ETHNOPHARMACOLOGY OF EASTERN EUROPEAN COUNTRIES

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ETHNOPHARMACOLOGY OF EASTERN EUROPEAN COUNTRIES

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REVIEW
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Turning Meadow Weeds Into Valuable Species for the Romanian Ethnomedicine While Complying With the Environmentally Friendly Farming Requirements of the European Union's Common Agricultural Policy

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The cross-compliance mechanism of the European Union (EU)'s common agricultural policy (CAP) makes the approval of the direct payments to the European farmers subject to compliance with the requirement to maintain the land in good agricultural and environmental condition. One of the obligations of the Romanian land owners and farmers is to avoid the installation of unwanted vegetation on their land plots. This vegetation is represented by some species of herbaceous or woody plants, annual or perennial, that spontaneously invade the agricultural lands, diminishing the production capacity of the cultivated plants. Included in this category are 10 meadow weeds, without fodder value or even toxic to animals: *Arctium lappa* L., *Carduus nutans* L., *Conium maculatum* L., *Eryngium campestre* L., *Euphorbia cyparissias* L., *Pteridium aquilinum* (L.) Kuhn, *Rumex acetosella* L., *Veratrum album* L., *Xanthium spinosum* L., and *Xanthium strumarium* L. Various and multiple uses in traditional medicine of these meadow weed species have been reported for Romania and other nine neighboring East European countries, *i.e.* Bosnia and Herzegovina, Bulgaria, Czech Republic, Estonia, Kosovo, Russia, Turkey, Serbia, and Ukraine. For *A. lappa* were recorded the highest number of ethnomedicinal uses, in the largest number of East European countries, including Romania. *C. maculatum* and *V. album* are not recommended for human consumption but can be further investigated as potential sources of pharmaceutically active compounds. Once removed by landowners and farmers from their land, the raw plant material of these 10 species become readily and easily available to the Romanian local communities and the industry of herbal food supplements, while the biodiversity of the agro-ecosystems is maintained.

Keywords: meadow weed, medicinal plant, ethnomedicine, *Arctium lappa*, *Eryngium campestre*, *Rumex acetosella*, *Xanthium spinosum*, *Xanthium strumarium*

INTRODUCTION

The European Union (EU)'s common agricultural policy (CAP) is one of the world's largest agricultural policies and the EU's longest-prevailing one (Pe'er et al., 2019). Besides producing food and stimulating rural community development, the CAP defines one more condition that allows farmers to fulfill their functions in society—environmentally sustainable farming—to produce food while simultaneously protecting nature and safeguarding biodiversity (European Commission, 2020a). Cross-compliance is a CAP's mechanism that links direct payments to compliance by farmers with basic standards concerning the environment, food safety, animal and plant health and animal welfare, as well as the requirement of maintaining land in good agricultural and environmental condition (GAEC). Evidence has shown that agriculture is the single largest cause of biodiversity loss but already nearly one-third of the world's farms have adopted more environmentally friendly practices while continuing to be productive (Pretty et al., 2018). In Europe, since 2005, all EU farmers receiving direct payments under CAP are subject to compulsory cross-compliance which is an important tool for integrating environmental requirements into the CAP (European Commission, 2020b). The good GAECs and the statutory management requirements (SMR), refer to a set of EU standards and requirements aiming at sustainable agriculture (European Parliament, 2013). In the area "Environment, climate change, good agricultural condition of land," when referring to the issue of "Landscape, minimum level of maintenance," GAEC 7 requires "Retention of landscape features, including where appropriate, hedges, ponds, ditches, trees in line, in group or isolated, field margins and terraces, and including a ban on cutting hedges and trees during the bird breeding and rearing season and, as an option, measures for avoiding invasive plant species" (European Parliament, 2013). After they are defined and detailed at national or regional level, all these standards are to be respected by all the European farmers receiving direct payments or some of the rural development payments.

METHODS: LITERATURE SEARCH STRATEGY

We systematically searched four databases (Web of Science, PubMed, Scopus, and ScienceDirect) for relevant, peer reviewed publications, using a combination of relevant keywords and Boolean operators which included the name of the 10 plant species and of the Eastern European countries, with no other filters applied (in July 2019). The number of retrieved abstracts was below 10, irrespective of the species searched for. This result was somehow anticipated by the guest editors of the research topic when they expected that "ethnopharmacological research in this area is quite limited, many of the existing studies being published in national or local journals, thus being less visible to the scientific community." To expand the literature search, Google Scholar database was interrogated, and all the retrieved publication was selected based in the information provided by their abstracts and subsequently by their full text version. Only peer-reviewed articles were included in our review. To access the literature published in the Romanian language, the online catalog of the County Library "G. T. Kirileanu" Neamt was searched using the eBibliophil search engine (https://bibgtkneamt.ebibliophil.ro). All the relevant specialty books retrieved were searched for the 10 plant species and if they contain any recommended use in ethnomedicine.

Selection criteria: all the publications referring to the 10 plant species and their use in the ethnomedicine of any of the Eastern European countries (from the former communist countries in the west till Russia, Ukraine and Turkey in the east) have been included in our literature review.

ROMANIA'S PROVISIONS FOR AVOIDING THE INSTALLATION OF UNWANTED VEGETATION ON THE FARMING LAND

Romania covers an area of 238,391 km² of which around 61% agricultural land (14.6 million ha) (64.2% arable land, 32.9% meadows and natural grasslands and 2.7% plantations of trees and vineyard), and 28.3% forests and other forestry vegetation lands. The Rural Development Programme (RDP) is outlining Romania's priorities for using the nearly € 9.5 billion of public money that is available for the CAP 2014–2020 (European Commission, 2015).

In agreement with the EU's regulations, Romania has defined three measures to be followed in order to implement the GAEC 7, one of which imposes that farmers have to prevent unwanted vegetation to install on the farming land, including on the uncultivated one. The Agency for Payments and Intervention in Agriculture (APIA) is the national authority responsible for coordinating the control activity on the cross-compliance norms within the schemes and support measures for the Romanian farmers (Ministry of Agriculture and Rural Development, 2015).

The details regarding the application of the measures of GAEC 7 have been modified along the years but its main objective was to maintain a minimum level of maintenance of agricultural land (regardless of the category of use, including land which is no longer used for production) by avoiding the installation of unwanted vegetation. In this context, the unwanted vegetation is represented by some species of herbaceous or woody plants, annual or perennial, that spontaneously invade the agricultural lands, diminishing the production capacity of the cultivated plants. Also included in this category are some meadow plant species, without fodder value or even toxic to animals.

The legal provisions recommend that the unwanted vegetation should not dominate the culture in more than 30% of the plot area, regardless of the land use category (arable land, permanent meadow, or permanent crops). However, when the

percentage of weediness exceeds the threshold of 80% of the surface of the plot, the entire plot is considered ineligible and excluded from payment. Also, the uncultivated agricultural land, one or more years, on which the unwanted vegetation was installed on more than 80% of the area, is excluded from payment. This decision is taken after on-site inspections carried out by APIA representatives (Agency for Payments and Intervention in Agriculture, 2019b).

THE UNWANTED MEADOW WEEDS IN ROMANIA

The main weed species that make up the unwanted vegetation on meadows (plants without fodder value or toxic plants) are annual, biennial or perennial plants. The APIA has listed 10 biannual or perennial weeds, all to be removed by the Romanian land owners or farmers from the meadows for which direct payments (EU subsidies) are requested: *Arctium lappa* L., *Carduus nutans* L., *Conium maculatum* L., *Eryngium campestre* L., *Euphorbia cyparissias* L., *Pteridium aquilinum* (L.) Kuhn, *Rumex acetosella* L., *Veratrum album* L., *Xanthium spinosum* L., and *Xanthium strumarium* L. (Agency for Payments and Intervention in Agriculture, 2019b). The same regulating and control body has prepared and publicly released a guide for identifying these 10 species that represent unwanted vegetation growing on the meadows (Agency for Payments and Intervention in Agriculture, 2019a). For each of the 10 species, a detailed description of the plant (roots, stem, leaves, flowers, and seeds), seed dispersal mechanism, and the general ecology of the species is provided, visually supported by four to six pictures. All these details are meant to facilitate the on-the-field identification for removal by the interested individuals.

To achieve its final goal, *i.e.* the removal of these 10 species from the EU-subsidized fields, APIA should simultaneously act by stopping and reversing the continuous degradation of grasslands due to overgrazing, a consequence of the larger, and increasing, number of grazing animals, also receiving a per-capita EU subsidy from the same governmental body: APIA. Overgrazing leads to the invasion of ruderal plant species, later on categorized as "unwanted" weed species by APIA. From the phytosociological point of view, two of the 10 species, *i.e. E. cyparissias* and *R. acetosella*, are natural components of most of the grasslands in the vegetation classes Festuco–Brometea (*E. cyparissias*) and Molinio–Arrhenatheretea (*R. acetosella*) (Sanda et al., 2008; Chifu, 2014), and they were erroneously categorized as unwanted vegetation in APIA's guide (Agency for Payments and Intervention in Agriculture, 2019a). Moreover, some of the other 10 species are not the most common and widespread species of their genus in Romania, *e.g. Carduus acanthoides* invades many more hectares of degraded grasslands than *C. nutans* (Sarateanu et al., 2008). All the above considered, the APIA's documents with respect to the invasive and unwanted vegetation should be critically reviewed and updated for the future post-2020 CAP.

PHYTOCHEMICAL CONSTITUENTS AND ETHNOMEDICINAL USES OF THE MEADOW WEEDS

All 10 species have ethnomedicinal uses which were reported around the world, including in Romania and other neighboring Eastern European countries, some of them dating back for centuries or even millennia. Their traditional use as edible and medicinal species has triggered investigations on their phytochemical composition.

A. lappa L.—Greater Burdock, *Asteraceae*

A. lappa has been used therapeutically in Europe, North America and Asia for hundreds of years (Wang et al., 2019). *A. lappa* is used in Eastern European folk medicine as an adjuvant in diabetes therapy (European Medicine Agency, 2011; Tousch et al., 2014; Tabassum et al., 2019), to treat digestive, renal, lung, and skin affections, having also depurative, diuretic, and diaphoretic properties (European Medicine Agency, 2011). Traditionally, the roots, buds, and seeds serve as blood purifier, and have been used as remedy for rheumatism, scurvy, gravel, venereal disease, and sores (Di Novella et al., 2013). An improvement of oxidative stress and inflammatory status was observed in patients with knee osteoarthritis treated with *A. lappa* root tea (Maghsoumi-Norouzabad et al., 2016), as well as in treating inflammatory acne (Miglani and Manchanda, 2014). In Greece, *A. lappa*'s root is used for musculoskeletal diseases, in particular against joint pains and rheumatism (Tsioutsiou et al., 2019). In Bosnia and Herzegovina this plant is used to treat skin conditions, to strengthen the hair root, against intestinal parasites, for dissolution of kidney stone, for improved urination, against diabetes and rabies, dog's bites, to release stomach gasses, against sexually transmitted diseases (STDs), and to treat facial nerve inflammation etc. (Redzic, 2010).

The main bioactive compounds identified in all parts of *A. lappa* are the lignans (arctigenin, artiin, matairesinol) and polyphenolic acids (caffeic acid derivatives) (Ferracane et al., 2010; da Silva et al., 2013; Edwards et al., 2015). These two groups possess high antioxidant activity (Liu et al., 2012). Arctigenin is reducing the inflammatory process through inhibiting iNOS expression and promoting cytokines (TNF-α and IL-6) productions (Zhao et al., 2009). *In vitro* studies show that lignans extracted from *A. lappa* have anti-proliferative effect against cancerous cells, inducing apoptosis and limiting migration of metastasis (Lou et al., 2017; Wang et al., 2019).

Several studies showed that the seeds are rich in caffeic acid, chlorogenic acid and cynarin, the leaves contain high amounts of phenolic acids, quercetin, quercitrin, luteolin, sesquiterpenes, and eudesmol, while the roots are rich in carbohydrates (including inulin up to 45%–50%, mucilage, pectin and sugars), arctic acid, polyacetylenes, arctiin, luteolin, quercetin rhamnoside (Ferracane et al., 2010; European Medicine Agency, 2011; Edwards et al., 2015), and caffeoylquinic acids derivatives (Tousch et al., 2014).

In Romanian ethnomedicine *A. lappa* is recommended for its detoxifying (due to the high fiber composition), hepatoprotective

(due to caffeic acid derivatives), cholesterol lowering (reduces the absorption of cholesterol and lipids at intestine level), diuretic, antiinflammatory, antioxidative (due to caffeic acid derivatives), hypoglycemic (due to high innulin content) (Bojor and Pop, 2010), and antibiotic effects (Stanescu et al., 2014). This plant is recommended for hepatobiliary and liver protection, hypercholesterolemia, dermatitis, eczema, skin infection, hyperglycemia, cholelithiasis, oily and seborrheic skin, hair regrowth (Bojor and Alexan, 1984; Parvu, 2000; Ardelean et al., 2008; Mohan, 2008; Bojor and Pop, 2010), intoxications, flu, high blood pressure, hemorrhages (Parvu, 2000; Mohan, 2008), muscular atrophy, gout, enterocolitis (Mohan, 2008), herpes, dandruff (Parvu, 2000; Ardelean et al., 2008), cystitis (Bojor and Alexan, 1984), burns (Bojor, 2018), hepatitis, bronchitis, scurvy (Milica et al., 2012), corns, typhoid fever, heart pain, chest pain, rheumatism, STDs, skin rashes (Stanescu et al., 2014), epilepsy, arthritis, constipation, abdominal colic, hepatitis, and insect bites (Grigore and Grigore, 2007).

C. nutans L. —Musk Thistle, Asteraceae

C. nutans is used as dietary supplement (Isik and Yucel, 2017). In Turkish folk medicine, this species has been used as tonic to stimulate liver function, detoxifying herb and to ameliorate fever (Aktay et al., 2000). In Bulgaria, *C. nutans* is used for its antihemorroidal, cardiotonic and diuretic properties (Zheleva-Dimitrova et al., 2011).

The main biochemical compounds of musk thistle are the flavonoids (apigenin, luteolin, kempferol and derivatives, rutin, tilianin, and isorhamnetin), sterols and triterpenes (β-sitosterol acetate, sitosterol-O-xyloside, taraxasterol acetate), polyacetylenes (vinylpentacetylene), phenolic acids, and anthocyanins (Bain and Desrochers, 1988; Abdallah and Ramadan, 1989; Jordon-Thaden and Louda, 2003; Zhelev et al., 2013). Flavonoids represents an important class of compounds known to have a positive impact in ameliorating symptoms of cancer, cardiovascular disease, and neurodegenerative disorders (Spencer et al., 2004; Salvamani et al., 2014). In the last decade, studies on apigenin use as an adjuvant in cancer treatment had shown tremendous results, this plant originated flavone acting as a chemoprotective agent, inhibiting tumor development through inducing cycle arrest and apoptosis (Banerjee and Mandal, 2015; Kashyap et al., 2018).

In Romanian traditional medicine (TM) the *C. nutans*'s seeds are used to prevent atherosclerosis, the flowers as febrifuge, blood purifier (Daraban et al., 2013) and used for treating polyarticular ankilosis, and myalgia, while the leaves are recommended for hypertension and liver diseases (Grigore and Grigore, 2007).

C. maculatum L.—Poison Hemlock, *Apiaceae*

C. maculatum is one of the most poisonous plants for laboratory animals, farm animals, and human beings, due to the presence of piperidine alkaloids in all its parts (Al-Snafi, 2016). Despite its poisonous nature, *C. maculatum* is included in several herbals as *Succus conii*, described as a narcotic, sedative, analgesic, spasmolytic, anti-aphrodisiac, and anti-cancer agent (Reynolds, 2005). *C. maculatum* extract is used as a traditional homeopathic remedy for cervix carcinoma (Mondal et al., 2014). This species has been used in ethnomedicine as an analgesic and anti-inflammatory agent (De Landoni and Conium maculatum, 1990; Arihan et al., 2009; Al-Snafi, 2016,; Madaan and Kumar, 2012), in Turkey to treat diabetes (Paksoy et al., 2016), and in Morocco as an alternative to treat typhoid fever and sterility, and also to ease labor (Kharchoufa et al., 2018). Externally, it has been used to treat herpes and swelled joints (Bloch, 2001). Some studies highlight the antispasmodic property of *C. maculatum* and it was reported to have a positive impact on patients with epilepsy, asthma, angina, rheumatism, and tetanus (Mitich, 1998; Hotti and Rischer, 2017).

The chemical composition of poison hemlock has been widely studied and the main toxic alkaloids are coniine and γ-coniceine identified in all plant parts, but mostly in roots and seeds, and they are also responsible for the sedative and anti-inflammatory properties of the species (Vetter, 2004; Panter et al., 2011; Cortinovis and Caloni, 2015; Al-Snafi, 2016; Kharchoufa et al., 2018). Besides alkaloids, *C. maculatum* is rich in flavonoids (anti-oxidative), coumarins (anti-microbial, anti-inflammatory), polyacetylenes, vitamins, and oils (Al-Snafi, 2016).

In Romanian ethnomedicine, *C. maculatum* was used as sedative (Bojor, 2018) and for reducing neuralgia (Ardelean et al., 2008; Bojor, 2018).

E. campestre L.—Field Eryngo, *Apiaceae*

E. campestre has been used in folk medicine (Zhang et al., 2008). In the European herbal medicine this plant was used as an infusion for the treatment of whooping cough, as well as in the treatment of kidney and urinary tract inflammations (Medbouhi et al., 2018). In Eastern European TM field eryngo roots and leaves are used for their anti-inflammatory, antiscorbutic, diaphoretic, antitussive, diuretic, expectorant, appetite-stimulant, and aphrodisiac properties, and to treat hemorrhoids, rheumatism, and infertility (Küpeli et al., 2006; Belda et al., 2013; Güneş et al., 2014; Conea et al., 2015; Kikowska et al., 2016; Soumia, 2018). In Turkey is used to treat intestinal disorders, flatulence, hepatitis, digestion disorders, and muscle pain (Akgul et al., 2018) but is also used fresh for human consumption (Demirci and Özkan, 2014). A study by Hawas et al., 2013 suggests its potential application in the treatment of Alzheimer's disease.

E. campestre represents an important source of multi-target antimicrobial essential oils, with spathulenol being the main chemical compound of the species (Erdem et al., 2015). Several studies showed that *E. campestre* is rich in flavonoids (quercetin glycosides, isorhamnetin glycosides, and myricetin glycosides), phenolic acids, acetylenes, saponins, steroids, terpenoids, and coumarins (Abou El-Kassem et al., 2013; Marčetić et al., 2014; Matejić et al., 2018; Soumia, 2018). The flavonoids and phenolic compounds contribute to the antioxidant properties of the plant (Küpeli et al., 2006; Matejić et al., 2018; Soumia, 2018).

In Romanian ethnomedicine *E. campestre* is recommended for its cholagogue, diuretic, appetite-stimulant (Craciun et al., 1976; Grigore and Grigore, 2007), expectorant, antitussive, bronchial antiseptic, antispasmodic, antibiotic, hypotensive, and carminative effects (Milica et al., 2012), and is used for treating gallbladder, urinary retention, gonococcal urethritis,

ascites, amenorrhea, scurvy, tuberculosis, acne, oliguria, colicky nephritis, seborrheic dermatitis, leg edema (Grigore and Grigore, 2007), amenorrhea, constipation, tooth pain, periodontitis, tooth decay (Milica et al., 2012), and nephrolithiasis (Ardelean et al., 2008). In the Romanian rural areas is used as ingredient in various dishes, especially in soups (Parvu, 2000).

E. cyparissias L.—Cypress Spurge, *Euphorbiaceae*

The leaves of *E. cyparissias* are used as anti-warts (Pieroni and Vandebroek, 2007). The flowers, stems, and leaves of cypress spurge are used in folk medicine in the treatment of dermatological diseases (psoriasis, eczema), respiratory diseases (asthma, bronchitis, chest congestion, throat spasm), hay fever, and against tumor development (Özbilgin et al., 2012; Stanković and Zlatić, 2014). Its seed oil is purgative. In the past, vapor of the chloroform extract was used as anesthetic agent. It is recommended for insomnia and ear constipation. Its condensed milky sap is one of the components of "emplastrum cantharides" (blister bug plaster) which is used principally to relieve deep-seated inflammation and to promote the absorption of effusions (Papp, 2004).

The whole plant has antioxidant properties given by the high amount of secondary metabolites, mainly isoprenoids (diterpenoids, triterpenoids, sesquiterpenoids—elemene and caryophyllene), phenolic acids, and flavonoids (Hemmer and Gülz, 1989; Özbilgin et al., 2012; Stanković and Zlatić, 2014). Two new diterpenoids have been identified in extracts of *E. cyparissias* (*i.e.*, cyparissin A and B) that exhibit important activity against cancerous cell lines in *in-vitro* studies (Lanzotti et al., 2015).

In Romanian ethnomedicine *E. cyparissias* is recommended for its purging, emetic (Craciun et al., 1976), antispasmodic, sedative, antifungal, anti-rheumatic, revulsive, and febrifuge effects (Milica et al., 2012) and is used for treating warts, heir loss and sciatic neuralgia (Grigore and Grigore, 2007), gastric distress, constipation, stomach pains, intestinal worms, emphysema, pleuritis, hay fever, tuberculosis, pharyngitis, mange, eczemas, arthritis, ringworm, anthrax, impetigo, nail mycosis, tooth pain, and freckles (Milica et al., 2012).

P. aquilinum (L.) Kuhn—Bracken Fern, *Polypodiaceae*

Dioscorides (ca. 50 AD) in his de *Materia Medica* referred to several ferns, including *P. aquilinum*, as having medicinal values. *P. aquilinum* is thought to be a fern with potent anti-cancer properties. In India, decoctions from the rhizomes of *P. aquilinum* are drunk as an herbal tea (Baskaran et al., 2018). Dried rhizomes mixed with milk are used to relieve diabetic disorders, and tender fronds are used as vegetables (Baskaran et al., 2018). Moreover, the strong rhizomes of plant have been used directly as a food or as an ingredient of bread (by Australian, British, French, Japanese populations or by Lapp and Siberian cultures) (Vetter, 2010). It is the most common edible pteridophyte in sub-Saharan Africa, used as human food in Angola, Cameroon, DRC, Gabon, Madagascar, Nigeria, and South Africa (Maroyi, 2014).

The high concentration of phenol, flavonoid, and terpenoid compounds gives to bracken fern extracts antioxidant properties and antimicrobial activity, justifying its traditional use to treat skin diseases and gastrointestinal disorders (May, 1978; Mannan et al., 2008; Piluzza and Bullitta, 2011; Kardong et al., 2013). The presence of tannins, cardiac glycosides, anthraquinone, and cyanogenic glycosides in this species may have a negative impact on liver function (Hassan et al., 2007) and vitamin B_1 metabolism (Fenwick, 1989; Vetter, 2010).

R. acetosella L.—Red Sorrel, *Polygonaceae*

In TM the leaves of *R. acetosella* are used by the native populations of North America as treatment for warts and bruises. The aerial parts contribute to amelioration of diarrhea and stomach disorders in North America and Hungary, while seeds are used to treat diarrhea and dysentery in Hungary (Chevalier, 1996; Shale et al., 1999; Foster et al., 2000; Wegiera et al., 2007; Vasas et al., 2015). An ethnobotanical survey of medicinal plants used in Turkey revealed that leaves of *R. acetosella* are used traditionally as an analgesic and diuretic (Cakilcioglu and Turkoglu, 2010). In Iran, *R. acetosella* is widely used by traditional healers for the treatment of jaundice and fever (Amiri et al., 2014). In Poland, this species is still in use today as potherb (Łuczaj and Szymański, 2007).

The main chemical compounds in the genus *Rumex* are antraquinones (emodin, physcion, and chrysophanol in fruits and leaves, and sennoside A in fruits and roots), nepodin, and flavanoids (quercetin-3-O-glucoside) with important antioxidant activity, while the stilbenoids demonstrated to have a positive impact in cancer therapy and inflammatory diseases (Wegiera et al., 2007; Vasas et al., 2015).

In Romanian ethnomedicine *R. acetosella* is recommended for its digestive, stomachic, laxative, cleansing, antidiarrheal, cholagogue, diuretic, emmenagogue, anthelmintic, astringent, appetite-stimulant, nutritious, anti-anemic, and antiscorbutic properties (Milica et al., 2012). This plant was recommended for treating paralysis (Craciun et al., 1976), hepatitis, jaundice, cholelithiasis, constipation, pulmonary illnesses, asthma, insect bites, gout, leucorrhea, nephritis, wounds, acne, boils, ringworms, cancerous ulcerations, and fever and as mineralizing treatment and for blood purification in the spring (Milica et al., 2012).

V. album L.—White Hellebore, *Melanthiaceae*

V. album has a long history of medicinal use, dating back as far as Hippocrates in which dilutions of the plant are prescribed to produce upward purging. Through the 1700s, preparations of *V. album* root and rhizomes were used medicinally in Europe primarily for their emetic properties (Chandler and McDougal, 2014). In the folk medicine *V. album* is used to treat rheumatism, toothache, gout, herpes, trigeminal neuralgia, and catarrh, and as

an hypotensive agent (Furbee, 2009; Ujváry, 2010; Wiart, 2012; Roberts and Wink, 2013).

The medicinal, and the toxic properties of white hellebore are given by the presence of steroidal alkaloids including proveratrine, cevadine, protoveratrine, jervine, veratramine, and veratridine, the last being the most poisonous of all. Some of the symptoms of intoxication with white hellebore are hypotension, bradycardia, and paralysis (Furbee, 2009). Similar to *C. maculatum*, the alkaloids presence in all plant parts gives the species sedative and anti-inflammatory properties, antihypertensive activity being also described, especially linked to the presence of protoveratrine A and B (Krayer and Meilman, 1977).

In the Romanian ethnomedicine *V. album* is used for its neuro-sedative (Craciun et al., 1976; Parvu, 2000; Ardelean et al., 2008), hypotensive, narcotic, hypnotic, antipyretic (Parvu, 2000), anthelmintic, and insecticidal effects (Milica et al., 2012). The plant is used for treating malignant hypertension, hypertensive crisis, eclampsia, fever, eczemas, mange, itchy skin (Parvu, 2000), rheumatism, gout, eczema (Ardelean et al., 2008; Milica et al., 2012), pneumonia, whooping cough, intermittent fever, mental illnesses, psychiatric disorders, mania, mange, pruritus, psoriasis, and herpes zoster (Milica et al., 2012).

X. spinosum L.—Bathurst Burr, *Asteraceae*

In ethnomedicine *X. spinosum* is used in renal disorders, for its antibacterial, antifungal, and anthelminthic properties; calming influence; wound healing properties; and the efficacy of infusions in treating benign prostate hyperplasia (Domokos et al., 2016; Aldibekova et al., 2018). It was used against rabies, to relieve chronic fevers, to abate diabetes effects, even to stimulate saliva production for its diuretic effect (Andreani et al., 2017) and "it exhibits" noticeable antioxidant activity (Aldibekova et al., 2018). In Pakistani local communities the leaves and fruits are reported to be diaphoretic, diuretic, and sedative and used for hydrophobia while the infusion of root is emetic (Aziz et al., 2018).

The main chemical compounds are polyphenols, flavones, diterpenes, sesquiterpene lactones (xanthatin), phytosterols (Domokos et al., 2016), tannins, and essential oils (Aldibekova et al., 2018). It has been showed that xanthatin can inhibit the growth of Gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and fungi (*Colletotrichum gloesporoides* and *Trichothecium roseum*) (Ginesta Peris et al., 1994). *In vitro* and *in vivo* studies showed that xanthatin and xanthinosin have chemopreventive properties, inhibiting tumor cells proliferation (Ramírez-Erosa et al., 2007; Romero et al., 2015). The therapeutic properties of xanthatin can be extended also to antiviral (herpes simplex virus, feline corona virus, influenza A H_1N_1 and A H_3N_2, influenza B, repvirus, respiratory syncytial virus etc.) and anti-angiogenic activities (Romero et al., 2015).

In Romanian ethnomedicine *X. spinosum* is recommended for its anti-inflammatory, diuretic, diaphoretic, and disinfectant effects (Bojor and Pop, 2010). This plant is recommended for treating polyuria, prostatitis, selective electrolyte retention (K^+ and Mg^{2+}) in the blood serum, protection of the myocardium, prostate adenoma, nephrolithiasis (Bojor and Pop, 2010), rabies, and hyperthyroidism (Ardelean et al., 2008).

X. strumarium L.—Common Cocklebur, *Asteraceae*

Although the fruit is the predominant part of *X. strumarium* used in folk medicine (Fan et al., 2019), the leaves and roots have been used for their diaphoretic, diuretic, emollient, appetite-stimulant, laxative, antirheumatic, antisyphilitic, anodyne, and sedative properties (Chopra et al., 1956). Traditionally, this species is also used as febrifuge drug and as an immunostimulant, against malaria, as well as dysentery cure, astringent, sedative, analgesic, against leucorrhea and urinary diseases, eczema and skin disease, bleeding, insect bite, to treat boils and pimples, against smallpox and stomach diseases, earache and strumous disease, leprosy, headache, and fever (Katewa, 2008; Kozuharova et al., 2019). In Yemen and Russia, *X. strumarium* is used as a medicine soothing illness, hemolytic, reducing temperature, skin diseases, antimicrobial, for ulcers, and antifungal (Aldibekova et al., 2018).

In common cocklebur the aerial parts' main compounds are the sesquiterpene lactones (guianolides, elemanolides, germacranolides, xanthinium, xanthumin, xanthatin) with important anti-inflammatory, antiviral, antitumor, and antimicrobial properties (Kamboj and Saluja, 2010; Fan et al., 2019).

A series of compounds with pharmacological importance have been identified in all parts of *X. strumarium*: glycosides (xanthostrumarin, atractyloside, carboxyatractyloside, the last being also the most toxic compound) (Kamboj and Saluja, 2010), phytosterols (xanthanol, isoxanthonol, xanthinosin, 4-oxo-bedfordia acid) (Olivaro et al., 2016), caffeoylquinic acid, thiazinodine, diacetyl xanthumin (antifungal compound), linoleic acid, and triterpenoids (botulin, betulinic acid, erythrodiol, lupeol acetate, oleanolic acid, amyrin) (Kamboj and Saluja, 2010; Yadav et al., 2015; Fan et al., 2019). In n-butanol fraction of the ethanolic extract of *X. strumarium* has been proven to possess the highest analgesic and anti-inflammatory activity (Han et al., 2007).

In Romanian ethnomedicine, the whole *X. strumarium* plant is recommended for treating diabetes (Grigore and Grigore, 2007).

RECOMMENDED ETHNOMEDICINAL USES OF SOME MEADOW WEEDS IN ROMANIA AND OTHER NEIGHBORING EAST EUROPEAN COUNTRIES

The weed species growing on the Romanian meadows, which have to be removed by land owners or farmers if public subsidies under EU's CAP are expected, have multiple and documented uses in TM. Yet, two of these species, *C. maculatum* and *V. album* are very toxic and they are not recommended for human use, but they can be potential valuable sources of pharmaceutically active compounds.

For the majority of these meadow species have been reported details of their recommended ethnomedicinal uses in Romania (**Table 1**). On the Romanian market many commercial herbal products that have ingredients derived from the meadow weeds are on sale, including through e-commerce (**Supplementary Table 1**).

The same meadow weeds have been used in the TM (**Table 2**) in many neighboring European Eastern countries (**Supplementary Figure 1**).

From all 10 species, *A. lappa* has recorded the highest number of ethnomedicinal recommended uses, both in Romania and neighboring East European countries. On the contrary, we were not able to find any documented use of *P. aquilinum* in Romanian ethnomedicine, but in Kosovo its leaves decoction is used as antibacterial and diuretic treatment (Mustafa et al., 2012).

Medicinal plants have historically proven their value as a source of molecules with therapeutic potential, and still represent an important pool for the identification of novel drug leads. Particularly relevant examples of plant-derived natural compounds that have become indispensable for modern pharmacotherapy are the anti-cancer agents, e.g., paclitaxel and its derivatives from *Taxus* species, vincristine and vinblastine from *Catharanthus roseus* (L.) G. Don, and camptothecin and its analogs initially discovered in *Camptotheca acuminata* Decne. (Atanasov et al., 2015). There were an estimated 18.1 million new cases of cancer and 9.6 million deaths from cancer worldwide in 2018 from which in Romania alone been registered 83,461 new cases and 50,902 deaths (Ferlay et al., 2019). As a highly relevant aspect related to human health, the reviewed peer-reviewed publications reported the use of seven out of the 10 species, *i.e. A. lappa*, *C. maculatum*, *E. cyparissias*, *P. aquilinum*, *R. acetosella, X. spinosum*, and *X. strumarium*, as treatments in ethnomedicine for different types of cancer (e.g. prostatic, pulmonary, endometrial), cancerous ulcerations and tumor development in Eastern European countries, suggesting further investigations. From all the above-mentioned species, only three of them have been reported in Romanian for treatment of cancerous ulcerations (*R. acetosella*) and prostate cancer (*X. spinosum*, and *X. strumarium*). At least one commercial herbal product containing both herba Xanthii and herba Xanthii spinosi is sold on the Romanian market.

THE RAW PLANT MATERIAL AND ITS INFLUENCE ON THE AUTHENTICITY OF THE DERIVED HERBAL PRODUCTS

The herbal products, sold as medicines or food supplements, represent a core part of the TM which has been has recognized by the World Health Organization (WHO) as a growing and expanding global phenomenon (World Health Organization, 2013). The rapidly expanding global market is expected to reach US$ 115 billion in 2020 (Raclariu et al., 2018) while the trade of medicinal plants will grow at the rate of 15%–25% annually and will reach US$ 5 trillion by 2050 (Booker et al., 2012). The increasing demand for herbals and the limited

TABLE 1 | Ethnomedicinal uses of the meadow weeds in Romania.

Species	Part used	Medicinal use or ailments treated	Preparation or administration	References
Arctium lappa L.	Leaves	Headache	Tea	(Papp et al., 2014)
	Roots	Sore throat, respiratory diseases	Tea	(Fierascu et al., 2017)
	Seeds	Cough, gastrointestinal disorders (reflux)	Tea	(Papp et al., 2011; Papp et al., 2017)
	Leaves, roots, seeds	Skin diseases (acne, dermatitis, dry scalp seborrhea, alopecia) Antimicrobial effect	Decoction	(Gilca et al., 2018) (Segneanu et al., 2019)
	Roots	Expectorant, antitussive, emollient, diuretic, anti-inflammatory, digestive, renal disorders, cough, bronchitis, wounds, sores	Decoction	(Tita et al., 2009)
Euphorbia cyparissias L.	Aerial parts	Alopecia, dermatitis	Homeopathic remedies	(Parvu, 2000; Gilca et al., 2018)
Eryngium campestre L.	Rhizomes	Detoxifying, diuretic, cicatrizing, eupeptic, carminative, sedative, abdominal distention, urinary lithiasis, anorexia, gastric ulcer, convulsive cough, wounds	Decoction	(Tita et al., 2009)
Rumex acetosella L.	Herb	Pneumonia	Cataplasm	(Papp et al., 2011)
	Leaves	Warts, bruises	Poultice	(Butura, 1979)
Veratrum album L.	Roots, rhizomes	Dermatitis, scabies Anti-inflammatory, antispastic, antibacterial, hypotensive	Tincture	(Parvu, 2000; Gilca et al., 2018; Segneanu et al., 2019)
Xanthium spinosum L.	Aerial parts	Rabies, hyperthyroidism, prostate adenoma	Decoction	(Parvu, 2000)
Xanthium strumarium L.		Diuretic	2% tincture of the cocklebur germ	(Aldibekova et al., 2018)
		Bladder and urethra diseases	Adenostop$^{TM(*)}$ herbal product (squeeze of the cocklebur spiny)	(Klimakhin et al., 2015)

(*)Disclaimer: Mention of proprietary products is solely for the purpose of providing specific information, and does not constitute an endorsement or a recommendation for their use.

TABLE 2 | Ethnomedicinal use of the meadow weeds in neighboring East European countries.

Species	Country	Part used	Medicinal use or ailments treated	Preparation or administration	References
Arctium lappa L.	Czech Republic	Roots	Adjuvant therapy in diabetes	Herbal tea	(European Medicine Agency, 2011)
	Estonia	Roots	Cancer treatment	Herbal tea	(Sak et al., 2014)
	Russia	Roots	Cancer treatment, antioxidant activity	Flor-Essence™(*) herbal tonic Infusions, decoctions, extracts or tinctures	(Tamayo et al., 2000) (Spiridonov, 2008)
		Leaves	Skin wounds, dermatological disorders	Crude	(Mamedov et al., 2004)
	Ukraine	Leaves	Headache	Fresh or dried	(Sõukand and Pieroni, 2016)
		Roots	Knee ache, hair loss Blood cleansing	Infusion Tea	
	Bosnia and Herzegovina, Serbia, Kosovo	Leaves	Wound healing	Powder or boiled in milk	(Jarić et al., 2018)
	Kosovo	Aerial parts	Gastrointestinal disorders, bronchitis, lithontriptic	Decoction	(Mustafa et al., 2012)
	Bulgaria	Roots	Diuretic, ulcer	Decoction	(Leporatti and Ivancheva, 2003)
Carduus nutans L.	Kosovo	Inflorescence	Eczemas	Extracted with cold water for 10 days and then used as tea	(Mustafa et al., 2012)
	Turkey	Seeds (crushed)	Liver diseases	Infusion	(Bulut et al., 2017)
Euphorbia cyparissias L.	Kosovo	Stem	Warts	Fresh leaves topically applied	(Mustafa et al., 2012)
Eryngium campestre L.	Bulgaria	Roots	Diuretic, spasmolytic, prostatitic	Infusion	(Leporatti and Ivancheva, 2003)
	Turkey	Aerial and root parts	Antitussive, diuretic, appetite-stimulant, stimulant, and aphrodisiac	Infusion	(Wang, 2012)
		Root	Vulnerary	Powdered (external)	(Altundag and Ozturk, 2011)
		Leaf	Carminative, jaundice	Decoction	
		Roots	Anti-inflammatory	Crushed	(Karakaya et al., 2019)
Pteridium aquilinum (L.) Kuhn	Kosovo	Leaves	Anti-bacterial, diuretic	Decoction	(Mustafa et al., 2012)
Rumex acetosella L.	Russia	Roots	Skin wounds, dermatological disorders	Powder	(Mamedov et al., 2004)
	Turkey	Leaf	Abscess	Pounded (external)	(Altundag and Ozturk, 2011)
		Herb	Stomachic	Internal	
Veratrum album L.	Bulgaria	Roots	Hypotensive	Tincture	(Leporatti and Ivancheva, 2003)
	Kosovo	Leaves	Anti-lice	Decoction	(Mustafa et al., 2012)
Xanthium strumarium L.	Russia	Whole plant	Hepatoprotector, pulmonary and uterus cancer	Infusions, decoctions, extracts, tinctures	(Spiridonov, 2008)

(*)*Disclaimer: Mention of proprietary products is solely for the purpose of providing specific information, and does not constitute an endorsement or a recommendation for their use.*

supply of many species that are harvested from the wild (Coghlan et al., 2015) is stimulating the economically-motivated adulteration (EMA) (Simmler et al., 2018). It was recently reported that 27% of almost 6,000 commercial herbal products sold in 37 countries were found to be adulterated, while in Europe almost half (47%) were adulterated. In Romania, 94% of the herbal products analyzed (n = 70) were reported to be adulterated, when their composition was compared with the labeled ingredient species (Ichim, 2019). The availability of new plant biomass while is removed from the Romanian meadows by its owners or farmers can become readily available to the interested users and could reduce the pressure on the existing sources of raw materials. Moreover, because only selected species have to be searched, identified and removed, a supplementary quality check is added to the plant raw material, before being further used or processed, which represents additional benefits for the quality of the herbal products and their benefits for the human health.

CONCLUSION

The Romanian landowners or farmers have to remove from their meadows 10 plant species without fodder value or toxic to animals as a compulsory condition to receive the public subsidies under EU's CAP. For these unwanted meadow weeds have reported many and various uses in Romanian ethnomedicine, as well as in other nine neighboring East European countries. Some of the ethnopharmacological uses are highly relevant for the modern medicine, supporting the use of the removed biomass from the meadows as additional or alternative source of pharmacologically active ingredients. The local communities and the industry of

herbal food supplements can take immediate advantage from these raw plant materials, readily and easily available, while assuring their environmentally-friendly, sustainable, and supportive of local traditions and commercial use.

AUTHOR CONTRIBUTIONS

MI conceived and supervised the research. MI and EG wrote the manuscript. MI and EG read and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Abdallah, O., and Ramadan, M. (1989). Phytochemical study of Carduus nutans L. (Asteraceae). *Bull. Pharm. Sci. Assiut Univ.* 18, 69–76.

Abou El-Kassem, L., Hawas, U., Awad, H., and Taie, H. (2013). Flavonoids from the aerial parts of Eryngium campestre L. with antioxidant and anti-Alzheimer activities. *Planta Med.* 79, PJ2. doi: 10.1055/s-0033-1352206

Agency for Payments and Intervention in Agriculture (2019a). Ghid pentru identificarea principalelor specii de buruieni care alcatuiesc "vegetatia nedorita" pe pajisti [Guide to identify the main weed species which make up the "unwanted vegetation" on the meadows].

Agency for Payments and Intervention in Agriculture (2019b). Ghidul fermierului privind ecoconditionalitatea [The farmer's guide regarding the cross-compliance]. Bucharest.

Akgul, A., Akgul, A., Senol, S. G., Yildirim, H., Secmen, O., and Dogan, Y. (2018). An ethnobotanical study in Midyat (Turkey), a city on the silk road where cultures meet. *J. Ethnobiol. Ethnomed.* 14, 12. doi: 10.1186/s13002-017-0201-8

Aktay, G., Deliorman, D., Ergun, E., Ergun, F., Yeşilada, E., and Çevik, C. (2000). Hepatoprotective effects of Turkish folk remedies on experimental liver injury. *J. Ethnopharmacol.* 73, 121–129. doi: 10.1016/S0378-8741(00)00286-5

Aldibekova, D. A., Kizaibek, M., Aisijiang, M., Dyuskaliyeva, G., Taldau, A., and Erkinbek, M. (2018). Morphology, anatomy, chlorogenic acid content and antioxidant capacity of Xanthium strumarium L. and Xanthium spinosum L. *Online J. Biol. Sci.* 18, 237–246. doi: 10.3844/ojbsci.2018.237.246

Al-Snafi, A. E. (2016). Pharmacology and toxicology of Conium maculatum - A review. *Pharmaceut. Chem. J.* 3, 136–142.

