Nakai; IA, *Ilex asprella* (Hook. & Arn.) Champ.; TD, *Tithonia diversifolia* (Hemsl.) A. Gray; MR, *Mallotus repandus* (Willd.) Müll. Arg.; BM, *Bombax malabarica* DC.; SI, *Solanum incanum* L.; AP, *Andrographis paniculata* (Burm. f.) Nees.

whereas those often matched with *T. diversifolia* were *A. nipponensis*, *I. asprella*, and *M. repandus* surrounded by *T. diversifolia*, *M. repandus*, and *S. incanum*.

DISCUSSION

In ethnopharmacological research, the method of field surveys have been used to explore the medicinal plants and traditional medicines prescribed for certain diseases in different regions. Topics related to medicinal botanicals that employed field investigation in the past include quality control of authentic medicinal materials (Zhao et al., 2012), green herb tea (Huang et al., 2020), herbal tea (Liu et al., 2013; Li et al., 2017), medicinal diets (Tan et al., 2017), and herbal compositions of lactation promoting herbs (Chao et al., 2020). In the present study, the field survey was adopted on Taiwanese bitter tea. We surveyed and analyzed the formulations of Taiwanese bitter tea, a special herbal tea commonly consumed in Taiwan, and revealed its current status in terms of ingredients and function.

Most of the identified plant materials in the study belonged to the family Asteraceae and Lamiaceae. The high involvement of Asteraceae and Lamiaceae in Taiwanese bitter tea is consistent with some other investigations in folk medicine outside Taiwan. They are two of the "hot families" in the medical plants (Saslis-Lagoudakis et al., 2011). Medicinal plants of the two families are dominant in Mediterranean (Gras et al., 2018), Ugandan (Tugume and Nyakoojo, 2019), Turkish (Güneş et al., 2017), Lebanese (Baydoun et al., 2015), Peruvian (Rehecho et al., 2011), Jordanian (Alzweiri et al., 2011), and Algerian (Rachid et al., 2012) folk medicines. Asteraceae exhibits high environmental adaptability and hosts a large number of naturalized and invasive plants globally. In addition, the climate in Taiwan is conducive for Asteraceae growth, and plants in this family are the third most abundant vascular plants in Taiwan, making them very easy to obtain in the wild (Rolnik and Olas, 2021). Asteraceae plants also possess numerous and diverse flavors. The Lamiaceae family is the second largest source of plant raw materials used in Taiwanese bitter tea. Lamiaceae family plants are characterized by high amounts of volatile oils, and thus the plants are extensively used as culinary herbs (Napoli et al., 2020). Previous studies have reported that the volatile oils of these plants have antioxidant, anti-inflammatory, and antibacterial properties (Nieto, 2017), which may explain why Lamiaceae is widely used in herbal tea. This study also found several naturalized plants that were introduced from America and Africa used in bitter tea. American native plants introduced into Taiwan and used in bitter tea included *Physalis angulata* L., *Bidens pilosa* L., and *Tithonia diversifolia*, and African native plants included *Solanum incanum* L. and *Momordica charantia* L. (Wu et al., 2004).

The diversity analysis for the northern, central, and southern Taiwan revealed that the more the north, the more diversity of medicinal materials were. Northern Taiwan is the political and economic center of Taiwan, with very little land to grow and correct plants. The medicinal materials used in northern Taiwan are usually imported from other areas. On the other hand, the central and southern areas of Taiwan grow more plants, including medicinal plants, and have more wild land, where wild medicinal plants could be collected. Therefore, medicinal materials used by stores in the central and southern Taiwan were more readily available and more restricted locally than those in northern Taiwan. This is the reason we speculated for the more number of medicinal materials and higher diversity in Northern Taiwan than in other areas.

All participants in the survey reported that the main function of bitter tea is to protect the liver. A previous study on green herb tea in Taiwan also reported similar results (Chen and Lin, 2012). We suspected that this result is associated with the past high prevalence of hepatitis B in Taiwan, with 15–20% of the population being chronic hepatitis B carriers. Although the Taiwanese government promoted large-scale vaccination initiatives for newborns that reduced the incidence of hepatitis B (Wait and Chen, 2012), hepatitis C—a major cause of liver cirrhosis and liver cancer—is still prevalent in Taiwan (Yu et al., 2020). According to the cause of death statistics, chronic liver disease and liver cirrhosis remain among the top 10 causes of death, with liver cancer part of the top 10 cancers in Taiwan (Ministry of Health and Welfare, 2019). Therefore, people seek local plant materials that can "protect the liver" and provide "liver protection." Therefore, Taiwanese bitter tea may have developed as a medicinal drink under such circumstances.

Taiwanese bitter tea was mainly composed of cold and cool plant materials. According to theory of traditional Chinese medicine, cold and cool plants have a "heat clearing" effect, which can treat "heat syndrome". Heat syndrome refers to symptoms such as redness, fever, inflammation, yellow and red urine, and dry stool (Su et al., 2013). In the survey, many storekeepers mentioned that the components rendering the bitter flavor can enter the liver and that plant raw materials of bitter plants have liver "fire reduction" effects. Therefore, Taiwanese bitter tea is used to reduce "liver fire" and improve liver health. Other commonly used traditional Chinese medicines with bitter flavor for treating liver diseases include *Gentiana scabra* Bunge, *Artemisia capillaris*, and *S. baicalensis* (Tsai et al., 2020). In addition, bitterness-imparting compounds have been extracted from *Artemisia* absinthium L., a medicinal plant with hepatoprotective effects and a strong bitter taste that has been traditionally used in Europe.

During our analysis of the use and properties of Taiwanese bitter tea plant materials in the literature, we found that hepatoprotection was the fifth most published aspect for the 24 most commonly used plant materials and the fourth for the top 15 plant materials by frequency. Plants with high UV values, such as *A. paniculate* (Hossain et al., 2014), *A. nipponensis* (Hsieh et al., 2016), and *T. diversifolia* (Mabou Tagne et al., 2018) were reported to have hepatoprotective effects in pharmacological studies, whereas *I. chinensis* was reported to have antiviral effects against the hepatitis B virus. Meanwhile, hepatoprotection was ranked sixth for Taiwanese green herb tea. The most frequently reported pharmacological action of plant materials in bitter tea was anti-inflammation, followed by anticancer, antioxidant, antimicrobial, and anti-glycosuria effects. Long-term chronic inflammation can cause excessive oxidative stress, which is a major cause of cancer development (Bishayee, 2014); therefore, anti-inflammatories and antioxidants are considered to have potential roles in preventing cancer. For Taiwanese green herb tea, the most reported pharmacological action for the plant materials was anti-inflammation, followed by antioxidant, antimicrobial, and anticancer (Huang et al., 2020). According to these data, Taiwanese green herb tea has similar benefits to those of bitter tea; however, bitter tea may have stronger hepatoprotective effects than does green herb tea.

A comparison of the composition of Taiwanese bitter tea with three popular herbal drinks from Lingnan (Liu Y. et al., 2013), Chaoshan (Li et al., 2017), and Fujian (Lin, 2014) revealed that 11 (15.1%) plants were used in common. Moreover, there are 35 (48%) plants used only in Taiwanese bitter tea but not used in the cool teas of the above-mentioned three areas of southern mainland China. Nevertheless, the functions of herbal teas advertised in these three regions in southern China are similar to those of herbal teas in Taiwan, all of which are used to "clear internal heat". Similarly, several medicinal plants used in herbal teas in the Lingnan, Chaoshan, and Fujian were been reported to have antioxidant, anti-inflammatory (Wu et al., 2020), and hepatoprotective (Bao et al., 2008) properties in pharmacological studies. However, given that almost half of the plant materials used in Taiwanese bitter tea are not used in herbal teas from Lingnan, Chaoshan, and Fujian, Taiwanese bitter tea can be regarded as a unique herbal tea.

The store owners selling Taiwanese bitter tea had a wide age distribution. Seventy-six percent of the source of knowledge on plant materials used in Taiwanese bitter tea was information passed down over generations. Generally, the herbal tea industry is traditional and conservative, and the recipes are often restricted within families and are not shared with external parties. Such conservative practices in the tea industry are gradually being abandoned. According to a study on herbal tea culture in Taiwan, the sale of herbal tea in Taiwan has decreased, with the number of traditional stores gradually declining (Chen and Lin, 2012). The findings of the present study can facilitate the preservation of these ancient family recipes as well as the cultural heritage of Taiwanese bitter tea through the Taiwanese government, the field of ethnopharmacology, and the food and pharmaceutical industries.

CONCLUSION

Bitter tea is a major traditional drink in Taiwan. The findings of the present study indicate that Taiwanese bitter tea is a very diverse mixture without a clear core species used in all these mixture. Moreover, the plant materials used in Taiwanese bitter tea are unique and that the constituent plants have numerous functions. More follow-up studies are required to ascertain the pharmacological effects of Taiwanese bitter tea; facilitate safer and more effective use of bitter tea in Taiwan; and preserve bitter tea as a traditional culture and resource.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CRREC-107–019. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

S-SH, T-YC, J-SD, L-HP, Y-CC, S-YS, and JC designed the study. S-SH, T-YC, J-SD, S-YS, and JC conducted the field work. S-SH, T-YC, L-HP, and JC performed the data analysis. S-SH, T-YC, Y-CC, and JC wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Ministry of Science and Technology (grant numbers MOST 107-2320-B-039-030-MY3, MOST 107-2320-B-039-063, MOST 108-2813-C-039-061-B and MOST 109-2320-B-039-026); China Medical University (Grant Number CMU106-N-24, CMU107-N-33, CMU109-MF-95, CMU108-MF-116, CMU-108-SR-101, and CMU108-N-22); Chang Gung Memorial Hospital (Chang Gung Medical Research Program grant number CMRPF1D0123); and the Higher Education Sprout Project and Teaching Practice Research Program of China Medical University (Grant Number 1077170A) and Ministry of Education, Taiwan.

ACKNOWLEDGMENTS

The authors are grateful to the herbarium of China Medical University for providing us with a space for storing the medicinal materials collected during this study. The authors would like to thank Editage for editorial assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.816029/full#supplementary-material

REFERENCES

Adewoye, E. O., Oguntola, M. A., and Ige, A. O. (2016). Anti-oxidative and reno-restorative Effects of Physalis Angulata (Whole Plant Extract) in Alloxan-Induced Diabetic Male Wistar Rats. *Afr. J. Med. Med. Sci.* 45 (1), 99–108.

Adisakwattana, S., Thilavech, T., and Chusak, C. (2014). Mesona Chinensis Benth Extract Prevents AGE Formation and Protein Oxidation against Fructose-Induced Protein Glycation *In Vitro*. *BMC Complement. Altern. Med.* 14, 130. doi:10.1186/1472-6882-14-130

Afolayan, F. I. D., Adegbolagun, O. M., Irungu, B., Kangethe, L., Orwa, J., and Anumudu, C. I. (2016). Antimalarial Actions of Lawsonia Inermis, Tithonia Diversifolia and Chromolaena Odorata in Combination. *J. Ethnopharmacol.* 191, 188–194. doi:10.1016/j.jep.2016.06.045

AkyÜz, E., TÜrkoĞlu, S., SÖzgen BaŞkan, K., TÜtem, E., and Apak, M. R. (2020). Comparison of Antioxidant Capacities and Antioxidant Components of Commercial Bitter Melon (*Momordica Charantia* L.) Products. *Turk. J. Chem.* 44 (6), 1663–1673. doi:10.3906/kim-2007-67

Al-Dualimi, D. W., Shah Abdul Majid, A., Al-Shimary, S. F. F., Al-Saadi, A. A., Al Zarzour, R., Asif, M., et al. (2018). 50% Ethanol Extract of *Orthosiphon Stamineus* Modulates Genotoxicity and Clastogenicity Induced by Mitomycin C. *Drug Chem. Toxicol.* 41 (1), 82–88. doi:10.1080/01480545.2017.1317785

Al-Emam, A., Al-Shraim, M., Eid, R., Alfaifi, M., Al-Shehri, M., Moustafa, M. F., et al. (2018). Ultrastructural Changes Induced by *Solanum Incanum* Aqueous Extract on HCT 116 colon Cancer Cells. *Ultrastruct. Pathol.* 42 (3), 255–261. doi:10.1080/01913123.2018.1447623

Alvarez, A., Pomar, F., Sevilla, M. A., and Montero, M. J. (1999). Gastric Antisecretory and Antiulcer Activities of an Ethanolic Extract of Bidens Pilosa L. Var. Radiata Schult. Bip. *J. Ethnopharmacol* 67 (3), 333–340. doi:10.1016/s0378-8741(99)00092-6

Alzweiri, M., Sarhan, A. A., Mansi, K., Hudaib, M., and Aburjai, T. (2011). Ethnopharmacological Survey of Medicinal Herbs in Jordan, the Northern Badia Region. *J. Ethnopharmacol.* 137 (1), 27–35. doi:10.1016/j.jep.2011.02.007

Arafa, A. F., Foda, D. S., Mahmoud, A. H., Metwally, N. S., and Farrag, A. R. H. (2019). *Bombax ceiba* Flowers Extract Ameliorates Hepatosteatosis Induced by Ethanol and Relatively Moderate Fat Diet in Rats. *Toxicol. Rep.* 6, 401–408. doi:10.1016/j.toxrep.2019.04.008

Arantes, D. A. C., Silva, A. C. G. D., Lima, E. M., Alonso, E. C. P., Marreto, R. N., Mendonça, E. F., et al. (2021). Biological Effects of Formulation Containing Curcuminoids and *Bidens Pilosa* L. In Oral Carcinoma Cell Line. *Braz. Oral Res.* 35, e063. doi:10.1590/1807-3107bor-2021.vol35.0063

Aslam, M. S., Ahmad, M. S., Mamat, A. S., Ahmad, M. Z., and Salam, F. (2016). Antioxidant and Wound Healing Activity of Polyherbal Fractions of *Clinacanthus Nutans* and *Elephantopus Scaber*. *Evid. Based Complement. Alternat. Med.* 2016, 4685246. doi:10.1155/2016/4685246

Bai, M., Chen, J. J., Xu, W., Dong, S. H., Liu, Q. B., Lin, B., et al. (2020). Elephantopinolide A-P, Germacrane-type Sesquiterpene Lactones from Elephantopus Scaber Induce Apoptosis, Autophagy and G2/M Phase Arrest in Hepatocellular Carcinoma Cells. *Eur. J. Med. Chem.* 198, 112362. doi:10.1016/j.ejmech.2020.112362

Bao, L., Yao, X. S., He, R. R., and Kurihara, H. (2008). [Protective Effects of Guangdong Liangcha Grandes on Restraint Stress-Induced Liver Damage in Mice]. *Zhongguo Zhong Yao Za Zhi* 33 (6), 664–669.

Bensky, D., Clavey, S., and Stoger, E. (2004). *Chinese Herbal Medicine Materia Medica*. Seattle, United States: Eastland Press.

Bhargava, S., and Shah, M. B. (2020). Evaluation of Efficacy of *Bombax ceiba* Extract and its Major Constituent, Mangiferin in Streptozotocin (STZ)-induced Diabetic Rats. *J. Complement. Integr. Med.* 18 (2), 311–318. doi:10.1515/jcim-2020-0027

Bilanda, D. C., Dzeufiet, P. D. D., Kouakep, L., Aboubakar, B. F. O., Tedong, L., Kamtchouing, P., et al. (2017). *Bidens Pilosa* Ethylene Acetate Extract Can Protect against L-NAME-Induced Hypertension on Rats. *BMC Complement. Altern. Med.* 17 (1), 479. doi:10.1186/s12906-017-1972-0

Bishayee, A. (2014). The Role of Inflammation and Liver Cancer. *Adv. Exp. Med. Biol.* 816, 401–435. doi:10.1007/978-3-0348-0837-8_16

Boonyaketgoson, S., Rukachaisirikul, V., Phongpaichit, S., and Trisuwan, K. (2018). Naphthoquinones from the Leaves of *Rhinacanthus Nasutus* Having Acetylcholinesterase Inhibitory and Cytotoxic Activities. *Fitoterapia* 124, 206–210. doi:10.1016/j.fitote.2017.11.011

Boueroy, P., Saensa-Ard, S., Siripong, P., Kanthawong, S., and Hahnvajanawong, C. (2018). Rhinacanthin-C Extracted from R*hinacanthus Nasutus* (L.) Inhibits Cholangiocarcinoma Cell Migration and Invasion by Decreasing MMP-2, uPA, FAK and MAPK Pathways. *Asian Pac. J. Cancer Prev.* 19 (12), 3605–3613. doi:10.31557/APJCP.2018.19.12.3605

Brimson, J. M., Brimson, S. J., Brimson, C. A., Rakkhitawatthana, V., and Tencomnao, T. (2012). Rhinacanthus Nasutus Extracts Prevent Glutamate and Amyloid-β Neurotoxicity in HT-22 Mouse Hippocampal Cells: Possible Active Compounds Include Lupeol, Stigmasterol and β-sitosterol. *Int. J. Mol. Sci.* 13 (4), 5074–5097. doi:10.3390/ijms13045074

Brimson, J. M., and Tencomnao, T. (2011). *Rhinacanthus Nasutus* Protects Cultured Neuronal Cells against Hypoxia Induced Cell Death. *Molecules* 16 (8), 6322–6338. doi:10.3390/molecules16086322

Broering, M. F., Nunes, R., De Faveri, R., De Faveri, A., Melato, J., Correa, T. P., et al. (2019). Effects of *Tithonia Diversifolia* (Asteraceae) Extract on Innate Inflammatory Responses. *J. Ethnopharmacol.* 242, 112041. doi:10.1016/j.jep.2019.112041

Burgos, R. A., Alarcón, P., Quiroga, J., Manosalva, C., and Hancke, J. (2020). Andrographolide, an Anti-inflammatory Multitarget Drug: All Roads lead to Cellular Metabolism. *Molecules* 26 (1), 5. doi:10.3390/molecules26010005

Chairissy, M. D., Wulandari, L. R., and Sujuti, H. (2019). Pro-apoptotic and Anti-proliferative Effects of *Physalis Angulata* Leaf Extract on Retinoblastoma Cells. *Int. J. Ophthalmol.* 12 (9), 1402–1407. doi:10.18240/ijo.2019.09.05

Chang, C. Z., Wu, S. C., Kwan, A. L., and Lin, C. L. (2016). Rhinacanthin-C, a Fat-Soluble Extract from *Rhinacanthus Nasutus*, Modulates High-Mobility Group Box 1-Related Neuro-Inflammation and Subarachnoid Hemorrhage-Induced Brain Apoptosis in a Rat Model. *World Neurosurg.* 86, 349–360. doi:10.1016/j.wneu.2015.08.071

Chang, C.-C., Ling, X.-H., Hsu, H.-F., Wu, J.-M., Wang, C.-P., Yang, J.-F., et al. (2016). Siegesbeckia Orientalis Extract Inhibits TGFβ1-Induced Migration and Invasion of Endometrial Cancer Cells. *Molecules* 21 (8), 1021. doi:10.3390/molecules21081021

Chang, Y. S. (2004). *Investigation of the Current Status of Green Herb Stores in Taiwan*. Taipei: Ministry of Health and Welfare, 337–373.

Chang, Y. S. (2005). Investigation of the Current Status of Green Herb Stores in Taiwan. *Yearb. Chin. Med. Pharm.*. Taipei 23 (5), 337–448.

Chao, J., Ko, C. Y., Lin, C. Y., Tomoji, M., Huang, C. H., Chiang, H. C., et al. (2020). Ethnobotanical Survey of Natural Galactagogues Prescribed in Traditional Chinese Medicine Pharmacies in Taiwan. *Front. Pharmacol.* 11, 625869. doi:10.3389/fphar.2020.625869

Chauhan, S., Sharma, A., Upadhyay, N. K., Singh, G., Lal, U. R., and Goyal, R. (2018). *In-vitro* Osteoblast Proliferation and *In-Vivo* Anti-osteoporotic Activity of *Bombax ceiba* with Quantification of Lupeol, Gallic Acid and β-sitosterol by HPTLC and HPLC. *BMC Complement. Altern. Med.* 18 (1), 233. doi:10.1186/s12906-018-2299-1

Chen, C. C., Kao, C. P., Chiu, M. M., and Wang, S. H. (2017). The Anti-cancer Effects and Mechanisms of *Scutellaria Barbata* D. Don on CL1-5 Lung Cancer Cells. *Oncotarget* 8 (65), 109340–109357. doi:10.18632/oncotarget.22677

Chen, C. C., Lii, C. K., Lin, Y. H., Shie, P. H., Yang, Y. C., Huang, C. S., et al. (2020). Andrographis Paniculata Improves Insulin Resistance in High-Fat Diet-Induced Obese Mice and TNFα-Treated 3T3-L1 Adipocytes. *Am. J. Chin. Med.* 48 (5), 1073–1090. doi:10.1142/S0192415X20500524

Chen, K. H., and Lin, H. L. (2012). Herb tea Culture in Taiwan. *J. Isl. Tour. Res.* 5 (3), 35–73. doi:10.29859/JITR.201209.0003

Chen, L. J., Hsu, T. C., Yeh, P. J., Yow, J. L., Chang, C. L., Lin, C. H., et al. (2021). Differential Effects of *Wedelia Chinensis* on Human Glioblastoma Multiforme Cells. *Integr. Cancer Ther.* 20, 15347354211000119. doi:10.1177/15347354211000119

Chen, R., He, J., Tong, X., Tang, L., and Liu, M. (2016). The *Hedyotis Diffusa* Willd. (Rubiaceae): a Review on Phytochemistry, Pharmacology, Quality Control and Pharmacokinetics. *Molecules* 21 (6), 710. doi:10.3390/molecules21060710

Chiang, L. C., Cheng, H. Y., Chen, C. C., and Lin, C. C. (2004). *In Vitro* anti-leukemic and Antiviral Activities of Traditionally Used Medicinal Plants in Taiwan. *Am. J. Chin. Med.* 32 (5), 695–704. doi:10.1142/S0192415X04002284

Chiavari-Frederico, M. O., Barbosa, L. N., Carvalho Dos Santos, I., Ratti da Silva, G., Fernandes de Castro, A., de Campos Bortolucci, W., et al. (2020). Antimicrobial Activity of Asteraceae Species against Bacterial Pathogens Isolated from Postmenopausal Women. *PLoS One* 15 (1), e0227023. doi:10.1371/journal.pone.0227023

Chien, S. C., Young, P. H., Hsu, Y. J., Chen, C. H., Tien, Y. J., Shiu, S. Y., et al. (2009). Anti-diabetic Properties of Three Common Bidens Pilosa Variants in Taiwan. *Phytochemistry* 70 (10), 1246–1254. doi:10.1016/j.phytochem.2009.07.011

Chu, J. M. T., Xiong, W., Linghu, K. G., Liu, Y., Zhang, Y., Zhao, G. D., et al. (2018). Siegesbeckia Orientalis L. Extract Attenuates Postoperative Cognitive Dysfunction, Systemic Inflammation, and Neuroinflammation. *Exp. Neurobiol.* 27 (6), 564–573. doi:10.5607/en.2018.27.6.564

Chuang, K. A., Li, M. H., Lin, N. H., Chang, C. H., Lu, I. H., Pan, I. H., et al. (2017). Rhinacanthin C Alleviates Amyloid-Beta Fibrils' Toxicity on Neurons and Attenuates Neuroinflammation Triggered by Lps, Amyloid-Beta, and Interferon-Gamma in Glial Cells. *Oxid. Med. Cel Longev.* 2017, 5414297. doi:10.1155/2017/5414297

Chung, T. W., Choi, H., Lee, J. M., Ha, S. H., Kwak, C. H., Abekura, F., et al. (20172017). Corrigendum to "Oldenlandia Diffusa Suppresses Metastatic Potential through Inhibiting Matrix Metalloproteinase-9 and Intercellular Adhesion Molecule-1 Expression via P38 and ERK1/2 MAPK Pathways and Induces Apoptosis in Human Breast Cancer MCF-7 Cells" [J. Ethnopharmacol. 195 (2017) 309-317]. *J. Ethnopharmacol.* 204, 309189–309317. doi:10.1016/j.jep.2017.04.016

Chusak, C., Thilavech, T., and Adisakwattana, S. (2014). Consumption of *Mesona Chinensis* Attenuates Postprandial Glucose and Improves Antioxidant Status Induced by a High Carbohydrate Meal in Overweight Subjects. *Am. J. Chin. Med.* 42 (2), 315–336. doi:10.1142/S0192415X14500219

Da Silva, B. J. M., Pereira, S. W. G., Rodrigues, A. P. D., Do Nascimento, J. L. M., and Silva, E. O. (2018). *In Vitro* antileishmanial Effects of *Physalis Angulata* Root Extract on *Leishmania Infantum*. *J. Integr. Med.* 16 (6), 404–410. doi:10.1016/j.joim.2018.08.004

Dai, W. P., Li, G., Li, X., Hu, Q. P., Liu, J. X., Zhang, F. X., et al. (2014). The Roots of *Ilex Asprella* Extract Lessens Acute Respiratory Distress Syndrome in Mice Induced by Influenza Virus. *J. Ethnopharmacol.* 155 (3), 1575–1582. doi:10.1016/j.jep.2014.07.051

Damsud, T., Grace, M. H., Adisakwattana, S., and Phuwapraisirisan, P. (2014). Orthosiphol A from the Aerial Parts of *Orthosiphon Aristatus* Is Putatively Responsible for Hypoglycemic Effect via Alpha-Glucosidase Inhibition. *Nat. Prod. Commun.* 9 (5), 639–641. doi:10.1177/1934578x1400900512

Darah, I., Lim, S. H., and Nithianantham, K. (2013). Effects of Methanol Extract of *Wedelia Chinensis* Osbeck (Asteraceae) Leaves against Pathogenic Bacteria with Emphasise on *Bacillus Cereus*. *Indian J. Pharm. Sci.* 75 (5), 533–539.

Ehigie, A. F., Wei, P., Wei, T., Yan, X., Olorunsogo, O. O., Ojeniyi, F. D., et al. (2021). Momordica Charantia L. Induces Non-apoptotic Cell Death in Human MDA-MB-436 Breast and A549 Lung Cancer Cells by Disrupting Energy Metabolism and Exacerbating Reactive Oxygen Species' Generation. *J. Ethnopharmacol.* 277, 114036. doi:10.1016/j.jep.2021.114036

Ejelonu, O. C., Elekofehinti, O. O., and Adanlawo, I. G. (2017). Tithonia Diversifolia Saponin-Blood Lipid Interaction and its Influence on Immune System of normal Wistar Rats. *Biomed. Pharmacother.* 87, 589–595. doi:10.1016/j.biopha.2017.01.017

Engels, N. S., Gierlikowska, B., Waltenberger, B., Chang, F. R., Kiss, A. K., and Stuppner, H. (2020). A New Diterpene and Anti-inflammatory Sesquiterpene Lactones from *Sigesbeckia Orientalis*. *Planta Med.* 86 (15), 1108–1117. doi:10.1055/a-1232-6869

Fan, M., Lee, J. I., Ryu, Y. B., Choi, Y. J., Tang, Y., Oh, M., et al. (2021). Comparative Analysis of Metabolite Profiling of *Momordica Charantia* Leaf and the Anti-obesity Effect through Regulating Lipid Metabolism. *Int. J. Environ. Res. Public Health* 18 (11), 5584. doi:10.3390/ijerph18115584

Ferreira Farias, A. L., Lobato Rodrigues, A. B., Lopes Martins, R., de Menezes Rabelo, É., Ferreira Farias, C. W., and Moreira da Silva de Almeida, S. S. (2019). Chemical Characterization, Antioxidant, Cytotoxic and Microbiological Activities of the Essential Oil of Leaf of *Tithonia Diversifolia* (Hemsl) A. Gray (Asteraceae). *Pharmaceuticals (Basel)* 12 (1), 34. doi:10.3390/ph12010034

Fouladi, S., Masjedi, M., Ganjalikhani Hakemi, M., and Eskandari, N. (2019). The Review of *In Vitro* and *In Vivo* Studies over the Glycyrrhizic Acid as Natural Remedy Option for Treatment of Allergic Asthma. *Iran J. Allergy Asthma Immunol.* 18 (1), 1–11. doi:10.18502/ijaai.v18i1.626

Fu, L., Pei, D., Yu, M., Shang, H., Si, J. G., Zhang, H. W., et al. (2020). New Phenolic Acids from the Whole Herb of *Elephantopus Scaber* Linn. And Their Anti-inflammatory Activity. *Nat. Prod. Res.* 35, 1–8. doi:10.1080/14786419.2020.1723086

Fu, Y., Yang, J. C., Cunningham, A. B., Towns, A. M., Zhang, Y., Yang, H. Y., et al. (2018). A Billion Cups: the Diversity, Traditional Uses, Safety Issues and

Potential of Chinese Herbal Teas. *J. Ethnopharmacol.* 222, 217–228. doi:10.1016/j.jep.2018.04.026

Gao, J., Lu, W. F., Dai, Z. J., Lin, S., Zhao, Y., Li, S., et al. (2014). Induction of Apoptosis by Total Flavonoids from *Scutellaria Barbata* D. Don in Human Hepatocarcinoma MHCC97-H Cells via the Mitochondrial Pathway. *Tumour Biol.* 35 (3), 2549–2559. doi:10.1007/s13277-013-1336-4

Gertsch, J. (2011). Botanical Drugs, Synergy, and Network Pharmacology: Forth and Back to Intelligent Mixtures. *Planta Med.* 77 (11), 1086–1098. doi:10.1055/s-0030-1270904

Gras, A., Parada, M., Rigat, M., Vallès, J., and Garnatje, T. (2018). Folk Medicinal Plant Mixtures: Establishing a Protocol for Further Studies. *J. Ethnopharmacol.* 214, 244–273. doi:10.1016/j.jep.2017.12.014

Güneş, S., Savran, A., Paksoy, M. Y., Koşar, M., and Çakılcıoğlu, U. (2017). Ethnopharmacological Survey of Medicinal Plants in Karaisalı and its Surrounding (Adana-Turkey). *J. Herb. Med.* 8, 68–75. doi:10.1016/j.hermed.2017.04.002

Gupta, P., Goyal, R., Chauhan, Y., and Sharma, P. L. (2013). Possible Modulation of FAS and PTP-1B Signaling in Ameliorative Potential of *Bombax ceiba* against High Fat Diet Induced Obesity. *BMC Complement. Altern. Med.* 13, 281. doi:10.1186/1472-6882-13-281

Hasan, M. M., Uddin, N., Hasan, M. R., Islam, A. F., Hossain, M. M., Rahman, A. B., et al. (2014). Analgesic and Anti-inflammatory Activities of Leaf Extract of Mallotus Repandus (Willd.) Muell. Arg. *Biomed. Res. Int.* 2014, 539807. doi:10.1155/2014/539807

Hassan, W. R. M., Basir, R., Ali, A. H., Embi, N., and Sidek, H. M. (2019). Anti-malarial and Cytokine-Modulating Effects of Andrographolide in a Murine Model of Malarial Infection. *Trop. Biomed.* 36 (3), 776–791.

Hiradeve, S. M., and Rangari, V. D. (2014). A Review on Pharmacology and Toxicology of *Elephantopus Scaber* Linn. *Nat. Prod. Res.* 28 (11), 819–830. doi:10.1080/14786419.2014.883394

Hong, M., Fan, X., Liang, S., Xiang, W., Chen, L., Yang, Y., et al. (2021). Total Flavonoids of *Bidens Pilosa* Ameliorates Bone Destruction in Collagen-Induced Arthritis. *Planta Med.* 87, 550-559. doi:10.1055/a-1352-5124

Horii, H., Suzuki, R., Sakagami, H., Umemura, N., Ueda, J. Y., and Shirataki, Y. (2012). Induction of Non-apoptotic Cell Death in Human Oral Squamous Cell Carcinoma Cell Lines by *Rhinacanthus Nasutus* Extract. *In Vivo* 26 (2), 305–309.

Horiuchi, M., Wachi, H., and Seyama, Y. (2010). Effects of *Bidens Pilosa* L. Var. Radiata Scherff on Experimental Gastric Lesion. *J. Nat. Med.* 64 (4), 430–435. doi:10.1007/s11418-010-0426-5

Hossain, M. S., Urbi, Z., Sule, A., and Hafizur Rahman, K. M. (2014). *Andrographis Paniculata* (Burm. f.) Wall. Ex Nees: a Review of Ethnobotany, Phytochemistry, and Pharmacology. *ScientificWorldJournal* 2014, 274905. doi:10.1155/2014/274905

Hossain, S., Urbi, Z., Karuniawati, H., Mohiuddin, R. B., Moh Qrimida, A., Allzrag, A. M. M., et al. (2021). *Andrographis Paniculata* (Burm. f.) Wall. Ex Nees: an Updated Review of Phytochemistry, Antimicrobial Pharmacology, and Clinical Safety and Efficacy. *Life (Basel)* 11 (4), 348. doi:10.3390/life11040348

Hsiao, C. Y., Chen, Y. M., Hsu, Y. J., Huang, C. C., Sung, H. C., and Chen, S. S. (2017). Supplementation with Hualian No. 4 Wild Bitter Gourd (*Momordica Charantia* Linn. Var. Abbreviata ser.) Extract Increases Anti-fatigue Activities and Enhances Exercise Performance in Mice. *J. Vet. Med. Sci.* 79 (6), 1110–1119. doi:10.1292/jvms.17-0079

Hsieh, C. W., Ko, W. C., Ho, W. J., Chang, C. K., Chen, G. J., and Tsai, J. C. (2016). Antioxidant and Hepatoprotective Effects of *Ajuga Nipponensis* Extract by Ultrasonic-Assisted Extraction. *Asian Pac. J. Trop. Med.* 9 (5), 420–425. doi:10.1016/j.apjtm.2016.03.029

Hsieh, C. Y., Lin, Y. T., Huang, S. S., Hsu, P. C., Lu, F. L., Tsai, C. F., et al. (2017). Analysis of the Confusion and Misuse of Chinese Herbs in the Registration of Chinese Medicinal Preparations. *Ann. Rept. Food Drug Res.* 8, 103–116.

Hsu, C. L., Hong, B. H., Yu, Y. S., and Yen, G. C. (2010). Antioxidant and Anti-inflammatory Effects of *Orthosiphon Aristatus* and its Bioactive Compounds. *J. Agric. Food Chem.* 58 (4), 2150–2156. doi:10.1021/jf903557c

Hu, S. Y. (2005). *Food Plants of China*. Hong Kong: The Chinese University Press, 3–46p.

Hu, X. Y., Shu, X. C., Guo, Y., and Ma, Y. (2012). Effect of an *Ilex Asprella* Root Decoction on the Related Genes of Lipid Metabolism from Chronic Stress and Hyperlipidemic Fatty Liver in Rats. *Chin. Med. J. (Engl)* 125 (19), 3539–3542.

Huang, L., Huang, M., Shen, M., Wen, P., Wu, T., Hong, Y., et al. (2019). Sulfated Modification Enhanced the Antioxidant Activity of Mesona Chinensis Benth Polysaccharide and its Protective Effect on Cellular Oxidative Stress. *Int. J. Biol. Macromol.* 136, 1000–1006. doi:10.1016/j.ijbiomac.2019.06.199

Huang, L., Shen, M., Wu, T., Yu, Y., Yu, Q., Chen, Y., et al. (2020). *Mesona Chinensis* Benth Polysaccharides Protect against Oxidative Stress and Immunosuppression in Cyclophosphamide-Treated Mice via MAPKs Signal Transduction Pathways. *Int. J. Biol. Macromol.* 152, 766–774. doi:10.1016/j.ijbiomac.2020.02.318

Huang, S. S., Chen, T. Y., Deng, J. S., Pao, L. H., Cheng, Y. C., and Chao, J. (2020). An Ethnobotanical Study on Qīng-Cǎo-Chá Tea in Taiwan. *Front. Pharmacol.* 11, 931. doi:10.3389/fphar.2020.00931

Huang, W., Liang, Y., Wang, J., Li, G., Wang, G., Li, Y., et al. (2016). Anti-angiogenic Activity and Mechanism of Kaurane Diterpenoids from *Wedelia Chinensis*. *Phytomedicine* 23 (3), 283–292. doi:10.1016/j.phymed.2015.12.021

Ide, M., Yoshida, I., Kumagai, M., Mishima, T., Takahashi, Y., Fujita, K., et al. (2020). *Tithonia Diversifolia*-derived Orizabin Suppresses Cell Adhesion, Differentiation, and Oxidized LDL Accumulation by Akt Signaling Suppression via PTEN Promotion in THP-1 Cells. *J. Food Biochem.* 44 (7), e13268. doi:10.1111/jfbc.13268

Jadhav, A. K., and Karuppayil, S. M. (2021). *Andrographis Paniculata* (Burm. F) Wall Ex Nees: Antiviral Properties. *Phytother. Res.* 35 (10), 5365–5373. doi:10.1002/ptr.7145

Jeenkeawpieam, J., Yodkeeree, S., Andrianopoulos, A., Roytrakul, S., and Pongpom, M. (2020). Antifungal Activity and Molecular Mechanisms of Partial Purified Antifungal Proteins from *Rhinacanthus Nasutus* against *Talaromyces marneffei*. *J. Fungi (Basel)* 6 (4), 333. doi:10.3390/jof6040333

Jiang, M., Zhao, S., Yang, S., Lin, X., He, X., Wei, X., et al. (2020). An "essential Herbal Medicine"-Licorice: A Review of Phytochemicals and its Effects in Combination Preparations. *J. Ethnopharmacol.* 249, 112439. doi:10.1016/j.jep.2019.112439

Jin, B., Liu, Y., Xie, J., Luo, B., and Long, C. (2018). Ethnobotanical Survey of Plant Species for Herbal tea in a Yao Autonomous County (Jianghua, China): Results of a 2-year Study of Traditional Medicinal Markets on the Dragon Boat Festival. *J. Ethnobiol. Ethnomed* 14 (1), 58. doi:10.1186/s13002-018-0257-0

Jin, Y., Chen, W., Yang, H., Yan, Z., Lai, Z., Feng, J., et al. (2017). Scutellaria Barbata D. Don Inhibits Migration and Invasion of Colorectal Cancer Cells via Suppression of PI3K/AKT and TGF-β/Smad Signaling Pathways. *Exp. Ther. Med.* 14 (6), 5527–5534. doi:10.3892/etm.2017.5242

Joubert, E., Gelderblom, W. C., Louw, A., and de Beer, D. (2008). South African Herbal Teas: Aspalathus Linearis, Cyclopia Spp. And Athrixia Phylicoides-Aa Review. *J. Ethnopharmacol* 119 (3), 376–412. doi:10.1016/j.jep.2008.06.014

Kan, X., Zhang, W., You, R., Niu, Y., Guo, J., and Xue, J. (2017). *Scutellaria Barbata* D. Don Extract Inhibits the Tumor Growth through Down-Regulating of Treg Cells and Manipulating Th1/Th17 Immune Response in Hepatoma H22-Bearing Mice. *BMC Complement. Altern. Med.* 17 (1), 41. doi:10.1186/s12906-016-1551-9

Kernan, M. R., Sendl, A., Chen, J. L., Jolad, S. D., Blanc, P., Murphy, J. T., et al. (1997). Two New Lignans with Activity against Influenza Virus from the Medicinal Plant *Rhinacanthus Nasutus*. *J. Nat. Prod.* 60 (6), 635–637. doi:10.1021/np960613i

Kim, J. W., Kim, H. P., and Sung, S. H. (2017). Cytotoxic Pterosins from *Pteris Multifida* Roots against HCT116 Human colon Cancer Cells. *Bioorg. Med. Chem. Lett.* 27 (14), 3144–3147. doi:10.1016/j.bmcl.2017.05.034

Kim, J. W., Seo, J. Y., Oh, W. K., and Sung, S. H. (2016). Anti-neuroinflammatory Ent-Kaurane Diterpenoids from *Pteris Multifida* Roots. *Molecules* 22 (1), 27. doi:10.3390/molecules22010027

Kim, K. W., Jin, U. H., Kim, D. I., Lee, T. K., Kim, M. S., Oh, M. J., et al. (2008). Antiproliferative Effect of *Scutellaria Barbata* D. Don. On Cultured Human Uterine Leiomyoma Cells by Down-Regulation of the Expression of Bcl-2 Protein. *Phytother. Res.* 22 (5), 583–590. doi:10.1002/ptr.1996

Komati, A., Anand, A., Shaik, H., Mudiam, M. K. R., Suresh Babu, K., and Tiwari, A. K. (2020). *Bombax ceiba* (Linn.) Calyxes Ameliorate Methylglyoxal-Induced Oxidative Stress via Modulation of RAGE Expression: Identification of Active Phytometabolites by GC-MS Analysis. *Food Funct.* 11 (6), 5486–5497. doi:10.1039/c9fo02714a

Kulkarni, P., Lohidasan, S., and Mahadik, K. (2021). Bioanalytical Method Development for Momordicinin and its Application to Long-Term Pharmacokinetics in Diabetic Rats. *Drug Dev. Ind. Pharm.* 47, 1064–1071. doi:10.1080/03639045.2021.1908337

Kupradinun, P., Siripong, P., Chanpai, R., Piyaviriyagul, S., Rungsipipat, A., and Wangnaitham, S. (2009). Effects of *Rhinacanthus Nasutus* Kurz on colon Carcinogenesis in Mice. *Asian Pac. J. Cancer Prev.* 10 (1), 103–106.

Kwak, H. J., Park, S., Kim, N., Yoo, G., Park, J. H., Oh, Y., et al. (2018). Neuraminidase Inhibitory Activity by Compounds Isolated from Aerial Parts of *Rhinacanthus Nasutus*. *Nat. Prod. Res.* 32 (17), 2111–2115. doi:10.1080/14786419.2017.1365067

Lashin, I., Fouda, A., Gobouri, A. A., Azab, E., Mohammedsaleh, Z. M., and Makharita, R. R. (2021). Antimicrobial and *In Vitro* Cytotoxic Efficacy of Biogenic Silver Nanoparticles (Ag-NPs) Fabricated by Callus Extract of *Solanum Incanum* L. *Biomolecules* 11 (3), 341. doi:10.3390/biom11030341

Lee, D. S., Keo, S., Cheng, S. K., Oh, H., and Kim, Y. C. (2017). Protective Effects of Cambodian Medicinal Plants on Tert-butyl H-ydroperoxide-induced H-epatotoxicity via Nrf2-mediated H-eme O-xygenase-1. *Mol. Med. Rep.* 15 (1), 451–459. doi:10.3892/mmr.2016.6011

Lee, H. J., Choi, E. J., Park, S., and Lee, J. J. (2020). Laxative and Antioxidant Effects of Ramie (Boehmeria Nivea L.) Leaf Extract in Experimental Constipated Rats. *Food Sci. Nutr.* 8 (7), 3389–3401. doi:10.1002/fsn3.1619

Li, D. L., Zheng, X. L., Duan, L., Deng, S. W., Ye, W., Wang, A. H., et al. (2017). Ethnobotanical Survey of Herbal tea Plants from the Traditional Markets in Chaoshan, China. *J. Ethnopharmacol.* 205, 195–206. doi:10.1016/j.jep.2017.02.040

Li, H., Xie, W., Qiao, X., Cui, H., Yang, X., and Xue, C. (2020). Structural Characterization of Arabinogalactan Extracted from *Ixeris Chinensis* (Thunb.) Nakai and its Immunomodulatory Effect on RAW264.7 Macrophages. *Int. J. Biol. Macromol.* 143, 977–983. doi:10.1016/j.ijbiomac.2019.09.158

Li, Q., Hao, Z., Hong, Y., He, W., and Zhao, W. (2018). Reprogramming Tumor Associated Macrophage Phenotype by a Polysaccharide from *Ilex Asprella* for Sarcoma Immunotherapy. *Int. J. Mol. Sci.* 19 (12), 3816. doi:10.3390/ijms19123816

Lim, J. Y., Lee, J. H., Lee, B. R., Kim, M. A., Lee, Y. M., Kim, D. K., et al. (2020). Extract of Boehmeria Nivea Suppresses Mast Cell-Mediated Allergic Inflammation by Inhibiting Mitogen-Activated Protein Kinase and Nuclear Factor-Kb. *Molecules* 25 (18), 4178. doi:10.3390/molecules25184178

Lin, F. M., Chen, L. R., Lin, E. H., Ke, F. C., Chen, H. Y., Tsai, M. J., et al. (2007). Compounds from *Wedelia Chinensis* Synergistically Suppress Androgen Activity and Growth in Prostate Cancer Cells. *Carcinogenesis* 28 (12), 2521–2529. doi:10.1093/carcin/bgm137

Lin, J., Feng, J., Yang, H., Yan, Z., Li, Q., Wei, L., et al. (2017). Scutellaria Barbata D. Don Inhibits 5-fluorouracil Resistance in Colorectal Cancer by Regulating PI3K/AKT Pathway. *Oncol. Rep.* 38 (4), 2293–2300. doi:10.3892/or.2017.5892

Lin, J. M., Lin, C. C., Chen, M. F., Ujiie, T., and Takada, A. (1995). Scavenging Effects of *Mallotus Repandus* on Active Oxygen Species. *J. Ethnopharmacol.* 46 (3), 175–181. doi:10.1016/0378-8741(95)01246-a

Lin, K. H., Marthandam Asokan, S., Kuo, W. W., Hsieh, Y. L., Lii, C. K., Viswanadha, V., et al. (2020). Andrographolide Mitigates Cardiac Apoptosis to Provide Cardio-protection in High-Fat-Diet-Induced Obese Mice. *Environ. Toxicol.* 35 (6), 707–713. doi:10.1002/tox.22906

Lin, R. S. (2014). *Study on the Medicinal Value and Resources of Fujian Local Herbal Tea Plants*. Fujian: Fujian Agriculture and Forestry University. Fujian Agriculture and Forestry University.

Lin, W. L., Wang, S. M., Ho, Y. J., Kuo, H. C., Lee, Y. J., and Tseng, T. H. (2014). Ethyl Acetate Extract of *Wedelia Chinensis* Inhibits Tert-Butyl Hydroperoxide-Induced Damage in PC12 Cells and D-Galactose-Induced Neuronal Cell Loss in Mice. *BMC Complement. Altern. Med.* 14, 491. doi:10.1186/1472-6882-14-491

Liu, H. L., Kao, T. H., Shiau, C. Y., and Chen, B. H. (2018). Functional Components in *Scutellaria Barbata* D. Don with Anti-inflammatory Activity on RAW 264.7 Cells. *J. Food Drug Anal.* 26 (1), 31–40. doi:10.1016/j.jfda.2016.11.022

Liu, J., Feng, W., and Peng, C. (2020). A Song of Ice and Fire: Cold and Hot Properties of Traditional Chinese Medicines. *Front. Pharmacol.* 11, 598744. doi:10.3389/fphar.2020.598744

Liu, M., Wang, W., Li, X., Shi, D., Mei, H., Jin, X., et al. (2013). *Wedelia Chinensis* Inhibits Nasopharyngeal Carcinoma CNE-1 Cell Growth by Inducing G2/M Arrest in a Chk1-dependent Pathway. *Am. J. Chin. Med.* 41 (5), 1153–1168. doi:10.1142/S0192415X1350078X

Liu, S., Lin, L., Shen, M., Wang, W., Xiao, Y., and Xie, J. (2018). Effect of *Mesona Chinensis* Polysaccharide on the Pasting, thermal and Rheological Properties of Wheat Starch. *Int. J. Biol. Macromol.* 118 (Pt A), 945–951. doi:10.1016/j.ijbiomac.2018.06.178

Liu, W., Fan, T., Li, M., Zhang, G., Guo, W., Yang, X., et al. (2020). Andrographolide Potentiates PD-1 Blockade Immunotherapy by Inhibiting COX2-Mediated PGE2 Release. *Int. Immunopharmacol.* 81, 106206. doi:10.1016/j.intimp.2020.106206

Liu, Y., Ahmed, S., and Long, C. (2013). Ethnobotanical Survey of Cooling Herbal Drinks from Southern China. *J. Ethnobiol. Ethnomed.* 9, 82. doi:10.1186/1746-4269-9-82

Lu, F. J., Ding, L. Q., Cao, S. J., Zhang, D. Q., Zhang, B. L., and Qiu, F. (2018). [Chemistry and Biology Research on Bitter-Taste Chinese Materia Medica with Function of Regulating Glycolipid Metabolism]. *Zhongguo Zhong Yao Za Zhi* 43 (19), 3834–3840. doi:10.19540/j.cnki.cjcmm.20180709.006

Lu, J., Ma, Y., Wu, J., Huang, H., Wang, X., Chen, Z., et al. (2019). A Review for the Neuroprotective Effects of Andrographolide in the central Nervous System. *Biomed. Pharmacother.* 117, 109078. doi:10.1016/j.biopha.2019.109078

Lu, K. H., Chang, Y. F., Yin, P. H., Chen, T. T., Ho, Y. L., Chang, Y. S., et al. (2010). *In Vitro* and *In Vivo* Apoptosis-Inducing Antileukemic Effects of Mucuna Macrocarpa Stem Extract on HL-60 Human Leukemia Cells. *Integr. Cancer Ther.* 9 (3), 298–308. doi:10.1177/1534735410378661

Lu, M. R., Huang, H. L., Chiou, W. F., and Huang, R. L. (2017). Induction of Apoptosis by *Tithonia Diversifolia* in Human Hepatoma Cells. *Pharmacogn. Mag.* 13 (52), 702–706. doi:10.4103/0973-1296.218113

Ma, T., Zhang, Y., Zhang, C., Luo, J. G., and Kong, L. Y. (2017). Downregulation of TIGAR Sensitizes the Antitumor Effect of Physapubenolide through Increasing Intracellular ROS Levels to Trigger Apoptosis and Autophagosome Formation in Human Breast Carcinoma Cells. *Biochem. Pharmacol.* 143, 90–106. doi:10.1016/j.bcp.2017.07.018

Mabou Tagne, A., Marino, F., and Cosentino, M. (2018). *Tithonia Diversifolia* (Hemsl.) A. Gray as a Medicinal Plant: a Comprehensive Review of its Ethnopharmacology, Phytochemistry, Pharmacotoxicology and Clinical Relevance. *J. Ethnopharmacol.* 220, 94–116. doi:10.1016/j.jep.2018.03.025

Malik, Z., Parveen, R., Parveen, B., Zahiruddin, S., Aasif Khan, M., Khan, A., et al. (2021). Anticancer Potential of Andrographolide from *Andrographis Paniculata* (Burm.f.) Nees and its Mechanisms of Action. *J. Ethnopharmacol.* 272, 113936. doi:10.1016/j.jep.2021.113936

Manjamalai, A., and Berlin Grace, V. M. (2012). Antioxidant Activity of Essential Oils from *Wedelia Chinensis* (Osbeck) *In Vitro* and *In Vivo* Lung Cancer Bearing C57BL/6 Mice. *Asian Pac. J. Cancer Prev.* 13 (7), 3065–3071. doi:10.7314/apjcp.2012.13.7.3065

Marconett, C. N., Morgenstern, T. J., San Roman, A. K., Sundar, S. N., Singhal, A. K., and Firestone, G. L. (2010). BZL101, a Phytochemical Extract from the *Scutellaria Barbata* Plant, Disrupts Proliferation of Human Breast and Prostate Cancer Cells through Distinct Mechanisms Dependent on the Cancer Cell Phenotype. *Cancer Biol. Ther.* 10 (4), 397–405. doi:10.4161/cbt.10.4.12424

Maregesi, S., van Miert, S., Pannecouque, C., Feiz Haddad, M. H., Hermans, N., Wright, C. W., et al. (2010). Screening of Tanzanian Medicinal Plants against *Plasmodium Falciparum* and Human Immunodeficiency Virus. *Planta Med.* 76 (2), 195–201. doi:10.1055/s-0029-1186024

Matsubara, T., Bohgaki, T., Watarai, M., Suzuki, H., Ohashi, K., and Shibuya, H. (1999). Antihypertensive Actions of Methylripariochromene A from *Orthosiphon Aristatus,* an Indonesian Traditional Medicinal Plant. *Biol. Pharm. Bull.* 22 (10), 1083–1088. doi:10.1248/bpb.22.1083

Matsumoto, T., Horiuchi, M., Kamata, K., and Seyama, Y. (2009). Effects of *Bidens Pilosa* L. Var. Radiata SCHERFF Treated with Enzyme on Histamine-Induced Contraction of guinea Pig Ileum and on Histamine Release from Mast Cells. *J. Smooth Muscle Res.* 45 (2–3), 75–86. doi:10.1540/jsmr.45.75

Mei, X. Y., Zhou, L. Y., Zhang, T. Y., Lu, B., and Ji, L. L. (2017). *Scutellaria Barbata* Attenuates Diabetic Retinopathy by Preventing Retinal Inflammation and the Decreased Expression of Tight junction Protein. *Int. J. Ophthalmol.* 10 (6), 870–877. doi:10.18240/ijo.2017.06.07

Meira, C. S., Guimarães, E. T., Dos Santos, J. A., Moreira, D. R., Nogueira, R. C., Tomassini, T. C., et al. (2015). *In Vitro* and *In Vivo* Antiparasitic Activity of *Physalis Angulata* L. Concentrated Ethanolic Extract against Trypanosoma Cruzi. *Phytomedicine* 22 (11), 969–974. doi:10.1016/j.phymed.2015.07.004

Meng, F., Li, Q., Qi, Y., He, C., Wang, C., and Zhang, Q. (2018). Characterization and Immunoregulatory Activity of Two Polysaccharides from the Root of *Ilex Asprella*. *Carbohydr. Polym.* 197, 9–16. doi:10.1016/j.carbpol.2018.05.066

Ministry of Health and Welfare (2019). Analysis on Statistical Results of Death Causes in 2019. Available at: https://dep.mohw.gov.tw/DOS/cp-4927-54466-113.html (Accessed June 16, 2020).