Altundag, E., and Ozturk, M. (2011). Ethnomedicinal studies on the plant resources of east Anatolia, Turkey. *Proc. Soc. Behav. Sci.* 19, 756–777. doi: 10.1016/j.sbspro.2011.05.195

Amiri, M. S., Joharchi, M. R., and Taghavizadeh Yazdi, M. E. (2014). Ethno-medicinal plants used to cure jaundice by traditional healers of Mashhad, Iran. *Iranian J. Pharmaceut. Res.* 13, 157–162. doi: 10.22037/ijpr.2014.1445

Andreani, S., Paolini, J., Costa, J., and Muselli, A. (2017). Chemical composition of essential oils of Xanthium spinosum L., an invasive species of Corsica. *Chem. Biodiversity* 14, e1600148. doi: 10.1002/cbdv.201600148

Ardelean, A., Mohan, G., and Mohan, D. A. (2008). *Flora medicinala a Romaniei [Romania's medicinal flora]* (Bucharest: ALL Publishing House).

Arihan, O., Boz, M., Iskit, A. B., and Ilhan, M. (2009). Antinociceptive activity of coniine in mice. *J. Ethnopharmacol.* 125, 274–278. doi: 10.1016/j.jep.2009.06.032

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P., et al. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* 33, 1582–1614. doi: 10.1016/j.biotechadv.2015.08.001

Aziz, M. A., Adnan, M., Khan, A. H., Shahat, A. A., Al-Said, M. S., and Ullah, R. (2018). Traditional uses of medicinal plants practiced by the indigenous communities at Mohmand Agency, FATA, Pakistan. *J. Ethnobiol. Ethnomed.* 14, 2. doi: 10.1186/s13002-017-0204-5

Bain, J. F., and Desrochers, A. M. (1988). Flavonoids of Carduus nutans and C. acanthoides. *Biochem. Systematics Ecol.* 16, 265–268. doi: 10.1016/0305-1978(88)90005-1

Banerjee, K., and Mandal, M. (2015). Oxidative stress triggered by naturally occurring flavone apigenin results in senescence and chemotherapeutic effect in human colorectal cancer cells. *Redox Biol.* 5, 153–162. doi: 10.1016/j.redox.2015.04.009

Baskaran, X.-R., Geo Vigila, A.-V., Zhang, S.-Z., Feng, S.-X., and Liao, W-b (2018). A review of the use of pteridophytes for treating human ailments. *J. Zhejiang University Sci. B* 19, 85–119. doi: 10.1631/jzus.B1600344

Belda, A., Zaragozí, B., Belda, I., Martínez, J., and Seva, E. (2013). Traditional knowledge of medicinal plants in the Serra de Mariola Natural Park, South-Eastern Spain. *Afr. J. Tradit. Complementary Altern. Med.* 10, 299–309. doi: 10.4314/ajtcam.v10i2.15

Bloch, E. (2001). Hemlock poisoning and the death of Socrates: Did Plato tell the truth? *Plato J.* 1, 255–278. doi: 10.14195/2183-4105_1_1

Bojor, O., and Alexan, M. (1984). *Plantele medicinale si aromatice de la A la Z [The medicinal and aromatic plants from A to Z]* (Bucharest: Recoop Publishing House).

Bojor, O., and Pop, M. (2010). *Fitoterapia in folosul tuturor [Phytotherapy for the benefit of all]* (Bucharest: Medical Publishing House).

Bojor, O. (2018). *Plantele medicinale si aromatice de la A la Z [The medicinal and aromatic plants from A to Z]* (Bucharest: Dharana Publishing House).

Booker, A., Johnston, D., and Heinrich, M. (2012). Value chains of herbal medicines - Research needs and key challenges in the context of ethnopharmacology. *J. Ethnopharmacol.* 140, 624–633. doi: 10.1016/j.jep.2012.01.039

Bulut, G., Zahid Bozkurt, M., and Tuzlacı, E. (2017). The preliminary ethnobotanical study of medicinal plants in Uşak (Turkey). *Marmara Pharmaceut. J.* 21, 305–310. doi: 10.12991/marupj.300795

Butura, V. (1979). *Enciclopedie de etnobotanica romaneasca [Romanian ethnobotany encyclopedia]* (Bucharest: Scientific and Encyclopedic Publishing House).

Cakilcioglu, U., and Turkoglu, I. (2010). An ethnobotanical survey of medicinal plants in Sivrice (Elaziğ-Turkey). *J. Ethnopharmacol.* 132, 165–175. doi: 10.1016/j.jep.2010.08.017

Chandler, C. M., and McDougal, O. M. (2014). Medicinal history of North American Veratrum. *Phytochem. Rev.* 13, 671–694. doi: 10.1007/s11101-013-9328-y

Chevalier, A. (1996). *The encyclopedia of medicinal plants* (New York: DK Pub).

Chifu, T. (2014). *Diversitatea fitosociologica a vegetatiei Romaniei [Phytosociological diversity of Romania's vegetation] (vol.I-III)* (Iasi, Romania: Editura Institutul European Iasi).

Chopra, R. N., Nayar, S. L., and Chopra, I. C. (1956). *Glossary of Indian medicinal plants* (New Delhi: Council of Scientific and Industrial Research).

Coghlan, M. L., Maker, G., Crighton, E., Haile, J., Murray, D. C., White, N. E., et al. (2015). Combined DNA, toxicological and heavy metal analyses provides an auditing toolkit to improve pharmacovigilance of traditional Chinese medicine (TCM). *Sci. Rep.* 5, 17475. doi: 10.1038/srep17475

Conea, S., Pârvu, A. E., Taulescu, M. A., and Vlase, L. (2015). Effects of Eryngium planum and Eryngium campestre extracts on ligature-induced rat periodontitis. *Digest J. Nanomat. Biostructures* 10, 693–704.

Cortinovis, C., and Caloni, F. (2015). Alkaloid-containing plants poisonous to cattle and horses in Europe. *Toxins* 7, 5301–5307. doi: 10.3390/toxins7124884

Craciun, F., Bojor, O., and Alexan, M. (1976). *Farmacia naturii [Nature's pharmacy] (vol. I)* (Bucharest: Ceres Publishing House).

da Silva, L. M., Allemand, A., Mendes, D. A. G. B., Dos Santos, A. C., André, E., de Souza, L. M., et al. (2013). Ethanolic extract of roots from Arctium lappa L. accelerates the healing of acetic acid-induced gastric ulcer in rats: Involvement of the antioxidant system. *Food Chem. Toxicol.* 51, 179–187. doi: 10.1016/j.fct.2012.09.026

Daraban, I. N., Arsene, G. G., Turcus, V., and Ardelean, A. (2013). Assessment on bioeconomical potential for medicinal plants in salty meadows from the aradului plain (W. Romania). *Studia Universitatis Vasile Goldis Arad Seria Stiintele Vietii* 23, 71–78.

De Landoni, J. H., and Conium maculatum, L. (1990). (PIM 144). Available at: http://www.inchem.org/documents/pims/plant/conium.htm [Accessed March 5, 2020].

Demirci, S., and Özkan, E. E. (2014). Ethnobotanical studies of some Apiaceae plants in Kahramanmaras and a review of their phytochemical studies. *Istanbul Ecz. Fak. Derg.* 44, 241–250.

Di Novella, R., Di Novella, N., De Martino, L., Mancini, E., and De Feo, V. (2013). Traditional plant use in the National Park of Cilento and Vallo di Diano, Campania, Southern, Italy. *J. Ethnopharmacol.* 145, 328–342. doi: 10.1016/j.jep.2012.10.065

Domokos, E., Kursinszki, L., Kelemen, H., and Varga, E. (2016). [Phytopharmacological review of bathurst burr (Xanthium spinosum L.)]. *Acta Pharm. Hungarica* 86, 35–40.

Edwards, S. E., Rocha, I., Williamson, E. M., and Heinrich, M. (2015). *Phytopharmacy: An evidence-based guide to herbal medical products* (Hoboken, NJ: Wiley-Blackwell).

Erdem, S. A., Nabavi, S. F., Orhan, I. E., Daglia, M., Izadi, M., and Nabavi, S. M. (2015). Blessings in disguise: A review of phytochemical composition and antimicrobial activity of plants belonging to the genus Eryngium. *DARU J. Pharmaceut. Sci.* 23, 53 doi: 10.1186/s40199-015-0136-3

European Commission (2015). Factsheet on 2014-2020 Rural Development Programme for Romania.

European Commission (2020a). Common agricultural policy.

European Commission (2020b). Cross-compliance.

European Medicine Agency (2011). Assessment report on Arctium lappa L., radix. London.

European Parliament (2013). Regulation (EU) No 1306/2013 of the European Parliament and of the Council of 17 December 2013 on the financing, management and monitoring of the common agricultural policy.

Fan, W., Fan, L., Peng, C., Zhang, Q., Wang, L., Li, L., et al. (2019). Traditional uses, botany, phytochemistry, pharmacology, pharmacokinetics and toxicology of xanthium strumarium L.: A review. *Molecules* 24 (2), 359. doi: 10.3390/molecules24020359

Fenwick, G. R. (1989). Bracken (Pteridium aquilinum) - toxic effects and toxic constituents. *J. Sci. Food Agric.* 46, 147–173. doi: 10.1002/jsfa.2740460204

Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D. M., Piñeros, M., et al. (2019). Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int. J. Cancer* 144, 1941–1953. doi: 10.1002/ijc.31937

Ferracane, R., Graziani, G., Gallo, M., Fogliano, V., and Ritieni, A. (2010). Metabolic profile of the bioactive compounds of burdock (Arctium lappa) seeds, roots and leaves. *J. Pharmaceut. Biomed. Anal.* 51, 399–404. doi: 10.1016/j.jpba.2009.03.018

Fierascu, R. C., Fierascu, I., Ortan, A., Avramescu, S. M., Dinu-Pirvu, C. E., and Ionescu, D. (2017). "Romanian aromatic and medicinal plants: From tradition to science," in *Aromatic and Medicinal Plants - Back to Nature* (London: InTech), 149–173. doi: 10.5772/66513

Foster, S., Duke, J. A., and Foster, S. (2000). *A field guide to medicinal plants and herbs of eastern and central North America* (Boston: Houghton Mifflin Co).

Furbee, B. (2009). "Neurotoxic plants," in *Clinical Neurotoxicology: Syndromes, Substances, Environments* (Elsevier Inc.), 523–542. doi: 10.1016/B978-032305260-3.50053-8

Güneş, M. G., İşgör, B. S., İşgör, Y. G., Shomali Moghaddam, N., Geven, F., and Yildirim, Ö. (2014). The effects of Eryngium campestre extracts on glutathione-S-transferase, glutathione peroxidase and catalase enzyme activities. *Turk J. Pharm. Sci.* 11, 339–346.

Gilca, M., Tiplica, G. S., and Salavastru, C. M. (2018). Traditional and ethnobotanical dermatology practices in Romania and other Eastern European countries. *Clinics Dermatol.* 36, 338–352. doi: 10.1016/j.clindermatol.2018.03.008

Ginesta Peris, E., Garcia Breijo, F. J., and Primo Yúfera, E. (1994). Antimicrobial activity of xanthatin from Xanthium spinosum L. *Lett. Appl. Microbiol.* 18, 206–208. doi: 10.1111/j.1472-765X.1994.tb00848.x

Grigore, G., and Grigore, C. (2007). *Ghidul plantelor medicinale si al bolilor tratate cu plante [Guide to the medicinal plants and plant treated-diseases]* (Bucharest: BIC ALL Publishing House).

Han, T., Li, H. L., Zhang, Q. Y., Han, P., Zheng, H. C., Rahman, K., et al. (2007). Bioactivity-guided fractionation for anti-inflammatory and analgesic properties and constituents of Xanthium strumarium L. *Phytomedicine* 14, 825–829. doi: 10.1016/j.phymed.2007.01.010

Hassan, S. W., Umar, R. A., Dabai, Y. U., Ebbo, A. A., and Faruk, U. Z. (2007). Antibacterial, phytochemical and toxicity studies of Pteridium aquilinum L. (Dennstaedtiaceae) in rabbits. *J. Pharmacol. Toxicol.* 2, 168–175. doi: 10.3923/jpt.2007.168.175

Hawas, U., El-Kassem, L., Awad, H., and Taie, H. (2013). Anti-Alzheimer, antioxidant activities and flavonol glycosides of Eryngium campestre L. *Curr. Chem. Biol.* 7, 188–195. doi: 10.2174/2212796811307020010

Hemmer, H., and Gülz, P. G. (1989). Tetra-and pentacyclic triterpenoids from epicuticular wax of Euphorbia cyparissias L., Euphorbiaceae. *Z. Für Naturforschung* 44c, 563–567. doi: 10.1515/znc-1989-7-804

Hotti, H., and Rischer, H. (2017). The killer of Socrates: Coniine and related alkaloids in the plant kingdom. *Mol. (Basel Switzerland)* 22 (11), 1962. doi: 10.3390/molecules22111962

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

Isik, G., and Yucel, E. (2017). Fatty acid composition of Carduus nutans seeds. *Int. J. Agri Env. Res.* 3, 19–25.

Jarić, S., Kostić, O., Mataruga, Z., Pavlović, D., Pavlović, M., Mitrović, M., et al. (2018). Traditional wound-healing plants used in the Balkan region (Southeast Europe). *J. Ethnopharmacol.* 211, 311–328. doi: 10.1016/j.jep.2017.09.018

Jordon-Thaden, I. E., and Louda, S. M. (2003). Chemistry of Cirsium and Carduus: A role in ecological risk assessment for biological control of weeds? *Biochem. Syst. Ecol.* 31, 1353–1396. doi: 10.1016/S0305-1978(03)00130-3

Küpeli, E., Kartal, M., Aslan, S., and Yesilada, E. (2006). Comparative evaluation of the anti-inflammatory and antinociceptive activity of Turkish Eryngium species. *J. Ethnopharmacol.* 107, 32–37. doi: 10.1016/j.jep.2006.02.005

Kamboj, A., and Saluja, A. K. (2010). Phytopharmacological review of Xanthium strumarium L. (Cocklebur). *Int. J. Green Pharm. (IJGP)* 4, 129–139. doi: 10.22377/IJGP.V4I3.133

Karakaya, S., Karakaya, S., Polat, A., Aksakal, Ö., Sümbüllü, Y. Z., and İncekara, Ü. (2019). Plants used in traditional medicine and other uses in South of Erzurum (Turkey): An ethnobotanical study. *Ethnobot. Res. Appl.* 18, 1–18.

Kardong, D., Upadhyaya, S., and Saikia, L. R. (2013). Screening of phytochemicals, antioxidant and antibacterial activity of crude extract of Pteridium aquilinum Kuhn. *JOPR: J. Pharm. Res.* 6, 179–182. doi: 10.1016/j.jopr.2012.11.037

Kashyap, D., Sharma, A., Tuli, H. S., Sak, K., Garg, V. K., Buttar, H. S., et al. (2018). Apigenin: A natural bioactive flavone-type molecule with promising therapeutic function. *J. Funct. Foods* 48, 457–471. doi: 10.1016/j.jff.2018.07.037

Katewa, S. S. (2008). "Indigenous people and forests: Perspectives of an ethnobotanical study from Rajasthan (India)," in *Herbal Drugs: Ethnomedicine to Modern Medicine*. Ed. K. Ramawat (Berlin Heidelberg: Springer), 33–56. doi: 10.1007/978-3-540-79116-4_3

Kharchoufa, L., Merrouni, I. A., Yamani, A., and Elachouri, M. (2018). Profile on medicinal plants used by the people of North Eastern Morocco: toxicity concerns. *Toxicon: Off. J. Int. Soc. Toxicol.* 154, 90–113. doi: 10.1016/j.toxicon.2018.09.003

Kikowska, M., Thiem, B., Sliwinska, E., Rewers, M., Kowalczyk, M., Stochmal, A., et al. (2016). Micropropagation of Eryngium campestre L. via shoot culture provides valuable uniform plant material with enhanced content of phenolic acids and antimicrobial activity. *Acta Biol. Cracoviensia Ser. Bot.* 58, 43–56. doi: 10.1515/abcsb-2016-0009

Klimakhin, G. I., Fonin, V. S., Maslyakov, V. Y., Fadeev, N. B., Semikin, V. V., and Pel'gunova, L. A. (2015). Biochemical features of common Cocklebur (Xanthium strumarium L.). *Pharmaceut. Chem. J.* 49, 547–550. doi: 10.1007/s11094-015-1324-7

Kozuharova, E., Ionkova, I., and Spadaro, V. (2019). Xanthium strumarium-a potential cheap resource of plant substances for medicinal use. *Flora Mediterr.* 29, 93–102. doi: 10.7320/FlMedit29.093

Krayer, O., and Meilman, E. (1977). "Veratrum alkaloids with antihypertensive activity," in *Antihypertensive agents* (Berlin Heidelberg: Springer Berlin Heidelberg), 547–570. doi: 10.1007/978-3-642-66309-3_12

Łuczaj, Ł., and Szymański, W. M. (2007). Wild vascular plants gathered for consumption in the Polish countryside: a review. *J. Ethnobiol. Ethnomed.* 3, 17. doi: 10.1186/1746-4269-3-17

Lanzotti, V., Barile, E., Scambia, G., and Ferlini, C. (2015). Cyparissins A and B, jatrophane diterpenes from Euphorbia cyparissias as Pgp inhibitors and cytotoxic agents against ovarian cancer cell lines. *Fitoterapia* 104, 75–79. doi: 10.1016/j.fitote.2015.05.012

Leporatti, M. L., and Ivancheva, S. (2003). Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy. *J. Ethnopharmacol.* 87, 123–142. doi: 10.1016/S0378-8741(03)00047-3

Liu, J., Cai, Y. Z., Wong, R. N. S., Lee, C. K. F., Tang, S. C. W., Sze, S. C. W., et al. (2012). Comparative analysis of caffeoylquinic acids and lignans in roots and seeds among various burdock (Arctium lappa) genotypes with high antioxidant activity. *J. Agric. Food Chem.* 60, 4067–4075. doi: 10.1021/jf2050697

Lou, C., Zhu, Z., Zhao, Y., Zhu, R., and Zhao, H. (2017). Arctigenin, a lignan from Arctium lappa L., inhibits metastasis of human breast cancer cells through the downregulation of MMP-2/-9 and heparanase in MDA-MB-231 cells. *Oncol. Rep.* 37, 179–184. doi: 10.3892/or.2016.5269

Madaan, R., and Kumar, S. (2012). Screening of alkaloidal fraction of Conium maculatum L. aerial parts for analgesic and antiinflammatory activity. *Indian J. Pharmaceut. Sci.* 74, 457–460. doi: 10.4103/0250-474X.108423

Maghsoumi-Norouzabad, L., Alipoor, B., Abed, R., Eftekhar Sadat, B., Mesgari-Abbasi, M., and Asghari Jafarabadi, M. (2016). Effects of Arctium lappa L. (Burdock) root tea on inflammatory status and oxidative stress in patients with knee osteoarthritis. *Int. J. Rheumatic Dis.* 19, 255–261. doi: 10.1111/1756-185X.12477

Mamedov, N., Gardner, Z., and Craker, L. E. (2004). Medicinal plants used in Russia and Central Asia for the treatment of selected skin conditions. *J. Herbs Spices Med. Plants* 11, 191–222. doi: 10.1300/J044v11n01_07

Mannan, M., Maridass, M., and Victor, B. (2008). A review on the potential uses of ferns. *Ethnobot. Leaflets* 12, 281–285.

Marčetić, M. D., Petrović, S. D., Milenković, M. T., and Niketić, M. S. (2014). Composition, antimicrobial and antioxidant activity of the extracts of Eryngium palmatum Pančić and Vis. (Apiaceae). *Cent. Eur. J. Biol.* 9, 149–155. doi: 10.2478/s11535-013-0247-0

Maroyi, A. (2014). Not just minor wild edible forest products: Consumption of pteridophytes in sub-Saharan Africa. *J. Ethnobiol. Ethnomed.* 10, 78. doi: 10.1186/1746-4269-10-78

Matejić, J. S., Stojanović-Radić, Z. Z., Ristić, M. S., Veselinović, J. B., Zlatković, B. K., Marin, P. D., et al. (2018). Chemical characterization, in vitro biological activity of essential oils and extracts of three Eryngium L. species and molecular docking of selected major compounds. *J. Food Sci. Technol.* 55, 2910–2925. doi: 10.1007/s13197-018-3209-8

May, L. W. (1978). The economic uses and associated folklore of ferns and fern allies. *Bot. Rev.* 44, 491–528. doi: 10.1007/BF02860848

Medbouhi, A., Tintaru, A., Beaufay, C., Naubron, J.-V., Djabou, N., Costa, J., et al. (2018). Structural elucidation and cytotoxicity of a new 17-membered ring lactone from Algerian Eryngium campestre. *Molecules* 23 (12), 3250. doi: 10.3390/molecules23123250

Miglani, A., and Manchanda, R. K. (2014). Observational study of Arctium lappa in the treatment of acne vulgaris. *Homeopathy* 103, 203–207. doi: 10.1016/j.homp.2013.12.002

Milica, C., Roman, C. N., and Troia, D. (2012). *Flora medicinala a Romaniei [Romania's medicinal flora]* (Iasi: Doxologia Publishing House).

Ministry of Agriculture and Rural Development (2015). Order no. 352 of February 10, 2015 for the approval of the rules on cross compliance in the support schemes and measures for farmers in Romania (published in the Official Gazette with the number 363 of May 26, 2015).

Mitich, L. W. (1998). Poison-Hemlock (Conium maculatum L.). *Weed Technol.* 12, 194–197. doi: 10.1017/S0890037X00042792

Mohan, G. (2008). *Tratarea bolilor cu plante medicinale [Treating diseases with medicinal plants]* (Bucharest: Corint Publishing House).

Mondal, J., Panigrahi, A. K., and Khuda-Bukhsh, A. R. (2014). Anticancer potential of Conium maculatum extract against cancer cells in vitro: Drug-DNA interaction and its ability to induce apoptosis through ROS generation. *Pharmacog. Magazine* 10, S524–S533. doi: 10.4103/0973-1296.139792

Mustafa, B., Hajdari, A., Krasniqi, F., Hoxha, E., Ademi, H., Quave, C. L., et al. (2012). Medical ethnobotany of the Albanian Alps in Kosovo. *J. Ethnobiol. Ethnomed.* 8, 6. doi: 10.1186/1746-4269-8-6

Özbilgin, S., Sal, G., and Citoğlu, T. (2012). Uses of some Euphorbia species in traditional medicine in Turkey and their biological activities. *Turk J. Pharmaceut. Sci.* 9, 241–256.

Olivaro, C., Rostan, V., Bandera, D., Moyna, G., and Vazquez, A. (2016). Xanthane sesquiterpenoids from the roots and flowers of Xanthium cavanillesii. *Natural Prod. Res.* 30, 2238–2242. doi: 10.1080/14786419.2016.1149709

Paksoy, M. Y., Selvi, S., and Savran, A. (2016). Ethnopharmacological survey of medicinal plants in UlukIşla (Niğde-Turkey). *J. Herbal Med.* 6, 42–48. doi: 10.1016/j.hermed.2015.04.003

Panter, K. E., Welch, K. D., and Gardner, D. R. (2011). "Toxic plants," in *Reproductive and Developmental Toxicology*. Ed. C. G. Ramesh (Elsevier Inc.), 689–705. doi: 10.1016/B978-0-12-382032-7.10051-7

Papp, N., Bartha, S., Boris, G., and Balogh, L. (2011). Traditional uses of medicinal plants for respiratory diseases in Transylvania. *Natural Product Commun.* 6, 1459–1460. doi: 10.1177/1934578x1100601012

Papp, N., Birkás-Frendl, K., Bencsik, T., Stranczinger, S., and Czégényi, D. (2014). Survey of traditional beliefs in the Hungarian Csángó and Székely ethnomedicine in Transylvania, Romania. *Braz. J. Pharmacog.* 24, 141–152. doi: 10.1016/j.bjp.2014.03.005

Papp, N., Tóth, M., Dénes, T., Gyergyák, K., Filep, R., Bartha, S. G., et al. (2017). Ethnomedicinal treatment of gastrointestinal disorders in Transylvania, Romania. *Acta Ethnographica Hungarica* 62, 207–220. doi: 10.1556/022.2017.62.1.10

Papp, N. (2004). Antimicrobial activity of extracts of five Hungarian Euphorbia species and some plant metabolits. *Acta Bot. Hungarica* 46, 363–371. doi: 10.1556/ABot.46.2004.3-4.8

Parvu, C. (2000). *Universul plantelor: Mica enciclopedie [Plants' universe: small encyclopedia]* (Bucuresti: Enciclopedica Publishing House).

Pe'er, G., Zinngrebe, Y., Moreira, F., Sirami, C., Schindler, S., Müller, R., et al. (2019). A greener path for the EU Common Agricultural Policy. *Science* 365, 449–451. doi: 10.1126/science.aax3146

Pieroni, A., and Vandebroek, I. (2007). *Traveling cultures and plants: the ethnobiology and ethnopharmacy of migrations* (New York-Oxford: Berghahn Books).

Piluzza, G., and Bullitta, S. (2011). Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. *Pharmaceut. Biol.* 49, 240–247. doi: 10.3109/13880209.2010.501083

Pretty, J., Benton, T. G., Bharucha, Z. P., Dicks, L. V., Flora, C. B., Godfray, H. C. J., et al. (2018). Global assessment of agricultural system redesign for sustainable intensification. *Nat. Sustainability* 1, 441–446. doi: 10.1038/s41893-018-0114-0

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

Ramírez-Erosa, I., Huang, Y., Hickie, R. A., Sutherland, R. G., and Barl, B. (2007). Xanthatin and xanthinosin from the burs of Xanthium strumarium L. as potential anticancer agents. *Can. J. Physiol. Pharmacol.* 85, 1160–1172. doi: 10.1139/Y07-104

Redzic, S. (2010). Wild medicinal plants and their usage in traditional human therapy (Southern Bosnia and Herzegovina, W. Balkan). *J. Med. Plants Res.* 4, 1003–1027. doi: 10.5897/JMPR09.254

Reynolds, T. (2005). Hemlock alkaloids from Socrates to poison aloes. *Phytochemistry* 66, 1399–1406. doi: 10.1016/j.phytochem.2005.04.039

Roberts, M. F., and Wink, M. (2013). *Alkaloids: Biochemistry, ecology, and medicinal applications*. (New York: Plenum Press).

Romero, M., Zanuy, M., Rosell, E., Cascante, M., Piulats, J., Font-Bardia, M., et al. (2015). Optimization of xanthatin extraction from Xanthium spinosum L. and its cytotoxic, anti-angiogenesis and antiviral properties. *Eur. J. Med. Chem.* 90, 491–496. doi: 10.1016/j.ejmech.2014.11.060

Sõukand, R., and Pieroni, A. (2016). The importance of a border: Medical, veterinary, and wild food ethnobotany of the Hutsuls living on the Romanian and Ukrainian sides of Bukovina. *J. Ethnopharmacol.* 185, 17–40. doi: 10.1016/j.jep.2016.03.009

Sak, K., Jürisoo, K., and Raal, A. (2014). Estonian folk traditional experiences on natural anticancer remedies: from past to the future. *Pharmaceut. Biol.* 52, 855–866. doi: 10.3109/13880209.2013.871641

Salvamani, S., Gunasekaran, B., Shaharuddin, N. A., Ahmad, S. A., and Shukor, M. Y. (2014). Antiartherosclerotic effects of plant flavonoids. *BioMed. Res. Int.* 2014, 480258. doi: 10.1155/2014/480258

Sanda, V., Öllerer, K., and Burescu, P. (2008). *Fitocenozele din Romania [The phytocenoses in Romania]* (Bucharest: Ars Docendi).

Sarateanu, V., Horablaga, M., Stroia, C., Butnariu, M., and Bostan, C. (2008). Assessing the invasive species impact on the grasslands from Western Romania. *Res. J. Agric. Sci.* 40, 319–326.

Segneanu, A.-E., Cepan, C., Grozescu, I., Cziple, F., Olariu, S., Ratiu, S., et al. (2019). "Therapeutic use of some Romanian medicinal plants," in *Pharmacognosy - Medicinal Plants* (London: IntechOpen). doi: 10.5772/intechopen.82477

Shale, T. L., Stirk, W. A., and Van Staden, J. (1999). Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. *J. Ethnopharmacol.* 67, 347–354. doi: 10.1016/S0378-8741(99)00035-5

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

Soumia, B. (2018). "Eryngium campestre L.: Polyphenolic and flavonoid compounds; Applications to health and disease," in *Polyphenols: Mechanisms of Action in Human Health and Disease* (Elsevier), 69–79. doi: 10.1016/b978-0-12-813006-3.00007-6

Spencer, J. P. E., Abd El Mohsen, M. M., and Rice-Evans, C. (2004). Cellular uptake and metabolism of flavonoids and their metabolites: Implications for their bioactivity. *Arch. Biochem. Biophysics* 423, 148–161. doi: 10.1016/j.abb.2003.11.010

Spiridonov, N. A. (2008). "Russian medicinal plants in treatment of cancer," in *Botanical medicine in clinical practice*. Eds. R. R. Watson and V. R. Preedy (Trowbridge: Cromwell Press), 421–429. doi: 10.1079/9781845934132.0421

Stanescu, U., Hancianu, M., Cioanca, O., Aprotosoaie, A. C., and Miron, A. (2014). *Plante medicinale de la A la Z [Medicinal plants from A to Z]* (Iasi: POLIROM Publishing House).

Stanković, M. S., and Zlatić, N. M. (2014). Antioxidant activity and concentration of secondary metabolites in the plant parts of Euphorbia cyparissias L. *Kragujevac J. Sci.* 36, 121–128. doi: 10.5937/KgJSci1436121S

Tabassum, S., Perk, A. A., Qureshi, M. Z., Sabitaliyevich, U. Y., and Tokmurziyeva, G. (2019). *"Arctium lappa," in Nonvitamin and Nonmineral Nutritional Supplements* (Elsevier Inc.), 277–281. doi: 10.1016/B978-0-12-812491-8.00039-4

Tamayo, C., Richardson, M. A., Diamond, S., and Skoda, I. (2000). The chemistry and biological activity of herbs used in Flor-Essence^{TM} herbal tonic and Essiac. *Phytother. Res.* 14, 1–14. doi: 10.1002/(sici)10.1002/(SICI)1099-1573(200002)14:13.3.CO;2-F

Tita, I., Mogosanu, G. D., and Tita, M. G. (2009). Ethnobotanical invenory of medicinal plants from the South-West of Romania. *FARMACIA* 57, 141–156.

Tousch, D., Bidel, L. P. R., Cazals, G., Ferrare, K., Leroy, J., Faucanié, M., et al. (2014). Chemical analysis and antihyperglycemic activity of an original extract from burdock root (arctium lappa). *J. Agric. Food Chem.* 62, 7738–7745. doi: 10.1021/jf500926v

Tsioutsiou, E. E., Giordani, P., Hanlidou, E., Biagi, M., De Feo, V., Cornara, L., et al. (2019). Ethnobotanical study of medicinal plants used in Central Macedonia, Greece. *Evid. Based Complementary Altern. Med.* 2019, 4513792. doi: 10.1155/2019/4513792

Ujváry, I. (2010). *"Pest control agents from natural products," in Hayes" Handbook of Pesticide Toxicology* (Elsevier Inc.), 119–229. doi: 10.1016/B978-0-12-374367-1.00003-3

Vasas, A., Orbán-Gyapai, O., and Hohmann, J. (2015). The Genus Rumex: Review of traditional uses, phytochemistry and pharmacology. *J. Ethnopharmacol.* 175, 198–228. doi: 10.1016/j.jep.2015.09.001

Vetter, J. (2004). Poison hemlock (Conium maculatum L.). *Food Chem. Toxicol.* 42, 1373–1382. doi: 10.1016/j.fct.2004.04.009

Vetter, J. (2010). "Toxicological and medicinal aspects of the most frequent fern species, pteridium aquilinum (L.) kuhn," in *Working with Ferns: Issues and Applications* (Springer), 361–375. doi: 10.1007/978-1-4419-7162-3_25

Wang, D., Badarau, A. S., Swamy, M. K., Shaw, S., Maggi, F., da Silva, L. E., et al. (2019). Arctium species secondary metabolites chemodiversity and bioactivities. *Front. Plant Sci.* 10, 834. doi: 10.3389/fpls.2019.00834

Wang, P. (2012). Phytochemical constituents and pharmacological activities of Eryngium L. (Apiaceae). *Pharmaceutical. Crops* 3, 99–120. doi: 10.2174/2210290601203010099

Wegiera, M., Smolarz, H. D., Wianowska, D., and Dawidowicz, A. L. (2007). Anthracene derivatives in some species of Rumex L. genus. *Acta Societatis Bot. Poloniae* 76, 103–108. doi: 10.5586/asbp.2007.013

Wiart, C. (2012). *Lead compounds from medicinal plants for the treatment of neurodegenerative diseases* (Academic Press).

World Health Organization (2013). *WHO traditional medicine strategy: 2014-2023* (Geneva).

Yadav, S., Yadav, S. S., Ganie, S. A., Gulia, S. S., and Yadav, N. (2015). Xanthium strumarium L.: An ethnomedicinal and phytochemical review. *Int. J. Phytomed.* 6, 471–476.

Zhang, Z., Li, S., Ownby, S., Wang, P., Yuan, W., Zhang, W., et al. (2008). Phenolic compounds and rare polyhydroxylated triterpenoid saponins from Eryngium yuccifolium. *Phytochemistry* 69, 2070–2080. doi: 10.1016/j.phytochem.2008.03.020

Zhao, F., Wang, L., and Liu, K. (2009). In vitro anti-inflammatory effects of arctigenin, a lignan from Arctium lappa L., through inhibition on iNOS pathway. *J. Ethnopharmacol.* 122, 457–462. doi: 10.1016/j.jep.2009.01.038

Zhelev, I., Dimitrova-Dyulgerova, I., Belkinova, D., and Mladenov, R. (2013). Content of phenolic compounds in the Genus Carduus L. from Bulgaria. *ECOLOGIA BALKANICA* 5, 13–21.

Zheleva-Dimitrova, D., Zhelev, I., and Dimitrova-Dyulgerova, I. (2011). Antioxidant activity of some Carduus species growing in Bulgaria. *Free Radicals Antioxid.* 1, 15–20. doi: 10.5530/ax.2011.4.4

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Traditionally Used *Sideritis cypria* Post.: Phytochemistry, Nutritional Content, Bioactive Compounds of Cultivated Populations

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Sideritis species are recognized as important medicinal plants and their commercial demand is continuously on the rise both in the European and in the global market. Consequently, the cultivation of *Sideritis* species has been occurred to successfully meet the need for mass production of high-quality plant material. The present study was undertaken in order to investigate the chemical composition of cultivated *S. cypria*. Infusions of flowers and leaves were prepared separately, according to the European Medicine Agency (EMA) monograph. The infusion of the flowers revealed the presence of four flavones, isoscutellarein-7-O-[6′″-O-acetyl-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside, its 4′-O-methyl-derivative, 4′-O-methyl-hypolaetin-7-O-[6′″-O-acetyl-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside, and isoscutellarein-7-O-[6′″-O-acetyl-β-D-allopyranosyl-(1→2)]-6″-O-acetyl-β-D-glucopyranoside; four phenylethanoid glucosides, acteoside, leucosceptoside A, lamalboside, and leonoside A; one iridoid, melittoside, and one phenolic acid, chlorogenic acid, while the infusion of the leaves of the same population afforded the same first two flavones; five phenylethanoid glucosides, acteoside, leucosceptoside A, lavandulifolioside, leonoside A, and lamalboside; melittoside and chlorogenic acid. The structural elucidation of the isolated compounds was undertaken by high-field NMR spectroscopy. Moreover, the essential oils of the flowers and leaves were studied by GC-MS, separately. In addition, the mineral, bioactive compounds, protein and carbohydrate contents were evaluated for both plant materials.

Keywords: ***Sideritis cypria*, cultivation, infusions, flowers, leaves, melittoside, leonoside A, lamalboside**

INTRODUCTION

Sideritis species (Lamiaceae) have been used as traditional medicine herbs for thousands of years (González-Burgos et al., 2011) and the last 30 years safe use (including 15 years in the EU) with well-defined posologies and mode of preparation (EMA, 2015). Nowadays, the infusion of *Sideritis scardica* Griseb.; *Sideritis clandestina* (Bory & Chaub.) Hayek; *Sideritis raeseri* Boiss. & Heldr.; and

Sideritis syriaca L., has been listed by European Medicine Agency (EMA) as a traditional medicine for the relief of mild gastrointestinal discomfort and against the common cold (EMA, 2015). Previous detailed studies underlie the important pharmacological activities of the genus such as the antioxidant, anti-inflammatory, antivirus, anticancer, hepatoprotective, antispasmodic, analgesic, neuroprotective activity, as well as its great effectiveness against diseases related to the central nervous and to the urinary system (Kirimer et al., 2004; González-Burgos et al., 2011; EMA, 2015; Hofrichter et al., 2016; Deveci et al., 2017; Aneva et al., 2019). Indeed, a clinical study that carried out by Wightman et al. (2018), showed that *S. scardica* (Greek mountain tea) improved the aspects of cognitive function and mood in a group of healthy, older adults. Therefore, these plants have been a subject of intensive phytochemical research and are characterized mainly by the presence of terpenes, flavonoids, phenylethanoid glucosides, phenolic acids, and essential oil (González-Burgos et al., 2011; EMA, 2015).

The genus *Sideritis* L. comprises around 150 species (Aneva et al., 2019); among them, the endemic species *S. cypria* Post. is a perennial herb belonging to the section *Empedoclia* Rafin., 60 cm high with bright yellow flowers, growing at altitude 300–925 m in Pentadactylos Mountains in Northern Cyprus (Meikle, 1985; Yildiz and Gücel, 2006; Tsintides et al., 2007). In particular, three *Sideritis* species have been found in Cyprus; *S. curvidens* Stapf, *S. perfoliata* L., and the endemic *S. cypria* Post (Hanoğlu et al., 2020). Traditionally, the infusion of *S. cypria* is locally used as diaphoretic, tonic, as well as against stomach disorders, headache and common cold (Yöney et al., 2010; Karousou and Deirmentzoglou, 2011; Hanoğlu et al., 2020). Hanoğlu et al. (2016) reported the antimicrobial efficacy of essential oils derived by *S. cypria*, and suggested to be used as a new medicinal resource particularly against *C. albicans* and Gram-positive bacteria.

However, the secondary metabolites of the traditional infusion of this plant are still undershadowed. The literature survey revealed only two publications concerning the chemical characterization of wild harvested *S. cypria* of its essential oil (Hanoğlu et al., 2016) and extracts (Hanoğlu et al., 2020). Therefore, for the purpose of this study, we investigated and compared for the first time the chemical composition of the traditional infusions of different plant parts, leaves and flowers, of cultivated *S. cypria*. In parallel, the essential oils and the mineral, bioactive compounds, protein, and carbohydrate contents of the flowers and the leaves of cultivated populations have been studied.

MATERIALS AND METHODS

Plant Material

Sideritis cypria was collected from the Cypriot National Agricultural Department, on June 2019. Species seeds are kept at the Agricultural Research Institute, national gene bank (Accession number: ARI02415), collected in 2018 (Universal Transverse Mercator-UTM with latitude 546102; longitude 3904418, altitude 640). This is the first registered time that the plant has been cultivated, and plants from the first (mother) plantation were collected at their flower stage, for this analysis. Plantation was established at 2018, frequently irrigated (~ weekly/biweekly during irrigation period) and common fertilizers applied, with 20-10-10 once a year as basic fertilizer and 19-19-19 every second month as fertigation. Representative images of the different plant parts are presented in **Figure 1**.

Mineral Content and Nutritional Value

In the present study we use the word “flowers” in a wide sense which is usual in pharmacy. In this case we refer as "flowers" the whole inflorescence, including corollas, calyces, bracts and axis. Thus, we do not use “flowers” in its strict botanical sense. Aerial parts of the plants were separated into leaves and flowers (including corollas, calyces and bracts) and dried until constant weight at 65°C, using an air-drying oven. Dried tissue was then milled to pass a 0.42 sieve and ashed in a furnace (Carbolite, AAF 1100, GERO, Germany) at 450°C for 6 h. Nitrogen was determined using the Kjeldahl method (BUCHI, Digest automat K-439 and Distillation Kjelflex K-360, Switzerland). For the determination of minerals, tissue was acidified with 2 N hydrochloric acid (HCl). Phosphorus (P) was assayed with the molybdate/vanadate method while potassium (K), calcium (Ca), sodium (Na), magnesium (Mg), copper (Cu), zinc (Zn), and iron (Fe) were determined using an atomic absorption spectrophotometer (PG Instruments AA500FG, Leicestershire, UK) as described previously (Chrysargyris et al., 2019a). The nutritional value of leaves and

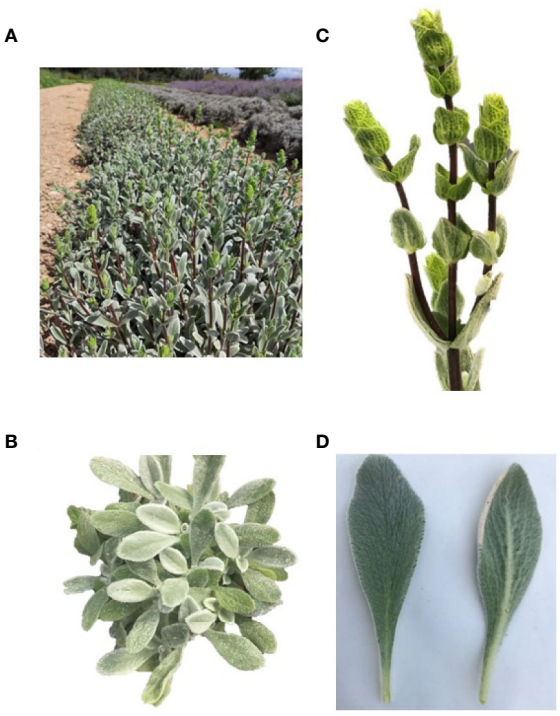

FIGURE 1 | *Sideritis cypria*, **(A)** cultivated crop, **(B)** plant, **(C)** flower, and **(D)** upper and bottom leaf area.

flowers was determined according to the methods described by AOAC (AOAC, 2016). The protein content (in dry weight; d.w.) was determined by using the Kjeldahl method (N × 6.25), petroleum ether Soxhlet extraction was used for total fat determination, and tissue incineration (600°C) for moisture. The total content of carbohydrates was determined by difference, and the energetic value was calculated using the following formula: Energy (kcal/100 g d.w.) = 4 × (g protein/100 g d.w. + g carbohydrate/100 g d.w.) + 9 × (g fat/100 g d.w.). Results were expressed in g/100 g d.w. Four replicates were analyzed for each plant part, while each replicate was a pool of three different plants (**Table 1**).

Bioactive Compounds of Plant Extracts

Both the infusion and the decoction of leaves and flowers were used for the estimation of the total phenolic and flavonoid content, as well as the evaluation of bioactive compounds of the plant (**Table 2**). The extracts were diluted with 50% methanol to reach a concentration of 2 mg/ml. The phenolic content was determined according to the Folin-Ciocalteu method. The reaction mixture consisted of 0.5 ml of each extract (2 mg/ml), 0.5 ml of Folin Ciocalteu reagent, and 5 ml of water. The reaction was incubated for 1 h before absorbance was measured at 765 nm (Goulas et al., 2014). Results were expressed as mg of gallic acids equivalents per g of dry material. Total flavonoids content was estimated as described by Chrysargyris et al. (2016), using the aluminum chloride colorimetric method, and results were expressed as mg of rutin equivalents per g of dried tissue.

Extracts bioactivity was evaluated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) and the ferric reducing antioxidant power (FRAP) assays. Serial dilutions of the extracts (0–1,000 μg/ml) were incubated with 0.3 mmol/L of DPPH for 30 min, and absorbance was measured at 517, as described by Goulas et al. (2014). The % of scavenging activity was determined using the formula: scavenging activity %=100-(Absorbance $_{samples}$-Absorbance $_{blank}$)100/Absorbance $_{control}$. IC50 values are the concentration of each extract that has the 50% of the antioxidant activity. The FRAP assay was also tested, as described by Chrysargyris et al. (2016), using serial dilutions of Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], as the reference standard. Results were expressed as mg Trolox per g of dried weight. All samples were tested in four replicates.

Essential Oils Extraction and Analysis

Aerial plant parts (leaves and flowers, separately) were placed in an air-drying oven at 42°C, for 48 h, until constant weight. Hydrodistillation was adopted for the extraction of the essential oils, using Clevenger apparatus. The duration of the extraction was 3 h for each sample, while four samples (pool of three plants per sample) were subjected for extraction and then for oil analysis. The essential oil yield was measured as μl of oil per 100 g of dried tissue.

The analysis of the essential oil components was carried out using a Shimadzu GC2010 gas chromatograph interfaced with a Shimadzu GC/MS QP2010plus mass spectrometer. A sample of each essential oil (1 μl) diluted in ethyl acetate (1:1,000) was injected in the autosampler, in split mode (1:20). The injector temperature was set at 230°C and the column temperature was programmed to rise from 60°C to 240°C at a rate of 5°C/min, with a 5 min hold at 240°C. Compound separation was performed on a Zebron ZB-5 (Phenomenex, USA) capillary column (0.25 μm x 30.0 m x 0.25 mm). Helium was used as the carrier gas with a flow set at 1.03 ml/min. Electron mass

TABLE 1 | Mineral analysis and nutritional value of aerial plant tissue, leaves, and flowers.

	Minerals			Nutritional Value	
	Flowers	**Leaves**		**Flowers**	**Leaves**
N (g/kg Dw)	14.43 ± 0.15a	12.91 ± 0.20b	Dry Matter (%)	28.75 ± 039a	29.19 ± 0.14a
K (g/kg Dw)	15.73 ± 0.40b	28.40 ± 0.14a	Moisture (%)	71.24 ± 0.39a	70.80 ± 0.14a
P (g/kg Dw)	4.00 ± 0.08a	3.49 ± 0.04b	Ash (%)	6.47 ± 0.06b	17.21 ± 0.20a
Mg (g/kg Dw)	2.54 ± 0.05a	2.04 ± 0.13b	Protein (%)	9.02 ± 0.09a	8.06 ± 0.12b
Ca (g/kg Dw)	14.65 ± 0.47b	20.13 ± 0.49a	Total Fats (%)	3.85 ± 0.06a	1.78 ± 0.87b
Na (g/kg Dw)	0.55 ± 0.01b	2.01 ± 0.14a	Carbohydrates (%)	80.65 ± 0.18a	72.90 ± 1.32b
Cu (mg/kg Dw)	64.95 ± 8.63b	113.09 ± 7.60a	Energy (kcal/100g Dw)	393.35 ± 0.29a	335.88 ± 18.11b
Zn (mg/kg Dw)	62.12 ± 3.77a	59.79 ± 1.67a			
Fe (mg/kg Dw)	153.77 ± 14.96b	398.87 ± 20.23a			

Data are presented as the mean of four replicates ± SE. Values in column for each extraction method followed by the same letter are not significantly different, P ≤ 0.05.

TABLE 2 | Total phenolics and flavonoids content and bioactive compounds of infusion and decoction of *S. cypria*.

		Total phenolics (mg GAE/g Dw)	**DPPH (IC50 mg/mL)**	**FRAP (mg TROLOX/g Dw)**	**Flavonoids (mg RUTIN/g Dw)**
Infusion	Leaves	3.022 ± 0.039b	792.992 ± 2.096a	75.926 ± 1.494b	7.721 ± 0.164b
	Flowers	8.019 ± 0.173a	267.891 ± 1.630b	199.374 ± 1.364a	32.004 ± 0.169a
Decoction	Leaves	1.396 ± 0.018b	605.536 ± 9.145a	98.418 ± 3.775b	3.604 ± 0.034b
	Flowers	3.972 ± 0.031a	404.995 ± 6.884b	159.729 ± 3.073a	9.961 ± 0.188a

Data are presented as the mean of four replicates ± SE. Values in column for each extraction method followed by the same letter are not significantly different, P ≤ 0.05.

spectra with ionization energy of 70 eV was recorded at 35–400 m/z. The identification of oil components (**Table 2**) was assigned by comparison of their retention indices relative to (C_8–C_{20}) n-alkanes with those of literature or with those of authentic compounds available in our laboratory, by matching their recorded mass spectra with those stored in the NIST08 mass spectral library of the GC–MS data system and other published mass spectra (Adams, 2012).

Isolation and Identification of Secondary Metabolites of Flowers and Leaves Infusions

General Experimental Procedures

1D- and 2D-NMR spectra were recorded in CD_3OD on Bruker DRX 400 instrument at 295 K. Chemical shifts are given in ppm (δ) and were referenced to the solvent signals at 3.31 and 49.0 ppm. COSY (COrrelation SpectroscopY), HSQC (Heteronuclear Single Quantum Correlation), HMBC (Heteronuclear Multiple Bond Correlation), and NOESY (Nuclear Overhauser Effect SpectroscopY) (mixing time 950 ms) experiments were performed using standard Bruker microprograms. The $[\alpha]_D$ values were obtained in CH_3OH on Perkin Elmer 341 Polarimeter. UV-Vis spectra were recorded on a Shimadzu UV-160A spectrophotometer, according to Mabry et al. (1970). Column chromatography (CC): silica gel (Merck, Art. 9385, Darmstadt, Germany), gradient elution with the solvent mixtures indicated in each case. Preparative-thin-layer chromatography (TLC) plates pre-coated with silica gel (Merck, Art. 5721). Fractionation was always monitored by TLC silica gel 60 F- 254, (Merck, Art. 5554) and cellulose (Merck, Art. 5552) with visualization under UV (254 and 365 nm) and spraying with vanillin-sulphuric acid reagent and with Neu's reagent (Neu, 1957) for phenolic compounds. In the whole process, all the obtained infusions, fractions, and isolated compounds were evaporated to dryness in vacuum under low temperature and then were put in activated desiccators with P_2O_5 until their weights had stabilized, in order to eliminate the moisture from the samples that might influence pre-saturation performance and then lead to an intense water signal in the ^{1}H-NMR spectra, making it difficult to observe near signals.

Fractionation and Isolation Procedure

Infusions of cultivated *S. cypria* Post were prepared based on the monograph of EMA, namely, 4.0 g of air-dried flowers and 4.0 g of air-dried leaves were separately dropped into 200 ml boiled distilled water each for 5 min, then were filtrated and concentrated to dryness. The infusion of flowers (1.1 g) was fractionated by CC over silica gel using as eluent mixtures of CH_2Cl_2:MeOH:H_2O 9.9:0.1:0.01-0:1:0 to yield finally 27 fractions (A-Z_1). Fractions J and K were combined (6.0 mg; eluted with CH_2Cl_2:MeOH:H_2O 8:2:0.2-7.8:2.2:0.2) and afforded compound **4** (4.1 mg). Fraction N (eluted with CH_2Cl_2:MeOH:H_2O 7.8:2.2:0.2) yielded compound **1** (3.3 mg). Fraction Q (10.2 mg; eluted with CH_2Cl_2:MeOH:H_2O 7.5:2.5:0.2) was further purified by preparative TLC plates pre-coated with silica gel and gave compounds **3** (1.7 mg) and **6** (3 mg). Combined fractions R, S, T, and U (108.0 mg; eluted with CH_2Cl_2:MeOH:H_2O 7.5:2.5:0.25-6.8:3.2:0.32) were further fractionated by CC over silica gel using as eluent mixtures of CH_2Cl_2:MeOH:H_2O 9.7:0.3:0.03-0:1:0 and afforded compound **1** (0.7 mg), a mixture of compounds **2** and **6** (0.7 mg), as well as compounds **5** (3.4 mg) and **8** in mixture with free sugars (5.2 mg). Compound **8** was further purified by preparative TLC plates pre-coated with silica gel and gave pure compound **8** (2.4 mg). Fraction Y (58.8 mg; eluted with CH_2Cl_2:MeOH:H_2O 6:4:0.4) was subjected to CC over Sephadex (MeOH:H_2O 100:0-90:10) and yielded compounds **9** (5.1 mg), **10** (15.3 mg) and **11** (1.6 mg).

The infusion of leaves (0.7 g) was fractionated by CC over silica gel using as eluent mixtures of CH_2Cl_2:MeOH:H_2O 9.9:0.1:0.01-0:1:0 to afford finally 23 fractions (A–W). Fraction I (0.9 mg; eluted with CH_2Cl_2:MeOH:H_2O 7.8:2.2:0.2) was identified as compound **1** (0.9 mg). Fractions K and M were eluted with CH_2Cl_2:MeOH:H_2O 7.5:2.5:0.2 and gave compounds **2** (0.7 mg) and **6** (1.2 mg), respectively. Fraction Q (56.3 mg; eluted with CH_2Cl_2:MeOH:H_2O 7:3:0.3-6.8:3.2:0.32) was applied to CC over silica gel using as eluent mixtures of CH_2Cl_2:MeOH:H_2O 9.9:0.1:0.01-0:1:0 and afforded compounds **5** (2.5 mg) and **8** (0.7 mg). Fraction S was eluted with CH_2Cl_2:MeOH:H_2O 6.5:3.5:0.35 and yielded compound **7** (9.0 mg). Fraction U (61.0 mg; eluted with CH_2Cl_2:MeOH:H_2O 6:4:0.4) was subjected to CC over Sephadex (MeOH:H_2O 100:0-90:10) and afforded compounds **9** (3.4 mg), **10** (14.8 mg) and **11** (1.4 mg).

RESULTS

Minerals, Nutritional Content, and Extracts Bioactivity

The mineral composition and the nutritional traits of both flowers and leaves of *S. cypria* plants are illustrated on **Table 1**. Flowers appeared to have significantly higher nutritional value than leaves, in terms of protein levels, total fats and carbohydrates, resulting in higher energy content. As for the minerals, flowers are richer in N, P, and Mg, while leaves have higher content of K, Ca, and Na. For the cases of K and Ca, concentration in leaves is almost two times higher than in flowers. Micronutrients as Fe and Cu were found in bigger amounts in leaves when compared to flowers but Zn remained at the same levels in leaves and flowers.

For the evaluation of the extract's bioactivity (**Table 2**), two different extracts were assayed; the infusion and the decoction of both flowers and leaves. In both cases of extracts, bioactive compounds and the correlated phenolic content appeared significantly higher for the flower extracts. In all cases, the infusion values were higher than the ones obtained from the decoction extracts. Total phenolic content in the infusion of flowers appeared more than double compared to the corresponding leaf extract. The same trend was revealed for all the bioactive compounds (DPPH and FRAP) assays tested, with the most remarkable differences to be evidenced for the total flavonoid content. Here flowers had more than 4 times higher values, in terms of rutin equivalents; 32.004 mg rutin per g of

dried tissue for the flowers while the leaf content was found at 7.721 mg rutin per g of dried tissue.

Essential Oils Yield and Compound Identification

The essential oil yield of the two plant parts tested in this study appeared significantly different. Flowers exhibited higher yield with 0.25% (± SE 0.029) while the essential oils of leaves were in relatively low level and did not exceed 0.03% (± SE 0.003). The GC/MS analysis that followed delivered 34 compounds in the leaves' oils and 31 compounds in the oils obtained from the flowers; 29 of these compounds appeared in the oils from both plant parts, but with great variability in terms of contribution to the oil profile (**Table 3**). The five and two compounds found only in leaves and flowers respectively, represent components with less than 0.350% (nonanal) each, out of the total chromatogram. In the oils from the leaves, the major terpenes were the monoterpenes hydrocarbons with 51.45%, followed by sesquiterpenes hydrocarbons (23.41%) and oxygenated sesquiterpenes (22.18%), while in flowers the total monoterpenes hydrocarbons reached 88.38%, and oxygenated compounds in total did not exceed 4.35% (1.34% and 2.99%, for mono- and sesquiterpenes, respectively). The major compounds identified in leaves were β-phellandrene (25.11%), β-caryophyllene (22.52%), α-pinene (11.92%), 14 hydroxy-β-caryophyllene (9.88%), and caryophyllene oxide (8.32%). In flowers, the dominant compound is α-pinene (37.97%), followed by β-phellandrene (25.81%), β-pinene (14.67%), β-caryophyllene (6.83%) and sabinene (5.58%). With the exception of β-phellandrene, the other major compounds differ significantly in terms of percentage (%) between the two tested oils. Comparing the total chromatographs of the two tested oils, the majority of the compounds (in terms of percentage participation), are significantly different, except eight compounds (**Table 3**). Considering the essential oil analysis in leaves and flowers separately, this is the first report of the analysis of the essential oils from *S. cypria* flowers.

TABLE 3 | Chemical composition (%) of essential oils of *Sideritis cypria* leaves and flowers.

Compound	RI[a]	Leaves	Flowers
α-Thujene	926	0.677 ± 0.016b	0.847 ± 0.002a
α-Pinene	933	11.924 ± 0.29b	37.97 ± 0.294a
Camphene	948	0.018 ± 0.000b	0.084 ± 0.001a
Sabinene	973	5.404 ± 0.090a	5.580 ± 0.017a
β-Pinene	977	4.979 ± 0.091b	14.671 ± 0.05a
β-Myrcene	989	0.773 ± 0.008b	1.016 ± 0.005a
α-Phellandrene	1004	1.891 ± 0.017a	1.577 ± 0.006b
a Terpinene	1017	0.061 ± 0.001a	0.110 ± 0.027a
o Cymene	1024	0.405 ± 0.004a	0.463 ± 0.026a
β-Phellandrene	1029	25.114 ± 0.325a	25.818 ± 0.056a
Eucalyptol	1031	0.017 ± 0.017a	0.042 ± 0.021a
Benzeneacetaldeyhe	1041	0.012 ± 0.006	–
γ-Terpinene	1058	0.153 ± 0.002a	0.205 ± 0.044a
Cis Sabinenehydrate	1066	0.095 ± 0.002a	0.083 ± 0.003b
1 octanol	1067	0.049 ± 0.003	–
Terpinolene	1089	0.053 ± 0.001a	0.040 ± 0.003b
trans Sabinenehydrate	1100	0.030 ± 0.001a	0.037 ± 0.005a
Nonanal	1104	0.350 ± 0.002	–
α-Campholenal	1127	0.021 ± 0.004b	0.081 ± 0.003a
trans Pinocarveol	1139	0.015 ± 0.000b	0.114 ± 0.006a
trans Verbenol	1143	0.028 ± 0.002b	0.142 ± 0.007a
Pinocarvone	1163	0.012 ± 0.006b	0.075 ± 0.003a
Terpinen-4-ol	1178	0.130 ± 0.003a	0.140 ± 0.006a
Cryptone	1187	0.160 ± 0.002a	0.069 ± 0.004b
α-Terpineol	1191	0.454 ± 0.008b	0.500 ± 0.008a
Myrtenol	1195	–	0.074 ± 0.005
Cumin aldehyde	1241	0.175 ± 0.002a	0.023 ± 0.001b
Carvone	1244	0.138 ± 0.053	–
Thymol	1299	–	0.034 ± 0.018
β-Caryophyllene	1425	22.525 ± 0.026a	6.836 ± 0.056b
α-Humulene	1462	0.890 ± 0.011a	0.176 ± 0.011b
Caryophyllene oxide	1587	8.325 ± 0.149a	1.498 ± 0.036b
Humulene epoxide	1607	0.177 ± 0.010	–
Camphoric Acid	1636	0.219 ± 0.023a	0.063 ± 0.008b
14 Hydroxy-β-caryophyllene	1666	9.884 ± 0.327a	1.388 ± 0.069b
14 Hydroxy-α-humulene	1707	3.803 ± 0.173a	0.108 ± 0.056b
Total Identified		98.955 ± 0.080	99.864 ± 0.024
Monoterpenes hydrocarbons		51.452 ± 0.836b	88.382 ± 0.240a
Sesquiterpenes hydrocarbons		23.415 ± 0.028a	7.012 ± 0.063b
Oxygenated monoterpenes		1.109 ± 0.048b	1.344 ± 0.043a
Oxygenated sesquiterpenes		22.188 ± 0.658a	2.995 ± 0.158b
Others		0.791 ± 0.027a	0.131 ± 0.010b

[a]*Components listed in order of elution from a Zebron ZB-5 capillary column*

[b]*values (n=3) in rows followed by the same letter are not significantly different, P ≤ 0.05.*

Identification of Non Volatile Secondary Metabolites

The infusion of the flowers of cultivated *S. cypria* yielded four flavones, isoscutellarein-7-O-[6′′-O-acetyl-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside (**1**) (Lenherr and Mabry, 1987), 4′-O-methyl-isoscutellarein-7-O-[6′′-O-acetyl-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside (**2**) (Venditti et al., 2014), isoscutellarein-7-O-[6′′-O-acetyl-β-D-allopyranosyl-(1→2)]-6′′-O-acetyl-β-D-glucopyranoside (**3**) (Halfon et al., 2013), and 4′-O-methyl-hypolaetin-7-O-[6′′-O-acetyl-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside (**4**) (Halfon et al., 2013); four phenylethanoid glucosides, acteoside (**5**) (Kawada et al., 1999), leucosceptoside A (**6**) (Miyase et al., 1982), leonoside A (**8**) (Çaliş et al., 1992), and lamalboside (**9**) (Budzianowski and Skrzypczak, 1995); one iridoid, melittoside (**10**) (Śawiątek et al., 1981), and one quinic acid derivative, chlorogenic acid (**11**) (Armata et al., 2008).

Moreover, the infusion of the leaves of the same population afforded two flavones, isoscutellarein-7-O-[6′′-O-acetyl-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside (**1**) and 4′-O-methyl-isoscutellarein-7-O-[6′′-O-acetyl-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside (**2**); five phenylethanoid glucosides, namely the four previously found in the infusion of flowers and lavandulifolioside (**7**) (Başaran et al., 1988), moreover the same iridoid and quinic acid derivative have been isolated (**Figure 2**).

Leonoside A (**8**) was first isolated from *Stachys sieboldii* in 1991 (Nishimura et al., 1991) as stachysoside B. Later, Çaliş et al. (1992) isolated it from *Leonurus glaucescens* and it was given the name "leonoside A". From the genus *Sideritis* L., it has been previously isolated only from *S. trojana* Bornm. (Kirmizibekmez

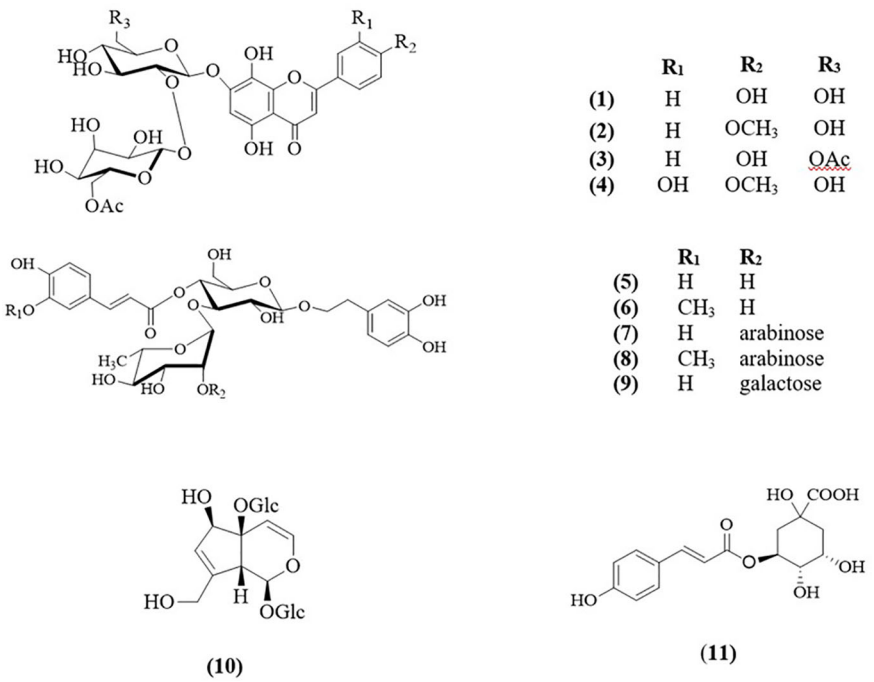

FIGURE 2 | Structures of isolated compounds **1–11**.

et al., 2012). Our study confirms the presence of leonoside A in *S. cypria* (Hanoğlu et al., 2020) and we report for the first time the isolation of this compound from the infusion of both, leaves and flowers. It is interesting to point out that compounds **2**- **4**, **9** - **11** had been mentioned previously in the genus *Sideritis* L., but not in *S. cypria*. Thus, the aforementioned compounds are reported as components of *S. cypria* for the first time. The structures of the isolated compounds were elucidated by spectroscopic data. Intriguingly, the NMR data of compounds **8**, **9,** and **10** are not fully recorded in the literature, therefore, herein, are presented (**Tables S1**–**S3**).

It is important to mention that the chemical characterization of the total infusions and the sub fractions were monitored by NMR techniques, which enabled us to identify and isolate all the main and the minor components.

DISCUSSION

The mineral content of *S. cypria* leaves differs from that of the flowers. As it was mentioned previously, leaves are richer in potassium and calcium while flowers in magnesium and phosphorus, and this is of great importance as reported by Bojovic et al. (2011), as water tea-infusions had large portions of K, P, Na, and Cu, and different parts of the plants can contribute in various ways on human nutrition. Comparing to other species of the genus, *S. cypria* leaves have similar levels of potassium (28.40 g/kg) with the leaves of *S. perfoliata*, ranging from 20.65 to 29.84 g/kg, depending on the cultivation method (Chrysargyris et al., 2019b). *S. cypria* has almost two times higher content of phosphorus and calcium, in both plant parts, than *S. perfoliata* (Chrysargyris et al., 2019b; Lall et al., 2019). Species *S. scardica* and *S. raeseri* appear poorer in terms of mineral content, and demonstrate almost half the quantities of the majority of nutrients as K, P, Ca, Mg (Karapandzova et al., 2013), compared to *S. cypria*. Trikka et al. (2019) evaluated different species of *Sideritis* genus from different geographical regions of Greece and plants exhibited high variability, even within species (*S. perfoliata, S. scarica, S. raeseri*), and had lower contents in minerals than *S. cypria*. These variations may be the effect of the cultivation practices applied or/and the harvesting periods (Chrysargyris et al., 2019b).

Regarding the nutritional value, the flowers of *S. cypria* are richer in protein, fats, and carbohydrates than leaves. *S. perfoliata* aerial parts (leaves) demonstrate protein content up to 14.64%, while in *S. cypria* the corresponding content was 8.06% and 9.02% for leaves and flowers. Total fats and carbohydrates demonstrated almost similar values for *S. cypria* (1.78% and 72.90%) and *S. perfoliata* (1.76% and 73.53%). A research conducted to investigate the chemical profile and metabolite content of *S. italica,* revealed that the protein content of the plant parts were 6% and 10% for the leaves and flowers, respectively (Menghini et al., 2014), values similar to *S. cypria* protein content. Generally, in the Lamiaceae family, protein content may vary among different species of the same genus. As it was assayed by Tunçtürk et al. (2017), *Thymus* species had protein

content varying from 6.85-14.34%, while *Mentha* species as *M. longifolia* and *M. spicata* demonstrated protein content of 13.86% and 15.13%, respectively.

The bioactive substances of the two types of aqueous extracts for both plant parts demonstrated the same trend; flowers are richer in compounds as phenolics and flavonoids, and thus, exhibiting higher bioactivity (Phatak and Hendre, 2014). Additionally, the infusions demonstrated higher bioactivity and appear to be richer than decoctions, due to the higher polarity of the solvent used (Martins et al., 2015). In general, plant's extracts appear to be less strong compared to other species of genus. *S. syriaca*'s decoction demonstrated total phenolic compounds almost 18 mg GAE/g Dw (Goulas et al., 2014), while *S. cypria* decoction ranged between 1.39 and 3.92 mg GAE/g Dw (for leaves and flowers, respectively). Nevertheless, plant species, cultivation practices, environmental conditions, and extraction methodology are parameters, to name a few, that affect the bioactive status of plants (González-Burgos et al., 2011; Chrysargyris et al., 2019b).

According to literature, *Sideritis* genus species yield low essential oil quantities, compared to other Lamiaceae species (González-Burgos et al., 2011). There are reports that indicate this yield variation among species, that range from traces (< 0.01%) of essential oil in many *Sideritis* species (Kirimer et al., 2004; Bojovic et al., 2011) to 0.94% (*S. lanata)* (Koutsaviti et al., 2013). *S. cypria* follows the same trend, with the flower's essential oil yield to be significantly higher (0.25%) than leaves' yield, which was measured at considerably low numbers of 0.03%. Hanoğlu et al. (2016; 2020) recorded a yield of 0.49%, when hydrodistilled *S. cypria* plants, during or post-flowering that were collected from mountains in north Cyprus (500–750 m altitude). This variation with the present results might be attributed that wild harvested plant tissue in mountain areas (Hanoğlu et al., 2016; Hanoğlu et al., 2020) can differ on the essential oil yield and aromatic profile, compared with cultivated crop in lowland.

As for the compounds, of the two oils tested (leaves and flowers), analysis showed remarkable differences in composition. The major compounds of leaves are β-phellandrene (25.11%) and β-caryophyllene (22.52%), followed by α-pinene (11.92%), 14 hydroxy-β-caryophyllene (9.88%), and caryophyllene oxide (8.32%). All these components were identified as well by previous studies of the plant during flowering phase, but with different percentages, while β-phellanderene was identified again as the major compound (17.83%) (Hanoğlu et al., 2020). The same authors referenced their previous work (Hanoğlu et al., 2016) mentioning that when oils from post flowering stage were analyzed, the compounds and their amounts varied, compared to those during flowering. Plants evaluated in this study were collected during flowering stage, and leaves' oil profile is similar to the report of Hanoğlu et al. (2020), but with differences mostly in secondary compounds, in terms of their participation in the oil profile. These differences can be ascribed to the separate analysis of leaves and flowers, and to the differences that occur due to plant's origin and cultivation practices. Hanoğlu et al. (2020) collected plants from the mountains of north Cyprus, while plants tested in this study are plants from the first official cultivation reported in Cyprus for *S. cypria*. That means that plants are subjected to specific irrigation regimes and cultivation practices, while plants collected from wild exhibit great variability. Todorova and Trendafilova (2014) reviewed this variation in other *Sideritis* species oils as well, mentioning region as a factor of this variation. Chrysargyris et al. (2019b) also reported that cultivation practices and harvesting periods affect components of *Sideritis* species oils.

To our knowledge, this is the first report of *S. cypria* oils analysis that separates oils from different plant parts, and is the first analysis of the flower oil. The major compounds found in flower oil are α-pinene (37.97%), β-phellandrene (25.81%), β-pinene (14.67%), and β-caryophyllene (8.83%). These differences between flowers and leaves, concern not only single components, but can be examined as differences in the grouped compounds, as leaves appear richer in sesquiterpenes hydrocarbons (23.41%) and oxygenated sesquiterpenes (22.18%), while in flower the corresponding percentages are together almost 10% (7.02% and 2.99%, respectively). This is of great importance, as consumer can use either leaves or flowers or mixture of them during their usage of *S. cypria*, reflecting different aromatic profile and properties. Bojovic et al. (2011) reviewed the phytochemical profile of *Sideritis* species, indicating that essential oils are characterised by high contents of monoterpene hydrocarbons with α-pinene, β-pinene, sabinene, myrcene or limonene and of sesquiterpene hydrocarbons, particularly δ-cadinene and β-caryophyllene, as the main compounds. However, *S. cypria* is not on that extensive review list of species, highlighting the importance of the present study.

Monoterpenes such as pinenes and phellandrenes, among others are the main components of the odour of medicinal plants with biocidal activities. For example, β-phellandrene has been tested as a strong antimicrobial terpene while it exhibits insect repellent activities (Harrewijn et al., 2001). It has shown *in vitro* activity against *Bacillus* sp.*, Candida albicans, Escherichia coli, Pseudomonas aeruginosa, and S. aureus* (Zhang et al., 2017). In plants, pinenes show fungicidal activity and have been used for centuries to produce flavors and fragrances, while the antimicrobial activity of some essential oils is attributed to the presence of α- and/or β-pinene (Rivas da Silva et al., 2012). α-Pinene is a monoterpene and member of hydrocarbon group of bicyclic terpenes that has not only a wide range of uses as a food additive (Yang et al., 2016), but also exhibits great biological activities as insecticidal, spasmolytic, anti-listerial, and anticholinesterase (Orhan et al., 2006). Additionally, Akutsu et al. (2002), found that α-pinene has a potential anti-stress activity. Moreover, β-pinene has also exhibited antidepressant activity, and furthermore it's mode of action could be connected to the mechanism of the action of the most frequently used antidepressant drugs (Guzman-Guttierez et al., 2015). Orhan et al. (2006) also reported the anti-inflammatory activity of the compound, while reviewing the synergistic effect of α-pinene with β-caryophyllene, as anti- inflammatory compounds. Interestingly, β-caryophyllene alone is a compound which has been demonstrated to possess a great potential application for

various pathological conditions, as central nervous system diseases (Parkinson's disease, Alzheimer's disease), osteoporosis, cancer, and antibacterial (Francomano et al., 2019). Importantly, it is also approved as food additive, taste enhancer, and flavoring agent by U.S. Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) (Fidyt et al., 2016; Machado et al., 2018).

Continuing our study on *Sideritis* genus, this work reports for the first time the composition of the traditional infusions of *S. cypria* Post. The genus *Sideritis* L. is characterized by the presence of several different secondary metabolites, mainly terpenes and various phenolic derivatives. In the present study, overall, eleven compounds were isolated from both infusions of the flowers and the leaves of cultivated *S. cypria*. It is interesting to note that the two plant parts showed similar chemical composition. Precisely, from the sample of the flowers were isolated more flavones, compared to the sample of the leaves in which were identified more phenylethanoid glucosides.

Numerous studies over the last years have revealed the presence of a great number of glycosides of 8-hydroxy flavones in *Sideritis* species, such as acetylated or non-acetylated 7-O-allosyl (1→2)glucosides of isoscutellarein (8-hydroxy apigenin) and hypolaetin (8-hydroxy luteolin), as well as their 4′-methoxy derivatives (EMA, 2015; Stanoeva et al., 2015). In the current study, three isoscutellarein and one hypolaetin derivatives were found. The results are in agreement with previously well studied *Sideritis* species belonging to the section *Empedoclia*, i.e., *S. scardica* (Todorova and Trendafilova, 2014; EMA, 2015), *S. perfoliata* L. subsp. *perfoliata* (Charami et al., 2008; Chrysargyris et al., 2019b), *S. euboea* (Tomou et al., 2019), *S. raeseri* (Gabrieli et al., 2005; Romanucci et al., 2017), and *S. syriaca* (Plioukas et al., 2010; Goulas et al., 2014).

Hanoğlu et al. (2020) studied the chemical composition of non polar and polar extracts of *S. cypria* wild populations and reported the presence of four flavones, four phenylethanoid glucosides and one iridoid glucoside in the polar extracts. Comparing our results to the literature data, we confirm the presence of the same phenylethanoid glucosides, with the only difference the isolation of lamalboside in both infusions. It is noteworthy that lamalboside has been isolated previously only from two *Sideritis* species belonging to the *Empedoclia* section; *S. germanicopolitana* Bornm. and *S. trojana* Bornm. (Kirmizibekmez et al., 2012; Kirmizibekmez et al., 2019). Lamalboside, also known as lamiuside A, was first isolated and described from the genus *Lamium* L. (Budzianowski and Skrzypczak, 1995; Ito et al., 2006; Yalçın et al., 2007). Furthermore, considering the identification of the flavonoid derivatives, the two infusions were characterized by isoscutellarein derivatives. However, Hanoğlu et al. (2020) reported the isolation of four flavones; one isoscutellarein derivative and three apigenin derivatives. This distinguishment could be attributed not only in the fact that our plant material is originated from cultivation, but also to the different polarity of the investigated samples between the two studies. Importantly, our research also revealed the presence of chlorogenic acid, which has been previously found in many *Sideritis* spp. (Armata et al., 2008; Samanidou et al., 2012; Goulas et al., 2014; Irakli et al., 2018; Chrysargyris et al., 2019b; Tomou et al., 2019). It is interesting to note that in both infusions the main iridoid derivative was melittoside. In the specific section (*Empedoclia*), melittoside have been reported in the species *S. euboea* Heldr. (Tomou et al., 2019), *S. montana* L. (Koleva et al., 2003; Fraga, 2012), *S. montana* L. subsp. *montana* (Venditti et al., 2016), *S. germanicopolitana* Bornm (Kirmizibekmez et al., 2019), *S. perfoliata* L. subsp. *perfoliata* (Chrysargyris et al., 2019b), and *S. syriaca* L. (Koleva et al., 2003; Fraga, 2012). Acteoside and leucosceptoside A, found in our study, have been also isolated from *S. euboea* Heldr. (Tomou et al., 2019), *S. perfoliata* L. subsp. *perfoliata* (Charami et al., 2008; Chrysargyris et al., 2019b), *S. scardica* Gris. (Fraga, 2012; Todorova and Trendafilova, 2014), *S. lysia* Boiss et. Heldr. (Fraga, 2012), and *S. raeseri* Boiss et. Heldr. (Petreska et al., 2011), while lavandulifolioside has been isolated from *S. euboea* Heldr. (Tomou et al., 2019), *S. perfoliata* L. subsp. *perfoliata* (Charami et al., 2008; Chrysargyris et al., 2019b), and *S. lysia* Boiss et. Heldr (Fraga, 2012). Moreover, a survey of four infusions of cultivated *S. raeseri* subsp. *raeseri* was carried out and underlay the great antioxidant activity due to the high phenolic content, including chlorogenic acid, phenylethanoid glucosides and flavonoid derivatives (Pljevljakušić et al., 2011).

Chrysargyris and co-workers (2019b) have revealed the presence of three isoscutellarein derivatives, two phenylethanoids, one quinic acid derivative and six iridoids from the infusion of the cultivated *S. perfoliata* L. subsp. *perfoliata* from Cyprus. Of note, our results are in accordance with this study. It is interesting to point out that cultivated *S. cypria* is very poor in iridoids compared to cultivated *S. perfoliata* L. subsp. *perfoliata*, while on the other hand *S. cypria* consists more phenylethanoid glucosides. In the context of the presence of phenylethanoid glucosides, bearing three sugar moieties, are common group of secondary metabolites of this genus, but the trisaccharide derivatives with galactose as one of the sugars are rare.

Several studies have mentioned the antioxidant activity of isoscutellarein and hypolaetin derivatives (Gabrieli et al., 2005; Armata et al., 2008; Charami et al., 2008; Kirmizibekmez et al., 2012). It is well established that flavonoids with at least one hydroxyl-group in B ring exhibit high antioxidant activity (Charami et al., 2008; Sarian et al., 2017). However, the existence of a hydroxyl group in ring A and catechol structure or 4′-hydroxyl group in ring B improve more the antioxidant activity (Charami et al., 2008; Sarian et al., 2017). Furthermore, phenylethanoid glucosides are known for various pharmacological activities (Fu et al., 2008). Precisely, Charami et al. (2008) reported the antioxidant and anti-inflammatory activity of some isolated phenylethanoid glucosides and flavones derivatives from the species *S. perfoliata* subsp. *perfoliata*. Phenylethanoid glucosides exhibited better anti-inflammatory activity, compared to flavonoids due to their structures (caffeoyl group and to their *o*-dihydroxyphenyl group) (Charami et al., 2008). Moreover, a survey of four infusions of cultivated *S. raeseri* subsp. *raeseri* was carried out and underlay the great antioxidant activity due to the high phenolic content, including chlorogenic acid, phenylethanoid glucosides, and flavonoid derivatives

(Pljevljakušić et al., 2011). Therefore, we could assume that the presence of flavones derivatives in combination with the abundance of phenylethanoid glucosides and chlorogenic acid in our samples could enhance the pharmacological activity, as well as it could justify the ethnopharmacological uses of *S. cypria*.

The present study revealed the chemical variation of the flowers and leaves essential oils (EOs) in comparison to the wild population of *S. cypria* previously investigated (Hanoğlu et al., 2016). The main constituents of the aerial parts of wild *S. cypria* EO were epi-cubebol (11.9%), *trans*-piperitol (8.9%), α-pinene (4.3%), and β-pinene (3.6%), while the main constituents of the flowers and leaves EOs were α-/β-pinenes in significant higher concentration, sabinene, β-phellandrene (ca. 25% in both EOs), β-caryophyllene, caryophyllene oxide, and 14-hydroxy-β-caryophyllene. In accordance with the EOs of Greek *Sideritis* species, where monoterpenes are the dominant components (Aligiannis et al., 2001; González-Burgos et al., 2011; Kloukina et al., 2019), the present samples are also rich in monoterpene hydrocarbons followed by sesquiterpenes. We assume that different factors (altitude, cultivation methods, and distillation methods) play an important role on the observed chemical variations.

CONCLUSION

The present study illustrates the phytochemical investigation of leaves and flowers of cultivated *S. cypria*, is a very rare and vulnerable species endemic to Cyprus with a small distribution area restricted to the Pentadaktylos Range (Tsintides et al., 2007; Bilz, 2011). The present study reveals that the cultivation of *S. cypria* is feasible without affecting its chemical profile. Cyprus ironwort has a chemical profile rich in bioactive secondary metabolites and can be used as culinary herb, but the plant material sold in local markets should not be collected from the field and should come from crops. *S. cypria* has a high phosphorus and calcium levels in both leaves and flowers. The nutritional value is mainly oriented on flowers which are richer in protein, fats, and carbohydrates than leaves. Flowers exhibited higher essential oil yield comparing to the leaves. The five major constituents identified in leaves were β-phellandrene, β-caryophyllene, α-pinene, 14 hydroxy-β-caryophyllene, and caryophyllene oxide, while in flowers the dominant compound was α-pinene, β-phellandrene, β-pinene, β-caryophyllene, and sabinene. Moreover, the infusion of the flowers was more abundant in flavones, though the infusion of the leaves was mainly consisted by phenylethanoid glucosides, based on the isolated compounds and NMR data.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

KL carried out the chemical analyses of the infusions. E-MT contributed to the chemical analyses of the infusions and to the writing of the article. HS supervised the chemical analyses of the infusions and contributed to the writing of the article. AC and CD carried out the analyses of EOs, of mineral and nutritional contents of the leaves and flowers infusions/decoctions. NT supervised the latter analyses and contributed to the writing of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00650/full#supplementary-material

REFERENCES

Çaliş, İ., Ersöz, T., Taşdemir, D., and Rüedi, P. (1992). Two phenylpropanoid glycosides from *Leonurus glaucescens*. *Phytochemistry* 31, 357–359. doi: 10.1016/0031-9422(91)83078-Y

Śawiątek, L., Lehmann, D., Chaudhuri, R. K., and Sticher, O. (1981). Occurrence of melittoside in the seeds of *Plantago media*. *Phytochemistry* 20, 2023–2024. doi: 10.1016/0031-9422(81)84058-7

Adams, R. P. (2012). *Identification of essential oil components by gas chromatography and mass spectroscopy. 4rth edition* (Carol Stream, IL, USA: Allured Publishing Corp.).

Akutsu, H., Kikusuia, T., Takeuchia, Y., Sanob, K., Hatanakac, A., and Moria, Y. (2002). Alleviating effects of plant-derived fragrances on stress-induced hyperthermia in rats. *Physiol. Behav.* 75, 355–360. doi: 10.1016/S0031-9384(01)00670-9

Aligiannis, N., Kalpoutzakis, E., Chinou, I. B., Mitakou, S., Gikas, E., and Tsarbopoulos, A. (2001). Composition and antimicrobial activity of the essential oils of five taxa of *Sideritis* from Greece. *J. Agr. Food Chem.* 49, 811–815. doi: 10.1021/jf001018w

Aneva, I., Zhelev, P., Kozuharova, E., Danova, K., Nabavi, S. F., and Behzad, S. (2019). Genus *Sideritis*, section *Empedoclia* in southeastern Europe and Turkey – studies in ethnopharmacology and recent progress of biological activities. *DARU J. Pharm. Sci.* 27, 407–421. doi: 10.1007/s40199-019-00261-8

AOAC (2016). "Official methods of analysis of AOAC International," in *Official Methods of Analysis of AOAC International*, *20th ed.*). Eds. W. Horwitz and G. Latimer (Gaithersburg: MD: AOAC International).