Ministry of Health and Welfare (2021). The Illustration Database of Common Medicinal Plants in Taiwan. Available at: https://www.cmtcmp.mohw.gov.tw/INDEX_ENG.ASPX (accessed December 31, 2021).

Miura, T., Nosaka, K., Ishii, H., and Ishida, T. (2005). Antidiabetic Effect of Nitobegiku, the Herb *Tithonia Diversifolia*, in KK-Ay Diabetic Mice. *Biol. Pharm. Bull.* 28 (11), 2152–2154. doi:10.1248/bpb.28.2152

Mondal, M., Hossain, M. M., Hasan, M. R., Tarun, M. T. I., Islam, M. A. F., Choudhuri, M. S. K., et al. (2020). Hepatoprotective and Antioxidant Capacity of *Mallotus Repandus* Ethyl Acetate Stem Extract against D-Galactosamine-Induced Hepatotoxicity in Rats. *ACS Omega* 5 (12), 6523–6531. doi:10.1021/acsomega.9b04189

Mussard, E., Jousselin, S., Cesaro, A., Legrain, B., Lespessailles, E., Esteve, E., et al. (2020). *Andrographis Paniculata* and its Bioactive Diterpenoids against Inflammation and Oxidative Stress in Keratinocytes. *Antioxidants (Basel)* 9 (6), 530. doi:10.3390/antiox9060530

Nadia, N. A. C., Cédric, Y., Raoul, S. N. S., Christian, N. O., Azizi, M. A., Diane, G. D. C., et al. (2020). Antimalarial Activity of Ethyl Acetate Extract and Fraction of *Bidens Pilosa* against *Plasmodium Berghei* (ANKA). *J. Parasitol. Res.* 2020, 8832724. doi:10.1155/2020/8832724

Napoli, E., Siracusa, L., and Ruberto, G. (2020). New Tricks for Old Guys: Recent Developments in the Chemistry, Biochemistry, Applications and Exploitation of Selected Species from the Lamiaceae Family. *Chem. Biodivers.* 17 (3), e1900677. doi:10.1002/cbdv.201900677

Nayak, A. G., Kumar, N., Shenoy, S., and Roche, M. (2021). Evaluation of the merit of the Methanolic Extract of *Andrographis Paniculata* to Supplement Anti-snake Venom in Reversing Secondary Hemostatic Abnormalities Induced by *Naja naja* Venom. *3 Biotech.* 11 (5), 228. doi:10.1007/s13205-021-02766-z

Ngoc, T. M., Phuong, N. T. T., Khoi, N. M., Park, S., Kwak, H. J., Nhiem, N. X., et al. (2019). A New Naphthoquinone Analogue and Antiviral Constituents from the Root of *Rhinacanthus Nasutus*. *Nat. Prod. Res.* 33 (3), 360–366. doi:10.1080/14786419.2018.1452004

Nguepi, I. S. T., Ngueguim, F. T., Gounoue, R. K., Mbatchou, A., and Dimo, T. (2021). Curative Effects of the Aqueous Extract of *Tithonia Diversifolia* (Hemsl.) A. gray (Asteraceae) against Ethanol Induced-Hepatotoxicity in Rats. *J. Basic Clin. Physiol. Pharmacol.* 32 (6), 1137–1143. doi:10.1515/jbcpp-2019-0370

Nguyen, T. D., Thuong, P. T., Hwang, I. H., Hoang, T. K., Nguyen, M. K., Nguyen, H. A., et al. (2017). Anti-hyperuricemic, Anti-inflammatory and Analgesic Effects of *Siegesbeckia Orientalis* L. Resulting from the Fraction with High Phenolic Content. *BMC Complement. Altern. Med.* 17 (1), 191. doi:10.1186/s12906-017-1698-z

Nieto, G. (2017). Biological Activities of Three Essential Oils of the Lamiaceae Family. *Medicines* 4 (3), 63. doi:10.3390/medicines4030063

Ohashi, K., Bohgaki, T., and Shibuya, H. (2000). [Antihypertensive Substance in the Leaves of Kumis Kucing (Orthosiphon Aristatus) in Java Island]. *Yakugaku Zasshi* 120 (5), 474–482. doi:10.1248/yakushi1947.120.5_474

Owoyele, V. B., Wuraola, C. O., Soladoye, A. O., and Olaleye, S. B. (2004). Studies on the Anti-inflammatory and Analgesic Properties of Tithonia Diversifolia Leaf Extract. *J. Ethnopharmacol.* 90 (2–3), 317–321. doi:10.1016/j.jep.2003.10.010

Ozmen, A., Madlener, S., Bauer, S., Krasteva, S., Vonach, C., Giessrigl, B., et al. (2010). *In Vitro* anti-leukemic Activity of the Ethno-Pharmacological Plant *Scutellaria Orientalis* Ssp. Carica Endemic to Western Turkey. *Phytomedicine* 17 (1), 55–62. doi:10.1016/j.phymed.2009.06.001

Pandey, V., Tripathi, A., Rani, A., and Dubey, P. K. (2020). Deoxyelephantopin, a Novel Naturally Occurring Phytochemical Impairs Growth, Induces G2/M Arrest, ROS-Mediated Apoptosis and Modulates lncRNA Expression against Uterine Leiomyoma. *Biomed. Pharmacother.* 131, 110751. doi:10.1016/j.biopha.2020.110751

Pegoraro, C. M. R., Nai, G. A., Garcia, L. A., Serra, F. M., Alves, J. A., Chagas, P. H. N., et al. (2021). Protective Effects of *Bidens Pilosa* on Hepatoxicity and Nephrotoxicity Induced by Carbon Tetrachloride in Rats. *Drug Chem. Toxicol.* 44 (1), 64–74. doi:10.1080/01480545.2018.1526182

Perera, W. H., Shivanagoudra, S. R., Pérez, J. L., Kim, D. M., Sun, Y., K Jayaprakasha, G., et al. (2021). Anti-inflammatory, Antidiabetic Properties and In Silico Modeling of Cucurbitane-type Triterpene Glycosides from Fruits of an Indian Cultivar of *Momordica Charantia* L. *Molecules* 26 (4), 1038. doi:10.3390/molecules26041038

Poswal, F. S., Russell, G., Mackonochie, M., MacLennan, E., Adukwu, E. C., and Rolfe, V. (2019). Herbal Teas and Their Health Benefits: A Scoping Review. *Plant Foods Hum. Nutr.* 74 (3), 266–276. doi:10.1007/s11130-019-00750-w

Punturee, K., Wild, C. P., Kasinrerk, W., and Vinitketkumnuen, U. (2005). Immunomodulatory Activities of *Centella Asiatica* and *Rhinacanthus Nasutus* Extracts. *Asian Pac. J. Cancer Prev.* 6 (3), 396–400.

Puttarak, P., Charoonratana, T., and Panichayupakaranant, P. (2010). Antimicrobial Activity and Stability of Rhinacanthins-Rich *Rhinacanthus Nasutus* Extract. *Phytomedicine* 17 (5), 323–327. doi:10.1016/j.phymed.2009.08.014

Qi, R., Li, X., Zhang, X., Huang, Y., Fei, Q., Han, Y., et al. (2020). Ethanol Extract of Elephantopus Scaber Linn. Attenuates Inflammatory Response via the Inhibition of NF-Kb Signaling by Dampening P65-DNA Binding Activity in Lipopolysaccharide-Activated Macrophages. *J. Ethnopharmacol.* 250, 112499. doi:10.1016/j.jep.2019.112499

Rachid, A., Rabah, D., Farid, L., Zohra, S. F., Houcine, B., and Nacéra, B. (2012). Ethnopharmacological Survey of Medicinal Plants Used in the Traditional Treatment of Diabetes Mellitus in the North Western and South Western Algeria. *J. Med. Plants Res.* 6 (10), 2041–2050. doi:10.13040/IJPSR.0975-8232.5(5).2006-13

Rehecho, S., Uriarte-Pueyo, I., Calvo, J., Vivas, L. A., and Calvo, M. I. (2011). Ethnopharmacological Survey of Medicinal Plants in Nor-Yauyos, a Part of the Landscape Reserve Nor-Yauyos-Cochas, Peru. *J. Ethnopharmacol.* 133 (1), 75–85. doi:10.1016/j.jep.2010.09.006

Rolnik, A., and Olas, B. (2021). The Plants of the Asteraceae Family as Agents in the protection of Human Health. *Ijms* 22 (6), 3009. doi:10.3390/ijms22063009

Roulette, C. J., Njau, E. A., Quinlan, M. B., Quinlan, R. J., and Call, D. R. (2018). Medicinal Foods and Beverages Among Maasai Agro-Pastoralists in Northern Tanzania. *J. Ethnopharmacol* 216, 191–202. doi:10.1016/j.jep.2018.01.022

Sa-Ngiamsuntorn, K., Suksatu, A., Pewkliang, Y., Thongsri, P., Kanjanasirirat, P., Manopwisedjaroen, S., et al. (2021). Anti-SARS-CoV-2 Activity of *Andrographis Paniculata* Extract and its Major Component Andrographolide in Human Lung Epithelial Cells and Cytotoxicity Evaluation in Major Organ Cell Representatives. *J. Nat. Prod.* 84 (4), 1261–1270. doi:10.1021/acs.jnatprod.0c01324

Saleem, U., Gull, Z., Saleem, A., Shah, M. A., Akhtar, M. F., Anwar, F., et al. (2021). Appraisal of Anti-parkinson Activity of Rhinacanthin-C in Haloperidol-Induced Parkinsonism in Mice: a Mechanistic Approach. *J. Food Biochem.* 45 (4), e13677. doi:10.1111/jfbc.13677

Sánchez-Mendoza, M. E., Reyes-Ramírez, A., Cruz Antonio, L., Martínez Jiménez, L., Rodríguez-Silverio, J., and Arrieta, J. (2011). Bioassay-guided Isolation of an Anti-ulcer Compound, Tagitinin C, from *Tithonia Diversifolia*: Role of Nitric Oxide, Prostaglandins and Sulfhydryls. *Molecules* 16 (1), 665–674. doi:10.3390/molecules16010665

Saslis-Lagoudakis, C. H., Williamson, E. M., Savolainen, V., and Hawkins, J. A. (2011). Cross-cultural Comparison of Three Medicinal Floras and Implications for Bioprospecting Strategies. *J. Ethnopharmacol* 135 (2), 476–487. doi:10.1016/j.jep.2011.03.044

Sato, Y., Suzaki, S., Nishikawa, T., Kihara, M., Shibata, H., and Higuti, T. (2000). Phytochemical Flavones Isolated from *Scutellaria Barbata* and Antibacterial Activity against Methicillin-Resistant *Staphylococcus aureus*. *J. Ethnopharmacol.* 72 (3), 483–488. doi:10.1016/s0378-8741(00)00265-8

Shah, M. A., Muhammad, H., Mehmood, Y., Khalil, R., Ul-Haq, Z., and Panichayupakaranant, P. (2017). Superoxide Scavenging and Antiglycation Activity of Rhinacanthins-Rich Extract Obtained from the Leaves of *Rhinacanthus Nasutus*. *Pharmacogn. Mag.* 13 (52), 652–658. doi:10.4103/pm.pm_196_17

Shah, S. S., Shah, S. S., Iqbal, A., Ahmed, S., Khan, W. M., Hussain, S., et al. (2018). Report: Phytochemical Screening and Antimicrobial Activities of Red Silk Cotton Tree (*Bombax ceiba* L.). *Pak. J. Pharm. Sci.* 31 (3), 947–952.

Shih, K. N., Huang, W. T., Chang, C. L., and Feng, C. C. (2014). Effects of *Ixeris Chinensis* (Thunb.) Nakai Boiling Water Extract on Hepatitis B Viral Activity and Hepatocellular Carcinoma. *Afr. J. Tradit. Complement. Altern. Med.* 11 (1), 187–193. doi:10.4314/ajtcam.v11i1.30

Siripong, P., Hahnvajanawong, C., Yahuafai, J., Piyaviriyakul, S., Kanokmedhakul, K., Kongkathip, N., et al. (2009). Induction of Apoptosis by Rhinacanthone Isolated from *Rhinacanthus Nasutus* Roots in Human Cervical Carcinoma Cells. *Biol. Pharm. Bull.* 32 (7), 1251–1260. doi:10.1248/bpb.32.1251

Siripong, P., Yahuafai, J., Piyaviriyakul, S., Kanokmedhakul, K., Koide, H., Ishii, T., et al. (2012). Inhibitory Effect of Liposomal Rhinacanthin-N Isolated from *Rhinacanthus Nasutus* on Pulmonary Metastasis in Mice. *Biol. Pharm. Bull.* 35 (7), 1197–1200. doi:10.1248/bpb.b12-00244

Siripong, P., Yahuafai, J., Shimizu, K., Ichikawa, K., Yonezawa, S., Asai, T., et al. (2006). Antitumor Activity of Liposomal Naphthoquinone Esters Isolated from Thai Medicinal Plant: *Rhinacanthus Nasutus* KURZ. *Biol. Pharm. Bull.* 29 (11), 2279–2283. doi:10.1248/bpb.29.2279

Sõukand, R., and Kalle, R. (2013). Where Does the Border Lie: Locally Grown Plants Used for Making tea for Recreation And/or Healing, 1970s-1990s Estonia. *J. Ethnopharmacol* 150 (1), 162–174. doi:10.1016/j.jep.2013.08.031

Sõukand, R., Quave, C. L., Pieroni, A., Pardo-de-Santayana, M., Tardío, J., Kalle, R., et al. (2013). Plants Used for Making Recreational tea in Europe: a Review Based on Specific Research Sites. *J. Ethnobiol. Ethnomed* 9 (1), 58. doi:10.1186/1746-4269-9-58

Su, S. Y., Yang, C. H., Chiu, C. C., and Wang, Q. (2013). Acoustic Features for Identifying Constitutions in Traditional Chinese Medicine. *J. Altern. Complement. Med.* 19 (6), 569–576. doi:10.1089/acm.2012.0478

Sulistyani, N., and Nurkhasanah, - (2020). Screening of Anticancer, Hepatoprotective and Nephroprotective Effects of Ethanol Extract of *Elephantopus Scaber* L. *Pak J. Pharm. Sci.* 33 (3), 901–907.

Sun, C. P., Oppong, M. B., Zhao, F., Chen, L. X., and Qiu, F. (2017a). Unprecedented 22,26-seco Physalins from *Physalis Angulata* and Their Anti-inflammatory Potential. *Org. Biomol. Chem.* 15 (41), 8700–8704. doi:10.1039/c7ob02205k

Sun, C. P., Qiu, C. Y., Zhao, F., Kang, N., Chen, L. X., and Qiu, F. (2017b). Physalins V-IX, 16,24-Cyclo-13,14-Seco Withanolides from *Physalis Angulata* and Their Antiproliferative and Anti-inflammatory Activities. *Sci. Rep.* 7 (1), 4057. doi:10.1038/s41598-017-03849-9

Sun, H. X., and Wang, H. (2006). Immunosuppressive Activity of the Ethanol Extract of *Siegesbeckia Orientalis* on the Immune Responses to Ovalbumin in Mice. *Chem. Biodivers.* 3 (7), 754–761. doi:10.1002/cbdv.200690077

Sun, P., Sun, D., and Wang, X. (2017). Effects of *Scutellaria Barbata* Polysaccharide on the Proliferation, Apoptosis and EMT of Human colon Cancer HT29 Cells. *Carbohydr. Polym.* 167, 90–96. doi:10.1016/j.carbpol.2017.03.022

Tan, F., Chen, Y., Tan, X., Ma, Y., and Peng, Y. (2017). Chinese Materia Medica Used in Medicinal Diets. *J. Ethnopharmacol.* 206, 40–54. doi:10.1016/j.jep.2017.05.021

Tewtrakul, S., Tansakul, P., and Panichayupakaranant, P. (2009). Anti-allergic Principles of *Rhinacanthus Nasutus* Leaves. *Phytomedicine* 16 (10), 929–934. doi:10.1016/j.phymed.2009.03.010

Thao, N. P., Binh, P. T., Luyen, N. T., Hung, T. M., Dang, N. H., and Dat, N. T. (2018). α-Amylase and α-Glucosidase Inhibitory Activities of Chemical Constituents from Wedelia Chinensis (Osbeck.) Merr. Leaves. *J. Anal. Methods Chem.* 2018, 2794904. doi:10.1155/2018/2794904

The Committee on Chinese Medicine and Pharmacy, D.o.H., Executive Yuan, Taiwan. (2003). The Catalogue of Medicinal Plant Resources in Taiwan, Taipei. 660.

The Committee on Chinese Medicine and Pharmacy, D.o.H., Executive Yuan, Taiwan. (2011). The Illustration of Common Medicinal Plants in Taiwan, Taipei. 467.

The Royal Botanic Gardens (2013). The Plant List. Available at: http://www.theplantlist.org/ (Accessed May 1, 2019).

The Royal Botanic Gardens (2021). Plants of the World Online. Available at: https://powo.science.kew.org/ (Accessed May 2021).

Toppo, E., Al-Dhabi, N. A., Sankar, C., Kumar, S. N., Buvanesvaragurunathan, K., Darvin, S. S., et al. (2021). Hepatoprotective Effect of Selected Isoandrographolide Derivatives on Steatotic HepG2 Cells and High Fat Diet Fed Rats. *Eur. J. Pharmacol.* 899, 174056. doi:10.1016/j.ejphar.2021.174056

Tsai, C. H., Lin, F. M., Yang, Y. C., Lee, M. T., Cha, T. L., Wu, G. J., et al. (2009). Herbal Extract of *Wedelia Chinensis* Attenuates Androgen Receptor Activity and Orthotopic Growth of Prostate Cancer in Nude Mice. *Clin. Cancer Res.* 15 (17), 5435–5444. doi:10.1158/1078-0432.CCR-09-0298

Tsai, C. H., Tzeng, S. F., Hsieh, S. C., Lin, C. Y., Tsai, C. J., Chen, Y. R., et al. (2015). Development of a Standardized and Effect-Optimized Herbal Extract of *Wedelia Chinensis* for Prostate Cancer. *Phytomedicine* 22 (3), 406–414. doi:10.1016/j.phymed.2015.01.013

Tsai, C. H., Tzeng, S. F., Hsieh, S. C., Tsai, C. J., Yang, Y. C., Tsai, M. H., et al. (2017a). A Standardized *Wedelia Chinensis* Extract Overcomes the Feedback Activation of HER2/3 Signaling upon Androgen-Ablation in Prostate Cancer. *Front. Pharmacol.* 8, 721. doi:10.3389/fphar.2017.00721

Tsai, C. H., Tzeng, S. F., Hsieh, S. C., Yang, Y. C., Hsiao, Y. W., Tsai, M. H., et al. (2017b). A Standardized Herbal Extract Mitigates Tumor Inflammation and Augments Chemotherapy Effect of Docetaxel in Prostate Cancer. *Sci. Rep.* 7 (1), 15624. doi:10.1038/s41598-017-15934-0

Tsai, F. J., Yang, P. Y., Chen, C. J., Li, J. P., Li, T. M., Chiou, J. S., et al. (2020). Decreased Overall Mortality Rate with Chinese Herbal Medicine Usage in Patients with Decompensated Liver Cirrhosis in Taiwan. *BMC Complement. Med. Ther.* 20 (1), 221. doi:10.1186/s12906-020-03010-6

Tsai, T. H., Huang, C. J., Wu, W. H., Huang, W. C., Chyuan, J. H., and Tsai, P. J. (2014). Antioxidant, Cell-Protective, and Anti-melanogenic Activities of Leaf Extracts from Wild Bitter Melon (Momordica Charantia Linn. Var. Abbreviata Ser.) Cultivars. *Bot. Stud.* 55 (1), 78. doi:10.1186/s40529-014-0078-y

Tsai, T. H., Huang, W. C., Ying, H. T., Kuo, Y. H., Shen, C. C., Lin, Y. K., et al. (2016). Wild Bitter Melon Leaf Extract Inhibits *Porphyromonas Gingivalis*-induced Inflammation: Identification of Active Compounds through Bioassay-Guided Isolation. *Molecules* 21 (4), 454. doi:10.3390/molecules21040454

Tugume, P., and Nyakoojo, C. (2019). Ethno-pharmacological Survey of Herbal Remedies Used in the Treatment of Paediatric Diseases in Buhunga parish, Rukungiri District, Uganda. *BMC Complement. Altern. Med.* 19 (1), 353. doi:10.1186/s12906-019-2763-6

Tundis, R., Rashed, K., Said, A., Menichini, F., and Loizzo, M. R. (2014). *In Vitro* cancer Cell Growth Inhibition and Antioxidant Activity of *Bombax ceiba* (Bombacaceae) Flower Extracts. *Nat. Prod. Commun.* 9 (5), 691–694. doi:10.1177/1934578x1400900527

Ubillas, R. P., Mendez, C. D., Jolad, S. D., Luo, J., King, S. R., Carlson, T. J., et al. (2000). Antihyperglycemic Acetylenic Glucosides from Bidens Pilosa. *Planta Med.* 66 (1), 82–83. doi:10.1055/s-0029-1243117

Vieceli, P. S., Juiz, P. J. L., Lauria, P. S. S., Couto, R. D., Tomassini, T. C. B., Ribeiro, I. M., et al. (2021). *Physalis Angulata* Reduces the Progression of Chronic Experimental Periodontitis by Immunomodulatory Mechanisms. *J. Ethnopharmacol.* 273, 113986. doi:10.1016/j.jep.2021.113986

Visweswara Rao, P., Madhavi, K., Dhananjaya Naidu, M., and Gan, S. H. (2013a). *Rhinacanthus Nasutus* Ameliorates Cytosolic and Mitochondrial Enzyme Levels in Streptozotocin-Induced Diabetic Rats. *Evid. Based Complement. Alternat. Med.* 2013, 486047. doi:10.1155/2013/486047

Visweswara Rao, P., Madhavi, K., Dhananjaya Naidu, M., and Gan, S. H. (2013b). *Rhinacanthus Nasutus* Improves the Levels of Liver Carbohydrate, Protein, Glycogen, and Liver Markers in Streptozotocin-Induced Diabetic Rats. *Evid. Based Complement. Alternat. Med.* 2013, 102901. doi:10.1155/2013/102901

Wait, S., and Chen, D. S. (2012). Towards the Eradication of Hepatitis B in Taiwan. *Kaohsiung J. Med. Sci.* 28 (1), 1–9. doi:10.1016/j.kjms.2011.10.027

Wang, G. K., Lin, B. B., Rao, R., Zhu, K., Qin, X. Y., Xie, G. Y., et al. (2013). A New Lignan with Anti-HBV Activity from the Roots of *Bombax ceiba*. *Nat. Prod. Res.* 27 (15), 1348–1352. doi:10.1080/14786419.2012.740032

Wang, H., Qiu, C., Chen, L., Abbasi, A. M., Guo, X., and Liu, R. H. (2019). Comparative Study of Phenolic Profiles, Antioxidant and Antiproliferative Activities in Different Vegetative Parts of Ramie (*Boehmeria Nivea* L.). *Molecules* 24 (8), 1551. doi:10.3390/molecules24081551

Wang, H., Teng, X., Zhang, Y., Gu, Q., and He, L. (2021). Diterpenoids from the Whole Plants of *Ajuga Nipponensis* and Their Inhibition of RANKL-Induced Osteoclastogenesis. *Chem. Biodivers.* 18 (1), e2000780. doi:10.1002/cbdv.202000780

Wang, L., Lu, S., Wang, L., Xin, M., Xu, Y., Wang, G., et al. (2021). Anti-inflammatory Effects of Three Withanolides Isolated from Physalis Angulata L. In LPS-Activated RAW 264.7 Cells through Blocking NF-Kb Signaling Pathway. *J. Ethnopharmacol.* 276, 114186. doi:10.1016/j.jep.2021.114186

Wang, Q., Liang, Y. Y., Li, K. W., Li, Y., Niu, F. J., Zhou, S. J., et al. (2021). Herba Siegesbeckiae: A Review on its Traditional Uses, Chemical Constituents, Pharmacological Activities and Clinical Studies. *J. Ethnopharmacol.* 275, 114117. doi:10.1016/j.jep.2021.114117

Wang, T. C., Lin, C. C., Lee, H. I., Yang, C., and Yang, C. C. (2010). Anti-hyperlipidemic Activity of Spider Brake (*Pteris Multifida*) with Rats Fed a High Cholesterol Diet. *Pharm. Biol.* 48 (2), 221–226. doi:10.3109/13880200903085458

Wang, T. C., Ti, M. C., Lo, S. C., and Yang, C. C. (2007). Free Radical-Scavenging Activity of Aqueous Extract of *Pteris Multifida* Poiret. *Fitoterapia* 78 (3), 248–249. doi:10.1016/j.fitote.2006.11.004

Wediasari, F., Nugroho, G. A., Fadhilah, Z., Elya, B., Setiawan, H., and Mozef, T. (2020). Hypoglycemic Effect of a Combined *Andrographis Paniculata* and *Caesalpinia Sappan* Extract in Streptozocin-Induced Diabetic Rats. *Adv. Pharmacol. Pharm. Sci.* 2020, 8856129. doi:10.1155/2020/8856129

Wu, D., Zhang, X., Liu, L., and Guo, Y. (2019). Key CMM Combinations in Prescriptions for Treating Mastitis and Working Mechanism Analysis Based on Network Pharmacology. *Evid. Based Complement. Alternat. Med.* 2019, 8245071. doi:10.1155/2019/8245071

Wu, J. N. (2005). *An Illustrated Chinese Materia Medica*. China: Oxiford University Press, 11p.

Wu, S. H., Hsieh, C. F., and Rejmanek, M. (2004). Catalogue of the Naturalized Flora of Taiwan. *Taiwania* 49 (1), 16–31. doi:10.6165/tai.2004.49(1).16

Wu, T., Wang, Q., Jiang, C., Morris-Natschke, S. L., Cui, H., Wang, Y., et al. (2015). Neoclerodane Diterpenoids from *Scutellaria Barbata* with Activity against Epstein-Barr Virus Lytic Replication. *J. Nat. Prod.* 78 (3), 500–509. doi:10.1021/np500988m

Wu, X. G., Wang, S. S., Miao, H., Cheng, J. J., Zhang, S. F., and Shang, Y. Z. (2016). Scutellaria Barbata Flavonoids Alleviate Memory Deficits and Neuronal Injuries Induced by Composited Aβ in Rats. *Behav. Brain Funct.* 12 (1), 33. doi:10.1186/s12993-016-0118-8

Wu, Y., and Chen, D. F. (2009). Anti-complementary Effect of Polysaccharide B3-PS1 in Herba Scutellariae Barbatae (*Scutellaria Barbata*). *Immunopharmacol. Immunotoxicol.* 31 (4), 696–701. doi:10.3109/08923970903095314

Wu, Y., Zhang, Z., Chen, T., Cheng, C., Zhang, Z., Zhou, H., et al. (2020). Comparison of Two *Polygonum Chinense* Varieties Used in Chinese Cool tea in Terms of Chemical Profiles and Antioxidant/anti-Inflammatory Activities. *Food Chem.* 310, 125840. doi:10.1016/j.foodchem.2019.125840

Wu, Y. H., Chiu, W. T., Young, M. J., Chang, T. H., Huang, Y. F., and Chou, C. Y. (2015). *Solanum Incanum* Extract Downregulates Aldehyde Dehydrogenase 1-mediated Stemness and Inhibits Tumor Formation in Ovarian Cancer Cells. *J. Cancer* 6 (10), 1011–1019. doi:10.7150/jca.12738

Wu, Z., Yang, L., He, L., Wang, L., and Peng, L. (2020). Systematic Elucidation of the Potential Mechanisms of Core Chinese Materia Medicas in Treating Liver Cancer Based on Network Pharmacology. *Evid. Based Complement. Alternat. Med.* 2020, 4763675. doi:10.1155/2020/4763675

Xin, Y.-J., Choi, S., Roh, K.-B., Cho, E., Ji, H., Weon, J. B., et al. (2021). Anti-inflammatory Activity and Mechanism of Isookanin, Isolated by Bioassay-Guided Fractionation from *Bidens Pilosa* L. *Molecules* 26 (2), 255. doi:10.3390/molecules26020255

Xu, J., Chen, D., Liu, C., Wu, X. Z., Dong, C. X., and Zhou, J. (2016). Structural Characterization and Anti-tumor Effects of an Inulin-type Fructan from *Atractylodes Chinensis*. *Int. J. Biol. Macromol.* 82, 765–771. doi:10.1016/j.ijbiomac.2015.10.082

Yang, J. Y., Kim, M. G., and Lee, H. S. (2013). Acaricidal Toxicities of 1-hydroxynaphthalene from *Scutellaria Barbata* and its Derivatives against House Dust and Storage Mites. *Planta Med.* 79 (11), 946–951. doi:10.1055/s-0032-1328631

Yang, W. C., Yang, C. Y., Liang, Y. C., Yang, C. W., Li, W. Q., Chung, C. Y., et al. (2019). Anti-coccidial Properties and Mechanisms of an Edible Herb, Bidens Pilosa, and its Active Compounds for Coccidiosis. *Sci. Rep.* 9 (1), 2896. doi:10.1038/s41598-019-39194-2

Yang, X., Gao, X., Du, B., Zhao, F., Feng, X., Zhang, H., et al. (2018). Ilex Asprella Aqueous Extracts Exert *In Vivo* Anti-inflammatory Effects by Regulating the NF-Kb, JAK2/STAT3, and MAPK Signaling Pathways. *J. Ethnopharmacol.* 225, 234–243. doi:10.1016/j.jep.2018.06.037

Ye, C. L., and Huang, Q. (2012). Extraction of Polysaccharides from Herbal Scutellaria Barbata D. Don (Ban-Zhi-Lian) and Their Antioxidant Activity. *Carbohydr. Polym.* 89 (4), 1131–1137. doi:10.1016/j.carbpol.2012.03.084

Yin, M. C., Chang, C. H., Su, C. H., Yu, B., and Hsu, Y. M. (2018). Pteris Multifida, Cortex Phellodendri, and Probiotics Attenuated Inflammatory Status and Immunity in Mice with a *Salmonella enterica* Serovar Typhimurium Infection. *Biosci. Biotechnol. Biochem.* 82 (5), 836–847. doi:10.1080/09168451.2018.1447356

Yu, J., Lei, J., Yu, H., Cai, X., and Zou, G. (2004). Chemical Composition and Antimicrobial Activity of the Essential Oil of *Scutellaria Barbata*. *Phytochemistry* 65 (7), 881–884. doi:10.1016/j.phytochem.2004.02.005

Yu, M. L., Chen, P. J., Dai, C. Y., Hu, T. H., Huang, C. F., Huang, Y. H., et al. (2020). 2020 Taiwan Consensus Statement on the Management of Hepatitis C: Part (I) General Population. *J. Formos. Med. Assoc.* 119 (6), 1019–1040. doi:10.1016/j.jfma.2020.04.003

Yu, S., Sheu, H. M., and Lee, C. H. (2017). *Solanum Incanum* Extract (SR-T100) Induces Melanoma Cell Apoptosis and Inhibits Established Lung Metastasis. *Oncotarget* 8 (61), 103509–103517. doi:10.18632/oncotarget.21508

Yuris, A., Goh, K. K. T., Hardacre, A. K., and Matia-Merino, L. (2019). The Effect of Gel Structure on the *In Vitro* Digestibility of Wheat Starch-Mesona Chinensis Polysaccharide Gels. *Food Funct.* 10 (1), 250–258. doi:10.1039/c8fo01501e

Zhang, L., Ren, B., Zhang, J., Liu, L., Liu, J., Jiang, G., et al. (2017). Anti-tumor Effect of Scutellaria Barbata D. Don Extracts on Ovarian Cancer and its Phytochemicals Characterisation. *J. Ethnopharmacol.* 206, 184–192. doi:10.1016/j.jep.2017.05.032

Zhang, R., Ma, C., Wei, Y., Wang, X., Jia, J., Li, J., et al. (2021). Isolation, Purification, Structural Characteristics, Pharmacological Activities, and Combined Action of Hedyotis Diffusa Polysaccharides: A Review. *Int. J. Biol. Macromol.* 183, 119–131. doi:10.1016/j.ijbiomac.2021.04.139

Zhang, W., Chen, S. T., He, Q. Y., Huang, L. Q., Li, X., Lai, X. P., et al. (2018). Asprellcosides B of Ilex Asprella Inhibits Influenza A Virus Infection by Blocking the Hemagglutinin- Mediated Membrane Fusion. *Front. Microbiol.* 9, 3325. doi:10.3389/fmicb.2018.03325

Zhang, Y., and Li, Y. (2016). Effect of Total Flavonoids of *Scutellaria Barbata* on Cognitive Function and Nogo-A Expression in the hippocampus in Cerebral Ischemia Model in Gerbils. *Pak. J. Pharm. Sci.* 29 (6 Suppl. l), 2373–2376.

Zhao, L. L., Makinde, E. A., Shah, M. A., Olatunji, O. J., and Panichayupakaranant, P. (2019). Rhinacanthins-rich Extract and Rhinacanthin C Ameliorate Oxidative Stress and Inflammation in Streptozotocin-Nicotinamide-Induced Diabetic Nephropathy. *J. Food Biochem.* 43 (4), e12812. doi:10.1111/jfbc.12812

Zhao, Z., Guo, P., and Brand, E. (2012). The Formation of Daodi Medicinal Materials. *J. Ethnopharmacol* 140 (3), 476–481. doi:10.1016/j.jep.2012.01.048

Zhou, X., Seto, S. W., Chang, D., Kiat, H., Razmovski-Naumovski, V., Chan, K., et al. (2016). Synergistic Effects of Chinese Herbal Medicine: A Comprehensive Review of Methodology and Current Research. *Front. Pharmacol.* 7, 201. doi:10.3389/fphar.2016.00201

Zhu, H., Liang, Q. H., Xiong, X. G., Wang, Y., Zhang, Z. H., Sun, M. J., et al. (2018). Anti-Inflammatory Effects of P-Coumaric Acid, a Natural Compound of *Oldenlandia Diffusa*, on Arthritis Model Rats. *Evid. Based Complement. Alternat. Med.* 2018, 5198594. doi:10.1155/2018/5198594

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 08 March 2022
doi: 10.3389/fphar.2022.816498

Moringa oleifera Seed at the Interface of Food and Medicine: Effect of Extracts on Some Reproductive Parameters, Hepatic and Renal Histology

Alfred F. Attah[1,2]*, *Opeyemi O. Akindele*[3], *Petra O. Nnamani*[4], *Ugochukwu J. Jonah*[5], *Mubo A. Sonibare*[1] and *Jones O. Moody*[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria, [2]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, University of Ilorin, Ilorin, Nigeria, [3]Laboratory for Reproductive Physiology and Developmental Programming, Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria, [4]Drug Delivery and Nanomedicines Research Unit/Public Health and Environmental Sustainability (PHES) Research Group, Department of Pharmaceutics, University of Nigeria, Nsukka, Nigeria, [5]Riyadh Al Khabra General Hospital, Riyadh, Saudi Arabia

OPEN ACCESS

Edited by:
Massimo Lucarini,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Xianli Wu,
United States Department of Agriculture, United States
Kennedy Honey Erlwanger,
University of the Witwatersrand, South Africa

***Correspondence:**
Alfred F. Attah
attah.fau@unilorin.edu.ng

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 16 November 2021
Accepted: 09 February 2022
Published: 08 March 2022

Citation:
Attah AF, Akindele OO, Nnamani PO, Jonah UJ, Sonibare MA and Moody JO (2022) Moringa oleifera Seed at the Interface of Food and Medicine: Effect of Extracts on Some Reproductive Parameters, Hepatic and Renal Histology. Front. Pharmacol. 13:816498. doi: 10.3389/fphar.2022.816498

The lipid-rich Seed of *Moringa oleifera* has been promoted as an effective water clarifier. Aside its vital nutritional application as an emerging food additive, the seed has continued to gain a wider acceptance in various global ethnomedicines for managing several communicable and lifestyle diseases, howbeit, its potential toxic effect, particularly on fertility and pregnancy outcomes has remained uninvestigated; the effect of *Moringa oleifera* seed (MOSE) aqueous-methanol extracts on fertility and pregnancy outcome, was investigated *in vivo* using female Wistar rats that were divided into 50, 100, 300 and 500 mg per kilogram body weight. Group six was given *Moringa oleifera* seed treated water *ad-libitum* (*ad-libitum* group). Organs harvested for histological assessment included ovary, uterus, liver and kidney. In addition to HPLC fingerprint and a preliminary peptide detection, we determined the physico-chemical characteristics and mineral content of MOSE using standard methods. Data were analyzed with significance at $p \leq 0.05$. There was no significant difference in the estrus cycle, mating index, gestation survival index, gestation index, fertility index and sex ratio among all groups. Gestation length was reduced in some groups. While the male pup birth weight was comparable among the different groups, female pups birth weights were significantly reduced in 50 and 100 mg groups. Anogenital distance indices of female pups in *ad libitum* group were significantly increased. Pathologies were observed in liver and kidneys of dams while kidneys of pups presented a dose dependent reduction in the number of glomeruli. There were no observed pathological changes in the ovary and uterus. This study showed for the first time in rodents, that the lipid-rich MOSE is unsafe to the kidney of rodents while the lipid-free MOSE appears to be safe at doses up to 300 mg/kg body weight. Findings from this study suggested that the female pups were masculinized. In conclusion, the lipid-rich seed extracts of MOSE appear to be unsafe during pregnancy, induce hepatic and renal toxicity while the lipid-free MOSE excludes inherent toxicity as the hydrophobic part has been linked to toxicity as observed in this study due to the developmental programming effect on female offspring in rodents.

Keywords: *Moringa oleifera*, seed, Wistar rats, hepatic toxicity, renal histology, reproductive parameters, developmental programming

1 INTRODUCTION

Based on accumulating scientific evidence, increasing number of plants are gaining acceptance as important sources of nutrients, required for growth, development, and prevention of lifestyle diseases (Witkamp and van Norren, 2018; Cisneros-Zevallos, 2021; Marques et al., 2021). Plants constitute an amazingly rich source of secondary metabolites, which have served as templates or active molecules for the prevention, treatment or cure for several diseases threatening mankind (Ashwin et al., 2021; Leuci et al., 2021; Pizzi, 2021). The application of edible plant species as both food and medicine has been well documented in countries endowed with rich Traditional Medicine and plant biodiversity (Cisneros-Zevallos, 2021; de Medeiros et al., 2021). Interestingly, the advancement in science, improved understanding of the crucial role of nutrition or more generally, lifestyle, in disease prevention/management tend to lend support to the practice of utilizing wild and cultivated botanicals at the interface of food and medicine. Thus, the popular phrase "Let thy food be thy medicine and thy medicine thy food" is increasingly gaining relevance in the 21st century (Witkamp and van Norren, 2018). *Moringa oleifera* (MO) Lam. (Moringaceae) and related 12 species within the genus Moringa is a typical example representing one of the most valued and widely used ethnomedicinal plant species occupying the food-medicine interface (Palada, 2019; Jattan et al., 2021).

Moringa oleifera (MO) is a resilient tree cultivated mainly within the tropical and sub-tropical regions of the world whose origin has been linked to two continents of Asia and Africa (Lakshmidevamma et al., 2021). More striking is the oral histories recorded by Lowell Fuglie in Senegal and many other parts of West Africa where the lifesaving nutritional applications of MO (Fuglie, 1999; Fahey, 2005) has continued to be harnessed. The mature and dry seeds from MO have been found useful in several traditional cultures especially those at the base of the economic ladder who lack access to clean drinking water. These native people have historically been applying crushed seeds obtained from MO to effect the clarification of turbid water for household use. The traditional technology was frequently observed with herdsmen whose main sources of drinking water have been muddy and cloudy lakes or running water. Thus, the use of crushed *Moringa oleifera* seeds (MOSE) as a cheap, accessible, affordable, sustainable and acceptable alternative for the treatment of unsafe water from multiple sources is well documented and scientifically validated (Gassenschmidt et al., 1995; Ghebremichael et al., 2005). Moringa seed is rich in lipids, with over 35% of each kernel containing mainly unsaturated fatty acids and other lipophilic metabolites (Ayerza, 2012), it is therefore logical to assume that crushed seeds locally applied to clarify polluted water will inadvertently leave some suspended oils and related metabolites in the treated water. It is reported in the literature that flocculant phytochemicals released from the seeds coagulate 80.0–99.5% turbidity with a concomitant bacterial load reduction of 90.0–99.99% (Madsen et al., 1987; Ueda Yamaguchi et al., 2021). Polypeptides present in the seeds have been implicated as the main flocculant molecules (Ghebremichael et al., 2005). Other secondary compounds isolated from the seed are lipids and glycosides including *O*-ethyl-4-(α-L-rhamnosyloxy) benzyl carbamate, 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, niazimicin, 3-*O*-(6′-*O*-oleoyl-β-D-glucopyranosyl)-β-sitosterol, β-sitosterol-3-*O*-β-D-glucopyranoside, niazirin, β-sitosterol and glycerol-1-(9-octadecanoate) (Guevara et al., 1999; Dzuvor et al., 2022). Some of the Moringa fatty acids and lipids have been reported as phytochemical agents with uterotonic potential (Gruber and O'Brien, 2011) making their intake potentially unsafe during pregnancy.

Many phytochemicals (chemical compounds of botanical origin) including steroidal alkaloids, pyridine-pyrrolidine alkaloids, substituted phenolic compounds, steroidal and triterpenoid glycosides, have been reported to affect growth and reproduction in animals (Francis et al., 2002). For instance, nicotinic alkaloids have been reported to produce morphological and behavioral changes in rodents (Khalki et al., 2012). Robert and colleagues (Kavlock et al., 1991) reported the *in vitro* developmental toxicity of phenolic compounds and demonstrated that once a phenol successfully gains entry into the embryo, the embryotoxicity of the compound is dependent on the degree of its lipophilicity. In other words, hydrophobic phenols appear to be more toxic to the developing embryo than highly polar phenols (Selassie et al., 1999). Using an effective concentration of less than 0.5 mM, the group reported the effect of 13 phenolic compounds in producing growth retardation and structural abnormalities *in vitro*. However, the findings from *in vivo* investigation suggest that only few of these substituted phenols, the lipophilic type actually produced statistically relevant developmental toxicity. The investigators used a dose range of 0–1000 mg/kg body weight of rodents. Furthermore, the potential *in vivo* embryotoxicity and teratogenicity of some botanical terpenes has also been reported; employing the embryonic *Danio rerio* (family: Cyprinidae) model, the teratogenic potentials of terpenes has been demonstrated following documented morphological defects, decreased hatchability and reduced heart rate (Thitinarongwate et al., 2021).

Raising scientific curiosity for further *in vivo*-inspired investigation is the report by Al-Anizi et al. (2014) regarding the cytotoxic and genotoxic potentials of the hydrophobic fraction (basically lipids) of MOSE. Meanwhile, folkloric claims regarding the abortifacient potential of some aerial parts of *Moringa oleifera* plant have been documented and scientifically validated (Nath et al., 1992; Fahey, 2005; Attah et al., 2020) and this should inform an elaborate *in vivo* reproductive toxicity studies on the seed of the plant which has now occupied an important global position at the

interphase of food and medicine. However, until now, there are currently no reported scientific findings on the potential effects of MOSE on fertility and pregnancy outcome. Yet the application of lipid-rich Moringa whole seed as well as the aqueous extracts of MOSE (containing suspended lipids) as an anti-infective and restorative nutraceutical (Xiong et al., 2020; Mohanty et al., 2021; Oyeleye et al., 2021; Reyes-Becerril et al., 2021; Santos et al., 2021; Xiong et al., 2021), as food additive (Milla et al., 2021; Okereke et al., 2021; Oyeleye et al., 2021) as well as for polluted water treatment in deprived rural settings (Lea, 2010; Palada, 2019; Aboagye et al., 2021; Ueda Yamaguchi et al., 2021) has continued to gain momentum across different regions of the world without any attention to its potential toxicities. Here, the effects of the lipid-containing MOSE aqueous-methanol extracts on fertility and pregnancy outcome have been elaborately investigated *in vivo* using rodents for their potential effects on reproductive parameters, ovary, uterus, renal and hepatic histology. Furthermore, the potential safety of the extract of the lipid-free seedcake as well as its physicochemical characteristics have been documented.

2 MATERIALS AND METHODS

2.1 Plant Materials and Extract Preparation

Approximately 3 kg of *Moringa oleifera* seeds freshly harvested from organic farmland in Zaria, Nigeria were obtained from Herbal Point, a local herbal medicinal store and the main distributor of MO products located in Zaria, Kaduna State, Nigeria. Viable seeds were further cultivated in a household plant garden in Ibadan, Nigeria and at least 3-months old growing plant samples were collected fresh and together with the seeds submitted for authentication at Forest Herbarium Ibadan (FHI). Voucher number was given (FHI: 109853) while the voucher specimen was deposited in the same herbarium. Further, seeds were mechanically hulled to remove the hulls in order to obtain the clean whitish seed kernels. To obtain the lipid-containing aqueous-methanol extract, the powdered material was extracted in distilled water/methanol (1:1 v/v) (Attah et al., 2016) for 24 h following occasional agitation. Freeze-dried and crispy samples were refrigerated until required for use. In preparing the lipid-free fraction, the lipid content was first removed using hexane. Briefly, the finely powdered kernels was extracted in food grade N-hexane for 24 h with occasional shaking at a sample/solvent ratio of 1:10 (w/v); filtration was carried out to separate out the marc (representing the seedcake). The well dried and crispy marc was further extracted with distilled water/methanol as described above. The freeze-dried aqueous extracts were kept refrigerated for the single dose 300 mg/kg body weight administration of the lipid-free MOSE. In addition, ~100 mg of powdered unextracted whole seed kernel (representing one seed kernel) was used to treat 1 L of well water sourced from a neighboring rural community at the University of Ibadan, Nigeria. The duration of 30 min treatment time was considered. The resultant filtrate represents the Moringa treated water which has been prepared according to its indigenous application in various African households. This was administered to the *ad libitum* group that had unlimited free access to the treated water.

2.2 Experimental Animals

Mature female Wistar rats weighing between 250–300 g and 150–170 g were respectively used for this *in vivo* studies. Animals were fed with a standard rat diet (Ladokun feeds, Ibadan, Nigeria) and allowed free access to water *ad libitum*. Distilled water was used to reconstitute freeze dried extract prior to administration. Administration of all extracts used has been achieved via the oral route (oral gavage) once daily using a maximum volume of 2 ml, administered at 1 ml at a time. All procedures used in this study conformed to the guideline of the care and use of animals in research and teaching (NIH publication, revised 1996) and were in line with the University of Ibadan Animal Welfare and Ethics Committee guidelines. All animals were therefore handled humanely in line with the ethics committee guidelines (approval number: FVM/UI/A.6/201301).

2.3 *In-Vivo* Experimental Procedure

Thirty female Wistar rats were randomly divided into six groups and treated with *Moringa oleifera* seed extract (MOSE) as follows: Group one received 1 ml of distilled water per kilogram body weight (1 ml/kg body weight). Groups two, three, four, and five received 50, 100, 300 and 500 mg of MOSE extract per kilogram body weight. Group six was given *Moringa oleifera* seed treated water *ad-libitum* constituting the *ad-libitum* group.

2.4 Determination of Estrous Cycle Pattern

Estrous cycle pattern was studied for 4 weeks; 2 weeks prior to the commencement of the administration of MOSE (pre-treatment period) and 2 weeks during which MOSE was administered before the animals were paired for mating (post-treatment period). The estrous cycle was monitored during the pre-treatment period to establish the estrous pattern of each animal. Briefly, vaginal smears were obtained using sterile cotton-tipped swab wetted with physiological saline under an ambient temperature. This was introduced about 1 cm deep into the vagina orifice ensuring the lowest possibility for excessive cervical stimulation. The swab was quickly but carefully turned and rolled once against the wall of the vagina and then gently removed. Collected epithelial cells of the vaginal wall were further transferred to a clean dry glass slide by moving the swab across the slide. The slide was air-dried, stained, and viewed under the light microscope. Morphometric analysis of stained vaginal smears was carried out under a light microscope followed by the determination of the number of epithelial cell types in each phase of the estrous cycle; where one estrous cycle has been defined as the number of days from one estrus to the next estrus. Analyzed cell types within the vaginal secretion include leucocytes, cornified epithelial cells and nucleated epithelial cells. The phase of estrous cycle during the experiment was based on the proportion of these cells detected in the vaginal secretion (Marcondes et al., 2002; Ajayi and Akhigbe, 2020).

2.5 Mating Procedure

The animals were paired with proven male breeders at ratio 2:1 (female: male). Vaginal lavage of the paired female animal was examined every morning after mating using the microscope to determine the presence of sperm cells. The day spermatozoa were seen in the vagina smear of the rat was designated as day one of pregnancy. All female rats spent 14 days with the male rats. The animals were allowed to litter naturally and the day of parturition was designated as postnatal day one. In summary, MOSE was administered to the experimental animals for a maximum period of 35 days, starting from the post treatment period till parturition. Animals were weighed weekly throughout the experiment. Anogenital distance and other morphometric indices of the pups were measured within 24 h after parturition using a digital Vernier caliper (Donguan Hust Tony Instrument, China). Mating index, fertility index, gestation survival index, gestation index, gestation length, litter size and sex ratio were evaluated using the following formulae;

$$\text{Mating index} = \frac{\text{Number of females mated}}{\text{Number of females cohabited}} \times 100$$

$$\text{Pregnancy index} = \frac{\text{Number of females delivering live newborn}}{\text{Number of females with evidence of pregnancy}} \times 100$$

$$\text{Delivery index} = \frac{\text{Number of delivering females}}{\text{Number of pregnant females}} \times 100$$

$$\text{Life birth index} = \frac{\text{Number of offspring living}}{\text{Number of offspring delivered}} \times 100$$

2.6 Sacrifice

All pups were sacrificed on postnatal day 14 while the dams were sacrificed during their first proestrus after the sacrifice of their pups. All sacrifice was done by cervical dislocation. The kidneys, liver, ovaries and uteri were carefully harvested from the dams while only the kidneys and liver were harvested from the pups. Organs were fixed in 10% formal saline in preparation for histological examination.

2.7 Procedure for Histology

The liver, kidney, uterus and ovary obtained from study animals were fixed in 10% formalin. They were routinely processed, using paraffin-wax embedding method. During sectioning, 5 μm thick sections were mounted on glass slides stained with Haematoxylin and Eosin. Histopathological assessment and photomicrography of the prepared slides was done. Photomicrographs were taken at ×100 and ×400 magnifications with a Digital Microscope, VJ-2005 DNMODEL BIO-MICROSCOPE®. using TS View CX Image® Software, File version 6.2.4.3. (Gunadi et al., 2020).

2.8 Physicochemical Characterization of Whole Seed Particles and the Lipid-Free *M. oleifera* Seed (MOSE)

2.8.1 Particle Morphology

The surface morphology of particles of MOSE that was previously freed from the potentially toxic lipid portion (lipid-free seedcake) as well as whole seed powder (lipid-rich) was observed by scanning electron microscopy (Hitachi S-4000 microscope) according to an earlier method (Nnamani et al., 2014; Attah et al., 2020). In brief, samples diluted with double-distilled water were deposited on film-coated copper grids and the samples were allowed an overnight drying at room temperature. The dried samples were visualized under the microscope and images of the particle microstructure were captured.

2.8.2 Particle Size and Polydispersity Index

The mean diameter and polydispersity index of the MOSE particles powder was measured using a Zetasizer Nano-ZS (Malvern Instruments, Worceshtire, United Kingdom) equipped with a 10 mW He–Ne laser employing the wavelength of 633 nm and a backscattering angle of 173° at 25°C.

2.8.3 Zeta Potential Analysis

The zeta potential of the powdered MOSE particles was determined via electrophoreticmobility measurements using a Zetasizer Nano-ZS (Malvern Instruments, Worceshtire, United Kingdom). The zeta potentialwas calculated applying the Helmholtz–Smoluchowski equation (n = 3).

2.9 Mineral Composition and Preliminary Peptidomic Analysis of MOSE

The mineral elemental composition of MOSE including calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) as well as heavy metals such as lead (Pb), cadmium (Cd), cobalt (Co), chromium (Cr) and nickel (Ni) were determined following the method described by the Association of Official Analytical Chemists (AOAC, 1990). All the determinations were carried out in triplicates. Briefly, after sample dehydration for 24 h at 105°C, 2 g each were digested with 8 ml of concentrated nitric acid, 2 mL concentrated sulphuric acid, and 2 mL hydrogen peroxide. This was followed by heat treatment for 4 h at 70°C and cooled; 20 m of distilled water was added, and further digestion was achieved by heat treatment in the presence of nitric acid and sulphuric acid. Drops of concentrated nitric acid was added until the complete oxidation of the organic matter assumed to have been reached when no further darkening of the solution occurs followed by a clear yellow colour solution on persistent heat application. Resulting mixture was cooled, insoluble solids filtered out with Whatman No. 42 filter paper and then transferred to suitable containers. The pH of the solutions was determined while the digested samples were read on Buck Scientific's Atomic Absorption Spectrophotometer (AAS: Model 210/211 VGP, United States). The values of calcium, magnesium and potassium have been reported in percentage while iron, zinc, manganese, copper and the analyzed heavy metals have been presented in mg/kg of the dry weight of MOSE (Diyaolu et al., 2021).