Armata, M., Gabrieli, C., Termentzi, A., Zervou, M., and Kokkalou, E. (2008). Constituents of *Sideritis syriaca* ssp. *syriaca* (Lamiaceae) and their antioxidant activity. *Food Chem.* 111, 179–186. doi: 10.1016/j.foodchem.2008.03.061

Başaran, A. A., Çaliş, İ., Anklin, C., Nishibe, S., and Sticher, O. (1988). Lavandulifolioside: A New Phenylpropanoid Glycoside from *Stachys lavandulifolia*. *HCA* 71, 1483–1490. doi: 10.1002/hlca.19880710612

Bilz, M. (2011). The IUCN Red List of Threatened Species 2011. *Sideritis Cypria*, e.T162296A5570289. doi: 10.2305/IUCN.UK.2011-1.RLTS.T162296A5570289.en

Bojovic, D., Jankovic, S., Potpara, Z., and Tadic, V. (2011). Summary of the phytochemical research performed to date on Sideritis species. *Ser. J. Exp. Clin. Res.* 12, 109–122. doi: 10.5937/sjecr1103109B

Budzianowski, J., and Skrzypczak, L. (1995). Phenylpropanoid esters from *Lamium album* flowers. *Phytochemistry* 38, 997–1001. doi: 10.1016/0031-9422(94)00727-B

Charami, M.-T., Lazari, D., Karioti, A., Skaltsa, H., Hadjipavlou-Litina, D., and Souleles, C. (2008). Antioxidant and antiinflammatory activities of *Sideritis perfoliata* subsp. *perfoliata* (Lamiaceae). *Phytother. Res.* 22, 450–454. doi: 10.1002/ptr.2333

Chrysargyris, A., Panayiotou, C., and Tzortzakis, N. (2016). Nitrogen and phosphorus levels affected plant growth, essential oil composition and antioxidant status of lavender plant (*Lavandula angustifolia* Mill.). *Ind. Crop Prod.* 83, 577–586. doi: 10.1016/j.indcrop.2015.12.067

Chrysargyris, A., Antoniou, O., Athinodorou, F., Vassiliou, R., Papadaki, A., and Tzortzakis, N. (2019a). Deployment of olive-stone waste as a substitute growing medium component for *Brassica* seedlings production in nurseries. *Environ. Sci. Pollut. Res.* 26, 35461–35472. doi: 10.1007/s11356-019-04261-8

Chrysargyris, A., Kloukina, C., Vassiliou, R., Tomou, E.-M., Skaltsa, H., and Tzortzakis, N. (2019b). Cultivation strategy to improve chemical profile and anti-oxidant activity of *Sideritis perfoliata* L. subsp. *perfoliata*. *Ind. Crop Prod.* 140, 111694. doi: 10.1016/j.indcrop.2019.111694

Deveci, E., Tel-Çayan, G., Yıldırım, H., and Duru, M. E. (2017). Chemical composition, antioxidant, anticholinesterase and anti-urease activities of *Sideritis pisidica* Boiss. & Heldr. endemic to Turkey. *Marmara Pharm. J.* 21, 898–905. doi: 10.12991/mpj.2017.13

EMA and /HMPC/39455/ (2015). Assessment report on *Sideritis scardica* Griseb.; *Sideritis clandestina* (Bory & Chaub.) Hayek; *Sideritis raeseri* Boiss. & Heldr.; *Sideritis syriaca* L., herba.

Fidyt, K., Fiedorowicz, A., Strządała, L., and Szumny, A. (2016). β-caryophyllene and β-caryophyllene oxide-natural compounds of anticancer and analgesic properties. *Cancer Med.* 5 (10), 3007–3017. doi: 10.1002/cam4.816

Fraga, B. M. (2012). Phytochemistry and chemotaxonomy of *Sideritis* species from the Mediterranean region. *Phytochemistry* 76, 7–24. doi: 10.1016/j.phytochem.2012.01.018

Francomano, F., Caruso, A., Barbarossa, A., Fazio, A., LaTorre, C., Ceramella, J., et al. (2019). β-Caryophyllene: A Sesquiterpene with Countless Biological Properties. *Appl. Sci.* 9, 5420. doi: 10.3390/app9245420

Fu, G., Pang, H., and Wong, Y. H. (2008). Naturally Occurring Phenylethanoid Glycosides: Potential Leads for New Therapeutics. *Curr. Med. Chem.* 15 (25), 2592–2613. doi: 10.2174/092986708785908996

Gabrieli, C. N., Kefalas, P. G., and Kokkalou, E. L. (2005). Antioxidant activity of flavonoids from *Sideritis raeseri*. *J. Ethnopharmacol.* 96, 423–428. doi: 10.1016/j.jep.2004.09.031

González-Burgos, E., Carretero, M. E., and Gómez-Serranillos, M. P. (2011). *Sideritis* spp.: uses, chemical composition and pharmacological activities-a review. *J. Ethnopharmacol.* 135, 209–225. doi: 10.1016/j.jep.2011.03.014

Goulas, V., Exarchou, V., Kanetis, L., and Gerothanassis, I. P. (2014). Evaluation of the phytochemical content, antioxidant activity and antimicrobial properties of mountain tea (*Sideritis syriaca*) decoction. *J. Funct. Foods* 6, 248–258. doi: 10.1016/j.jff.2013.10.014

Guzman-Guttierez, S. L., Bonilla-Jaime, H., Gomez-Cansino, R., and Reyes-Chilpa, R. (2015). Linalool and β-pinene exert their antidepressant-like activity through the monoaminergic pathway. *Life Sci.* 128, 24–29. doi: 10.1016/j.lfs.2015.02.021

Halfon, B., Çiftçi, E., and Topçu, G. (2013). Flavonoid constituents of *Sideritis caesarea*. *Turk. J. Chem.* 37, 464–472. doi: 10.3906/kim-1206-45

Hanoğlu, D. Y., Hanoğlu, A., Güvenir, M., Süer, K., Demirci, B., Başer, K. H. C., et al. (2016). Chemical composition and antimicrobial activity of the essential oil of *Sideritis cypria* Post endemic in Northern Cyprus. *J. Essent. Oil Res.* 29, 228–232. doi: 10.1080/10412905.2016.1251503

Hanoğlu, D. Y., Hanoğlu, A., Yusufoğlu, H., Demirci, B., Başer, K. H. C., Çalış, İ., et al. (2020). Phytochemical Investigation of Endemic *Sideritis cypria* Post. *Rec. Nat. Prod.* 14, 105–115. doi: 10.25135/rnp.140.18.11.1079

Harrewijn, P., Van Oosten, A. M., and Piron, P. G. M. (2001). "Natural Terpenoids as Messengers," in *A multidisciplinary study of their production, biological functions and practical applications* (Dordrecht: Kluwer Academic Publishers), 440.

Hofrichter, J., Krohn, M., Schumacher, T., Lange, C., Feistel, B., Walbroel, B., et al. (2016). *Sideritis* spp. Extracts Enhance Memory and Learning in Alzheimer's β-Amyloidosis Mouse Models and Aged C57Bl/6 Mice. *J. Alzheimers Dis.* 53, 967–980. doi: 10.3233/JAD-160301

Irakli, M., Tsifodimou, K., Sarrou, E., and Chatzopoulou, P. (2018). Optimization infusions conditions for improving phenolic content and antioxidant activity in *Sideritis scardica* tea using response surface methodology. *J. Appl. Res. Med. Aromat. Plants* 8, 67–74. doi: 10.1016/j.jarmap.2017.12.001

Ito, N., Nihei, T., Kakuda, R., Yaoita, Y., and Kikuchi, M. (2006). Five new Phenylethanoid Glycosides from the whole plants of *Lamium purpureum* L. *Chem. Pharm. Bull.* 54, 1705–1708. doi: 10.1248/cpb.54.1705

Karapandzova, M., Qazimia, B., Stefkova, G., Bačevac, K., Stafilovc, T., Kadifkova Panovskab, T., et al. (2013). Chemical characterization, mineral content and radical scavenging activity of *Sideritis scardica* and *S. raeseri* from R. Macedonia, and R. Albania. *Nat. Prod. Commun.* 8 (5), 639–644.

Karousou, R., and Deirmentzoglou, S. (2011). The herbal market of Cyprus: Traditional links and cultural exchanges. *J. Ethnopharmacol.* 133, 191–203. doi: 10.1016/j.jep.2010.09.034

Kawada, T., Asano, R., Hayashida, S., and Sakuno, T. (1999). Total synthesis of the Phenylpropanoid Glycoside, Acteoside. *J. Org. Chem.* 64, 9268–9271. doi: 10.1021/jo9906983

Kirimer, N., Baser, K. H. C., Demirci, B., and Duman, H. (2004). Essential oils of *Sideritis* species of Turkey belonging to the section *Empedoclia*. *Chem. Nat. Compd.* 40, 19–23. doi: 10.1023/B:CONC.0000025458.00475.cf

Kirmizibekmez, H., Ariburnu, E., Masullo, M., Festa, M., Capasso, A., Yesilada, E., et al. (2012). Iridoid, phenylethanoid and flavonoid glycosides from *Sideritis trojana*. *Fitoterapia* 83, 130–136. doi: 10.1016/j.fitote.2011.10.003

Kirmizibekmez, H., Erdoğan, M., Kúsz, N., Karaca, N., Erdem, U., Demirci, F., et al. (2019). Secondary metabolites from the aerial parts of *Sideritis germanicopolitana* and their *in vitro* enzyme inhibitory activities. *Nat. Prod. Res.*, 1–4. doi: 10.1080/14786419.2019.1586700

Kloukina, C., Tomou, E.-M., and Skaltsa, H. (2019). Essential oil composition of two Greek cultivated. *Sideritis Spp. Nat. Volatiles Essent. Oils* 6 (3), 16–23.

Koleva, I. I., Linssen, J. P., van Beek, T. A., Evstatieva, L. N., Kortenska, V., and Handjieva, N. (2003). Antioxidant activity screening of extracts from *Sideritis* species (Labiatae) grown in Bulgaria. *J. Sci. Food Agric.* 83, 809–819. doi: 10.1002/jsfa.1415

Koutsaviti, A., Bazon, I., Milenkovic, M., Pavlovic-Drobac, M., and Tzakou, O. (2013). Antimicrobial activity and essential oil composition of five *Sideritis* taxa of Empedoclia and Hesiodia Sect. from Greece. *Rec. Nat. Prod.* 7 (1), 6–14.

Lall, N., Chrysargyris, A., Lambrechts, I., Fibrich, B., van Staden, A. B., Twilley, D., et al. (2019). *Sideritis perfoliata* (subsp. *perfoliata*) nutritive value and its potential medicinal properties. *Antioxidants* 8 (11), 521. doi: 10.3390/antiox8110521

Lenherr, A., and Mabry, T. J. (1987). Acetylated allose-containing flavonoid glucosides from *Stachys anisochila*. *Phytochemistry* 26, 1185–1188. doi: 10.1016/S0031-9422(00)82375-4

Mabry, T. G., Markham, K. R., and Thomas, M. B. (1970). The Systematic Identification of Flavonoids. *Springer-Verlag New Y.*, 354. doi: 10.1007/978-3-642-88458-0

Machado, K., da, C., Islam, M. T., Ali, E. S., Rouf, R., Uddin, S. J., et al. (2018). A systematic review on the neuroprotective perspectives of beta-caryophyllene. *Phytother. Res.* 32, 2376–2388. doi: 10.1002/ptr.6199

Martins, N., Barros, L., Santos-Buelga, C., Silva, S., Henriques, M., and Ferreira, I. C. F. R. (2015). Decoction, infusion and hydroalcoholic extract of cultivated thyme: Antioxidant and antibacterial activities, and phenolic characterization. *Ind. Crop Prod.* 167, 131–137. doi: 10.1016/j.foodchem.2014.06.094

Meikle, R. D. (1985). *Flora of Cyprus Vol. 2. Kew Bentham-Moxon Trust* (UK: Royal Botanic Gardens).

Menghini, L., Pintore, G., Tirillini, B., and Leporini, L. (2014). Chemical composition, antioxidant activities and protective effects of *Sideritis italica* extract on C_2C1_2 oxidative stress. *Eur. J. Med. Plant* 4 (4), 365–382. doi: 10.9734/EJMP/2014/7090

Miyase, T., Koizumi, A., Ueno, A., Noro, T., Kuroyanagi, M., Fukushima, S., et al. (1982). Studies on the acyl glycosides from *Leucoseptrum japonicum* (Miq.) Kitamura et Murata. *Chem. Pharm. Bull.* 30, 2732–2737. doi: 10.1248/cpb.30.2732

Neu, R. (1957). Chelate von Diarylborsäuren mit aliphatischen Oxyalkylaminen als Reagenzien für den Nachweis von Oxyphenyl-benzo-γ-pyronen. *Naturwissenschaften* 44, 181–183. doi: 10.1007/BF00599857

Nishimura, H., Sasaki, H., Nobuyuki, I., Chin, M., and Mitsuhashi, H. (1991). Nine phenethyl alcohol glycosides from Stachys sieboldii. *Phytochemistry* 30 (3), 965–969. doi: 10.1016/0031-9422(91)85288-b

Orhan, I., Küpeli, E., Aslan, M., Kartal, M., and Yesilada, E. (2006). Bioassay-guided evaluation of anti-inflammatory and antinociceptive activities of pistachio, *Pistacia vera* L. *J. Ethnopharmacol.* 105, 235–240. doi: 10.1016/j.jep.2005.10.023

Petreska, J., Stefova, M., Ferreres, F., Moreno, D. A., Tomás-Barberán, F. A., Stefkov, G., et al. (2011). Potential bioactive phenolics of Macedonian *Sideritis* species used for medicinal "mountain tea". *Food Chem.* 125, 13–20. doi: 10.1016/j.foodchem.2010.08.019

Phatak, R. S., and Hendre, A. S. (2014). Total antioxidant capacity (TAC) of fresh leaves of *Kalanchoe pinnata*. *J.Pharmacogn. Phytochem.* 2, 32–35.

Plioukas, M., Termentzi, A., Gabrieli, C., Zervou, M., Kefalas, P., and Kokkalou, E. (2010). Novel acylflavones from *Sideritis syriaca* ssp. *syriaca*. *Food Chem.* 123, 1136–1141. doi: 10.1016/j.foodchem.2010.05.076

Pljevljakušić, D., Šavikin, K., Janković, T., Zdunić, G., Ristić, M., Godjevac, D., et al. (2011). Chemical properties of the cultivated *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*. *Food Chem.* 124, 226–233. doi: 10.1016/j.foodchem.2010.06.023

Rivas da Silva, A. C., Lopes, P. M., Barros de Azevedo, M. M., Machado Costa, D. C., Alviano, C. S., and Alviano, D. S. (2012). Biological Activities of α-Pinene and β-Pinene enantiomers. *Molecules* 17, 6305–6316. doi: 10.3390/molecules17066305

Romanucci, V., Di Fabio, G., D'Alonzo, D., Guaragna, A., Scapagnini, G., and Zarrelli, A. (2017). Traditional uses, chemical composition and biological activities of *Sideritis raeseri* Boiss. & Heldr.: Uses, composition and activities of *S. raeseri*. *J. Sci. Food Agric.* 97, 373–383. doi: 10.1002/jsfa.7867

Samanidou, V., Tsagiannidis, A., and Sarakatsianos, I. (2012). Simultaneous determination of polyphenols and major purine alkaloids in Greek *Sideritis* species, herbal extracts, green tea, black tea, and coffee by high-performance liquid chromatography-diode array detection. *J. Sep. Sci.* 35, 608–615. doi: 10.1002/jssc.201100894

Sarian, M. N., Ahmed, Q. U., Mat So'ad, S. Z., Alhassan, A. M., Murugesu, S., Perumal, V., et al. (2017). Antioxidant and Antidiabetic Effects of Flavonoids: A Structure-Activity Relationship Based Study. *BioMed. Res. Int.* 2017, 1–14. doi: 10.1155/2017/8386065

Stanoeva, J. P., Stefova, M., Stefkov, G., Kulevanova, S., Alipieva, K., Bankova, V., et al. (2015). Chemotaxonomic contribution to the *Sideritis* species dilemma on the Balkans. *Biochem. Syst. Ecol.* 61, 477–487. doi: 10.1016/j.bse.2015.07.008

Todorova, M., and Trendafilova, A. (2014). *Sideritis scardica* Griseb., an endemic species of Balkan peninsula: Traditional uses, cultivation, chemical composition, biological activity. *J. Ethnopharmacol.* 152, 256–265. doi: 10.1016/j.jep.2014.01.022

Tomou, E. M., Chatzopoulou, P., and Skaltsa, H. (2019). NMR analysis of cultivated *Sideritis euboea* Heldr. *Phytochem. Anal.* 31, 1–9. doi: 10.1002/pca.2874

Trikka, F., Michailidou, S., Makris, A. M., and Argiriou, A. (2019). Biochemical fingerprint of Greek *Sideritis* spp.: Implications for potential drug discovery and advanced breeding strategies. *Med. Aromat. Plants.* 8, 335. doi: 10.35248/2167-0412.19.8.335

Tsintides, T., Christodoulou, C. S., Delipetrou, P., and Georghiou, K. (2007). *The red data book of the flora of Cyprus, Lefkosia, CY : Cyprus Forestry Association.*

Tunçtürk, M., Eryiğit, T., and Rahmi Kaya, A. (2017). Nutritional properties, minerals, and selected heavy metal contents in herby cheese plants of Lamiaceae. *Appl. Biol. Chem.* 60 (1), 41–44. doi: 10.1007/s13765-016-0245

Venditti, A., Bianco, A., Nicoletti, M., Quassinti, L., Bramucci, M., Lupidi, G., et al. (2014). Characterization of secondary metabolites, biological activity and glandular trichomes of *Stachys tymphaea* Hausskn. from the Monti Sibillini National Park (Central Apennines, Italy). *Chem. Biodiver.* 11, 245–261. doi: 10.1002/cbdv.201300253

Venditti, A., Bianco, A., Frezza, C., Serafini, M., Giacomello, G., Giuliani, C., et al. (2016). Secondary metabolites, glandular trichomes and biological activity of *Sideritis montana* L. subsp. *montana* from Central Italy. *Chem. Biodiver.* 13, 1380–1390. doi: 10.1002/cbdv.201600082

Wightman, E. L., Jackson, P. A., Khan, J., Forster, J., Heiner, F., Feistel, B., et al. (2018). The acute and chronic cognitive and cerebral blood flow effects of a *Sideritis scardica* (Greek mountain tea) extract: A double blind, randomized, placebo controlled, parallel groups study in healthy humans. *Nutrients* 10, 955. doi: 10.3390/nu10080955

Yöney, A., Prieto, J. M., Lardos, A., and Heinrich, M. (2010). Ethnopharmacy of Turkish-speaking Cypriots in greater London. *Phytother. Res.* 24, 731–740. doi: 10.1002/ptr.3012

Yalçın, F. N., Kaya, D., and Kılıç, E. (2007). Antimicrobial and free radical scavenging activities of some *Lamium* species from Turkey. *Hacettepe Univ. Eczacı. Fak. Derg.* 27, 11–22.

Yang, H., Woo, J., Nim Pae, A., Young Um, M., Cho, N. C., Park, K. D., et al. (2016). a-Pinene, a major constituent of pine tree oils, enhances non-rapid eye movement sleep in mice through GABAA-benzodiazepine receptors. *Mol. Pharmacol.* 90, 530–539. doi: 10.1124/mol.116.105080

Yildiz, K., and Gücel, S. (2006). Chromosome numbers of 16 endemic plant taxa from Northern Cyprus. *Turk. J. Bot.* 30, 181–192.

Zhang, J. H., Sun, H. L., Chen, S. H., Zeng, L., and Wang, T. T. (2017). Anti-fungal activity, mechanism studies on α-phellandrene and nonanal against *Penicillium cyclopium*. *Bot. Stud.* 58, 13. doi: 10.1186/s40529-017-0168-8

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REVIEW
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Essential Oil-Bearing Plants From Balkan Peninsula: Promising Sources for New Drug Candidates for the Prevention and Treatment of Diabetes Mellitus and Dyslipidemia

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Metabolic diseases like diabetes mellitus or dyslipidemia have a complex etiology characterized by the interference of genetic predisposition and environmental factors like diet or lifestyle. Over time they can cause significant vascular complications, leading to dysfunction or failure of key organs (brain, heart), with possible fatal consequences or a severe reduction of life quality. Although current authorized drugs may successfully control blood glucose or cholesterol level, their use is often associated with severe side effects, therefore the development of new drug candidates is necessary for a better management of metabolic diseases. Among potential new drug sources, aromatic plants rich in essential oils like *Melissa officinalis* L., *Mentha x piperita* L., *Cuminum cyminum* L. or *Pistacia lentiscus* L. var. *chia* are very promising due to their diverse chemical composition and multiple mechanisms of action. This review describes a series of recent experimental studies investigating antidiabetic and hypolipemic effects of essential oils extracted from several aromatic plant species with an ethnopharmacological relevance in the Balkan peninsula. The pharmacological models used in the studies together with the putative mechanisms of action of the main constituents are also detailed. The presented data clearly sustain a potential administration of the studied essential oils for the prevention and treatment of metabolic diseases. Further research is needed in order to ascertain the therapeutic importance of these findings.

Keywords: essential oils, Balkans, terpenoids, antidiabetic, antihyperlipidemic, metabolic diseases

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INTRODUCTION

Metabolic diseases have a complex etiology dominated by genetic predisposition and environmental factors like diet or lifestyle (Barroso and McCarthy, 2019). The most important metabolic diseases with a significant impact on global health are diabetes mellitus (type 1 or insulin-dependent and type 2 or non-insulin-dependent) and dyslipidemias (hypercholesterolemia, hypertriglyceridemia, and mixed dyslipidemia). Diabetes mellitus affected at least 422 million people worldwide in 2014, and caused 1.5 million deaths in 2012, according to an official report of the World Health Organization (WHO) from 2016, which also stated that the global prevalence of the disease has nearly doubled from 1980, reaching an alarming level of 8.5% in the adult population (World Health Organization, 2016). Dyslipidemia is a major risk factor for ischemic heart disease and stroke, causing 2.6 million deaths and 29.7 million disability adjusted life years (DALYS) in 2008 (World Health Organization, 2008). In diabetes mellitus (DM), chronic hyperglycemia may result from an impaired insulin secretion and/or resistance of key peripheral tissues to the effects of insulin (Tan et al., 2019). In dyslipidemia, elevated concentrations of total cholesterol and low-density lipoproteins (LDL) can induce endothelial cell dysfunction and smooth muscle cell proliferation leading to the genesis of atherosclerotic plaque (Helkin et al., 2016). Over time, chronic hyperglycemia or hypercholesterolemia associated with the two diseases which can also coexist, can cause significant vascular complications, leading to dysfunction or failure of multiple organs (brain, heart, kidneys), with possible fatal consequences or a severe reduction of life quality (De Fronzo et al., 2015).

Despite recent advances in medical sciences and the development of multiple antidiabetic or antihyperlipidemic drug classes, the prevalence of diabetes mellitus and dyslipidemia and the associated risk of premature death have constantly increased in low- and medium-income countries (Magliano et al., 2019). The ever-growing treatment cost of metabolic diseases may have a huge economic impact in several parts of the world, limiting the access of patients to effective treatments. In this context, the discovery of new molecules of natural origin, capable of being translated into cost-effective antidiabetic and antihyperlipidemic drugs could be important for large categories of patients, having the potential advantages of being readily available and better tolerated (Yatoo et al., 2017).

In the Balkans and Eastern Mediterranean region, plants have been used in traditional medicine since Antiquity, for the treatment of various ailments of different organs and systems, including metabolic diseases. The influential works of Dioscorides or Galen highlighted the ethnopharmacological relevance of medicinal plants in the Greek and Roman civilizations, setting the foundations of modern medicine. In the last two decades, several active constituents of medicinal plants like flavonoids, saponins, alkaloids or coumarins have been extensively researched for the treatment of metabolic diseases, but essential oils were rarely studied for this purpose (Gushiken et al., 2016).

Essential oils are complex mixtures of aromatic terpenes (mainly monoterpenes and sesquiterpenes) formed as secondary metabolites in specialized secretory tissues of aromatic plants (Sharifi-Rad et al., 2017). Nowadays, essential oils as main constituents of aromatic plants, are widely used for their flavoring properties in food industry or as fragrances in cosmetology, but there is also a growing interest for the evaluation of their complex pharmacological effects. Multiple studies proved antioxidant, antimicrobial, anti-inflammatory, or antinociceptive effects of essential oils (Bakkali et al., 2008), but there are limited data concerning possible effects on glucose and lipid metabolism. Therefore, the aim of this review was to evaluate the antidiabetic and antihyperlipidemic effect of essential oils, presenting the experimental pharmacological models used for their study, with the subsequent mechanistic explanations.

ESSENTIAL OIL-BEARING PLANTS FROM THE BALKANS

Ethnobotanical Data and Chemical Composition

Balkan peninsula is a vast territory extending from Central Europe in the north to the Eastern Mediterranean region in the south and from Black Sea in the east to the Adriatic Sea in the west. It includes the modern states of Albania, Bosnia-Herzegovina, Bulgaria, Croatia, Greece, Montenegro, North Macedonia, Romania (Dobrogea region), Serbia, Slovenia, and Turkey (European region). The complexity of Balkan geography favored a significant biodiversity in the plant kingdom (Griffiths et al., 2004), with a number of around 8,000 plant species and subspecies being catalogued, of which 2,600 to 2,700 are considered endemic (Stevanović et al., 2007). Not surprisingly, traditional medicine using plants for the treatment of various ailments, plays an important role in many Balkan countries, especially in remote, rural areas where access to healthcare system is difficult (Dzamic and Matejic, 2017). An ethnobotanical study from Bosnia-Herzegovina identified 228 plant species used in traditional medicine for the treatment of respiratory, gastrointestinal but also metabolic disorders, among which aromatic plants like *Foeniculum vulgare* Mill., or *Rosmarinus officinalis* L. are used in herbal infusions in rural areas from the central part of the country (Saric-Kundalic et al., 2010). In Greece, a field study found 109 plant species known for medicinal purposes, some of them being used for essential oil extraction, sometimes by traditional methods from aromatic plants cultivated in small areas like *Lavandula stoechas* L., used for skin infections and burns (Axiotis et al., 2018). Another study mentioned an endemic essential oil-bearing plant species, *Pistacia lentiscus* L. var. *chia*, uniquely present on Chios Island in the Eastern Aegean region of Greece, used in traditional medicine over the last 2,500 years in the treatment of gastrointestinal disorders which recently proved significant antihyperlipidemic and cardioprotective effects (Pachi et al., 2020). A study from Serbia identified 83 plant species including two aromatic plants present in the wild flora of the Kopaonik Mountain region (*Melissa officinalis* L. and *Thymus vulgaris* L.)

which were used in the treatment of gastrointestinal, respiratory, or cardiac disorders but also in metabolic diseases (Jaric et al., 2007). In Turkey, a survey study found that of 64 plant species used in traditional medicine, 9 species were used for essential oil production which are usually sold in local markets as remedies for urinary, respiratory or cardiovascular diseases but also for diabetes like the essential oil from *Myrtus communis* L., endemic in the Mediterranean area (Gurdal and Kultur, 2013).

Our review of published data identified sixteen aromatic plant species, cultivated or wild in the Balkan region, belonging to nine families which presented antidiabetic and antihyperlipidemic properties demonstrated by experimental models. The plants were organized alphabetically by family and botanical name, the main chemical constituents being also presented (**Table 1**).

The concentration of main components from essential oils may be variable according to environmental conditions, plant chemotype or methods of harvesting. Also, other minor constituents from EOs could contribute to their biological effects. The most representative active compounds individually tested in several pharmacological models, are presented in **Figure 1**.

Preclinical Studies Investigating Antidiabetic Effect of Essential Oils

This review showed that essential oils from fifteen aromatic plant species belonging to eight families presented antidiabetic properties demonstrated by specific *in vivo* or *in vitro* preclinical experimental models. The aromatic plant families with the highest proportion of

TABLE 1 | Chemical composition of essential oils (EO) from Balkan region with antidiabetic and antihyperlipidemic activity.

No.	Plant species with essential oils (part used)	Main chemical constituents	C/W	References
1.	**Apiaceae** *Foeniculum vulgare* Mill. – fennel (seeds)	*trans*-anethole, 50.0–90.0%; limonene, 1.4–26.44%; γ-terpinene, 10.5%; α-pinene, 0.4–10.0%; 1,8-cineole, 1.0–6.0%	c	Miguel et al., 2010; Raal et al., 2012
2.	*Cuminum cyminum* L.—cumin (seeds)	cuminaldehyde, 19.25–27.02%; p-mentha-1,3-dien-7-al, 4.29–12.26%; γ-terpinene, 7.06–14.10%; p-cymene, 4.61–12.01%	c	Can Baser et al., 1992
	Asteraceae			
3.	*Tanacetum praeteritum* (Horw.) Heywood (flowers)	α-thujone, 0–79.4%; camphor, 0.7–37.6%; 1,8-cineole, 4.3–19.5%; bornyl acetate, 0–10.0%; terpinen-4-ol, 1.0–9.3%	w	Özek, 2018
	Fabaceae			
4.	*Trigonella foenum-graecum* L.—fenugreek (seeds)	neryl acetate, 17.32%; ß-pinene, 15.05%; β-caryophyllene, 14.63%; geranial, 4.81%; camphor, 16.32%	c	Hamden et al., 2011
	Lamiaceae			
5.	*Lavandula stoechas* L.—Spanish lavender (aerial part)	pulegone, 0–40.4%; α-pinene, 1.0–23.18%; camphor, 0–22.4%; menthol, 0–18.1%; menthone, 0–12.6%; lavandulyl acetate, 0–3.0%	c	Kirmizibekmez et al., 2009
6.	*Melissa officinalis* L.—lemon balm (leaves)	geranial, 0–65.42%; citronellal, 0.7–39.6%; neral, 3.28–31.5%; caryophyllene oxide, 0.2–10.26%; eugenol, 0.05–0.5%	w	Fahima et al., 2014
7.	*Mentha* × *piperita* L.—peppermint (aerial part)	menthol, 31.52%; menthone, 18.35%; carvone, 13.03%; isomenthol acetate, 7.63%; p-menthan-3-one, 6.21%	c	Abdellatief et al., 2017
8.	*Rosmarinus officinalis* L.—rosemary (aerial part)	α-pinene, 7.9–38.1%; verbenone, 15–37%; camphor, 1–22.35%; bornyl acetate, 0.9–12%	w	Satyal et al., 2017
9.	*Salvia sclarea* L.—clary sage (leaves)	germacrene D, 0.6–10.60%; geranyl acetate, 3.45–5.8%; neryl acetate, 1.8–3.0%; caryophyllene oxide, 0.50–2.2%	w	Souleles and Argyriadou, 1997
10.	*Thymus vulgaris* L.—common thyme (aerial part)	thymol, 30–48.2%; p-cymene, 2.2–42.8%; γ-terpinene, 0.3–30.90%; linalool, 1.3–12.4%; terpinen-4-ol, 0.3–9.5%; carvacrol, 0.5–5.5%	w	Borugă et al., 2014
	Lauraceae			
11.	*Laurus nobilis* L.—laurel, bay tree (leaves)	1,8 cineole, 24.2–68.82%; α-terpinenyl acetate, 4.8–18.65%; methyl eugenol, 0.2–16.7%; linalool, 0.7–16.0%; sabinene, 2.1–12.2%	w	Taban et al., 2018
	Myrtaceae			
12.	*Myrtus communis* L.—myrtle (leaves)	α-pinene, 8.1–56.7%; 1,8-cineole, 8–37%; myrtenyl acetate, 0.1–36%; limonene, 4.1–19%; linalool, 0.5–18.4%	w	Zomorodian et al., 2013
13.	**Pistaciaceae** *Pistacia lentiscus* L. var. *chia*—mastic tree (mastic gum)	α-pinene, 58.86–77.10%; myrcene, 0.23–12.27%; linalool, 0.45–3.71%; camphene, 0.75–1.04%	w	Papageorgiou et al., 1991
	Ranunculaceae			
14.	*Nigella sativa* L.—black cumin (seeds)	p-cymene, 18.46–52.64%; thymoquinone, 0.14–29.7%; carvacrol, 0.87–11.5%; α-terpineol, 5.11–9.72%	c	Ghanavi et al., 2018
	Rutaceae			
15.	*Citrus x aurantiifolia* (Christm.) Swingle—lime (leaves)	limonene, 57.84%; neral, 7.81%; linalool, 4.75%; isogeraniol, 3.48%; citronellal, 2.19%	c	Ibrahim et al., 2019
16.	*Citrus x limon* (L.) Osbeck—lemon (pericarps)	limonene, 53.07–80.0%; β-pinene, 9.53%; borneol, 5.57%; neral, 4.7%; sabinene, 4.18%; linalool, 3.70%	c	Oboh et al., 2017

C/W, cultivated/wild species.

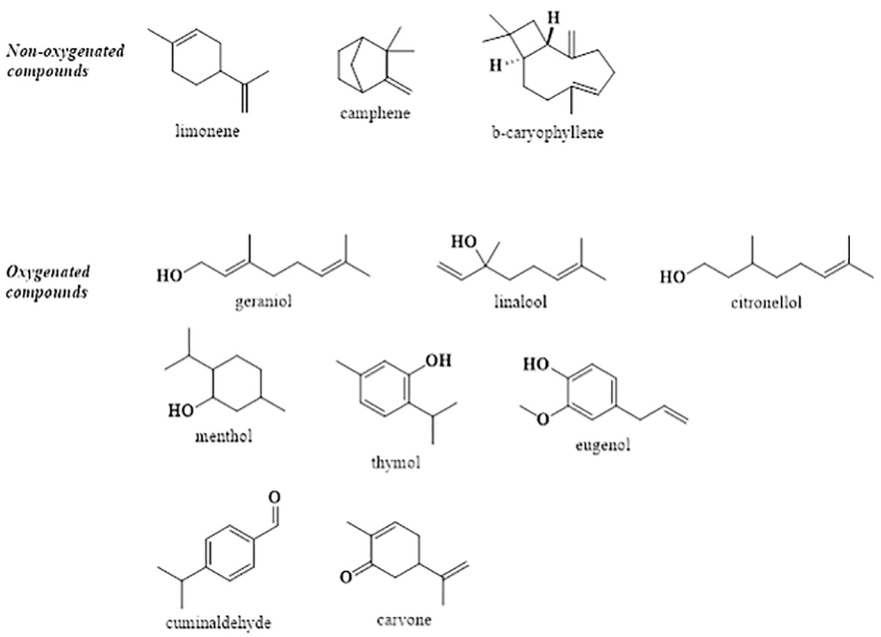

FIGURE 1 | Chemical structures of main compounds with antidiabetic and antihyperlipidemic activity.

species with antidiabetic essential oils (EO) were Lamiaceae (six species), Apiaceae (two species) and Rutaceae (two species). Other identified families were Asteraceae, Fabaceae, Lauraceae, Myrtaceae, and Ranunculaceae, each with only one plant species with antidiabetic essential oils (**Table 2**).

Previous reviews investigated the antidiabetic effects of natural compounds and medicinal plants. An extended study screened *in silico* a library of over 2,300 compounds derived from 30 common herb species, showing that liquorice, hops, fennel, and rosemary are a reach source of antidiabetic compounds like flavonoids and alkaloids (Perreira et al., 2019). Another study identified 111 medicinal plants who were reported to have a beneficial effect in diabetes mellitus, finding flavonoids, glycosides, and oligosaccharides as main active compounds (Eddouks et al., 2014). Our data confirmed some of the findings from these studies, demonstrating that α-amylase, α-glucosidase or glucose-6-phosphatase are effective molecular targets not only for the mentioned phytochemical classes but also for essential oils, equally capable of reducing blood glucose in a variety of experimental models.

The analysis of the aforementioned studies showed that the majority of experimental models used for the assessment of antidiabetic effect of essential oils were *in vivo* techniques (10 studies from 16). Among these, alloxan and streptozotocin-induced diabetes methods were preferred, being capable of reproducing the destruction of pancreatic beta-cells from diabetes with subsequent hyperglycemia and glucose intolerance. Alloxan enters the pancreatic beta cells where it suppresses ATP-mediated insulin release while streptozotocin alkylates the DNA of beta cells inducing their necrosis (Hamden et al., 2011). In both models, the administration of several essential oils reduced hyperglycemia and protected pancreatic beta cells against chemical aggression. Other methods employed genetically modified laboratory animals like *db/db* mice which can also reproduce some of the pathological features of diabetes mellitus. These mice have a mutation in their leptin receptor, with the subsequent development of insulin resistance and obesity which was partially corrected by the administration of lemon balm essential oil (Chung et al., 2010). Five experimental models used *in vitro* techniques for evaluating the effect of essential oils on important enzymes from glucose metabolism like α-amylase or α-glucosidase which are involved in the release of glucydic fractions in the digestive system (Özek, 2018). In these models, the administration of essential oils inhibited the two enzymes with IC50 ranging from 0.5 to 8.16 μg/ml. Although the experimental models used for the study of antidiabetic essential oils are quite diverse, having generated important data, further studies using additional techniques are necessary, for a more accurate evaluation.

Preclinical Studies Investigating Antihyperlipidemic Effect of Essential Oils

This review showed that seven essential oils-bearing aromatic plant species belonging to five families presented antihyperlipidemic effects confirmed by specific *in vivo* or *in vitro* preclinical experimental models (**Table 3**).

Six of the presented essential oils-bearing plants (fennel, cumin, lemon balm, rosemary, black cumin, and lemon) have also proved a priorly discussed antidiabetic activity, the co-existence of antidiabetic and antihyperlipidemic properties being a possible advantage in the development of new drug candidates. The presented data showed that predominantly, antihyperlipidemic effect of essential oil was evaluated using *in*

TABLE 2 | Plant species containing essential oils (EO) with antidiabetic activity demonstrated in preclinical studies (*in vivo* and in *vitro*).

No.	Plant species with essential oils	Experimental model/Dose	Findings	References
1.	**Apiaceae** *Foeniculum vulgare* Mill.—fennel	Streptozotocin induced diabetes in rats/30 mg/kg EO	Reduction of hyperglycemia	Abou et al., 2011
2.	*Cuminum cyminum* L.—cumin	In vitro assessment of alpha-glucosidase In vitro assessment of insulin secretagogue action on isolated rat pancreatic islets	Inhibition of alpha-glucosidase with IC50 of 0.5 µg/ml 3.34-fold increase of insulin secretion	Lee, 2005 Patil et al., 2013
	Asteraceae			
3.	*Tanacetum praeteritum* (Horw.) Heywood	In vitro assessment of α-amylase	Inhibition of α-amylase with IC50 of 1.02 µg/ml	Özek, 2018
	Fabaceae			
4.	*Trigonella foenum-graecum* L.—fenugreek	Alloxan induced diabetes in rats/5% in food	Lowering of blood glucose, improvement of lipid profile, protective effect on pancreatic beta-cells	Hamden et al., 2011
	Lamiaceae			
5.	*Lavandula stoechas* L.—Spanish lavender	Alloxan induced diabetes in rats/50 mg/kg EO	Reduction of blood glucose, decrease of lipoperoxidation	Sebai et al., 2013
6.	*Melissa officinalis* L.—lemon balm	db/db mice model/0.015 mg/day EO	Reduction of blood glucose, improvement of glucose tolerance, increase of serum insulin, increase of glucokinase and GLUT-4 gene expression	Chung et al., 2010
7.	*Mentha* × *piperita* L.—peppermint	Streptozotocin induced diabetes in rats/40–80 mg/kg EO	Reduction of blood glucose, increase of serum insulin and C-peptide, upregulation of Bcl-2 expression	Abdellatief et al., 2017
8.	*Rosmarinus officinalis* L.—rosemary	Alloxan induced diabetes in rats/mg/kg EO	Reduction of blood glucose, hepatic and renal protective effects	Selmi et al., 2017
9.	*Salvia sclarea* L.—clary sage	Alloxan induced diabetes in mice/50–200 mg/kg EO	Reduction of blood glucose	Raafat and Habib, 2018
10.	*Thymus vulgaris* L.—common thyme	Alloxan induced diabetes in rats/0.4 ml/kg mg/kg EO	Reduction of blood glucose, improvement of lipid profile	Tuama, 2016
	Lauraceae			
11.	*Laurus nobilis* L. –laurel, bay tree	In vitro assessment of α-glucosidase	Inhibition of α-glucosidase with IC50 of 1.748 µg/ml	Sahin Basak and Candan, 2013
	Myrtaceae			
12.	*Myrtus communis* L.—myrtle	Alloxan induced diabetes in rabbits/50 mg/kg EO	Reduction of blood glucose, normalization of oral glucose tolerance test	Sepici et al., 2004
	Ranunculaceae			
13.	*Nigella sativa* L.—black cumin	Streptozotocin induced diabetes in rats/0.3% EO in diet	Reduction of blood glucose, increase of insulin serum concentration	Sultan et al., 2014
	Rutaceae			
14.	*Citrus* × *aurantiifolia* (Christm.) Swingle—lime	Alloxan induced diabetes in rats/50 ml/kg EO	Reduction of fasting blood and hepatic glucose, increase of glycogen in the liver	Ibrahim et al., 2019
15.	*Citrus x limon* (L.) Osbeck—lemon	In vitro assessment of α-amylase and α-glucosidase	Inhibition of α-amylase and α-glucosidase with IC50 of 8.16 and 7.56 µg/ml	Oboh et al., 2017

vivo experiments in which dyslipidemia was induced either by a cholesterol-enriched diet or by the administration of Triton WR1339, an inhibitor of lipoprotein lipase which favors the accumulation of VLDL lipoproteins (Vallianou et al., 2011). In both models, the administration of the mentioned essential oils decreased the concentration of total cholesterol, LDL, and triglycerides. A study evaluated also the aortic intima thickness an important parameter in cardiovascular diseases, noting favorable effects after the administration of lemon essential oil for 8 weeks (Lee et al., 2018). Another experimental model used APOE2 transgenic mice, which develop hyperlipoproteinemia with elevated cholesterol and triglycerides. The administration of lemon balm essential oil produced in these animals a decrease of plasma triglycerides by inhibiting fatty acids synthesis following a down-regulation of Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) (Jun et al., 2012). Also, two experimental models were performed *in vitro* using HepG2 cells, in which treatment with lemon balm and mastic tree essential oils reduced intracellular cholesterol biosynthesis. Due to the complexity of cholesterol metabolism, further studies are necessary to understand the molecular basis of antihyperlipidemic effect of essential oils.

Clinical Studies With Essential Oils Used in Metabolic Diseases

The antidiabetic and antihyperlipidemic effects of selected essential oils were investigated in several clinical studies, summarized in **Table 4**. All selected studies used patients with diabetes, dyslipidemia, or metabolic syndrome, the essential oils being administered by oral route with a treatment duration ranging from 40 to 90 days.

The analysis of data resulted from the selected clinical studies showed that cumin (*Cuminum cyminum* L.*)* essential oil was predominantly used for the treatment of diabetes and dyslipidemia, only one study using another essential oil,

TABLE 3 | Plant species containing essential oils (EO) with antihyperlipidemic activity demonstrated in preclinical studies (*in vivo* and in *vitro*).

No.	Plant species with essential oils	Experimental model/Dose	Findings	References
1.	**Apiaceae** *Foeniculum vulgare* Mill.—fennel	Diet induced dyslipidemia in rats/30 mg/kg EO	Reduction of total cholesterol, LDL and TG, slight increase of HDL	Al-Okbi et al., 2018
2.	*Cuminum cyminum* L.—cumin	Diet induced dyslipidemia in rabbits/500 mg/kg EO	Reduction of total cholesterol, LDL and TG	Chouhan and Purohit, 2018
	Lamiaceae			
3.	*Melissa officinalis* L.—lemon balm	APOE2 transgenic mice In vitro assessment of cholesterol biosynthesis on HepG2 cells	Reduction of plasma TG Reduction of cellular cholesterol and TG	Jun et al., 2012
4.	*Rosmarinus officinalis* L.—rosemary	Diet induced dyslipidemia in rats/10 mg/kg EO	Reduction of total cholesterol, LDL and TG, slight increase of HDL	Al-Okbi et al., 2018
	Pistaciaceae			
5.	*Pistacia lentiscus* L. var. *chia*—mastic tree	Triton WR1339-induced dyslipidemia in rats In vitro assessment of cholesterol biosynthesis on HepG2 cells	Reduction of total cholesterol, LDL and TG Reduction of intracellular cholesterol biosynthesis	Vallianou et al., 2011
	Ranunculaceae			
6.	*Nigella sativa* L.—black cumin	Spontaneously hypertensive rats/800 mg/kg EO	Reduction of total cholesterol, LDL and TG	El-Dakhakhny et al., 2000
	Rutaceae			
7.	*Citrus x limon* (L.) Osbeck—lemon	Diet induced dyslipidemia in rabbits/250 mg/kg EO	Reduction of total cholesterol, LDL and TG; Reduction of aortic intima thickness	Lee et al., 2018

TABLE 4 | Clinical studies with antidiabetic and antihyperlipidemic essential oils.

Disease	Authors	Type of clinical study	Number of patients	Treatment	Results
Diabetes mellitus	Jafari et al., 2017	Randomized, control study	99 type 2 DM patients with suboptimal glycemic control	Cumin essential oil 50 and 100 mg/day for 8 weeks	Reduction of fasting blood glucose and glycosylated hemoglobin (HbA1C)
Diabetes mellitus	Bilal et al., 2009	Randomized control study	41 type 2 DM patients	Black cumin essential oil 700 mg/day for 40 days	Reduction of fasting blood glucose
Diabetes mellitus	Jafari et al., 2017	Randomized control study	64 prediabetic patients	Cumin essential oil 75 mg/day for 10 weeks	No significant change in glycemic indices; significant improvement of lipid profile
Diabetes mellitus, Dyslipidemia	Keihan et al., 2016	Randomized control study	95 diabetic and dyslipidemic patients	Cumin essential oil 25 mg/day for 90 days	Reduction of fasting blood glucose, HbA1C, triglycerides and oxidized LDL
Dyslipidemia	Hadi et al., 2018	Meta-analysis	6 RCTs with 376 patients	Cumin essential oil	Significant reduction of total cholesterol and LDL
Metabolic syndrome	Morovati et al., 2019	Randomized control study	56 patients with metabolic syndrome	Cumin essential oil 75 mg t.i.d. for 8 weeks	Reduction of fasting blood glucose and total cholesterol

extracted from the black cumin (*Nigella sativa* L.). According to the majority of these studies, the administered essential oils managed to reduce fasting blood glucose and glycosylated hemoglobin in diabetic patients, decreasing also total cholesterol and LDL concentration in dyslipidemia. Nevertheless, the presented clinical studies with antidiabetic and antihyperlipidemic essential oils have several limitations. They were represented mainly by randomized control studies (RCTs) using small numbers of patients with an insufficient statistical significance. Only one study was a meta-analysis with superior statistical power, although itself used a limited number of randomized controlled trials (Hadi et al., 2018). Therefore, additional clinical studies on larger populations are needed to ascertain the therapeutic value of essential oils with antidiabetic or antihyperlipidemic potential.

Toxicological Evaluation of Essential Oils With Antidiabetic and Antihyperlipidemic Potential and Their Main Chemical Constituents

Essential oils are characterized by a high content in monoterpenes, compounds with low molecular weight and high lipophilicity which enable them to easily pass through biological barriers and potentially affect multiple organs. Despite their promising impact for health sciences, only a few studies investigated the toxicological aspects of essential oils with antidiabetic and antihyperlipidemic potential and their main chemical constituents. A study on female Wistar rats evaluated the repeated oral toxicity of essential oil from *Cuminum cyminum* L. and found no evidence of clinical signs of toxicity or pathological modifications at organ level, defining a non-

observed adverse effect level (NOAEL) of 500 mg/kg/day (Taghizadeh et al., 2017). Another study evaluating the sub-chronic toxicity of DL-menthol in male B6C3F1 mice, also found no clinical signs or histopathological evidence of toxicity and established a NOAEL of 1956 mg/kg/day. In male Fischer 344 rats, the same study did not demonstrate significant signs of toxicity for DL-menthol, calculating a NOAEL of 937 mg/kg/day (OECD SIDS programs, 2003). The toxicity of eugenol was evaluated by an *in vitro* method using isolated rat hepatocytes, which demonstrated that an exposure to high doses of this compound present in several essential oils can cause significant hepatotoxicity, attenuated by acetylcysteine, showing some similarity with paracetamol intoxication (Nejad et al., 2017). For geraniol, a CYP-mediated metabolic pathway could generate geranial and 6,7-epoxygeraniol which proved significant sensitizing properties in the murine local lymph node assay (Hagvall et al., 2008).

In humans, several chemical constituents from essential oils like 1,8-cineole, and thujone have been shown to induce epileptiform convulsions, in children with a history of epileptic syndromes, according to a report (Burkhard et al., 1999). Additionally, the meta-analysis which investigated antidiabetic and antihyperlipidemic effects of cumin (*Cuminum cyminum* L.) essential oil in clinical trials, identified rare adverse effects like respiratory complications and dermatitis (Hadi et al., 2018). Due to insufficient and sometimes contradictory data, further research is needed to rigorously evaluate the safety profile of essential oils with antidiabetic and antihyperlipidemic potential and their main chemical constituents.

MECHANISMS OF ACTION OF ANTIDIABETIC AND ANTIHYPERLIPIDEMIC ESSENTIAL OILS WITH A FOCUS ON ACTIVE COMPOUNDS

Current Pharmacological Approach in Diabetes and Dyslipidemia

The main objective of antidiabetic medication is the reduction of hyperglycemia and the risk of vascular complications, which can be achieved by multiple pharmacological mechanisms, due to the complexity of glucose metabolism (Tan et al., 2019). Various types of insulins, with different pharmacokinetic properties are administered subcutaneously in type 1 diabetes, acting as agonists on specific receptors expressed by a variety of cells including hepatocytes, adipocytes, or muscular cells. Subsequently, key glucose transporters (GLUT) are activated at cellular level, inducing a significant reduction of blood glucose. For type 2 diabetes, multiple classes of antidiabetic drugs are currently on the market. Sulfonylureas and meglitinides act as insulin secretagogues, amplifying the exocytosis of insulin granules from pancreatic beta-cells. Other classes like biguanides increase the uptake and metabolism of glucose in peripheral tissues while inhibitors of alpha-glucosidase limit the amount of absorbed glucose in the digestive tract. Another approach was to target peroxisome proliferator-activated receptors gamma (PPAR-γ) with thiazolidinedione drugs, in order to obtain an increased expression of glucose transporters in key peripheral cells. The more recently developed incretin mimetic drugs can either act as agonists on incretin receptors or inhibit incretin metabolism by blocking dipeptidyl-peptidase IV enzyme, both with complex beneficial effects on glucose metabolism. Finally, the inhibitors of sodium-glucose cotransporter 2 (SGLT-2) act by augmenting the urinary excretion of glucose (De Fronzo et al., 2015; Tan et al., 2019).

In dyslipidemia, the main objective of the medication is to minimize the risk of major vascular events like stroke or heart attack by reducing the level of LDL, the most atherogenic lipoprotein fraction. The backbone of antihyperlipidemic drugs is represented by statins which inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, reducing the formation of mevalonate, a precursor of cholesterol. Additionally, statins may increase the number of LDL receptors on hepatocytes which also contributes to their cholesterol lowering effect (Chang and Robidoux, 2017). Fibrates are used especially in hypertriglyceridemia and mixed dyslipidemia, acting as agonists on peroxisome proliferator-activated receptor-alpha (PPAR-alpha) which leads to the activation of lipoprotein lipase with the subsequent degradation of VLDL fraction rich in triglycerides. Bile acid sequestrants like cholestyramine act by removing bile acids from enterohepatic circuit with the slow development of a cholesterol lowering effect. Ezetimibe, a more recent drug, can reduce intestinal absorption of cholesterol by blocking its main transporter, the Nieman-Pick C1-Like1 (NPC1L1) protein, being frequently used in association with statins (Tiwari and Khokhar, 2014). A novel class is represented by monoclonal antibodies against proprotein convertase subtilisin/kexin type 9 (PCSK9), a protein involved in lysosomal degradation of LDL receptors in the hepatocytes. They are reserved for selected cases of familial heterozygous or homozygous hypercholesterolemia in patients insufficiently controlled with the maximal tolerable doses of statins (Toth et al., 2017).

Unfortunately, the beneficial effects of many existing antidiabetic or antihyperlipidemic drugs are accompanied by numerous adverse effects, sometimes severe, which can limit patient's compliance and treatment efficacy. Hence, there is a growing need for new drug candidates with improved characteristics, among which naturally occurring molecules like essential oils could play an important part. Essential oils have a complex chemical composition which allows them to target multiple physiological structures and processes involved in glucose metabolism, several mechanisms of action being described. Although some aspects of the antidiabetic effect of essential oils and their constituents have been explained, further research is needed for a better understanding of their cellular and molecular actions.

Antidiabetic Mechanisms of Essential Oils

Inhibition of Glucose Absorption From the Digestive Tract

Glucose is the main energy source in the human body, being obtained generally by degrading more complex molecules like

starch, sucrose, or lactose. After the initial action of salivary and pancreatic α-amylase, the digestion is continued in the small intestine epithelium by α-glucosidase (along with maltase and lactase) with the release of absorbable glucose, fructose, or galactose. Therefore, inhibition of α-glucosidase and α-amylase can reduce the amount of absorbable glucydic fractions from the digestive tract, being capable of preventing postprandial hyperglycemic peaks. The mechanism is clinically validated for acarbose and miglitol, two α-glucosidase inhibitors which are used in the treatment of type 2 diabetic patients. A significant inhibitory effect on α-glucosidase was demonstrated *in vitro* for cuminaldehyde a main component in cumin essential oil. Cuminaldehide showed an IC50 value of 0.5 μg/ml, being only slightly inferior to acarbose, proving also a strong inhibitory effect on aldose reductase (IC50 of 0.085 μg/ml) which may suggest additional protective effects against toxic actions of sorbitol in diabetic neuropathy (Lee, 2005). Also, the inhibition of α-glucosidase was demonstrated by another *in vitro* study investigating the essential oil from *Laurus nobilis* L., which showed a 90% inhibition of the enzyme (IC50 of 1.748 μg/ml), the main chemical constituent responsible for the effect being 1,8-cineole (Sahin Basak and Candan, 2013). The research of Özek (2018) identified a significant inhibition of α-amylase for the essential oil from *Tanacetum praeteritum* subsp. *praeteritum* (Horw.) Heywood, in the I_2/KI method with IC50 of 1.02 μg/ml, suggesting that **thujone** is the main component responsible for the effect.

Increase of Glycogen Synthesis in the Liver and Decrease of Gluconeogenesis

Glucose homeostasis is a multi-faceted process in which the liver has an important role, being capable of storing or releasing glucose. In diabetic patients, a decreased glycogen synthesis coupled with increased gluconeogenesis and glycogenolysis are detected, which lead to elevated blood glucose. Therefore, stimulation of glucokinase and more importantly glycogen synthase is capable of reducing hyperglycemia by stimulating the conversion of glucose into glycogen. The *in vivo* study of Ibrahim et al. (2019) proved that essential oil from *Citrus x aurantiifolia* (Christm.) Swingle presented a hypoglycemic effect in an alloxan-induced diabetes model. In this study, intraperitoneal administration of 100 mg/kg EO to diabetic rats for 14 days, significantly reduced fasting blood glucose levels, decreased hepatic glucose production, and increased hepatic glycogen, suggesting that reduction of gluconeogenesis and augmentation of glycogenesis are the main mechanism of action of the essential oil. On the other hand, a new *in vivo* study showed that oral administration of **geraniol** a natural monoterpene found in several essential oils, in doses between 100 and 400 mg/kg, to STZ-induced diabetic rats for 45 days, reduced plasma glucose, and glycosylated hemoglobin (HbA1C). The analysis of liver tissue from the treated rats proved that geraniol was capable of inhibiting hepatic glucose-6-phosphatase activity, thus reducing gluconeogenesis (Babukumar et al., 2017). Also, other three studies found that citronellol, d-limonene, and caryophyllene inhibited both glucose-6-phosphatase and fructose-1-6-phosphatase, reducing gluconeogenesis in STZ-induced diabetic rats, with a significant reduction of plasma glucose and HbA1C (Murali and Saravanan, 2012; Srinivasan and Muruganathan, 2016; Basha and Sankaranarayanan, 2014).

Increase of Insulin Sensitivity

Insulin intracellular signaling is a complex process finalized with the translocation of GLUT2 and GLUT4 glucose transporters from the cytoplasm of target cells to their membrane. Phosphatidylinositol 3-kinase (PI3K) and adenosine monophosphate-activated protein kinase (AMPK) contribute to this process. An *in vivo* study using genetically modified diabetic mice (db/db) demonstrated that oral administration of essential oil from *Melissa officinalis* L. in a dose of 0.015 mg/day for 6 weeks, significantly reduced blood glucose and improved glucose tolerance. The RT-PCR and Western blotting methods performed in this study proved that the essential oil was able to increase the expression of glucose transporter (GLUT4) genes probably by the activation of PI3K cascade, leading to an increased glucose uptake in key cells of treated animals (Chung et al., 2010). Also, an *in vitro* study on cultivated astrocytes originating from C57BL/6 mice pups, proved that **eugenol**, a main constituent in several essential oils, was capable of enhancing intracellular insulin signaling mechanism by increasing AKT phosphorylation, without changing PTP-1B expression (Sartorius et al., 2014).

Increase of Insulin Secretion

An increase of insulin secretion could be achieved by depolarizing beta cell membrane by modulating potassium channels or by protecting beta cells against aggression and apoptosis. An *in vivo* study proved that **menthol**, present in the chemical composition of several essential oils, administered orally to STZ-induced diabetic rats in doses of 25 to 100 mg/kg for 45 days, reduced blood glucose and glycosylated hemoglobin (HbA1C). The substance showed a mechanism of action similar with sulphonylurea drugs, being able to inhibit ATP-sensitive K channels on beta cells membrane, increasing the exocytosis of insulin. Additionally, menthol increased the survivability of beta cells by stimulating the expression of Bcl-2, an antiapoptotic factor (Muruganathan et al., 2017). The results were confirmed by Abdellatief et al. (2017) who demonstrated that essential oil from *Mentha x piperita* L. also increased the expression of Bcl-2 and hence protected pancreatic beta cells against apoptosis in a STZ-induced diabetes model in rats.

Antihyperlipidemic Mechanisms of Essential Oils

Activation of Lipoprotein Lipase

Lipoprotein lipase (LPL) is capable of hydrolyzing triacylglycerols from VLDL fraction, with the apparition of monoacylglycerol used by specific tissues. The activation of the enzyme is produced by fibrates, a well-established class of drugs used in the treatment of hypertriglyceridemia. An *in vivo* study using Triton WR1339-induced dyslipidemia model in rats, proved that essential oil from the plant *Pistacia lentiscus* L. var. *chia*, endemic in the island of Chios in Greece, was capable of activating lipoprotein lipase in rats with a subsequent 34.5%

reduction of triglycerides (Vallianou et al., 2011). The study showed also that **camphene** was the active substance responsible for this effect, no significant changes in plasma lipids of treated animals being observed for other terpenoids present in the essential oil, which were individually tested. Additionally, camphene reduced also total cholesterol and LDL, suggesting that this monoterpene could be a possible drug candidate in the development of new antihyperlipidemic molecules. Another *in vivo* study using high fed diet (HFD) mice found that administration of **thymol** (10–40 mg/kg for 5 weeks), an important active compound from thyme essential oil, caused a reduction of triglycerides and total cholesterol in treated animals, producing also an increase in HDL concentration. The study suggested that the augmentation of lipoprotein lipase (LPL) activity and a reduction in leptin concentration could be main mechanisms responsible for the antihyperlipidemic effect (Saravanan and Pari, 2015).

Modulation of TRPV1 Receptor

The transient receptor potential vanilloid 1 (TRPV1) was considered to have important roles on sensory neurons, however other research demonstrated its presence on hepatocytes with possible roles in reducing blood cholesterol level. A recently published study investigated the effects of **eugenol**, an active compound present in several essential oils, including lemon balm EO, on lipid profile in rats fed with cholesterol-enriched diet. The obtained data showed that **eugenol** administered orally to the rats, in doses of 10 to 100 mg/kg, reduced total cholesterol, LDL, and atherogenic index in treated animals. Immunohistochemical methods proved that menthol down-regulated TRPV1 receptor in the liver of treated animals, mechanism supported by collateral molecular docking studies. Additionally, a histopathological examination performed in the same study found that eugenol protected the liver, reducing hepatic steatosis and the level of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activity (Harb et al., 2019).

Reduction of Fatty Acid Synthesis by Modulation of SREBP-1c

Excessive production of VLDL in which fatty acid synthesis plays an important role is involved in dyslipidemia and metabolic syndrome. A key enzyme in the process is fatty acid synthase (FAS) which is regulated by complex nuclear mechanisms. An *in vivo* study investigating the effects of essential oil from *Melissa officinalis* L. in APOE2 transgenic mice proved a decrease of plasma triglycerides by inhibiting fatty acids synthesis. The obtained data showed that the treatment with essential oil inhibited the nuclear translocation of Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) which in turn led to the suppression of key genes involved in lipid metabolism, primarily fatty acid synthase (FAS). The study did not investigate which of the essential oil active constituents could be responsible for this effect (Jun et al., 2012).

CONCLUSION

This review identified sixteen essential oil-bearing plant species from Balkan region with antidiabetic and antihyperlipidemic effects demonstrated in preclinical and clinical studies. The main mechanisms of antidiabetic effect of individual chemical components identified in the selected species were represented by an inhibition of α-amylase and α-glucosidase by cuminaldehyde, 1,8-cineole, and thujone, a decrease of gluconeogenesis by geraniol and an increase of insulin sensitivity by eugenol or insulin secretion by menthol. Activation of lipoprotein lipase by camphene and thymol and modulation of TRPV1 receptor by eugenol were considered responsible for the antihyperlipidemic effect. The active terpenoid components identified in the chemical composition of the studied essential oils could become promising new drug candidates, but future studies are needed in order to evaluate their potential.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology: SH, LF, and OV. Validation and formal analysis: CM, DM, CI, and MM.

REFERENCES

Abdellatief, S. A., Beheiry, R. R., and El-Mandrawy, S. A. M. (2017). Peppermint essential oil alleviates hyperglycemia caused by streptozotocin-nicotinamide-induced type 2 diabetes in rats. *Biomed. Pharmacother.* 95, 990–999. doi: 10.1016/j.biopha.2017.09.020

Abou, N., El-Soud, N. A., El-Laithy, N., El-Saeed, G., Wahby, M. S., Khalil, M., et al. (2011). Antidiabetic activities of *Foeniculum vulgare* Mill. essential oil in streptozotocin-induced diabetic rats. *Maced. J. Med. Sci.* 4 (2), 139–146. doi: 10.3889/MJMS.1857-5773.2011.0173

Al-Okbi, S. Y., Hussein, A. M. S., Elbakry, H. F. H., Fouda, K. A., Mahmoud, K. F., and Hassan, M. E. (2018). Health benefits of fennel, rosemary volatile oils and their nano-forms in dyslipidemic rat model. *Pak. J. Biol. Sci.* 21 (7), 348–358. doi: 10.3923/pjbs.2018.348.358

Axiotis, E., Halabalaki, M., and Skaltsounis, L. A. (2018). An ethnobotanical study of medicinal plants in the Greek islands of north Aegean region. *Front. Pharmacol.* 9, 409. doi: 10.3389/fphar.2018.00409

Babukumar, S., Vinothkumar, V., Sankaranarayanan, C., and Srinivasan, S. (2017). Geraniol, a natural monoterpene, ameliorates hyperglycemia by attenuating the key enzymes of carbohydrate metabolism in streptozotocin-induced diabetic rats. *Pharm. Biol.* 55, 1442–1449. doi: 10.1080/13880209.2017.1301494

Bakkali, F., Averbeck, S., Averbeck, D., and Idaomar, M. (2008). Biological effects of essential oils-a review. *Food Chem. Toxicol.* 46 (2), 446–475. doi: 10.1016/j.fct.2007.09.106

Barroso, I., and McCarthy, M. I. (2019). The genetic bases of metabolic disease. *Cell* 177, 146–161. doi: 10.1016/j.cell.2019.02.024

Basha, R. H., and Sankaranarayanan, C. (2014). β-Caryophyllene, a natural sesquiterpene, modulates carbohydrate metabolism in streptozotocin-

induced diabetic rats. *Acta Histochem.* 116, 1469–1479. doi: 10.1016/j.acthis.2014.10.001

Bilal, A., Masud, T., Uppal, A. M., and Naveed, A. K. (2009). Effects of *Nigella sativa* oil on some blood parameters in type 2 diabetes patients. *Asian J. Chem.* 21 (7), 5373–5381.

Borugă, O., Jianu, C., Mişcă, C., Goleţ, I., Gruia, A. T., and Horhat, F. G. (2014). *Thymus vulgaris* essential oil: chemical composition and antimicrobial activity. *J. Med. Life.* 7 (3), 56–60.

Burkhard, P. R., Burkhardt, K., Haenggeli, C.-A., and Landis, T. (1999). Plant-induced seizures: reappearance of an old problem. *J. Neurol.* 246, 667–670. doi: 10.1007/s004150050429

Can Baser, K. H., Kurkcuoglu, M., and Ozek, T. (1992). Composition of the Turkish cumin seed oil. *J. Essent. Oil Res.* 4, 133–138. doi: 10.1080/10412905.1992.9698034

Chang, Y., and Robidoux, J. (2017). Dyslipidemia management update. *Curr. Opin. Pharmacol.* 33, 47–55. doi: 10.1016/j.coph.2017.04.005

Chouhan, H., and Purohit, A. (2018). Protective effect of cumin (*Cuminum cyminum*) on cardiovascular system, toxicity and hematology on hyperlipidemic rabbits: an experimental study. *Asian J. Pharm. Clin. Res.* 11 (10), 353–356. doi: 10.22159/ajpcr.2018.v11i10.27400

Chung, M. J., Cho, S.-Y., Haque Bhuiyan, M. J., Kim, K. H., and Lee, S.-J. (2010). Anti-diabetic effects of lemon balm (*Melissa officinalis*) essential oil on glucose and lipid regulating enzymes in type 2 diabetic mice. *Br. J. Nutr.* 104, 180. doi: 10.1017/S0007114510001765

De Fronzo, R. A., Ferrannini, E., Groop, L., Henry, R. P., Herman, W. H., Holst, J. J., et al. (2015). Type 2 diabetes mellitus. *Nat. Rev. Dis.* 1, 15019. doi: 10.1038/nrdp.2015.19

Dzamic, A., and Matejic, J. S. (2017). *Aromatic plants from western Balkans: a potential source of bioactive natural compounds.* (Rijeka: Intech).

Eddouks, M., Bidi, A., El Bouhali, B., Hajji, L., and Zeggwagh, N. A. (2014). Antidiabetic plants improving insulin sensitivity. *J. Pharm. Pharmacol.* 66, 1197–1214. doi: 10.1111/jphp.12243

El-Dakhakhny, M., Mady, N. I., and Halim, M. A. (2000). *Nigella sativa* L. oil protects against hepatotoxicity and improves serum lipid profile in rats. *Arzneimittelforschung* 50 (9), 832–836. doi: 10.1055/s-0031-1300297

Fahima, A., Hadjira, B., Abdelghani, Z., and Aicha, H. (2014). Chemical composition and antimicrobial activity of the essential oil from leaves of algerian *Melissa officinalis* L. *Exp. Clin. Sci. J.* 13, 772–781.

Ghanavi, Z., Velayati, A. A., Farnia, P., Naji, A. M., and Kalatehjari, S. (2018). Essential Oil Composition of *Nigella sativa* Seeds from Different Populations of Iran. *J. Essent. Oil-Bear. Plants.* 21, 1652–1659. doi: 10.1080/0972060X.2018.155115

Griffiths, H. I., Krystufek, B., and Reed, J. M. (2004). *Balkan Biodiversity. Pattern and Process in the European Hotspot* (Rotterdam: Springer Netherlands).

Gurdal, B., and Kultur, S. (2013). An ethnobotanical study of medicinal plants in Marmaris (Turkey). *J. Ethnopharmacol.* 146, 113–126. doi: 10.1016/j.jep.2012.12.012

Gushiken, L. F., Beserra, F. P., Rozza, A. L., Bergamo, P. L., Bergamo, P. L., and Pellizzon, C. H. (2016). Chemical and biological aspects of extracts from medicinal plants with antidiabetic effects. *Rev. Diabetes Stud.* 13 (2-3), 96–112. doi: 10.1900/RDS.2016.13.96

Hadi, A., Mohammadi, H., Roshanravan, N., and Kafeshani, M. (2018). Cumin (*Cuminum cyminum* L.) is a safe approach for management of lipid parameters: a systematic review and meta-analysis of randomized controlled trials. *Phytother. Res.* 32 (11), 2146–2154. doi: 10.1002/ptr.6162

Hagvall, L., Baron, J. M., Borje, A., Weidolf, L., Merk, H., and Karlberg, A.-T. (2008). Cytochrome P450-mediated activation of fragrance compound geraniol forms potent contact allergens. *Toxicol. Appl. Pharmacol.* 233 (2), 308–313. doi: 10.1016/j.taap.2008.08.014

Hamden, K., Keskes, H., Belhaj, S., Mnafgui, K., Feki, A., and Allouche, N. (2011). Inhibitory potential of omega-3 fatty and fenugreek essential oil on key enzymes of carbohydrate digestion and hypertension in diabetes rats. *Lipids Health Dis.* 10, 226. doi: 10.1186/1476-511X-10-226

Harb, A. A., Bustanji, Y. K., Almasri, I. M., and Abdalla, S. S. (2019). Eugenol Reduces LDL Cholesterol and Hepatic Steatosis in Hypercholesterolemic Rats by Modulating TRPV1 Receptor. *Sci. Rep.* 9, 14003. doi: 10.1038/s41598-019-50352-4

Helkin, A., Stein, J. J., Lin, S., Siddiqui, S., Maier, K. G., and Gahtan, V. (2016). Dyslipidemia part1-review of lipid metabolism and vascular cell physiology. *Vasc. Endovasc. Surg.* 50 (2), 107–118. doi: 10.1177/1538574416628654

Ibrahim, F. A., Usman, L. A., Akolade, J. O., Idowu, O. A., Abdulazeez, A. T., and Amuzat, A. O. (2019). Antidiabetic Potentials of *Citrus aurantifolia* Leaf Essential Oil. *Drug Res. (Stuttg.)* 69, 201–206. doi: 10.1055/a-0662-5607

Jafari, S., Sattari, R., and Ghavamzadeh, S. (2017). Evaluation of the effect of 50 and 100 mg doses of *Cuminum cyminum* essential oil on glycemic indices, insulin resistance and serum inflammatory factors on patients with diabetes type II: a double-blind randomized placebo controlled clinical trial. *J. Tradit. Compl. Med.* 7 (3), 332–338. doi: 10.1016/j.jtcme.2016.08.004

Jaric, S., Popovic, Z., Macukanovic-Jocic, M., Djurdjevic, L., Mijatovic, M., Karadzic, B., et al. (2007). An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). *J. Ethnopharmacol.* 111, 160–175. doi: 10.1016/j.jep.2006.11.007

Jun, H.-J., Lee, J. H., Jia, Y., Hoang, M.-H., Byun, H., and Kim, K. H. (2012). *Melissa officinalis* essential oil reduces plasma triglycerides in human apolipoprotein E2 transgenic mice by inhibiting sterol regulatory element-binding protein-1c-dependent fatty acid synthesis. *J. Nutr.* 142, 432–440. doi: 10.3945/jn.111.152538

Keihan, S. G., Gharib, M. H., Momeni, A., Hemati, Z., and Sedighin, R. (2016). A comparison between the effect of *Cuminum cyminum* and vitamin E on the level of leptin, paraoxonase 1, HbA1C and oxidized LDL in diabetic patients. *Int. J. Mol. Cell Med.* 5 (4), 229–235. doi: 10.22088/acadpub.BUMS.5.4.229

Kirmizibekmez, H., Demirci, B., Yeşilada, E., Başer, K. H. C., and Demirci, F. (2009). Chemical composition and antimicrobial activity of the essential oils of *Lavandula stoechas* L. ssp. stoechas growing wild in Turkey. *Nat. Prod. Commun.* 4, 1001–1006. doi: 10.1177/1934578X0900400727

Lee, H., Woo, M., Kim, M., Noh, J. S., and Song, Y. O. (2018). Antioxidative and cholesterol-lowering effects of lemon essential oil in hypercholesterolemia-induced rabbits. *Prev. Nutr. Food Sci.* 23 (1), 8–14. doi: 10.3746/pnf.2018.23.1.8

Lee, H. S. (2005). Cuminaldehyde: aldose reductase and alpha-glucosidase inhibitor derived from *Cuminum cyminum* L. seeds. *J. Agric. Food Chem.* 53 (7), 2446–2450. doi: 10.1021/jf048451g

Magliano, D. J., Islam, R. M., Barr, E. L. M., Gregg, E. W., Pavkov, M. E., Harding, J. L., et al. (2019). Trends in incidence of total or type 2 diabetes: systematic review. *BMJ* 366, l5003. doi: 10.1136/bmj.l5003

Miguel, M. G., Cruz, C., Faleiro, L., Simões, M. T. F., Figueiredo, A. C., Barroso, J. G., et al. (2010). *Foeniculum vulgare* essential oils: chemical composition, antioxidant, and antimicrobial activities. *Nat. Prod. Commun.* 5, 319–328. doi: 10.1177/1934578X1000500231

Morovati, A., Pourghassem Gargari, B., and Sarbakhsh, P. (2019). Effects of cumin (*Cuminum cyminum* L.) essential oil supplementation on metabolic syndrome components: a randomized triple blind, placebo-controlled clinical trial. *Phytother. Res.* 33 (12), 3261–3269. doi: 10.1002/ptr.6500.

Murali, L., and Saravanan, L. (2012). Antidiabetic effect of d-limonene, a monoterpene in streptozotocin-induced diabetic rats. *Biomed. Prev. Nutr.* 2, 269–275. doi: 10.1016/j.bionut.2012.08.008

Muruganathan, U., Srinivasan, S., and Vinothkumar, V. (2017). Antidiabetogenic efficiency of menthol, improves glucose homeostasis and attenuates pancreatic β-cell apoptosis in streptozotocin–nicotinamide induced experimental rats through ameliorating glucose metabolic enzymes. *Biomed. Pharmacother.* 92, 229–239. doi: 10.1016/j.biopha.2017.05.068

Nejad, S. M., Ozgunes, H., and Basaran, N. (2017). Pharmacological and toxicological properties of eugenol. *Turk. J. Pharm. Sci.* 14 (2), 201–206. doi: 10.4274/tjps.62207

Özek, G. (2018). Chemical diversity and biological potential of *Tanacetum praeteritum* subsp. *praeteritum* essential oils. *J. Turkish Chem. Soc Sect. A Chem.* 5, 493–510. doi: 10.18596/jotcsa.389075

Oboh, G., Olasehinde, T. A., and Ademosun, A. O. (2017). Inhibition of enzymes linked to type-2 diabetes and hypertension by essential oils from peels of orange and lemon. *Int. J. Food Prop.* 20 (1), S586–S594. doi: 10.1080/10942912.2017.1303709

OECD SIDS programs (2003). Menthols. Downloaded from: https://hpvchemicals.oecd.org/ui/handler.axd?id=463CE644-E5C8-42E8-962D-3A917F32AB90.

Pachi, V. K., Mikropoulou, E. V., Gkiouvetidis, P., Siafakas, K., Argyropoulou, A., Angelis, A., et al. (2020). Traditional uses, phytochemistry and pharmacology of Chios mastic gum (*Pistacia lentiscus* var. Chia, Anacardiaceae): a review. *J. Ethnopharmacol.* 254, 112485. doi: 10.1016/j.jep.2019.112485

Papageorgiou, V. P., Mellidis, A. S., and Argyriadou, N. (1991). The chemical composition of the essential oil of mastic gum. *J. Essent. Oil Res.* 3 (2), 107–110. doi: 10.1080/10412905.1991.9697918

Patil, S. B., Takalikar, S. S., Joglekar, M. M., Haldavnekar, V. S., and Aryindekar, A. U. (2013). Insulinotropic and beta-cell protective action of cuminaldehyde, cuminol and inhibitor isolated from *Cuminum cyminum* in streptozotocin-induced diabetic rats. *Br. J. Nutr.* 110 (8), 1434–1443. doi: 10.1017/S0007114513000627

Perreira, A. S. P., Banegas-Luna, A. J., Pena-Garcia, J., Perez-Sanchez, H., and Apostolides, Z. (2019). Evaluation of the anti-diabetic activity of some common herbs and spices: providing new insights with inverse virtual screening. *Molecules* 24, 4030. doi: 10.3390/molecules24224030

Raafat, K., and Habib, J. (2018). Phytochemical compositions and antidiabetic potentials of *Salvia sclarea* L. essential oils. *J. Oleo. Sci.* 67 (8), 1015–1025. doi: 10.5650/jos.ess17187

Raal, A., Orav, A., and Arak, E. (2012). Essential oil composition of *Foeniculum vulgare* Mill. fruits from pharmacies in different countries. *Nat. Prod. Res.* 26, 1173–1178. doi: 10.1080/14786419.2010.535154

Sahin Basak, S., and Candan, F. (2013). Effect of *Laurus nobilis* L. Essential Oil and its Main Components on α-glucosidase and Reactive Oxygen Species Scavenging Activity. *Iran. J. Pharm. Res.* 12, 367–379. doi: 10.22037/ijpr.2013.1315

Saravanan, S., and Pari, L. (2015). Role of thymol on hyperglycaemia and hyperlipidemia in high fat diet-induced type 2 diabetic C57BL/6J mice. *Eur. J. Pharmacol.* 761, 279–287. doi: 10.1016/j.ejphar.2015.05.034

Saric-Kundalic, B., Dobes, C., Klatte-Asselmeyer, V., and Saukel, J. (2010). Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina. *J. Ethnopharmacol.* 131, 33–55. doi: 10.1016/j.jep.2010.05.061

Sartorius, T., Peter, A., Schulz, N., Drescher, A., Bergheim, I., Machann, J., et al. (2014). Cinnamon Extract Improves Insulin Sensitivity in the Brain and Lowers Liver Fat in Mouse Models of Obesity. *PloS One* 9, e92358. doi: 10.1371/journal.pone.0092358

Satyal, P., Jones, T. H., Lopez, E. M., McFeeters, R. L., Ali, N. A. A., Mansi, I., et al. (2017). Chemotypic Characterization and Biological Activity of *Rosmarinus officinalis*. *Foods* 620, 1–20. doi: 10.3390/foods6030020

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

Selmi, S., Rtibi, K., Grami, D., Sebai, H., and Marzouki, L. (2017). Rosemary (*Rosmarinus officinalis*) essential oil components exhibit anti-hyperglycemic, anti-hyperlipidemic and antioxidant effects in experimental diabetes. *Pathophysiology* 24 (4), 297–303. doi: 10.1016/j.pathophys.2017.08.002

Sepici, A., Gurbuz, I., Cevik, C., and Yesilada, E. (2004). Hypoglycaemic effects of myrtle oil in normal and alloxan-diabetic rabbits. *J. Ethnopharmacol.* 93, 311–318. doi: 10.1016/j.jep.2004.03.049

Sharifi-Rad, J., Sureda, A., Tenore, G. C., Daglia, M., Sharifi-Rad, M., Valussi, M., et al. (2017). Biological activities of essential oils: From plant chemo ecology to traditional healing systems. *Molecules* 22, E70. doi: 10.3390/molecules22010070

Souleles, C., and Argyriadou, N. (1997). Constituents of the essential oil of *Salvia sclarea* growing wild in Greece. *Pharm. Biol.* 35, 218–220. doi: 10.1076/phbi.35.3.218.13295

Srinivasan, S., and Muruganathan, U. (2016). Antidiabetic efficacy of citronellol, a citrus monoterpene by ameliorating the hepatic key enzymes of carbohydrate metabolism in streptozotocin-induced diabetic rats. *Chem. Biol. Interact.* 250, 38–46. doi: 10.1016/j.cbi.2016.02.020

Stevanović, V., Tan, K., and Petrova, A. (2007). Mapping the endemic flora of the Balkans-a progress report. *Bocconea* 21, 131–137.

Sultan, M. T., Butt, M. S., Karim, R., Zia-Ul-Haq, M., Batool, R., Ahmad, S., et al. (2014). *Nigella sativa* fixed, and essential oil supplementation modulates hyperglycemia and allied complications in streptozotocin induced diabetes mellitus. *Evid. Based Complement. Alternat. Med.* 2014, 826380. doi: 10.1155/2014/826380

Taban, A., Saharkhiz, M. J., and Niakousari, M. (2018). Sweet bay (*Laurus nobilis* L.) essential oil and its chemical composition, antioxidant activity and leaf micromorphology under different extraction methods. *Sustain. Chem. Pharm.* 9, 12–18. doi: 10.1016/j.scp.2018.05.001

Taghizadeh, M., Ostad, S. N., Asemi, Z., Mahboubi, M., Sharafati-Chaleshtori, R., Rashidi, A., et al. (2017). Sub-chronic oral toxicity of *Cuminum cyminum* L.'s essential oil in female Wistar rats. *Regul. Pharmacol. Toxicol.* 88, 138–143. doi: 10.1016/j.yrtph.2017.06.007

Tan, S. Y., Wong, J. L. M., Sim, Y. J., Wong, S. S., Elhassan, S. A. M., Tan, S. H., et al. (2019). Type 1 and 2 diabetes mellitus: a review of current treatment approach and gene therapy as potential intervention. *Diabetes Metab. Syndr. Clin. Res. Rev.* 13, 364–372. doi: 10.1016/j.dsx.2018.10.008

Tiwari, V., and Khokhar, M. (2014). Mechanism of action of anti-hypercholesterolemia drugs and their resistance. *Eur. J. Pharmacol.* 741, 156–170. doi: 10.1016/j.ejphar.2014.07.048

Toth, P. P., Worthy, G., Gandra, S. R., Sattar, N., Bray, S., Cheng, L. I., et al. (2017). Systematic Review and Network Meta-Analysis on the Efficacy of Evolocumab and Other Therapies for the Management of Lipid Levels in Hyperlipidemia. *J. Am. Heart Assoc.* 6, 1–13. doi: 10.1161/JAHA.116.005367

Tuama, R. J. (2016). Effect of thyme (*Thymus vulgaris*) oil on some biochemical parameters of diabetic female rats. *World J. Pharm. Sci.* 4 (6), 320–325.

Vallianou, I., Peroulis, N., Pantazis, P., and Hadzopoulou-Cladaras, M. (2011). Camphene, a plant-derived monoterpene reduces plasma cholesterol and triglycerides in hyperlipidemic rats independently of HMG-CoA reductase activity. *PloS One* 6 (11), e20516. doi: 10.1371/journal.pone.0020516

World Health Organization. (2008). *Global Health Observatory Report 2008*. Downloaded from: https://www.who.int/gho/ncd/risk_factors/cholesterol_text/en/.

World Health Organization. (2016). *Global Report on Diabetes 2016. Downloaded from*: https://www.who.int/diabetes/global-report/en/.

Yatoo, M. I., Saxena, A., Gopalakrishnan, A., Alagawany, M., and Dhama, K. (2017). Promising antidiabetic drugs, medicinal plants, and herbs: an update. *Int. J. Pharmacol.* 13, 732–745. doi: 10.3923/ijp.2017.732.745

Zomorodian, K., Moein, M., Lori, Z. G., Ghasemi, Y., Rahimi, M. J., Bandegani, A., et al. (2013). Chemical Composition and Antimicrobial Activities of the Essential Oil from *Myrtus communis* Leaves. *J. Essent. Oil-Bear. Plants.* 16, 76–84. doi: 10.1080/0972060X.2013.764183

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Antioxidant, Anti-Inflammatory and Antiproliferative Effects of the *Vitis vinifera* L. var. Fetească Neagră and Pinot Noir Pomace Extracts

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The pathophysiology of inflammation and oxidative stress generated during different types of cancers and anticancer treatments is well documented. Traditionally, grape pomace is used for animal feed, organic fertilizers, ethanol production or is disposed as waste. Because grape pomace is a rich source of antioxidant compounds, the purpose of the study was to evaluate the antioxidant, anti-inflammatory, and antiproliferative effects of fresh and fermented grape pomace extracts of two *Vitis vinifera* L. varieties Fetească neagră and Pinot noir cultivated in Romania. Firstly, grape pomace phytochemical analysis and *in vitro* antioxidant tests were performed. Secondly, the effect of a seven-day pretreatment with grape pomace extracts on the turpentine oil-induced inflammation in rats was assessed by measuring total oxidative status, total antioxidant response, oxidative stress index, malondialdehyde, total thiols, nitric oxide and 3-nitrotyrosine. Thirdly, the antiproliferative properties were evaluated on human lung carcinoma (A549), human breast adenocarcinoma (MDA-MB-231), murine melanoma (B164A5), and keratinocyte (HaCat) cell lines. Fetească neagră and Pinot noir grape pomace extracts have a rich content of polyphenols and *in vitro* antioxidant effect. Fermented samples had higher polyphenol content, but fresh samples had better antioxidant activity. Pretreatment with grape pomace extracts reduced inflammation-induced oxidative stress in a concentration-dependent way, fresh samples being more efficient. The malignant cells' proliferation was inhibited by all grape pomace extracts, fermented Fetească neagră extracts having the strongest effect. Conclusion: fresh and fermented pomace extracts of *Vitis vinifera* L. varieties Fetească neagră and Pinot noir cultivated in a Romanian wine region have antioxidant, anti-inflammatory and antiproliferative effects.

Keywords: grape pomace, Fetească neagră, Pinot noir, antioxidant, anti-inflammatory, antiproliferative

INTRODUCTION

During tumor development tissue hypoxia occurs, which activates signaling pathways stimulating cell proliferation, angiogenesis, and death. Hypoxia in the tumor microenvironment causes tumor cells to secrete chemokines, such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-alpha), or interleukin 8 (IL-8), which activates neutrophils to produce more pro-oncogenetic and immunosuppressive factors (Ardi et al., 2007). Lymphocytes are an important part of the immune response to tumors because they inhibit tumorigenesis and kill cancer cells. However, in a proinflammatory tumor state, neutrophils can suppress the efficient lymphocyte-mediated immune response. Moreover, there are tumor-derived factors that induce myelopoiesis, accumulation, and differentiation of tumor-associated macrophages (TAMs). These TAMs produce ROS and RNS in the tumor microenvironment, triggering a tumor-induced inflammation, and creating a vicious cycle between inflammation and cancer (Ardi et al., 2007; Reuter et al., 2010; Andrisic et al., 2018; de Souza et al., 2018). As for platelets, thrombocytosis is common in cancer because tumor cells secrete thrombopoietic cytokines, such as interleukin-6 (IL-6). In turn, platelets can promote angiogenesis through vascular endothelial growth factor (VEGF) secretion and thus protect tumor cells from the immune response. Cancer cells' adaption to hypoxia is part of the malignant phenotype and aggressive tumor progression mechanism (Bambace and Holmes, 2011; Petrillo et al., 2018).