The pre-purified aqueous extract of MOSE was preliminarily analyzed for isotopically resolved groups of peptide peaks performed on an ABSciex 4800 reflector TOF/TOF (time-of-flight) analyzer (Framingham, MA). Approximately 0.5 μL of the peptide-rich extract was mixed with 3 μL of saturated alpha-

TABLE 1 | Effects of *Moringa oleifera* Lam. Aqueous Seed Extracts on Mating Index, Fertility index, Gestation Survival Index, Gestation Index and Gestation Length.

Groups	0 mg	50 mg	100 mg	300 mg	500 mg	Ad libitum
MI (%)	80 ± 1.80	80 ± 2.80	80 ± 1.30	80 ± 2.50	80 ± 3.50	80 ± 2.30
FI (%)	80 ± 1.40	80 ± 2.10	80 ± 2.50	80 ± 2.90	80 ± 2.80	80 ± 3.80
GSI (%)	100 ± 1.20	100 ± 3.30	100 ± 2.80	100 ± 3.20	100 ± 3.30	100 ± 2.50
GI (%)	100 ± 1.90	100 ± 1.80	100 ± 1.70	100 ± 3.00	100 ± 2.60	100 ± 3.10
GL (Days)	21 ± 1.30	21 ± 1.40	21 ± 1.10	20.8 ± 1.40	20.3 ± 1.0*	20.2 ± 1.20**

Values have been presented as percentages/Means ± SEM; n = *5. *p ≤ 0.05 and **p ≤ 0.01 compared with control. MI: Mating Index; FI: Fertility Index; GSI: Gestation Survival Index; GI: Gestation Index; GL: Gestation Length (20.3 and 20.2 may not be biologically significant due to the difference in mating time which was monitored once in a day).*

cyano-hydroxylcinnamic acid (matrix) prepared in 50% double distilled water (DDW), 50% acetonitrile, 0.1% trifluoroacetic acid (v/v/v) purchased from Sigma-Aldrich, St Louis, Missouri. This was spotted on the MALDI target plate and analyzed after allowing spot to dry in the dark. Acquired spectra were processed using the Data Explorer software v. 4.9 (Koehbach et al., 2013). To determine the general chemical nature of aqueous extracts of MOSE, the RPHPLC fingerprint of the extract was done. The lyophilized extract was reconstituted in double distilled water and the solution was filtered using further syringe filter with a pore size of 0.45 mm followed by analytical RP-HPLC analysis on a Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA), applying a flow rate of 1 mL/min, 2% gradient in a 60 min run time.

2.10 Statistical Analysis

Values were expressed as mean ± standard error of mean and as percentage. Data were analyzed using one-way analysis of variance and Student's *t*-test where applicable. $p \leq 0.05$ was taken as the level of significance while Bonferroni's post hoc multiple comparison test at 95% confidence Interval of difference was used.

3 RESULTS

To obtain extracts of the lipid-rich MOSE containing some lipid component, an equal volume of distilled water and methanol was used to extract the powdered seeds resulting in a yield of 4.7% of the dry weight containing some lipophilic and hydrophilic constituents; as methanol is known to lyse cells to release its entire content. The 24 h hexane extraction resulted in removing 30% of lipids from MOSE. While seedcake extraction produced a yield of 3%. The administered extracts or MOSE-treated water has been carefully prepared considering both aspects of the traditional application of the seed in water treatment, as a food supplement and as a phytomedicine. The effect of the administration of MOSE on some indices in Dams has been presented in **Table 1**, while effect on pups is presented in **Tables 2** and **3**. **Figure 1** presents the effect of MOSE on weight variation of Dams over the entire period of administration. **Figures 2**–**7** presents the effects of lipid-rich MOSE aqueous extracts on selected key organs while **Figure 8** demonstrates the effect of the extracts of lipid-free seedcake on selected organs. The physicochemical characters of the oil-rich and oil-free MOSE has been presented graphically in **Figure 9**.

3.1 Effects of *Moringa oleifera* Lam. Aqueous Seed Extracts on Body Weight

Throughout the 2 weeks of administration (pretreatment period), there were no significant differences in the weight gained by the test groups compared with control. 50 and 100 mg groups had higher weight gain than control but this was not statistically significant. The weight gained by 500 mg and *ad-libitum* groups was significantly lower than that of the 50 mg group (**Figure 1**). Although the 50 mg, 100 mg, and 300 mg groups had increased weight gain during the post-treatment period, these changes were not significant compared with weight gain during the pre-treatment period. Likewise, 500 mg and *ad-libitum groups* had reduced weight gain during the post-treatment period but these differences were not statistically significant.

3.2 Effects of *Moringa oleifera* Lam. Aqueous Seed Extracts on Frequency of Estrous Phases

The length of estrous cycle during the pre-treatment phase and post-treatment phase did not change significantly in all groups. There were no significant differences in frequency of post-treatment proestrus, estrus and diestrus in all treated groups compared with the corresponding pre-treatment phases. The frequency of post-treatment metestrus in the 50 mg group was however reduced compared with the pre-treatment phase ($p \leq 0.05$).

3.3 Effects of *Moringa oleifera* Lam. Aqueous Seed Extracts on Mating Index, Fertility Index, Gestation Survival Index, Gestation Index and Gestation Length

Mating index, fertility index and gestational survival index were not different among all groups (**Table 1**). Gestation length was significantly reduced in the 500 mg and *ad-libitum* groups compared with control.

3.4 Effects of *Moringa oleifera* Lam. Aqueous Seed Extracts on Litter Size and Sex Ratio

Sex ratio in the test groups, which ranged from 1.0–5.0 was not significantly different from that of control (3.0). Litter size of 6–7 was documented for the test groups which has not shown any significant difference from the control group (litter size = 7).

TABLE 2 | Effects of *Moringa oleifera* Lam. Aqueous seed extracts on morphometric indices of male pup.

Treatment	0 mg	50 mg	100 mg	300 mg	500 mg	*Ad-libitum*
Birth Weight (g)	5.5 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.3	5.5 ± 0.2	5.1 ± 0.1
ADI (mm/g)	1.5 ± 0.2	1.4 ± 0.1	2.1 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	2.2 ± 0.1
HAR	0.90 ± 0.03	0.84 ± 0.03	0.85 ± 0.02	0.87 ± 0.03	0.91 ± 0.03	0.91 ± 0.01

ADI: Anogenital distance Index; HAR: head to Abdominal ratio; Values presented as mean ± SEM; n = *5.*

TABLE 3 | Effects of *Moringa oleifera* Lam. Aqueous seed extracts on female pup morphometric indices.

Treatment	0 mg	50 mg	100 mg	300 mg	500 mg	Ad-libitum
Birth Weight (g)	5.8 ± 0.1	4.3 ± 0.4*	4.7 ± 0.2*	4.8 ± 0.2	5.4 ± 0.2	5.5 ± 0.4
ADI (mm/g)	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.1**
HAR	0.77 ± 0.04	0.89 ± 0.02	0.86 ± 0.04	0.89 ± 0.04	0.94 ± 0.02	0.87 ± 0.02

Values presented as mean ± SEM; n = *5.* *p ≤ *0.05,* **p ≤ *0.01 compared with control. ADI: Anogenital distance Index; HAR: head to Abdominal ratio.*

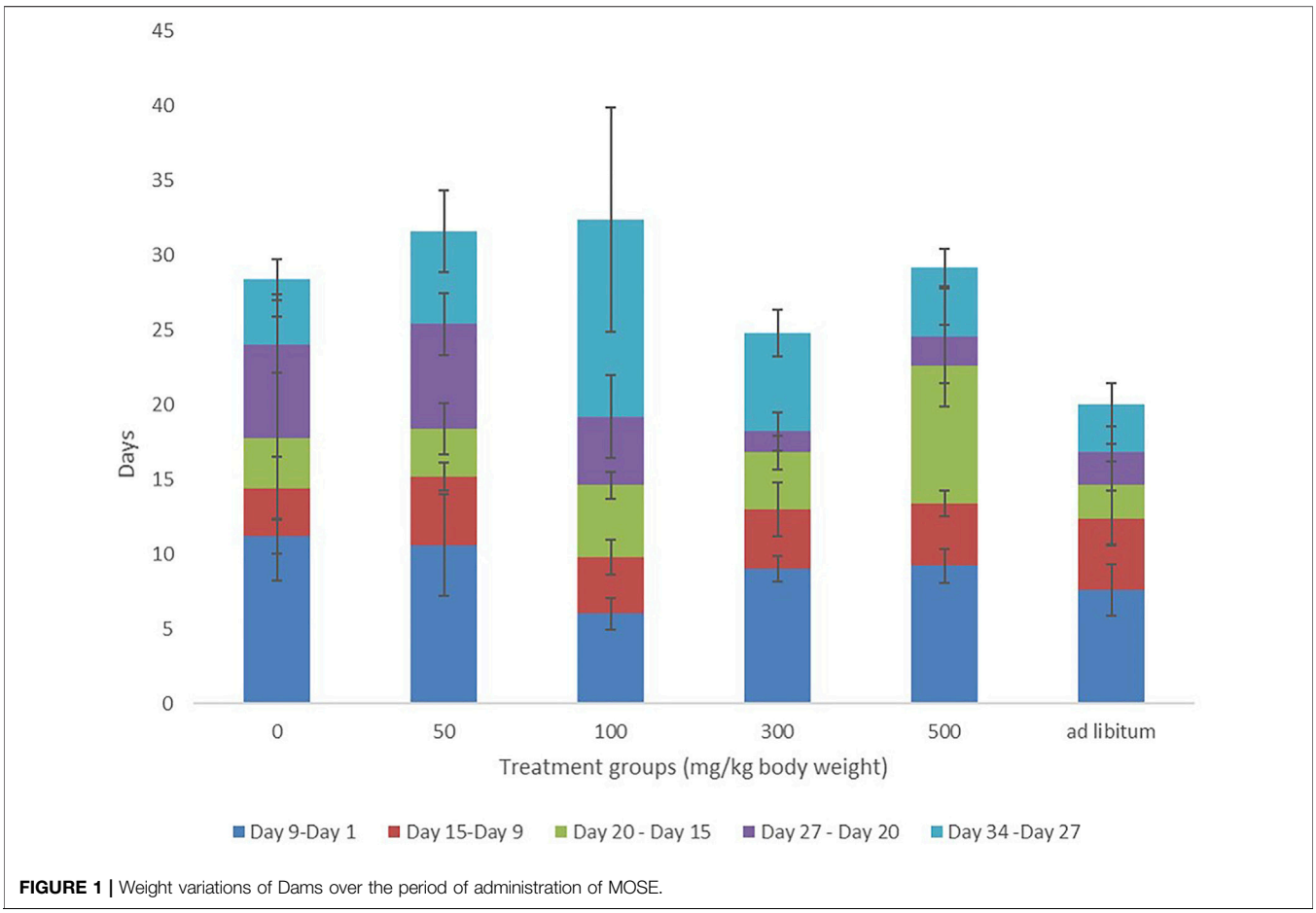


FIGURE 1 | Weight variations of Dams over the period of administration of MOSE.

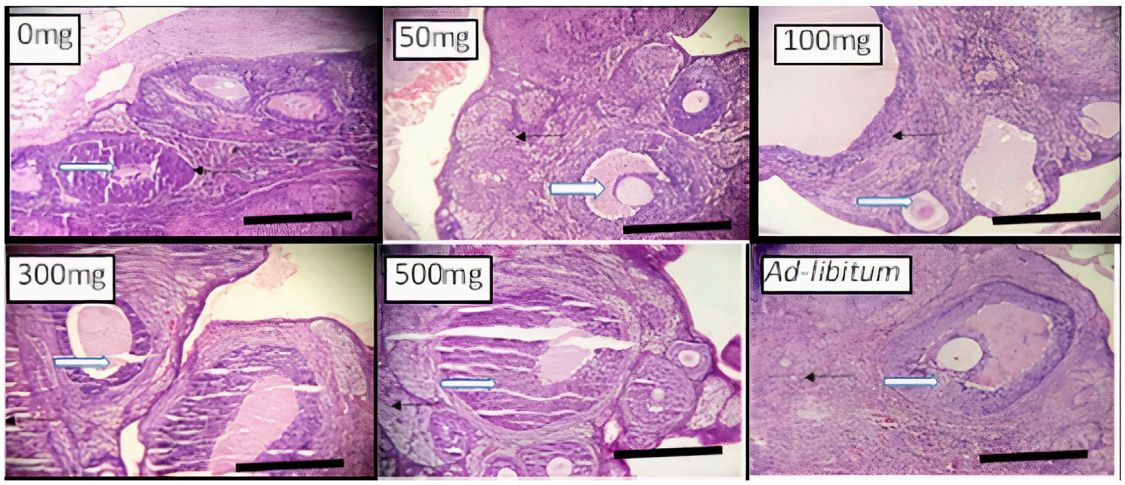


FIGURE 2 | Effects of *Moringa oleifera* Aqueous Seed Extracts on Ovarian tissue of Dams; H&E, X100, scale bar = 50 μm. Photomicrograph of an ovarian sections showing normal, developing follicles (white arrow) within the cortex. Moderate infiltration of inflammatory cells is seen in stroma (slender arrow) of 0 mg only. The stroma (slender arrows) appears normal in all other groups.

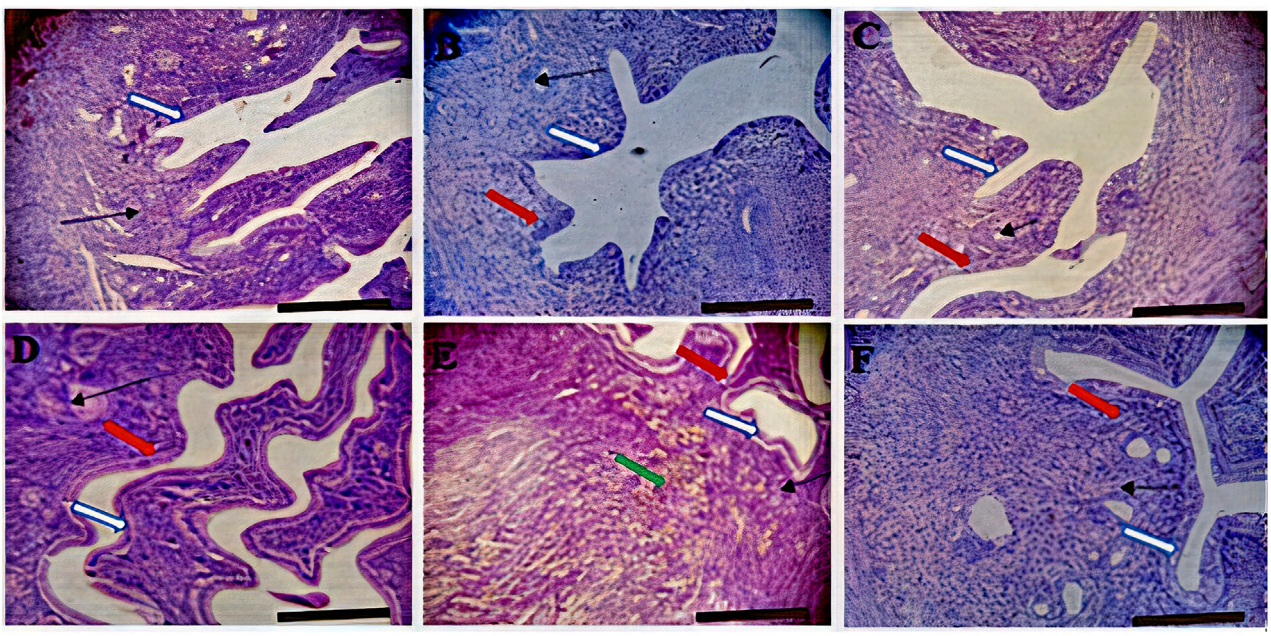


FIGURE 3 | Effects of *Moringa oleifera* Aqueous Seed Extracts on Uterine Tissue of Dams; H&E, X100, scale bar = 50 μm. **(A)**—0 mg: Photomicrograph of uterus showing normal epithelial layer (white arrow) and normal endometrial glands (slender arrow). The lumen is normal. **(B)**—50 mg: Endometrial layer with normal epithelial layer (white arrow) and normal endometrial glands (slender arrow). The endometrium is infiltrated by inflammatory cells including lymphocytes and polymorphonuclear cells (red arrow). **(C)**—100 mg: Endometrium with normal epithelial layer (white arrow) and normal endometrial glands (slender arrow). The endometrium is infiltrated by inflammatory cells including lymphocytes and polymorphonuclear cells (red arrow). **(D)**—300 mg: Endometrial layer with normal epithelial layer (white arrow) and proliferated endometrial cells and glands (slender arrow). The endometrium is very mildly infiltrated by inflammatory cells (red arrow) including lymphocytes and polymorphonuclear cells. **(E)**—500 mg: Endometrial layer with normal epithelial layer (white arrow) moderately proliferated endometrial cells and glands (slender arrow) with mild infiltration of inflammatory cells (red arrow). Mild haemorrhage seen (green arrow). **(F)**—*Ad-libitum*: Endometrial tissue with normal glandular cells (slender arrow) and moderate infiltration of inflammatory cells (red arrow). The epithelial linings are normal (white arrow).

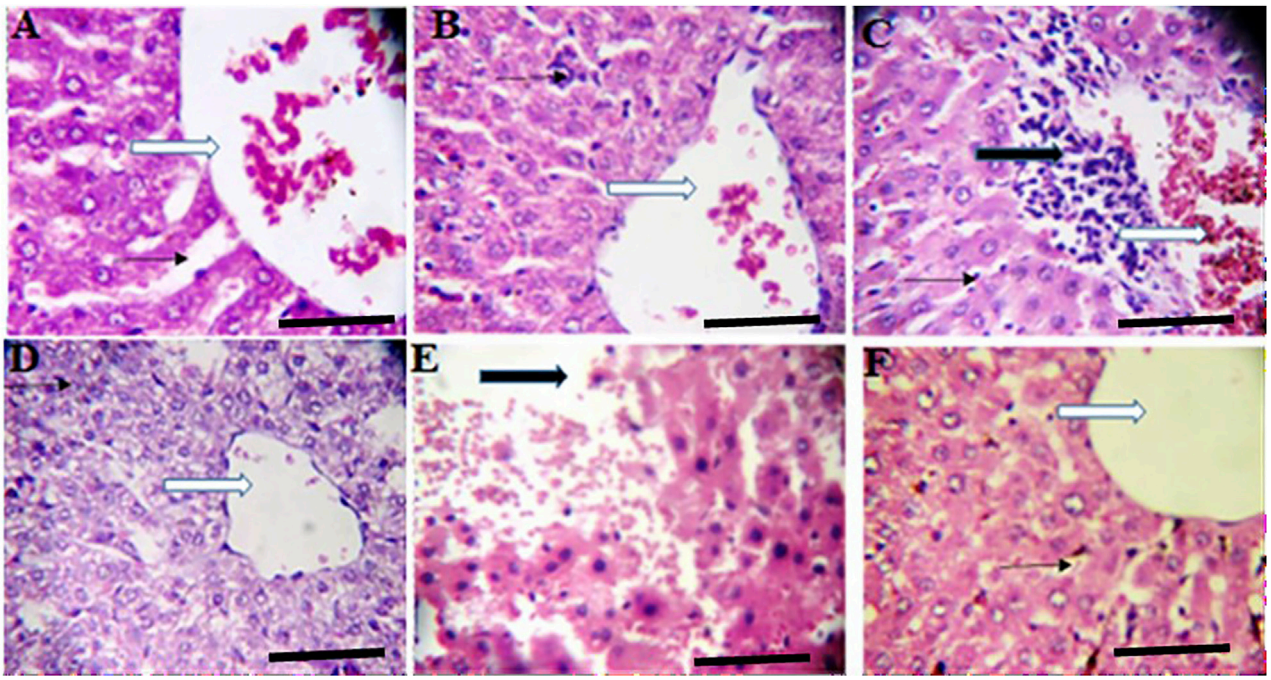


FIGURE 4 | Effects of *Moringa oleifera* Aqueous Seed Extracts on Liver of Dams; H&E, X400, scale bars = 50 μm. **(A)**—0 mg: Photomicrograph of normal liver section showing normal architecture, the central vessel appears normal (white arrow), the sinusoids (slender arrow) appear normal there is no infiltration. The morphology of the hepatocytes appear normal and no pathological leision is seen. **(B)**—50 mg: Normal liver architecture, the central venule appear normal (white arrow), no vascular congestion seen, the sinusoids show mild infiltration of inflammatory cells (slender arrow), the morphology of the hepatocytes appear normal. **(C)**—100 mg: Moderately normal liver architecture with normal central venule (white arrow), there are some focal areas of mild diffusion of red cells infiltrating the liver parenchyma and sinusoids (slender arrow). There is periportal infiltration (black arrow). The morphology of the hepatocytes appear normal. **(D)**—300 mg: Poor liver architecture. The central venule (white arrow) appear normal, no vascular congestion seen, the sinusoids appear mildly infiltrated (slender arrow). The morphology of the hepatocytes appears normal. **(E)**—500 mg: Poor architecture with loss of liver plate and diffused red cells (black arrow). There are hepatocytes with hyperchromic nuclei (blue arrow), others appear normal. **(F)**—*Ad-libitum:* Normal liver architecture, the central venule (white arrow). No vascular congestion is seen and the sinusoids appear normal (slender arrow). The morphology of the hepatocytes appear normal.

3.5 Effects of *Moringa oleifera* Lam. Aqueous Seed Extracts on Pup Morphometric Indices

There were no significant differences in the birth weight, anogenital distance index and head to abdominal ratio of male pups belonging to MOSE-treated groups compared with control group (**Table 2**). Birth weights of female pups belonging to 50 mg, 100 mg groups were reduced compared with that of control pups. Anogenital distance index of the female pups from *ad-libitum* dams was increased significantly compared with the anogenital distance index of the control group ($p \leq 0.01$). No significant difference was seen in the female pup head to abdominal ratio of test groups as compared with control group (**Table 3**). The weight variation documented during the treatment period has been presented in **Figure 1**.

3.6 Effects of Lipid-Rich *Moringa oleifera* Lam. Aqueous Seed Extracts on Selected Key Organs

3.6.1 Safety Evaluation of Lipid-free MOSE

Following the organ toxicity observed with the lipid-rich MOSE, we carried out a follow-up single dose (300 mg/kg body weight) *in vivo* experiment to fully validate previously reported *in vitro* findings and our hypothesis: that MOSE whole seed may be toxic and the separation of the lipid portion from the metabolite-rich seedcake will exclude the reported *in vitro* cytotoxicity/genotoxicity and the *in vivo* organ toxicity of MOSE documented in this study. The chosen dose of 300 mg/kg body weight was selected as it represents the median dose at which pathologies were observed during this *in vivo* study. Result from this experiment excluded the renal and hepatic pathologies observed with the lipid-containing MOSE extract. The liver, kidney and uterus studied showed normal architecture with no observable pathologies (**Figure 8**).

3.6.2 Physicochemical Characterization of MOSE Particles

The shape and surface morphology of both lipid-rich (whole seed powder) and lipid-free (seedcake) MOSE particles revealed a smooth and super porous spongy appearance (**Figure 9**), as seed-cake (images ACE) or whole seed powder (images BDF). Lower measurements of 1 μm showed somewhat more pronounced tubular channels in the whole seed powder than in the seed cake, perhaps due to presence of lipids in the whole seed powder. The particle size of

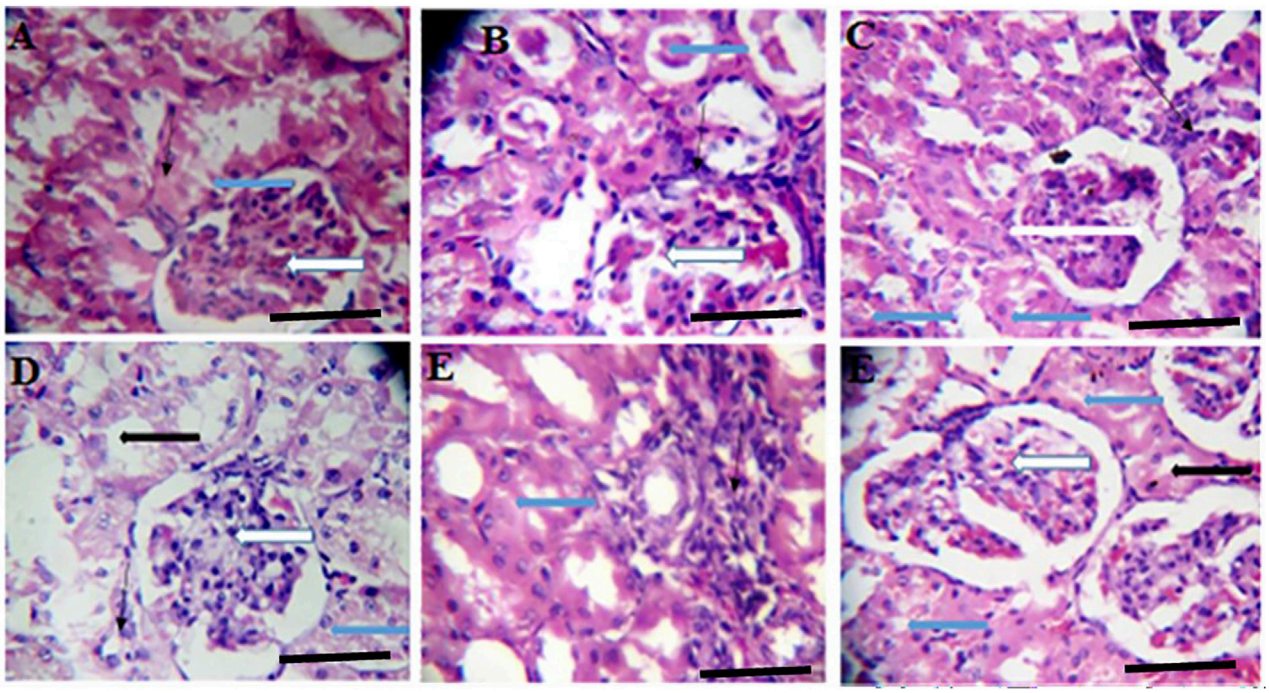


FIGURE 5 | Effects of *Moringa oleifera* Aqueous Seed Extracts on the Kidney of Dams; H&E, X400, scale bars = 50 μm. **(A)**—0 mg: photomicrograph of normal kidney section showing normal renal cortex with normal glomeruli (white arrow) containing normal mesangial cells and capsular spaces. The renal tubules shows normal epithelial distribution and appear normal (blue arrow), the interstitial spaces (slender arrow) are not infiltrated. **(B)**—50 mg: Some glomeruli with mild mesangial cells hyperplasia (white arrow). The renal tubules show mild tubular necrosis and eosinophilic cast (black arrow) within their lumen. The interstitial spaces are midly infiltrated (slender arrow). **(C)**—100 mg: Normal glomeruli containing normal mesangial cells and capsular spaces (white arrow). The renal tubules show moderate tubular necrosis with loss of brush border (blue arrow). There is very mild infiltration of interstitial spaces (slender arrow). **(D)**—300 mg: Normal glomeruli containing normal mesangial cells and capsular spaces (white arrow). The renal tubules shows moderate tubular necrosis with loss of brush border (blue arrow) while other tubules are intact (black arrow). The interstitial spaces appear normal (slender arrow). **(E)**—500 mg: Tubules with moderate tubular necrosis (blue arrow). The interstitial spaces show scanty infiltration (slender arrow). **(F)**—*Ad-libitum*: Normal glomeruli containing normal mesangial cells and capsular spaces (white arrow). There is mild tubular necrosis (blue arrow) while other renal tubules appear normal (black arrow).

MOSE particles was 848.6 nm with polydispersity index (pdi) of 0.75 and zeta potential measurement of −12 mV compared to the larger-sized whole seed powder (8.88 μm) with more variation in size (0.89 pdi) and slightly lower zeta potential value of −10.50 mV. However, it is clear that MOSE particles (seed cake powder or whole seed powder) were stable and had numerous pores and/or openings. Obviously, MOSE particles from the seedcake (without lipid portion) existed at nanoscale (~849 nm size) and can be said to be a nanomaterial. Nanomaterials have been shown to have more interesting properties than bulk materials (such as MOSE lipid-rich whole seed powder). This could explain why MOSE seedcake particles (without lipid portion) had lower reproductive toxicity than the larger particles from the whole seed particles containing oil portions. Hence, oil removal from MOSE results in a seedcake whose particle characteristics implies a safer reproductive effects than whole seed particles. Additionally, this favors the storage stability of MOSE particles of the seekcake over the whole seed powder.

Results obtained from the mineral analysis indicate that MOSE is rich in essential minerals like Ca (0.077%), Mg (0.160%), K (0.30%), Mn (10.43 mg/kg), Cu (1.92 mg/kg), Fe (159 mg/kg), and Zn (20.92 mg/kg). The heavy metal component of MOSE including Pb (0.03 mg/kg), Cd (not detected), Co (not detected), Cr (0.021 mg/kg) and Ni (0.019 mg/kg) were all found to be lower than the limit set by WHO/FAO (Diyaolu et al., 2021). The RP-HPLC fingerprint of the aqueous extract of MOSE indicated early to late early elution of MOSE metabolites within 4–23 min retention time (**Figure 10**). The MALDI-TOF MS was further used to screen for peptide occurrence leading to the detection of abundant number of peptides ranging from 1.0—4.0 kDa within the mass window analyzed.

4 DISCUSSION

Often ignored are the claims regarding the abortifacient potentials of some aerial parts of *Moringa oleifera* plant particularly the well valued fruit and potential toxicity of MOSE white kernels. Meanwhile, MOSE are now increasingly used as food additives, polluted water clarifier and as a phytomedicine without considering its potential toxicity. For instance, the *in vitro* cytotoxicity and genotoxicity of MOSE

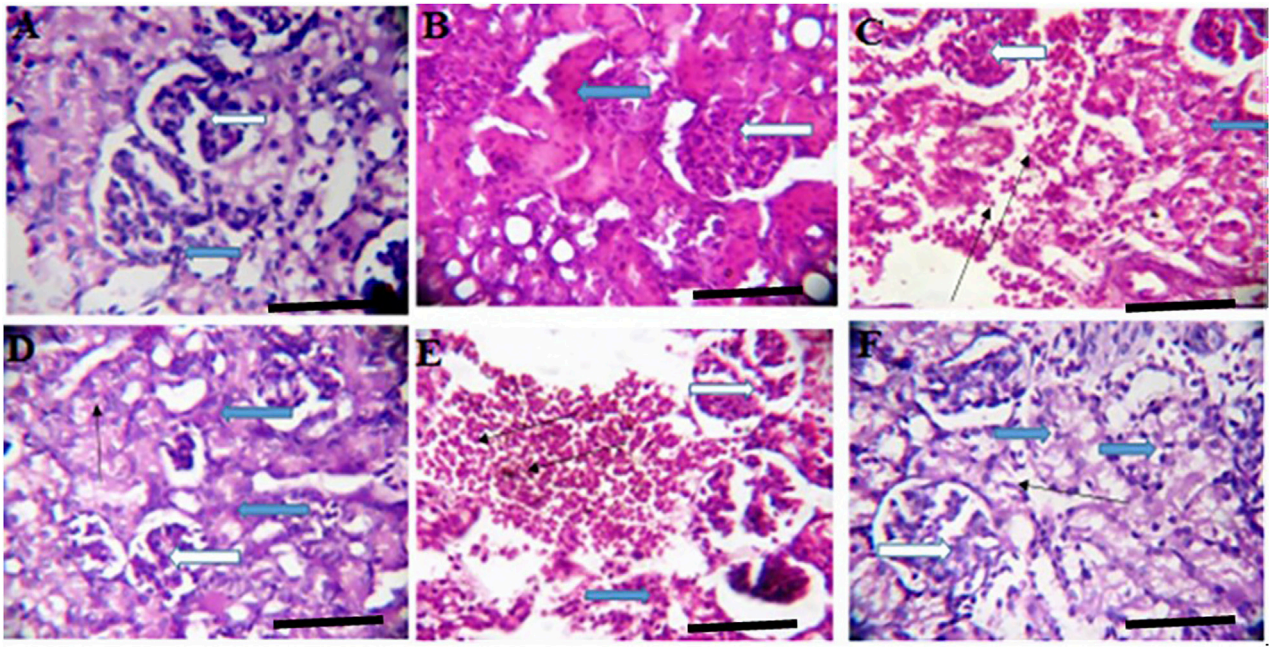


FIGURE 6 | Effects of *Moringa oleifera* Aqueous Seed Extracts on the Kidney of Pup; H&E, X400, scale bars = 50 μm. **(A)**—0 mg: Photomicrograph of normal kidney section showing normal multiple renal cortex with normal glomeruli (white arrow) containing normal mesangial cells and capsular spaces. The interstitial spaces show mild infiltration (slender arrow). **(B)**—50 mg: Numerous normal glomeruli within the renal cortex (white arrow). The renal tubules show attenuation and some tubules appear collapsed with lack of luminar spaces (black arrow). The interstitial spaces appear normal (slender arrow). **(C)**—100 mg: Numerous normal glomeruli within the renal cortex (white arrow). Some tubules are collapsed with diminished lumen (blue arrow) while others appear normal. The interstitial space is moderately widened and infiltrated by red cells (slender arrow) and few inflammatory cells. **(D)**—300 mg: Numerous compact glomeruli within the renal cortex (white arrow). The renal tubules shows acute tubular necrosis with loss of brush border (blue arrow). There are limiting lumen in the tubules as some tubules appear collapsed, the interstitial space is hardly defined (slender arrow). **(E)**—500 mg: Compact glomeruli within the renal cortex (white arrow). The renal parenchyma shows moderate fibrosis and tubules shows mild tubular necrosis (blue arrow). Some tubules are collapsed. There is vascular congestion seen in the interstitium (slender). The interstitial space is mildly infiltrated. **(F)**—*Ad-libitum*: Normal glomeruli containing normal mesangial cells and capsular spaces (white arrow). The renal tubules appear normal (blue arrow), the interstitial spaces appear normal and not infiltrated (slender arrow).

using the cell bioreporter model has been reported (Al-Anizi et al., 2014). To the best of our knowledge, this work represents the first report on the effect of MOSE aqueous extracts on fertility and pregnancy outcomes in rodents; although flocculant high molecular weight proteins have been characterized in Moringa seed kernel (Gassenschmidt et al., 1995; Ghebremichael et al., 2005; Dzuvor et al., 2022), we further report that MOSE seed kernel is rich in peptides and proteins as indicated by the findings from protein proximate analysis and the preliminary MALDI TOF-guided peptide detection. These numerous peptides are likely hydrolysates of bigger proteins which have been released during the harsh solvent-based extraction process.

This study investigated the effects of aqueous-methanol extracts of MOSE on, body weight gain, estrous cycle pattern, mating index, fertility index, gestation survival index, gestation index, gestation length, litter size, sex ratio, pup birth morphometric indices and selected organ histology. Here, the aqueous-methanol crude extracts of oil-rich and oil-free MOSE were used for the assessment of reproductive toxicity in female rats.

Aqueous MOSE extracts did not adversely affect the pregnancy length and the estrus cycle of the animals. This result tends to support the use of MOSE and its abundant polypeptides as flocculent molecules. However, it is of note that many bioactive agents reported in MOSE have been described as uterotonic compounds (Gruber and O'Brien, 2011). The very mild *in vivo* uterine contractility activities of MOSE aqueous extracts may be associated with the low occurrence of these uterine toning compounds in the seeds. More so, beta-sitosterol (a uterotonic agent) present in MOSE has been reported to be poorly absorbed in the gut (Babu and Jayaraman, 2020). This reduces the physiological beta-sitosterol and by extension reduces the abortifacient potential of the seed.

Also, MOSE did not have any statistically significant effect on body weight of rats. However, there was a dose dependent decrease in weight gain and the lower doses seemed to cause an increased weight gain while the higher doses caused reduction in weight gain. Several reports have shown that many phytochemicals present in medicinal plants negatively impact weight gain in animals (Jamroz et al., 2009; Van Hul and Cani, 2019). For instance, phenolic compounds such as tannins, which are reported present in MOSE (Santos et al., 2005) are capable of inhibiting digestive enzymes (Barrett et al., 2018) affecting the availability of amino acids, fatty acids and monosaccharides for absorption thereby leading to decreased weight gain. Foods rich in tannin are considered to be of low

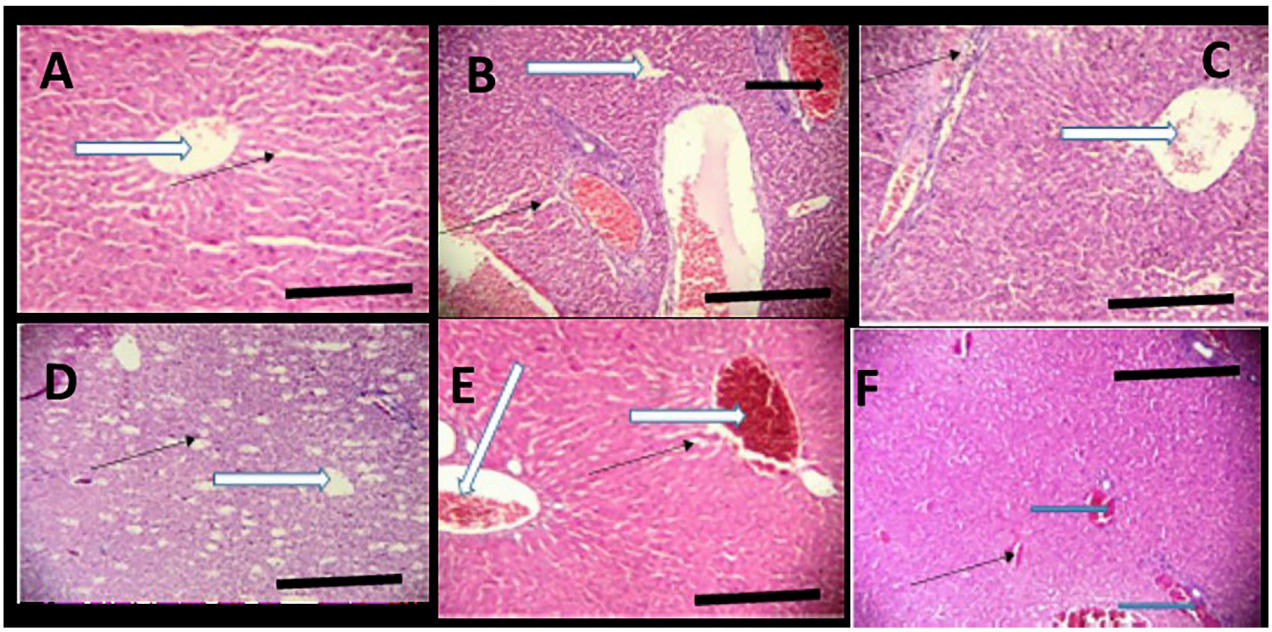


FIGURE 7 | Effects of *Moringa oleifera* Lam. Aqueous Seed Extracts on the Liver of Pup; H&E, X100, scale bars = 50 μm. **(A)**—0 mg: Photomicrograph of normal liver section showing normal architecture, the central vessel appear normal (white arrow), the sinusoids appear normal (slender arrow) and not infiltrated. No pathological leision seen. **(B)**—50 mg: Poor liver architecture. There is moderate vascular congestion seen (black arrow). The sinusoids show no infiltration of inflammatory cells (slender arrow). **(C)**—100 mg: Moderately normal liver architecture with normal central venule (white arrow). There is mild focal area of mild diffusion of red cells and inflammatory cells infiltrating the liver parenchyma and sinusoids (slender arrow). **(D)**—300 mg: Central venule (white arrow) without congestion. The sinusoids arewide and mildly infiltrated (slender arrow). **(E)**—500 mg: Poor liver architecture. The venule and portal vein are congested (white arrow), the sinusoids show moderate dilatation with mild infiltration of inflammatory cells, (slender arrow). **(F)**—*Ad-libitum*: Normal liver architecture. There is mild vascular congestion seen (blue arrow), the sinusoids appear normal with no infiltration.

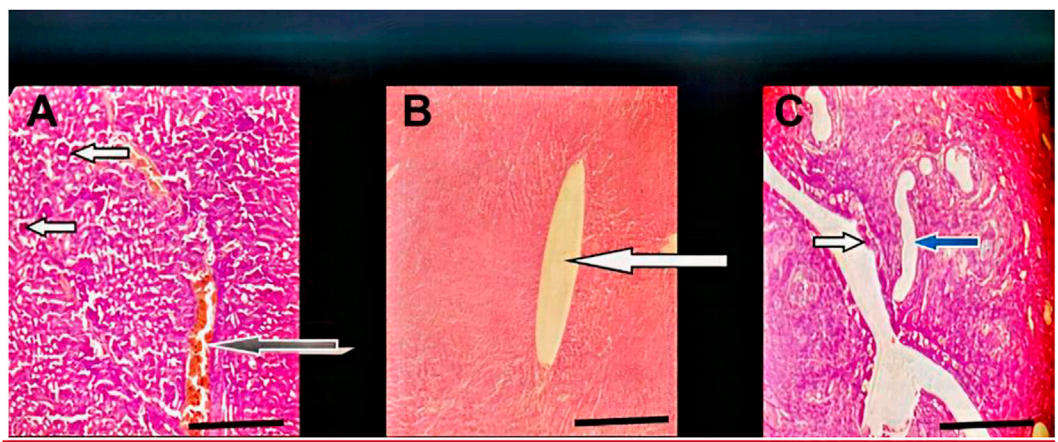


FIGURE 8 | Effect of administration of 300 mg/kg body weight MOSE Cake freed of oil on some selected organs of Wistar rats; H&E, X100, scale bar = 50 μm. **(A)** Photomicrographs of kidney sections showing moderately normal architecture, the renal cortex shows normal glomeruli with normal capsular spaces and mesengial cells (white arrow), most the renal tubules appear normal (blue arrow), however, few area s with collapsed tubules are noted (red arrow). the interstitial spaces appear normal and not infiltrated (slender arrow). **(B)** Moderate architecture of the central venules and portal tracts are not congested (white arrows), the sinusoids show scanty infiltration of inflammatory cells (slender arrow). Hepatocytes show normal morphology (blue arrow). **(C)** Normal uterus, the epithelium (spanned) of the uterine mucosa layer (endometrium) appears normal (white arrow), the myometrium and perimetrium appear normal, the lumen is clean (red arrow), the uterine glands show mild dilatation (blue arrow) (blue arrow).

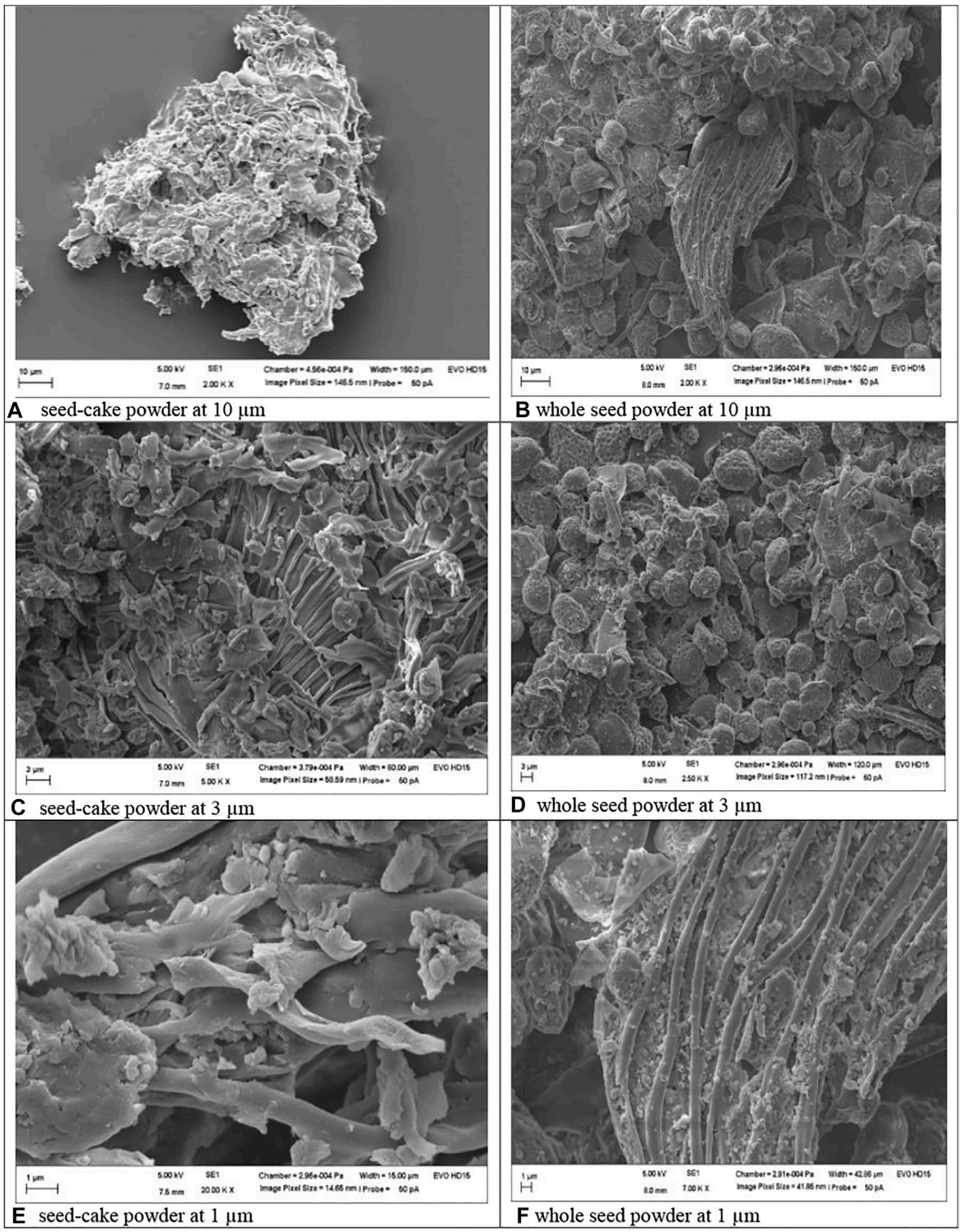


FIGURE 9 | Morphology of MOSE as seed-cake powder (images ACE) and whole seed powder (images BDE). Mineral, heavy metal and RP-HPLC fingerprint of MOSE aqueous extract.

nutritional value (Chung et al., 1998; Manzoor et al., 2020) and the addition of a high percentage of tannin to animal diet has been reported to reduce weight gain while addition of low tannin showed no such effect (Mkhize et al., 2018). The chemical markers for West African MOSE include proteins, oleic acid and flavan-3-ols, indicative of lower tannin content in

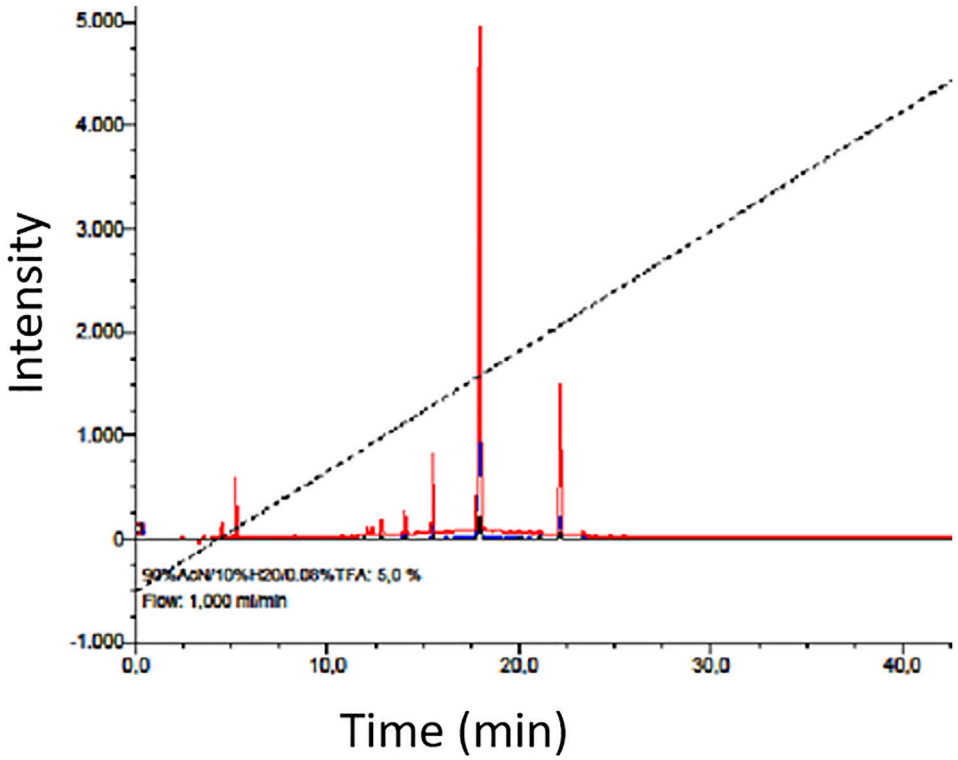


FIGURE 10 | RP-HPLC chromatogram showing a general fingerprint and chemical nature of the prepurified aqueous extract of MOSE; mainly indicating early and late early eluting metabolites as expected which have been preliminarily analyzed by MALDI-TOF MS (Koehbach et al., 2013) to contain abundant peptide fragments (ranging from 1000 Da to 4.0 kDa within the mass window analyzed).

MOSE (Fernandes et al., 2021). However, a high dosage of MOSE may lead to a cumulative effect and this may be one of the reasons why the animals that took higher doses had significant reduction in body weight gain compared with the 50 mg group. The question still remains, why was there an increase in body weight gain of the 50 mg group? MOSE is rich in carbohydrate, protein and fat and oil (Fernandes et al., 2021) and should naturally nourish the body. These rich dietary constituents, especially the fat content may be responsible for the increase in weight gain seen in the 50 mg group. However, as the dosage increases, so does the tannin content that naturally acts to inhibit digestive enzymes and prevents the availability of the end products of these rich dietary constituents.

The length of the estrous cycle was not affected by MOSE intake; however, the frequency of metestrus phase was reduced in the 50 mg group while the frequencies of other phases were not affected. Since the length of the cycle remained the same and reduction in metestrus frequency was not accompanied by alteration in the frequency of the other phases, MOSE did not seem to have any adverse effect on the length or regularity of estrous cycle. More so, metestrus is a transient phase immediately following ovulation in rats and this period may be very short or even absent (Ajayi and Akhigbe, 2020).

Histology of the ovary suggests that MOSE prevented pre-ovulatory inflammation. The infiltration in the ovary of the control rat was not a pathological reaction because proestrus seems to be associated with mast cell degranulation resulting in inflammation (Xu et al., 2020) that may be needed for the initiation of ovulation or estrus. Results from this study corroborates the results of other studies that reported the anti-inflammatory effects of *Moringa oleifera* seed extract (Xiong et al., 2020). This result also suggests that an increase in the frequency of metestrus was the outcome of prolonged proestrus. Though the alteration in frequencies of proestrus was not significant during the 2 weeks of administration, the deviation pattern of the cycle suggests that chronic intake could cause a significant effect which may eventually alter the pattern of the cycle. Contrary to the anti-inflammatory activity exerted by MOSE in the ovaries, the uterine and liver tissues of all test groups had infiltration of inflammatory cells. Why this is so being not understood. Administration of MOSE induced renal toxicity as tubular necrosis was observed in all the groups that took it. Pathological changes were also observed in the liver of rodents investigated. These *in vivo* findings suggest that the lipid-rich MOSE extract may be toxic to the liver and kidney. This contradicts several reports in local journals regarding the hepato-protective and nephroprotective effects of MOSE but is in agreement with well investigated *in vitro* reports by Al-Anizi et al. (Al-Anizi et al, 2014). These opposing reports may be associated with the part of the plant used for the assay, geographical variations or other extant factors. In this

experiment, only the aqueous extracts of the husked seed (called kernel) of MOSE was used. This approach is most similar to how the seed is used in ethnomedicine, as food additive and as polluted water clarifier.

Fertility indices of the test animals in this study were not different from that of control. The result on litter size also suggests that implantation was not affected since the MOSE-treated groups had similar litter size as the control group. Gestation length appeared not to be significantly affected across all the experimental groups. Thus, all pups born to the MOSE-treated mothers were viable at birth and till sacrifice. This finding is in line with a report on the improvement of poultry egg characteristics without any effect on fertility when given MOSE (Ashour et al., 2020).

Dietary method of preconceptional selection of fetal sex relies on the ionic content of the female diet in humans (Stolkowski and Lorrain, 1980; Alhimaidi et al., 2021). MOSE is rich in potassium, calcium, magnesium and phosphorus and did not affect sex ratio in this work. Diets with a high ratio of sodium and potassium to calcium and magnesium favour the birth of male offspring while the low ratio of these ions favours the birth of female offspring (Alhimaidi et al., 2021). The MOSE used during this study are richer in calcium and potassium than in magnesium. The distribution of these ions in MOSE appear not to be in conformity with the classical pattern of ion distribution that has been suggested to lead to more male or female births.

The environment encountered *in-utero* by a developing fetus exerts a profound influence on physiological function and risk of disease in adult life (Calkins and Devaskar, 2011; Hsu and Tain, 2019). Anthropometry in humans and morphometry in animals could provide useful information for determining the risk of disease in adult life (Tuvemo et al., 1999). The male pup morphometry was not affected by MOSE treatment. The reduction in birth weight of the female pups belonging to the 50 and 100 mg groups suggests that maternal MOSE-treatment had a fetal programming effect on these offspring. The implication of this is that these pups may develop a range of diseases later in life. The female pups of the *ad-libitum* groups also showed a sign of masculinization as they had increased anogenital distance index. Alterations in the human anogenital distance were first seen in female infants that had congenital adrenal hyperplasia, and they were presented with longer anogenital distance than girls without the disorder (Hsieh et al., 2008). Anogenital distance measurement in humans is a non-invasive method of determining the degree of masculinization and androgen exposure during fetal life (Schwartz et al., 2019). *Moringa oleifera* seed extract was reported to exert an androgenic effect in a study (Obembe, 2019) and such effect during gestation may be associated with the masculinization of the female reproductive organs as well as the female brain.

Findings from this study indicate that the liver and kidney of pups belonging to the treated groups were programmed *in-utero* by maternal MOSE intake. This was evident in the varying degrees of pathologies seen in these tissues as early as 2 weeks' post-natal life. This suggests that intake of MOSE during gestation may be toxic to fetal development. The effects exerted on the kidney and liver were not gender specific and did not seem to be dose-dependent. Result obtained from the heavy metal occurrence in MOSE which were all below standard limits set by the WHO/FAO suggests that the toxicity of lipid-rich MOSE used in this study was not associated with the occurrence of toxic metals. This further provides support for the work of Al-alnizi (Al-Anizi et al., 2014) regarding the cytotoxicity and genotoxicity of lipid-rich MOSE which was excluded when MOSE was freed of its lipid content and administered to the test rodents at a dose of 300 mg/kg body weight. Findings from this study are also consistent with a recent report on the toxicity of *Moringa stenopetala*, a close member of the genus Moringa, on the reproductive indices of rodents (Teshome et al., 2021).

Aside providing insight to the nature of metabolites present, the RP-HPLC fingerprint as well as the abundant peptide masses detected by the MALDI TOF peptidomics provide a useful link between the hydrophilic less toxic component of MOSE and the lypohpilic toxic component of MOSE and further validated the efficiency of the extraction of the two (i.e aqueous lipid-free and lipid-rich) components of MOSE used in this study; hence, the analytical RP-HPLC chromatogram of aqueous extract of the lipid-free MOSE indicated presence of hydrophilic compounds (with early retention times) as well as less hydrophilic metabolites (hydrophobic behaviour) as judged by the major signals observed. This result is consistent with a large body of literature on the characterization of hydrophilic and less hydrophilic compounds (including, phenolics, glycosides and alkaloids) from MOSE (Xiong et al., 2020; Dzuvor et al., 2022; Xiong et al., 2021). Together, these results provide starting point and could guide future further mechanistic studies.

The physicochemical characterization of MOSE particles demonstrated clearly that MOSE particles (seed cake powder or whole seed powder) were stable and had numerous pores and/or openings. These pores could possibly be responsible for the efficient occlusion, entrapment, adhesion or attachment of moieties to MOSE particles when used as medicine, nutraceutical, food and for water clarification. The particles were more amorphous in the seed-cake forms (images A, C and E) compared to the whole seed powder (images B, D and F) possibly due to the absence of lipids which in other words were responsible for the bigger size, more tubular appearance and possibility far less storage stability of the whole seed powder particles. Meanwhile, MOSE particles of the seekcake existed at the nanoscale (~849 nm) and as a nanomaterial could be expected to have more attractive properties than the bulk whole seed powder. This could explain why the former had safer reproductive effects that the later. A link between the physicochemical characteristics of a compound or nanoparticle and its toxic manifestation has been reported and this suggest that physicochemical properties generally influence the toxic manifestations of these phytochemical compounds and nanomaterials (Price et al., 2009). It has been documented that the physicochemical properties of food are mainly responsible for the final quality of the product which could make it more or less toxic (Mat Lazim et al., 2021). Moreover, the analysis of these properties is

crucial for design and quality control during food processing. Here we present MOSE as food and medicine thus supporting the need for the physicochemical characterisation of MOSE particles.

In the absence of the toxic lipid portion, our findings suggest that MOSE seedcake possess far more interesting particle characteristics suitable for optimal product development and efficient manufacturing and this lends support to the potential efficiency and effectiveness in the application of the seedcake at the interphase of food and medicine. Additionally, findings from the physicochemical characterization of MOSE suggest that the application of the lipid-free seedcake of MOSE at the interface of food and medicine will not only make it safer and healthier, but will extend the shelf life of the product.

5 CONCLUSION

It is concluded in this study that oral administration of lipid-rich *Moringa oleifera* seed (MOSE) did not produce any serious adverse effect on estrous cycle and fertility of female Wistar rats. However, female offspring of the dams showed evidence of intra-uterine growth restriction and masculinization. Documented in this study is intrauterine programming of hepatic and renal organs of the offspring of MOSE-treated dams and this effect seemed to be more profound on the female offspring. Furthermore, the kidney and liver tissues showed varying degrees of pathologies in both male and female offspring suggestive of toxicity. Interestingly, the observed toxicity was excluded when lipid-free MOSE was administered at a dose of 300 mg/kg body weight. At the interface of food and medicine, common anecdotal claims on the intake of MOSE for medicinal purposes may be beneficial, however, this is discouraged except after excluding the lipid portion. The addition of the nutrient-laden MOSE powder yet to be defatted as a nutritional food additive may be unsafe. We suggest the safe use of lipid free but peptide rich seedcake for nutritional fortification.

This work therefore supports the safe use of lipid free MOSE extract and polypeptides for achieving clean drinking water in economically deprived settings as previous *in vitro* and our first reported *in vivo* evidence supports the toxicity of the lipid-rich kernels. The hydrophobic constituents have been associated with observed cytotoxicity and genotoxicity, and should be removed prior to its use for water treatment, as Food additive or for formulation into an effective phytomedicine. For polluted water treatment, the lipid-free seed cake may equally be adsorbed unto sand particles that have been innovatively parked into a stainless metal filter tube for point-of-use in households as well as for small rural community application; however, a pilot study on this is suggested for proof-of-concept.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Ibadan Animal Welfare and Ethics Committee (approval number: FVM/UI/A.6/201301).

AUTHOR CONTRIBUTIONS

AA: Conceptualisation, methodology, data curation, formal analysis, writing—original draft, review and editing. OA: Data curation, raw material acquisition, writing—review and editing. PN: Methodology, Writing—review and editing. UJ: Review and editing; MS and JM: Supervision, review and editing.

FUNDING

This work was supported by Grand Challenges Canada's Stars in Global Health Round 5, Phase I, grant number S5 0431-01 and Round 4 Phase I, Grant Number S4 0251-01. Grand Challenges Canada is funded by the Government of Canada and is dedicated to supporting Bold Ideas with Big Impact in global health. Authors also acknowledge the support from the Nigerian Government through the Tertiary Education Trust Fund (TETFUND/DR and D/CE/SI/CoV-19/UI/VOL.1).

ACKNOWLEDGMENTS

Authors thank Assoc. Prof. Christian W. Gruber, Center for Physiology and Pharmacology, Medical University of Vienna, Austria, for access to the mass spectrometry facility. Authors also acknowledge and thank Prof. Y. Raji, Laboratory for Reproductive Physiology and Developmental Programming, Department of Physiology, College of Medicine, University of Ibadan, Nigeria and many thanks to Gbeoluwaga, Department of Physiology, University of Ibadan, Nigeria for their assistance during the experiment.

REFERENCES

Aboagye, G., Navele, M., and Essuman, E. (2021). Protocols for Assessing Antibacterial and Water Coagulation Potential of Moringa Oleifera Seed Powder. *MethodsX* 8, 101283. doi:10.1016/j.mex.2021.101283

Ajayi, A. F., and Akhigbe, R. E. (2020). Staging of the Estrous Cycle and Induction of Estrus in Experimental Rodents: an Update. *Fertil. Res. Pract.* 6 (1), 5–15. doi:10.1186/s40738-020-00074-3

Al-Anizi, A. A., Hellyer, M. T., and Zhang, D. (2014). Toxicity Assessment and Modelling of Moringa Oleifera Seeds in Water Purification by Whole Cell Bioreporter. *Water Res.* 56, 77–87. doi:10.1016/j.watres.2014.02.045

Alhimaidi, A. R., Ammari, A. A., Alghadi, M. Q., Al Saiady, M. Y., Amran, R. A., and Swelum, A. A. (2021). Sex Preselection of Sheep Embryo by Altering the Minerals of Maternal Nutrition. *Saudi J. Biol. Sci.* 28 (1), 680–684. doi:10.1016/j.sjbs.2020.10.059

Ashour, E. A., El-Kholy, M. S., Alagawany, M., Abd El-Hack, M. E., Mohamed, L. A., Taha, A. E., et al. (2020). Effect of Dietary Supplementation with Moringa Oleifera Leaves And/or Seeds Powder on Production, Egg Characteristics, Hatchability and Blood Chemistry of Laying Japanese Quails. *Sustainability* 12 (6), 2463. doi:10.3390/su12062463

Ashwin, K., Pattanaik, A. K., and Howarth, G. S. (2021). Polyphenolic Bioactives as an Emerging Group of Nutraceuticals for Promotion of Gut Health: A Review. *Food Biosci.* 44, 101376. doi:10.1016/j.fbio.2021.101376

Attah, A. F., Hellinger, R., Sonibare, M. A., Moody, J. O., Arrowsmith, S., Wray, S., et al. (2016). Ethnobotanical Survey of Rinorea Dentata (Violaceae) Used in South-Western Nigerian Ethnomedicine and Detection of Cyclotides. *J. Ethnopharmacol* 179, 83–91. doi:10.1016/j.jep.2015.12.038

Attah, A. F., Moody, J. O., Sonibare, M. A., Salahdeen, H. H., Akindele, O. O., Nnamani, P. O., et al. (2020). Aqueous Extract of Moringa Oleifera Leaf Used in Nigerian Ethnomedicine Alters conception and Some Pregnancy Outcomes in Wistar Rat. *South Afr. J. Bot.* 129, 255–262. doi:10.1016/j.sajb.2019.07.041

Ayerza, R. (2012). Seed and Oil Yields of Moringa Oleifera Variety Periyakalum-1 Introduced for Oil Production in Four Ecosystems of South America. *Ind. Crops Prod.* 36 (1), 70–73. doi:10.1016/j.indcrop.2011.08.008

Babu, S., and Jayaraman, S. (2020). An Update on β-sitosterol: A Potential Herbal Nutraceutical for Diabetic Management. *Biomed. Pharmacother.* 131, 110702. doi:10.1016/j.biopha.2020.110702

Barrett, A. H., Farhadi, N. F., and Smith, T. J. (2018). Slowing Starch Digestion and Inhibiting Digestive Enzyme Activity Using Plant Flavanols/tannins- A Review of Efficacy and Mechanisms. *Lwt* 87, 394–399. doi:10.1016/j.lwt.2017.09.002

Calkins, K., and Devaskar, S. U. (2011). Fetal Origins of Adult Disease. *Curr. Probl. Pediatr. Adolesc. Health Care* 41 (6), 158–176. doi:10.1016/j.cppeds.2011.01.001

Chung, K.-T., Wong, T. Y., Wei, C.-I., Huang, Y.-W., and Lin, Y. (1998). Tannins and Human Health: A Review. *Crit. Rev. Food Sci. Nutr.* 38 (6), 421–464. doi:10.1080/10408699891274273

Cisneros-Zevallos, L. (2021). The Power of Plants: How Fruit and Vegetables Work as Source of Nutraceuticals and Supplements. *Int. J. Food Sci. Nutr.* 72 (5), 660–664. doi:10.1080/09637486.2020.1852194

de Medeiros, P. M., Figueiredo, K. F., Gonçalves, P. H. S., de Almeida Caetano, R., da Costa Santos, É. M., Dos Santos, G. M. C., et al. (2021). Wild Plants and the Food-Medicine Continuum—An Ethnobotanical Survey in Chapada Diamantina (Northeastern Brazil). *J. Ethnobiol. Ethnomed* 17 (1), 1–10. doi:10.1186/s13002-021-00463-y

Diyaolu, O. A., Attah, A. F., Oluwabusola, E. T., Moody, J. O., Jaspars, M., and Ebel, R. (2021). Heavy Metals, Proximate Analysis and Brine Shrimp Lethality of Vernonia Amygdalina and Ocimum Gratissimum Growing in Crude Oil-Rich Delta State, Nigeria. *Foods* 10 (12), 2913. doi:10.3390/foods10122913

Dzuvor, C. K., Pan, S., Amanze, C., Amuzu, P., Asakiya, C., and Kubi, F. (2022). Bioactive Components from Moringa Oleifera Seeds: Production, Functionalities and Applications–A Critical Review. *Critical Reviews in Biotechnology* 42 (2), 271–293.

Fahey, J. W. (2005). Moringa Oleifera: A Review of the Medical Evidence for its Nutritional, Therapeutic, and Prophylactic Properties Part 1. *Trees for life Journal*, 1 (5), 1–15.

Fernandes, Â., Bancessi, A., Pinela, J., Dias, M. I., Liberal, Â., Calhelha, R. C., et al. (2021). Nutritional and Phytochemical Profiles and Biological Activities of Moringa Oleifera Lam. Edible Parts from Guinea-Bissau (West Africa). *Food Chem.* 341, 128229. doi:10.1016/j.foodchem.2020.128229

Francis, G., Kerem, Z., Makkar, H. P., and Becker, K. (2002). The Biological Action of Saponins in Animal Systems: a Review. *Br. J. Nutr.* 88 (6), 587–605. doi:10.1079/BJN2002725

Fuglie, L. J. (1999). *The Miracle Tree: Moringa Oleifera, Natural Nutrition for the Tropics*. Senegal: Dakar (Senegal) CWS.

Gassenschmidt, U., Jany, K. D., Tauscher, B., and Niebergall, H. (1995). Isolation and Characterization of a Flocculating Protein from Moringa Oleifera Lam. *Biochim. Biophys. Acta* 1243 (3), 477–481. doi:10.1016/0304-4165(94)00176-x

Ghebremichael, K. A., Gunaratna, K. R., Henriksson, H., Brumer, H., and Dalhammar, G. (2005). A Simple Purification and Activity Assay of the Coagulant Protein from Moringa Oleifera Seed. *Water Res.* 39 (11), 2338–2344. doi:10.1016/j.watres.2005.04.012

Gruber, C., and O'Brien, M. (2011). Uterotonic Plants and Their Bioactive Constituents. *Planta Med.* 77 (03), 207–220. doi:10.1055/s-0030-1250317

Guevara, A. P., Vargas, C., Sakurai, H., Fujiwara, Y., Hashimoto, K., Maoka, T., et al. (1999). An Antitumor Promoter from Moringa Oleifera Lam. *Mutat. Res.* 440 (2), 181–188. doi:10.1016/s1383-5718(99)00025-x

Gunadi, J. W., Tarawan, V. M., Daniel Ray, H. R., Wahyudianingsih, R., Lucretia, T., Tanuwijaya, F., et al. (2020). Different Training Intensities Induced Autophagy and Histopathology Appearances Potentially Associated with Lipid Metabolism in Wistar Rat Liver. *Heliyon* 6 (5), e03874. doi:10.1016/j.heliyon.2020.e03874

Hsieh, M. H., Breyer, B. N., Eisenberg, M. L., and Baskin, L. S. (2008). Associations Among Hypospadias, Cryptorchidism, Anogenital Distance, and Endocrine Disruption. *Curr. Urol. Rep.* 9 (2), 137–142. doi:10.1007/s11934-008-0025-0

Hsu, C-N., and Tain, Y-L. J. N. (2019). The Good, the Bad, and the Ugly of Pregnancy Nutrients and Developmental Programming of Adult Disease. *Nutrients* 11 (4), 894. doi:10.3390/nu11040894

Jamroz, D., Wiliczkiewicz, A., Skorupińska, J., Orda, J., Kuryszko, J., and Tschirch, H. (2009). Effect of Sweet Chestnut Tannin (SCT) on the Performance, Microbial Status of Intestine and Histological Characteristics of Intestine wall in Chickens. *Br. Poult. Sci.* 50 (6), 687–699. doi:10.1080/00071660903191059

Jattan, M., Kumari, N., Kumar, R., Kumar, A., Rani, B., Phogat, D. S., et al. (2021). Moringa (Moringa Oleifera L.): An Underutilized and Traditionally Valued Tree Holding Remarkable Potential. *J. Hortic. Sci.* 16 (1), 1–13. doi:10.24154/jhs.2021.v16i01.001

Kavlock, R. J., Oglesby, L. A., Hall, L. L., Fisher, H. L., Copeland, F., Logsdon, T., et al. (1991). *In Vivo* and *In Vitro* Structure-Dosimetry-Activity Relationships of Substituted Phenols in Developmental Toxicity Assays. *Reprod. Toxicol.* 5 (3), 255–258. doi:10.1016/0890-6238(91)90059-o

Khalki, H., Khalki, L., Aboufatima, R., Ouachrif, A., Mountassir, M., Benharref, A., et al. (2012). Prenatal Exposure to Tobacco Extract Containing Nicotinic Alkaloids Produces Morphological and Behavioral Changes in Newborn Rats. *Pharmacol. Biochem. Behav.* 101 (3), 342–347. doi:10.1016/j.pbb.2012.01.020

Koehbach, J., Attah, A. F., Berger, A., Hellinger, R., Kutchan, T. M., Carpenter, E. J., et al. (2013). Cyclotide Discovery in Gentianales Revisited-Iidentification and Characterization of Cyclic Cystine-Knot Peptides and Their Phylogenetic Distribution in Rubiaceae Plants. *Biopolymers* 100 (5), 438–452. doi:10.1002/bip.22328

Lakshmidevamma, T. N., Ugalat, J., Apoorva, K. A., Suresh, S. P., Doddamani, M., Kadam, S., et al. (2021). Genetic Diversity of Moringa (Moringa Oleifera Lam.). *The Moringa Genome*, 57–65. Compendium of Plant Genomes, Cham: Springer.

Lea, M. (2010). Bioremediation of Turbid Surface Water Using Seed Extract from Moringa Oleifera Lam. (Drumstick) Tree. *Curr. Protoc. Microbiol.* 1, Unit1G. doi:10.1002/9780471729259.mc01g02s16

Leuci, R., Brunetti, L., Poliseno, V., Laghezza, A., Loiodice, F., Tortorella, P., et al. (2021). Natural Compounds for the Prevention and Treatment of Cardiovascular and Neurodegenerative Diseases. *Foods* 10 (1), 29. doi:10.3390/foods10010029

Madsen, M., Schlundt, J., and Omer, E. F. (1987). Effect of Water Coagulation by Seeds of Moringa Oleifera on Bacterial Concentrations. *J. Trop. Med. Hyg.* 90, 101–109.

Manzoor, F., Nisa, M. U., Hussain, H. A., Ahmad, N., and Umbreen, H. (2020). Effect of Different Levels of Hydrolysable Tannin Intake on the Reproductive Hormones and Serum Biochemical Indices in Healthy Female Rats. *Sci. Rep.* 10 (1), 20600–20608. doi:10.1038/s41598-020-77672-0

Marcondes, F. K., Bianchi, F. J., and Tanno, A. P. (2002). Determination of the Estrous Cycle Phases of Rats: Some Helpful Considerations. *Braz. J. Biol.* 62, 609–614. doi:10.1590/s1519-69842002000400008

Marques, E. B., Souza, K. P. d., Alvim-Silva, T., Martins, I. L. F., Pedro, S., and Scaramello, C. B. V. (2021). Nutrition and Cardiovascular Diseases: Programming and Reprogramming. *Int. J. Cardiovasc. Sci.* 34, 197–210. doi:10.36660/ijcs.20200031

Mat Lazim, A., Elmi Sharlina, M. S., Azfaralariff, A., Yaacob, W. A., Lim, S. J., Fazry, S., et al. (2021). Structure, Physicochemical and Toxicity Properties of

Underused Malaysian Native Tuber's Starch (Dioscorea Pentaphylla). *J. King Saud Univ. - Sci.* 33 (6), 101501. doi:10.1016/j.jksus.2021.101501

Milla, P. G., Peñalver, R., and Nieto, G. (2021). Health Benefits of Uses and Applications of Moringa Oleifera in Bakery Products. *Plants* 10 (2), 318. doi:10.3390/plants10020318

Mkhize, N. R., Heitkönig, I. M. A., Scogings, P. F., Dziba, L. E., Prins, H. H. T., and De Boer, W. F. (2018). Effects of Condensed Tannins on Live Weight, Faecal Nitrogen and Blood Metabolites of Free-Ranging Female Goats in a Semi-arid African savanna. *Small Ruminant Res.* 166, 28–34. doi:10.1016/j.smallrumres.2018.07.010

Mohanty, M., Mohanty, S., Bhuyan, S. K., and Bhuyan, R. (2021). Phytoperspective of Moringa Oleifera for Oral Health Care: An Innovative Ethnomedicinal Approach. *Phytotherapy Res.* 35 (3), 1345–1357. doi:10.1002/ptr.6896

Nath, D., Sethi, N., Singh, R. K., and Jain, A. K. (1992). Commonly Used Indian Abortifacient Plants with Special Reference to Their Teratologic Effects in Rats. *J. Ethnopharmacol* 36 (2), 147–154. doi:10.1016/0378-8741(92)90015-j

Nnamani, P., Kenechukwu, F., Anugwolu, C., and Attama, A. (2014). Evaluation of Hydrogels Based on Poloxamer 407 and Polyacrylic Acids for Enhanced Topical Activity of Gentamicin against Susceptible Infections. *Trop. J. Pharm. Res.* 13 (9), 1385–1391. doi:10.4314/tjpr.v13i9.2

Obembe, O. O. (2019). Sex Hormones and Oxidative Stress Biomarkers of Male Wistar Rats Treated with Moringa Oleifera Seed Fractions. *JBRA Assist. Reprod.* 23 (4), 408–413. doi:10.5935/1518-0557.20190047

Okereke, G. O., Igbabul, B. D., and Ikya, J. K. (2021). Evaluation of Breads Made from Wheat Flour, Modified white Yam/trifoliate Yam/sweet Potato Starches and Moringa Oleifera Seed Flour Blends. *International Journal of Food Science and Nutrition*, 6 (2), 107–119.

Oyeleye, S. I., Ojo, O. R., and Oboh, G. (2021). Moringa Oleifera Leaf and Seed Inclusive Diets Influenced the Restoration of Biochemicals Associated with Erectile Dysfunction in the Penile Tissue of STZ-induced Diabetic Male Rats Treated With/without. *Acarbose drug* 45 (3), e13323. doi:10.1111/jfbc.13323

Palada, M. C. (2019). *The Miracle Tree: Moringa Oleifera*. Philippine: Xlibris Corporation.

Pizzi, A. (2021). Tannins Medical/Pharmacological and Related Applications: A Critical Review. *Sustain. Chem. Pharm.* 22, 100481. doi:10.1016/j.scp.2021.100481

Price, D. A., Blagg, J., Jones, L., Greene, N., and Wager, T. (2009). Physicochemical Drug Properties Associated with *In Vivo* Toxicological Outcomes: a Review. *Expert Opin. Drug Metab. Toxicol.* 5 (8), 921–931. doi:10.1517/17425250903042318

Reyes-Becerril, M., Angulo, C., and Silva-Jara, J. (2021). Antibacterial and Immunomodulatory Activity of Moringa (Moringa Oleifera) Seed Extract in Longfin Yellowtail (Seriola Rivoliana) Peripheral Blood Leukocytes. *Aquaculture Research* 52 (9), 4076–4085.

Santos, A. F., Argolo, A. C., Coelho, L. C., and Paiva, P. M. (2005). Detection of Water Soluble Lectin and Antioxidant Component from Moringa Oleifera Seeds. *Water Res.* 39 (6), 975–980. doi:10.1016/j.watres.2004.12.016

Santos, L. M. M., Silva, P. M., Moura, M. C., Carvalho Junior, A. R., Amorim, P. K., Procópio, T. F., et al. (2021). Anti-Candida Activity of the Water-Soluble Lectin from Moringa Oleifera Seeds (WSMoL). *J. Mycol. Med.* 31 (2), 101074. doi:10.1016/j.mycmed.2020.101074

Schwartz, C. L., Christiansen, S., Vinggaard, A. M., Axelstad, M., Hass, U., and Svingen, T. (2019). Anogenital Distance as a Toxicological or Clinical Marker for Fetal Androgen Action and Risk for Reproductive Disorders. *Arch. Toxicol.* 93 (2), 253–272. doi:10.1007/s00204-018-2350-5

Selassie, C., Shusterman, A., and Verma, R. (1999). On the Toxicity of Phenols to Fast Growing Cells. A QSAR Model for a Radical-Based Toxicity. *J. Chem. Soc. Perkin Trans* 2 (12), 2729–2733. doi:10.1039/a905764a

Stolkowski, J., and Lorrain, J. (1980). Preconceptional Selection of Fetal Sex. *Int. J. Gynecol. Obstet.* 18 (6), 440–443. doi:10.1002/j.1879-3479.1980.tb00537.x

Teshome, D., Tiruneh, C., and Berihun, G. (2021). Toxicity of Methanolic Extracts of Seeds of Moringa Stenopetala, Moringaceae in Rat Embryos and Fetuses. *BioMed Research International*.

Thitinarongwate, W., Mektrirat, R., Nimlamool, W., Khonsung, P., Pikulkaew, S., Okonogi, S., et al. (2021). Phytochemical and Safety Evaluations of Zingiber Ottensii Valeton Essential Oil in Zebrafish Embryos and Rats. *Toxics* 9 (5), 102. doi:10.3390/toxics9050102

Tuvemo, T., Cnattingius, S., and Jonsson, B. (1999). Prediction of Male Adult Stature Using Anthropometric Data at Birth: a Nationwide Population-Based Study. *Pediatr. Res.* 46 (5), 491–495. doi:10.1203/00006450-199911000-00001

Ueda Yamaguchi, N., Cusioli, L. F., Quesada, H. B., Camargo Ferreira, M. E., Fagundes-Klen, M. R., Salcedo Vieira, A. M., et al. (2021). A Review of Moringa Oleifera Seeds in Water Treatment: Trends and Future Challenges. *Process Saf. Environ. Prot.* 147, 405–420. doi:10.1016/j.psep.2020.09.044

Van Hul, M., and Cani, P. D. (2019). Targeting Carbohydrates and Polyphenols for a Healthy Microbiome and Healthy Weight. *Curr. Nutr. Rep.* 8 (4), 307–316. doi:10.1007/s13668-019-00281-5

Witkamp, R. F., and van Norren, K. (2018). Let Thy Food Be Thy medicine.... when Possible. *Eur. J. Pharmacol.* 836, 102–114. doi:10.1016/j.ejphar.2018.06.026

Xiong, Y., Rajoka, M. S. R., Mehwish, H. M., Zhang, M., Liang, N., Li, C., et al. (2021). Virucidal Activity of Moringa A from Moringa Oleifera Seeds against Influenza A Viruses by Regulating TFEB. *Int. Immunopharmacol* 95, 107561. doi:10.1016/j.intimp.2021.107561

Xiong, Y., Riaz Rajoka, M. S., Zhang, M., and He, Z. (2020). Isolation and Identification of Two New Compounds from the Seeds of Moringa Oleifera and Their Antiviral and Anti-inflammatory Activities. *Nat. Product. Res.* 34, 1–9. doi:10.1080/14786419.2020.1851218

Xu, S., Wang, X., Zhao, J., Yang, S., Dong, L., Qin, B., et al. (2020). GPER-mediated, Oestrogen-dependent Visceral Hypersensitivity in Stressed Rats Is Associated with Mast Cell Tryptase and Histamine Expression. *Fundam. Clin. Pharmacol.* 34 (4), 433–443. doi:10.1111/fcp.12537

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 09 March 2022
doi: 10.3389/fphar.2022.826712

Prunus mira Koehne in Sichuan, China: Recorded History as a Medicine and Food, Modern Applications, Distribution, and Ethnobotanical Investigations

*Jingwen Zhang[1], Wanyue Chen[1], Weijun Sun[1], You Zhou[1], Xiaoli Li[1], Jing Zhang[2], Gang Fan[2], Hongxiang Yin[2], Ju Qin[1], Yongcui Yuan[2], Wei Xu[3] and Zhang Wang[2]**

[1]College of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2]College of Ethnomedicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [3]College of Medical Information Engineeringe, Chengdu University of Traditional Chinese Medicine, Chengdu, China

OPEN ACCESS

Edited by:
Massimo Lucarini,
Council for Agricultural Research and Economics, Italy

Reviewed by:
Jen-Tsung Chen,
National University of Kaohsiung, Taiwan
Prashant Kaushik,
University of Valencia, Spain
Erna Karalija,
University of Sarajevo, Bosnia and Herzegovina

***Correspondence:**
Zhang Wang
wangzhangcqcd@cdutcm.edu.cn

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 01 December 2021
Accepted: 20 January 2022
Published: 09 March 2022

Citation:
Zhang J, Chen W, Sun W, Zhou Y, Li X, Zhang J, Fan G, Yin H, Qin J, Yuan Y, Xu W and Wang Z (2022) Prunus mira Koehne in Sichuan, China: Recorded History as a Medicine and Food, Modern Applications, Distribution, and Ethnobotanical Investigations. Front. Pharmacol. 13:826712. doi: 10.3389/fphar.2022.826712

Prunus mira Koehne, a *Prunus* plant in the Rosaceae family, is named ཁམས་ཆུ། in Tibetan and "Guang he tao" in Chinese. It is mainly distributed in Tibet Autonomous Region, Yunnan Province, and Sichuan Province in China. It is also a rare "living fossil group" of peach genetic resources in the world. It is used in traditional Chinese medicine for the treatment of dysmenorrhea, injury, intestinal dryness, constipation, and other diseases, and is used in Tibetan medicine for the treatment of hair, eyebrows, and beard shedding. In this article, the botanical characteristics, medicinal history, modern applied research, and ethnobotanical investigation of *P. mira* were recorded and evaluated. *P. mira* was first recorded in *Dumu Materia Medica*. *P. mira* in Sichuan Province is mainly distributed in Ganzi Tibetan Autonomous Prefecture, and has certain economic and medicinal value. *P. mira* has high nutritional composition. It is made into high-quality edible oil, cosmetic base oil, fruit juice, fruit wine, fruit vinegar, "Liang guo", and other products. Oleic acid and linoleic acid are the main fat-soluble components of *P. mira*, which has an anti-inflammatory medicinal value and promotes hair growth. Its longevity and cold resistance can bring great genetic value and play an important role in maintaining peach genetic diversity. At present, there are few studies on the pharmacological effects of specific active components of *P. mira* and there are also few clinical studies. We can continue to study these aspects in the future. At the same time, products of *P. mira* have great market potential. All in all, *P. mira* is very worthy of further research and development.

Keywords: *Prunus mira* Koehne, medicinal record history, botanical charateristics, modern research and application, ethnobotanical investigation, medicinal and edible, traditional Chinese medicine

INTRODUCTION

P. mira, a *Prunus* plant in Rosaceae, is also known as *Amygdalus mira* (Koehne) Ricker. The Tibetan name is ཁམས་ཅུ། (transliteration for: Kangbu, Ximu, Kangkang, Shukan) (Gawu, 1995). The Chinese name is "Guang he tao", "Maotao", and "Tibetan peach", because its core is smoother than *Prunus persica* (L.) Batsch, and so it is named *P. mira*. It is not only the original variety of wild peach in Tibetan areas of China, but also one of the most widely distributed varieties of wild fruit tree germplasm resources in Tibetan areas of China. *P. mira* has excellent characteristics such as strong cold resistance, drought resistance, barren resistance, disease resistance, and high concentration CO_2 resistance (Ji et al., 2019), and its life span can reach thousands of years. It is the "living fossil group" of rare and precious peach germplasm resources in China and the world (Tan et al., 2012). *P. mira* is the most widely distributed peach in Tibet and has a high ornamental value. Because of the high elevation of *P. mira*, when the flowers of *P. mira* are in full bloom, the mountain opposite the forest is still covered with snow, and the scenery of the flowers of *P. mira* reflecting the snow-capped mountains opening is beautiful. Its fruit has a unique flavor and a high sugar content, which can be processed to make juice. The kernel of *P. mira* was included in *Sichuan traditional Chinese Medicine Standard* (1992 edition) (Health, 1991) and was used in traditional Chinese medicine to treat amenorrhea, dysmenorrhea, fall injury, intestinal dryness, and constipation. *Jingzhu Materia Medica* (1735) of Tibetan medicine (Tamar, 1986) and "Zang yao zhi" (1991) (Northwest Institute of Plateau Biology, 1991) recorded "seed extraction, oil and rub to treat yellow water disease, hair, eyebrow, beard and other prolapse"; now, it is also included in *the Standard of Tibetan Medicinal Materials of Sichuan Province* (2020 Edition). According to the "Quality arrangement and quality research of commonly used Chinese Medicinal Materials" (1997), Xu and Xu (1997) recorded that *P. mira* is distributed in the border areas of Sichuan Province, Yunnan Province, and Tibet Autonomous Region, mainly distributed in Batang County, Derong County, Xiangcheng County, Daocheng County, Yajiang County, Li County, Markang County, Jinchuan County, Muli County, and other counties in Sichuan Province; Zhongdian County and Ninglang County in Yunnan Province; and Bomi County, Chayu County, Mangkang County, Jiangda County, Basu County, and other counties in Tibet Autonomous Region. Because the book was published earlier, the distribution and reserves of *P. mira* recorded in the book may have changed greatly with the changes in society and the environment.

Ethnobotany is a comprehensive study of the relationship between botanical drugs in traditional ethnic and folk medicine and humankind in the region. It contains all plants that are culturally and economically important (Pei, 1988). The concept of ethnobotany was first put forward by Professor Hashburger, a botanist at the University of Pennsylvania in 1896 (Sun and Sheng, 2004). The purpose is to collect, screen, and seek the raw materials needed for industrialization through relevant knowledge of national plants (Pei, 2008). In the past 50 years, ethnobotany has paid more attention to the important knowledge in biodiversity conservation and sustainable utilization of native plants (Bhattarai et al., 2010), and the research field has also changed from resource development to traditional knowledge such as biodiversity conservation and sustainable utilization of plant resources (Pei, 2003). It is important to study traditional ethnic medicine at present. It has important theoretical value and practical significance to promote the inheritance of plant resources, diversity and characteristic culture in ethnic minority areas, and the sustainable development of regional economy and society (Pei, 2011). Kunming Institute of Botany, Chinese Academy of Sciences, the first institution specializing in ethnobotany in China, was established in 1987; the second international national biology conference was held in Kunming in 1990, which marked the preliminary launch of ethnobotany research in China (Li and Long, 2019). Tibetan areas of Sichuan are not only vast in territory, but also rich in plant resources. Its higher plants account for about one-third of China's total, second only to Yunnan Province in China. The traditional Chinese medicine produced in the province accounts for 1/3 of the total output of traditional Chinese medicine in China, and it is the largest base of traditional Chinese medicine in China (Committee, 2018). Sichuan Province is located in southwest China; it borders Yunnan Province and Tibet Autonomous Region, and is the second largest Tibetan area in China, mainly including Ganzi Tibetan Autonomous Prefecture, Aba Tibetan and Qiang Autonomous Prefecture, and Muli Tibetan Autonomous County and Liangshan Yi Autonomous Prefecture, accounting for 51.49% of the total area of Sichuan Province (Census Office of the State Council, 2012). The ethnic medicinal plants in Sichuan Tibetan areas have a diverse variety, have wide applications and national characteristics, and have an important utilization and development value (Qiu, 2020). Among them, the Tibetan medicine *P. mira* is mainly produced in the Tibetan area of Sichuan, China. However, there are few ethnobotanical studies on Sichuan Tibetan areas, especially Tibetan medicine ethnobotany.

The data on the distribution of *P. mira* resources have been recorded for more than 30 years. During this period, due to pests and diseases (Xiang et al., 2019), social changes, climate and environmental changes, farmland reclamation, and other reasons, *P. mira* is under threat, and the natural population area of *P. mira* continues to decrease (Tang, 2012; Fachun et al., 2014; Tian et al., 2015). In a survey, it was found that about 90% of Tibetan residents knew *P. mira*, but more than half of Tibetan residents did not know it is a protected plant (Wang et al., 2021). At the same time, the main distribution area of *P. mira* has the tradition of raising yaks and Tibetan pigs, so the fruit is eaten in large quantities when it is ripe, and the wild *P. mira* tends to be aging seriously (Fang et al., 2008). Therefore, it is particularly important to conduct in-depth scientific and detailed protective research on *P. mira*.

With more in-depth medical research and the expansion of people's cognition of ethnobotanical drugs, more and more scholars have carried out research on *P. mira*. Therefore, this paper expounds the medicinal record history, botanical characteristics, modern research and application, and ethnobotany research in Sichuan Province, to further develop

the potential value of *P. mira* and provide reference for future research.

MEDICINAL RECORD HISTORY OF *P. MIRA*

The Tibetan medical works *Dumu Materia Medica* (750) (Xi, 2016) and *Gan lu ben cao ming jing* (1993) (Gama, 1993) provide the earliest records of *P. mira*. Their shape descriptions are that the leaves are lanceolate and the flowers are white, providing clues for the identification of *P. mira*. *Dumu Materia Medica* records that "Kangbu" was born in gullies, plains, and other places. Its trunk is tall and hard, with leaves like willow leaves. Its flowers are white and the fruit is red when ripe. According to *Gan lu ben cap ming jing*, "Kangbu" is a small perennial tree with purple stems, and hard and many branches; the new leaves are green and soft, the back of the leaf is light-colored, the leaves are alternate and lanceolate, the apex is long and pointed, the margin is serrated, and the petiole is long; the flower is pink with many petals. In spring, they grow leaves and then bloom. The summer fruits are green with yellow short hairs on the surface, and they become purple-red when they are mature. *Jingzhu Materia Medica (*1735) (Tamar, 1986) separates apricot and peach and describes them as two different plants; almond oil has the effect of curing hair loss, while semen persicae is used to ward off evil spirits, remove poison, and clear the throat, but has no effect on hair loss. In contrast, cores of "Zang tao" and "Kang mu tao" are smooth, which need to be further compared. *Jingzhu Materia Medica* (1735) reveals that apricot is divided into three kinds: "Shan xing" and "Chuan xing"; "Chuan xing" is further divided into "Han xing" and "Zang xing". "Shan xing" tastes bitter, and "Chuan xing" tastes sweet. "Han xing" is large and sweet. "Zang xing" is inferior in flavor to "Han xing", and its core is smooth. Almond oil promotes hair growth and darkens hair. There are three kinds of semen persicae: "Ru tao", "Ci tao", and "Kang mu tao". There are four, five, and six joint seams on the surface of the core of the "Ru tao". The core of "Ci tao" is like the *Phyllanthus emblica* L.; the surface has an abrupt grain. "Kang mu tao" is smooth and looks like the fruit of *Quercus robur* L. According to the *Zang yao zhi* (1991) (Northwest Institute of Plateau Biology, 1991), the Tibetan medical medicine "Kangbu" should belong to two plants of the Rosaceae family, and it involves two categories, namely, "Shan sheng" and "Chuan sheng". "Shan sheng" includes *Prunus persica* (L.) Batsch and *P. mira*, which tastes bitter and is used in treating hair loss. "Chuan sheng" is divided into two kinds, which are produced in India and in Tibet, China, and has a sweet taste. In *Chinese Materia Medica* (1999) (Tibetan Medicine Volume) (Editorial Board of Chinese Materia Medica, 2002), it is believed that "Kangbu" has two kinds of Rosaceae plants: *P. mira* and *Prunus persica* (L.) Batsch. The kernel of *Prunus persica* (L.) Batsch is capable of treating hair loss, but the effect of the kernel of *P. mira* has not been recorded. According to Tibetan medicine, *P. mira* is generally called "Kangbu" and the *Prunus persica* (L.) Batsch is called "Kangburexia". The fruit of *P. mira* is green with yellow fluff and turns reddish purple when ripe. The kernel of *P. mira* can promote hair growth and make the hair black, treat grasserie, hair, eyebrows, and other shedding disease.

Both *Chinese Materia Medica* (Tibetan Medicine Volume) and *Zang yao zhi* believe that *P. mira* belongs to "Kangbu", and it may be that "*Chinese Materia Medica*" (Tibetan Medicine Volume) agrees with the view of *Zang yao zhi*. Through the textual research of these two herbs, it can be concluded that *P. mira* belongs to "Kangbu", which has a therapeutic effect on alopecia, but it needs further identification. *Sichuan Standard of Chinese Medicinal Materials* (supplement) (Health, 1991) includes the dried and mature seeds of the *P. mira* of the Rosaceae family. After ripening, the seeds of *P. mira* were collected; the pulp and shell were removed and then dried. It can be used for amenorrhea, dysmenorrhea, lumps, bruises, intestinal dryness, and constipation. It is not recorded for hair loss. In *Newly revised Jingzhu Materia Medica* (Luo, 2004), it is believed that "Kang mu tao" is the original plant of today's semen persicae, and the kernel oil of "Kang mu tao" has the effect of curing alopecia. According to the *Newly revised Jingzhu Materia Medica,* there are three kinds of peach: "Ru tao", "Ci tao", and "Kang mu tao". The first two are not seen in today's varieties, and the "Kang mu tao" is the original plant of today's *Prunus persica* (L.) Batsch. There are three kinds of basic sources: *Prunus persica* (L.). Batsch., *P. mira*, and *Prunus davidiana* (Carrière) Franch, the kernel oil of all of which can promote hair growth and darken hair. According to the *Dictionary of Chinese ethnic Medicine*, *P. mira* is a plant of the Rosaceae family (Jia and Zhang, 2016). The oil extracted from seeds can cure baldness, and eyebrows and beard shedding, and the ash from pulp, core, and seed burning can cure various wounds, grasserie, and constipation. *The Standard of Tibetan Medicinal Materials of Sichuan Province* (2020 edition) includes *P. mira*, but uses its synonym (*Amygdalus mira* Koehne Yü et Lu). This standard recognizes the role of kernel of *P. mira* in the treatment of hair loss and establishes a relatively high-quality standard.

According to the above herbal authentication, it can be seen that different ancient Tibetan medical books have different understandings of the base source of "Kangbu", which may be due to the complex geographical environment and the communication constraints in Tibetan areas. Both *Zang yao zhi* and *Chinese Materia medica* (Tibetan Medicine Volume) recognize *P. mira* as one of the sources of Tibetan medicine "Kangbu". In addition, traditional Chinese medicine mainly uses the kernel of *P. mira* to treat gynecological and traumatic diseases, while Tibetan medicine mainly uses it to treat hair loss diseases, which expands the clinical application of the kernel of *P. mira*. However, the clinical experience and literature records have not been widely promoted.

BOTANICAL CHARACTERISTICS OF *P. MIRA*

Distribution and Genetic Diversity of *P. mira*

P. mira is mainly born in coniferous and broadleaved mixed forest or hillside forest edge at 2,600–4,000 m altitude. It is highly adaptable, drought-tolerant, and light-loving and grows rapidly in favorable habitats. It is distributed in 20 counties between 31°10′-29°58′ north latitude and 91°50′-98°48′ east longitude,

especially in the lower reaches of Yarlung Zangbo River and its tributaries Niyang River and Parlung Tsangpo River basin (Dong, 1991). It is produced in Sichuan Province (Batang County, Derong County, Xiangcheng County, Daocheng County, Yajiang County, Li County, Markang County, Jinchuan County, Muli County, and Yanyuan County), Yunnan Province (Shangri-La city, Deqin County, Ninglang County, etc.), Tibet (Bomi County, Mangkang County, Jiangda County, Badu County, and other counties) (Xu and Xu, 1997), and also in Nepal (Online, 2021). Zhou et al. (Zhou et al., 1994; Zhou et al., 1995) of the National Crop Germplasm Resources Investigation Team investigated the traces of *P. mira* in the study of rare core fruit trees in southwest Sichuan. They proposed that the kernel of *P. mira* could be divided into smooth type, shallow groove type, and deep groove type, and found that the three types of *P. mira* varied with the altitude. There are many types of variations of *P. mira* in Muli County, Yanyuan County and Mianning County of Sichuan Province. The annual average temperature for *P. mira* is 6–14°C. The average temperature of the coldest month (January) is −2.7°C, and the average temperature of the hottest month (July) is 18–19°C. It mainly grows in alpine shrubs and brown forest soil, alpine cultivated meadow soil, etc., and the soil quality is sandy loam, light sandy soil, or loam. The pH value of the land is between 6.4 and 7.5, and the habitat belongs to the sub-humid and semi-arid type (Zhong, 2008). Dong Guozheng made a preliminary investigation on *P. mira* resources in Tibet and estimated that there were about 3×10^5 trees of *P. mira* in the whole region. The area was about 150–200,000 mu, and the annual yield of kernel of *P. mira* is about 5,000 kg (Dong, 1991). Only in the Nyingchi area of Tibet was the annual output about 5×10^6 million kg (Cai et al., 1997a), and the total reserves were 53×10^5–69×10^5 kg (Zhong et al., 2010) according to the harvest year. However, due to farmland reclamation and overgrazing, the genetic diversity of *P. mira* was destroyed, and the area of *P. mira* in natural population was decreasing. Therefore, it is important to strengthen the resource conservation of *P. mira* in Tibet.

Cultivation of *P. mira*

At present, the aging of *P. mira* is a serious problem in Nyingchi, Tibet. In field investigation, the regeneration of the seedlings in the native forests is rarely recorded (Fang et al., 2008). Scholars' research on artificial cultivation of *P. mira* can provide a lot of seedling data for the cultivation of local *P. mira*. The seeds were treated with different concentrations of gibberellic acid (GA3), 6-benzyladenine (6-BA), and NaCl, and the effects on seed germination were observed. It was found that GA3, 6-BA, and NaCl could improve the germination rate, germination potential, and germination index of seeds of *P. mira* (Zhang et al., 2011). Geng et al. conducted a study on the seedling cultivation technology of *P. mira* (Geng et al., 2008). It was shown that the sowing and seedling of *P. mira* are relatively easy to operate, and the reproductive rate of *P. mira* can be effectively guaranteed. Seeds of *P. mira* had a higher germination rate after treatment, and asexual reproduction of *P. mira* can be carried out. The stem segments of the *P. mira* were cultured *in vitro* to study the effects of different sampling times, different sterilization methods, different media, different hormone types and concentrations on germination, axillary bud proliferation, and rooting. The results showed that the proliferation coefficient of the *P. mira* was higher under LP+6-BA 1.0 mg/L + IBA 0.2 mg/L. Rooting condition was 1/2 ms + IAA 1.0 mg/L + NAA 1.0 mg/L (Li et al., 2009). Li et al. studied the grafting technology of *P. mira* in Lhasa, Tibet, and discussed the best time for grafting of *P. mira* in a solar greenhouse. It was found that the best grafting period was from June 30 to July 10, and either too early or too late was not good for germination (Li et al., 2010). The effects of basic culture medium, sampling time, and disinfection method and plant growth regulator on the germination, proliferation, rooting, and transplanting of the bud stem segments of *P. mira* were studied. The results showed that the bud stem segments of *P. mira* were disinfected with 75% alcohol for 20 s and 0.1% mercury for 13 min on MS culture medium, and the bud stem segments of *P. mira* were disinfected with 75% alcohol for 20 s and 0.1% mercury for 13 min. Yellow loam:humus = 1:2 under transplanting matrix optimal conditions (Gao, 2019). In semi-arid areas such as Lhasa, Tibet, wood inlaid bud technology, peach t-shaped bud technology, and pipe protection technology have also been applied to achieve better adaptability of trees of *P. mira* (Mima et al., 2018).

Morphological Characteristics of *P. mira*

P. mira is a small deciduous tree; the tree height is 15–20 m, and the crown is 20–30 m. The root system is more developed (see **Figure 1A** for details). The diameter at the breast of the tree is 40 cm at 1 m above the ground; there are many transverse lenticels. Branchlets and buds have no villi or spines. Shoots are green and grayish brown when old with purplish-brown lenticels, but few lenticels are found on flowering branches (see **Figure 1A** for details). Leaf is blade lanceolate or ovate-lanceolate, 10–11.2 cm long, 2.5–3.6 cm wide, apex long acuminate, base obtuse or broadly cuneate, margin obtuse; leaf both surfaces glabrous or flanked tomentose on lower midvein surface; petiole is 1.2–1.3 cm long, apically with two to three glands, stipules are caducous. Rust-like insect pests are relatively less. The flowering period is from early March to mid-April, opening before the leaves; flowers are 3–3.5 cm in diameter, with short stems. Calyx is tube campanulate, glabrous, purplish red in color, 5 calyx lobes, ovoid, margin slightly pilose; 5 petals, white or pale pink, petal base pink, obovate, apex rounded obtuse, middle slightly concave. Some petals are not fully expanded and slightly overlapping. The stamens (about 41) are slightly longer than the style, not fully expanded, and the pistil is 1. The fruit ripening period is from mid-September to early October. It is suborbicular, densely fluffy, yellow green, and purple red on the sunny side, sour and juicy, with a long diameter of about 3.0–3.3 cm, a short diameter of about 2.9–3.2 cm, and a thickness of about 3.0–3.3 m; fruiting pedicel is about 4–5 mm long; the pulp and core are easy to separate. The core is oblate, hard, indehiscent, 1.9–2.1 cm long, 1.7–1.9 cm short, and 1.1–1.3 cm thick. The surface is yellowish brown, slightly compressed on both sides, asymmetrical, acute at the top, non-acute at the base, slightly oblique, with sharp ridges, smooth surface, only on the back and ventral surface with a

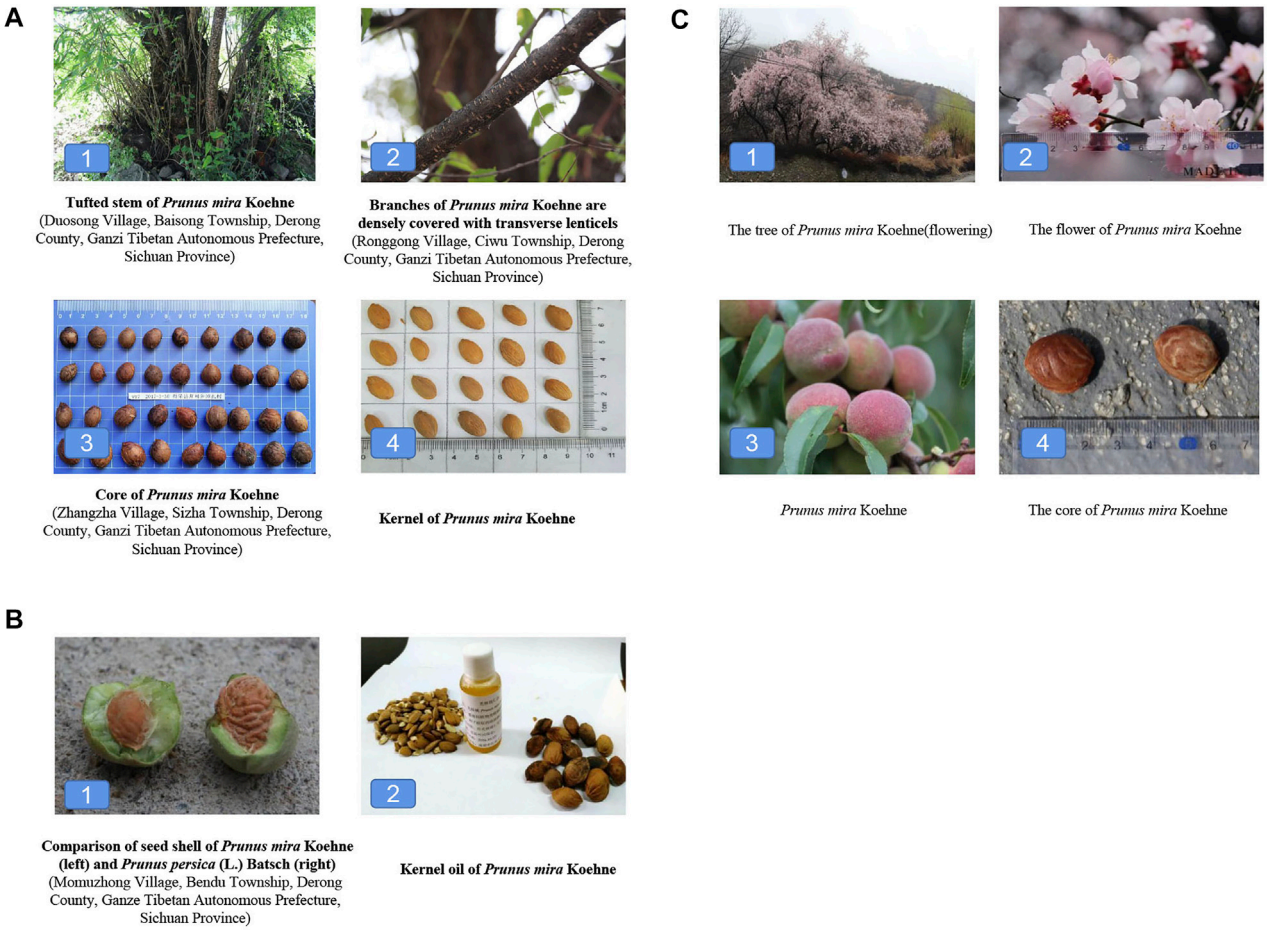


FIGURE 1 | Morphological characteristics, identification characteristics, and ethnobotany of *P. mira* [**(A)**. Morphological characteristics of *P. mira*; **(B)**. Identification characteristics of *P. mira*; **(C)**. The tree, flower, fruit, and core of *P. mira*].

few insignificant longitudinal shallow grooves, and no holes (see **Figure 1A** for details). The kernel is long oval or short oval, with a long diameter of about 1.3–1.5 cm, a short diameter of about 1.0–1.2 cm, and a thickness of about 0.4–0.5 cm. The surface is yellowish brown or brown, with fine granular protrusions. The tip is sharp, the base is blunt, slightly oblique, and the edge is thin. There is a linear hilum on one side of the tip, and most brown vascular bundle veins are scattered from the base chalazal, forming longitudinal concave lines covered with the seed coat. The seed coat is thin with 2 cotyledons, white in color, and oil-rich. The breath is slight and the taste is bitter and astringent, as detailed in **Figure 1A**.