Another issue related to the tumor-induced inflammation is that it modulates cancer responsiveness or resistance to anticancer therapies. In some cancers, elevated basal nuclear transcription factor NF-κB activity and inflammatory mediator production were associated with tumor resistance to chemotherapy and radiation. Chemotherapy with cisplatin, daunomycin, doxorubicin, 5-fluorouracil, paclitaxel, tamoxifen, vinblastine, and vincristine may cause chemoresistance by activating the NF-κB, and NF-κB inhibition acts as radiosensitizer of the tumor cells (Silva et al., 2018). Because oxygen is the best radiosensitizer, tumor-induced hypoxia is considered to be the most important cause of radioresistance (Reuter et al., 2010; Kim et al., 2018).

In order to interrupt the vicious circle between inflammation, nitro-oxidative stress, and cancer, the endogenous enzymatic and nonenzymatic antioxidant molecules may be supplemented with exogenous antioxidant molecules, such as plant-derived polyphenolic compounds. In high concentrations, high pH, and the presence of redox-active metals, polyphenolic compounds can exert a pro-oxidant effect with cytotoxic consequences (Pizzino et al., 2017).

Grape pomace (GP) is a residue of the winemaking process and represents an important ecological and economic problem of waste management, since around 20% of the grapes weight remains as GP (Beres et al., 2017). Due to the incomplete extraction during the winemaking process, around 70% of polyphenolic compounds remain in fermented GP. Traditionally it is mainly used for animal feed, organic fertilizers, ethanol production or is disposed as waste. Over the last decades many products were obtained from grape pomace, and the most common approach was to prepare GP extracts (García-Lomillo and González-SanJosé, 2017). In some countries GP is included in functional foods and cosmetic preparations. Due to its polyphenol content with strong antioxidant effect, GP seemed to be efficient for the prevention of disease-associated oxidative stress. Other GP effects are the antimicrobial and anti-inflammatory activities (Teixeira et al., 2014).

The aims of the study were to evaluate the antioxidant, anti-inflammatory, and antiproliferative effects of the fresh and fermented GP ethanol extracts from two *Vitis vinifera* L. varieties, Fetească neagră and Pinot noir, cultivated in Romania.

MATERIALS AND METHODS

Reagents and Cell Cultures

Sulfanylamide (SULF), N-(1-Naphthyl) ethylenediamine dihydrochloric acid (NEDD), vanadium chloride (III) (VCl3), methanol, diethyl ether, xylenol orange [o-cresosulfonphthalein-3,3-bis (sodium methyliminodiacetate)], orthodianisidine dihydrochloric acid (3-3′-dimethoxybenzidine), ferrous ammonium sulfate, hydrogen peroxide (H2O2), sulfuric acid, hydrochloric acid, glycerol, trichloroacetic acid (TCA), ethylenediaminetetra-acetic acid, sodium dodecal, sulfate butylated hydroxytoluene, thiobarbituric acid, 1,1,3,3-tetraethoxypropane, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB), 1,1-diphenyl-2-picrylhydrazyl (DPPH), o-phthalaldehyde (Darmstadt, Germany) were purchased from Merck and Sigma-Aldrich (Taufkirchen, Germany), 96% ethanol (SA, Iaşi, Romania), ascorbic acid (Lach-Ner, Czech Republic). All chemicals were of analysis grade.

A549—human lung carcinoma, MDA-MB-231—human breast adenocarcinoma, B164A5—murine melanoma cell lines, and HaCat keratinocyte cell lines were purchased from the European Cell Culture Collection (ECACC). Dulbecco's Modified Eagle Environment (DMEM), fetal calf serum (FCS) and resazurine sodium salt were purchased from Sigma Aldrich (Munich, Germany). The phosphate buffer solution (PBS) and the mixture of penicillin/streptomycin and trypsin-EDTA antibiotics were purchased from Gibco (Karlsruhe, Germany).

The ELISA kit for 3-nitrotyrosine (3NT) (KA0445) was purchased from ABNOVA EMBLEM (Heidelberg, Germany).

Grape Samples

The *Vitis vinifera* L. variety Fetească neagră (clone 762 grafted on rootstock SO4, Austria), and *Vitis vinifera* L. variety Pinot noir (clone 828 grafted on rootstock SO4, France) planted in 2006, in Mureș County, Mica parish, part of Târnavelor Plateau (46°21′ 44.5″N and 24°23′55.7″E; 330–350 m above sea level), Romania, were used in our study. Grapes were harvested manually at full maturity level during the 2018 vintage. The GP samples were collected in two winemaking stages: the fresh unfermented GP

was supplied immediately after pressing the grapes, and the fermented GP was supplied after 20 days of fermentation at 20°C and must separation. The samples were stored in vacuum bags at −22°C prior to the analysis and use in the experiments.

Plant Extract Preparation

Fetească neagră and Pinot noir fresh and fermented GP extracts were obtained with 70% ethanol (Merck, București, Romania) by a modified Squibb repercolation method (1/1 g/ml) (Andreicut et al., 2018).

Determination of Total Polyphenols Content

The total polyphenol content (TPC) of the extracts was measured using the Folin–Ciocâlteu method, with some modifications. The absorbance was measured at 760 nm using a JASCO UV-VIS spectrophotometer. Standard curve was prepared using different concentrations of gallic acid (GAE). TPC was expressed as mg GAE/g dry plant material (Toiu et al., 2018).

LC/MS Analysis of Polyphenolic Compounds

A HPLC-MS method was used for the qualitative and quantitative polyphenol determination. The analysis was carried out with an Agilent 1100 Series HPLC system (Agilent, USA) consisting of a G1322A degasser, G1311A binary gradient pump and a G1313A autosampler, and a UV detector. The chromatographic separation was performed using a reversed-phase analytical column (Zorbax SB-C18 100 mm × 3.0 mm i.d., 3.5 μm particle) maintained at 48° C. The mobile phase consisted of a binary gradient: methanol and acetic acid 0.1% (v/v). The mobile phase was delivered with a flow rate of 1 ml/min and the injection volume was 5 μl. Polyphenol detection was performed on UV (330 and 370 nm) and MS mode. The MS system operated using an ion trap mass spectrometer with electrospray negative ionization. The chromatographic data were processed using Chem station and Data Analysis software from Agilent, USA. The calibration curves in the 0.5–5 μg/ml range showed good linearity ($R2 < 0.999$) for a five point plot (Toiu et al., 2018; Farcaș et al., 2019).

For the LC/MS profile polyphenolic compounds standards were: caftaric acid, hyperoside, isoquercitrin, rutoside, miricetol, quercitrin, quercetol, and kaempferol.

In Vitro Antioxidant Activity Analysis

The antioxidant activity (AOA) of the extracts was evaluated by DPPH radical scavenging assay (Blois, 1958). DPPH is considered a stable radical because of the paramagnetism conferred by its odd electron. DPPH solution in ethanol 96% with a concentration of 1 mM was used as a standard antioxidant stock solution. In each reaction, 0.5 ml of the GP extracts was mixed with 0.5 ml of 1 mM DPPH and with 2 ml of 0.167 mM ascorbic acid in ethanol 96%. The mixture was analyzed using a UVI Line 9400 spectrophotometer (SI Analytics), for 20 min at 10 s intervals. Ascorbic acid was used as positive control. The reduction of DPPH free radicals was measured by reading the absorbance at 516 nm. DPPH is a purple colored stable free radical and when reduced, it becomes yellow. The AOA-percentage was calculated with the following formula:

$$AOA(\%) = 100 - \frac{A_{\,sample}}{A_{\,control}} \times 100$$

where: AOA = antioxidant activity (%); A *control* = absorbance of DPPH measured at 516 nm, for 20 min at an interval of 10 s (without sample); A *sample* = absorbance of the sample measured at 516 nm, for 20 min at an interval of 10 s.

Animals and Experimental Protocol

The experiments were performed in triplicate on 14 groups (n = 5) of male albino Wistar rats, weighing 200–250 g that were bred in the Animal Facility of the Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania. The animals were housed in standard polypropylene cages (five per cage) under controlled conditions (12 h light/dark cycle at an average temperature of 21–22°C) and with *ad libitum* access to standard pellet diet (Cantacuzino Institute, Bucharest, Romania) and water. Experimental protocols have been approved by the Ethics Committee (nr. 26/16.12.2015) of the Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania. Four ethanolic extracts of GP were tested: FNfr—Fetească neagră fresh GP extract; FNfe—Fetească neagră fermented GP extract; PNfr—Pinot noir fresh GP extract; PNfe—Pinot noir fermented GP extract. The extracts were administered orally by gavage (1 ml/animal/day) in three dilutions, respectively 100, 50, and 25%, for seven days. Animals from the negative control group (CONTROL) and the inflammation group (INFLAM) received tap water (1 ml/animal/day) by gavage for seven days (He and Mu, 2015). On day eight, except for the CONTROL group, inflammation was induced by injecting turpentine oil (6 ml/kg b.w.) intramuscularly (Toyohara et al., 2013). On day nine, under general anesthesia induced by pentobarbital (50 mg/kg IP) (Zatroch et al., 2017), blood was withdrawn by retro-orbital puncture, serum was separated and stored at −80°C until use, and animals were euthanized by cervical dislocation.

In Vivo Oxidative Stress Assessment

Oxidative stress was assessed using global and specific tests. The global oxidative stress tests were total oxidative status (TOS), total antioxidant reactivity (TAR), and the oxidative stress index (OSI). Specific oxidative stress tests were malondialdehyde (MDA), total thiols (SH), total serum nitrates and nitrates (NOx), and 3-nitrotyrosine (3NT) (Balea et al., 2018b; Farcas et al., 2019).

Alamar Blue Cell Proliferation Assay

The four cell lines were seeded on 96-well microplates (1 × 104 cells/well). After 24 h incubation (37°C, 5%CO_2 and 95% humidity), 200 μl of medium containing DMEM supplemented with 10% FCS, 1% mixture of penicillin/streptomycin (100 U/ml penicillin and 100 pg/ml streptomycin) and ethanolic extracts of

GP (1,000 μg/L) were added to each well and incubated for 48 h (Lo et al., 2011). After 48 h incubation, 20 μl of Alamar blue (AB) was added to each well, and cells were incubated for 4 h at 37°C. AB staining was used to determine the cell viability of both cancer cells (A549, MDA-MB-231, B164A5) and healthy HaCat cells after they were stimulated with GP extracts. The plate was then placed under a microplate reader to determine the absorbance value of each well at 570 and 600 nm; untreated cell wells were used as controls. All *in vitro* experiments were performed in triplicate (Sadej and Skladanowski, 2012). Cell proliferation was calculated by the formula:

$$\%\ \text{AB reduction} = \frac{(\varepsilon\text{OX})\lambda 2(\text{A}\lambda 1) - (\varepsilon\text{OX})\lambda 1(\text{A}\lambda 2)}{(\varepsilon\text{RED})\lambda 1(\text{A}^\circ\lambda 2) - (\varepsilon\text{RED})\lambda 2(\text{A}^\circ\lambda 1)} \times 100$$

where: εOX = the molar extinction coefficient of the oxidized AB form (blue), A = absorption of test wells, A° = absorption of the positive growth control well (cells without tested compounds), λ1 = 570 nm, λ2 = 600 nm.

Statistical Analysis

All results were expressed as mean ± standard deviation (SD) whenever data were normally distributed. Comparisons between the different experimental groups were performed using the ANOVA test and the *post hoc* Bonferroni–Holm test. The correlation analysis was performed with the Pearson test. Values of $p < 0.05$ were considered statistically significant. The analysis was performed using IBM SPSS Statistics, version 20 (SPSS Inc. Chicago, IL, USA).

RESULTS AND DISCUSSION

Cancer remains a leading cause of death worldwide despite considerable progress in basic research and clinical studies. Early diagnosis and chemoprevention are essential for reducing the incidence of cancers. In addition, the side effects of conventional therapies contribute to diminishing patients' life quality and imply the need to develop a safe and effective therapeutic alternative. Although research has been conducted to combat cancer in terms of natural therapy, a satisfactory and complete therapeutic agent has not been found.

Polyphenols Analysis

The differences between the polyphenol content depend on the grape variety, grape maturity, environmental factors, and the technological processes used during the vinification (Xu et al., 2016). The TPC of the extracts varied with the GP product, FNfe having the higher TPC (15.03 ± 0.84 mg GAE/g), followed by PNfe (9.23 ± 0.85 mg GAE/g), PNfr (8 ± 0.10 mg GAE/g), and FNfr (6 ± 0.75 mg GAE/g).

The LC/MS analysis identified the compounds from the GP extracts and confirmed the TPC results, respectively fermented GP samples had a higher content of polyphenols than the fresh GP samples, FNfe having the highest concentration of polyphenols.

Caftaric acid is a phenolic acid found in grapes and gives the white wine color (Song et al., 2018a). In this study, caftaric acid was below the limit of detection (LOD) for all analyzed samples (**Table 1**, **Figures 1–4**).

Kaempferol is a dietary antioxidant flavonol that reduces the risk of chronic diseases, including cancer. At the molecular level, kaempferol has been reported to modulate a number of key elements in cell signal transduction related to apoptosis, angiogenesis, inflammation, and metastasis (Chen and Chen, 2014; Beres et al., 2017). It was found that exposure to solar radiation increase kaempferol concentration. It was detected only in the FNfr (3.679 ± 0.04 μg/ml) and Fnfe (5.740 ± 0.78 μg/ml) GP extracts, and the concentration was comparable to the one determined in South African Shiraz (0.36 mg/100 ml) and Cabernet Sauvignon (0.35 mg/100 ml), but higher than the concentration from three Calabrian red wines (Gidaro et al., 2016) (**Table 1**, **Figures 1–4**).

Miricetol is a flavonol with potent antioxidant, anticancer, analgesic, antidiabetic, hepatoprotective and anti-inflammatory activities. Extensive research into the anticancer activities of miricetol has shown that the compound is cytotoxic to a number of human cancer cell lines, including liver, skin, colon, and pancreas cancer cells. The antioxidant property of miricetol was higher than that of vitamin E. The anti-inflammatory activity of miricetol has been demonstrated in acute and chronic *in vivo* animal models by preventing NF-kB activation, NO, proinflammatory cytokines and PGE2 production (Semwal

TABLE 1 | The: polyphenolic compounds content in the Fetească neagră and Pinot noir grape pomace extracts.

Compound (μg/ml)	FNfr	FNfe	PNfr	PNfe
Caftaric acid	NF	NF	NF	NF
Kaempferol	3.679 ± 0.04	5.740 ± 0.78	NF	NF
Miricetol	0.341 ± 0.01	1.029 ± 0.12	NF	NF
Isoquercitrin	2.429 ± 0.18	65.698 ± 7.11	3.685 ± 0.35	42.042 ± 1.35
Hyperoside	0.804 ± 0.06	10.813 ± 0.18	NF	NF
Rutoside	NF	NF	NF	2.136 ± 0.21
Quercitrin	NF	14.952 ± 1.54	NF	3.272 ± 0.169
Quercetol	8.407 ± 0.54	15.637 ± 1.18	2.473 ± 0.22	3.936 ± 0.27

FNfe, Fetească neagră fermented grape pomace extract; FNfr, Fetească neagră fresh grape pomace extract; PNfe, Pinot noir fermented grape pomace extract; PNfr, Pinot noir fresh grape pomace extract; NF, not found, below the Limit of Detection. Values are expressed as mean ± SD (n = 3).

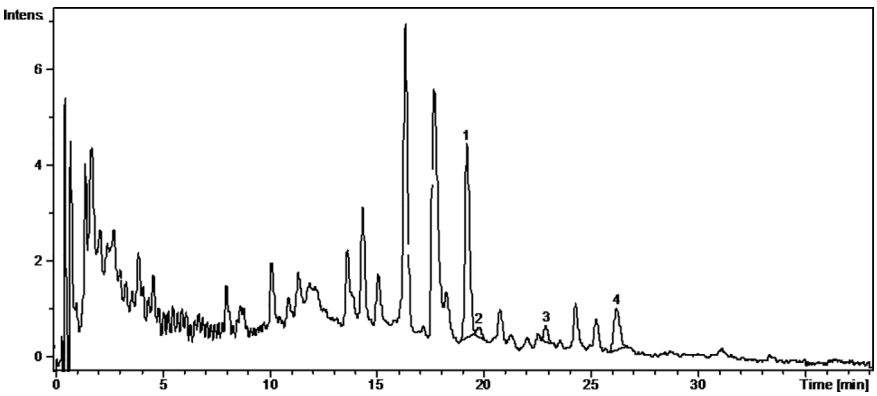

FIGURE 1 | The UV chromatogram of fresh Pinot noir 2015 grape pomace extract. 1, Isoquercitrin; 2, Rutoside; 3, Quercitrin; 4, Quercetin.

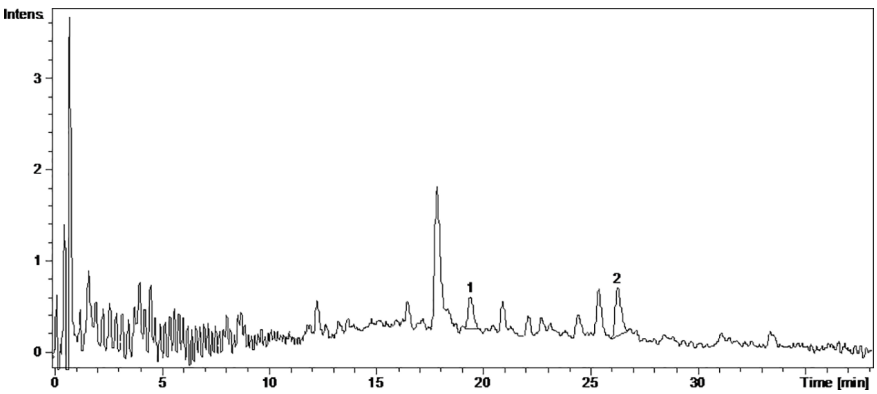

FIGURE 2 | The UV chromatogram of frtmented Pinot noir 2015 grape pomace extract. 1, Isoquercitrin; 2, Quercetin.

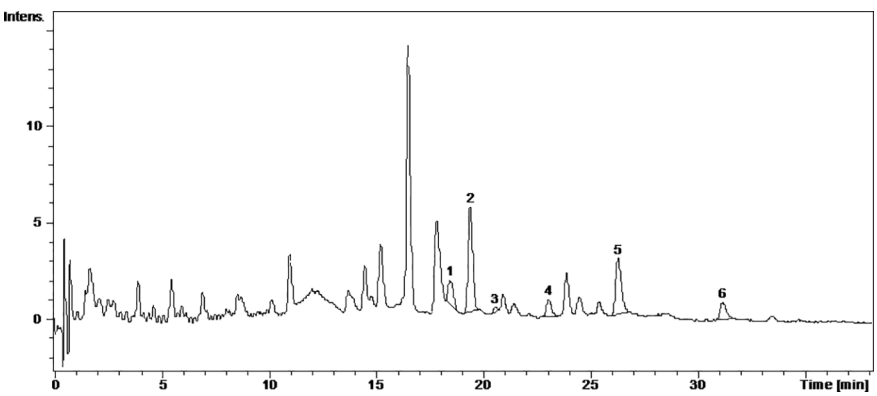

FIGURE 3 | The UV chromatogram of fresh Fetească neagră 2015 grape pomace extract. 1, Hyperoside; 2, Isoquercitrin; 3, Miricetol; 4, Quercitrin; 5, Quercetin; 6, Kaempferol.

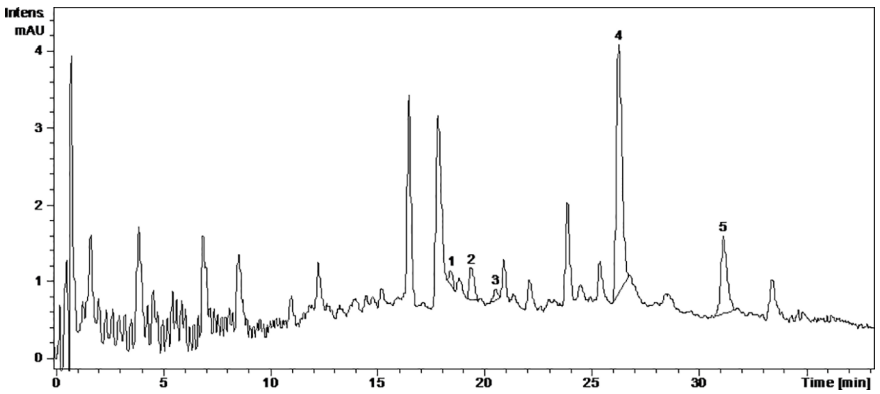

FIGURE 4 | The UV chromatogram of fermented Fetească neagră 2015 grape pomace extract. 1, Hyperoside; 2, Isoquercitrin; 3, Miricetol; 4, Quercetin; 5, Kaempferol.

et al., 2016). In the Pinot noir GP extracts, kaempferol and miricetol were under the LOD, in FNfe moderate amounts of kaempferol and small amounts of miricetol were detected, and in FNfr only small amounts of miricetol were found (**Table 1**, **Figures 1–4**).

Quercetin exists mostly in its quercetin glycosides, which occur naturally and are among the most common flavonoids in the human diet. They have neuroprotective, cardioprotective, chemopreventive, antioxidant, anti-inflammatory, and antiallergic effects. The antioxidant and anti-inflammatory effects were associated with reduced expression of iNOS and inhibition of NF-κB expression (Dai et al., 2013). Quercetin has poor bioavailability, but quercetin glycosides have the same *in vivo* therapeutic effects and better bioavailability. The quercetin glycosides evaluated in our study were quercitrin, isoquercetin, hyperoside, and rutoside (Song et al., 2018b). Quercitrin was above the LOD only in the fermented GP samples, FNfe having a significantly higher concentration of quercitrin than PNfe ($p < 0.001$). Isoquercetin was present in all the samples, having a significantly higher concentration in FNfe and PNfe ($p < 0.001$). Hyperoside was found above the LOD only in Fetească neagră samples, FNfe having a more important content of hyperoside than FNfr ($p < 0.001$). Rutoside has been shown to have immunomodulatory (Ganeshpurkar and Saluja, 2017), antioxidant (Shahid et al., 2016), anti-inflammatory, neuroprotective (Song et al., 2018b), antitumor (Chen et al., 2015), and cardioprotection (Wang et al., 2017) effects. As flavonol, rutoside has low bioavailability due to poor absorption, high metabolism, and rapid excretion, which limits its potential therapeutic use (Martinez-zapata et al., 2016). Rutoside was detected only in the PNfe samples (**Table 1**, **Figures 1–4**).

Quercetol has antioxidant properties by inhibing lipid peroxidation and xanthine oxidase, by scavenging ROS *in vitro* and can inhibit cancer (Ali et al., 2016). Quercetol was found in all GP samples, FNfe having the higher concentration (**Table 1**, **Figures 1–4**).

In a previous study we found significant concentrations of resveratrol in the Fetească neagră and Pinot noir GP extracts, the fresh GP extracts having a higher concentration (Balea et al., 2018a; Balea et al., 2018b). Several *in vitro* studies have shown that resveratrol has antitumor, antioxidant, anti-inflammatory, cardioprotective and antiplatelet activity, and glycosylated stilbenes have antifungal and antioxidant effects (Flamini et al., 2013). Studies conducted with resveratrol showed that it improves the effectiveness of cisplatin and doxorubicin chemotherapy, suggesting that it can be used in cervical cancer treatment (Silva et al., 2018).

In conclusion, the polyphenol analysis of the fresh and fermented pomace of *Vitis vinifera* L. var. Fetească neagră and var. Pinot noir extracts performed in the present and previous studies (Balea et al., 2018b) suggested antioxidant, anti-inflammatory and antiproliferative activities.

In Vitro Antioxidant Activity

All ethanolic extracts of GP proved to have lower antioxidant effects compared to the ascorbic acid. The fresh extracts had a higher AOA after 1,200 s than in the initial moment, FNfr increasing AOA to around 65%, and PNfr increasing AOA to around 60%. The PNfe extracts initially had no antioxidant effects, but after 1,200 s AOA increased to 50%. The FNfe was the single sample that revealed a slight decrease of the AOA after 1,200 s, from around 44% initially to 40%. The AOA results do not correlate with the polyphenol content because the fresh GP samples had a higher AOA and the fermented GP samples had a higher polyphenol concentration (**Figure 5**).

In Vivo Antioxidant Effects

Polyphenols can reduce oxidative stress directly by preventing free radical formation and indirectly by increasing the activity of key antioxidant enzymes (Annunziata et al., 2020). While some

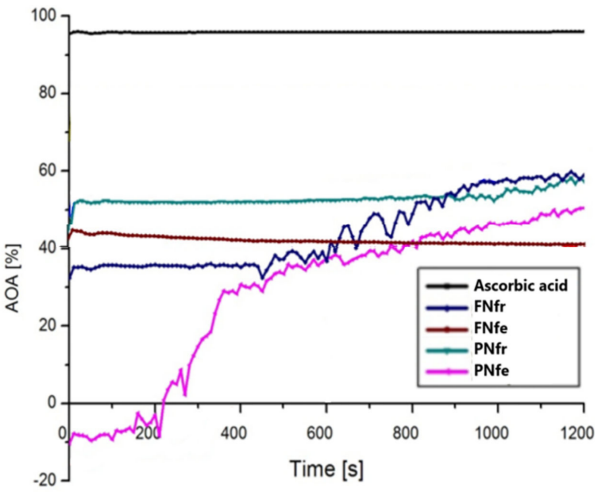

FIGURE 5 | Antioxidant activity evaluation with DPPH test. FNfe, Feteasca neagra fermented grape pomace extract; FNfr, Feteasca neagra fresh grape pomace extract; PNfe, Pinot noir fermented grape pomace extract; PNfr, Pinot noir fresh grape pomace extract.

studies have shown that natural extracts have antioxidant properties both *in vitro* and *in vivo*, other studies have shown that *in vitro* antioxidant activity does not always apply to *in vivo* models because polyphenols may also act as prooxidants (Veskoukis et al., 2012). Therefore, after demonstrating the antioxidant capacity by DPPH test, the *in vivo* antioxidant activity was tested on a turpentine-induced acute inflammation model (Lim et al., 2013). Because ROS have a short life, the *in vivo* oxidative stress assessment is generally based on the measurement of indirect markers. There are global parameters, like TOS, TAR, OSI, and NOx, and specific tests, such as molecules modified by free radicals, antioxidant enzymes, and transcription factors (Alam et al., 2013).

Turpentine administration resulted in a significant increase in TOS and OSI ($p < 0.001$) plus a TAR reduction ($p < 0.001$). Overall, the global oxidative stress parameters revealed important antioxidant properties for the evaluated GP extracts. The GP extract effects on TOS and OSI were concentration-dependent and decreased significantly TOS and OSI ($p < 0.001$), but there were no important changes of TAR ($p > 0.05$). Related to TOS and OSI, between the two *Vitis vinifera* L. varieties or between fresh and fermented GP extracts, there were no significant differences. The fermented GP samples had higher polyphenol content but that did not correlate with TOS and OSI changes (**Table 2**).

An important cellular effect of ROS is peroxidation of the phospholipids and fatty acids in the membrane, resulting in modified membrane fluidity, protein structure, and cell signaling. Such a lipid peroxidation product is MDA. Some studies indicated that MDA has mutagenic and tumor promoter potential. Therefore, we evaluated the effects of GP extracts on

TABLE 2 | *In vivo* antioxidant global tests results.

GROUP	OSI	TOS (μM H_2O_2/L)	TAR (mM TROLOX/L)
CONTROL	0.25 ± 0.02	26.92 ± 10.30	1.09 ± 0.0005
INF	0.37 ± 0.03	40.51 ± 3.34	1.09 ± 0.0008
FNfe 100%	0.26 ± 0.05	28.06 ± 5.39	1.09 ± 0.0009
FNfe 50%	0.21 ± 0.03	23.38 ± 2.80	1.09 ± 0.0007
FNfe 25%	0.27 ± 0.08	29.35 ± 8.25	1.09 ± 0.0001
FNfr 100%	0.26 ± 0.04	28.84 ± 4.44	1.09 ± 0.0005
FNfr 50%	0.24 ± 0.04	25.72 ± 4.64	1.09 ± 0.0006
FNfr 25%	0.29 ± 0.08	31.41 ± 8.25	1.09 ± 0.0012
PNfr 100%	0.26 ± 0.03	28.08 ± 3.39	1.09 ± 0.0004
PNfr 50%	0.27 ± 0.03	29.41 ± 2.87	1.09 ± 0.0006
PNfr 25%	0.28 ± 0.02	30.16 ± 1.97	1.09 ± 0.0003
PNfe 100%	0.26 ± 0.02	28.05 ± 2.82	1.09 ± 0.0010
PNfe 50%	0.28 ± 0.05	30.21 ± 4.93	1.09 ± 0.0010
PNfe 25%	0.29 ± 0.06	31.31 ± 7.01	1.09 ± 0.0007

FNfe, Feteasca neagra fermented grape pomace extract; FNfr, Feteasca neagra fresh grape pomace extract; PNfe, Pinot noir fermented grape pomace extract; PNfr, Pinot noir fresh grape pomace extract; TOS, total oxidative status; TAR, total antioxidant reactivity; OSI, oxidative stress index.

the MDA formation induced by the experimental inflammation. Turpentine administration increased MDA significantly ($p < 0.01$). All GP extracts had moderate inhibitory effect on MDA formation ($p < 0.01$). These results may be linked to the finding that flavan-3-ols monomers reduce LDL oxidizability through their incorporation into the LDL particles and the radical trapping effects (Annunziata et al., 2018; Annunziata et al., 2019) and to our previous study which reported an important content of flavan-3-ols monomers in the GP extracts (Balea et al., 2018b; Balea et al., 2018a). There were no significant differences between the effects on MDA of the two grape varieties or between fresh and fermented GP extracts of the same variety ($p > 0.05$) (**Table 3**).

In the plasma there are two major groups of thiols: protein thiols, mainly albumin thiols, and nonprotein thiols or small molecules thiols, such as cysteine (Cys), cysteinylglycine, glutathione, homocysteine and γ-glutamylcysteine. Under oxidative stress conditions Cys residues are oxidized resulting in mixed disulphides between protein thiol groups and small molecules thiols, preventing protein thiol oxidation (Yang and Guan, 2015). These disulphide bonds are reversible, creating a dynamic thiol–disulphide homeostasis which is important in antioxidant protection. The dynamic thiol–disulphide dysbalance is implicated in the pathophysiology of many diseases, including cancer (Emre et al., 2017). Turpentine-induced oxidative stress significantly reduced SH ($p < 0.001$). The pretreatment with GP extracts increased SH ($p < 0.001$) in a concentration-dependent way. The fresh GP extracts were more efficient, with no significant differences between the two *Vitis vinifera* L. varieties (**Table 3**).

In inflammation NF-kB is a pleiotropic transcription factor that regulates the expression of genes like those for chemokines, cytokines, cell adhesion molecules, growth factors, antioxidant enzymes, iNOS, and others (Silva et al., 2018). NO produced under these conditions is an effector molecule that may have beneficial or harmful effects. At nontoxic concentrations, NO is

TABLE 3 | *In vivo* antioxidant specific tests results.

GROUP	MDA (nM/L)	SH (mM GSH/L)	NO (μM/L)	3NT (nmol/L)
CONTROL	1.09 ± 0.22	0.54 ± 0.06	33.31 ± 4.50	29.78 ± 2.39
INF	4.46 ± 0.50	0.47 ± 0.11	40.04 ± 5.44	34.76 ± 6.32
FNfe 100%	4.15 ± 0.71	0.68 ± 0.18	28.97 ± 1.76	37.80 ± 3.14
FNfe 50%	4.45 ± 0.97	0.47 ± 0.14	36.59 ± 3.79	41.80 ± 6.48
FNfe 25%	3.95 ± 0.65	0.59 ± 0.16	39.77 ± 6.29	50.40 ± 7.42
FNfr 100%	3.84 ± 0.62	0.58 ± 0.09	44.07 ± 6.20	30.70 ± 2.42
FNfr 50%	3.74 ± 0.40	0.43 ± 0.10	47.31 ± 2.41	40.50 ± 5.01
FNfr 25%	3.97 ± 0.49	0.51 ± 0.15	48.96 ± 7.64	60.20 ± 7.92
PNfr 100%	3.90 ± 0.40	0.65 ± 0.11	29.73 ± 2.91	35.54 ± 4.83
PNfr 50%	3.86 ± 1.15	0.66 ± 0.10	38.59 ± 12.02	39.87 ± 5.61
PNfr 25%	4.65 ± 0.71	0.61 ± 0.05	41.13 ± 9.27	61.55 ± 2.98
PNfe 100%	4.46 ± 1.14	0.59 ± 0.12	41.18 ± 7.11	40.52 ± 4.03
PNfe 50%	4.78 ± 0.40	0.70 ± 0.11	45.72 ± 9.98	42.30 ± 3.74
PNfe 25%	4.87 ± 1.08	0.50 ± 0.10	43.01 ± 5.48	55.21 ± 4.91

FNfe, Feteasc neagr fermented grape pomace extract; FNfr, Feteasc neagr fresh grape pomace extract; PNfe, Pinot noir fermented grape pomace extract; PNfr, Pinot noir fresh grape pomace extract; MDA, malondialdehyde; SH, total thiol; NOx, total serum nitrates and nitrates; 3NT, 3-nitrotyrosine.

an effective antioxidant *in vitro* and *in vivo*. If the synthesis is excessive, NO reacts with O_2^- producing high quantities of peroxynitrite (ONOO−), a strong oxidant, which can induce oxidative stress, nitrosative stress, and nitration stress (Lacza et al., 2009). The toxicity hypothesis indicates that high levels of NO induce mitochondrial respiratory inhibition, ATP depletion, DNA deamination, oxidation, and nitration. The hypothesis of the cytoprotective role of NO states that NO protects cells against lipid peroxidation by reaction with sulfhydryl groups in proteins (Jiang et al., 2018). Because flavonols inhibit NO production (Semwal et al., 2016) and NO accumulation (Dai et al., 2013), the reduction of NO may be correlated with the polyphenol content. It was reported that polyphenols can suppress NF-kB activation and translocation into the nucleus of the activated B cells (Annunziata et al., 2020). Plant extracts with anti-inflammatory effects mediated by iNOS inhibition and nitro-oxidative stress reduction may be an adjuvant alternative therapeutic option for tumor cells proliferation and metastasis inhibition (Jiang et al., 2018). Turpentine administration significantly increased NOx ($p < 0.001$), and pretreatment with GP extracts caused a concentration-dependent reduction of NOx, the fresh GP extracts having a stronger inhibitory effect than the fermented GP extracts. These results correlated with the DPPH test. There were no significant differences between the two *Vitis vinifera* L. varieties in the case of fresh GP extracts (**Table 3**).

3NT is a product of tyrosine nitration mediated by RNS, and it is a marker of inflammation, NO production, nitrative stress, and oxidative stress induced cellular damage (Knight et al., 2018). Induction of inflammation increased 3NT significantly ($p < 0.001$), and pretreatment with GP extracts reduced 3NT in a concentration-dependent way. There were no important differences between the *Vitis vifera* L. varieties or fresh and fermented GP samples. 3NT correlated with TOS and OSI (**Table 3**).

Because a vicious circle between inflammation, oxidative stress, and ROS formation develops, (Park et al., 2015), the antioxidant therapy involves consecutive anti-inflammatory effects and the analysis of the antioxidant activity indirectly analyzes the anti-inflammatory effects (Shahid et al., 2016). That is the reason why the antioxydant activity of the tested GP extracts can be also considered an anti-inflamatory activity.

Antiproliferative Effects

Previous data on the effect of GP extracts on cancer cells are limited and the mechanisms are not fully understood. Studies performed on colon cancer cells proved that GP extracts rich in polyphenols had cytotoxic and antiproliferative effects (De Sales et al., 2018; Pérez-Ortiz et al., 2019). Their activity depends on the concentration, target molecule, and environmental conditions. GP extracts have cytoprotective effects toward normal cells and cytotoxic effects toward cancerous cells (Brglez Mojzer et al., 2016). Many data show that polyphenols have anticancer effects due to their antioxidant and anti-inflammatory effects. Their ROS scavenging effects decrease cell proliferation and DNA oxidative damage (Lizarraga et al., 2011). Through the prooxidative effect polyphenols may induce apoptosis of the cancer cells. By inhibiting angiogenesis, polyphenols reduce tumor growth, and by reducing the adhesiveness and invasiveness of cancer cells, reduce the metastatic potential (Brglez Mojzer et al., 2016). Moreover, polyphenols such as resveratrol, quercetin, catechin, and curcumin, proved to influence mitochondrial function. Because cancer cells are high ATP consumers in order to support accelerated proliferation and associated processes, mitochondrial energy metabolism seems to be a proper target in order to cause dysfunction in cancer cells (De Sales et al., 2018). A study performed with Pinot noir GP extract from Brazil, rich in polyphenols with high antioxidant activity, on human hepatocarcinoma HepG2 cells showed that a short-term incubation increased mitochondrial respiration and antioxidant capacity and lowered glycolytic metabolism, and a long-term incubation was cytotoxic and cells died by necrosis (Brglez

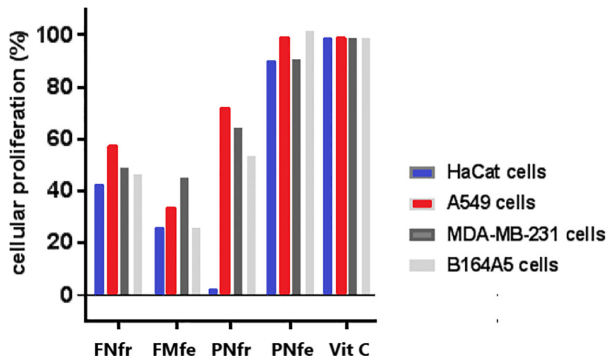

FIGURE 6 | Alamar blue cell proliferation assay. HaCat, keratinocyte; A549, human lung carcinoma; MDA-MB-231, human breast adenocarcinoma; B164A5, murine melanoma; FNfe, Fetească neagră fermented grape pomace extract; FNfr, Fetească neagră fresh grape pomace extract; PNfe, Pinot noir fermented grape pomace extract; PNfr, Pinot noir fresh grape pomace extract.

Mojzer et al., 2016). There are pieces of evidence that the antiproliferative activity of the polyphenol involves also epigenetic mechanisms, such as DNA methylation, histone changes, and micronucleotic acids (miRNAs) that modulate gene expression in cancer (Arora et al., 2019). Therefore, the antiproliferative activity of our fresh and fermented GP extracts with a rich polyphenol content and antioxidant activity was tested on cancer cell lines A549, MDA-MB-231, B164A5, and normal cells HaCat using the Alamar blue viability test.

Proliferation of all four cell lines treated with FNfr was significantly reduced: HaCat (42.58%), A549 (57.27%), MDA-MB-231 (48.89%), and B164A5 (46.59%). FNfe greatly reduced cell proliferation of all cell lines too: HaCat (26.17%), A549 (33.32%), MDA-MB-2 (41%), and B164A5 (29.95%). The antiproliferative effects of FNfe were significantly higher than those of FNfr. PNfr inhibited all cell line proliferation: HaCat (2.39%), A549 (72.36%), MDA-MB-231 (64.47%), and B164A5 (53.75%). Normal cells were found to be more sensitive to stimulation with PNfr. The smallest reductions in cell proliferation were observed for all four cell types after exposure to PNfe: HaCat (89.74%), A549 (98.82%), MDA-MB-231 (90.32%), and B164A5 (101.23%). The antiproliferative effects of PNfr proved to be much stronger than those of PNfe (**Figure 6**). These results were correlated with the polyphenols identified in the GP extracts, respectively FNfe had the highest polyphenol content and the strongest antiproliferative effect. Because the antioxidant activity of the GP extracts was better in the fresh samples, we hypothesized that the prooxidant properties of the GP fermented extracts were involved in the better anti-proliferative effect.

CONCLUSIONS

The phytochemical analysis revealed rich polyphenol content in the *Vitis vinifera* L. var. Fetească neagră and var. Pinot noir pomace extracts, the fermented GP samples having the higher polyphenol concentration. The *Vitis vinifera* L. var. Fetească neagră and var. Pinot noir pomace extracts have antioxidant and anti-inflammatory effects, and the *in vitro* and *in vivo* antioxidant activity were better in the fresh pomace extracts. *In vivo* NO reduction seems to be the cause of a stronger antioxidant effect for the fresh GP extracts. *Vitis vinifera* L. var. Fetească neagră and var. Pinot noir pomace extracts have antiproliferative effects on tested cancer cells and normal cells, and these effects correlate with the higher polyphenol content in the fermented pomace samples.

In conclusion, due to the antioxidant, anti-inflammatory and antiproliferative effects of the *Vitis vinifera* L. var. Fetească neagră and var. Pinot noir pomace extracts, these products can be considered potential agents for nutraceutical formulation in cancer prevention and treatment. Due to the higher polyphenol content the fermented GP extract might be better nutraceuticals.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca.