The distinguishing feature of *P. mira* is that the kernel shell of this species is smooth (there are no holes on the surface of the kernel, with wide and shallow longitudinal grooves), which is easy to distinguish from other species (see **Figure 1B** for details). It is similar to *Prunus kansuensis* Rehder, but *Prunus kansuensis* Rehder has a cuneate leaf base, widest leaf above middle, and pilose outside calyx tube and sepal. There are also differences in distribution area.

Other Biological Characteristics of *P. mira*

Under the special climatic conditions of long sunshine and strong radiation in Tibet, *P. mira* gradually evolved into having different photosynthetic characteristics from *Prunus persica* (L.) Batsch. In exploring whether high-intensity UV radiation can inhibit the photosynthesis and growth of *P. mira*, Hou et al. (2012) reached a preliminary conclusion after simulating the treatment of seedlings of *P. mira* with different intensities of UV-B radiation. In a short period of time, UV-B radiation can reduce the stomatal conductance and photosynthetic efficiency and then inhibit the growth of seedlings of *P. mira*. The annual average temperature in the distribution area of *P. mira* is 6–13°C, which is sensitive to high temperature. The growth and development of 1-year-old seedlings of *P. mira* were poor due to high temperature when they were transplanted in warmer areas of Beijing (the average temperature in summer is 25°C, with a high temperature of 37°C and an extremely high temperature of 42°C). The accumulation of ABA in leaves of seedlings of *P. mira* induced by HRT was increased, which may help to enhance the heat resistance of PSII (Hao et al., 2012). In recent years, most

studies on the physiological characteristics of *P. mira* are focused on its photosynthesis, and the resistance of *P. mira* is also one of its important biological characteristics. Plant resistance refers to the evolution characteristics of plants in changing environments (climate, soil, water and nutrient supply, etc.). It is the result of adaptation to different environmental conditions. The research on plant resistance has an important guiding value for plant introduction and breeding. In recent years, the average annual temperature in Tibet has been gradually rising, the annual precipitation has been gradually declining, and the climate drought has become increasingly obvious (Smith, 2011). Then, drought has occurred frequently, which has brought great losses to the ecological environment and the economy in Tibet (Liu and Yuan, 2015). Drought stress is a common problem of plants. Some scholars have studied the effects of drought stress on seedlings of *P. mira* and analyzed the seedlings of *P. mira* in different soil moisture content of the protective enzyme activity and the change of photosynthetic characteristics, and it was found that under drought stress, to a certain extent, *P. mira* can increase water use efficiency; the soil moisture content is 10.1%–12.7%, and drought stress was the most significant (Guo et al., 2010). Under drought stress, both leaf (Cao, 2017) and root (Tian, 2016) had drought resistance and water retention ability, and all data were recovered following rehydration after severe drought, indicating that *P. mira* had strong drought resistance ability. By measuring the physiological indexes of *P. mira* under drought conditions, it was found that the drought resistance of *P. mira* was different with different provenances, among which shannan provenances of *P. mira* had a better drought resistance (Huang et al., 2018). However, studies have shown that the waterlogging resistance of *P. mira* is weak (Zhou et al., 2017; Zhang, Cuiling et al., 2019).

Genetic Characteristics of *P. mira*

P. mira has excellent characteristics such as drought resistance, disease resistance, and longevity. It has high ecological, economic, and ornamental value. It has made a lot of contributions to promoting the evolution of peach resources and cultivating new varieties with high resistance. The product value, intellectual property, and patent value of *P. mira* to be developed will be considerable. Under the influence of many factors, the existing resource development and research are not enough, and the research on the protection and sustainable utilization of its genetic resources still needs more effort (Zhang et al., 2013). Most studies have focused on the ecology and genetic diversity of *P. mira* (Bortiri et al., 2001; Fang et al., 2008). *P. mira* not only is considered as an important gene bank for improving germplasm resources of cultivated peach, but also can be used to control soil erosion and restore vegetation because of its high tolerance to harsh environments (drought, cold, and barren soil) (Hao et al., 2009). Because of its high tolerance to harsh environments and high yield, it has great potential in peach breeding (Li et al., 2014; Tian et al., 2015).

P. mira has a long life, is disease resistant, and has barren characteristics. Through research, it is found that the life span of *P. mira* is 5–20 times longer than that of ordinary peach species, and it has the potential to become one of the longest peach species. It can not only contribute to extending the life of other peach species, but also increase the output value of *P. mira* (Bao et al., 2018a). Because of its high disease resistance, *P. mira* can survive and produce better results under environmental stress, which can significantly reduce the capital investment in labor costs and pesticide costs. At present, the improper use of pesticides leads to the continuous decline of land quality, which has attracted great attention from all over the country. The improper use of chemical fertilizers (such as the use of excessive amount of nitrogen and phosphorus) also occurs in the peach-growing areas in China (Wang et al., 2009). Improper fertilization also restricts the development of the peach industry. At present, less than 1% of pesticides can be used when fruit trees are planted and sprayed, and most pesticides are wasted or remained on fruit, which is harmful to human health (Xue, 2019). *P. mira* grown on the plateau are rarely contaminated by pesticides. The resistance of different species and types of peach to root-knot nematodes was determined through years of natural disease nursery sowing and pot experiments, and it was found that some *P. mira* have the potential to become resistant (Zuo et al., 1988).

P. mira has important genetic resource value. Resources of *P. mira* have the value of primitive species. Some scholars have determined that *P. mira* is the original species of peach resources through studies on its distribution and morphological changes of fruit cores. It is produced in Tibet, northwestern Yunnan Province, western Sichuan Province, and other places in China, and the above areas are the origin centers of all peach species (Wang and Zhou, 1990). At present, the genetic evaluation of peach germplasm resources was carried out from the aspects of morphology, SSR marker, palynology, isoenzyme, RAPD marker, etc., and some conclusions about the occurrence and phylogenetic evolution of peach population were obtained. Through electron microscope scanning of pollen grains, Guo et al. found that the pollen grains of *P. mira* were elliptical in shape, and the outer wall feature had simple parallel straight stripes without perforation, and it was the most original wild species of peach subgenus (Guo et al., 2006). The pollen morphology of five Tibet wild *P. mira* was observed using an scanning electron microscope, the pollen morphology diversity of four petal variant materials was significant, and the pollen patterns of the five materials were all primitive (Zeng, 2016). Zhou analyzed the anther water-soluble proteins of *P. mira*, *Prunus kansuensis* Rehder, Prunus davidiana (Carrière) Franch, and *Prunus persica* (L.) Batsch by isoelectric aggregation (IEF). It was found that *P. mira* had the simplest protein band and showed primitiveness (Zhou et al., 1998). Zongxue et al. analyzed the pollen protein by sodium dodecyl sulfate (SDS) electrophoresis. It is also believed that the primordiality of *P. mira* is the strongest (Zong et al., 1995). In order to provide basic genetic resources for comparative population genomics studies, the complete chloroplast genome sequence of *P. mira* was studied (Amar et al., 2018). The complete chloroplast (Cp) genome is assembled from scratch using low-coverage whole genome sequencing data, and phylogenetic analysis showed that *P. mira* was the most primitive and basic lineage in the subgenus *Pmygdalus* (subfamily *Prunoideae*),

consistent with traditional classification (Bao et al., 2019). Genetic diversity is the basis for long-term survival and evolution of species (Nei, 1978). Wild *P. mira* has rich genetic diversity and great potential for exploitation and utilization. At the second International Biodiversity Ecology and Environment Conference in 2013, some scholars aimed to use simple sequence repeat (SSR) technology to study the diversity and genetic relationship of peach species such as *P. mira*, and provided experience for improving breeding programs. Thus, the economic value and ornamental value of *P. mira* can be improved (Xing et al., 2013). The genetic diversity level of *P. mira* should be consistent with the characteristics of wide distribution, strong adaptability, and high genetic diversity level of outcrossing species believed by most scholars (Liu et al., 2012). Two molecular marker methods (SSR and ISSR) are used to study 72 materials. The results show that there is a certain correlation between geographical location and genetic distance. Wild *P. mira* and "Henan tao" are closely related, so *P. mira* could be considered as a resource for the development of peach germplasm, which provides a theoretical basis for the development and protection of peach germplasm resources to a certain extent (Xing, 2014). Amplification fragment length polymorphism (AFLP) was used to analyze the genetic relationship of 83 germplasm resources of *P. mira* from five populations, and it was found that the genetic diversity of germplasm resources of *P. mira* was high, which provided a new gene source for peach rootstock variety breeding (Li et al., 2014). Bao et al. used SSR markers to deeply study the genetic diversity and genetic structure of *P. mira* in Tibet. It is found that the genetic diversity of *P. mira* was high, with genetic variation of geographical isolation and elevation gradient among different populations, and the level of genetic differentiation was relatively good (Bao et al., 2018b). Tan et al. used SRAP molecular marker technology to analyze the genetic diversity of natural populations of *P. mira* in Tibet, and clarified its genetic diversity level and genetic structure at the molecular level. It was found that *P. mira* had a higher level of genetic diversity, less genetic differentiation among populations, and a higher degree of gene communication among populations (Tan et al., 2012). According to domestic and foreign research materials, *Prunus amygdalus* Batsch can be crossed with *Prunus persica* (L.) Batsch, *P. mira*, *Prunus davidiana* (Carrière) Franch, and other resources; many hybrid offspring have characteristics such as stress resistance, and *P. mira* has provided its own genetic characteristics (Ma et al., 2002).

MODERN RESEARCH AND APPLICATION OF *P. MIRA*

Chemical Composition

There are many studies on the chemical constituents of the kernel of *Prunus persica* (L.) Batsch and the kernel of *Prunus davidiana* (Carrière) Franch in the *Pharmacopoeia of the People's Republic of China*, but there are few studies on the chemical constituents of the kernel of *P. mira*. According to the comparison of HPLC fingerprints of the kernel of *P. mira* and the kernel of *Prunus persica* (L.) Batsch, it was found that the chemical components of the kernel of *P. mira* and the kernel of *Prunus persica* (L.) Batsch were similar (Du et al., 2019). After the analysis of seed oils of six plants in Tibet, it was found that the main fatty oils of *P. mira* were myromytic acid (0.1%), palmitic acid (7.4%), palmitoleic acid (0.3%), stearic acid (2.8%), oleic acid (60.7%) and linoleic acid (28.7%), and the main fatty acid component of the seed oil of *P. mira* was oleic acid (Zhang et al., 1979). After optimizing the extraction process of the kernel oil of *P. mira*, it was found that it had excellent physical and chemical properties, and 12 kinds of fatty acid components were detected; the main fatty acids were cis-oleic acid (57.32%), linoleic acid (31.65%), palmitic acid (6.49%), and stearic acid (2.29%), and unsaturated fatty acids (89.43%) were the main fatty acids. Among them, polyunsaturated fatty acids (31.76%) and monounsaturated fatty acids (57.67%) were the main types (Kan et al., 2020). Some studies had shown that oleic acid and linoleic acid are the main fatty acids in *Prunus* plant in the Rosaceae family (Kodad and Socias I Company, 2008; Sathe et al., 2008). Fatty acid composition of the kernel of *P. mira* from different provenations in Tibet was analyzed, and it was found that unsaturated fatty acid content of the oil of *P. mira* from different regions in Tibet was more than 90% (Wang and Wei, 2016), which was mainly oleic acid and linoleic acid, similar to the results of previous studies (Wei et al., w). Wei et al. conducted qualitative and quantitative analysis on the fatty acids of the oil of *P. mira* in Nyingchi, Tibet. The oil yield of the kernel of *P. mira* was as high as 51.4%, and the content of unsaturated fatty acids was 91.98%. Its main components were oleic acid and linoleic acid, while the main components of saturated fatty acids were palmitic acid and stearic acid. The fat-soluble components of different varieties of semen persicae were identified by the GC-MS method. It was found that there were significant differences in fat-soluble components of different varieties and origins of semen persicae, among which there were 16 chemical components (benzaldehyde, trans-2,4-decadienal, tedocane, cyclododecene, 8-heptatene, Ethyl palmitate, methyl oleate, oleic acid, 9,17-octadecadienal, linoleic acid, methyl linoleate, squalene, β-tocopherol, Vitamin E, β-sitosterol, and 1-eicosene) of fat-soluble components in the kernel of *P. mira* (Liang et al., 2012). Sun et al. (2018) analyzed the fat-soluble components of the kernel oil of *P. mira* by GC-MS, and the 35 compounds of the kernel oil of *P. mira* were mainly oleic acid, β-sitosterol, trans-squalene, γ-tocopherol, and vitamin E. Then, the contents of vitamin E, squalene, β-sitosterol, and α-tocopherol were determined by HPLC (Liang, 2011). A total of 41 compounds were identified by GC-MS, mainly oleic acid, β-sitosterol, trans squalene, γ-tocopherol, and vitamin E, and an HPLC method was established for the determination of α-tocopherol, vitamin E, and β-sitosterol of the kernel oil of *P. mira* (Sun, 2018). In addition, HPLC method was used to establish a method for the determination of oleic acid, linoleic acid, and amygdalin of *P. mira* (Zhou, 2020). Compared with commercial peach, *P. mira* of Tibet is rich in polyphenols, flavonoids, polysaccharides, and other functional substances, and the polyphenol content (2,716.09 ± 14.10 mg GAE/100 g d.b.) was 3–5 times higher than that of commercial peach; monophenols mainly included catechin, chlorogenic acid, and neochlorogenic acid. The contents of flavonoids and polysaccharides were 2,179.21 ± 11.20 mg RE/100 g d.b. and

TABLE 1 | Chemical composition of *P. mira*.

Plant part	Extraction method	Detect method	Main chemical compositions	References
The kernel of *P. mira*	Ethanol extraction	HPLC	Oleic acid, linoleic acid, D-Amygdalin hydrate	Du et al. (2019)
The kernel of *P. mira*	Petroleum ether extraction	Solvent reflux method	Myromytic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid	Zhang et al. (1979)
The kernel of *P. mira*	Aqueous extraction	GC	Cis-oleic acid, linoleic acid, palmitic acid, stearic acid	Kan et al. (2020)
The kernel of *P. mira*	Petroleum ether extraction	Solvent reflux method, gas chromatography	Oleic acid, linoleic acid	Wang and Wei (2016)
The kernel of *P. mira*	Petroleum ether extraction	GC	Oleic acid, linoleic acid, palmitic acid, stearic acid	Wei et al. (2013)
The kernel of *P. mira*	Petroleum ether extraction	GC-MS	Benzaldehyde, trans-2, 4-decadienal, tedocane, cyclododecene, 8-heptatene, Ethyl palmitate, methyl oleate, oleic acid, 9,17-octadecadienal, linoleic acid, methyl linoleate, squalene, β-tocopherol, vitamin E, β-sitosterol, 1-eicosene	Laing et al. (2012)
The kernel of *P. mira*	Petroleum ether extraction	GC-MS	Oleic acid, β-sitosterol, trans-squalene, γ-tocopherol, vitamin E	Sun et al. (2018)
The kernel of *P. mira*	Petroleum ether extraction	HPLC	Vitamin E, squalene, β-sitosterol, α-tocopherol	Liang (2011)
The kernel of *P. mira*	Petroleum ether extraction	GC-MS	Oleic acid, β-sitosterol, trans squalene, γ-tocopherol, vitamin E	Sun (2018)
The kernel of *P. mira*	Potassium hydroxide ethanol extraction, methanol extraction	HPLC	Oleic acid, linoleic acid, amygdalin	Zhou (2020)
The fruit of *P. mira*	Methanol extraction, ethanol extraction	Vacuum freeze drying combined with ultrafine grinding technology	Polyphenols, flavonoids, polysaccharides, catechin, chlorogenic acid, neochlorogenic acid	Zuo (2019)
The kernel of *P. mira*	—	Atomic absorption spectrophotometer	Cu, Fe, Mn, Zn, Ca, Mg	Wei et al. (2017)

9,393.81 ± 284.97 mgGE/100 g d.b., respectively, which were about three times and two times that of commercial peaches (Zuo, 2019). *P. mira* is also rich in a variety of trace elements, and the contents of Cu, Fe, Mn, Zn, Ca, and Mg in the kernel of *P. mira* from 10 provenance areas in Tibet were determined by atomic spectrophotometry. The results showed that the mean value of trace element content among provenances was Mg > Ca > Zn > Fe > Mn > Cu, and the variation coefficient of trace element content among different provenances was Ca > Mn > Cu > Fe > Zn > Mg (Wei et al., 2017). The relevant information is shown in **Table 1**.

Pharmacological Effects

The fat-soluble components of the kernel of *P. mira* have obvious anti-inflammatory and vasodilating effects (Xu and Xu, 1997). The kernel of *P. mira* (Liu et al., 1989) could significantly increase the flow of isolated rabbit ear vasoperfusion fluid, eliminate the vasoconstriction effect of norepinephrine, and also show significant anti-inflammatory effects on rat paw swelling caused by egg white. The LD50 of the kernel of *P. mira* decoction on mice was 42.81 ± 0.02 g/kg, which was 238 times the commonly used dose (0.18 g/kg) in clinical patients. The long-term toxicity test of rats showed no significant effect on hematology, blood biochemistry, and histopathology. In the acute toxicity test of *P. mira*, the maximum dose of the kernel oil of *P. mira* orally in rats and mice was 144.612 and 289.224 g crude drug/(kgd), respectively, and the maximum dose of the kernel oil of *P. mira* orally in rabbits was 482.28 mg crude drug/(cm^2 d). It had good security (Sun et al., 2017). Two kinds of hair removal methods (sodium sulfide hair removal cream and hair removal instrument) were used to study the effects of different doses of the kernel oil of *P. mira* on hair growth in KM mice, and it was found that the kernel oil of *P. mira* could promote the transformation of hair follicles into growth stage and promote hair growth in mice (Zhou et al., 2020). The fat-soluble components of the kernel of *P. mira* can promote hair growth in the range of 15.06–60.26 mg/cm^2/d (Sun, 2018), but the material basis of its efficacy is still unclear. The results showed that vitamin E (3.125 mg/cm^2/d), β-glutenosteroid (0.061 mg/cm^2/d), and linoleic acid (0.156 mg/cm^2/d) could promote the hair growth of mice. Its mechanism may be related to the Wnt/β-catenin pathway (Zhou, 2020). Amygdalin from the kernel of *P. mira* was extracted by ultrasonic oscillation method, and different concentrations of amygdalin from the kernel of *P. mira* extract had different killing effects on different insects (slugs, cabbage worms, and aphids) (Yang et al., 2020). The relevant information is shown in **Table 2**.

Product Development

P. mira resources are rich in Tibet, but due to its self-generation and self-extinction, the resources have not been fully utilized. Biological test method was used to analyze the contents of soluble sugars, organic acids, and mineral elements in batches of *P. mira* from different producing areas. Due to the differences in basic quality characteristics and physiological and biochemical characteristics of fruits of *P. mira* from different producing areas, most of them lean towards the general level (Liu and Meng, 2013). Many scholars take into account the taste

TABLE 2 | Pharmacological effects of *P. mira*.

Plant part	Extractive	Animal	Administration method	Animal model	Dose	Activity	References
The kernel of *P. mira*	Decoction	Wistar rat	Gastric irrigation	Foot swelling model	5.084 g crude drug/kg	Anti inflammation and vasodilation	Liu et al. (1989)
The kernel of *P. mira*	Oil	SD rat; KM mice; Rabbit	Gastric irrigation; Skin administration	Depilation model	144.612 g crude drug/(kg·d); 289.224 g crude drug/(kg·d); 482.28 mg crude drug/(cm^2·d)	Good safety	Sun et al. (2017)
The kernel of *P. mira*	Oil	KM mice; C57BL/6 mice	Skin administration	Depilation model	Vitamin E 3.125 mg/cm^2/d, β-sitosterol 0.061 mg/cm^2/d, linoleic acid 0.156 mg/cm^2/d	Promote hair growth	Zhou et al. (2020)
The kernel of *P. mira*	Amygdalin	Slugs, cabbage worms, aphids	—	—	10 g crude drug/L	Insecticidal	Yang et al. (2020)

characteristics of *P. mira*, so they explore the processing technology to improve the taste of their products. EDTA, citric acid, VB-Na, phytic acid, and NaCl were selected as composite preservatives for storage of pulp of *P. mira*. U12 (64 × 2) uniform design was adopted. The optimal comprehensive formula was obtained as follows: EDTA dosage was 0.002%, citric acid was 0.01909%, VB-Na was 0.04%, phytic acid was 0.019%, and NaCl was 0.159% (Zhong et al., 2014). Compared with ordinary *Juglans regia* L., the bitter taste of *P. mira* was obvious. The pulsed vacuum debitter process was used to remove 95.77 ± 0.116% amygdalin in wild kernel of *P. mira*, and can significantly shorten the debitter time and improve the edible ability of kernel of *P. mira* (Zhang, Chaoqi et al., 2019). The mineral and vitamin C content of *P. mira* is higher than that of ordinary peach, and its pulp is rich in pectin, cellulose, and dietary fiber. The potential of developing functional products of dietary fiber cannot be underestimated (Zeng et al., 2009). The commercialization of the fruit *P. mira* and its processed juice has produced considerable economic benefits. The traditional processing method is to make dried peach. At present, after *P. mira* was debittered and most of the crude cellulose was degraded, highly nutritious mixed juice and preserves with a suitable taste could be made, providing new ideas for the product development of *P. mira* in Tibet (Cai et al., 1997a). Three products such as "juice of *P. mira*", "preserved fruit of *P. mira*" and "Liuhe" fruit tea of *P. mira* had also been successfully developed (Cai et al., 2002). In the trial production of mixed juice of *P. mira* in Tibet, it was found that 15% juice of *P. mira* and 5% apple juice could be mixed to obtain a mixed juice with a rich flavor (Cai et al., 1997b). Response surface methodology (RSM) was used to optimize the fermentation process of wine of *P. mira*. It was found that the optimal fermentation conditions of wine of *P. mira* were as follows: after juicing the fresh fruit of *P. mira*, the initial sugar content was adjusted to 18.84%, pH 3.96, the yeast inoculation amount was 0.76‰, and the volume fraction of alcohol was 11.33%. After fermentation and aging, the wine has a golden color and a mellow and sweet taste (Zhong et al., 2012). Some scholars took pectinase as a clarifier to determine the optimal clarification process parameters and obtain the clarified juice of *P. mira*, which laid a foundation for the development of the juice, wine, and fruit vinegar of *P. mira* (Kan et al., 2018). The liquid–solid string leaching fermentation process was used to obtain *P. mira* fruit vinegar with 86.4% alcohol fermentation rate, 75.3% acetic acid conversion rate, and a fermentation period of 15–16 days (Zhong and Fang, 2011). Many scholars have also explored the processing technology to ensure the antioxidant capacity of *P. mira*. Steam hot treatment could significantly improve the content of nutrients and antioxidant capacity of *P. mira*, which could be used as an effective pretreatment method for deep processing of *P. mira* (Zuo et al., 2018). The ultra-fine powder of *P. mira* with excellent quality could be prepared by vacuum freeze drying technology and ultra-fine grinding technology on the premise of retaining the main active components and antioxidant capacity of *P. mira* to a large extent (Zuo et al., 2019). "Liang guo" of *P. mira* (Zhong and Pu, 2001) was also a good product of *P. mira*. *P. mira* has high oil content and is mainly composed of oleic acid and linoleic acid. At the same time, it is rich in Ve and other active substances (Wang and Wei, 2016). It could be used as edible oil (Luo and Zheng, 1998), as well as cosmetics base oil and advanced lubricating oil. Flesh color is one of the important traits affecting the value of peach fruit products. The study on the main metabolites and transcripts related to peach fruit coloring provides useful information for the improvement of peach fruit quality and provides theoretical support for improving the attractiveness and quality of peach varieties.

INVESTIGATION ON ETHNOBOTANY OF *P. MIRA* IN SICHUAN PROVINCE

Survey Methods

China is in the east of Asia. Sichuan Province is located in southwest China and is the second largest Tibetan area in China. Sichuan Ganzi Tibetan Autonomous Prefecture, Aba Tibetan and Qiang Autonomous Prefecture, and Liangshan Yi Autonomous Prefecture covered most of the production areas of *P. mira*, so the counties and cities of these three prefectural regions were mainly selected for research (see **Figure 2** for details). Books related to the history, identification, medicinal value, resource distribution, and usage of *P. mira* had been widely collected, including modern herbal works, medicinal materials standards, and flora and literature data. Meanwhile, researchers examined specimens at the Chengdu Institute of Biology, Chinese Academy of Sciences to determine the scientific name, origin,

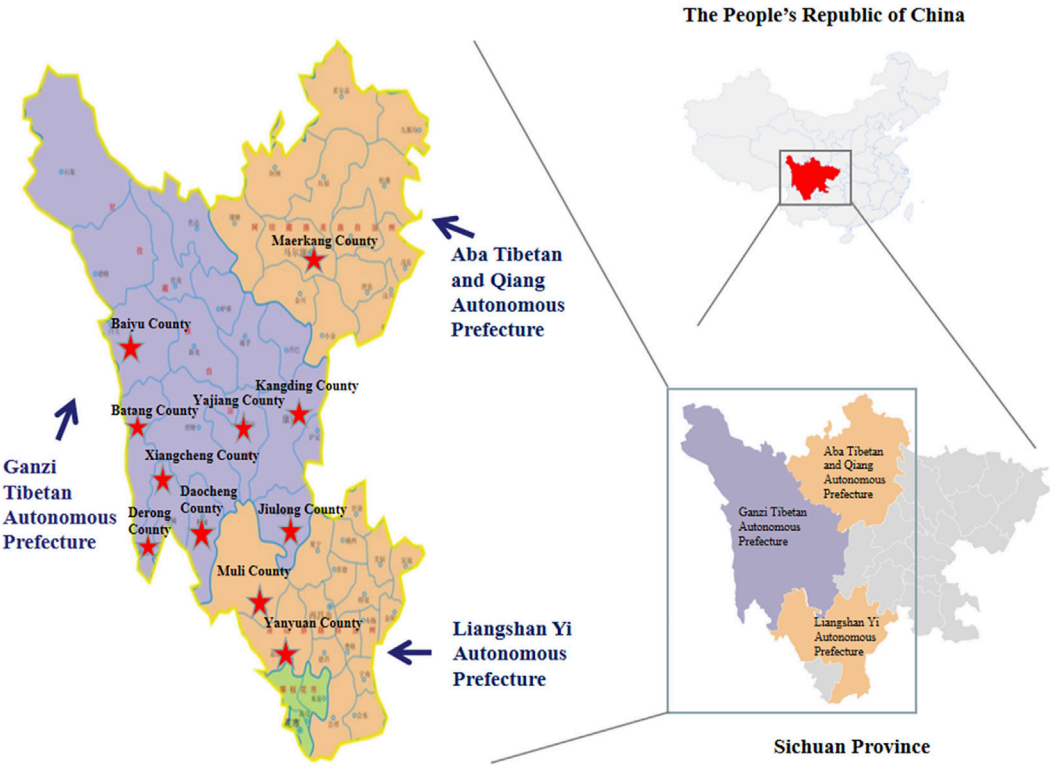


FIGURE 2 | Distribution of survey sites. (★ The survey site is marked with red pentagonal stars in the figure)

distribution, botanical characteristics, distribution area, and ecological environment of the *P. mira*. The geographical topography, climate environment, species distribution, and other data of the three prefectural counties along 318 National Highway, including Derong County, Xiangcheng County, Daocheng County, Batang County, Yajiang County, Markang County, Muli County, and Yanyuan County, were consulted to clarify the basic situation of the survey area and the possible growth area of *P. mira*. The second step was to interview key people. The researchers learned about the quantity, distribution, cultivation area, medicinal material yield, market price, and other information from local forestry bureau, agricultural bureau and other units, planting bases, farmers, and herdsmen, and further determined the investigation sites at township and village levels. The third step was field investigation and specimen collection. Through field investigation, the quantity, distribution, morphology, habitat, wild resources, planting situation, use situation, and market situation of *P. mira* in Sichuan Province were understood and recorded. At the same time, the plants were photographed and proof specimens were made for identification purposes.

Basic Information

The relevant information of the surveyed areas is shown in **Table 3**. Specimens collected from various places were identified as *P. mira* by Professor Jia Minru of Chengdu University of Traditional Chinese Medicine (see **Figure 1C** for details). The condition of the tree of wild *P. mira* was recorded in detail, which included tree age, tree height, crown width, trunk diameter, leaf length and width, fruit, core and kernel length, width, and thickness, all quantifiable indicators. The results showed that *P. mira* mainly grew in 2,500- to 3100-m mountain valleys, mountain slopes, vegetable fields on the edge of fields, and on the front yard of houses. Because the national road and provincial road land utilization rate is higher, more trees of *P. mira* were cut down and the semi-high mountains and less inhabited forest more, meadow and pine forest is rarely seen *P. mira* distribution. The age of the tree of wild *P. mira* ranges from 15 to 100 years; the tree height is between 3 and 15 m, the crown diameter is between 5 and 15 m, and the circumference of the trunk 1 m above the ground is 50–500 cm. The flowering period is from March to April, and the fruiting period is from September to October. The leaf length is 5–15 cm, the width is 1.0–4.0 cm, the number of glands is 2–7, the fruit weight is 27.70 ± 13.29 g, the long diameter is 2.2–5.0 cm, the short diameter is 1.7–4.0 cm, and the thickness is 1.0–4.0 cm. The weight of the kernel is 1.32 ± 0.69 g, the average nucleation rate is about 10%, the long diameter of nucleus is 1.2–2.6 cm, the short diameter of nucleus is 1.0–2.0 cm, the thickness of the kernel is 0.8–1.7 cm, the weight of the kernel is 0.24 ± 0.13 g, the diameter of the kernel is 1.1–2.1 cm, the diameter of kernel is 0.7–1.8 cm, the thickness of kernel is 0.4–0.8 cm, and the average rate of kernel emergence is about 17%. Associated plants are *Juglans regia* L., *Fagopyrum esculentum* Moench, and so on. In the course

TABLE 3 | Information and number of survey sites in Sichuan Province.

Number	Serial number	Autonomous prefecture	County	Township/ Town	Village	Altitude (m)	Longitude	Latitude	Survey date
1	160518DRXY	Ganzi Tibetan autonomous prefecture	Derong county	Guxue township	Xiayong village	2,589	99°18′5706″	28°24′4128″	2016.5.18
2	160723DRMU	Ganzi Tibetan autonomous prefecture	Derong county	Bendu township	Momushang village	2,600	99°21′1667″	28°36′8444″	2016.7.23
3	160724DRMZ	Ganzi Tibetan autonomous prefecture	Derong county	Baisong township	Menza village	2,568	99°22′5640″	28°54′4644″	2016.7.24
4	160917DRDS	Ganzi Tibetan autonomous prefecture	Derong county	Baisong township	Duosong village	2,889	99°42′5068″	28°93′3574″	2016.9.17
5	160724DRLD	Ganzi Tibetan autonomous prefecture	Derong county	Ciwu township	Langda village	3,095	99°16′5692″	28°57′5104″	2016.7.24
6	160917DRRG	Ganzi Tibetan autonomous prefecture	Derong county	Ciwu township	Ronggong village	3,275	99°29′8785″	28°00′0438″	2016.9.17
7	170917DRKS	Ganzi Tibetan autonomous Prefecture	Derong county	Ciwu township	Kase village	2,783	99°20′1049″	28°55′1383″	2017.9.17
8	160724DRQY	Ganzi Tibetan autonomous prefecture	Derong county	Songmai town	Quya village	3,300	99°19′4115″	28°46′5876″	2016.7.24
9	160724DRZZ	Ganzi Tibetan Autonomous Prefecture	Derong county	Sizha township	Zhangzha village	3,000	99°15′3838″	28°48′5699″	2016.7.24
10	160724DRKG	Ganzi Tibetan autonomous prefecture	Derong county	Sizha township	Kalong village	2,500	99°17′13.38″	28°48′3599″	2016.7.24
11	160725DRRD	Ganzi Tibetan autonomous prefecture	Derong county	Rilong township	Ridui village	3,028	99°11′1158″	28°41′2357″	2016.7.25
12	160916DRRD	Ganzi Tibetan autonomous prefecture	Derong county	Rilong township	Ridui village	2,934	99°18′8960″	28°68′9485″	2016.9.16
13	170720DRLR	Ganzi Tibetan autonomous prefecture	Derong county	Rilong township	Longrong village	3,196	99°12′3309″	28°41′3902″	2017.7.20
14	170918DRLR	Ganzi Tibetan autonomous prefecture	Derong county	Rilong township	Longrong village	3,196	99°12′3309″	28°41′3902″	2017.9.18
15	160925DRZR	Ganzi Tibetan autonomous prefecture	Derong county	Xulong township	Zhangren village	2,935	99°15′7005″	28°74′1808″	2016.9.15
16	170401DRJL	Ganzi Tibetan autonomous prefecture	Daocheng county	Chitu township	Jiala village	3,164	100°16′1899″	27°37′3276″	2017.4.1
17	170916XCZD	Ganzi Tibetan autonomous prefecture	Xiangcheng county	Zhengdou township	Yongde village	2,750	99°31′2148″	29°05′4042″	2017.9.16
18	170916XCML	Ganzi Tibetan autonomous prefecture	Xiangcheng County	Dingbo township	Mala village	2,841	99°31′4347″	29°13′2911″	2017.9.16
19	170917BTDQ	Ganzi Tibetan autonomous prefecture	Batang county	Zhongzan town	Duoqiang village	2,929	99°19′1089″	29°21′2186″	2017.9.17
20	170917BTXB	Ganzi Tibetan autonomous prefecture	Batang county	Zhongzan town	Xuebo village	2,867	99°14′1627″	29°19′1000″	2017.9.17
21	170922KDCC	Ganzi Tibetan autonomous prefecture	Kangding county	Pusharong township	Changcaoping village	2,922	101°19′1446″	29°32′1447″	2017.9.22
22	170923YJWX	Ganzi Tibetan autonomous prefecture	Yajiang county	Bajiaolou Township	Wangjiayi village	2,719	101°06′1872″	30°06′0709″	2017.9.23
23	170924JLCE	Ganzi Tibetan autonomous prefecture	Jiulong county	Xiaer town	Chaer village	2,823	101°30′3892″	28°59′1512″	2017.9.24
24	180821BYJS	Ganzi Tibetan autonomous prefecture	Baiyu county	Jinsha township	Jisonggang village	2,924	100°48′1240′	31°16′4831″	2018.8.21
25	170926MLTB	Liangshan Yi autonomous prefecture	Muli county	Wachang town	Taoba village	2,577	100°50′2772″	28°09′4138″	2017.9.26
26	170927MLWJ	Liangshan Yi autonomous prefecture	Muli county	Wujiao township	Wujiao village	2,754	100°44′4022″	27°57′5178″	2017.9.27
27	170927YYDL	Liangshan Yi autonomous prefecture	Yanyuan county	Qiansuo township	Doule River	2,559	100°47′2188″	27°52′1083″	2017.9.27
28	170927YYDZ	Liangshan Yi autonomous prefecture	Yanyuan county	Luguhu town	Dazu village	2,636	100°47′0130″	27°45′1395″	2017.9.27
29	180611MEKS	Aba Tibetan and Qiang autonomous prefecture	Markang county	Songgang town	—	2,531	102°06′0027″	31°55′1044″	2018.6.11
30	180614MEKK	Aba Tibetan and Qiang autonomous prefecture	Markang county	—	—	2,571	102°14′3039″	31°53′3036″	2018.6.14

of investigation, it was also found that the main mixed varieties of *P. mira* in the Tibetan areas of Sichuan were *Prunus kansuensis* Rehder and *Prunus tangutica* (Batalin) Koehne. A large number of *Prunus kansuensis* Rehder were found in Heishui County, Aba Tibetan and Qiang Autonomous Prefecture, and local farmers also called it "Mao tao".

Distribution and Resource Overview

The survey results showed that the *P. mira* in Sichuan was mainly distributed in Ganzi Tibetan Autonomous Prefecture, with the largest number in Derong County, Batang County, and Xiangcheng County, followed by Baiyu County, Yajiang County, Jiulong County, Muli County, Daocheng County, and Kangding City, and the least number in Yanyuan County and Markang City. The planting area of Sichuan Province is about 2,800 mu, and the annual yield of *P. mira* is about 40 tons. The *P. mira* in Derong County is mainly distributed in the Jinsha River and its tributaries in the Dingqu River basin, namely, Ancient Township, Guxue Township, Bari Township, Ciwu Township, Rilong Township, Baisong Township, Xulong Township, and Sizha Township. There are about 16,000 plants, among which there are about 500 mu of artificial cultivation. Trees of wild *P. mira* are older, more than 30 years old, and most of them reach 100 years old. In the past, there were many trees of *P. mira*, but some trees of wild *P. mira* have been cut down due to busy farming, unmanned management, land development, and other reasons. The study group found that reserves of wild *P. mira* were very large in Derong County, and this may be related to the fact that Derong County is the second-to-the-last county in China with access to highways, which has resulted in little attention from scientists, slow economic development, and relatively little deforestation of *P. mira*. The trees of *P. mira* in Batang County are both wild and planted. Trees of wild *P. mira* are mainly distributed in Zhongzheng Town, Changbo Township, and Zhubalong Township, while the generally distributed villages and towns are Diwu Township, Zhongxinrong Township, Yarigong Township, Xia Qiong Town, Suwalong Township, Lawa Township, Dangba Township, and Moduo Township. The villages and towns without *P. mira* distribution are Bomi Township, Bogoxi Township, Lieyi Township, Deda Township, Cuola Township, Chaluo Township, Songduo Township, and Jiaying Township. Along the G215 line along the Jinsha River, *P. mira* is rarely seen due to human activities, but they can be seen in Mangkang City, Changdu, Tibet. Batang County government accords great importance to *P. mira* planting and has planned to build a planting base in Zhongza town. The trees of *P. mira* in the township are all wild, concentrated along the road from township to Daocheng County, mainly in Qingde Township, Qingyi Township, Zhengdou Township, Shuiwa Township, Shagong Township, etc. The counties with general distribution of *P. mira* are Jiulong County, Baiyu County, Kangding City, Yajiang County, Daocheng County, and Muli County. There are many trees of *P. mira* in Char Village and the nearby semi-alpine mountains in Jiulong County, so Char Village is also known locally as "Tao hua cun". The *P. mira* of Kangding city is mainly distributed in Pusharong Township and Jiju Township. The *P. mira* in Baiyu County is mainly wild, and it is distributed in 11–12 townships in Baiyu County, mainly including Magong Township, Zhangdu Township, Gaiyu Township, Shanyan Township, Shama Township, and beside the Jinsha River, especially near the Laser tunnel in Zhangdu Township, Dingqu River ditch, Rejia Township, and also kang Basi below until Jinsha River. There are about 4,000 plants of *P. mira* in Yajiang County, which have not been harvested and used in recent years. They are mainly distributed in villages and towns along the Yalong River, such as Murong Township, Xiala Township, Bajiao Lou Township, Hekou Town, Milong Township, Malangco Township, Egu Township, Bayirong Township, Poshihe Township, and Yanyihe Township. *P. mira* in Daocheng County is mainly distributed in Chitu Township, Shangrila Township, Dongyi District, and Mengzi Township, all of which are wild *P. mira*, without artificial planting or pest control. A large number of natural populations of *P. mira* were found in Muli County (Yang, 1989), but after investigation, it was found that the reserves had been greatly reduced, mainly because a large number of trees of *P. mira* were cut down and planted with other crops with more economic value, and it is also very likely that this investigation team has not yet entered the original forest for investigation. Our research group investigated Liziping Township, Boke Township, Qiaowa Township, Kerr Township, and other towns in Muli County, and there was no *P. mira* distribution; only three to five *P. mira* was sporadically distributed along National Highway 616, which only flowered but did not bear fruit. Beside the road, more crops or fruits with greater economic value are planted, such as *Prunus armeniaca* L., *Juglans regia* L., and *Prunus persica* (L.) Batsch. In the course of investigation, a villager surnamed Luo in Muli County reported that there were trees of *P. mira* in front and behind each house in seven villages in Kala Township of Muli County. The trees were of uneven age. Among them, there were about 110 households in Maolao Village, two to five trees per household, and a total of about 500 plants. There are sporadic *P. mira* growing in Lugu Lake Town, Yanyuan County, and the hanging fruit is very few and the fruit is small. There are few *P. mira* in Markang County, which are less distributed in the residential buildings along the banks of the river, and sporadically distributed in the nearby mountains. Information on resource distribution was also obtained during the interview. During the visit to the herbal medicine market in Yongquan Street, Xichang, Liangshan Yi Autonomous Prefecture, it was found that there was no kernel of *P. mira* for sale in the market, but according to merchants in the market, there was *P. mira* distribution in Yuexi County. However, due to the long distance of Yuexi County, the field investigation was not carried out. In addition, according to a staff member of the Health Bureau in Xinlong County, Ganzi Tibetan Autonomous Region, *P. mira* is distributed in Heping Township, Dagai Township, Dong'an Township, Sewei Township, Asequ Gou Township, Jialaxi Township, Pigao Township, Zituoxi Township, Rulong Town, Youlaxi Township, Mayue Township, and Luozuo Township in Xinlong County. It is also assumed that *P. mira* is distributed in Danba County, Mao County, Dege County, Jinchuan County, Wenchuan County, Xiaojin County, Luhuo County, Heishui County, and Li County. The grassland-dominated counties

TABLE 4 | Statistics of the use of *P. mira* in Ganzi Tibetan Autonomous Prefecture, Sichuan Province.

County	Part used	Usage	The yield of core (tons)	The unit price of core (RMB/kg)	The yield of kernel (tons)	The unit price of kernel (RMB/kg)	Peasant household income (RMB/year)
Derong County	Pulp, core	The pulp was fed to pigs and the core was sold	100–130	7–13	10	50–60	5,000–15,000
Batang County	Pulp, kernel	The pulp was fed and the kernel was used as medicine and sold	60	10–30	15	60	3,000–10,000
Xiangcheng County	Pulp	the pulp was fed to pigs and cows	—	14	7	30	150–500
Daocheng County	Core	The core was sold	4	14	1	45	—
Kangding County	Core	The core was sold	—	14	5	30	—
Yajiang County	Pulp	The pulp was fed	—	—	—	—	—
Baiyu County	Pulp	The pulp was fed to pigs	—	—	—	—	—

may have no *P. mira* distribution, including Shiqu County, Ganzi County, Seda County, Rangtang County, and Litang County. These data provided reference for the follow-up investigation on the distribution of resources of *P. mira*.

Artificial Planting and Seedling Rising

At present, there are three *P. mira* seedling and planting bases. First, the planting base of Derong County in Sichuan Province is located in Rilong Village and Riyong Village of Ciwu Township, covering an area of about 100 mu. Second, the planting base of Batang County in Sichuan Province is in Wave Village, Zhongza Town, with an area of about 1700 mu and Renmian Village with an area of about 600 mu. If 55 plants are planted per mu, there are about 120,000 plants planted. Batang County and Derong County seedlings were mainly from Benzilan Town, Deqin County, Yunnan Province. Third, the *P. mira* seedlings in Benzilan Town, Deqin County, Yunnan Province are about 10,000 plants, with an average height of 1.5 m, with a nutrition bowl and growing well. The seeds of *P. mira* need to be soaked in clean water for 4–7 days and disinfected with potassium permanganate; farm manure, especially sheep dung, is added to the soil, and then two to three nuts are poured into the nutrition bowl, which is beneficial to absorb the water and nutrition of the lower soil, and the nutrition bowl is placed in the greenhouse to be watered accordingly to prevent livestock and insect pests. The seedlings of *P. mira* are sown in January every year, and the seedlings of 2 years are the best for transplanting. At present, the main problems in planting bases are due to topography and climate, serious shortage of water resources, and weak water conservancy facilities, which affect the normal growth of artificial *P. mira* forest.

Economic Value and Medicinal Value

P. mira can be used as both medicine and food, but there is less in-depth development and utilization at present, as shown in **Table 4**. The pulp is mainly used to feed livestock (though a few farmers and herdsmen will eat it), the core is mainly sold to medicinal herbs, and the kernel can be used as medicine. During the visit, it was found that the villagers in Yajiang County would eat the dried kernel raw or fry it, and during hard times, they would pick *P. mira*, take out the kernel, mash it, and add it into butter tea (to remove the bitter taste) before eating it. In the past, the kernel of *P. mira* from Derong County, Batang County, and Xiangcheng County was mainly sold to herbal medicine merchants in Shangri-La city, and eventually went to the "*He hua chi* (Lotus Pond)" Chinese herbal medicine market in Chengdu. The price of peach cores was 7–14 yuan/kg, and the price difference of peach kernel was 30–60 yuan/kg. Because the *P. mira* has not been part of the "*Pharmacopoeia of the People's Republic of China*", it affects the scope of application and usage. At present, due to the reduction of the purchase volume, farmers and herdsmen mainly use the *P. mira* for feeding pigs, cattle, and other livestock, a small part for sale. Most farmers believe that the fruit of *P. mira* tastes sour, and they occasionally pick it to quench their thirst, but our research group tried to deep fry and stir fry the kernel of *P. mira* and found that it tasted good. After freezing the flesh with sugar, the flesh has an "original peach flavor", a crisp taste, and a suitable sour and sweet taste, and is edible.

DISCUSSION

P. mira was first recorded in *Dumu Materia Medica*, marking its presence in history after 1,300 years. From its modern application, it has been proven to be medicinal and edible. Its pulp is treated to have edible value. The fruit of *P. mira* is low in sugar, and high in minerals and vitamin C (Zeng et al., 2009); thus, its market potential is huge and has been growing in the low-sugar preserved fruit (Cai et al., 1997a), juice (Cai et al., 2002), fruit wine (Zhong et al., 2012), and fruit vinegar (Kan et al., 2018) market. Pharmacological studies have found that it has anti-inflammatory effects and can expand blood vessels (Xu and Xu, 1997); it can also promote hair growth (Zhou et al., 2020). Researchers visited and investigated some districts and counties of Ganzi Tibetan Autonomous Prefecture, Liangshan Yi Autonomous Prefecture, and Aba Tibetan and Qiang Autonomous Prefecture in Sichuan. The aim is to understand the resource distribution, growth environment, and application value of *P. mira*. It is found that due to social changes, man-made logging, farmland reclamation, and other factors, the main

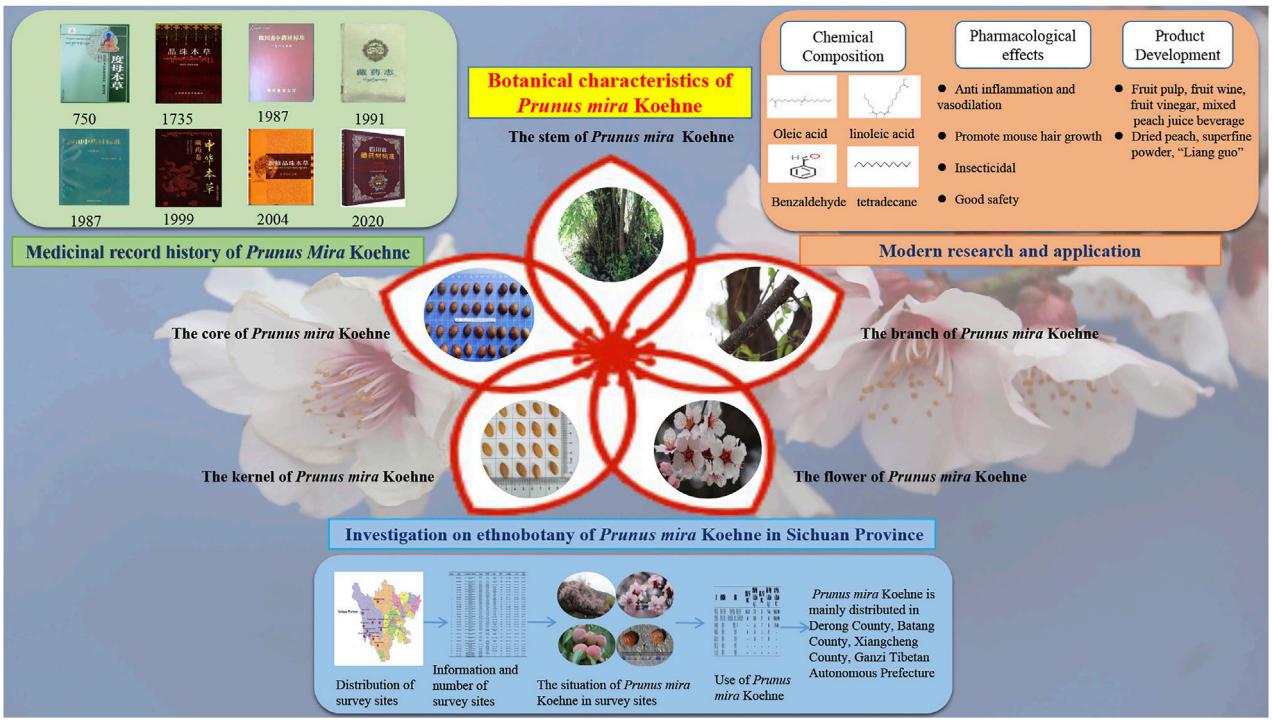


FIGURE 3 | Investigation and evaluation of *P. mira*.

distribution and quantity have greatly changed compared with the recorded data. At present, *P. mira* is mainly distributed in Ganzi Tibetan Autonomous Prefecture, and the largest number of *P. mira* is found in Derong County, Batang County, and Xiangcheng County. As shown in **Figure 3**, the local county government has gradually begun to pay attention to the utilization of resources and industrial development of *P. mira*, and established the planting base of *P. mira*. However, county-level funding is low and it may be difficult to support the investment. We can consider introducing social capital to adopt the form of equity for investment and income distribution. In addition, other features of *P. mira* are also worthy of attention. Its longevity and cold resistance characteristics can bring great genetic value, and it plays an important role and value in maintaining the genetic diversity of peaches and cultivating long-lived and cold-resistant peach varieties (Zhang et al., 2013).

In Sichuan Province, the kernels of *P. mira* were used as medicine of semen persicae in the market. Because *P. mira* is not included in the *Pharmacopoeia of the People's Republic of China*, its application scope and usage are affected. Semen persicae in Chinese Pharmacopoeia come from *Prunus persica* (L.) Batsch and *Prunus davidiana* (Carrière) Franch (Pharmacopoeia, 2015). It is distributed in Hebei, Yunnan, Gansu, Sichuan, and other provinces (Yang et al., 2018). At present, HPLC was used to compare the fingerprints of the kernel of *P. mira* and other semen persicae, and it was found that their chemical constituents were generally similar. It provides some basis for the mixed use of the kernel of *P. mira* and other semen persicae in the market (Du et al., 2019). The similarities and differences of the two herbs should be evaluated by quality control indicators (Li et al., 2019), safety evaluation, and system effectiveness evaluation. If *P. mira* can be used as one of the *Pharmacopoeia of the People's Republic of China* semen persicae source, it can greatly promote the development of *P. mira* harvest, sales, and downstream industries.

Natural ecology is the basis of local culture, which restricts the material conditions of daily life. If the environment and its cultural ecology are well handled, and the continuous accumulation of intangible capital and a virtuous cycle are ensured, the popularization and sustainable development of local knowledge depend on the development and interaction of academic achievements (Wang, 2020). From the perspective of China's consumption trend development, tourism consumption accounts for an increasing proportion of people's income. The way people travel from single to diversified development, rural tourism, agricultural tourism, and folk tourism were becoming more and more popular among tourists (Liu, 2007). Because the flower of *P. mira* has a certain ornamental value, local government could develop tourism. The local government can use the "combination of medicine and tourism" as the development idea. At the same time, it can use the help of the development plan of the "11th Five-Year Plan" and "Tourism Planning" (Province, 2017) of Sichuan Province to vigorously develop the tourism industry. Nyingchi in Tibet has had high-quality ecological resources linked by the flowers of *P. mira* as a link since ancient times, and the flowers of *P. mira* tourism and cultural festival that has gradually arisen attracts a large number of tourists every year (Luo and Wang, 2019). Nyingchi in Tibet makes full use of the superior resources of the flower of *P. mira* to develop tourism and improve economy. High-quality "ecological" resources

are the biggest advantage of Tibetan ecotourism, and ecotourism is the cornerstone of sustainable tourism (Guo, 1997).