AUTHOR CONTRIBUTIONS

SB, AP, MP, LV, CD, and TP conceived and designed the structure of the manuscript and data collection. SB, AP, MP, LV, and TP drafted and revised the manuscript. AP, MP, and LV critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Alam, M. N., Bristi, N. J., and Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharm. J.* 21, 143–152. doi: 10.1016/j.jsps.2012.05.002

Ali, H., Dixit, S., Ali, D., Alkahtane, A. A., Alarifi, S., Ali, B. A., et al. (2016). Isolation and evaluation of biological efficacy of quercetol in human hepatic carcinoma cells. *Drug Des. Devel. Ther.* 10, 155–162. doi: 10.2147/DDDT.S95275

Andreicut, A.-D., Pârvu, A. E., Mot, A. C., Pârvu, M., Fischer Fodor, E., Cătoi, A. F., et al. (2018). Phytochemical Analysis of Anti-Inflammatory and Antioxidant Effects of *Mahonia aquifolium* Flower and Fruit Extracts. *Oxid. Med. Cell. Longev.* 2018, 1–12. doi: 10.1155/2018/2879793

Andrisic, L., Dudzik, D., Barbas, C., Milkovic, L., Grune, T., and Zarkovic, N. (2018). Short overview on metabolomics approach to study pathophysiology of oxidative stress in cancer. *Redox Biol.* 14, 47–58. doi: 10.1016/j.redox.2017.08.009

Annunziata, G., Maisto, M., Schisano, C., Ciampaglia, R., Daliu, P., Narciso, V., et al. (2018). Colon bioaccessibility and antioxidant activity of white, green and black tea polyphenols extract after in vitro simulated gastrointestinal digestion. *Nutrients* 10 (11), 1711. doi: 10.3390/nu10111711

Annunziata, G., Maisto, M., Schisano, C., Ciampaglia, R., Narciso, V., Hassan, S. T. S., et al. (2019). Effect of grape pomace polyphenols with or without pectin on TMAO serum levels assessed by LC/MS-based assay: A preliminary clinical study on overweight/obese subjects. *Front. Pharmacol.* 10, 1–11. doi: 10.3389/fphar.2019.00575

Annunziata, G., Jimenez-García, M., Tejada, S., Moranta, D., Arnone, A., Ciampaglia, R., et al. (2020). Grape polyphenols ameliorate muscle decline reducing oxidative stress and oxidative damage in aged rats. *Nutrients* 12 (5), 1280. doi: 10.3390/nu12051280

Ardi, V. C., Kupriyanova, T. A., Deryugina, E.II, and Quigley, J. P. (2007). Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proc. Natl. Acad. Sci.* 104, 20262–20267. doi: 10.1073/pnas.0706438104

Arora, I., Sharma, M., and Tollefsbol, T. O. (2019). Combinatorial epigenetics impact of polyphenols and phytochemicals in cancer prevention and therapy. *Int. J. Mol. Sci.* 20. doi: 10.3390/ijms20184567

Balea, Ş. S., Pârvu, A. E., Pop, N., Marín, F. Z., Andreicuţ, A., and Pârvu, M. (2018a). Phytochemical profiling, antioxidant and cardioprotective properties of pinot noir cultivar pomace extracts. *Farmacia* 66 (3), 432–441. doi: 10.31925/farmacia.2018.3.7

Balea, Ş. S., Pârvu, A. E., Pop, N., Marín, F. Z., and Pârvu, M. (2018b). Polyphenolic Compounds, Antioxidant, and Cardioprotective Effects of Pomace Extracts from Feteasc Neagr Cultivar. *Oxid. Med. Cell. Longev.* 2018, 8194721. doi: 10.1155/2018/8194721

Bambace, N. M., and Holmes, C. E. (2011). The platelet contribution to cancer progression. *J. Thromb. Haemost.* 9, 237–249. doi: 10.1111/j.1538-7836.2010.04131.x

Beres, C., Costa, G. N. S., Cabezudo, I., da Silva-James, N. K., Teles, A. S. C., Cruz, A. P. G., et al. (2017). Towards integral utilization of grape pomace from winemaking process: A review. *Waste Manage.* 68, 581–594. doi: 10.1016/j.wasman.2017.07.017

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical [10]. *Nature.* 181, 1199–1200. doi: 10.1038/1811199a0

Brglez Mojzer, E., Knez Hrnčič, M., Škerget, M., Knez, Ž., and Bren, U. (2016). Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. *Molecules* 21 (7), 901. doi: 10.3390/molecules21070901

Chen, A. Y., and Chen, Y. C. (2014). A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention. *Food Chem.* 138, 2099–2107. doi: 10.1016/j.foodchem.2012.11.139.A

Chen, L., Yang, S., Zumbrun, E. E., Guan, H., Prakash, S., and Nagarkatti, M. (2015). Resveratrol attenuates lipopolysaccharide-induced acute kidney injury by suppressing inflammation driven by macrophages, *Mol. Nutr. Food Res.* 59, 853–864. doi: 10.1002/mnfr.201400819

Dai, X., Ding, Y., Zhang, Z., Cai, X., and Li, Y. (2013). Quercetin and quercitrin protect against cytokine-induced injuries in RINm5F β-cells via the mitochondrial pathway and NF-κB signaling. *Int. J. Mol. Med.* 31, 265–271. doi: 10.3892/ijmm.2012.1177

De Sales, N. F. F., Da Costa, L. S., Carneiro, T.II, Minuzzo, D. A., Oliveira, F. L., Cabral, L. M. C., et al. (2018). Anthocyanin-rich Grape Pomace Extract (Vitis vinifera L.) from wine industry affects mitochondrial bioenergetics and glucose metabolism in human hepatocarcinoma HepG2 cells. *Molecules* 23, 1–17. doi: 10.3390/molecules23030611

de Souza, T. A., de Campos, C. B., De Biasi Bassani Gonçalves, A., Nunes, F. C., Monteiro, L. N., de Oliveira Vasconcelos, R., et al. (2018). Relationship between the inflammatory tumor microenvironment and different histologic types of canine mammary tumors. *Res. Vet. Sci.* 119, 209–214. doi: 10.1016/j.rvsc.2018.06.012

Emre, S., Demirseren, D. D., Alisik, M., Aktas, A., Neselioglu, S., and Erel, O. (2017). Dynamic thiol/disulfide homeostasis and effects of smoking on homeostasis parameters in patients with psoriasis. *Cutan. Ocul. Toxicol.* 36, 393–396. doi: 10.1080/15569527.2017.1311339

Farcaş, A. D., Moţ, A. C., Pârvu, A. E., Toma, V. A., Popa, M. A., Mihai, M. C., et al. (2019). In Vivo Pharmacological and Anti-inflammatory Evaluation of Xerophyte Plantago sempervirens Crantz. *Oxid. Med. Cell. Longev.* 2019, 5049643 doi: 10.1155/2019/5049643

Farcas, A. D., Mot, A. C., Zagrean-Tuza, C., Ticolea, M., Sevastre, B., Kulak, M., et al. (2019). Remarkable rutin-rich Hypericum capitatum extract exhibits anti-inflammatory effects on turpentine oil-induced inflammation in rats. *BMC Complement. Altern. Med.* 19, 1–13. doi: 10.1186/s12906-019-2680-8

Flamini, R., Mattivi, F., De Rosso, M., Arapitsas, P., and Bavaresco, L. (2013). Advanced knowledge of three important classes of grape phenolics: Anthocyanins, stilbenes and flavonols. *Int. J. Mol. Sci.* 14, 19651–19669. doi: 10.3390/ijms141019651

Ganeshpurkar, A., and Saluja, A. K. (2017). Protective effect of rutin on humoral and cell mediated immunity in rat model. *Chem. Biol. Interact.* 273, 154–159. doi: 10.1016/j.cbi.2017.06.006

García-Lomillo, J., and González-SanJosé, M. L. (2017). Applications of Wine Pomace in the Food Industry: Approaches and Functions. *Compr. Rev. Food Sci. Food Saf.* 16, 3–22. doi: 10.1111/1541-4337.12238

Gidaro, M. C., Astorino, C., Petzer, A., Carradori, S., Alcaro, F., Costa, G., et al. (2016). Kaempferol as Selective Human MAO-A Inhibitor: Analytical Detection in Calabrian Red Wines, Biological and Molecular Modeling Studies. *J. Agric. Food Chem.* 64, 1394–1400. doi: 10.1021/acs.jafc.5b06043

Jiang, Y.-X., Dai, Y.-Y., Pan, Y.-F., Wu, X.-M., Yang, Y., Bian, K., et al. (2018). Total Flavonoids from Radix Glycyrrhiza Exert Anti-Inflammatory and Antitumorigenic Effects by Inactivating iNOS Signaling Pathways. *Evidence-Based Complement. Altern. Med.* 2018, 1–10. doi: 10.1155/2018/6714282

Kim, W., Son, B., Lee, S., Do, H., and Youn, B. (2018). Targeting the enzymes involved in arachidonic acid metabolism to improve radiotherapy. *Cancer Metastasis Rev.* 37 (2–3), 213–225. doi: 10.1007/s10555-018-9742-0

Knight, A. R., Taylor, E. L., Lukaszewski, R., Jensen, K. T., Jones, H. E., Carré, J. E., et al. (2018). A high-sensitivity electrochemiluminescence-based ELISA for the measurement of the oxidative stress biomarker, 3-nitrotyrosine, in human blood serum and cells. *Free Radic. Biol. Med.* 120, 246–254. doi: 10.1016/j.freeradbiomed.2018.03.026

Lacza, Z., Pankotai, E., and Busija, D. W. (2009). Mitochondrial nitric oxide synthase: Current concepts and controversies. *Front. Biosci.* 14, 4436–4443. doi: 10.2741/3539

Lim, K. H., Ko, D., and Kim, J. H. (2013). Cardioprotective potential of Korean red ginseng extract on isoproterenol-induced cardiac injury in rats. *J. Ginseng Res.* 37, 273–282. doi: 10.5142/jgr.2013.37.273

Lizarraga, D., Vinardell, M. P., Noé, V., van Delft, J. H., Alcarraz-Vizán, G., van Breda, S. G., et al. (2011). A Lyophilized Red Grape Pomace Containing Proanthocyanidin-Rich Dietary Fiber Induces Genetic and Metabolic Alterations in Colon Mucosa of Female C57BL/6J Mice. *J. Nutr.* 141 (9), 1597–1604. doi: 10.3945/jn.110.133199

Lo, C., Lai, T. Y., Yang, J. S., Yang, J. H., Ma, Y. S., Weng, S. W., et al. (2011). Gallic acid inhibits the migration and invasion of A375.S2 human melanoma cells through the inhibition of matrix metalloproteinase-2 and Ras. *Melanoma Res.* doi: 10.1097/CMR.0b013e3283414444

Martinez-Zapata, M., Vernooij, R., Uriona Tuma, S., Stein, A., Moreno, R., Vargas, E., et al. (2016). Phlebotonics for venous insufficiency (Review) Summary of

findings for the main comparison. *Cochrane Database Syst Rev.* 4 (4), CD003229. doi: 10.1002/14651858.CD003229

Pérez-Ortiz, J. M., Alguacil, L. F., Salas, E., Hermosín-Gutiérrez, I., Gómez-Alonso, S., and González-Martín, C. (2019). Antiproliferative and cytotoxic effects of grape pomace and grape seed extracts on colorectal cancer cell lines. *Food Sci. Nutr.* 7 (9), 2948–2957. doi: 10.1002/fsn3.1150

Park, M.-Y., Kim, J.-M., Kim, J.-S., Choung, M.-G., and Sung, M.-K. (2015). Chemopreventive Action of Anthocyanin-rich Black Soybean Fraction in APC (Min/+) Intestinal Polyposis Model. *J. Cancer Prev.* 20, 193–201. doi: 10.15430/JCP.2015.20.3.193

Petrillo, A., Laterza, M. M., Tirino, G., Pompella, L., Ventriglia, J., Pappalardo, A., et al. (2018). Systemic-inflammation-based score can predict prognosis in metastatic gastric cancer patients before first-line chemotherapy. *Futur. Oncol.* 16 (24), 2493–2505. doi: 10.2217/fon-2018-0167

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., et al. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxid. Med. Cell. Longev.* 2017, 8416763. doi: 10.1155/2017/8416763

Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic. Biol. Med.* 49, 1603–1616. doi: 10.1016/j.freeradbiomed.2010.09.006

Sadej, R., and Skladanowski, A. C. (2012). Dual, enzymatic and non-enzymatic, function of ecto-5'-nucleotidase (en, CD73) in migration and invasion of A375 melanoma cells. *Acta Biochim. Pol.* 59 (4), 647–652. doi: 10.18388/abp.2012_2105

Semwal, D. K., Semwal, R. B., Combrinck, S., and Viljoen, A. (2016). Myricetin: A dietary molecule with diverse biological activities. *Nutrients* 8, 1–31. doi: 10.3390/nu8020090

Shahid, A., Ali, R., Ali, N., Hasan, S. K., Rashid, S., Majed, F., et al. (2016). Attenuation of genotoxicity, oxidative stress, apoptosis and inflammation by rutin in benzo(a)pyrene exposed lungs of mice: Plausible role of NF-κB, TNF-α and Bcl-2. *J. Complement. Integr. Med.* 13, 17–29. doi: 10.1515/jcim-2015-0078

Silva, G. Á. F., Nunes, R. A. L., Morale, M. G., Boccardo, E., Aguayo, F., and Termini, L. (2018). Oxidative stress: therapeutic approaches for cervical cancer treatment. *Clinics (Sao Paulo).* 73, 1–10. doi: 10.6061/clinics/2018/e548s

Song, H., Zhang, X., Wang, W., Liu, R., Zhao, K., Liu, M., et al. (2018a). Neuroprotective mechanisms of rutin for spinal cord injury through anti-oxidation and anti-inflammation and inhibition of p38 mitogen activated protein kinase pathway. *Neural Regen. Res.* 13, 128. doi: 10.4103/1673-5374.217349

Song, H., Zhang, X., Wang, W., Liu, R., Zhao, K., Liu, M., et al. (2018b). Neuroprotective mechanisms of rutin for spinal cord injury through anti-oxidation and anti-inflammation and inhibition of p38 mitogen activated protein kinase pathway. *Neural Regen. Res.* 13, 128. doi: 10.4103/1673-5374.217349

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., et al. (2014). Natural bioactive compounds from winery by-products as health promoters: A review. *Int. J. Mol. Sci.* 15, 15638–15678. doi: 10.3390/ijms150915638

Toiu, A., Mocan, A., Vlase, L., Pârvu, A. E., Vodnar, D. C., Gheldiu, A.-M., et al. (2018). Phytochemical composition, antioxidant, antimicrobial and in vivo anti-inflammatory activity of traditionally used Romanian Ajuga laxmannii (Murray) Benth. ("nobleman's beard" - barba împăratului). *Front. Pharmacol.* 9. doi: 10.3389/fphar.2018.00007

Toyohara, J., Sakata, M., Oda, K., Ishii, K., and Ishiwata, K. (2013). Longitudinal observation of [11C]4DST uptake in turpentine-induced inflammatory tissue. *Nucl. Med. Biol.* 40, 240–244. doi: 10.1016/j.nucmedbio.2012.10.008

Veskoukis, A. S., Kyparos, A., Nikolaidis, M. G., Stagos, D., Aligiannis, N., Halabalaki, M., et al. (2012). The antioxidant effects of a polyphenol-rich grape pomace extract in vitro do not correspond in vivo using exercise as an oxidant stimulus. *Oxid. Med. Cell. Longev.* 2012, 185867. doi: 10.1155/2012/185867

Wang, W., Wu, Q., Sui, Y., Wang, Y., and Qiu, X. (2017). Rutin protects endothelial dysfunction by disturbing Nox4 and ROS-sensitive NLRP3 inflammasome. *Biomed. Pharmacother.* 86, 32–40. doi: 10.1016/j.biopha.2016.11.134

Xu, Y., Burton, S., Kim, C., and Sismour, E. (2016). Phenolic compounds, antioxidant, and antibacterial properties of pomace extracts from four Virginia-grown grape varieties. *Food Sci. Nutr.* 4, 125–133. doi: 10.1002/fsn3.264

Yang, Y., and Guan, X. (2015). Rapid and thiol-specific high-throughput assay for simultaneous relative quantification of total thiols, protein thiols, and nonprotein thiols in cells. *Anal. Chem.* 87, 649–655. doi: 10.1021/ac503411p

Zatroch, K. K., Knight, C. G., Reimer, J. N., and Pang, D. S. J. (2017). Refinement of intraperitoneal injection of sodium pentobarbital for euthanasia in laboratory rats (Rattus norvegicus). *BMC Vet. Res.* 13, 4–10. doi: 10.1186/s12917-017-0982-y

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Traditional Use of Medicinal Plants in South-Eastern Serbia (Pčinja District): Ethnopharmacological Investigation on the Current Status and Comparison With Half a Century Old Data

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Balkan Peninsula is one of the most important biodiversity centers in Europe. Despite that, the usage of plant species in the traditional medicine of some Balkan regions remained largely unexplored in the past. This study aimed to collect and document data on the traditional use of medicinal plants in Pčinja district in South-Eastern Serbia, which is among the least developed regions in Serbia. Also, comparison with data collected by Dr. Jovan Tucakov, in a book called Herbal therapy was conducted. The survey was carried out using semi-structured interviews and 113 informants were interviewed. Quantitative ethnobotany factors were calculated, allowing us discussing the results. The informants reported data on 86 medicinal plants belonging to 43 families in Pčinja district. *Lamiaceae, Asteraceae*, and *Rosaceae* were the dominant locally used families. The species with the highest number of use reports were *Mentha piperita*, *Matricaria chamomilla*, and *Hypericum perforatum*. Gastrointestinal ailments, respiratory problems and skin diseases were the most frequently reported indications. Usually, the administration was primarily oral followed by topical applications. Leaves were dominantly exploited plant parts and the most frequent preparation form was infusion. Medicinal plants in Pčinja district are mainly used as a mode of primary health care for treating minor health issues. After comparing our results with the ones collected half a century ago by Dr. Jovan

Tucakov we can conclude that plant species mentioned in our investigation previously had a much wider spectrum of application.

Keywords: medicinal plants, ethnobotany, Balkan traditional medicine, ethnopharmcology, gastrointestinal ailments, Serbia

INTRODUCTION

The use of medicinal plants in Serbia for the treatment of numerous health problems has a long history and the oldest documents about this topic date back from the 14th century (The Hodoch Codex) and the 16th century (The Chilandar Medicinal Codex) (Sarić, 1989). Much of the knowledge on the use of medicinal plants has been collected during the second half of the 20th century by a university professor, Dr. Jovan Tucakov, in a book called Herbal therapy (first time published in 1973). This book represents the essential guide-book in this area. According to this author, the history of the medical culture of people from the Balkan is very interesting and complex since in this part of Europe strong influences of East and West are present. Medicinal literature of Mediterranean and other countries has been mixed and intertwined with the folk medicine of the illiterate warriors and shepherds (Tucakov, 1997). Almost every home in Serbia possesses this book and family members rely on it while practicing self-medication (Stojanović et al., 2017). Today, in Serbia, the simultaneous use of herbal preparations together with conventional drug therapy is very frequent (Samojlik et al., 2013).

During the last decade of the 20th century, the health status of the Serbian population sharply deteriorated. This was due to several causes including the dramatic break up of socialist Yugoslavia, series of wars, international sanctions and a general decline of standards of living (Nagorni-Obradović and Vuković, 2014). According to research conducted in 2015 forty percent of Serbian citizens reported that a long-term illness or health problems are somewhat more common among residents of Southern and Eastern Serbia (Lončar, 2016).

In Serbia, the poverty level is the highest in South-east regions where the economy relies on small farming, poor infrastructures and inadequate public services. As a result of economic failures, the entire region is witnessing decade-long depopulation. According to the census performed in 2002, there were 64 inhabitants per km^2. However, the census of 2011 has indicated the palpable decrease as there were 45 inhabitants living per km^2 in Pčinja district. This fact testifies about the potential disappearance of traditional knowledge.

The aim of this paper was to analyze ethnomedicinal usage of medicinal plants in Pčinja district in South-Eastern Serbia using data obtained through the semi-structured interviews of autochthonous population and to compare results with previously published ethnomedicinal studies conducted on the territory of Serbia (Jarić et al., 2007; Šavikin et al., 2013; Zlatković et al., 2014; Jarić et al., 2015). Also, we have compared the obtained results with the data collected by Dr. Jovan Tucakov in the book called Herbal therapy. The purpose of this comparison was to investigate how the usage of medicinal plants has been changing over time. More precisely to determine are there any differences in the application of medicinal plants and can we notice any decrease or diversity of use. Also, comparison with previously published data collected from researchers from surrounding territories in Serbia was performed.

MATERIAL AND METHODS

Research Area

The research was conducted in the area of Pčinja district, which extends to the south of the Republic of Serbia covering an area of 3,520 square kilometers (3.98% of the territory of the Republic of Serbia). In the east, it borders with Bulgaria, in the south with North Macedonia and Albania, in the west with Kosovo and Metohija and in the north with the district of Jablanica. The district includes municipalities of Vranje, Bujanovac, Bosilegrad, Preševo, Trgovište, Vladičin Han, and Surdulica (Marković and Stevović, 2016).

Pčinja district is the region in South Serbia which is extremely poor. Population aging and negative demographic trends are present, literacy level is low (especially in women over 65 in rural households), socio-economic development is low and high level of depopulation is present. Absence or low quality of road infrastructures, limited access to social and public services, traditional agricultural production on small households characterize the majority of municipalities, especially rural ones. Although health care is available and free for all citizens regardless of social status, Serbian healthcare system has been severely underfunded for decades and therefore the standard of available healthcare has poor quality. Equipment and facilities were not modernized for many years. On the other hand, human resources strategy was not appropriate for decades and education policy was not coordinated with the needs of health care, so the number of unemployed doctors was constantly increasing. At the same time, there was insufficient number of some specialist (radiologist, anaesthesiologist and cardiac surgeons). Low salaries and high unemployed rates create an incentive for doctors to emigrate (Stošić and Karanović, 2014).

Due to that reason, self-medication is popular among inhabitants, as well as simultaneous use of herbal preparations together with conventional drug therapy. The most common causes for going to the Health Centre general practitioner are respiratory and cardiovascular diseases. Also, there is an increase in the number of patients with neurological diseases and substance abuse (which represent a major health and socio-economic problem) (Mužik and Karajičić, 2014).

Ethnomedicinal Survey

Data was collected using semi-structured ethnomedicinal interviews during the period March-September 2015. A total of

113 informants were interviewed. No special criteria for the selection of the informants were used. The majority of informants (109) were Serbian nationality, one was Macedonian and three did not declare. The age of informants was between 17 and 74, with an average value of 48 years (**Table 1**) and the number of male and female informants was 30 (27.4%) and 81 (72.6%).

Interviews were conducted by researchers of the Institute for medicinal plant research "Dr. Josif Pančić". They were equipped with appropriate terrain vehicles and with knowledge of common locations known for tradition in collecting medicinal plants since they have rich experience in conducting pharmacognostic and resource field research in Serbia. Also, researchers were familiar with the information provided from small and medium collectors of medicinal plants from various parts of Serbia with whom the Institute has long-term cooperation in medicinal plants trade.

For this study, the informants were selected based on the recommendations of local village leader or by known collectors of medicinal plants. Also, in each village, we look for potential informants in gathering places of elderly people and we asked for information about other potential informants through a snowball sampling. Explanations of the background and the aim of the study were provided to the potential informants before starting the surveys. Only those who claimed to know the plants and their medicinal uses were interviewed.

Interviews were conducted orally with all respondents. When it was possible, ethnomedicinal interviews were combined with guided tours, to the locations where the informants usually collect medicinal plants or to the market places, where the informants identified plant material. Moreover, for the identification, researchers were equipped with herbarium, photos of the medicinal plants as well as the relevant literature - Flora of Serbia (Josifović, 1970–1977; Sarić and Diklić, 1986) and Flora of Europe (Tutin et al., 1964–1980; Tutin et al., 1993).

The informants were asked to list all the plants they use in the treatment of different health issues. In particular, the interview included the following questions: respondent name, sex, age, residence, nationality, profession, local names of the plants they use, plant part(s) used, preparation/administration, and folk medicinal uses. Vouchers were collected and dried for herbarium preparations, while photographs were taken for easier identification with the help of standard literature: Flora of Serbia (Josifović, 1970–1977; Sarić and Diklić, 1986) and Flora of Europe (Tutin et al., 1964–1980; Tutin et al., 1993). Some of the plant species were bought in pharmacies. The identity of these commercial products was confirmed *via* their package leaflet and it was considered correct.

TABLE 1 | Demografic features of informants in Pčinja district.

	Pčinja district
Total number of informants	**113**
<20	7
20–40	21
41–60	64
61–80	21
>80	0

All reported plant species were indicated for the citations of 15 out of 17 ailment categories which were established according to the International Classification of Primary Care accepted by the WHO (Direktoratet for e-helse, 2018): general and unspecified (A), digestive (D), blood, blood-forming organs and immune mechanisms (B), endocrine/metabolic and nutritional (T), psychological (P), neurological (N), eye (F), ear (H), cardiovascular (K), respiratory (R), skin (S), musculoskeletal (L), urological (U), pregnancy, childbearing, family planning (W), female genital (X), and male genital (Y).

All plants cited by informants, even when only mentioned by a sole informant, have been considered. After collecting data, plants were ranked based on the number of times that their uses were mentioned by the participants.

Each time a plant was mentioned as "used" it was considered as one "use-report" (UR). If one informant used a plant to treat more than one disease in the same category, it was considered as a single use-report (Trotter and Logan, 1986).

Data Analysis

The collected ethnomedicinal data were analyzed to obtain information about frequency and percentage of families, the number of most cited plant species and their uses, the most commonly used plant parts as well as preparation methods. The data collected during the field study was sorted in Microsoft Excel and further evaluated by quantifying the use reports according to previously published literature (Trotter and Logan, 1986). Also, informant consensus factor was calculated according to the equitation: FIC = (Nur-Nt)/(Nur-1), where Nur presents the number of use citations in each ailment category and Nt presents the number of species used (Trotter and Logan, 1986). This means that FIC values would be low (near 0) if plants were chosen randomly or if informants do not exchange information about their use. FIC values would be high (near 1) if there were well-defined selection criteria and/or if informants exchange information about plant usage (Teklehaymanot and Giday, 2007).

The obtained data were statistically analyzed using appropriate tests for the determination of statistical significance (chi-square test of independence with correction of continuity according to Yeats).

We have compared the obtained results with the data collected by Dr. Jovan Tucakov in the book called Herbal therapy. The purpose of this comparison was to investigate how the usage of medicinal plant has been changing over time. Data presented in the book Herbal therapy represent knowledge gained through Dr. Jovan Tucakov's working lifetime, especially during his field research, compiled with the data from relevant scientific literature available at that time. The area of research was predominantly the territory of the today's Republic of Serbia, but data relating to other parts of the SFR Yugoslavia, the state that existed at the time, are also presented in the book. Unfortunately, precise methodology has not been described.

Also, in order to compare traditional plant use in investigated district with neighboring areas, four previously published ethnomedicinal studies conducted on the territory of Serbia were considered (Jarić et al., 2007; Šavikin et al., 2013; Zlatković et al., 2014; Jarić et al., 2015), having in mind these studies were performed in the closest proximity of the investigated region.

RESULTS AND DISCUSSION

Ethnomedicinal Survey

The results obtained during our study are presented in **Table 2** where plants are arranged in alphabetical order of their botanical names. For each plant, the botanical name and family, local names, part(s) used, voucher number, preparation/administration, folk medical uses and total number of use reports were reported. In the same table, we have provided data from Herbal therapy written by Dr. Jovan Tucakov that we have used for comparison.

According to our study, botanical remedies comprise 85 plant species belonging to 43 families. Number of plant species cited by younger (< 40 years) and older informants (> 40 years) significantly differed in Pcinja district (p=0.01). Also, a statistically significant difference was observed among males and females on the number of plant species used for treatment of various health disorders (p=0.02). The predominant botanical families were *Lamiaceae* (30% of species), *Asteraceae* (26% of species) and *Rosaceae* (19% of species). These families include many cosmopolitan medicinal plant species that can be easily reached in the investigated area's ecosystems. So, at least partially their wide application in traditional medicine can be attributed to their predominance in the flora of the investigated region. Around 15% of Serbian flora comprise Balkan endemic species. Still, in our study, the traditional use of endemic species was not recorded in the investigated district.

In Pčinja district species with the highest number of use reports were *Mentha piperita* (74), *Hypericum perforatum* (48), *Matricaria chamomilla* (43), *Salvia officinalis* (33), *Urtica dioica* (31), *Thymus serpyllum* (28), and *Tilia cordata* (20). Folk medicine is primarily used for healing minor diseases with some exceptions (e.g., *Viola tricolor* and *Viola odorata* for treating malignant diseases). The most frequent medicinal uses were for treating diseases of the digestive system, respiratory system and diseases of the skin and subcutaneous tissue, followed by general and unspecified diseases (such as pain, fever, malignancy and health prevention). The prominence gastrointestinal applications of medicinal plants in our study could be due to the prevalence of these ailments but also the absence of efficacious pharmaceuticals in those diverse conditions, e.g. digestive problems, diarrhea, constipation, abdominal pain, flatulence, colic. Also, among the most common causes for going to the Health Centre general practitioner are respiratory diseases and that is reflected in the uses of herbal medicines.

Table 3 indicates FIC values of the category of ailment. The level of informants' agreement was high for most ailment categories and these points out towards the great uniformity in the selection of species used to treat diseases belonging to these ailment categories. The knowledge interchange and homogenization due to knowledge transmission can be seen as a possible explanation for the existing low number of medicinal plants used in investigated districts (Cavalli-Sforza et al., 1982). The majority of the plants (72) were reported to have 1–3 different usages and the species with the most diverse uses were *Hypericum perforatum* and *Urtica dioica*. These plant species hold important place in the Serbian traditional medicine, as well as in common beliefs. It is thus believed that on the St. Georges Day (6th of May) you need to take a bath with the water in which nettle was submerged. This would contribute to general health. For the St. Johns Worth, also known in the folk medicine as Virgin Mary's grass, there is a belief existing on the South of Serbia that it got its spots after drops of water falling from the hands of the Virgin Mary to its leaves (Čajkanović, 1985).

Several different ways for preparation and administration of medicinal plants were reported. The most of all reported plants were consumed internally (79.52%), whereas 14.46% of all plants in Pčinja district were used both internally and externally and 6.02% of medicinal plants were used only externally. As far as internal consumption is concerned, in the Pčinja district, the dominant form was infusion, followed by eating fresh plant parts, using juice, tincture or decoction. Although infusions should be prepared with lighter parts of plants (leaves, herb, flowers) and decoction should be applied for extracting constituents from roots, bark, seeds, and berries, we have noticed that informants do not make such a difference. External application included infusions for gargling and rinsing, compress or fresh plant parts as cataplasm, ointments, oil extracts and tinctures. Minor preparation forms were syrup and macerate. Several medicinal plants were used as a spice.

The most used plant part was leaf (31.58%). This partially can be explained by their collection convenience. Other parts used were: flower, fruit, herb, root, seed, stem, bark, bulb, stigma, needles, and thallus.

Sixty-nine informants (64.5%) in Pčinja district confirmed that there was tradition of collecting medicinal plants in their family and 55 (51.4%) informants collect plants by themselves. In Serbia, special environmental legislation is protecting numerous medicinal and aromatic plant species. This way their rational and moderate use has been guaranteed. Some of these plant species are also mentioned by informants in investigated districts (*Alchemilla* sp., *Arctostaphylos uva-ursi*, *Betula pendula*, *Gentiana lutea*, *Juniperus communis*, *Satureja* sp., and *Vaccinium myrtillus*) (Jarić et al., 2014).

It should be noted that some of very well-known plants (e.g. *Betula pendula, Verbascum densiflorum, Aesculus hippocastanum, Humulus lupulus, Juglans regia* and *Morus nigra*) were not reported in the investigated district.

Comparison With Previously Published Data and Novelty of Uses in the Studied Area

In order to compare traditional plant use in the investigated district with neighboring areas, four previously published ethnomedicinal studies conducted on the territory of Serbia were considered (Jarić et al., 2007; Šavikin et al., 2013; Zlatković et al., 2014; Jarić et al., 2015). Though the research areas and methods are different in these studies, similarity regarding plant use and modes of application can be expected due to the fact that these areas share similar flora, and also due to the cross-cultural knowledge exchange in the past. According to our results, considering 86 plant species recorded in Pčinja

TABLE 2 | Plant species used in modern and traditional medicine of Pčinja district.

Scientific name, local Serbian name, family name, voucher number	Part(s) used	Medicinal use[a]	Preparation form	Medical use according to Tucakov (Tucakov, 1997)	Number of use reports
Achillea millefolium L., hajdučka trava, sporiš, hajdučica, *Asteraceae*, IPLB36/13	Herb	D: 8 (I: loss of appetite, stomach disorders, liver problems) R: 4 (I: cough and cold) U: 1 (I: renal complaints) K: 2 (I: circulation improvement, against hemorrhoids and palpitations) S: 1 (E: against wounds)	Infusion, Tincture	D: tonic, stomachic, antispasmodic, excessive intestinal gas and flatulence, chronic constipation and gallstones R: bronchial asthma, febrifuge U: kidney stones K: hemorrhoids S: wounds and stab wounds X: emmenagogue	16
Acorus calamus L., iđirot, *Acoraceae*, IPLB44/13	Root	D: 2 (I: digestive problems, ulcers)	Decoction	D: improving appetite, digestion, against colic, diarrhea and intestinal gas R: bronchitis	2
Allium sativum L., beli luk, *Amarillydaceae*, IPLB28/14	Bulb	T: 2 (I: high lipid level)	Fresh	D: (I: against diarrhea and parasites) K: (I: heart pain) A: (I: malaria, against typhus and infectious diseases that are results of floods) N: (E: headache, unconsciousness) S: (E: for skin diseases, against hair loss)	1
Allium ursinum L., sremuš, *Amarillydaceae*, IPLB26/14	Leaf Root Bulb	D: 3 (I: stimulation of digestive system) T: 1 (I: source of vitamin C)	Fresh, Tincture	/	4
Althaea officinalis L., beli slez, *Malvaceae*, IPLB32/14	Root Leaf Flower	R: 11 (I: cough) U: 1 (I: urinary tract infections)	Macerate, Infusion	Data only for root: R: (I: inflammation of respiratory tract) X: (E: flushing of genital tract) G: (I: against diarrhea)	12
Anethum graveolens L., mirođija, *Apiaceae*, IPLB21/14	Leaf	D: 1 (I: digestive complaints)	Spice	G: (I: better digestion, against flatulence) N: (I: insomnia) C: (I: hemorrhoids)	1
Arctostaphylos uva-ursi (L.) Spreng., medveđe grožđe, uva, *Ericaceae*, IPLB17/14	Leaf	U: 3 (I: urinary tract infections)	Infusion	U: (I: urinary infections, kidney stone)	3
Armoracia rusticana P.Gaertn., B.Mey. & Scherb., hren, ren, *Brassicaceae*, IPLB18/13	Root	D: 1 (E: stomach pain)	Compress	/	1
Aronia melanocarpa (Michx.) Elliott, aronija, *Rosaceae*, IPLB25/13	Leaf Fruit	T: 1 (I: diabetes) K: 1 (I: high blood pressure) B: 3 (I: anemia, weakened immune system)	Fresh, Juice, Infusion	/	5
Artemisia absinthium L., beli pelin, *Asteraceae*, IPLB17/13	Flower	D: 2 (I: gastrointestinal and liver complains) U: 1 (I: renal complaints)	Infusion	/	3
Beta vulgaris L., cvekla, *Amaranthaceae*, IPLB67/14	Root	B: 1 (I: anemia)	Juice, Fresh	/	1
Brassica nigra (L.) K. Koch., crna slačica, *Brassicaceae*, IPLB33/13	Seed	D: 1 (I: liver complaints) T: 1 (I: diabetes) L: 1 (I: rheumatism)	Decoction	R: (E: bronchitis, mild pneumonia) L: (E: rheumatism)	3
Calendula officinalis L., neven, *Asteraceae*, IPLB53/13	Flower, Herb	S: 8 (E: against burns, wounds and skin complaints) D: 3 (I: digestive and liver complaints) K: 1 (E: vein problems) L: 2 (E: bone pain)	Fat based ointments, infusion, tincture	S: (E: scaly tinea, impetigo, wounds and skin ulcers, bee and wasp sting) X. (I: irregular menstrual bleeding; E: vaginal irrigation) D: (I: stomach, gut and gallbladder diseases)	14
Camellia sinensis L. Kuntze, zeleni čaj, *Theaceae*, IPLB1-T/13	Leaf	T: 4 (I: detoxification, antioxidant) S: 1 (E: burns) K: 1 (I: high blood pressure)	Infusion, compress	T: (I: antidote in cases of alkaloids and heavy metals poisoning) D: (I: for some stomach diseases) U: (I: mild diuretic)	6
Capsicum annuum L., crvena paprika, Solanaceae, IPLB73/13	Fruit	L: 1 (E: rheumatism)	Compress	D: (I: improves digestion, stomach pain) P: (I: alcoholism)	1

(Continued)

TABLE 2 | Continued

Scientific name, local Serbian name, family name, voucher number	Part(s) used	Medicinal use[a]	Preparation form	Medical use according to Tucakov (Tucakov, 1997)	Number of use reports
Carum carvi L., kim, *Apiaceae*, IPLB101/13	Fruit	D: 2 (I: flatulence, meteorism) U: 1 (I: diuretic)	Decoction	D: (I: improves digestion) U: (I: diuretic) X: (I: galactagogue)	3
Cetraria islandica L., islandski lišaj, *Parmeliaceae*, IPLB86/14	Thallus	R: 2 (I: cough)	Decoction	R: (I: cough relief) G: (I: against nausea and vomiting, tonic and stomachic, against chronic diarrhea)	2
Chelidonium majus L., rusopas, rusa, *Papaveraceae*, IPLB8/13	Leaf Flower	D: 1 (I: liver complaints)	Infusion, Juice	S: (E: cutaneous tuberculosis, warts removing) D: (I: bile production stimulation, gastrointestinal pain) R: (I: asthma)	1
Cichorium intybus L., vodopija, cikorija, gologuza, *Asteraceae*, IPLB40/14	Stem	D: 1 (I: diarrhea)	Infusion	D: (I: improving of appetite, bile ducts cleaning, inflammation of gut and stomach inflammation)	1
Cinnamomum verum J. Presl, cimet, *Lauraceae*, IPLB12-T/14	Bark	A: 1 (E: antiseptic)	Infusion	/	1
Citrus x limon (L.) Osbeck., limun, *Rutaceae*, IPLB7-T/14	Fruit Leaf	T: 2 (I: source of vitamin C) B: 1 (I: weakened immune system)	Juice	/	3
Cornus mas L., dren, drenjina, *Cornaceae*, IPLB14/14	Fruit	D: 1 (I: diarrhea)	Infusion	D: (I: diarrhea, treatment of gastrointestinal organs)	1
Crataegus monogyna Jacq., glog, *Rosaceae*, IPLB10/13	Leaf Flower Fruit	K: 8 (I: heart failure) R: 1 (I: respiratory complaints) T: 1 (I: rich source of vitamin C)	Infusion, Juice	K: (I: arrhythmia, arteriosclerosis and hypertension)	10
Cucurbita pepo L., tikva, *Cucurbitaceae*, IPLB114/14	Seed	Y: 1 (I: benign prostate hyperplasia)	Fresh, Mixed with honey	S: fresh or cooked pulpa for treatment of psoriasis)	1
Cydonia oblonga Mill., dunja, *Rosaceae*, IPLB99/13	Leaf	D: 1 (I: diarrhea)	Infusion	R: (I: antitussive agent) S: (E: for burns)	1
Cynara scolymus L., artičoka, *Asteraceae*, IPLB82/14	Flower	T: 1 (I: high lipid level)	Tincture	G: (I: improves digestion, choleretic and cholagogue) U: (I: mild diuretic)	1
Daucus carota L., šargarepa, *Apiaceae*, IPLB86/13	Root	F: 1 (I: good for improving eyesight)	Fresh	D: (I: juice is used for threadworms, against diarrhea) T: (I: juice is used for diabetes)	1
Equisetum arvense L., rastavić, preslica *Equisetaceae*, IPLB6/13	Herb	U: 2 (I: urinary tract ailments and infections) B: 1 (I: anemia) R: 1 (I: respiratory complaints)	Infusion	U: (I: diuretic)	4
Ficus carica L., smokva, *Moraceae*, IPLB116/14	Fruit	D: 1 (I: digestive complaints)	Fresh, Juice	R: (I: expectorant, as a component in tea mixtures for lungs) D: (in combination with senna leaves)	1
Foeniculum vulgare Mill., morač, komorač, *Apiaceae*, IPLB85/13	Fruit	D: 3 (I: digestive complaints)	Infusion, decoction, fresh	D: (I: improving of appetite, beneficial for good digestion, diminish consequences of incomplete and slow digestion)	3
Fragaria vesca L., jagoda, *Rosaceae*, IPLB77/14	Fruit	T: 1 (I: high lipid level)	Fresh, Juice	Rhizome: D: (I: against diarrhea, dysentery, gastrointestinal inflammations) K: (E: hemorrhoids) U: (I: diuretic)	1
Gentiana lutea L., lincura, *Gentianaceae*, IPLB12/12	Root	D: 3 (I: digestive problems, loss of appetite) K: 2 (E: vein complaints)	Tincture	D: (for stomach, against fever) S: (I: for wounds) R: (I: root is cooked and tea is used against cough)	5
Geranium macrorrhizum L., zdravac, *Geraniaceae*, IPLB53/14	Leaf Flower	D: 1 (I: digestive disorders)	Infusion	D: (I: treatment of inflamed gastrointestinal mucosa)	1
Ginkgo biloba L., ginko, *Ginkgoaceae*, IPLB3-T/14	Leaf	K: 1 (I: circulation disorder)	Infusion	/	1
Hedera helix L., baršljan, *Araliaceae*, IPLB105/13	Leaf	R: 1 (I: cough)	Syrup	S: (E: treatment of skin diseases, warts) L: (treatment of rheumatism)	1
Hibiscus sabdariffa L., hibiscus, *Malvaceae*, IPLB2-T/14	Flower	R: 2 (I: cold)	Infusion	/	2
Hypericum perforatum L., kantarion, *Hypericaceae*, IPLB9/12	Herb	S: 25 (E: against burns, wounds and skin complaints) D: 13 (I: gastrointestinal ailments)	Infusion, Oil extract	S: (E: cuts, burns, wounds) K: (E: hemorrhoids) D: (I: liver and gastric pain, diarrhea)	48

(Continued)

TABLE 2 | Continued

Scientific name, local Serbian name, family name, voucher number	Part(s) used	Medicinal use[a]	Preparation form	Medical use according to Tucakov (Tucakov, 1997)	Number of use reports
		K: 3 (E: circulation disorders) R: 3 (I: cold) P: 3 (I: depression, sedative) U: 1 (I: urinary complaints)			
Hyssopus officinalis L., miloduh, *Lamiaceae*, IPLB83/14	Leaf Flower	R: 1 (I: bronchitis)	Infusion	R: (I: chronic bronchitis, asthma) G: (I: stomachic)	1
Inula helenium L., oman, *Asteraceae*, IPLB60/13	Leaf Flower	R: 1 (I: cough)	Infusion	R: (I: bronchitis, cough) X: (I: irregular menstrual bleeding) S: (E: dry tinea infections and itchiness) G: (I: stomachic) L: (I: rheumatism)	1
Juniperus communis L., kleka, *Cupressaceae*, IPLB12/13	Fruit	U: 1 (I: diuretic)	Juice, Infusion	U: (I: diuretic) R: (I: common cold, cough, asthma) X,Y: (I: gonorrhea)	1
Laurus nobilis L., lovor, *Lauraceae*, IPLB47/13	Leaf	R: 1 (I: cough)	Infusion	D: (I: essential oil of leaves – carminative)	1
Lavandula angustifolia Mill., lavanda, *Lamiaceae*, IPLB19/13	Herb	P: 1 (I: anxiety, insomnia) D: 1 (I: gastrointestinal spasms and flatulence)	Infusion	S: (E: external application, for bath, rubefacient)	2
Linum usitatissimum L., lan, *Linaceae*, IPLB23/14	Seed	R: 1 (I: cough)	Infusion	D: (I: treatment of inflamed mucosa of mouth, stomach and gut, gastrointestinal inflammatory diseases)	1
Lycopodium clavatum L., prečica, *Lycopodiaceae*, IPLB41/14	Herb	D: 1 (I: liver disorders)	Infusion	S: (E: non-infectious skin rashes and various skin diseases) U: (I: bladder and kidney diseases)	1
Matricaria chamomilla L., kamilica, *Asteraceae*, IPLB74/13	Herb	S: 3 (E: skin inflammation, as mouth and eyewash, for burns) D: 22 (I: digestive problems, liver disorders) R: 15 (I: cough) L: 3 (I: anxiety and insomnia)	Infusion	S: (E: burns, wounds, skin ulcers, non-infectious skin rashes) X: (E: washing genital organs) D: (E: digestion complaints) L: (I: insomnia)	43
Melissa officinalis L., matičnjak, *Lamiaceae*, IPLB43/13	Leaf	P: 7 (I: anxiety and insomnia, depression) N: 1 (I: migraine) L: 2 (E: rheumatism, gout) S: 1 (E: wound) D: 1 (I: digestive problems)	Infusion, Tincture, Oil extract	P: (I: hysteria) D: (I: flatulence, vomiting, diarrhea) P: (I: neurasthenia)	12
Mentha x piperita L., pitoma nana, *Lamiaceae*, IPLB15/13	Herb	D: 60 (I: digestive problems, meteorism, flatulence, spasms) L: 7 (I: anxiety, insomnia) N: 1 (I: headache) A: 4 (I: fever) S: 2 (E: mouthwash)	Infusion, Spice	D: (I: intestinal gas, flatulence and cramps, digestion problems as a stomachic, mild diarrhea) P: (I: anxiolytic)	74
Mentha spicata L., divlja nana, *Lamiaceae*, IPLB30/13	Herb	D: 1 (I: stomach disorders) R: 1 (I: cough)	Infusion	/	2
Morinda citrifolia L., noni, *Rubiaceae*, IPLB10-T/14	Fruit	T: 1 (I: tonic)	Tincture	/	1
Ocimum basilicum L., bosiljak, *Lamiaceae*, IPLB24/13	Herb, Flower, Leaf	R: 10 (I: inhalation agent, cough, asthma) D: 5 (I: gastrointestinal ailments) U: 1 (I: urinary complaints) P: 1 (I: anxiety) S: 1 (E: warts)	Infusion, Compress, Spice	P: (I: for calming of exhilarated nervous system) D: (E: against intestinal gas, flatulence, and gastrointestinal complaints, for improving of appetite) U: (I: stimulates urination, against kidney inflammation) X: (I: increasing breast milk production at nursing mothers, regulation of menstrual cycle)	18
Origanum majorana L., majoran, *Lamiaceae*, IPLB39/14	Flower	D: 1 (I: digestive complaints) N: 1 (I: headache)	Infusion	S: (E: skin inflammation and wound healing)	2

(Continued)

TABLE 2 | Continued

Scientific name, local Serbian name, family name, voucher number	Part(s) used	Medicinal use[a]	Preparation form	Medical use according to Tucakov (Tucakov, 1997)	Number of use reports
Origanum vulgare L., vranilova trava, *Lamiaceae*, IPLB27/13	Herb	U: 1 (I: urinary tract infections)	Infusion	D: (I: gastrointestinal diseases, especially diarrhea) S: (E: skin and mucosal inflammation)	1
Petroselinum crispum (Mill.) Fuss, peršun, *Apiaceae*, IPLB34/13	Leaf Root Seed	U: 6 (I: urinary tract infections)	Infusion, Spice	U: (I: diuretic)	6
Pinus sylvestris L., beli bor, *Pinaceae*, IPLB38/14	Needle	R: 1 (I: respiratory disorders)	Syrup	R: (I: chronic bronchitis) L: (E: rheumatism)	1
Plantago major L., ženska bokvica, *Plantaginaceae*, IPLB16/13	Leaf	R: 3 (I: cough, bronchitis) S: 2 (E: skin complaints)	Infusion, Compress	R: (I: expectorant) S: (E: skin and mucosal inflammation) D: (I: beneficial effect on gastrointestinal system, for diarrhea, stomach cramps, gastric and duodenal ulcers)	5
Potentilla anserina L., steža, *Rosaceae*, IPLB48/14	Stem Flower	D: 1 (I: diarrhea)	Infusion	K: (I: heart pain) D: (diarrhea)	1
Primula veris L., jaglika, jagorčevina, *Primulaceae*, IPLB29/13	Root Flower Leaf	R: 5 (I: cough) K: 1 (I: vein complaints)	Decoction Infusion Tincture Syrup	R: (I: cough)	6
Prunus armeniaca L., kajsija, *Rosaceae*, IPLB35/13	Seed	T: 1 (I: source of vitamin B17)	Fresh	/	1
Pulmonaria officinalis L., plućnjak *Boraginaceae*, IPLB45/13	Leaf Flower Root	R: 1 (I: respiratory disorders)	Infusion	R: (I: expectorants) U: (I: diuretic) G: (I: antidiarrhoeic agent)	1
Punica granatum L., nar, *Lythraceae*, IPLB52/13	Bark	D: 1 (I: diarrhea)	Infusion	D: (I: against tapeworms)	1
Rosa canina L., šipurak, divlja ruža, *Rosaceae*, IPLB20/13	Fruit	A: 4 (I: fever) D: 2 (I: stomach complaints) T: 2 (I: source of vitamin C) B: 1 (I: weakened immune system)	Decoction	T: (I: source of vitamin C) D: (I: astringent in gut diseases, especially at kids diarrhea) U: (I: diuretic – Cynosbati semen)	9
Rosmarinus officinalis L., ruzmarin *Lamiaceae*, IPLB5/13	Leaf Flower	D: 2 (I: stomach complaints) K: 1 (I: cardiovascular complaints)	Infusion Spice	S: (E: stimulates hair growth) D: (I: carminative) X: (I: abortifacient)	3
Rubus vulgaris Weihe & Nees, kupina, *Rosaceae*, IPLB31/13	Leaf, Fruit	B: 1 (I: anemia)	Infusion	D: (I: chronic diarrhea) K: (I: hemmorhoides)	1
Salix caprea L., vrba iva, *Salicaceae*, IPLB61/14	Bark	L: 1 (I: rheumatic pain)	Infusion	–	1
Salvia officinalis L., žalfija, *Lamiaceae*, IPLB4/13	Herb	R: 28 (E: mouth and throat wash, cold) P: 2 (I: anxiety) D: 2 (I: for "warm" stomach) B: 1 (I: weakened immune system)	Infusion	R: (E: for mouth and throat washing in inflammatory diseases accompanied with secretion)	33
Sambucus nigra L., zova, *Adoxaceae*, IPLB23/13	Flower, Fruit, Leaf,	A: 12 (I: diaphoretic, fever) U: 1 (I: renal complaints) T: 2 (I: diabetes) K: 1 (I: cleaning the blood)	Infusion, Juice	A: (I: diaphoretic) D: (I: laxative) U: (I: diuretic)	16
Sempervivum tectorum L., čuvarkuća, *Crassulaceae*, IPLB49/14	Leaf	H: 5 (E: earache) S: 2 (E: wounds and warts)	Juice Compress, Tincture	S: (E: skin inflammation)	7
Senna alexandrina Mill., sena, *Fabaceae*, IPLB5-T/14	Leaf	D: 3 (I: laxative)	Infusion	/	3

(Continued)

TABLE 2 | Continued

Scientific name, local Serbian name, family name, voucher number	Part(s) used	Medicinal use[a]	Preparation form	Medical use according to Tucakov (Tucakov, 1997)	Number of use reports
Silybum marianum (L.) Gaertn., gujina trava, *Asteraceae*, IPLB76/14	Fruit	D: 1 (I: liver disorders)	Decoction	R: (I: asthma) A: (I: headache, tonic) D: (I: alleviates gallbladder attack, constipation) T: (I: diabetes) K: (I: hemorrhoids)	1
Stevia rebaudiana (Bertoni) Bertoni, stevia, *Asteraceae*, IPLB13-T/14	Leaf	T: 1 (I: sweetener instead of sugar)	Fresh	–	1
Symphytum officinale L., gavez, *Boraginaceae*, IPLB50/13	Flower, leaf	L: 1 (E: bone fractures, rheumatism)	Compress	L: (E: bone fracture) S: (E: for stabs, skin ulcers, old and abscess wounds) R: (I: as expectorant and for inflammation of mouth)	1
Taraxacum campylodes G.E.Haglund, maslačak, *Asteraceae*, IPLB70/13	Root, leaf, flower	D: 2 (I: laxative, liver disorder) T: 5 (I: diabetes, antioxidant) R: 1 (I: respiratory problems)	Infusion, Decoction, Spice	–	8
Teucrium chamaedrys L., podubica, *Lamiaceae*, IPLB54/14	Leaf Herb	D: 3 (I: digestive and liver complaints)	Infusion	D: (I: gallbladder problems) K: (I: hemorrhoids) S: (E: wounds) X: (E: increased vaginal discharge with no infections)	3
Teucrium montanum L., trava iva, *Lamiaceae*, IPLB12/14	Herb	D: 2 (I: digestive complaints)	Infusion	D: (I: gallbladder problems) K: (I: hemorrhoids) S: (E: wounds) X: (E: increased vaginal discharge with no infections)	2
Thymus serpyllum L., majkina dušica, *Lamiaceae*, IPLB3/13	Herb	R: 13 (I: cold, bronchitis) D: 11 (I: gastrointestinal disorders) P: 3 (I: anxiety and insomnia) X: 1 (I: gynecological complaints in women)	Infusion Syrup	D: (I: diarrhea) A: (I: infectious diseases)	28
Thymus vulgaris L., timijan, *Lamiaceae*, IPLB2/13	Leaf	R: 1 (I: respiratory disorders)	Infusion	D: (I: intestinal parasites, diarrhea) R: (I: component of mixture for whooping cough and regular cough)	1
Tilia cordata Mill., lipa, *Malvaceae*, IPLB64/13	Flower	A: 16 (I: fever, diaphoretic) P: 4 (I: anxiety)	Infusion	A: (I: diaphoretic) D: (I: for diminishing pain that accompanies stomach cramps)	20
Trifolium pratense L., detelina, *Fabaceae*, IPLB58/14	Leaf Flower	T: 1 (I: rich source of vitamins)	Salad	/	1
Tussilago farfara L., podbel, *Asteraceae*, IPLB37/13	Leaf	R: 2 (I: cough, bronchitis)	Infusion	S: (E: leaves covered with lard or oil for stabs, cuts, abscessed and inflamed skin places/area) R: (I: asthma)	2
Urtica dioica L., kopriva, *Urticaceae*, IPLB14/13	Herb Leaf Seed	T: 7 (I: detoxification) K: 10 (I: cleaning the blood, hemorrhoids) B: 7 (I: anemia) S: 3 (E: mouthwash, loss of hair) L: 2 (E: rheumatic disorders) R: 1 (I: respiratory problems) D: 1 (I: digestive problems)	Infusion, spice, tincture, juice, seed mixed with honey	D: (I: diarrhea) X: (E: increased vaginal discharge with no infections) K: (I: for hemorrhoids, against bleeding) S: (E: stimulates hair growth, against hair loss and dandruff) L: (E: rheumatism, sciatica and neuralgia)	31
Vaccinium myrtillus L., borovnica, *Ericaceae*, IPLB51/13	Fruit Leaf	B: 4 (I: anemia) U: 1 (I: diuretic)	Infusion Juice	D: (I: mild antidiarrheal agent, for acute enterocolitis in adults) U: (I: diuretic) T: (I: diabetes)	5
Vaccinium vitis-idea L., brusnica, *Ericaceae*, IPLB8-T/14	Fruit	U: 5 (I: urinary tract infections, diuretic)	Infusion	U: (I: diuretic, treatment of kidney and bladder inflammation)	5
Valeriana officinalis L., odoljen, macina trava, valerijana, *Caprifoliaceae*, IPLB55/13	Leaf Flower	P: 1 (I: sedative)	Infusion, Tincture	Data only for root P: (I: calming of nervous system) D: (I: carminative) K: (I: beneficial effect on heart)	1

(Continued)

TABLE 2 | Continued

Scientific name, local Serbian name, family name, voucher number	Part(s) used	Medicinal use[a]	Preparation form	Medical use according to Tucakov (Tucakov, 1997)	Number of use reports
Vitis vinifera L., vinova loza, *Vitaceae*, IPLB62/14	Leaf	L: 1 (I: rheumatism)	Infusion	X: (I: regulation of menstrual cycle) Juice obtained during vine pruning – U: (I: kidney stones); F: (I: for inflamed eyes)	1
Zea mays L., kukuruzna svila, *Poaceae*, IPLB90/13	Stigma	U: 3 (I: urinary complaints) L: 1 (I: rheumatic pain, gout)	Infusion	U: (I: mild diuretic and calming agent for neutralization of pressure in bladder, bladder sand, chronic cystitis, nephritis) L: (I: reducing of rheumatic pain)	4
Zingiber officinale Roscoe, đumbir, *Zingiberaceae*, IPLB6-T/14	Root	R: 2 (I: sinus disorders) B: 2 (I: weakened immune system) K: 1 (I: circulation disorder) L: 1 (I: for better muscle mobility)	Spice, Mixed with honey	D: (I: carminative, stimulation of appetite)	6

[a]*Methods of employment: I, internally; E, externally;* •*Not reported in previously conducted ethnobotanical studies in Serbia (Jarić et al., 2007; Šavikin et al., 2013; Zlatković et al., 2014; Jarić et al., 2015); IPLB – Herbarium of the Institute for Medicinal Plants Research.*

TABLE 3 | Informants consensus factor (FIC) for different ailment categories (according to International Classification of Primary Care).

Ailment category	Abbreviation	Pčinja district			
		% of use reports	Nt	Nur	FIC
General and unspecified	**A**	7.0	5	37	0.89
Blood, blood-forming organs and immune mechanism	**B**	3.4	8	18	0.59
Endocrine/metabolic and nutritional	**T**	6.4	17	34	0.51
Psychological	**P**	4.2	8	22	0.67
Neurological	**N**	0.6	3	3	0.00
Eye	**F**	0.2	1	1	0.00
Ear	**H**	1.0	1	5	1.00
Cardiovascular	**K**	6.3	11	33	0.69
Respiratory	**R**	22.5	26	113	0.78
Digestive	**D**	33.4	37	171	0.79
Skin	**S**	9.4	12	49	0.77
Musculoskeletal	**L**	2.5	10	13	0.25
Urological	**U**	2.7	14	27	0.50
Female genital	**X**	0.2	1	1	0.00
Male genital	**Y**	0.2	1	1	0.00
Pregnancy, childbearing, family planning	**W**	0.0	0	0	–

district, 23 of them were not mentioned in four cited studies from neighboring areas. Several of these species are not characteristic for the Serbian flora: *Camellia sinensis*, *Senna alexandrina*, *Cinnamomum verum* and *Morinda citrifolia*. Species mentioned in investigated area, as well as in areas used for comparison are: *Achillea millefolium*, *Allium ursinum*, *Althaea officinalis*, *Cichorium intybus*, *Equisetum arvense*, *Hypericum perforatum*, *Matricaria chamomilla*, *Mellisa officinalis*, *Plantago major*, *Primula veris*, *Sambucus nigra*, *Sempervivum tectorium*, and *Urtica dioica*. The similarity between data on medicinal plants obtained in current as well as in studies previously conducted in surrounding regions is presented in **Table 4**. Similarly, to Zlatibor district number of cited plant species per informant is lower compared to surrounding areas (Kopaonik, Suva planina, and Rtanj), suggesting the disappearance of ethnobotanical practice.

Also during this research comparison has been made with the knowledge about traditional medicine that had been recorded by Dr. Jovan Tucakov. In the book called Herbal therapy he sees traditional medicine as a "treasure chest". According to this comparison 17 plant species recorded during ethnobotanical investigation in Pčinja district have not been mentioned in the Jovan Tucakovs' book. However, for plant species mentioned by Tucakov much more diverse application is elaborated compared to modern-day accounts from Pčinja district. Examples for this are the following species: *Inula helenium*, *Juniperus communis*, *Pulmonaria officinalis*, *Symphytum officinale*, *Teucrium chamaedrys*, *Chelidonium majus* (**Table 2**). In this respect, Tucakov says for the *Teucrium chamaedrys* that it is one of the most favorable folk medicine of Serbs, especially in mountainous regions. There is a practice of making wine from *T. chamaedrys* (200 g is poured with liter of red wine and is left to stay for 8 days) which is used by the people as the cure against weaknesses, anemia and for wound washing. Another plant that Tucakov describes as one of the most favorite plants of the Serbian people is garlic. For this species, he says there are only a few local plants which acquired so much importance and in which people have such firm confidence as is the case with garlic. Consequently, Tucakov lists several examples recorded among Serbian population:„it is useful to eat garlic on a daily basis in order to fight cholera and typhus"; there is no such diarrhoea or heart pain which can not be cured with garlic and oak peel" (Tucakov, 1997).

Also, for different plant species, various plant organisms are recorded. For example, Tucakov emphasizes the use of *Fragaria vesca* rhizome in traditional medicine, while respondents in the Pčinja region use strawberry fruit for medicinal purposes. Also, while Tucakov states the application of the valerian root as a sedative, the modern-day inhabitants of the Pčinja district region use valerian leaf and flower. According to our knowledge, there are no data from investigations that could justify such an application.

TABLE 4 | Comparison between the medicinal plant uses in Pčinja district and those previously recorded in ethnobotanical studies conducted in surrounding regions.

Area	Year(s) when the field studies were conducted	Number of study participants	Number of recorded plant species	Number of plant species cited per informant	% of plant species also quoted in Pčinja district
Zlatibor district (Šavikin et al., 2013)	2011	220	69	0.31	68.12
Kopaonik (Jarić et al., 2007)	2002–2005	60	83	1.38	48.19
Suva planina (Jarić et al., 2015)	2012–2014	66	128	1.94	39.06
Rtanj (Zlatković et al., 2014)	2011–2012	37	45	1.27	40.00
Pčinja district	2015	107	86	0.80	–

Another conclusion which can be made from this comparison is that half a century ago a greater number of plant species have been used for so-called female diseases. Tucakov (1997) names as much as twelve plant species (*Achillea millefolium*, *Althaea officinalis*, *Calendula officinalis*, *Carum carvi*, *Inula helenium*, *Juniperus communis*, *Matricaria chamomilla*, *Ocimum basilicum*, *Rosmarinus officinalis*, *Teucrium chamaedrys*, *Teucrium montanum*, *Urtica dioica*, *Vitis vinifera*) that can be used for this group of diseases. One of the reasons for such a situation can be found in the fact that Serbia was in that time overwhelmingly peasants' society and visits to the doctor were not part of their culture. Also, the number of doctors was scarce.

In order to highlight new or rare uses of medicinal plants, collected data were compared with regional, national and global uses of plants. Most of the recorded species in both districts are traditionally well known, and after comparison with literature - PDR (Physicians Desk References) for Herbal Medicines (2000) some of them have shown new uses. Few examples are discussed below:

- Juice of *Sempervivum tectorum* has been used in earache treatment. This has also previously reported in traditional medicine of other regions in Serbia (Jarić et al., 2007; Šavikin et al., 2013). Also, Stojković et al. (2015) showed that the juice possesses antimicrobial activity towards clinical isolates of bacteria linked to otitis. Antimicrobial activity was tested using a microdilution assay on bacteria (*Proteus mirabilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) isolated from ear swabs of the patients suffering from the ear pain. Reference strain of *P. aeruginosa* (ATCC 27853) was the most sensitive to the influence of *S. tectorum* juice, with MIC of 0.153 mg/mL and MBC 0.290 mg/mL. In the same investigation quorum sensing functions in *Pseudomonas aeruginosa* were effectively controlled with juice obtained from leaf of *Sempervivum tectorum*.
- Corn silk is used for the treatment of gout and rheumatic pain. As a well-known diuretic corn silk (*Zea mays*) stimulates elimination of toxins and uric acid from the body, which can be a possible explanation for relieving gout, edema and arthritis. Nile and Park (2014) showed that polyphenolic compounds isolated from maize (peonidin-3-glucoside, 3'-methoxyhirsutrin, vanillic acid and ferulic acid) exhibited significant inhibition of xanthine oxidase *in vitro* in spectrophotometric assay and formation of hydroperoxide (8.2–8.7 μM) compared to standard compound allopurinol (7.5 μM).
- Gentian root is used for vein complaints. Previously, researchers showed that gentian extract as well as its compound isovitexin possess significant anti-atherogenic properties (Kesavan et al., 2016). Diet supplemented with 2% gentian root powder prevented atheroma formation in streptozotocin induced diabetic rats after 12 weeks of application. In the same study authors recorded a significant decrease of the circulating levels of total cholesterol as well as lipid deposition on the aortic arch.

CONCLUSIONS

The current study represents a useful documentation which can contribute to preserving ethnobotanical knowledge in South-Eastern Serbia. After comparing our results with the ones collected half a century ago by Dr. Jovan Tucakov, we can conclude that plant species mentioned in our investigation previously had a much wider spectrum of application. Also, information obtained during this investigation has shown lower number of used plant species when compared to other studies carried out in neighboring regions. For some well-known species new applications have been recorded what can present a good starting point for new investigations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JŽ conceived and designed the research, was enrolled in all the analyses and prepared the draft version of the

manuscript. MI and AI performed data collection, curation, and methodology. KŠ and GZ were enrolled in the analysis of data and edited the final manuscript. DS performed data collection, interpreted results, and edited the final draft of the manuscript.

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REFERENCES

Čajkanović, V. (1985). *Dictionary of Serbian popular believes about plants* (Belgrade, in Serbian.: SKZ, SANU).

Cavalli-Sforza, L. L., Feldman, M. W., Chen, K. H., and Dornbusch, S. M. (1982). Theory and observation in cultural transmission. *Science* 218, 19–27. doi: 10.1126/science.7123211

Direktoratet for e-helse (2018). ICPC-2e – English version. https://ehelse.no/icpc-2e-english-version#icpc-2e-download- (accessed 30 April 2019).

Jarić, S., Popović, Z., Mačukanović-Jocić, M., Đurđević, L., Mijatović, M., Kradžič, B., et al. (2007). An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). *J. Ethnopharmacol.* 111, 160–175. doi: 10.1016/j.jep.2006.11.007

Jarić, S., Mitrović, M., and Pavlović, P. (2014). "An ethnobotanical and ethnomedicinal study on the use of wild medicinal plants in rural areas of Serbia," in *Ethnobotany and Biocultural Diversities in the Balkans*. Eds. A. Pieroni and C. L. Quave (New York, Heidelberg, Dordrecht and London: Springer), 87–112.

Jarić, S., Mačukanović-Jocić, M., Đurđević, L., Mitrović, M., Kostić, O., Karadžić, B., et al. (2015). An ethnobotanical survey of traditionally used plants on Suva planina mountain (south-eastern Serbia). *J. Ethnopharmacol.* 175, 93–108. doi: 10.1016/j.jep.2015.09.002

Josifović, M. (1970–1977). *Flora of Serbia, I-IX* (in Serbian: Serbian Academy of Sciences and Art, Belgrade).

Kesavan, R., Chandel, S., Upadhyay, S., Bendre, R., Ganugule, R., Potunuru, U. R., et al. (2016). *Gentiana lutea* exerts anti-atherosclerotic effects by preventing endothelial inflammation and smooth muscle cell migration. *Nutr. Metab. Cardiovasc. Dis.* 26, 293–301. doi: 10.1016/j.numecd.2015.12.016

Lončar, D. (2016). Indicators of development of the health system of Serbia and the effectiveness of the current economic model in health care. *Ekonomika preduzeća* 1-2, 157–173. doi: 10.5937/ekopre1602157L

Marković, J., and Stević, S. (2016). Environmental soil quality research as prediction for sustainable orchards cultivation in Southern Serbia. *J. Agric. Sci.* 8, 151–158. doi: 10.5539/jas.v8n2p151

Mužik, R., and Karajičić, S. (2014). *Serbia: Brief health system review*. http://www.hpi.sk/en/2014/01/serbia-brief-health-system-review/ (accessed 2 April 2019).

Nagorni-Obradović, L., and Vuković, D. S. (2014). The prevalence of COPD co-morbidities in Serbia: results of a national survey. *NPJ Prim. Care Respir. Med.* 24, 14008. doi: 10.1038/npjpcrm.2014.8

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

PDR (Physicians Desk References) for Herbal Medicines (2000). Second ed., Medical Economics Company, Montvale NJ.

Samojlik, I., Mijatović, V., Gavarić, N., Krstić, S., and Božin, B. (2013). Consumers' attitude towards the use and safety of herbal medicines and herbal dietary supplements in Serbia. *Int. J. Clin. Pharm.* 35, 835–840. doi: 10.1007/s11096-013-9819-3

Sarić, M. (1989). *Medicinal plants of the Federal Republic of Serbia. special edition* (Belgrade, (in Serbian): Serbian Academy of Sciences and Art).

Šavikin, K., Zdunić, G., Menković, N., Živković, J., Ćujić, N., Tereščenko, M., et al. (2013). Ethnobotanical study on traditional use of medicinal plants in South-Western Serbia, Zlatibor district. *J. Ethnopharmacol.* 146, 803–810. doi: 10.1016/j.jep.2013.02.006

Sarić, M., and Diklić, N. (1986). *Flora of Serbia, X* (in Serbian: Serbian Academy of Sciences and Art, Belgrade).

Stošić, S., and Karanović, N. (2014). Health care in Serbia: current problems and changes. *Vojnosanit. Pregl.* 71 (11), 1055–1061. doi: 10.2298/VSP120205002S

Stojanović, N. M., Samardžić, L., Ranđelović, P. J., and Radulović, N. S. (2017). Prevalence of self-medication practice with herbal products among non-psychiatric patients from southearstern Serbia: A cross-secional study. *Saudi Pharm. J.* 25, 884–890. doi: 10.1016/j.jsps.2017.02.002

Stojković, D., Barros, L., Petrović, J., Glamočlija, J., Santos-Buelga, C., Ferreira, I. C. F. R., et al. (2015). Ethnopharmacological uses of *Sempervivum tectorum* L. in Southern Serbia: Scientific confirmation for the use against otitis linked bacteria. *J. Ethnopharmacol.* 176, 297–304. doi: 10.1016/j.jep.2015.11.014

Teklehaymanot, T., and Giday, M. (2007). Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. *J. Ethnobiol. Ethnomed.* 3, 12. doi: 10.1186/1746-4269-3-12

Trotter, R. T., and Logan, M. H. (1986). "Informant consensus: a new approach for identifying potentially effective medicinal plants," in *Plants in Indigenous Medicine and Diet, Behavioural Approaches*. Ed. N. L. Etkin (Bredfort Hills, New York: Redgrave Publishing Company), 91–112.

Tucakov, J. (1997). *Healing with plants* (Serbian: Rad, Belgrade).

Tutin, T. G., Heywood, V., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M., et al. (1964–1980). *Flora Europaea 1–5* (Cambridge: Cambridge University Press.).

Tutin, T. G., Heywood, V., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M., et al. (1993). *Flora Europaea 1. 2nd ed.* (Cambridge: Cambridge Univeristy Press).

Zlatković, B. K., Bogosavljević, S. S., Radivojević, A. R., and Pavlović, A. M. (2014). Traditional use of the native medicinal plant resource of Mt. Rtanj (Eastern Serbia): Ethnobotanical evaluation and comparison. *J. Ethnopharmacol.* 151, 704–713. doi: 10.1016/j.jep.2013.11.037

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frontiers in Pharmacology

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Alantolactone Enhances the Phagocytic Properties of Human Macrophages and Modulates Their Proinflammatory Functions

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Aim of the Study: Phagocytosis is a crucial element of innate immune defense involved in bacterial killing. The aim of our study was to evaluate the influence of alantolactone on phagocytosis and cytokines release by THP1-derived macrophages. We assessed whether antimicrobial compound alantolactone (a sesquiterpene lactone present in *Inula helenium L.*) is able to stimulate immune functions of macrophages by increase of *S. aureus* uptake, phagosome acidification and further stimulation of phago-lysosomes formation. Simultaneously, we tested influence of alantolactone on cytokines/chemokines production and p65 NF-κB concentration. The activity of alantolactone was compared with clarithromycin at concentration 20 μM.

Methods: The cytotoxicity of alantolactone as well as *S. aureus* uptake, pH of phagosomes and phago-lysosomes fusion were analysed with flow cytometry. Reactive oxygen species and superoxide production were evaluated spectrophotometrically. The efficiency of phagocytosis was evaluated *via* quantifying viable bacteria (CFU). The effect on p65 protein concentration and cytokine production by macrophages were measured by enzyme-linked immunosorbent assay (ELISA).

Results: Alantolactone enhanced phagocytosis *via* increase of *S. aureus* uptake, acidification of phagosomes, and later stimulation of phago-lysosomes fusion. Alantolactone treatment resulted in ROS and superoxide production decrease. Furthermore, alantolactone inhibited production of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 as well as decreased p65 concentration, the subunit responsible for NF-κB activation and cytokine production and simultaneously stimulated release of anti-inflammatory mediators (IL-10 and TGF-β).

Conclusion: Results of our study indicate that alantolactone enhances clearance of *S. aureus*, and simultaneously modulates immune response, preventing collateral damage of the surrounding tissues. Considering the importance of phagocytosis in the pathogen killing, alantolactone may have a great potential as the supportive treatment of *S. aureus* infections. Further *in vivo* studies are warranted to confirm this hypothesis.

Keywords: alantolactone, *Staphylococcus aureus*, THP-1, phagocytosis, intracellular killing, cytokines

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INTRODUCTION

Staphylococcus aureus, one of the world's most prolific pathogen, afflicts humans and animals' morbidity and mortality worldwide. As previously recognized, this bacteria commonly causes nosocomial infections, but some strains have a propensity to disseminate between otherwise healthy individuals, giving rise to community-acquired illnesses (Mediavilla et al., 2012). The most serious concern occurred with the emergence of multi-drug resistant strains, such as methicillin-resistant *S. aureus* (MRSA) demonstrating enhanced infectivity and virulence (Nimmo, 2012; Francis and Kuyyalil, 2018). Incredibly, these bacteria can colonize every tissue, causing pathologies varying from whole range of skin and soft tissue infections to fatal invasive diseases, such as necrotizing pneumonia and sepsis (Gonzalez et al., 2005; Labandeira-Rey et al., 2007; Saavedra-Lozano et al., 2008). The success of *S. aureus* as a pathogen can be attributed, among others, to the ability to weaken both the innate and adaptive immune responses of the host.

Phagocytic cells, mainly neutrophils and macrophages, are essential for effective host immune response to infections. The interaction of neutrophils with *S. aureus* has been thoroughly characterized (Spaan et al., 2013). Remarkably, *S. aureus* can withstand neutrophil-mediated killing, which is an impressive feature, considering the potent microbicidal capacity of the neutrophils. In contrast, the interaction of *S. aureus* with macrophages has not been studied thoroughly. Macrophages are professional phagocytes that possess large armamentarium of antimicrobial functions, and thus represent an important component of the innate immune response. What is more, macrophages can shape adaptive immunity through phagocytosis of pathogens and presentation of their antigens (Flannagan et al., 2015). Given the immune functions of the macrophages, it stands to a reason that evasion of macrophage-dependent killing of pathogens is essential for successful initiation and maintenance of an infection.

Phagocytosis is an example of canonical defense against pathogens (Tauber, 2003). Activation of membrane toll-like receptors (TLRs) by components of bacterial cell wall, such as lipoteichoic acid, leads to rearrangement of actin cytoskeleton of macrophages, and subsequently, to the internalization of a particle in the newly-formed phagosomes (Flannagan et al., 2012). Phagosome formation is not microbicidal *per se*, as the lumen of the nascent vacuole reflects the fluid phase outside the macrophage and the surrounding phagosomal membrane is derived directly from the cell membrane. However, the nascent phagosome rapidly undergoes significant biochemical remodeling, achieved by acquisition and removal of proteins and a marked decrease in pH (Pitt et al., 1992). This process of phagosome "maturation" is comprised of a series of strictly coordinated membrane fission/fusion events between the phagosome and endo/lysosomes, and leads to the formation of the mature phagolysosome, a degradative organelle possessing potent microbicidal properties (Fairn and Grinstein, 2012). The newly created phagolysosomes may be among the main effectors of pathogens killing. This statement is based on extensive correlative data associating phago-lysosomes formation with the efficiency of the pathogen killing (Sturgill-Koszycki et al., 1994; Clemens and Horwitz, 1995; Ullrich et al., 1999; Aberdein et al., 2013).

Killing pathogens in the phagolysosomes depends on activation of the phagocyte NADPH oxidase (Nox2). The NADPH oxidase catalyzes formation of highly unstable super oxide anion (O_2^-) that initiates a variety of chemical reactions, resulting in the generation of noxious reactive oxygen species (ROS) such as superoxide, peroxides, hydroxyl radical, α-oxygen and singlet oxygen (Pospíšil et al., 2019). ROS production in phagocytes serves multiple purposes, from cell signaling to microbial killing. The balance between intensity and timing of ROS production versus ROS scavenging appears to be critical for the pathogen clearance (Dupré-Crochet et al., 2013).

Intracellular killing is a highly sophisticated process, which requires a complex network of molecular pathways for successful pathogen damage (Aderem, 2003). Binding of pathogen to surface receptors, especially TLR2 and TLR4, initiates a rapid pro-inflammatory response mediated by pro-inflammatory transcription factors, mainly NF-κB (Schorey and Cooper, 2003). The effects typical for early events of the immune response mediated by NF-κB include release of both, pro- and anti-inflammatory cytokines (Hayden et al., 2006; Sur et al., 2019). It is clear that some cytokines, like IL-1β, IFNs, or IL-10, modulate phagocytic properties of macrophages (Zhai et al., 2019). They may intensify the maturation of phagosomes and intracellular killing inside of phagolysosomes (Zhai et al., 2019). However, it is poorly understood how *S. aureus* modulates the production of the other cytokines, such as IL-1β, IL-6, IL-8, INF-α, IL-12, IL-10, and TGF-β.

Stimulation of phagocytic properties of macrophages may be a promising treatment strategy. According to Hanckock et al., a novel approach involves host-directed immunomodulatory therapies, whereby natural mechanisms of the host are used to enhance the therapeutic benefit (Hancock et al., 2012). The objective is to initiate or enhance phagocytic properties of leukocytes while limiting inflammation-induced tissue injury. The latter needs proper balance between pro- and anti-inflammatory cytokines (Cicchese et al., 2018).

The aim of our study was to evaluate the biological activity of alantolactone, being the dominant compound occurring in Inula species, in context of *S. aureus* infection (Kim et al., 2017; Gierlikowska et al., 2020). We have evaluated influence of alantolactone on phagocytosis and cytokines release by macrophages. We assessed whether alantolactone, known as antimicrobial compound (Stojanović-Radić et al., 2012), is able to stimulate immune response of macrophages by increase of *S.*

Abbreviations: ROS, Reactive Oxygen Spices; SO, Superoxide; CFU, colony forming unit; MOI, multiplicity of infection; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TNFα, tumor necrosis alpha; IL-6, interleukin 6; IL-8, interleukin 8; IL-1β, interleukin 1β; IL-10, interleukin 10; IL-12, interleukin 12; TGF-β, transforming growth factor beta, INF-α, interferon alfa; PMA, Phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; PBS, phosphate buffered saline.

aureus uptake, phagosome acidification and further stimulation of phago-lysosomes formation. Simultaneously, we tested influence of alantolactone on NF-κB activity and release of cytokines/chemokines. The activity of alantolactone was compared with clarithromycin tested at 20 μM. Clarithromycin is a macrolide antibiotic, which has been shown to have the anti-microbial and immunomodulating properties (Simpson et al., 2008; Cervin et al., 2009). Some data highlight the ability of clarithromycin to stimulate phagocytic properties of macrophages (Xu et al., 1996) and neutrophils (Fietta et al., 1997).

MATERIALS AND METHODS

Chemicals and Reagents

RPMI 1640 Medium, L-glutamine, alantolactone ≥98% (HPLC), clarithromycin ≥95% (HPLC), Lysostaphin from *Staphylococcus staphylolyticus*, 12-myristate 13-acetate (PMA), DAPI solution were bought from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Fetal calf serum (FCS) and Phosphate buffered saline (PBS) were obtained from Gibco (Grand Island, NY, USA) and penicillin–streptomycin from PAA Laboratories (Pasching, Austria). Accutase™ Cell detachment solution from BD Biosciences (San Jose, CA, USA). Cellular ROS/Superoxide Detection Assays were obtained from Abcam (Cambridge, UK). *S. aureus* 25923 was bought from ATCC (Virginia, USA). Alexa 647 carboxylic acid, Alexa Fluor 633 phalloidin and TMR- and biotin-conjugated dextran were obtained from Thermo Fisher (Massachusetts, USA). NF-κB p65 Transcription Factor Assay kit was bought from Active Motif (CA, USA). Enzyme-linked immunosorbent assays (ELISA) were obtained from BD Biosciences (San Jose, USA).

Alantolactone Source and Preparation of Stock Solutions for Bioassays

For all experiments alantolactone purchased from Sigma Aldrich was used (Steinheim, Germany). The chemical structure of alantolactone is presented in **Figure 1A**. Alantolactone was dissolved in DMSO (10 mM stock solution) and tested at a concentration range of 1–20 μM. As a positive control clarithromycin tested at 20 μM was used.

Staphylococcus aureus Culture

The bacteria were cultured in basal LB medium, centrifuged and suspended in 1 mL of 0.1 M sodium bicarbonate (pH 8.3). Subsequently, 50 μL of Alexa Fluor 647 carboxylic acid (10 mg/mL DMSO) or Alexa Fluor 633 phalloidin (10 mg/mL DMSO) was added to the cell suspension and incubated for 1 h in the dark. After incubation, cells were washed 4 times in 100 mM glycine and 2 times in PBS. Eventually, bacteria were suspended in 200 μL of PBS containing 0.02% sodium azide.

Antimicrobial Properties of Alantolactone Against *Staphylococcus aureus*

Sensitivity of *S. aureus* was examined by the standard disc-diffusion method according to CLSI (previously NCCLS) guidelines (Watts et al., 2008). The results (diameter of the growth inhibition zone) were read after 18 h of incubation at 35°C. Minimal Inhibitory Concentrations (MIC) were tested by the two fold serial microdilution method (in 96-well microtiter plates) using Mueller-Hinton Broth medium (Beckton Dickinson) according to CLSI guidelines (Watts et al., 2008). Alantolactone and clarithromycin were both tested at 20 μM.

THP-1 Culture

THP-1, a monocytes cell line, was purchased from the ATCC collection. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin at concentrations of 100 units/mL and 100 μg/mL respectively. Cell culturing was performed at 37°C and 5% CO_2 in a humidified atmosphere. For differentiation, phorbol-12-myristate 13-acetate (PMA) was added to a final concentration of 10 nM. After 48 h, the PMA supplemented media were removed, cells were washed with PBS and rested in fresh PMA-free media for further 24 h in order to gain phenotypic characteristics of macrophages.

Determination of Cytotoxicity by PI Staining

Cytotoxicity of alantolactone was determined by a standard flow cytometric measurement using DAPI staining. Differentiated THP-1 cells were seeded on 12-well plates, 5×10^5 cells per well, and cultured for 24 h in a medium containing alantolactone

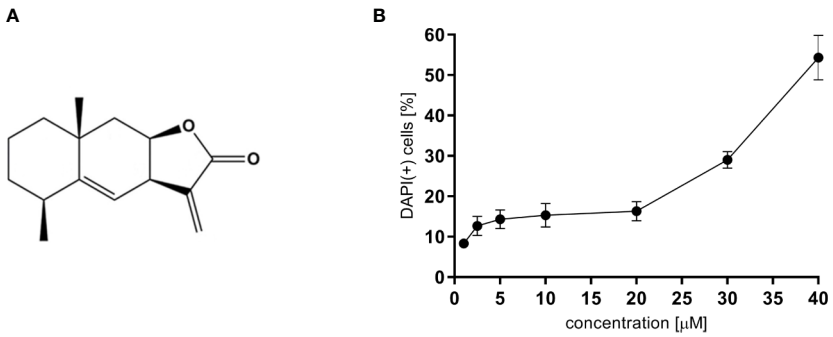

FIGURE 1 | (A) The chemical structure of alantolactone, and **(B)** the cytotoxicity of alantolactone in THP-1 cells.

in a concentration range 1-40 μM. After incubation, cells were harvested with Accutase, centrifuged (1300 RPM; 10 min; 4°C), labeled with DAPI and re-suspended in 400 μL of PBS. After 15 min of incubation with DAPI at room temperature, cytotoxic effect of alantolactone was analyzed by BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA) by recording 10,000 events per sample. Cells that displayed high permeability to DAPI were expressed as percentage of DAPI (+) cells with the loss of cell membrane integrity.

S. aureus Uptake Assay

Differentiated THP-1 cells were seeded on 6-well plates, 1.5×10^6 cells per well. Compounds were added as described above and cells were cultured for 24 h. Subsequently, pretreated cells were infected with Alexa 647-labeled *S. aureus* for 15, 30, and 60 min and 4 h. After infection, cells were washed twice on ice with ice-cold PBS, then suspended in 400 μL of cold PBS, scraped and left in tubes on ice until flow cytometry analysis. Analysis was performed on single cells as presented in the Supplementary Material (**Figure S1**). The value of geometric mean of fluorescence (GMF) as well as representative cytograms were shown in **Figures 2** and **3**. The % of positive-infected cells (cells phagocytosed one or more bacteria) has been included in the Supplementary Material (**Figure S2**). Differences between controls (uninfected vs *S. aureus*-infected cells) were visualized by Zeiss LSM 710/780 confocal microscopy after prior cell fixation with 4% formaldehyde and DAPI staining.

Phagosomal pH Measurements

For phagosomal pH measurement, macrophages were seeded on 12-well plates as described above, pretreated for 24 h with compounds (or calibration buffers) and then incubated for 1 h with heat-killed *S. aureus*-labeled with Alexa Fluor 647. Intracellular pH calibration was achieved by clamping phagosomal pH using potassium rich buffer solutions (120 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES with the pH adjusted from 4 to 8) for 30 min at 37°C. Changes in pH were measured by the intensity ratios of fluorescence using flow cytometry. The method was adapted from Di et all protocol (Di et al., 2006; Renna et al., 2011).

Phagosome-Lysosome Fusion Assay

For phagosome-lysosome fusion analysis, differentiated THP-1 cells were seeded on 12-well plates as described above, they were pretreated for 24 h with compounds and then incubated (1-h pulse, 4-h chase) with TMR- and biotin-conjugated dextran to load lysosomes and then fed IgG-coated fluorescent latex beads (30-min pulse, 2-h chase). Beads were recovered by cell disruption, the degree of bound fluorescent dextran was quantified by flow cytometry, and average geometric mean fluorescence was determined.

Superoxide and Reactive Oxygen Species (ROS) Production

For superoxide