Quality control and value chains should be ensured for such an important herb. There is a need to popularize this plant by government, national, internal, social, and media agencies to increase people's awareness of *P. mira* and protect its identity and quality. Data regarding many aspects of this plant such as mechanisms of action and pharmacological effects of specific active components are still limited. Additional clinical studies should be conducted in the future. The scientific community should also develop a more active role in developing its research, conservation, and cultivation strategies.

AUTHOR CONTRIBUTIONS

JZ and WC are mainly responsible for the research and the main work in this article; YZ and WS are responsible for assisting in the field research and data arrangement; XL, JZ, JQ, YY, WX, GF, and HY are responsible for assisting in the research. ZW functions as communication author.

FUNDING

This work was supported by the Special Program for Poverty Alleviation by Science and Technology in Sichuan Province (No. 2017NFP0080) and the Chengdu University of Traditional Chinese Medicine Xinglin Scholar subject talent research promotion plan project (No. QNXZ2018041).

ACKNOWLEDGMENTS

We thank Chengdu University of Traditional Chinese Medicine for providing us with a research platform, and thank all authors for their contributions to this article.

REFERENCES

Amar, M. H., Magdy, M., Zhou, H., Wang, L., and Han, Y. (2018). The Complete Chloroplast Genome of Amygdalus Mira (Rosaceae) a Threatened Wild Chinese Peach. *Conservation Genet. Resour.* 10 (4), 801–803. doi:10.1007/s12686-017-0934-7

Bao, W., Ao, D., Wuyun, T., Li, T., Wang, L., and Liu, H. (2019). The Complete Chloroplast Genome of Prunus Mira Koehne (Prunoideae, Rosaceae), a Wild and Indigenous Peach on Tibet, China. *Mitochondrial DNA B Resour.* 4 (2), 3731–3733. doi:10.1080/23802359.2019.1679048

Bao, W., Wuyun, T., Du, H., Li, T., Liu, H., and Bai, Y. (2018a). Genetic Diversity Analysis of Phenotypic Characters of *Prunus Mira* Regia in Tibet. *Mol. Plant Breed.* 16 (16), 5463–5473.

Bao, W., Wuyun, T., Du, H., Li, T., Liu, H., Wang, L., et al. (2018b). Genetic Diversity and Population Structure of Amygdalus Mira in the Tibet Plateau in China Based on SSR Markers. *Scientia Silvae Sinicae* 54 (02), 30–41.

Bhattarai, S., Chaudhary, R. P., Quave, C. L., and Taylor, R. S. (2010). The Use of Medicinal Plants in the Trans-himalayan Arid Zone of Mustang District, Nepal. *J. Ethnobiol. Ethnomed* 6, 14. doi:10.1186/1746-4269-6-14

Bortiri, E., Oh, S. H., Jiang, J., Baggett, S., Granger, A., Weeks, C., et al. (2001). Phylogeny and Systematics of Prunus (Rosaceae) as Determined by Sequence Analysis of ITS and the Chloroplast trnL-trnF Spacer DNA. *Syst. Bot.* 26 (4), 797–807.

Cai, C., Huang, B., Zhong, M., and Xiao, W. (1997a). Trial Production of Mixed Fruit Juice of Tibet Wild Peach. *Mod. Food Sci. Tech.* (02), 35–12.

Cai, C., Zhong, M., Huang, B., Wang, B., Xiao, W., and Qiu, J. (1997b). Wild Peach Resources and its Comprehensive Processing and Utilization in Tibet. *Guangdong Agric. Sci.* (06), 25–26.

Cai, C., Zhong, M., Xiao, W., Huang, B., Qiu, J., and Fang, J. (2002). Study on the Comprehensive Utilization of Wild Peach in Xizhang According to its Characteristics. *Food Sci.* 11, 73–76.

Cao, Y. (2017). Preliminary Study on Response Mechanism of Prunus Mira Leaves under Drought Stress. Master's thesis. Northeast Forestry University.

Census Office of the State Council (2012). *China's 2010 Census*. Beijing: China Statistics Press.

Committee (2018). *Sichuan Yearbook 2018*. ChengDu: Sichuan Yearbook Society.

Dong, G. (1991). *Investigation on Prunus Mira in Tibet*. Forest By-Product and Speciality in China, 44–45.03

Du, H., Xu, T., Li, Q., Wang, Z., Zhang, J., and Fan, G. (2019). Comparison of Photochromatographic Fingerprints of Prunus Mira Kernel and walnut Kernel by HPLC. *Chin. Traditional Patent Med.* 41 (11), 2796–2799.

Editorial Board of Chinese Materia Medica (2002). *Chinese Materia Medica*. Shanghai: Shanghai Science and Technology Press.

Fachun, G., Shiping, W., Rongqin, L., Mu, P., and Fanjuan, M. (2014). Genetic Diversity of Wild Peach (Prunus mira Koehne kov et. kpst) from Different Altitudes in the Tibetan Plateau by Pollen Morphous and RAPD Markers. *HortScience horts* 49 (8), 1017–1022.

Fang, J., Zhong, Z., and Zhong, G. (2008). Age Structure of Prunus Mira Population in Nyingchi Area. *J. For. Eng.* 22 (01), 53–56.

Gama, Q. (1993). *Gan Lu Ben Cao Ming Jing*. Tibet: Tibet people's Publishing House.

Gao, J. (2019). Establishment of Regeneration Technology System of Stem Segment with Bud of Prunus Mira. Master's thesis. Sichuan Agricultural University.

Gawu, D. (1995). *Jing Jing Ben Cao*. Beijing: Nationalities Publishing House.

Geng, Y., Zhang, J., and Li, Y. (2008). *Cultivation Technology of Prunus Mira Seedlings*. Beijing, China: Forest Science and Technology, 20–21.02

Guo, G., Wang, L., Yan, Z., Zhu, G., and Fang, W. (2006). Study on the Variation and Evolution of Peach and its Related Wild Species by Pollen Morphology. *J. Fruit Sci.* 23 (05), 664–669.

Guo, L. (1997). Chinese Ecotourism-The Foundation of Sustainable Tourism. *Prog. Geogr.* 16 (04), 3–12.

Guo, Q., Luo, D., Wang, Z., Fang, J., and Zhao, K. (2010). Response of Photosynthetic Characteristics and Protective Enzymes of Prunus Mira Seedlings to Drought Stress. *J. Northwest A F Univ. (Natural Sci. Edition)* 38 (06), 138–144.

Hao, H. P., Jiang, C. D., Shi, L., Tang, Y. D., and Yao, J. (2009). Effects of Root Temperature on Thermo-Stability of Photosynthetic Apparatus in Prunus Mira Seeding. *Chin. J. Plant Ecol.* 33, 984–992.

Hao, H. P., Jiang, C. D., Zhang, S. R., Tang, Y. D., and Shi, L. (2012). Enhanced thermal-tolerance of Photosystem II by Elevating Root Zone Temperature in Prunus Mira Koehne Seedlings. *Plant and Soil* 353 (1-2), 367–378. doi:10.1007/s11104-011-1037-y

Health (1991). *Standard of Traditional Chinese Medicine in Sichuan Province*. Chengdu, China: Chengdu University of science and Technology Press.

Hou, C., Bai, T., Wang, Y., Zhang, X., and Han, Z. (2012). Effects of UV-B Radiation on Photosynthesis and Endogenous Hormone Levels of Prunus Mira. *Chin. Agric. Sci. Bull.* 28 (22), 184–189.

Huang, X., Meng, F., Luo, Q., and Quan, H. (2018). Comparative Study on Drought Resistance of Prunus Mira from Different Provenances in Tibet. *Liaoning For. Sci. Tech.* (01), 8–12+64.

Ji, X., Lei, P., Sang, L., Wang, Y., Guan, F., and Meng, F. (2019). Comparison of Resistance of Wild Light walnut and Cultivated Peach to High Concentration CO2. *Econ. For. Researches* 37 (01), 167–172.

Jia, M., and Zhang, Y. (2016). *Chinese National Medicine Dictionary*. Beijing: China Medical Science and Technology Press.

Kan, J., Ren, H., Wu, H., Liu, Y., and Zhong, Z. (2018). Optimum Enzymolysis Technology of Prunus Mira Koehne Juice. *J. Plateau Agric.* 2 (06), 640–646+659.

Kan, J., Yuan, L., and Zhong, Z. (2020). Study on Synchronous Extraction Process of Oil and Protein from Prunus Mira Nut and the Oil Quality Analysis. *Food & Machinery* 36 (01), 210–215+236.

Kodad, O., and Socias I Company, R. (2008). Variability of Oil Content and of Major Fatty Acid Composition in almond (Prunus Amygdalus Batsch) and its Relationship with Kernel Quality. *J. Agric. Food Chem.* 56 (11), 4096–4101. doi:10.1021/jf8001679

Li, G., and Long, C. (2019). New Progress in Ethnobotany. *Science* 71 (02), 1–4+69.

Li, J., Yu, M., Ma, R., Shen, Z., and Xu, J. (2009). Study on Rapid Propagation of Three Peach Rootstocks *In Vitro*. *Jiangsu J. Agric. Sci.* 25 (03), 635–639.

Li, L., Liu, Y., Yan, Y., Tang, Z., Duan, J., Song, Z., et al. (2019). Research on Grade Prediction of Persicae Semen Based on Binary Logistic Regression Analysis. *Chin. Traditional Herbal Drugs* 50 (19), 4691–4696.

Li, T., Liu, J., Xie, Y., Wang, Q., and Meng, F. (2014). Analysis of Genetic Diversity in Prunus Mira Koehne Ex Sargent Populations Using AFLP Markers. *Plant Syst. Evol.* 300 (3), 475–482. doi:10.1007/s00606-013-0896-5

Li, Y., Zhu, R., Zhang, H., Wang, X., and Wang, Y. (2010). Study on Grafting Technology of Tibetan Prunus Mira. *Anhui Agric. Sci. Bull.* 16 (09), 117+175.

Liang, Y., Pei, J., Zhou, M., Fan, Z., Liu, W., and Wan, D. (2012). Analysis of Fat-Soluble Composition in Different Varieties of Semen Persicae by GC-MS. *West China J. Pharm. Sci.* 27 (02), 174–176.

Liang, Y. (2011). Study on Quality Evaluation of the Effective Components in Safflower-Semen Persicae and Their Preparations. Master's thesis. Chengdu University of TCM.

Liu, C., Li, B., and Xie, Y. (1989). Pharmacological Studies on Nuts of Prunus Mira Koehne and Prunus Davidiana. *Pharmacol. Clin. Chin. Materia Med.* (02), 46–47.

Liu, H., Wang, Z., Liu, J., Wang, S., Tang, Y., and Pang, X. (2012). Genetic Diversity and Genetic Structure of Siberian Apricot Populations in the Yan Mountains. *Scientia Silvae Sinicae* 48 (08), 68–74.

Liu, L., and Meng, F. (2013). Fruit Quality Analysis of Amygalus Mira in Tibet. *J. Anhui Agric. Sci.* 41 (35), 13740–13741+13743.

Liu, W. (2007). On the Present Situation and Trend of Domestic Tourism Consumption in China. *Social Sci. Hunan* (02), 118–120.

Liu, Y., and Yuan, L. (2015). *Occurrence Characteristics and Prevention Measures of Agrometeorological Disasters in Xigaze, Tibet*. Beijing Agriculture, 165–166.20

Luo, D. (2004). *Xin Xiu Jing Zhu Ben Cao*. Chengdu, China: Sichuan Science and Technology Press.

Luo, D., and Zheng, W. (1998). Wild Fruit Resources and Their Utilization prospect in Sejila Mountain, Tibet. *J. Fruit Sci.* (03), 283–289.

Luo, H., and Wang, L. (2019). 2019 Is the April Season when Peach Blossoms Bloom in Nyingchi. *Tibet Geogr.* (02), 16–17+178.

Ma, R., Ma, Y., Xu, Y., Jiang, Y., and Cao, M. (2002). *Analysis of International Genetic Resources of Almond, Annual Meeting of Young Agricultural Science of China Agricultural Association*. Shenzhen, China: Annual Report of Chinese Youth Agricultural Science, 328–332.

Mima, C., Gesang, Q., Danzeng, N., and Danzeng, L. (2018). Grafting and Management Techniques of Peach Trees under Climatic Conditions in Semi-arid Areas of Lhasa, Tibet. *Tibet Sci. Tech.* (04), 68–70.

Nei, M. (1978). Estimation of Average Heterozygosity and Genetic Distance from a Small Number of Individuals. *Genetics* 89 (3), 583–590. doi:10.1093/genetics/89.3.583

Northwest Institute of Plateau Biology (1991). *Tibetan Medicine Records*. Xining, China: Qinghai People's Publishing House.

Online (2021). Prunus Mira Koehne. Available at: https://eol.org/pages/240727.

Pei, S. (2003). Chinese Ethnobotany: Retrospect and Prospects. *The Chin. Acad. Med. Mag. Organisms* (02), 66–71.

Pei, S. (1988). Ethnobotany and Exploitation of Plant Resources. *J. Plant classification Resour.*, 135–144.

Pei, S. (2011). General Introduction to Three-Decades Development of Ethnobotany and Perspectives in China. *J. Minzu Univ. China (Natural Sci. Edition)* 20 (02), 5–9.

Pei, S. (2008). Review of Ethnobotany Research in the Past 20 Years. *Acta Botanica Yunnanica* 30 (04), 505–509.

Pharmacopoeia (2015). *Pharmacopoeia of the People's Republic of China :2015 Edition*. Beijing: China Medical Science and Technology Press.

Province (2017). *Tourism Development Plan of Sichuan Province in the 13th Five Year Plan*. Springer.

Qiu, T. (2020). Study on Medicinal Ethnobotany in Tibetan Areas of Sichuan. Master's thesis. Southwest Medical University.

Sathe, S. K., Seeram, N. P., Kshirsagar, H. H., Heber, D., and Lapsley, K. A. (2008). Fatty Acid Composition of California Grown Almonds. *J. Food Sci.* 73 (9), C607–C614. doi:10.1111/j.1750-3841.2008.00936.x

Smith, M. D. (2011). The Ecological Role of Climate Extremes: Current Understanding and Future Prospects. *J. Ecol.* 99 (3), 651–655. doi:10.1111/j.1365-2745.2011.01833.x

Sun, C., and Sheng, X. (2004). *Ethnobotany and the Development and Utilization of Chinese Herbal Medicine*. Crop research, 434–438.S1

Sun, W. (2018). Study on Chemical Constituents of Nuts Oil from Prunus Mira Koehne and the Mechanism of Promoting Hair Growth. Master's thesis. Chengdu University of TCM.

Sun, W., Zhou, X., Wang, Z., Zhang, J., Gan, D., LiAng, Y., et al. (2017). Study on the Acute Toxicity of Prunus Mira Oil in Rats and Rabbits. *China Med. Herald* 14 (21), 18–24.

Sun, W., Zhou, X., Wang, Z., Zhang, J., Yue, M., Liang, Y., et al. (2018). Analysis of Fat-Soluble Components of Nuts Oil from Prunus Mira in Derong County by GC-MS. *Chin. Traditional Patent Med.* 40 (01), 142–146.

Tamar, D. (1986). *Jing Zhu Ben Cao*. Shanghai: Shanghai Science and Technology Press.

Tan, J., Zeng, X., and Liao, M. (2012). Genetic Diversity of Natural Prunus Mira Populations Detected by SRAP. *J. grassland industry* 21 (06), 213–220.

Tang, J. (2012). Analysis of Fruit Characters and Genetic Diversity of Prunus Mira in Tibet. Master's thesis. Sichuan Agricultural University.

Tian, Y. (2016). Preliminary Study on Root Response Mechanism of Prunus Mira under Drought Stress. Master's thesis. Northeast Forestry University.

Tian, Y., Xing, C., Cao, Y., Wang, C., Guan, F., Li, R., et al. (2015). Evaluation of Genetic Diversity onPrunus miraKoehne by Using ISSR and RAPD Markers. *Biotechnol. Biotechnological Equipment* 29, 1053–1061. doi:10.1080/13102818.2015.1064780

Wang, C. (2020). Sustainable Development of Local Cultural Ecology:A Continuous Conceptual Discussion of Cultural Industry,Plants Culture and Ethnobotany. *Guizhou Ethnic Stud.* 41 (12), 148–155.

Wang, W., and Wei, L. (2016). Analysis of Fatty Acid Compositions in Prunus Mira Kernels from Different Growing Areas in Tibet. *Food Sci.* 37 (22), 107–111.

Wang, X., Peng, F., Wang, Z., and Li, G. (2009). "In Current Situation and Existing Problems of Fertilization in Peach Orchards in Shandong Province," in Proceedings of the Second Annual Academic Meeting of Peach branch of China Horticultural Society (Chengdu, Sichuan, China: PLoS ONE), 5.

Wang, Z., Zhao, W., Gesang, W., and Wei, L. (2021). Study on Tibetan Residents' Cognition of Prunus Mira Resources and Expectation of Planting Assistance. *J. Plateau Agric.* 5 (01), 101–108.

Wang, Z., and Zhou, J. (1990). Study on Genetic Evolution of Peach Germplasm -- Pollen Morphology Analysis. *Acta Horticulturae Sinica* (03), 161–168.

Wei, L., Yang, X., and Gong, W. (2017). Analysis and Evaluation of the Contents of Trace Elements in Nuts of Prunus Mira Koehne from Different Provenances. *Jiangsu Agric. Sci.* 45 (22), 195–197. doi:10.1002/dc.23716

Wei, L., Zhong, Z., and Li, M. (2013). Extraction of Fatty Oil from Prunus Mira Kernel and Analysis of its Physicochemical Properties. *Non-wood For. Res.* 31 (03), 136–139.

Xi, W. (2016). *Du Mu Ben Cao*. Qinghai: Qinghai People's Publishing House.

Xiang, D., Yang, J., Zhang, S., Wang, Z., and Deqing, Z. (2019). Investigation on Occurrence and Harm of Prunus Mira Perforation Disease in Lin Zhi. *J. Agric.* 9 (10), 13–17.

Xing, C. (2014). Genetic Diversity Analysis of Prunus Mira Germplasm Resources Northeast. Master's thesis. Forestry University.

Xing, C., Meng, F., Wang, C., Wang, Q., and Yi, J.-S. (2013). "Assenssing the Genetic Diversity of Prunus Mira Koehne from the Tibet Plateau Using SSR Marker," in 2013 2nd International Congress on Biodiversity Ecology and Environment (Nanjing, Jiangsu, China: Journal of Ecosystem & Ecography), 1.

Xu, G., and Xu, L. (1997). *Quality Arrangement and Quality Study of Common Chinese Medicinal Materials*. Fujian: Fujian science and Technology Press.

Xue, L. (2019). Economic Value Assessment and Protection of Amygdalus Mira Genetic Resources. *For. Sci. Tech. Inf.* 51 (01), 24–25.

Yang, S. (1989). Prunus Mira Koehne Resources Were Found in Muli Tibetan Autonomous County, Sichuan. *J. Fruit Sci.* (02), 120.

Yang, Y., Cheng, S., Jin, X., Hou, J., Du, C., and Hong, D. (2020). Insecticidal Effect of Amygdalin from Light Walnut Kernel Extract. *J. Plateau Agric.* 4 (06), 566–569.

Yang, Y., Di, S., Zhang, H., Zhao, L., and Tong, X. (2018). Study on Clinical Application and Dosage of Peach Kernel. *Jilin J. Chin. Med.* 38 (12), 1441–1446.

Zeng, X., Ciren, Z., Li, Q., and Gong, J. (2009). *Changes of Dietary Fiber in walnut Flesh of Xizang Wild Light*. Chengdu, Sichuan, China: The second annual conference of Peach Branch of Chinese Society of Horticulture, 3.

Zeng, X. (2016). Diversity of Wild Prunus Mirat Flowers in Tibet. *Anhui Agric. Sci. Bull.* 22 (01), 12–15.

Zhang, C., Li, Y., Yuan, L., Lan, X., and Zhong, Z. (2019). Pulse Vacuum Debittering Process of Optic Walnut Kernel. *J. Plateau Agric.* 3 (05), 559–565+585586.

Zhang, C., Wang, Z., Liu, F., Liu, Z., and Jiang, L. (2019). Pot experiment on Waterlogging Tolerance of Peach Rootstocks. *North. Fruits* (02), 11–12+18.

Zhang, L., Meng, R., and Lu, G. (2013). Economic Evaluation and protection ofAmygdalus Miragenetic Resource. *Acta Eco Sin* 33 (22), 7277–7287. doi:10.5846/stxb201207030928

Zhang, L., Liu, L., and Niu, X. (2011). Effects of Different Treatments on Seed Germination of Prunus Mira. *Seed* 30 (12), 83–84+87.

Zhang, Q., Meng, S., Wang, J., and He, G. (1979). Component Analysis of Seed Oil of Six Plants in Tibet. *J. Integr. Plant Biol.* (01), 80–81.

Zhong, Z., and Fang, J. (2011). Liquid & Solid Cross-Sprinkling Fermentation in Producing Prunus Mira Koehne Vinegar. *Food Res. Dev.* 32 (03), 94–96.

Zhong, Z., Fang, J., and Pu, Q. (2010). Investigation and Analysis on Fruit Growth Law and Yield of Prunus Mira in Nyingchi, Tibet. *J. Anhui Agric. Sci.* 38 (22), 12047–12049+12053.

Zhong, Z., and Pu, Q. (2001). Study on Processing Technology of Tibet Wild Peach Cold Fruit. *For. Sci. Tech.* (12), 35–36. doi:10.1108/09540910110385248

Zhong, Z. (2008). Study on Resource Ecology of Prunus Mira in Nyingchi, Tibet. Master's thesis. Tibet University.

Zhong, Z., Wang, T., and Fang, J. (2014). Optimum Ingredient of Compound Artistalingagent for Prunus Mira Koehne Pulps by Uniform Design Method. *Food Sci. Tech.* 39 (05), 47–52.

Zhong, Z., Wang, T., Gao, G., Fu, K., and Fang, J. (2012). Optimization of Fermentation Process for Prunus Mira Koehne Wine at Natural Temperature by Response Surface Methodology. *Food Sci.* 33 (13), 197–201.

Zhou, C., Bai, T., Wang, Y., Wu, T., Zhang, X., Xu, X., et al. (2017). Morpholoical and Enzymatic Responses to Waterlogging in Three Prunus Species. *Scientia Horticulturae* 221, 62–67. doi:10.1016/j.scienta.2017.03.054

Zhou, J., Guo, H., Zhao, M., and Lu, Z. (1998). "IEF Electrophoresis Analysis of Water-Soluble Proteins in Wild Peach Anthers," in Horticulture Satellite Conference of the 3rd Annual Youth Academic Conference of China Association for Science and Technology and the 2nd Youth Academic Symposium of Chinese Society of Horticulture (Nanjing, Jiangsu, China: ISHS), 3.

Zhou, J., Zhong, Y., Wang, T., Yang, S., and Guo, H. (1995). Rare Stone Fruit Resources in Southwest Sichuan. *China Seed Industry* (02), 21–22.

Zhou, J., Zhong, Y., Wang, T., Yang, S., Xu, J., and Guo, H. (1994). "In Types and Origin of Prunus Mira and Peach in Southwestern Sichuan," in The First Youth Symposium of China Horticultural Society (Hangzhou, Zhejiang, China: ISHS), 4.

Zhou, Y., Tang, G., Li, X., Sun, W., Liang, Y., Gan, D., et al. (2020). Study on the Chemical Constituents of Nut Oil from Prunus Mira Koehne and the Mechanism of Promoting Hair Growth. *J. Ethnopharmacol* 258, 112831. doi:10.1016/j.jep.2020.112831

Zhou, Y. (2020). Study on Effective Components Promoting Hair Growth in Tibetan Medicine Nuts of Prunus Mira Koehne and Their Mechanism of Action. Master's thesis. Chengdu University of TCM.

Zong, X., Yu, H., Wang, Z., and Qi, m. (1995). Phylogenetic Relationship and Evolution of Peach Species — Analysis of Pollen Protein by SDS Electrophoresis. *Acta Horticulturae Sinica* (03), 288–290.

Zuo, L., Bi, J., Liu, C., Lv, J., Jin, X., and Zhou, M. (2018). "Effects of Different Treatments on Functional Components and Antioxidant Activities of Prunus Mira Koehne," in The 15th Annual Conference of Chinese Institute of Food Science and Technology (Shandong, China: Qingdao), 2.

Zuo, L., Lv, J., Bi, J., Chen, Q., Jin, X., and Li, X. (2019). Research on Powder Characteristics and Active Components of Prunus Mira Koehne Powder Prepared by Superfine Grinding Technology. *Mod. Food Sci. Tech.* 35 (04), 182–189+135.

Zuo, L. (2019). Study on the Functional Components and Powder Technology of Prunus Mira Koehne. Master's thesis. Tianjin University of Science and Technology.

Zuo, Q., Gong, F., Zhu, G., Zong, X., and Zhang, X. (1988). Preliminary Report on Identification of Resistance to Root Knot Nematode of Different Peach Rootstocks. *J. Fruit Sci.* (03), 116–119.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 30 March 2022
doi: 10.3389/fphar.2021.789360

Traditional Dietary Knowledge of a Marginal Hill Community in the Central Himalaya: Implications for Food, Nutrition, and Medicinal Security

S. N. Ojha[1†], *Aryan Anand*[1‡], *R. C. Sundriyal*[1,2*] *and Deepshikha Arya*[3]

[1]*G.B. Pant National Institute of Himalayan Environment, Kosi-Katarmal, Almora, India,* [2]*Department of Forestry and Natural Resources, HNB Garhwal University, Srinagar, India,* [3]*Regional Ayurveda Research Institute, Ranikhet, India*

Himalayan communities illustrate a rich agriculture–medicine use system that not only provides adequate dietary diversity and nutrition but also delivers therapeutic security. This study explores the food–medicine interface as observed by the marginal hill communities in the central Himalaya with an aim to assess traditional agriculture and food plants with relation to dietary diversity and nutritional and medicinal values based on comprehensive research. A total of 445 respondents were interviewed to obtain data on food intakes using dietary recall methods and dietary diversity indices (DDIs). The ethnomedical use of plant species was gathered from respondents as well as from various published studies for respective species. Nutritional parameters were collected from the Indian Food Composition Table developed by the ICMR, India to analyze the average nutritional intake. The traditional food system achieves the dietary and nutritional needs of the community within the standard norms. The average household dietary diversity of 7.45, 7.34, and 8.39 in summer, monsoon, and winter seasons, respectively, sustain 79, 74, and 93% of energy requirements in respective, seasons. The average food consumption score (FCS) was 73.46, and all the food exhibited rich phytochemicals, such as amino acids, alkaloids, carotenoids, flavonoids, glycosides, and phenolic acids. These plants also provided effective treatments against several ailments and illnesses, such as cardiovascular diseases, diabetics, gastrointestinal issues, and inflammation The indigenous cuisines also have significant food and medicinal values. Considering that the community had significant knowledge of food systems with their nutritional and therapeutic utility, there is a need to protect and document this indigenous knowledge. Also, most of the crops are still under cultivation, so there is a need to create more awareness about the nutritional and therapeutic value of the system so that it could be retained intact and continued. The implications of this research are of both academic importance and practical significance to ensure food–medicine security and avoid malnutrition among rural communities. It is expected that the study would lead to renewed thinking and policy attention on traditional agriculture for its role in food and nutritional security that may lead to a sustainable food supply system.

Keywords: central Himalaya, traditional food crops, dietary intake, food–medicine interface, nutritional security, health care, traditional cultural knowledge, Uttarakhand

OPEN ACCESS

Edited by:
Michael Heinrich,
UCL School of Pharmacy,
United Kingdom

Reviewed by:
Arunachalam Karuppusamy,
Chinese Academy of Sciences (CAS), China
Giulia Mattalia,
Ca' Foscari University of Venice, Italy
Concepción Obon,
Miguel Hernández University of Elche, Spain
Yao Fu,
Kunming Institute of Botany (CAS), China

****Correspondence:***
R. C. Sundriyal
sundriyalrc@yahoo.com

†Present address:
S. N. Ojha,
Department of Botany, Uttarakhand Open University, Haldwani, India

‡Present address:
Aryan Anand,
Central University of Punjab, Ghudda, Bathinda, India

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *04 October 2021*
Accepted: *16 December 2021*
Published: *30 March 2022*

Citation:
Ojha SN, Anand A, Sundriyal RC and Arya D (2022) Traditional Dietary Knowledge of a Marginal Hill Community in the Central Himalaya: Implications for Food, Nutrition, and Medicinal Security. Front. Pharmacol. 12:789360. doi: 10.3389/fphar.2021.789360

INTRODUCTION

Notwithstanding significant growth in the agriculture sector in the past few decades, still, there is persistent hunger and malnutrition in many parts of the world (FAO 2017). Expanding the food production system to new areas comes with a heavy natural environmental cost, thus posing a challenge to sustainable food supply to the ever-growing population (Fróna et al., 2019). It is disheartening to note that despite the global commitment to bring food security and end hunger and malnutrition by 2030, the world is far from achieving these SDG objectives; on the contrary, the number of undernourished and hungry people has been increasing (FAO, IFAD, UNICEF, WFP and WHO, 2020). There is also a decline in the access of quantity and quality of food in many places. Disruptions in food supply and income greatly impact the access of poor and vulnerable people to nutritious foods and healthy food across the globe (FAO, IFAD, UNICEF, WFP and WHO, 2018). As per the FAO report, nearly 11% of the global population and 14.5% of India's population is undernourished. An Indian Council of Medical Research (ICMR) report emphasized that malnutrition is a major contributing factor behind the death of children below 5 years of age, and the incidences of malnutrition are high in rural and tribal areas (Narayan et al., 2019). The major cause of malnutrition is lack of adequacy of fresh fruits, vegetables, legumes, grains, meat, and milk. Fortunately, there are many traditional crops and food production systems that are in place for centuries and have been meeting food and nutritional requirement of a substantial population (Adhikari et al., 2019). Such agricultural systems are well-established and sustainable in food supply; thus, they can provide competitive benefits over the modern agriculture systems (Carl et al., 2017). All over the world, traditional food systems are supportive in maintaining local food habits and culture along with conserving vital food and fodder plants and their wild relatives (FAO 2011a; Khoury et al., 2016). It is largely practiced by smallholders, particularly by women, and is important in sustaining agricultural genetic diversity and soil fertility (FAO 2011b; Maikhuri et al., 2015). Other important aspects of traditional agriculture are withstanding nutritional and food security, ensuring optimum productivity and economic return, reducing the vulnerability of crops during adverse conditions, retaining natural resource base, and minimizing adverse environmental impacts (Sundriyal et al., 1994; ICMR-ICFT 2017). Traditional food systems are considered beneficial in terms of providing fewer calories and saturated fat, more iron, calcium, zinc, and vitamin A (IFPRI 2015). It also provides relief in selected health issues, such as allergies, asthma, digestive and cardiovascular illness, obesity, and even diabetes, thus acting as an interface of food and medicine (CINE 2021). The traditional crops and wild plants offer wide food diversity and nutritional security to local communities, thus safeguarding against hunger (Jones 2017). It secures access of marginal communities to adequate food especially in the low-income regions where a significant share of the population, especially women, is still engaged in agriculture (Bisht et al., 2018). Local food systems also offer enriched dietary diversity and quality that strengthen environmental sustainability (Fanzo et al., 2013) and therapeutic efficacy (Sarkar et al., 2015). Thus the traditional food and dietary systems constitute a backbone for the sustainable development of agriculture, food security, and poverty alleviation. Therefore, supporting the diversity of foods and species consumed in local diets has significant benefits for sustainable food systems and public health perspectives (Jennings et al., 2015; Hettiarachchi 2021). However, globally, there is a net decline in traditional food systems despite the fact that a significant population still depends on them (Khoury et al., 2014). The developing countries are undergoing a rapid change in land uses and governance with a shift from subsistence to commercial agriculture to accelerate economic growth that ultimately affects the access of local communities to food (Broegaard et al., 2017). A decline in crop diversity has significant worldwide implications for food and nutritional security. This increases the demand to make traditional agriculture more reliable by generating greater awareness regarding such systems that not only meet food demand but also serve as a better example of an interface of food and medicine. The present study was undertaken with a similar focus so as to create an enhanced understanding of the advantages and limitations of traditional agriculture in the central Himalayan region. Here, a large section of the population is still dependent on traditional agriculture, and women have been a major workforce to perform agricultural activities (Sundriyal et al., 2014). We argue that there is a need to highlight the health, nutritional, and therapeutic benefits of the traditional food system to revive it from disappearance. Considering this, the present study investigates the food–nutrition–medicine interface of marginal hill communities in the central Himalaya. The objectives are to assess 1) crops and wild plant diversity used to fulfill the dietary quality of the Central Himalayan community and 2) nutrient and therapeutic claims of the traditional diets essential for sustenance of the community. We expect that a better understanding of the intersection between traditional food, dietary diversity, nutritional quality, and medicinal efficacy along with some possible developmental options, thus, would receive greater attention from policy planners and developmental workers. In addition to this, it may salvage the dwindling traditional dietary system that is further leading the marginalization of the smallholder farming communities.

Study Region and Methods

For this study, the Uttarakhand state in the central Himalaya, India was targeted that comprised a total geographical area (TGA) of 53,483 km^2. The state shares borders with Himachal Pradesh in northwest & Uttar Pradesh in the south and international borders with Tibet in the north and Nepal in the west. Physiographically, Uttarakhand can be divided into three broad zones, viz., the Himalayas, the Siwalik, and the Terai region. Administratively, the state has 13 districts and houses a population of 10.09 million (69.77% rural and 30.23% urban) with a livestock population of 4.79 million and largely exhibits an agrarian and pastoralist economy with high dependence on forest resources. For detailed investigation, we selected the Bageshwar district (area of 2,302 km^2) of Uttarakhand that is most centrally located and represents all broad features of the state (**Figure 1**). Bageshwar is largely hilly terrain covering Siwalik ranges and the high Himalayas. Pindar, Saryu (Sarju), Gomati, and Pungar are the main rivers flowing across the district. Administratively, the

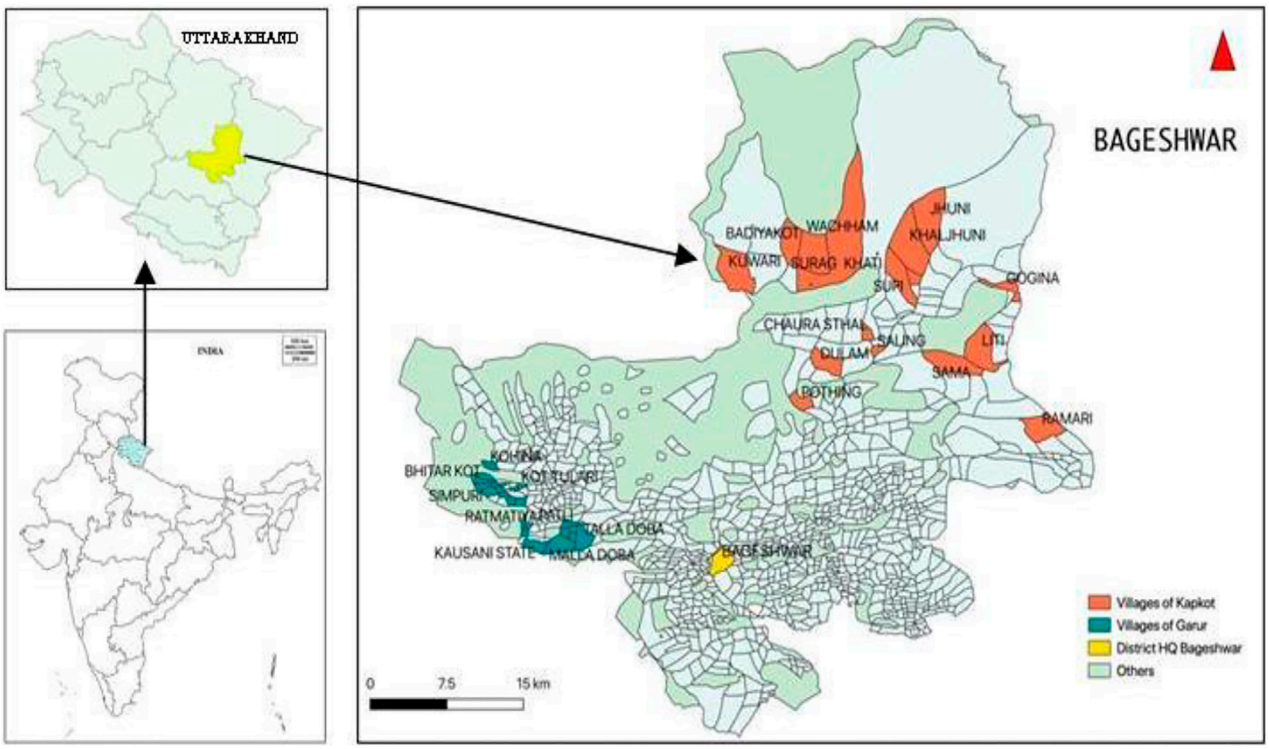


FIGURE 1 | Study area and villages in Garur and Kapkot Blocks of Bageshwar, Uttarakhand, India.

district comprised four Tehsils, viz., Bageshwar, Kapkot, Kanda, and Garur and three blocks, viz., Bageshwar, Garur, and Kapkot. As per the 2011 census, the total population is 259,898 (male 48% and female 52%) with 96% living in the rural areas. There are 874 inhabited villages in the district (Anonymous 2011). The community of the area is divided into three categories, viz., General, Scheduled Class, and Scheduled Tribe. The education status of the district is good, with an overall literacy rate of 80.01%, although it is much higher for males (92.33%) than females (69.03%). The district has 10.8% land area under agriculture, which is mainly rain-fed (average annual rainfall of 1,200–1,400 mm). Only 20% of agricultural land is irrigated. In addition, the district comprised 55% land area under forest, 5.42% cultivable barren land, 3.23% barren and uncultivable land, and 10.64% as permanent pasture and grazing land (Anonymous 2011). The district also has 273,051 livestock that forms an integral part of each household. These animals comprised cattle (37%), buffalo (10%), goats (40%), sheep (6.5%), and others (6.5%). In addition, there is also backyard poultry. As per the livestock census 2012, Uttarakhand exhibited low per capita availability of milk (387g/day/person), meat (2kg/person/year), and egg (27eggs/person/year); therefore, in rural places, there is high dependence on agriculture. However, the economy is collectively met from all these lands and largely subsistence-type. The majority of people are involved in the primary sector (agricultural activities), although some also work in secondary and tertiary sectors, such as private works, businesses, and government jobs. Generally, the community is greatly dependent on farming and natural resources and characterized as highly marginal with small and scattered land holdings, small production, and low income. The major foods of the community are rice, finger millet, wheat, barley, maize, pulses, and a wide variety of vegetables cultivated or collected from the wild. Occasionally, people also consume animal products (meat, ghee, buttermilk, milk, curd, etc.). Generally, the male population out migrates to earn better livelihoods, leading to dominance of womenfolk in the villages. It also results in a continuous increase in fallow lands and culturable wastelands. The district has limited health infrastructure mainly located in urban areas. As per the National Family Health Survey (2015–16), the prevalence of malnutrition in Uttarakhand among children under 5 years of age was 26.6% underweight and 33.5% stunting (Anonymous 2014). As per Food Policy Research data, the status of nutrition in Bageswar revealed that among the children <5 years, 23% exhibited stranded growth, 26.3% were underweight, and 45% anemic. In women of reproductive age, 41.3% were anemic while 24.9% had a body mass index <18.5 kg/m^2. For common health needs, rural communities are largely dependent on the traditional health care system (Ojha et al., 2020).

Assessing Traditional Food and Medicinal Usage

The study was conducted from 2017 to 2019. To collect field data, we randomly selected 24 villages covering Garur Ganga, Saryu,

TABLE 1 | Crops plants used as traditional foods, nutritional security, and primary healthcare by central Himalayan communities.

	Crop category and scientific name {family, (RKT no.#)}	Common name	Local name	[$]Availability season	Cultivated or collected from the wild	Additional use other than food (during illness)	Mode of use or application	Details of recipes or medicinal use
A	Cereals, pseudo-cereals and millets							
1	*Amaranthus caudatus* L. {Amaranthaceae (RKT 25885)}	Amaranth/ Ramdana	*Kedari chuwa*	S, W	C	Measles	DA	Seeds (25 g) are spread over the sleeping bed
2	*Echinochloa frumentacea* Link. {Poaceae (RKT 7475)}	Barnyard millet	*Jhangora/ Madira*	S, W	C	Anemia	Co	De-husked seeds and flour used as *chapati* (bread) and cooked rice, respectively
3	*Eleusine coracana* (L.) Gaertn. {Poaceae (RKT 7299)}	Finger millet	*Madua*	M, W	C	Cold and cough and high blood pressure	Co	Porridge of flour and breads (hot *chapatti* 2–3 nos. for 3–4 days)
4	*Fagopyrum esculentum* Moench {Polygonaceae (RKT 27688)}	Buckwheat/Kuttu	Ogal/phaphar	S, W	C	Energy booster	Co	Recipes (halwa, chapati, and vegetables) eaten for boosts of energy
5	*Fagopyrum cymosum* (Trev.) Meisn {Polygonaceae (RKT 12896)}	Wild buckwheat	Jhankara	S	W	Stomachic	Co	Leaves and tender twigs used as vegetable
6	*Hordeum vulgare* L. {Poaceae (RKT 7855)}	Barley	*Jau*	S, M	C	High blood pressure and throat disorders	S, Co	Breads (*Chapatis*-25 g/ person) for blood pressure normalization; smoke of burning grains (10 g) inhaled for throat cure
7	[a]*Oryza sativa* L. {Poaceae (RKT 4796)}	Paddy	*Dhan*	S, M, W	C	Leucorrhea	Co	Boiled rice of *Sanwdhan* (100–200 g/person) locally known as *Bhaat*
8	*Setaria italica* (L.) P. Beauv. {Poaceae (RKT 7389)}	Foxtail millet	*Kauni*	S, M, W	C	Measles	Co	De-husked grains (50–100 g/individual) are cooked as rice and served to the patients
9	[a]*Triticum aestivum* L. {Poaceae (RKT 26973)}	Wheat	*Gehun*	S, M, W	C	--	Co	Seed flour used as chapatti and other traditional dishes
10	*Zea mays* L. {Poaceae (RKT 7536)}	Maize	*Makka*	S, M	C	Whooping cough	AF	Blank cob's ash (20–30 g)
B	Vegetables							
11	*Asparagus filicinus* Buch.-Ham. ex D.Don {Asparagaceae (RKT 14469)}	*Asparagus*	*Kairuwa*	S	W	Energy booster and tonic	Co	Young shoots (40–100 g/ individual) vegetable
12	*Bauhinia variegata* L. {Fabaceae (--)}	Kachnar	*Kwairal*	S	W	Dysentery, diarrhea, and Stomachic	Co	Boiled flower buds used as a traditional *Rayata* and pickle
13	*Benincasa hispida* (Thunb.) Cogn. {Cucurbitaceae (RKT 26003)}	Wax gourd	*Paitha/Kumila/ Bhuj*	S, M	C	Stomachic	Co	Fruit used in traditional *Baris* and sweet dishes
14	*Brassica oleracea* var. capitata L. {Brassicaceae (--)}	Cabbage	*Band gobi*	W	C	--	Co	Vegetative bud used as vegetable
15	*Chenopodium album* L. {Amaranthaceae (RKT 19173)}	*Chenopodium*	*Bathua*	W	C	Cold and cough	Co	Soup of (100 ml) of matured grains (25 g) with normal spices
16	*Colocasia esculenta* (L.) Schott {Araceae (RKT 8706)}	Taro	*Gaderi/Pinalu*	M, W	C	--	Co	Corms, rolled leaf blade, and petiole or leaf stalk used as vegetable
17	*Cucumis sativus* L. {Cucurbitaceae (RKT 1040)}	Cucumber	*Kakari*	S, M	C	Sun stroke and malaria	In	Water of matured cucumber fruits
18	*Cucurbita moschata* Duch. ex Poir. {Cucurbitaceae (--)}	Pumpkin	*Kaddu*	M,W	C	--	Co	Green and matured (ripe) fruit vegetable (60–100 g/ individual)
19	*Cyclanthera pedata* (L.) Schrad {Cucurbitaceae (RKT 27159)}	Wild cucumber	*Meetha/ RamKarela*	S, M	C	Liver diseases and stomachic	Co	Fruit (100 g/individual) vegetable
20	*Diplazium* esculentum (Retz.) Sw. {Athyriaceae (--)}	Fern	*Lingura*	S, M	W	Constipation	Co	Young fronds (50 g/ individual) used as vegetable
21	*Dioscorea alata* L. () {Dioscoreaceae (RKT 11878)}	Winged yam	*Tarur/Tairu*	W	W	Stomachic	Co	Tuber used in traditional recipe—*Tarur ki sabzi* (50 g/ individual)
22	*Dioscorea bulbifera* L. {Dioscoreaceae (RKT 27263)}	Dioscorea, Yam	*Genthi*	W	C	Cold and cough	Co	Cooked vegetables of yams (150 g/individual)
23	*Lagenaria siceraria* (Molina) Standl. {Cucurbitaceae (--)}	Bottle gourd	*Lauki*	S, M	C	Low and high blood pressure	Co	Juice or soup (85–105 g/ individual) of vegetable
24	*Luffa acutangula* (L.) Roxb. {Cucurbitaceae (RKT 3602)}	Ridge gourd	*Torai*	S, M	C	Fever	Co	Fruit (85–105g/individual) vegetable
25	*Megacarpaea polyandra* Benth. {Brassicaceae (RKT 1378)}	Rooki	*Barmola/Rookhi/ Rugi*	S	W	Dysentery, fever, and stomach ache	Co, DA	Fresh leaves (70 g/ individual) used as vegetable; roots eaten raw
26	*Momordica charantia* L. {Cucurbitaceae (RKT 24932, RKT 27529)}	Bitter gourd	*Karela*	S,M	C	Stomach ache, diabetes, and antiparasitic	Co, In	Fresh fruit juice (10–20 ml/ day for 3–4 days in a week; Fruit (50 g/individual) vegetable
27	*Phytolacca acinosa* Roxb. {Phytolaccaceae (--)}	Indian pokeweed	*Jarag*	S, M	C	Cough, cold, and constipation	Co	The fresh tender leaves and twigs (40–50 g/individual) are cooked as vegetable
28	*Raphanus sativus L.* (Brassicaceae) {Brassicaceae (RKT 10925, RKT 27049)}	Radish	*Mooli*	M, W	C	Jaundice	Co	Green leaves and roots (50–70 g/individual) cooked as vegetables without oil and turmeric
29	*Solanum melongena* L. {Solanaceae (RKT 29242)}	Brinjal	*Baigan*	S, M	C	Dog bite/rabies	AF	Stem wood ash (20–30 g) powder is tied on the wounds of biting spot

(Continued on following page)

TABLE 1 | (*Continued*) Crops plants used as traditional foods, nutritional security, and primary healthcare by central Himalayan communities.

	Crop category and scientific name {family, (RKT no.#)}	Common name	Local name	$Availability season	Cultivated or collected from the wild	Additional use other than food (during illness)	Mode of use or application	Details of recipes or medicinal use
30	[a]*Solanum tuberosum* L. {Solanaceae (RKT 8138)}	Potato	*Aalu*	S, M, W	C	--	Co	Starchy food (king of the vegetable), ingredients of all type vegetable
31	*Spinacia oleracea* L. {Amaranthaceae (RKT 28871)}	Spinach	*Palak*	W	C	Hemoregulatory	Co	Leaf used as traditional dish—*Palak ka Kafa*
32	*Trichosanthes anguina* L. {Cucurbitaceae (RKT 2264)}	Snake gourd	*Chichanda*	S, M	C	Fever	Co	Vegetables of fruits (70 g/person)
33	*Urtica ardens* Link. {Urticaceae (RKT 24064)}	Himalayan nettle	*Bichhughas Kandali/*	S, M, W	W	Menorrhagia disorder and muscular pain	Co, DA	Young shoots are cooked as vegetable and eaten for smooth menstruation; young shoots applied in body cramp & external pains (muscular pain)
C	Pulses							
34	*Glycine max* (L.) Merrill (Fabaceae) {Fabaceae (RKT 29313)}	Soybean	*Bhatt*	S, M, W	C	Jaundice and piles	Co	Local recipes like *Jaula, Dubuke*, and *Chutkani* are prepared from the grains (40 g/individual)
35	*Glycine max subsp*. soja (Sieb. & Zucc.) H. Ohashi {Fabaceae (RKT 15664)}	Black soybean	*Kala Bhatt*	S, M, W	C	Jaundice and piles	Co	Local recipes like *Jaula, Dubuke*, and *Chutkani* are prepared from the grains (45 g/individual)
36	*Lens culinaris Medik* {Fabaceae (RKT 7781)}	Lentil	*Masoor*	S, M, W	C	Anemia	Co	Local recipes like *Dal and Dubuke* are prepared from the grains (40 g/persons)
37	*Phaseolus vulgaris* L. {Fabaceae (RKT 29059)}	French Bean	*Rajma*	S, M, W	C	Energy booster (protein rich)	Co	Local landraces are good sources of protein used as different recipes
38	*Vigna mungo* (L.) Hepper {Fabaceae (RKT 27199)}	Black gram/urad	*Maas*	S, M, W	C	Energy booster and for fracture	Co, In	Local recipes like *chaise, Baidu roti* etc are prepared from the grains (40–50/persons); Paste prepared by grinding of (50 g/person) seeds with water applied on the fractured part
39	*Vigna unguiculata* (L.) Walp {Fabaceae (RKT 16856)}	Cowpea	*Lobia/Sotta/ Sunt*	S, M, W	C	Diabetes and energy booster	Co	Taken as a traditional dish form (40–50 g/person)
40	*Macrotyloma uniflorum* (Lam.) Verde {Fabaceae (--)}	Horsegram	*Gahat*	S, M, W	C	Kidney stone, cold, and cough	Co	Pulse (*Daal*–40–50 g/person) soup; *Gahat ka Ras* (an indigenous dish) prepared by seeds (50 g/individual) cooked with water (1 ltr.) until the volume reduced (100 ml) and taken regularly
41	*Vigna umbellata* (Thunb.) Ohwi & Ohashi {Fabaceae (--)}	Rice bean	*Guruns/Rayans*	W	C	Jaundice and measles	Co	Pulse (*Daal*) of grains (50 g/individual)
D	Fruits							
42	*Citrus hystrix* DC. (Rutaceae) {Rutaceae (--)}	Jambhiri lemon	*Jamir*	W	C	Malaria, fever, and dehydration	Sy	Concentrated juice or extract (5–10 ml.) of matured fruits
43	*Citrus limon (L.)* Burm. f. {Rutaceae (RKT 2381)}	Lemon	*Nimbu*	W	C	Vitamin C, vomiting, and gastric disorder	In, Sy	Fruit taken as traditional nimbu *sanni*; fruit juice, or extract (5–10 ml) is ingredients of traditional chutneys
44	*Ficus palmata* Forssk. {Moraceae (RKT 8886, RKT 28094)}	Fig	*Bedu*	S, M	Wd	Gastric ulcer, cuts and wounds, and removal of thorn	Co, DA	Vegetables of fruits (50 g) for gastric ulcer; stem latex (4–5 drops) for removal of thorn form the bottom of the feet.
45	*Ficus auriculata* Lour. (Moraceae) {Moraceae (RKT 7524)}	Elephant ear fig	*Timila*	S, M	Wd	Acidity, blood pressure, and duodenal ulcer	Co	Vegetables of fruits (50 g/individual)
46	*Punica granatum L.* {Lythraceae (RKT 28845)}	Pomegranate	*Darim*	S, M, W	C, Wd	Anemia, cold and cough, and source of vitamin 'C'	Po, In	Fruit peels (5–10 g) used for 3–4 times or powder (50 g) of dried fruit peel taken orally with warm water for cold and cough; fruit juice (50 ml) given twice a day to anemic patient
47	*Psidium guajava* L. {Myrtaceae (RKT 13868)}	Guava	*Amrood*	W	C	Cold and cough and mouth blisters	In	Fruits (50 g) baked in the hot ash are served to the patients; fresh leaves are chewed as astringent
48	*Syzygium cumini* (L.) Skeels {Myrtaceae (RKT 8839)}	Blackberry	*Jamun*	S	Wd	Diabetes	In	Fruits and seeds (100 g)
E	Spices and condiments							
49	[a]*Allium cepa* L. {Alliaceae (RKT 7675)}	Onion	*Pyaz*	S, M, W	C	Pimples	O	Paste of bulbs (50 g) applied on the spot

(Continued on following page)

TABLE 1 | (*Continued*) Crops plants used as traditional foods, nutritional security, and primary healthcare by central Himalayan communities.

	Crop category and scientific name {family, (RKT no.#)}	Common name	Local name	$Availability season	Cultivated or collected from the wild	Additional use other than food (during illness)	Mode of use or application	Details of recipes or medicinal use
50	[a]*Allium sativum* L. (Alliaceae) {Alliaceae (RKT 19219)}	Garlic	*Lahsun*	S,M,W	C	Gastric problem and joint pain (Arthritis)	In, O	Cloves (2–3 nos.) are eaten in the morning before breakfast; paste prepared from 5 to 7 spilled cloves heated with 20 ml mustard oil and massage on joints
51	*Allium schoenoprasum* L. {Alliaceae (RKT 24974)}	Chives	*Dunn/Dhungar/ Panguri*	S,M,W	C	Cold and cough, gastric problem, and joint pain	Co	Soup of cloves (20 g) and fresh (20 g) and dried leaves (20 g) taken as a vegetable; tempering the dishes
52	[a]*Capsicum annuum* L. {Solanaceae (RKT 7675)}	Chilli	*Khursani*	S, M, W	C	Skin burn	O	Paste of powder of chilli capsules applied over burned parts
53	*Cannabis sativa* L. {Cannabaceae (RKT 27224)}	Hemp	*Bhang*	S, M, W	C	Constipation, stomach ache, and warm effect	Co	Seed milk (100 ml) extracted from 20 g seed and used as an ingredient of traditional dishes
54	[a]*Coriandrum sativum* L. {Apiaceae (RKT 29354, RKT 28118)}	Coriander	*Dhania*	S, M, W	C	Urine disorder	Inf	Coriander grains (20 g) are soaked in water (100 ml); this water is served to the patients
55	[a]*Trachyspermum ammi* (L.) Spr. {Apiaceae (--)}	Ammi	*Ajwain*	S, M	C	Stomach ache and gastric problem	In, Po	Seeds (5 g) are chewed or powder is consumed with lukewarm water
56	[a]*Curcuma longa* L. (Zingiberaceae) {Apiaceae (RKT 5970)}	Turmeric	*Haldi*	S, M, W	C	Cuts, internal injury, and wounds	O, Po, DA	Paste of rhizomes for cuts and wound healing; powder (5 g) mixed with a full glass of warm milk for internal injury; dry leaves (5–10 nos.) are used as bed for infants
57	*Trigonella foenum–*graecum L. {Fabaceae (RKT 29342, RKT 28507)}	Trigonella	*Methi*	S, M, W	C	Cold and cough, constipation, diabetes, indigestion, joint pain, and obesity	Co, Inf	Vegetable of grains (12 g/ individual) and leaves (50 g/ individual); leaf juice (5–10 ml) is taken orally for curing obesity, indigestion, joint pain and constipation; 25 g seeds are soaked overnight and filtered, the filtrate taken orally on empty stomach for gastric problems and diabetes
58	[a]*Zingiber officinale* Roscoe {Zingiberaceae (RKT 5921)}	Ginger	*Adrak*	S, M, W	C	Cold and cough and viral fever	In, Sy	Baked rhizomes and soup (50 g) or a piece (5–10 g) of broiled rhizome mixed with small amount of honey and chewed
F	Oilseeds							
59	[a]*Brassica campestris* L. {Brassicaceae (RKT 26286)}	Yellow mustard	*Pili sarson*	S, M, W	C	Paralysis treatment	O	Oil (100 ml) massage on the affected parts
60	[a]*B. juncea* (L.) Czern {Brassicaceae (RKT 2286)}	Brown mustard	*Bhuri sarson*	S, M, W	C	Paralytic limbs	O	Oil (100 ml) massage on the affected parts
61	*Brassica nigra* (L.) Koch {Brassicaceae (--)}	Black mustard	*Raituwa rai*	S, M, W	C	Stomachic, refreshing agent, and relieves tiredness	In	Ground seed (5 g for 500 ml curd) is a main ingredients of traditional *Pahari Raytas*
62	*Lepidium sativum L*. (Brassicaceae) {Brassicaceae (--)}	Pepper cress	*Chamsur, Halang*	W	C	Asthma, cold and cough, and massage (infant)	Co, O	Leafy vegetables (30 g) for cold cough; fatty oil (8–10 drops) body massage for infant
63	*Linum usitatissimum* L. {Linaceae (RKT 22549)}	Linseed	*Alsi*	S, M, W	C	Immunity enhancer, and joint pain	DA, Po, In	Seeds or seed powder (1 g/ day) taken early in the morning; Roasted seeds (25 g/individual) is an ingredient for traditional chutneys
64	*Perilla frutescens* (L.) Britton {Lamiaceae (RKT 28724)}	Perilla	*Bhangira*	S, M, W	C	Cough and joint pain	In	Roasted seeds (20g/ individual) are an ingredient for traditional chutneys
65	*Ricinus communis* L. {Euphorbiaceae (RKT 22941)}	Castor	*Arandi*	S, M, W	Wd	Vein and artery problems	O	Oil (10–20 drops) massage
66	*Sesamum indicum* L. {Pedaliaceae (RKT 25193)}	Sesame	*Til*	S, W	C	Washing hair, cold and cough, and muscular pain	DA, In	Crushed green leaves (20–25 g) are used as shampoo; dried seeds (100 g) or crushed seed powder mixed with jaggery, small balls are prepared, and consumed during the winter season as medicine, Oil used in massage

(Continued on following page)

TABLE 1 | (*Continued*) Crops plants used as traditional foods, nutritional security, and primary healthcare by central Himalayan communities.

	Crop category and scientific name {family, (RKT no.#)}	Common name	Local name	[$]Availability season	Cultivated or collected from the wild	Additional use other than food (during illness)	Mode of use or application	Details of recipes or medicinal use
G	Aromatic plants							
67	*Mentha arvensis* L. {Lamiaceae (RKT 1838; RKT 4355)}	Mint	*Pudina*	S, M	C	Diarrhea	In	Sauces (*Chutney*) of young tender leafy stem leaves and twigs (10–20 g)
68	*Ocimum basilicum* L. {Lamiaceae (RKT 1836, 19325)}	Basil	*Tulsi*	S, M	C	Cold and cough and viral fever	Inf	Tea of leaves or soup (10–15 nos. of leaves) of boiled leaves

[a]*Eaten on a daily basis;* [$]*Availability: crop season, storage, and market; S=summer (April to June); M = monsoon (July to September); Winter (December to March); cultivated (C); wild (Wd); ointment (O); ingestion (In); infusion (Inf); powder (Po); direct application (DA); cooking (Co); syrup (Sy); ash form (AF); smoke (S). #Matched vouchers with the herbarium specimen lodged in CCRAS-RARI, tarikhet, Ranikhet, Uttarakhand with the acronym (RKT).*

and Gomati Valley of Garur and Kapkot blocks of the district. The selection of villages covered an altitude from 1,200 to 2,700 m above sea level. Largely, the area has a similar sociocultural structure though crops and plants vary with elevation. The criterion for village selection was to maximize the probability of including respondents from different altitudes, social statuses, and castes. Owing to the topography of the region, the villages lie on the fringes of the hills as well as in the high peaks of the region. Thus, it was assured that a probabilistic approach was to be taken. A detailed questionnaire was prepared to cover various broad areas of food intake. In addition, focused interviews were also arranged regarding knowledge, attitude and practices, dietary intake, and food habits. Since most farmers owned small landholding and were categorized as smallholders, for selecting a household (participant), we initially conducted a household listing operation in each selected village to provide a frame to include in the sample. Accordingly, a total of 25% households or a maximum of 30 households were sampled in each village with equal probability. Altogether, we sampled a total of 445 households across villages. Of the total respondents, 65% were women being the major workforce as men migrated to towns and cities for finding alternate employment. The median age of the interviewees was 38. A detailed list of all traditional agricultural practices, crops, and foods eaten by the local community, the local name of the species, its source of availability (cultivated or collected from wild), plant part used, the month of collection, seasonal consumption pattern, uses other than food during illness, and mode of use and application was prepared. We also explored major crops and cropping patterns in rain-fed and irrigated fields. As the majority of crop species were common, they were simply matched with the existing herbarium for identification purposes. We used standard plant nomenclature using IPNI (2021). An important part of the study was to assess the food and medicine interface of traditional crops and plants that are most commonly used by the marginal hill community. These practices are being used since eternity, descended from the inherited knowledge of the locals and indigenous population of Uttarakhand.

As women commence most household doings and take key decisions in food making, of the total respondents, a total of 100 women were targeted for 24-h dietary recall survey (FAO, 2018). These women were of the age group of 25–50 years. The study was repeated consecutively for three major seasons, viz., summer (March to June), monsoon (July to October), and winter (November to February). Common village measuring units (such as *patha*) were standardized to obtain an actual quantity of food intake by the participants to estimate the usual daily intake of food, and the frequency of intake was combined to obtain the optimum digits. Information on main ingredients and cooking methods was gathered for different traditional food recipes, cuisines, or herbal preparations. We considered those dishes that have been traditional in nature and were in practice through generations. The frequency of food intake for different species is gathered in three categories: daily (consumed regularly), weekly (consumed at least once in a week), and occasional (consumed at least once in a month or season). We considered median values of intake instead of mean to avoid skewness of data. As some species are preferred for healthcare also, we also gathered information on the illness being treated, therapy applied, and techniques used in treatment. To further assess the potency of a treatment using local crops and plants, we also interviewed local *Vaidyas* (herbal healers or folk medicine practitioners) using snowball sampling. It was through discussion with locals and other practitioners that we identified 30 healers who agreed to share information. Later on, they were interviewed to elucidate the potency of crops/plants for therapeutic purposes. For this, two general meetings (one in each valley) were organized in which the information gathered from the community was shared with vaidyas to provide a common viewpoint on the effectiveness of the dietary habit for treatment of illness/ailments. In addition, the healers also have knowledge and experience on the use of different crops and wild plants for its use in curing diseases and ailments. Other than vaidyas, 20 members of the native community also participated in each meeting. Information was gathered on the illness being tackled, plant parts used, processing (if any), and mode of application. All the data collected as above were arranged by listing all crops and wild plants used as food, dietary diversity and food groups consumed in different seasons, preference of traditional food in terms of nutrition and medicinal utility, and sociocultural significance of food items, if any.

Assessing Dietary Diversity Indices

Dietary diversity indices (DDI) provide us with quantified variables with outcomes that define the quality of diet taken by the communities. The study used two major indices in the study to quantify the quality and diversity of food taken by the community. The household dietary diversity score (HDDS) was used to check the

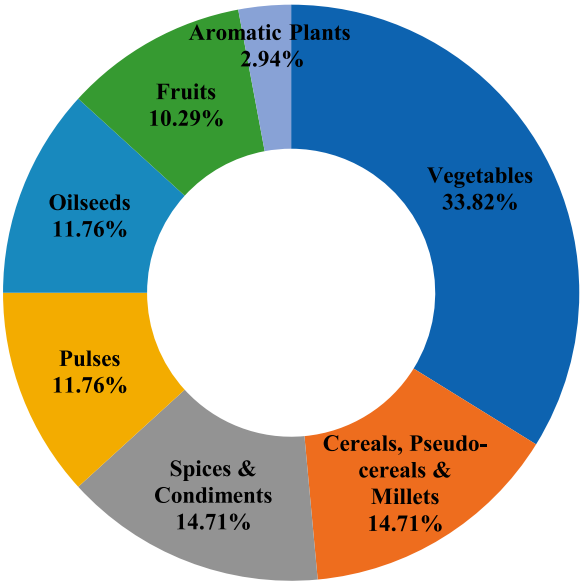


FIGURE 2 | Categories of agri-horticultural plant species used in food, nutrition, and primary health care.

food consumption and household access to a variety of food as well as a proxy for nutrient adequacy. Data were collected in accordance with the guidelines for measuring household and individual dietary diversity and with suggested 12-food group indicators (FAO, 2010). For all the three seasons (summer, monsoon, and winter), a 24-h dietary recall was taken from the respondents which was converted into Boolean variables (where 0 = food not taken and 1 = food taken) and finally summing up the values to obtain the HDDS. Another index implemented in the study is the Food Consumption Score (FCS) developed by the World Food Program to aggregate household-level data on diversity and frequency of food consumed during a specific duration (WFP, 2009). The indicator used a 7-day dietary recall and frequency of the food groups whose intake frequencies were noted in the questionnaire and after applying the respective weights to the food groups.

FCS was calculated using **Equation 1** as follows:

$$\begin{aligned} FCS = {} & A\_staple \;\; X\_staple + A\_pulses \;\; X\_pulses + A\_veg \\ & X\_veg + A\_fruits \;\; X\_fruits + A\_meat \\ & X\_meat + A\_sugar\, X\_sugar + A\_dairy\, X\_dairy + A\_oils\, X\_oils, \end{aligned} \quad (1)$$

where,

$a\neg i$ = Weight of each group (Starch staples = 2, Pulses = 3, Vegetables = 1, Fruits = 1, Meat/fish/eggs = 4, Milk = 4, Sugar/Sweets = 0.5, Fat = 0.5).

xi = frequency of food consumed in past 7 days.

The nutrient adequacy ratio (NAR) was also calculated for the 13 nutritional indicators (viz., energy, proteins, carbohydrates, fats, vitamin B1, vitamin C, vitamin D, vitamin E, Ca, Mg, Fe, Na, and Zn) used in the study by dividing the participant's actual intake of each nutrient by the recommended dietary allowance (RDA), and additionally the mean adequacy ratio (MAR) was calculated using the following equation (INDEX Project, 2018):

$$MAR = \Sigma NAR/(\text{Number of Nutrients})$$

Assessing Nutritional Status of Food and Medicinal Plants

Food plants comprise significant nutritional and health benefits as they contain all the essential nutrients in the leaves, roots, stems, flowers, and fruits of many plants. Therefore, after collecting the information on traditional food plants and food habits of the communities, selected crops were assessed for their nutritional values from the Indian food composition developed by the ICMR, India (ICMR-ICFT 2017; Longvah et al., 2017). The species-specific values for energy, proteins, fats and carbohydrates, water-soluble and insoluble vitamins (such as thiamine—vit. B1; ascorbic acid—vit. C; ergocalciferol—vit. D; and tocopherol—vit E), and minerals (such as Ca, Fe, Mg, Na, and Zn) were undertaken. For some species, nutritional parameters were assessed from other research sources derived by searching Google Scholar and PubMed sources. The intakes, as classified on the basis of daily, weekly, and occasionally, were filtered and analyzed with respect to the nutritional information to obtain the average nutritional intake of individuals, although the rarely eaten foods were not included in the consumption analysis.

RESULTS

Richness of the Food System

It was recorded that the community used as many as 68 food plant species comprising cereals, pseudo-cereals, millets, vegetables, pulses, fruits, spices and condiments, oilseeds, and medicinal and aromatic plants to fulfill their basic needs (**Table 1**). The main crops were rice (comprise 50% of agricultural land), finger millet (20%), wheat (19%), barley (5%), and maize (1.35%), and the cropping pattern differs in irrigated and unirrigated (rain-fed) agricultural lands. In irrigated fields, rice forms the major crop, although in some areas, wheat is also grown. In unirrigated fields, however, mixed cropping along with crop rotation has been the key feature. In such areas either dry rice, followed by wheat and millet, or millet followed by rice and wheat cultivation is followed on a 3-years crop rotation basis. Mix-cropping of pulses (lentil, urd, etc.) and oilseeds (mustard, sesame, soybean, etc.) was integral in rain-fed lands. Besides, the cultivation of a wide variety of minor crops, vegetables, and medicinal plants was also performed by the farmers. The main fruits of the study area were citrus, pear, mango, walnut, apple, peach, plum, apricot, litchi, etc. Most species were cultivated as vegetables (33.82%), followed by cereals and millets (15%), spices & condiments (14.71%), oilseeds (11.76%), fruits (10.29%), and aromatic species (2.9%) (**Figure 2**). A total of 35 species were also sold in local markets. In addition, a significant number of wild plants were collected from nearby forests to fulfill diverse needs.

TABLE 2 | Mean daily, weekly, and rare intake of food crops in different seasons.

Consumed common food crops	Parts used/consumed	[a]Average intake (g/day/individual)		
		Summer	Monsoon	Winter
Daily basis				
Allium cepa L	Bulb	69.00 ± 19.27	61.00 ± 10.99	57.00 ± 12.00
*Allium sativum*L	Bulb (cloves)	04.00 ± 2.20	11.00 ± 4.36	25.00 ± 08.46
Brassica juncea (L.) Czern	Seed oil	30.00 ± 06.89	32.00 ± 06.22	34.00 ± 08.03
Capsicum annuum L	Fruit capsule	08.00 ± 02.85	08.00 ± 02.56	12.00 ± 06.25
Coriandrum sativum L	Seed	24.00 ± 06.97	20.00 ± 07.54	20.00 ± 06.70
Cucumis sativus L	Fruit	105.0 ± 17.90	72.00 ± 20.36	--
Curcuma longa L	Rhizome	05.00 ± 03.41	05.00 ± 03.11	7.00 ± 02.95
Oryza sativa L	Seed (Grains)	190.00 ± 16.24	135.00 ± 17.03	160.00 ± 09.97
Solanum tuberosum L	Stem	75.00 ± 7.15	75.00 ± 11.12	90.00 ± 21.20
Trachyspermum ammi (L.) Spr	Seed	05.00 ± 0.82	05.00 ± 0.88	10.00 ± 0.83
Triticum aestivum L	Seed (grains)	150.00 ± 10.33	140.00 ± 18.54	100.00 ± 21.73
Zingiber officinale Roscoe	Rhizome	10.00 ± 03.82	15.00 ± 04.05	15.00 ± 04.90
Weekly (twice in a week)				
Allium schoenoprasum L	Cloves and leaf	10.00 ± 05.20	--	20.00 ± 05.98
Amaranthus caudatus L. (Amaranthaceae)	Seed (grains)	--	15.00 ± 12.62	60.00 ± 26.64
Brassica oleracea var. *capitata* L	Vegetative buds	--	--	65.00 ± 28.49
Chenopodium album L	Leaf twig	--	--	30.00 ± 12.84
Cucurbita moschata Duch. ex Poir	Fruit	--	100.00 ± 44.60	60.00 ± 32.13
Cyclanthera pedata (L.) Schrad	Fruit	--	130.00 ± 55.04	--
Colocasia esculenta L	Corms and petiole	--	80.00 ± 38.54	120.00 ± 60.97
Dioscorea bulbifera L	Aerial tuber	--	--	130.00 ± 28.65
Eleusine coracana (L.) Gaert	Seed (grains)	--	70.00 ± 18.86	110.00 ± 31.45
Fagopyrum esculentum Moench	Leaf	75.00 ± 28.67	--	--
Glycine max (L.) Merrill	Seed	38.00 ± 14.09	33.00 ± 11.11	50.00 ± 11.72
Glycine max subsp. *soja* (Sieb. & Zucc.) H. Ohashi	Seed	40.00 ± 23.30	35.00 ± 15.13	60.00 ± 31.22
Lagenaria siceraria (Molina) Standl	Fruit	80.00 ± 19.55	95.00 ± 21.82	--
Lens culinaris Medik	Seed	30.00 ± 18.47	35.00 ± 16.39	55.00 ± 30.53
Luffa acutangula (L.) Roxb	Fruit	85.00 ± 20.99	105.00 ± 23.42	--
Macrotyloma uniflorum (Lam.) Verde	Seed	25.00 ± 10.83	30.00 ± 11.26	75.00 ± 20.24
Momordica charantia L		--	50.00 ± 27.59	--
Phaseolus vulgaris L	Seed	45.00 ± 23.55	40.00 ± 20.01	70.00 ± 31.34
Raphanus sativus L	Whole plant	50.00 ± 9.5	65.00 ± 10	70.00 ± 8.4
Spinacia oleracea L	Leaf	--	--	100.00 ± 45.41
Trichosanthes anguina L	Fruit	--	70.00 ± 13.62	--
Trigonella foenum–graecum L	Seed and leaf	14.00 ± 02.47	12.00 ± 03.02	[b]50.00 ± 11.12
Vigna mungo (L.) Hepper	Seed	30.00 ± 16	35.00 ± 18	55.00 ± 30
Vigna unguiculata (L.) Walp	Seed	25.00 ± 16	45.00 ± 22	50.00 ± 30
Occasionally used (taken only on seasonal basis)				
Asparagus filicinus Buch.-Ham. ex D. Don	Tender shoots	70.00 ± 36.12	--	--
Bauhinia variegata L	Flower buds	10.00 ± 2.5	--	--
Benincasa hispida Thunb	Fruit	--	35.00 ± 17.41	--
Brassica nigra (L.) Koch	Seed	5.00 ± 2.53	5.00 ± 2.52	5.00 ± 4.20
Cannabis sativa L	Seed	10.00 ± 8.34	10.00 ± 7.27	20.00 ± 11.95
Citrus hystrix DC.	Fruit	--	--	90.00 ± 39.40
Citrus limon (L.) Burm. f	Fruit and extract	10.00 ± 7.14	10.00 ± 7.49	90.00 ± 44.03
Dioscorea alata L	Tuber	--	--	50.00 ± 29.39
Diplazium esculentum (Retz.) Sw	Young fronds	40.00 ± 22.95	40.00 ± 22.20	--
Echinochloa frumentacea Link	Seed (grains)	50.00 ± 29.39	--	50.00 ± 29.61
Fagopyrum esculentum Moench	Seed (grains)	40.00 ± 23.87	--	40.00 ± 24.13
Fagopyrum cymosum Trev	Leaf	70.00 ± 38.56	--	--
Ficus palmata Forssk	Fruit	35.00 ± 18.46	40.00 ± 20.44	--
Ficus auriculata Lour	Fruit	45.00 ± 27.46	53.00 ± 29.40	--
Hordeum vulgare L	Seed (grains)	20.00 ± 11.07	17.00 ± 08.95	--
Lepidium sativum L	Leaf	--	--	30.00 ± 12.84
Linum usitatissimum L	Seed	20.00 ± 12.58	20.00 ± 11.95	35.00 ± 21.37
Megacarpaea polyandra Benth	Leaf	70.00 ± 36.91	--	--
Mentha arvensis L	Leaf	20.00 ± 08.31	15.00 ± 07.80	--
Ocimum basilicum L	Leaf	08.00 ± 04.50	06.00 ± 03.29	--
Perilla frutescens (L.) Britton	Seed	20.00 ± 13.07	10.00 ± 5.03	20.00 ± 11.60
Phytolacca acinosa Roxb	Leaf	50.00 ± 29.39	40.00 ± 22.95	--
Psidium guajava L	Fruit	--	--	50.00 ± 22.46
Punica granatum L	Fruit	20.00 ± 12.14	50.00 ± 22.42	30.00 ± 13.31

(Continued on following page)

TABLE 2 | (*Continued*) Mean daily, weekly, and rare intake of food crops in different seasons.

Consumed common food crops	Parts used/consumed	[a]Average intake (g/day/individual)		
		Summer	Monsoon	Winter
Sesamum indicum L	Seed	10.00 ± 06.08	--	15.00 ± 08.79
Setaria italica (L.) P. Beauv	Seed	80.00 ± 37.88	68.00 ± 29.94	50.00 ± 23.80
Solanum melongena L	Fruit	80.00 ± 36.63	85.00 ± 42.56	--
Syzygium cumini (L.) Skeels	Fruit	30.00 ± 13.72	--	--
Urtica ardens Link	Leaf twig	--	--	50.00 ± 29.39
Vigna umbellata (Thunb.) Ohwi& Ohashi	Seed	--	--	50.00 ± 29.66
Zea mays L	Seed	--	65.00 ± 32.14	--

[a]*Average intake as per availability of the consumed part in different seasons.*
[b]*Used and consumed part only leaf; (--) no intake during respective season.*

The main characteristics of traditional farming are that it is performed on small and fragmented land holdings and is subsistence-type and labor-intensive, therefore practiced as family farming. It is largely organic in nature as the community uses only farmyard manure (FYM) to maintain soil fertility. The traditional food system comprised a combination of food plants, wild edibles, and other cuisines, etc., and there was a preference for traditional food items/dishes that maintained taste, nutrition, and cultural values. Women are at the center of the traditional food systems that undertake diverse roles from seed selection, storage, agriculture field preparation, weeding, harvesting, storage, cooking, etc., protecting farm diversity and maintaining dietary diversity within the community. The community strongly believed that the traditional food items substantiate their energy, protein, and carbohydrate requirements, particularly of women who performed extensive physical works at households and farms. An assessment of average food self-sufficiency revealed that communities would produce a significant share of their household need from their farms. For generations, the community has been successful in extending proper local governance in a decentralized manner to various resources, such as land, forest, and water, on which the traditional food system is intensely reliant. All these resources form an integral part of the local socioecological systems. All natural resources are maintained with the perspectives for proper access, harvest cycles, and equitable distribution, which is key to sustaining local food systems and diets.

Seasonal Dietary Analysis

It was noticed that the dietary habits of the local community are influenced by seasons as per the availability of crops and wild plants. Some seasonal food items are also brought to the local market, and people can procure them from the market if it is not grown by them. An assessment of 24-h dietary recall of the community against the recommended dietary allowance (RDA) indicated that a large share of daily energy requirements of a working woman was fulfilled by traditional diets only (**Figure 2**). The community prefers energy-rich foods in their diets. An assessment of energy requirement of the age group of 19–50 years against the RDA revealed that traditional crops fulfill 78.97% requirement during summer, 73.29% during monsoon, and 92.88% in winter (**Tables 2**, **3**). The energy intake was higher during winter than in monsoon. During winter, the community consumes more energy-rich foods such as *Eleusine coracana*, *Dioscorea bulbifera*, *Glycine max*, and *Amaranthus caudatus* (**Table 4**). Protein intake was recorded as 60.90 g/day in summer, 61.73 g/day in monsoon, and 77.85 g/day in winter, which is higher than the recommended intake; however, it can be attributed to the amount of labor performed for various works. Fat intake was confined within the RDA limits that reduce the risk of cholesterol-linked diseases. The traditional food also supports higher carbohydrate content in winter (345 g/day) than summer (298 g/day) and monsoon (277 g/day). Traditional food supports a higher intake of vitamin D within tolerable limits. Occasional consumption of meat and fish products also met nutrient demands. A low intake of calcium and sodium was observed, although the requirement of the latter element is met from natural salts. The study clearly revealed that traditional food crops are an important asset to food and nutritional security even in remotely located villages.

Dietary Diversity Scores

An investigation of the household dietary diversity score (HDDS) was also undertaken to assess the households' access to a variety of foods (**Table 5**). It was interesting to note that most households were consuming adequate nutritional food in all the seasons, and none of them were placed with low dietary scores. The HDDS ranged from 4 to 10, being maximum in winter, followed by summer and monsoon months. The communities were fed well in all the food groups with daily consumption of cereals, oils and fats, vegetables, and milk products as well as meat and related products in all the seasons. The consumption of meat products was low in monsoon. There was low consumption of sugar edibles and fruits in almost all the seasons, although vitamin C–rich fruits were consumed in ample quantity in winter.

The average nutrition adequacy ratio (NAR) for all the nutritional parameters was 9.51 with a mean adequacy ratio of 0.73, which promises good quality of food intake by the communities living in remote locations. Although the anthropometric was not undertaken, it was recorded that the community was not eating junk food, sugar additives, and other unhealthy dietary markers. It reduces the chances of obesity, diabetes, and other similar ailments. The population is found taking better intake of nutrition that can be an indicator of low risks of diabetes and other ailments as well.

It was interesting to note that traditional diets form a rich source of nutrients (**Table 4**). The most common mode to use

TABLE 3 | Nutritional requirements and uptake from traditional food crops commonly eaten by the communities.

Nutrients	[a]RDA (per day)	[$]TUL	Daily and weekly average intake of nutrients in different seasons										
			Summer				Monsoon				Winter		
			Daily	Weekly	Total	(%)[b]FRDA	Daily	Weekly	Total	(%)*FRDA	Daily	Weekly	Total	(%)*FRDA
Energy (KCal)	2,200.00	--	1,452.92	284.41	1737.33	78.97	1,225.08	387.31	1,612.39	73.29	1,223.56	819.69	2043.25	92.88
Protein (g)	46.00	--	44.16	16.74	60.90	132.39	38.77	22.95	61.73	134.19	38.31	39.55	77.85	169.24
Fat (g)	28.00	--	17.78	3.48	21.26	75.91	17.97	5.47	23.44	83.70	19.74	10.22	29.95	106.98
Carbohydrate. (g)	130.00	--	274.64	23.87	298.51	229.62	223.19	53.51	276.69	212.84	224.40	120.66	345.06	265.43
Vit. B1 (mg)	01.10	ND	1.07	0.25	1.32	119.66	1.00	0.38	1.38	125.62	0.87	0.65	1.53	139.06
Vit. C (mg)	75.00	2000.00	101.23	27.86	129.09	172.12	64.98	41.26	106.24	141.66	77.91	79.78	157.69	210.25
Vit. D (μg)	15.00	100.00	18.86	5.22	24.07	160.50	18.80	19.06	37.86	252.41	18.65	21.81	40.45	269.70
Vit. E (mg)	15.00	1,000.00	3.39	0.33	3.71	24.75	3.60	0.59	4.19	27.93	3.69	1.33	5.02	33.50
Ca (mg)	1,000.00	2,500.00	386.59	145.59	532.17	53.22	295.31	289.71	585.03	58.50	304.65	757.67	1,062.33	106.23
Fe (mg)	18.00	45.00	17.87	14.44	32.31	179.52	16.52	8.37	24.89	138.28	14.94	16.94	31.88	177.12
Mg (mg)	320.00	350.00	222.33	103.21	325.55	101.73	187.61	167.14	354.76	110.86	193.11	297.59	490.71	153.35
Na (mg)	1,500.00	2,300.00	55.86	17.84	73.71	4.91	53.70	20.95	74.65	4.98	42.05	84.56	126.62	8.44
Zn (mg)	08.00	40.00	8.44	2.31	10.75	134.38	7.34	3.47	10.82	135.22	6.81	6.92	13.73	171.58

[a]*Recommended dietary allowance (RDA); tolerable upper limit (TUL).*

[b]*Fulfillment against RDA; ND- not determined.*

species is in the form of cooked, direct ingestion, as an ointment, and applying it directly either by crushing, powder, or adding the part as it is (**Figure 3**). In terms of plant parts used for treating the ailments, they include seeds (32.89%), fruits (21.05%), and leaves (18.41%) followed by bulbs, seed oils, and others, etc. (**Figure 4**). Most species had multipurpose uses ensuring food and nutritional security as well as medicinal efficacy for curing minor ailments (**Figure 5**). The community had species-specific knowledge on the use and application of various species. A total of 43 species were reportedly used as dietary supplements and food additives with definite physiological benefits during different ailments. Such species are commonly consumed as cooked food; thus, it can be categorized as neutraceutical or bioceutical. Other common methods comprised ingestion (15 species), ointment (eight species), and direct application (seven species).

The average food consumption score of the communities was 73.46, which is a good sign of food diversity intake among communities ranging from a minimum score of 55.55 and a maximum score of 87.00. The average cumulative probability for the entire crop species intake in the FCS was above 0.6, showing higher diversity of intake (**Figure 6**).

The Interface of Food and Medicine

Traditional food plants are rich sources of nutrients and chemical compounds that are used by the body to function properly and maintain health. An assessment of the nutritional status of crops plants revealed the presence of diverse components, such as proteins, fats, carbohydrates, vitamins, and minerals, in traditional foods and diets (**Supplementary Annexure 1**). Many species comprised high energy content (>3.5–5.5 kcal/g), such as *Sesamum indicum*, *Trachyspermum ammi*, *Oryza sativa*, *Cannabis sativa*, *Brassica juncea*, *Glycine max* subsp. *soja*, *Lepidium sativum*, *Linum usitatissimum*, *Echinochloa frumentacea*, *Bauhinia variegata*, and *Glycine max*. While *Zea mays, Vigna mungo*, *Triticum aestivum*, *Setaria italica*, *Phaseolus vulgaris*, *Fagopyrum esculentum*, *Perilla frutescens*, *Vigna unguiculata*, *Vigna umbellata*, *Trigonella foenum–graecum*, and *Macrotyloma uniflorum* exhibits medium energy content (2.5–3.5 kcal/g), *Asparagus*, *Bauhinia variegata*, *Benincasa hispida*, *Brassica campestris*, *Brassica juncea*, *Brassica nigra*, *Brassica oleracea*, *Cannabis sativa*, *Capsicum annuum*, and *Chenopodium album* exhibited high protein content (>20–43 g/100 g). Similarly, *Cannabis sativa*, *Perilla frutescens*, *Sesamum indicum*, *Brassica juncea*, *Linum usitatissimum*, *Lepidium sativum*, and *Trachyspermum ammi* comprised high fat content (20–49 g/100 g). Many local species were also rich in vitamins and other nutrients (**Supplementary Annexure 1**). Thus, the data clearly reveal that the nutritional energy value (can be calculated as the sum of food energies of all components) of the traditional food systems was very high.

The use of plants and food recipes has been a fundamental component of all rural house treatment systems as it is the most easily accessible resource available to the local community at the time of medical urgency. The local communities are well aware of the potency of food crops and their utility for treating various ailments and illnesses. Although they may not be known for the

TABLE 4 | Key nutrient-rich food crops used in traditional diets.

Use purpose	Key sources in traditional diet (crop species)
Energy	*Lepidium sativum* L., *Macrotyloma uniflorum* (Lam.) Verde, *Setaria italica* (L.) P. Beauv., *Eleusine coracana* (L.) Gaert., *Glycine max* (L.) Merrill, *Vigna umbellata* (Thunb.) Ohwi & Ohashi, *Brassica juncea* (L.) Czern, *Triticum aestivum* L., and *Fagopyrum esculentum* Moench
Protein	*Amaranthus caudatus* L., *Brassica juncea* (L.) Czern, *Glycine max* (L.) Merrill, *Glycine max* subsp. *soja* (Sieb. & Zucc.) H. Ohashi, *Lepidium sativum* L., *Sesamum indicum* L., and *Trigonella foenum–graecum* L
Fat	*Sesamum indicum* L., *Glycine max* (L.) Merrill, *Trachyspermum ammi* (L.) Spr., *Brassica juncea* (L.) Czern, and *Glycine max* subsp. *soja* (Sieb. & Zucc.) H. Ohashi
Carbohydrate	*Amaranthus caudatus* L., *Dioscorea bulbifera* L., *Oryza sativa* L., *Setaria italica* (L.) P. Beauv., *Zea mays* L., *Hordeum vulgare* L., *Solanum tuberosum* L., *Phaseolus vulgaris* L., *Vigna unguiculata* (L.) Walp, and *Colocasia esculenta* L
Vitamin B1	*Lepidium sativum* L., *Sesamum indicum* L., *Glycine max* (L.) Merrill, *Vigna mungo* L., *Vigna unguiculata* (L.) Walp, and *Phaseolus vulgaris* L
Vitamin C	*Allium cepa* L., *Capsicum annuum* L., *Raphanus sativus* L.) Hook, *Citrus hystrix* DC, *Citrus limon* (L.) Burm. f., *Fagopyrum esculentum* Moench, and *Brassica oleracea* var. *capitata* L
Vitamin D	*Amaranthus caudatus* L., *Glycine max* (L.) Merrill, *Punica granatum* L., *Brassica juncea* (L.) Czern., *Eleusine coracana* (L.) Gaert, *Vigna mungo* (L.) Hepper, *Trichosanthes anguina* L., and *Cucurbita moschata* Duch. ex Poir
Vitamin E	*Brassica juncea* (L.) Czern, *Curcuma longa* L. and *Vigna umbellata* (Thunb.) Ohwi & Ohashi

TABLE 5 | Dietary diversity at the household level in central Himalaya.

Food groups (12-FGI)	Household dietary diversity distribution					
	Summer (HDDS = 7.45)		Monsoon (HDDS = 7.34)		Winter (HDDS = 8.39)	
	MDS (4–5) (*n* = 11)	HDS (>6) (*n* = 89)	MDS (4–5) (*n* = 10	HDS (>6) (*n* = 90)	MDS (4–5) (*n* = 8)	HDS (>6) (*n* = 92)
Cereals (%)	100.00	100.00	100.00	100.00	100.00	92.00
White roots & tubers (%)	54.55	98.88	60.00	100.00	62.50	90.00
Vegetables (%)	100.00	96.63	40.00	97.78	75.00	87.00
Fruits (%)	-	22.47	20.00	32.22	0.00	61.00
Meat (%)	-	53.93	-	24.44	12.50	40.00
Eggs (%)	-	34.83	-	31.11	-	48.00
Fish and other sea food (%)	-	16.85	-	-	-	39.00
Legumes, nuts, and seeds (%)	-	47.19	10.00	67.78	25.00	57.00
Milk and milk products (%)	36.36	98.88	60.00	94.44	25.00	83.00
Oil and fats (%)	72.73	100.00	90.00	100.00	100.00	91.00
Sweets (%)	-	6.74	-	14.44	12.5.00	19.00
Spices and condiments (%)	90.91	98.88	100.00	100.00	100.00	91.00

Medium dietary score (MDS); High dietary score (HDS).

scientific reason as to how these food crops work in the body in treating the ailment. Since such treatments have been in practice for centuries, they apply it as the best affordable means. A literature survey on traditional crops clearly exhibited high medicinal value ranging from antifungal, anti-inflammatory, decreasing risks of cancer, reducing the risk of diabetics, etc. (**Supplementary Annexure 2**). *Oryza sativa*, *Eleusine coracana*, *Glycine max*, *Vigna umbellata*, and *Macrotyloma uniflorum* were good sources of carbohydrates, while *Brassica juncea*, *Sesamum indicum*, and *Lepidium sativum* provided worthy fat sources. Some species also exhibited aphrodisiac, antimicrobial (fungal and bacterial), antidiabetic, cardioprotective, anticancer, analgesic, antianemic, hepatoprotective, immunomodulatory, swelling & cholesterol-reducing, blood sugar lowering, etc. characteristics. It clearly revealed that local food has enough provisions for appropriate diets to satisfy the nutritional and energy needs along with a good balance of vitamins and micronutrients to the local community. It is essential to perform diverse functions, such as from boosting the immunity to therapeutic actions and repair of cellular damage to the healing of wounds and ailments, thus acting as protective shields against different ailments.

An important aspect of the interface of food and medicine is the use of diverse food recipes and the community's hyperawareness of its potency for curing different illnesses (**Table 6**). The use of food recipes varies with seasons. The mode of preparation, ingredients, cooking process, and intake frequency differs. The community uses foods with warm potency in monsoon and winter. Such species are *Amaranthus virdis*, *Chenopodium album*, *Dioscorea bulbifera*, *Hordeum vulgare*, *Eleusine coracana*, and *Macrotyloma uniflorum*, while, cool potency food such as *Cucumis sativus*, *Coriandrum sativum*, *Oryza sativa*, and *Raphanus sativus* were taken usually in summer. Several other criteria also work in selecting the food, such as people prefer eating meat products and coarse grain cereals more at higher altitudes due to cold. Other than achieving wellness, the traditional recipes also help address seasonal health issues. There is significant scope to prepare many new food dishes from traditional recipes to attract the market.

The health status of Bageswar reveals that the children below 5 years are impacted with anemia, stranded growth, and are

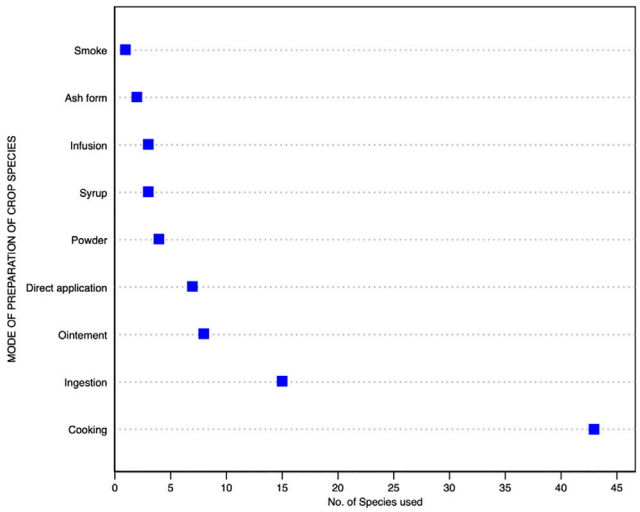


FIGURE 3 | Species-specific methods in terms of their preparation for health care.

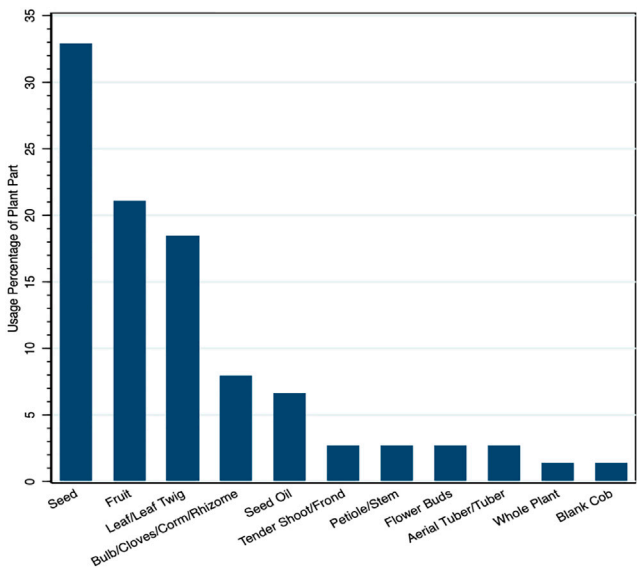


FIGURE 4 | Plant part used in preparation of medicine.

underweight. Similarly, the women of reproductive age are anemic with less bodyweight, thus influencing their lactation ability. A major reason for this is the lack of proteinaceous food and timely supply of tonics and other medicines. Fortunately, there are a large number of local food plants that can supplement such dietary needs of the community. For example, species such as *Eleusine coracana*, *Setaria italic*, *Glycine max*, *Amaranthus caudatus*, *Fagopyrum esculentum*, *Chenopodium album*, *Punica granatum*, *Vigna umbellata*, *Syzygium cumini*, *Spinacia oleracea*, and *Urtica ardens* are useful to avoid anemia. In addition, in case of stunted growth, consumption of *Eleusine coracana*, *Glycine max*, *Glycine soja*, *Setaria italica*, *Vigna umbellata*, *Brassica juncea*, *Fagopyrum esculentum*, and *Linum usitatissimum* is considered beneficial. Likewise, to enhance breast milk, consumption of *Eleusine coracana*, *Glycine max*, *Glycine soja*, *Phaseolus vulgaris*, *Spinacia oleracea*, *Amaranthus caudatus*, *Fagopyrum esculentum*, and *Brassica oleracea* var. *capitata* is advisable. There are also many protein-rich traditional recipes such as *Bhatia/Jaula*, *Bhatt ke dubake*, *Bhatt ka fana*, *Bhatt mathi ki sabzi*, *Bhatt papad ke sabzi*, *Chudkani*, and *Sonta (Lobia) ka chaise*. (**Table 6**). The traditional recipes can also help overcome anemic conditions by consuming *Chaulai ki roti*, *Jhangora/ Madira ki roti*, *Kutu ki roti*, *Kauni ki roti*, *Madua ki roti*, *Sonta (Lobia) ki bedu roti*, *Bathua ki sabzi*, *Bichhu/Kandali ka*

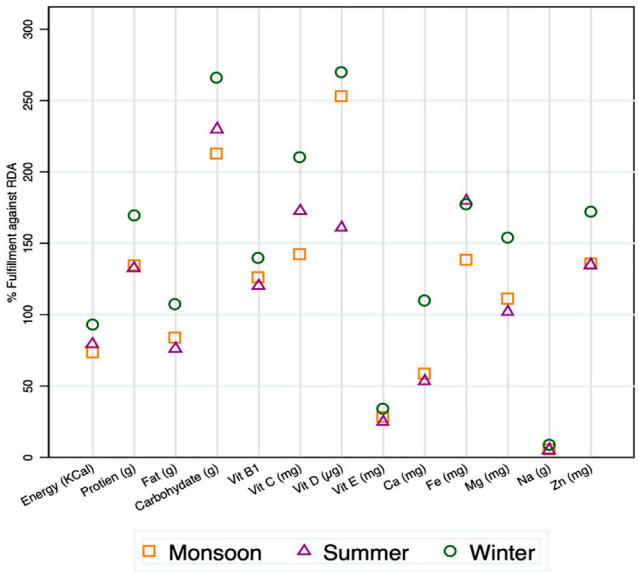


FIGURE 5 | Seasonal variation in fulfillment of nutrition against the RDA.

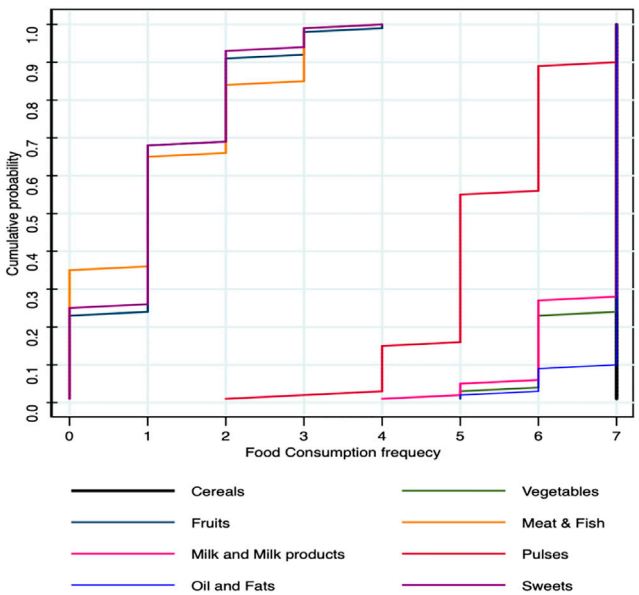


FIGURE 6 | Cumulative probabilty of the FCS.

saag, *Barmola/Rooki ki sabzi*, *Jhankara ka saag/Tinari*, etc. There is a need to make people aware of these plants and dishes to consume them for treating such illnesses.

DISCUSSION

Access to healthy food is a major global challenge; a major reason for this is changing food systems and steady decline in traditional diets (FAO, IFAD, UNICEF, WFP and WHO, 2020). As traditional food systems are well-adapted to the local ecological, sociocultural, and economic setting, they are best placed to carry forward the nutritional and health security among the masses (Niketan et al., 2018). In many places, such traditional systems are still prevalent, and there is a need to highlight and endorse the health, nutritional, and therapeutic benefits of the traditional food system to revive it from disappearance. This study explored a Central Himalayan community of the Bageshwar district, Uttarakhand (India) that is highly marginal; however, it still exhibits a significant

TABLE 6 | Traditional recipes with major ingredients and its special uses in the central Himalaya.

	Name of traditional recipes (*vernacular name*)	Common name and ingredients	Important uses	
			As food	As medicinal purposes
A	**Chapati (bread)**			
1	*Chaulai ki roti*	Amaranth seed flour	SF, TF	IE, SE
2	*Jhangora/Madira ki roti*	Barnyard millet flour	SF	SE
3	*Kutu ki roti*	Buckwheat flour	SF, TF	SE
4	*Kauni ki roti*	Foxtail millet flour	SF	SE
5	*Madua ki roti*	Finger millet flour	SF, TF	IE, SE
6	*Makke ki Roti*	Maize flour	SF	SE
7	*Choi/Chhola roti*	Rice	SF	--
8	*Lesuwa roti*	Finger millet and Wheat flour	SF, TF	IE, SE
9	*Gahat ki bedu roti*	Horse Gram and wheat flour	SF, TF	IE, SE
10	*Gurunsh ki bedu roti*	Rice bean and wheat flour	SF	IE, SE
11	*Mash ki bedu roti*	Black Gram and wheat flour	SF	IE, SE
12	*Sonta (Lobia) ki bedu roti*	Cowpea and wheat flour	SF	IE, SE
B	**Rice**			
13	*Chaulai ka bhat*	Amaranth seeds	SF, TF	IE, SE
14	*Jhangora/Madira ka bhat*	Barnyard millet seed	SF	IE, SE
15	*Kauni ka bhat*	Foxtail millet seed	SF	Iln, IE, SE
C	**Dishes related to pulses**			
16	*Bhatia/Jaula*	Black or white soybean (bhatt) and rice	SF	AF, IE, SE
17	*Bhatt ke dubake*	Black soybean (kala bhatt)	SF	AF, IE, SE
18	*Bhatt ka fana*	Black soybean (kala bhatt) and finger millet flour	SF	AF, IE, SE
19	*Bhatt mathi ki sabzi*	Black/white soybean (bhatt) and methi leaves	SF	AF, IE, SE
20	*Bhatt papad ke sabzi*	Black/white soybean (bhatt) and dried petiole/stalk of Taro	SF	IE, SE
21	*Chudkani*	Black soybean (kala bhatt) and rice flour	SF, TF	AF, Iln, IE, SE
22	*Gahat ki dal*	Horse gram and heeng	SF, TF	Iln, SE
23	*Gahat ke dubake*	Horse gram	SF	Iln, SE
24	*Gahat ka fana*	Horse gram and finger millet flour	SF, TF	Iln, SE
25	*Gahat gaderi ki dal*	Horse gram and Taro	SF	Iln, SE
26	*Gahat muli ki sabzi*	Horse gram and radish	SF, TF	AF, Iln, IE, SE
27	*Maas/Urad ka chaisa*	Black gram	SF, TF	SE
28	*Maas/Urad ki dool dal* (Khari dal)	Black gram	SF, TF	SE
29	*Maas/Urad ke bade*	Black gram paste	SF	SE
30	*Masur dal ke sabzi*	Lentil paste and onion and Garlic	SF	Iln, SE
31	*Ras* (extract of mixed pulses)	(Horse gram, black or white bhatt, cowpea, black gram, gram, rice bean, French bean) and rice flour	SF, TF	AF, IE, SE
32	*Sonta (Lobia) ka chaisa*	Cowpea	SF	SE
33	*Sonta (Lobia) ke bade*	Cowpea paste	SF	SE
D	**Vegetables**			
34	*Aalo or muli ka thinchwani*	Potato or raphanus and hemp seed milk	SF, TF	SE
35	*Aalu muli ki sabzi*	Potato and radish and curd/buttermilk	SF, TF	SE
36	*Aalu sarson ki sabzi*	Potato and Mustard seeds	SF, TF	SE
37	*Bathua ki sabzi*	Tender twigs of *Chenopodium*	SF, TF	Iln, IE, SE
38	*Barmola/Rooki ki sabzi*	Rooki leaf and root	SF	AF, AS, Iln, IE, SE
39	*Bichhu/Kandali ka saag*	Soft twigs *Urtica* sp. and garlic and heeng	SF, TF	AF, AS, Iln, IE, SE
40	*Bhang aur gaderi ki sabzi*	Hemp seed milk and taro tuber	SF, TF	AS, IE, SE
41	*Bhang aur genthi ki sabzi*	Hemp seed milk and genthi yams (aerial tuber)	SF, TF	AF, AS, Iln, IE, SE
42	*Bhang aur gobi ki sabzi*	Hemp seed milk and cabbage	SF, TF	AS, SE
43	*Dhungar/Dunna ke sabzi*	*Allium sp*. and curd/buttermilk	SF, TF	AF, AS, Iln, IE, SE
44	*Genthi ki sabzi*	Dioscorea/yam (aerial tuber)	SF, TF	AF, AS, Iln, IE, SE
45	*Jarag ka saag*	Jarag tender twigs	SF, TF	Iln, SE
46	*Jhankara ka saag/Tinari*	Wild buckwheat tender twigs and leaves	SF	AF, AS, IE, SE
47	*Kairua ka saag*	Tender shoots of *Asparagus* sp	SF, TF	IE, SE
48	*Kafa/Kapa (Palak ka Kapa)*	Spinach and rice flour	SF, TF	IE, SE
49	*Karela ka bharuwa/sabzi*	Bitter gourd and fennel	SF	Iln, IE, SE
50	*Kewral/Gwaral ki sabzi*	Tender flower bud of *Bauhimia* sp	SF	Iln, IE, SE
51	*Lahsun ki sabzi*	Garlic	SF, TF	Iln, IE, SE
52	*Lingura ki sabzi Lingura*	*Lingura* fern fronds	SF	IE, SE
53	*Meetha/Ram karela ki sabzi*	Fruits of wild cucumber	SF	IE, SE
54	*Patyude/Pinalu ka gunuwa*	Leaves of taro and lentil or gram flour	SF	--
55	*Pindalu/Dharud ke gabe/sabzi*	Taro rolled leaf blade and petiole or leaf stalk and radish	SF	--
56	*Tarur ki sabzi*	Tuber of winged yam	SF	SE

(Continued on following page)

TABLE 6 | (*Continued*) Traditional recipes with major ingredients and its special uses in the central Himalaya.

	Name of traditional recipes (*vernacular name*)	Common name and ingredients	Important uses	
			As food	As medicinal purposes
57	*Timila ki sabzi*	*Ficus auriculata* tender fruits	SF	AF, AS, Iln, IE, SE
58	*Ogal/Phafar ka saag*	Buckwheat tender twigs and leaves	SF, TF	IE, SE
E	**Bari (prepared by mixing black gram bean flour with vegetables and)**			
59	*Bhuj ki bari ke sabzi*	Bari (wax gourd and black gram) and rice/wheat flour/gram flour	SF, TF	--
60	*Kakadi ki bari ke sabzi*	Bari (matured cucumber and black gram) and rice/wheat flour/gram flour	SF	--
61	*Mooli ki bari ke sabzi*	Bari (radish and black gram) and rice/wheat flour/gram flour	SF, TF	--
62	*Pinalu ki bari ke sabzi*	Bari (taro tuber and black gram) and rice/wheat flour/gram flour	SF	--
63	*Pinalu ke danthal/dhare ki bari*	Bari (taro petiole or leaf stalk and black gram) and rice/wheat flour/gram flour	SF	--
F	**Raita (prepared with curd)**			
64	*raita*	Cucumber and curd and brassica seed (rai)	VS	AF, AS
65	*Kewral/Gwaral ka raita*	Tender flower buds of *Bauhinia* and curd and brassica seed (rai)	VS	AF, AS, Iln
66	*Lauki ka raita*	Bottle gourd fruits and curd and brassica seed (rai)	VS	AF, AS, Iln
67	*Mooli ka raita*	Radish and curd and brassica seed (rai)	VS	AF, AS
68	*Timila ka raita*	*Ficus auriculata* tender fruits and curd and brassica seed (rai)	VS	AF, AS, Iln
G	**Chutney (sauce, ketchup, and seasoning)**			
69	*Alsi ki chutney*	Linseed and lemon extract	SC	AF, AS, IE
70	*Bhang ki chutney*	Hemp seed and lemon extract	SC, TF	AF, AS
71	*Bhangeera ki chutney*	*Perilla* seeds and lemon extract	SC	AF, AS, IE
72	*Bhatt ki chutney*	Black/brown seeded soybean (bhat) and lemon extract	SC	AF, AS, IE, SE
73	*Darim ki chutney*	Wild pomegranate seeds and lemon extract	SC	AF, AS
74	*Kaddu ke meethi Chutney*	Pumpkin and hemp seed and jaggery and lemon extract	SC	AF, AS
75	*Nimbu ke saani*	Lemon and hemp seeds and curd and jaggery	SC, TF	AF, AS
76	*Til ki chutney*	Sesame seeds and lemon extract	SC, TF	AF, AS, IE
77	*Timila ki Chatni/Sanni*	*Ficus auriculata* fruit and lemon extract and mustard seed	SC	AF, AS, IE
H	**Pakories** (fritters))			
78	*Ogal/Phaphar ki Pakori*	Buckwheat flour and potato	S, TF	SE
79	*Palak ki pakori*	Spinach leaf and gram flour	S	SE
80	*Jarag ki Pakori*	Jarag tender twigs and gram flour	S	SE
I	**Sweet dishes**			
81	*Chaulai ki kheer*	Amaranth seeds and milk	D, TF	IE, SE
82	*Chaulai ka halwa*	Amaranth seeds and milk/water	D, TF	IE, SE
83	*Jhangora/Madira ki kheer*	Barnyard millet seed and milk	D	SE
84	*Jhangora/Madira ka halwa*	Barnyard millet flour	D	SE
85	*Kauni ki kheer*	Foxtail millet seed and milk	D	SE
86	*Khir-Khaja*	Rice and milk	D	SE
87	*Lapsi/Leta/Rautti*	Wheat flour and curd	D, TF	SE
88	*Meetha bhat*	Rice and jaggery	D	--
89	*Ogal/Phaphar ka halwa*	Buckwheat flour	D, TF	SE
90	*Rot*	Wheat flour and butter and milk	D	--
91	*Singal, Puwe*	Rice or suji flour and milk and curd and butter	D	--
92	*Shaya*	Rice or suji flour and curd and milk and butter	D	Iln
93	*Swanl-Ladao*	Wheat flour; rice flour and water and butter and sesame	D	--
J	**Other dishes**			
94	*Chhachhiya/Jaula*	Rice and curd/buttermilk	SF	Iln, SE
95	*Laina Jaul*	Buttermilk/curd and rice	SF	Iln, IE, SE
96	*Sattu*	Barley; wheat; and finger millet	SF	AF, AS, Iln, IE, SE
97	*Vigaud*	Colostrum of buffalo or cow	S	AF, AS, Iln, IE, SE

Antifatigue (AF); antistress (AS); dessert (D); illness (Iln); immunity enhancing (IE); snacks (S); spicy cuisine (SC); stamina enhancing (SE); staple food (SF); thermogenic food (TF); vegetable substitute (VS).

dependence on the traditional food system. The traditional farming practice is age-old and time-tested, which greatly helped maintain crop diversity along with ensuring food security. This study provided some valuable data in this regard:

i) The community uses as many as 68 food plants as part of their regular diet to fulfill their basic food needs, and the efficacy of the traditional food system can be judged from the perspectives of production, consumption, nutrition, and healing characteristics. The production perspective comprised a diversity of cereals, millets, vegetables, fruits, spices and condiments, medicinal plants, and meat sourced for food from farming or wild areas; the consumption perspective comprised the diversity of foods in local diets along with its cultural identity; the nutritional significance refers to the contents of food that fulfill the nutritional

requirements, such as proteins, carbohydrates, fats, minerals, and vitamins, of the local community, thus minimizing nutrition deficiency in the community; while the healing perspective comprised remedial treatments offered by food and wild plants to accomplish medicinal and health security. This clearly reflects that the native dietary pattern fulfills diverse requirements of the marginal hill community, and a large section still considers it as a healthy food system.

ii) The communities possess significant knowledge about the local plants and diets (cultivation or wild collection, storage, food preparation, seasonal uptake, nutritional traits, therapeutic efficacy, etc.), which makes it a highly resilient food system. The foods support diverse and nutritionally rich diets in different seasons holistically and comprehensively. Continuity of such a system is greatly required to maintain on-farm crop diversity and species richness.

iii) The community has been successful for generations in maintaining the food production system embedded in the local sociocultural and ecological context that safeguards local communities to afford healthy diets at their home, thus providing an answer to counter malnutrition in the community.

iv) Women are at the core of the traditional food system that plays a key role in maintaining dietary diversity within the community. Their voices can lead to expansion of traditional agriculture and diversification of local diets for ensuring nutrient adequacy within indigenous territories.

v) The community has been successful in providing proper governance to various resources, such as land, forest, and water, on which the traditional food system has strong dependence. The community manages these resources as an integral part of the local livelihood in a socially acceptable and decentralized manner by maintaining access, harvest cycles, and equitable distribution, which is key to sustaining local food systems and diets.

Given the abovementioned situation, it is clear that traditional food systems, local knowledge, sociocultural setup, women, and resource governance together can lead to maintaining a traditional agroecological system that can provide a basis for a future of holistic food and health system. A large share of global crop genetic diversity including landraces and wild plants and animals is under the communities' custody (Oldfield and Alcorn 1987). Traditional food systems are largely practiced by smallholders, particularly by women, who maintain local food habits and genetic diversity and soil fertility of agricultural fields (FAO 2011a; FAO 2011b; Sundriyal et al., 2014; Maikhuri et al., 2015). They are low-cost, energy-efficient, local resource–dependent, and climate-smart systems, thus contributing immensely to food supply and environmental sustainability (Sundriyal et al., 1994; CINE 2021). The dietary diversity index works as a proxy for nutritional security and varies in seasons, which is also a resemblance to our study (Hjertholm et al., 2019). Worldwide previous studies concluded that dietary diversity has a positive alliance with nutritional adequacy (Ruel, 2003; Steyn et al., 2006; Faber et al., 2009). The inclusion of a household healthy diet can reduce the risk of diseases in the community (Bezerra and Sichieri, 2011). A study on the relationship between food insecurity and diabetes in women found that they consume adequate diet, thus having a lower risk of diabetes than others (Seligman et al., 2007). Himalayan communities make good use of local herbs and spices that increase appetite and healing properties (Joshi et al., 2015). Allium species are used as spices and vegetables, and many of the species own strong medicinal purpose in the form of fresh paste or tonic (Keusgen et al., 2006). *Curcuma longa*, a major ingredient in the local kitchen, has miraculous effects on human ailments, thus being the consumer choice for cancer prevention, liver protection, treating wounds, and other activities in traditional medicine (Nita Chainani-Wu, 1982). Traditional food and recipes help address seasonal health issues, and diversity of recipes provide a supporting base for the nutritional security of the communities in the state of Uttarakhand (Mehta et al., 2010). Other than providing food security, the traditional food system also reduces the risk of deficiency in chronic health conditions such as obesity, high blood pressure, and higher cholesterol as well as nutrition increases the mental efficiency, physical development of children, etc. (Cole and Fox, 2004; Seligman et al., 2007; Borborah et al., 2014; Martin et al., 2016).

The health of women and children has been an enduring concern all over the state and the country (Anonymous 2014). As reported, the status of nutrition in Bageswar exhibited children influenced with stranded growth, underweight, and anemia. Also, the women of reproductive age were anemic. Uttarakhand comprised 25% of children with low birth weight (<2.5 kg) which is similar to the statistics in Bageshwar district (Pradhan 2017). A major reason for this is the lack of a health care delivery system to supply even the low-cost appropriate medical technology to all women and newborns. Moreover, the low purchasing power of the community for procuring tonics and related medicines is another reason. The state has been implementing *Janani Suraksha Yojana* (Women Safety Scheme) that provides financial assistance to mothers after delivery and *Janani–Shishu Suraksha Karyakram* (Mother–child safety scheme) for ensuring better facilities for women and child health services along with providing immunization, vitamin A, and iron supplement at childbirth (Anonymous 2012). The efficiency of such schemes needs to be improved in terms of ensuring universal access to all pregnant women and children, particularly in villages. Most of the dietary requirements are being met from traditional sources without which the magnitudes of malnutrition would have been much larger. Therefore, the role of traditional diets could not be undermined as, at least, it is helps maintain a certain dietary level, although it also necessitates strengthening the traditional dietary system to meet adequate nourishment requirements, particularly for lactating mothers and infants. The local community should be made aware of the nutritional role of local crops and plants for treating anemia, stunted growth, and lactation for which *Eleusine coracana*, *Setaria italica*, *Glycine max*, *Amaranthus caudatus*, *Fagopyrum esculentum*, *Vigna umbellata*, *Phaseolus vulgaris*, *Spinacia oleracea*, and *Chenopodium album* are greatly helpful. In addition, there are many traditional dishes that are supportive during such illnesses.

Despite clear distinguishing features and health benefits, increasing population, degradation of natural resources, biodiversity loss, soil degradation, and climate change, etc. are threatening to traditional agriculture systems. In addition, a change in cultural, social, and spiritual values has posed a significant threat to the traditional food system. A discussion with the community highlighted that the dependence on traditional food crops was more prevalent until the last decade. However, slowly, such practice is changing because of varied reasons. Traditional agriculture is labor-intensive and is practiced in small and fragmented landholdings. Because of its subsistence-type nature, farmers lack a surplus to sell in the market. This led to the switch on to other crops or the abandonment of agriculture fields. It creates a threat to a large variety of local crop gene pools. The young generation due to widespread education is looking for white-collar jobs, thus migrating to towns and cities. In addition, due to globalization and access to markets, food habit is changing. Some youths also feel that consuming traditional crops and food recipes is inferior in comparison to many other crops available in markets. All these factors influence traditional crops and food habits. Another possible reason for this is that diverse nutrient-rich local foods have gained limited consideration in the policy and decision-support process. Modern industrial agriculture and food systems have largely evolved through a centralized approach that brings in several hidden costs, including environmental as well as degradation of natural resources, etc. (Niketan et al., 2018). Such an approach has been a major threat to the biological and nutritional diversity of traditional diets (Fróna et al., 2019). High-cost market-driven and industrial crops are being promoted through a centralized approach. There is disconnect between agricultural and food policy and adequate nutrition supply (Pingali 2015). An analysis of India's agricultural and food policy showed an inclination toward industrial food system. To address the issue of malnutrition and hunger and achieve Integrated Child Development targets, the country implemented the public distribution system (PDS) and mid-day meal scheme; however, they are more market-driven and frequently complain about micronutrient deficiencies in many areas. Inconsiderately, the state agencies also promote a market-based agricultural system. There is a need to make traditional agriculture and farming systems more reliable and sustainable (FAO 2017). It would be highly disadvantageous if the community lost such an efficient system or changes their agroecological practices. This highlights a need to have a realistic way forward to protect native food systems and diets so that they can continuously support food, nutrition, and health benefits.

Way Forward

The implications of this research are of both academic importance and practical significance to ensure food–medicine security and avoid malnutrition among rural communities. For food security, a sustainable food production system is highly demanding. The study established a better understanding of the intersection between traditional food, dietary diversity, nutritional quality, and medicinal efficacy. It highlighted the need to conserve and continue with the traditional food systems as it is in the best interest of the people. We expect that the study would receive greater attention from policy planners and developmental workers that will not only salvage the dwindling traditional dietary system but also ensure a healthy dietary cover to the marginal hill communities. The local government should recognize the role of traditional food and dietary systems in meeting community necessities and support appropriate policies and programs to continue with it. It should support better financial incentives to farmers to continue traditional agriculture and dietary practices. It would empower the local communities to manage traditional food and dietary systems not only to feed themselves and preserve crop gene pools but also to sell produces in markets. Greater awareness of the masses and policy planners is desired on the beneficial aspects of traditional crops and wild plants. In addition, proper documentation of traditional knowledge related to local crops, cropping practices, local delicacies and recipes, and herbal preparation is highly desirable for future use. Such an approach will not only promote and nurture traditional food systems but also support local sociocultural and ecological systems and provide a sustainable solution for nutrition security. This requires location-specific strategies and trade-offs so as to develop a synergy in terms of food security and environmental gains with minimum transformations. Other than government support, public investment in various forms is also required. In addition, there is a need for proper scientific investigation on nutritional/anti-nutritional and therapeutic efficiencies in food items and herbal preparation as the current state of knowledge is highly limiting on these aspects. There is a need to make some traditional food, recipes, cuisines, and ingredients appropriate for recent times that may attract a significant population outside. The local government should incentivize the traditional system so that it creates new values and job opportunities for youngsters. It is expected that the study would lead to advancement of a renewed thinking on traditional agriculture for food and nutritional security as well as sustainable rural development all over. It is a global challenge to conserve traditional food systems and maintain healthy diets that have been sustainably used for generations and can have an important synergy with SDGs (Wang et al., 2018).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written information was given to the village head to undertake the study in a village involving local participants.

AUTHOR CONTRIBUTIONS

RS: conceptualized the study and prepared the manuscript. SO: undertook fieldwork and arranged the data. AA: undertook field work and typeset the material. DA assisted in identifying and matching plant species with registered herbarium.

ACKNOWLEDGMENTS

We owe our gratitude to the local community of Bageshwar, Uttarakhand, who shared valuable information and knowledge. The authors thankfully acknowledge the facilities received from GBP-NIHE, Kosi-Katarmal and Almora, India for undertaking this work. We sincerely thank the Director, CRAS-RARI, Tarikhet and Ranikhet, Uttarakhand for helping in the identification of plant species. We are thankful to DST, Govt. of India for the financial assistance through NMSHE, Task Force 5 sponsored project entitled "Network program on the convergence of traditional knowledge system for sustainable development in the Indian Himalayan Region". We sincerely thank Prof. S. C. Garkoti, JNU for his constant support and cooperation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.789360/full#supplementary-material

REFERENCES

Abdul, W., Hajrah, N., Sabir, J. M., Al-Garni, S., Sabir, M., Kabli, S., et al. (2018). Therapeutic Role of *Ricinus communis* L. And its Bioactive Compounds in Disease Prevention and Treatment. *Asian Pac. J. Trop. Med.* 11 (3), 177–185. doi:10.4103/1995-7645.228431

Adhikari, L., Tuladhar, S., Hussain, A., and Aryal, K. (2019). Are Traditional Food Crops Really 'Future Smart Foods?' A Sustainability Perspective. *Sustainability* 11, 5236. doi:10.3390/su11195236

Adi, B. S., and Reddy, R. S. (2017). *Momordica charantia* of Phytochemical Study: A Review. *Int. J. Homoeopath. Sci.* 1, 1–4.

Ahmad, I., Irshad, M., and Rizvi, M. M. A. (2011). Nutritional and Medicinal Potential of *Lagenaria siceraria*. *Int. J. Vegetable Sci.* 17 (2), 157–170. doi:10.1080/19315260.2010.526173

Ahmed, H. M. (2019). Ethnomedicinal, Phytochemical and Pharmacological Investigations of *Perilla frutescens* (L.) Britt. *Molecules* 24 (102), 1–23. doi:10.3390/molecules24010102

Akande, T. O., Odunsi, A. A., Olabode, O. S., and Ojediran, T. K. (2012). Physical and Nutrient Characterization of Raw and Processed castor (*Ricinus communis* L.) Seeds in Nigeria. *World J. Agric. Res.* 8 (1), 89–95.

Alam, K., Hoq, O., and Uddin, S. (2016). Medicinal Plant *Allium sativum*: A Review. *J. Med. Plants Stud.* 4 (6), 72–79.

Anand, U., Jacobo-Herrera, N., Altemimi, A., and Lakhssassi, N. (2019). A Comprehensive Review on Medicinal Plants as Antimicrobial Therapeutics: Potential Avenues of Biocompatible Drug Discovery. *Metabolites* 9, 258. doi:10.3390/metabo9110258

Anila, K. R., Pal, A., Khanum, F., and Bawa, A. S. (2010). Nutritional, Medicinal and Industrial Uses of Sesame (*Sesamum indicum* L.) Seeds - an Overview. *Agric. Conspec. Sci.* 75 (4), 159–168.

Anitha, J., and Miruthula, S. (2014). Traditional Medicinal Uses, Phytochemical Profile and Pharmacological Activities of *Luffa acutangula* Linn. *Int. J. Pharmacogn. Phytochem. Res.* 1 (3), 174–183. doi:10.13040/IJP.0975-8232

Annongu, A. A., and Joseph, J. K. (2008). Proximate Analysis of castor Seeds and Cake. *J. Appl. Sci. Environ. Manag.* 12, 39–41. doi:10.4314/jasem.v12i1.55567

Ezeabara, C. A., and Regina, R. O. (2018). Comparative Analyses of Phytochemical and Nutritional Compositions of Four Species of *Dioscorea*. *Act. Sci. Nutr. Health* 2 (7), 90–94.

Anonymous (2014). *Annual Health Survey 2011-12: Fact Sheet Uttarakhand*. India, New Delhi: Vital Statistics Division, Office of the Registrar General & Census Commissioner. www.censusindia.gov.

Anonymous (2011). *Perspective Plan of Bageshwar District-2021. Town & Country Planning Organisation*. New Delhi, India: Ministry of Urban Development.

Anonymous (2012). *Uttarakhand State Health Report*. Dehradun, India: Department of Health and Family welfare. Govt. of Uttarakhand.

Asgarpanah, J., and Kazemivash, N. (2012). Phytochemistry, Pharmacology and Medicinal Properties of *Coriandrum sativum* L. *Afr. J. Pharm. Pharmacol.* 6 (31), 2340–2345. doi:10.5897/ajpp12.901

Bairwa, R., Sodha, R. S., and Rajawat, B. S. (2012). *Trachyspermum ammi*. *Pharmacogn Rev.* 6 (11), 56–60. doi:10.4103/0973-7847.95871

Bansal, V., Malviya, R., DeekshaMalviya, T., and Sharma, P. K. (2014). Phytochemical, Pharmacological Profile and Commercial Utility of Tropically Distributed Plant *Bauhinia variegata*. *Afr. J. Pharm. Pharmacol.* 8 (2), 196–205. doi:10.5829/idosi.gjp.2014.8.2.82296

Bezerra, I. N., and Sichieri, R. (2011). Household Food Diversity and Nutritional Status Among Adults in Brazil. *Int. J. Behav. Nutr. Phys. Act* 8 (1), 22. doi:10.1186/1479-5868-8-22

Bhowmik, D., Gopinath, H., Kumar, B. P., Duraivel, S., Aravind, G., and Kumar, K. P. S. (2013). Medicinal Uses of *Punica granatum* and its Health Benefits. *Res. Rev. J. Pharmacogn. Phytochem.* 1 (5), 28. doi:10.22271/phyto

Bijauliya, R. K., Alok, S., Sabharwal, M., and Chanchal, D. K. (2018). *Syzygium cumini* (Linn.) - an Overview on Morphology, Cultivation, Traditional Uses and Pharmacology. *Int. J. Pharm. Sci. Res.* 9 (9), 3608–3620. doi:10.13040/IJPSR.0975-8232.9(9).3608-20

Bisht, I. S., Mehta, P. S., Negi, K. S., Verma, S. K., Tyagi, R. K., and Garkoti, S. C. (2018). Farmers' Rights, Local Food Systems and Sustainable Household Dietary Diversification: A Case of Uttarakhand Himalaya in north-western India. *Agroecol. Sustain. Food Syst.* 42, 73–113. doi:10.1080/21683565.2017.1363118

Borborah, K., Dutta, B., and Borthakur, S. K. (2014). Traditional Uses of *Allium* L . Species from North East India with Special Reference to Their Pharmacological Activities. *Am. J. Phytomedicine Clin. Ther.* 2 (8), 1037–1051.

Broegaard, R. B., Rasmussen, L. V., Dawson, N., Mertz, O., Vongvisouk, T., and Grogan, K. (2017). Wild Food Collection and Nutrition under Commercial Agriculture Expansion in Agriculture-forest Landscapes. *For. Pol. Econ.* 84, 92–101. doi:10.1016/j.forpol.2016.12.012

Burlando, B., and Cornara, L. (2014). Therapeutic Properties of rice Constituents and Derivatives (*Oryza sativa* L.): A Review Update. *Trends Food Sci. Tech.* 40 (1), 82–98. doi:10.1016/j.tifs.2014.08.002

Carl, L., Jessica, R. E., Smith, W. K., Patrick, K., Van, D., Verzelenc, K., et al. (2017). Dietary Species Richness as a Measure of Food Biodiversity and Nutritional Quality of Diets. *PNAS* 115 (1), 127–132. doi:10.1073/pnas.1709194115

Chahal, K., Alfonso, A. A. C., Dhaiwal, K., Kumar, A., Kataria, D., and Singla, N. (2017). Chemical Composition of *Trachyspermum ammi* L. And its Biological Properties: A Review. *Int. J. Pharmacogn. Phytochem.* 6 (3), 131–140.

Chainani-Wu, N. (1982). Safety and Anti-inflammatory Activity of Curcumin: a Component of Tumeric (*Curcuma longa*). *J. Altern. Complement. Med.* 9 (1), 161–168. doi:10.1089/107555303321223035

Chandra, S., Saklani, S., Semwal, R. B., and Semwal, D. K. (2018). Estimation of Nutritional and mineral Contents of *Eleusine coracana* and *Echinochloa frumentacea* – Two Edible Wild Crops of India. *Curr. Nutr. Food Sci.* 14, 1–4. doi:10.2174/1573401314666180327160353

Chauhan, H., and Singh, M. (2019). Phytochemical Characterization and Antibacterial Potential of Indian and Chinese Cabbage Genotypes against Human Pathogens in Uttarakhand, India. *Int. J. Recent Sci. Res.* 10 (12), 36462–36466. doi:10.4103/injms.injms_42_18

Chong, E. S., McGhie, T. K., Heyes, J. A., Stowell, K. M., and Kathryn, M. (2013). Metabolite Profiling and Quantification of Phytochemicals in Potato Extracts Using Ultra-high-performance Liquid Chromatography-Mass Spectrometry. *J. Sci. Food Agric.* 93 (15), 3801–3808. doi:10.1002/jsfa.6285

CINE (2021). *Centre for Indigenous Peoples' Nutrition and Environment (CINE), Macdonald Campus*. Ste-Anne-de-Bellevue, QC: McGill University. 21111 Lakeshore Rd Ste-Anne-de-Bellevue, QC H9X 3V9514-398-7757 (Accessed on 11 15, 2021).

Cole, N., and Fox, M. K. (2004). *Nutrition and Health Characteristics of Low-Income Populations: Volume II, WIC Participants and Non participants*. Washington, DC: US Department of Agriculture. Economic Research Service 04014-2.

Daniel, P., Supe, U., and Roymon, M. G. (2014). A Review on Phytochemical Analysis of *Momordica charantia*. *Int. J. Adv. Pharm. Biol. Chem.* 3, 214–220.

Das, M., and Barua, N. (2013). Pharmacological Activities of *Solanum melongena* Linn. (Brinjal Plant). *Int. J. Green. Pharm.* 7 (4), 274–277. doi:10.4103/0973-8258.122049

Dasgupta, T., Poddar, S., Ganguly, A., and Qais, N. (2016). Anti-inflammatory and Neuropharmacological Activities of the Seed Extract of *Setaria italica*. *J. App. Pharm. Sci.* 6 (5), 193–197. doi:10.7324/japs.2016.60530

Devi, N. (2017). Medicinal Values of *Trichosanthus cucumerina* L. (Snake Gourd) - A Review. *Bjpr* 16 (5), 1–10. doi:10.9734/bjpr/2017/33575

Devi, P. B., Vijayabharathi, R., Sathyabama, S., Malleshi, N. G., and Priyadarisini, V. B. (2014). Health Benefits of finger Millet (*Eleusine coracana* L.) Polyphenols and Dietary Fiber: A Review. *J. Food Sci. Technol.* 51 (6), 1021–1040. doi:10.1007/s13197-011-0584-9

Dhyani, D., and Dhyani, S. (2014). Nutritional, Food and Energy Value of *Perilla frutescens*: An Underutilized Traditional Oilseed Crop of Western Himalaya, India. Agro. *Food Industry Hi-Tech.* 25 (1), 24–27.

Faber, M., Schwabe, C., and Drimie, S. (2009). Dietary Diversity in Relation to Other Household Food Security Indicators. *Ijfsnph* 2 (1), 1–15. doi:10.1504/ijfsnph.2009.026915

FAO (2018). *Dietary Assessment: A Resource Guide to Method Selection and Application in Low Resource Settings*. Rome.

FAO (2010). *Guidelines for Measuring Household and Individual Dietary Diversity*. Rome, Italy: FAO, 53.

FAO (2011a). *Save and Grow: A Policymaker's Guide to the Sustainable Intensification of Smallholder Crop Production*. Rome.

FAO (2017). *The Future of Food and Agriculture: Trends and Challenges*. Rome: Food and Agriculture Organization of the United Nations.

FAO (2011b). *The State of Food and Agriculture 2010–11. Women in Agriculture: Closing the Gender gap for Development*. Rome.

FAO, IFAD, UNICEF, WFP & WHO (2018). *The State of Food Security and Nutrition in the World 2018. Building Climate Resilience for Food Security and Nutrition*. Rome: FAO.

FAO, IFAD, UNICEF, WFP and WHO (2020). *The State of Food Security and Nutrition in the World 2020. Transforming Food Systems for Affordable Healthy Diets*. Rome: FAO.

Fathima, S. N. (2015). A Systemic Review on Phytochemistry and Pharmacological Activities of Capsicum Annuum. *Int. J. Pharm. Pharm. Sci.* 4 (3), 51–68.

Fróna, D., Szenderák, J., and Harangi-Rákos, M. (2019). The challenge of Feeding the World. *Sustainability* 11, 5816. doi:10.3390/su11205816

Ganesan, K., and Xu, B. (2017). A Critical Review on Polyphenols and Health Benefits of Black Soybeans. *Nutrients* 9 (5), 455. doi:10.3390/nu9050455

Ghete, A. B., Duda, M. M., Varban, D. I., Varban, R., Moldovan, C., Muntean, S., et al. (2019). Maize (*Zea mays*), a Prospective Medicinal Plant in Romania. *Hop Med. Plants* 26 (1–2), 44–51.

Ghosh, S. (2015). Phytochemsitry and Therapeutic Potential of Medicinal Plant: *Dioscorea bulbifera*. *Med. Chem.* 5 (4), 160–172. doi:10.4172/2161-0444.1000259

Gul, S., Ahmed, S., Kifli, N., Uddin, Q. T., Batool Tahir, N., Hussain, A., et al. (2014). Multiple Pathways Are Responsible for Anti-inflammatory and Cardiovascular Activities of *Hordeum vulgare* L. *J. Transl. Med.* 12, 316. doi:10.1186/s12967-014-0316-9

Gupta, S. k., Sharma, A., and Sharma, A. (2014). Medicinal Properties of *Zingiber officinale* Roscoe - A Review. *Iosrjpbs* 9 (5), 124–129. doi:10.9790/3008-0955124129

Gupta, U. K., Das, S., Aman, S., and Nayak, A. (2016). Pharmacological Activities of *Vigna unguiculata*-A Review. *World J. Pharm. Res.* 5 (10), 337–345.

Hettiarachchi, C. A., and Wijesinghe, S. S. (2021). Food-Medicine Interface and its Application in International Level, Comparative Jurisdictions and Sri Lankan Legal Context. *Wn* 12 (1), 103–111. doi:10.26596/wn.2021121103-111

Hidayat, D. A., and Dwira, S. (2018). Phytochemical Analysis and *In Vitro* Cytotoxicity Test of Black Soybean (*Glycine soja* L.) Ethanolic Extract as a Growth Inhibitor of the HCT-116 colon Carcinoma Cell Line. *J. Phys. Conf. Ser.* 1073. doi:10.1088/1742-6596/1073/3/032041

Hjertholm, K. G., Holmboe-Ottesen, G., Iversen, P. O., Mdala, I., Munthali, A., Maleta, K., et al. (2019). Seasonality in Associations between Dietary Diversity Scores and Nutrient Adequacy Ratios Among Pregnant Women in Rural Malawi - A Cross-Sectional Study. *Food Nutr. Res.* 63, 2712. doi:10.29219/fnr.v63.2712

Ibrahim, S. V. K., Satish, S., Kumar, A., and Hegde, K. (2017). Review Article Pharmacological Activities of *Vigna unguiculata* (L) Walp: A Review. *Int. J. Pharm. Chem. Res.* 3 (1), 44–49.

ICMR-ICFT (2017). *Indian Food Composition Tables*. Hyderabad, India: National Institute Of Nutrition (Indian Council of Medical Research) Department of Health Research, Ministry of Health & Family Welfare, Government of India.

IFPRI (2015). *Global Nutrition Report 2015. Actions and Accountability to advance Nutrition and Sustainable Development*. Washington, DC: IFPRI.

INDEX Project (2018). *Data 4 Diets: Building Blocks for Diet-Related Food Security Analysis*. Boston, MA: Tufts University. (Accessed on August 26, 2020).

IPNI (2021). International Plant Names Index. Available at: http://www.ipni.org.

Jamil, M., and Anwar, F. (2016). Properties, Health Benefits, and Medicinal Uses of *Oryza sativa*. *Eur. J. Biol. Sci.* 8 (4), 136–141. doi:10.5829/idosi.ejbs.2016.136.141

Jennings, H. M., Merrell, J., Thompson, J. L., and Heinrich, M. (2015). Food or Medicine? the Food-Medicine Interface in Households in Sylhet. *J. Ethnopharmacol.* 167, 97–104. doi:10.1016/j.jep.2014.09.011

J. Fanzo, D. Hunter, T. Borelli, F. Mattei, and Diversifying food and diets: Using agricultural biodiversity to improve nutrition and health (Editors) (201310017). (New York, NY: Routledge), 711 Third Avenue.

Jones, A. D. (2017). Critical Review of the Emerging Research Evidence on Agricultural Biodiversity, Diet Diversity, and Nutritional Status in Low- and Middle-Income Countries. *Nutr. Rev.* 75 (10), 769–782. doi:10.1093/nutrit/nux040

Monica, S. J., and Joseph, M. (2016). Phytochemical Screening of Flaxseed (*Linum usitatissimum* L.). *Int. J. Sci. Res.* 3, 218–220.

Joshi, D. C., Chaudhari, G. V., Sood, S., Kant, L., Pattanayak, A., Zhang, K., et al. (2019). Revisiting the Versatile Buckwheat: Reinvigorating Genetic Gains through Integrated Breeding and Genomics Approach. *Planta* 250 (3), 783–801. doi:10.1007/s00425-018-03080-4

Joshi, P., Sharma, N., Roy, M. L., Kharbikar, H. L., Chandra, N., and Sanwal, R. (2015). Traditional Food Practices in Northwestern Himalayan Region: A Case of Uttarakhand. *J. Agric. Sci. Food Sci. Technol.* 2 (3), 170–174.

Joshi, R. K., Satyal, P., and Setzer, W. N. (2016). Himalayan Aromatic Medicinal Plants: A Review of Their Ethnopharmacology, Volatile Phytochemistry, and Biological Activities. *Medicines (Basel)* 3 (6), 1–55. doi:10.3390/medicines3010006

Joshi, Y., Joshi, A. K., Prasad, N., and Juyal, D. (2014). A Review on *Ficus palmata* (Wild Himalayan Fig). *J. Phytopharm.* 3 (5), 374–377.

Kanchana, P., Santha, M. L., and Dilip, R. K. (2016). A Review on *Glycine max* (L.) Merr. (Soybean). World. *J. Pharm. Pharm. Sci.* 5 (1), 356.

Keusgen, M., Fritsch, R. M., Hisoriev, H., Kurbonova, P. A., and Khassanov, F. O. (2006). Wild *Allium* Species (Alliaceae) Used in Folk Medicine of Tajikistan and Uzbekistan. *J. Ethnobiol. Ethnomed.* 2, 18–19. doi:10.1186/1746-4269-2-18

Khoury, C. K., Bjorkman, A. D., Dempewolf, H., Ramirez-Villegas, J., Guarino, L., Jarvis, A., et al. (2014). Increasing Homogeneity in Global Food Supplies and the Implications for Food Security. *Proc. Natl. Acad. Sci. U S A.* 111 (11), 4001–4006. doi:10.1073/pnas.1313490111

Khoury, C. K., Achicanoy, H. A., Bjorkman, A. D., Navarro-Racines, C., Guarino, L., Flores-Palacios, X., et al. (2016). Origins of Food Crops Connect Countries Worldwide. *Proc. R. Soc. B.* 283 (1832), 20160792. doi:10.1098/rspb.2016.0792

Klimek-Szczykutowicz, M., Szopa, A., and Ekiert, H. (2020). *Citrus lemon* (Lemon) Phenomenon—A Review of the Chemistry, Pharmacological Properties, Applications in the Modern Pharmaceutical, Food, and Cosmetics Industries, and Biotechnological Studies. *Plants* 9 (119). doi:10.3390/plants9010119

Kuddus, M., Ginawi, I., and AlHazimi, A. (2013). *Cannabis sativa*: An Ancient Wild Edible Plant of India. *Emir. J. Food Agric.* 25 (10), 736–745. doi:10.9755/ejfa.v25i10.16400

Kumar, P., Yadava, R. K., Gollen, B., Kumar, S., Verma, R. K., and Yadav, M. (2011). Nutritional Contents and Medicinal Properties of Wheat: A Review. *Life Sci. Med. Res.* 22. doi:10.1111/j.1467-8616.2011.00779.x

Kumar, S., and Andy, A. (2012). Health-promoting Bioactive Phytochemicals from *Brassica*. *Int. Food Res. J.* 19 (1), 141–152.

Kunwar, R. M., Shrestha, K. P., and Bussmann, R. W. (2010). Traditional Herbal Medicine in Far-West Nepal: a Pharmacological Appraisal. *J. Ethnobiol. Ethnomed.* 6, 35. doi:10.1186/1746-4269-6-35

Longvah, T., Ananthan, R., Bhaskarachary, K., and Venkaiah, K. (2017). *Indian Food Composition Tables*. Hyderabad: National Institute of Nutrition (Indian Council of Medical Research) Department of Health Research, Ministry of Health & Family Welfare, Government of India.

Mahendra, P., and Bisht, S. (2011). *Coriandrum sativum*: A Daily Use Spice with Great Medicinal Effect. *Pharmacognosy J.* 3 (21), 84–88. doi:10.5530/pj.2011.21.16

Maikhuri, R. K., Sundriyal, R. C., Negi, G. C. S., and Dhyani, P. P. (2015). Smallholders and Family Farming in the Himalayan Region of India: Policy Considerations. *Policy in Focus* 34, 21–23.

Mallik, J., Das, P., and Das, S. (2013). Pharmacological Activity of *Cucumis sativus* L. – a Complete Overview. *Asian J. Pharm. Res.* 1 (1), 6.

Mane, P. C., Kadam, D. D., Chaudhari, R. D., Varpe, K. A., Sarogade, D., Thorat, V. T., et al. (2015). Phytochemical Investigations of *Spinacia oleracea*: An Important Leafy Vegetable Used in Indian Diet. *Eur. J. Exp. Biol.* 4 (1), 1–4.

ShailManjari, D., Neeraj, K., and Gupta, L. N. (2016). Nutritional Importance of *Lepidium sativum* L. (Garden Cress/Chandrashoor): A Review. *Anal. Res.* 5 (1), 2016–2152.

Martin, K. S., Colantonio, A. G., Picho, K., and Boyle, K. E. (2016). Self-Efficacy is Associated with Increased Food Security in Novel Food Pantry Program. *SSM Popul. Health* 2, 62–67. doi:10.1016/j.ssmph.2016.01.005

Mehta, P. S., Negi, K. S., and Ojha, S. N. (2010). Native Plant Genetic Resources and Traditional Foods of Uttarakhand Himalaya for Sustainable Food Security and Livelihood. *Indian J. Nat. Prod. Resour.* 1 (1), 89–96.

Mishra, S., Mishra, A., Thakur, M., Sharma, A., and Alok, S. (2017). Investigations on the Hypolipidemic Activity of *Asparagus filicinus* Buch-Ham Ex D. Don. *Int. J. Pharm. Sci. Res.* 8 (2), 813–818. doi:10.13040/IJPSR.0975-8232.8(2).813-18

Mohamed El-Feky, F., El-Sherbiny, I. M., El-Fedawy, M. G., and Abdel-Mogib, M. (2016). Phytochemical Studies on *Linum usitatissimum* Seeds and the Nanoformulation of the Bioactive Butanol Extract. *Ijsea* 5, 76–91. doi:10.7753/ijsea0502.1006

Moradi kor, N., Didarshetaban, M. B., and Pour, H. R. S. (2013). Fenugreek (*Trigonella foenum-graecum* L.) as a Valuable Medicinal Plant. *Int. J. Adv. Biol. Biomed. Res.* 1 (8), 922–931.

Mustafa, A., Ahmad, A., Ahmad, A., Tantray, A. H., and Parry, P. A. (2018). Ethnopharmacological Potential and Medicinal Uses of Miracle Herb *Dioscorea* Spp. *J. Ayu. Her. Med.* 4 (2), 79–85. doi:10.31254/jahm.2018.4208

Mutalik, S., Paridhavi, K., Rao, C. M., and Udupa, N. (2003). Antipyretic and Analgesic Effect of Leaves of *Solanum melongena* Linn. In Rodents. *Indian J. Pharmacol.* 35 (5), 312–315.

Narayan, J., John, D., and Ramadas, N. (2019). Malnutrition in India: Status and Government Initiatives. *J. Public Health Pol.* 40 (1), 126–141. doi:10.1057/s41271-018-0149-5

Naseer, S., Hussain, S., Naeem, N., Pervaiz, M., and Rahman, M. (2018). The Phytochemistry and Medicinal Value of *Psidium guajava* (Guava). *Clin. Phytoscience* 4 (1). doi:10.1186/s40816-018-0093-8

Nasri, H., Sahinfard, N., Rafieian, M., Rafieian, S., Shirzad, M., and Rafieian-kopaei, M. (2014). Turmeric: A Spice with Multifunctional Medicinal Properties. *J. Herb. Med. Pharmacol.* 3 (1), 5–8.

Negi, J. S., Singh, P., Joshi, G. P., Rawat, M. S., and Bisht, V. K. (2010). Chemical Constituents of Asparagus. *Pharmacogn Rev.* 4 (8), 215–220. doi:10.4103/0973-7847.70921

Negi, K. S., Koranga, S. S., Ojha, S. N., Rayal, A., and Mehta, P. S. (2013). Cultural Significance of *Brassica nigra* (L.)Koch. In Central Himalayan Region. *Asian J. Agri-history.* 17 (3), 275–279.

Niketan, N., Ibrahim, G., Thar, R., Jain, A., Abraham, M., Valsangkar, S., et al. (2018). *Exploring the Potential of Diversified Traditional Food Systems to Contribute to a Healthy Diet*. Food Sovereignty Alliance India & Catholic Health Association India.

Ocho-Anin atchibri, A. L., Brou, K. D., Kouakou, T. H., Kouadio, Y. J., and Gnakri, D. (2010). Screening for Antidiabetic Activity and Phytochemical Constituents of Common Bean (*Phaseolus vulgaris* L.) Seeds. *J. Med. Plants Res.* 4, 1757–1761. doi:10.5897/JMPR10.280

Ojha, S. N., Tiwari, D., Anand, A., and Sundriyal, R. C. (2020). Ethnomedicinal Knowledge of a Marginal hill Community of Central Himalaya: Diversity, Usage Pattern, and Conservation Concerns. *J. Ethnobiol. Ethnomed.* 16, 29. doi:10.1186/s13002-020-00381-5

Okut, N., Yagmur, M., Selcuk, N., and Yildirim, B. (2017). Chemical Composition of Essential Oil of *Mentha longifolia* L. Subsp. *longifolia* Growing Wild. *Pak. J. Bot.* 49 (2), 525–529.

Oldfield, M. L., and Alcorn, J. B. (1987). Conservation of Traditional Agroecosystems. *BioScience* 37 (3), 199–208. doi:10.2307/1310519

Pagare, M. S., Patil, L., and Kadam, V. J. (2011). *Benincasa hispida*: A Natural Medicine. *Res. J. Pharm. Technol.* 4 (12), 1941–1944.

Parvu, A. E., Parvu, M., Vlase, L., Miclea, P., Mot, A. C., and Silaghi-dumitrescu, R. (2014). Anti-inflammatory Effects of *Allium schoenoprasum* L. Leaves. *J. Physiol. Pharmacol.* 65 (2), 309–315.

Pawar, A., Choudhary, P. D., and Kamat, S. R. (2018). An Overview of Traditionally Used Herb, *Colocasia esculenta*, as a Phytomedicine. *Med. Aromatic Plants* 7 (4). doi:10.4172/2167-0412.1000317

Pingali, P. (2015). Agricultural Policy and Nutrition Outcomes - Getting beyond the Preoccupation with Staple Grains. *Food Sec.* 7, 583–591. doi:10.1007/s12571-015-0461-x

Poonia, A., and Upadhayay, A. (2015). *Chenopodium album* Linn: Review of Nutritive Value and Biological Properties. *J. Food Sci. Technol.* 52 (7), 3977–3985. doi:10.1007/s13197-014-1553-x

Pour, H., Norouzzade, R., Heidari, M., Ogut, S., Yaman, H., and Gokce, S. (2014). Therapeutic Properties of *Zingiber officinale* Roscoe: A Review. *Ejmp* 4 (12), 1431–1446. doi:10.9734/ejmp/2014/11138

Pradhan, M. R. (2017). *National Family Health Survey – 4: 2015-16: District Fact Sheet, Bageshwar, Uttarakhand*. Mumbai: International Institute for Population Sciences.

Prajapati, R. P., Kalariya, M., Parmar, S. K., and Sheth, N. R. (2010). Phytochemical and Pharmacological Review of *Lagenaria sicereria*. *J. Ayurveda Integr. Med.* 1 (4), 266–272. doi:10.4103/0975-9476.74431

Prasad, S. K., and Singh, M. K. (2015). Horse Gram- an Underutilized Nutraceutical Pulse Crop: a Review. *J. Food Sci. Technol.* 52 (5), 2489–2499. doi:10.1007/s13197-014-1312-z

R, A. S., S, S., N, R., Ts, G., Karthikeyan, M., Gnanasekaran, A., et al. (2018). *Solanum tuberosum* L: Botanical, Phytochemical, Pharmacological and Nutritional Significance. *ijpm* 10 (3), 115–124. doi:10.5138/09750185.2256

Raghavan, K., Kumar, A., Pal, A., Khanum, F., and Bawa, A. S. (2010). Nutritional, Medicinal and Industrial Uses of Sesame (*Sesamum indicum* L.) Seeds - an Overview. *Agriculturae Conspectus Scientificus* 75 (4), 159–168.

Rahman, M., Khatun, A., Liu, L., and Barkla, B. J. (2018). Brassicaceae Mustards: Traditional and Agronomic Uses in Australia and New Zealand. *Molecules* 23, 231–248. doi:10.3390/molecules23010231

Rajput, P., Chaudhary, M., and Sharma, R. A. (2018). Phytochemical and Pharmacological Importance of Genus *Urtica* - a Review. *Int. J. Pharm. Sci. Res.* 9 (4), 1387–1396.

Ramadan, M., and Mörsel, J.-T. (2002). Oil Composition of Coriander (*Coriandrum sativum* L.) Fruit-Seeds. *Eur. Food Res. Tech.* 215, 204–209. doi:10.1007/s00217-002-0537-7

Reyad-ul-Ferdous, M. (2015). Present Biological Status of Potential Medicinal Plant of *Amaranthus viridis*: A Comprehensive Review. *Ajcem* 3 (5), 12. doi:10.11648/j.ajcem.s.2015030501.13

Rivas, M., Vignale, D., Ordoñez, R. M., Zampini, I. C., Alberto, M. R., Sayago, J. E., et al. (2013). Nutritional, Antioxidant and Anti-inflammatory Properties of *Cyclanthera pedata*, An Andinean Fruit and Products Derived from them. *Fns* 04, 55–61. doi:10.4236/fns.2013.48a007

Ruel, M. T. (2003). Operationalizing Dietary Diversity: A Review of Measurement Issues and Research Priorities. *J. Nutr.* 133, 3911S–3926S. doi:10.1093/jn/133.11.3911S

Saklani, S., and Chandra, S. (2011). Antimicrobial Activity Nutritional Profile and Quantitative Study of Different Fractions of *Ficus palmata*. *Int. Res. J. Plant Sci.* 2 (11), 332–337.

Saklani, S., and Chandra, S. (2012). *In-vitro* Anti-microbial Activity, Nutritional Profile and Phytochemical Screening of Wild Edible Fruit of Garhwal Himalaya (Ficus Auriculata). *Int. J. Pharm. Sci. Rev. Res.* 12 (2), 61–64.

Sanati, S., Razavi, B. M., and Hosseinzadeh, H. (2018). A Review of the Effects of *Capsicum annuum* L. and its Constituent, Capsaicin, in Metabolic Syndrome. *Iran J. Basic Med. Sci.* 21 (5), 439–448. doi:10.22038/IJBMS.2018.25200.6238

Sandhya Suresh, S., and S S Sisodia, S. S. (2018). Phytochemical and Pharmacological Aspects of *Cucurbita moschata* and *Moringa oleifera*. *Pbj* 6 (6), 45–53. doi:10.20510/ukjpb/6/i6/179239

Sarkar, P., Lohith Kumar Dh, L., Dhumal, C., Panigrahi, S. S., and Choudhary, R. (2015). Traditional and Ayurvedic Foods of Indian Origin. *J. Ethnic Foods* 2 (3), 97–109. doi:10.1016/j.jef.2015.08.003

Seligman, H. K., Bindman, A. B., Vittinghoff, E., Kanaya, A. M., and Kushel, M. B. (2007). Food Insecurity Is Associated with Diabetes Mellitus: Results from the National Health Examination and Nutrition Examination Survey (NHANES) 1999-2002. *J. Gen. Intern. Med.* 22 (7), 1018–1023. doi:10.1007/s11606-007-0192-6

Semwal, R., Semwal, D., Mishra, S., and Semwal, R. (2015). Chemical Composition and Antibacterial Potential of Essential Oils from *Artemisia capillaris*, *Artemisia nilagirica*, *Citrus limon*, *Cymbopogon flexuosus*, *Hedychium spicatum* and *Ocimum tenuiflorum*. *Npj* 5, 199–205. doi:10.2174/2210315505666150827213344

Shahana, S., and Nikalje, A. P. G. (2017). Brief Review on *Bauhinia variegata*: Phytochemistry, Antidiabetic and Antioxidant Potential. *Am. J. Pharmtech. Res.* 7 (1), 186–197.

Shankar, S., Segaran, G., Sundar, R. D. V., Settu, S., and Sathiavelu, M. (2019). Brassicaceae – A Classical Review on its Pharmacological Activities. *Int. J. Pharm. Sci. Rev. Res.* 55 (1), 107–113.

Sharma, A., and Rai, P. K. (2018). Assessment of Bioactive Compounds in *Brassica juncea* Using Chromatographic Techniques. *J. Pharmacogn. Phytochem.* 7 (3), 1274–1277.

Sidana, J., Saini, V., Dahiya, S., Nain, P., and Bala, S. (2013). A Review on Citrus - the Boon of Nature. *Int. J. Pharm. Sci. Rev. Res.* 18 (2), 20–27.

Singh, A., and Navneet (2018). Ethnobotanical Uses, Antimicrobial Potential, Pharmacological Properties and Phytochemistry of *Syzygium cumini* Linn. Syn. *Eugenia jambolana* (Jamun) – A Review. *Int. J. Iinnov. Pharm. Sci. Res.* 1 (6), 32–47. doi:10.21276/IJIPSR.2018.06.01.252

Singh, P., and Singh, J. (2013). Medicinal and Therapeutic Utilities of *Raphanus sativus*. *Int. J. Plant Anim. Env. Sci.* 3 (2), 12–105.

Sirisha, N., Sreenivasulu, M., Sangeeta, K., and Chetty, C. M. (2010). Antioxidant Properties of *Ficus* Species–A Review. *Int. J. Pharmtech Res.* 2 (4), 2174–2182. doi:10.5368/aedj.2010.2.3.86-89.pdf

Steyn, N. P., Nel, J. H., Nantel, G., Kennedy, G., and Labadarios, D. (2006). Food Variety and Dietary Diversity Scores in Children: Are They Good Indicators of Dietary Adequacy? *Public Health Nutr.* 9, 644–650. doi:10.1079/phn2005912

Suma, P. F., and Urooj, A. (2012). Antioxidant Activity of Extracts from Foxtail Millet (*Setaria italica*). *J. Food Sci. Technol.* 49 (4), 500–504. doi:10.1007/s13197-011-0300-9

Sundriyal, R. C., Negi, G. C. S., Maikhuri, R. K., Rawat, D. S., Rawal, R. S., and Dhyani, P. P. (2014). "Family and Smallholder Farming in Himalayan Communities," in *Deep Roots* (Rome, Italy, and Tudor Rose UK, 105–108. & 253, published by FAO.

Sundriyal, R. C., Rai, S. C., Sharma, E., and Rai, Y. K. (1994). Hill Agroforestry Systems in South Sikkim, India. *Agroforest Syst.* 26 (3), 215–235. doi:10.1007/bf00711212

Tewari, L. M., Tewari, G., Chopra, N., Tewari, A., Pandey, N. C., and Kumar, M. (2020). Phytochemical Screening and Antioxidant Potential of Some Selected Wild Edible Plants of Nainital District, Uttarakhand. In: *Natural Products and Their Utilization Pattern* (Edts G. Tewari, A. Tewari, and L. M. Tewari). New York, NY: Nova Science Publishers, Inc. pp 71–97.

Tongco, J. V. V., Villaber, R. A. P., Aguda, R. M., and Razal, R. A. (2014). Nutritional and Phytochemical Screening, and Total Phenolic and Flavonoid Content of *Diplazium esculentum* (Retz.) Sw. From Philippines. *J. Chem. Pharm. Res.* 6, 238–242.

Uzama, D., Orishadipe, A. T., and Danhalilu, R. L. (2016). Phytochemical, Nutritional and Antimicrobial Evaluations of the Aqueous Extract of *Brassica nigra* (Brassicaceae) Seeds. *Am. J. Appl. Chem.* 4 (4), 161–163. doi:10.11648/j.ajac.20160404.17

Varma, R. K., Garg, V. K., Singh, L., and Kumar, D. (2013). Pharmacognostic Evaluation and Phytochemical Analysis of Seeds of *Vigna mungo* (L.) Hepper. *J. Pharmacogn. Phytotherapy*. 1 (1), 01–09.

Verma, R., Awasthi, M., Modgil, R., and Dhaliwal, Y. S. (2012). Effect on the Physic-Chemical and Nutritional Characteristics of Kachnar (*Bauhinia variegata* Linn.) green Buds and Flowers. *Indian J. Nat. Prod. Resour.* 3 (2), 242–245.

Wang, L., Du, S., Wang, H., and Popkin, B. M. (2018). Influence of Dietary Fat Intake on Body Weight and Risk of Obesity Among Chinese Adults, 1991–2015: a Prospective Cohort Study. *The Lancet* 392, 420. doi:10.1016/s0140-6736(18)32649-7

World Food Programme (WFP) (2009). *Comprehensive Food Security & Vulnerability Analysis Guidelines*. Rome, Italy: United Nations World Food Programme.

Zhang, B., Peng, H., Deng, Z., and Tsao, R. (2018). Phytochemicals of Lentil (*Lens culinaris*) and Their Antioxidant and Anti-inflammatory Effects. *J. Food Bioact.* 1, 93–103. doi:10.31665/jfb.2018.1128

Zhao, G., Wang, A., Tang, yu., and Hu, Zhu. (20042004). "Research on the Nutrient Constituents and Medicinal Values of *Fagopyrum cymosum* Seeds," in Proceedings of the 9th International Symposium on Buckwheat (Prague).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership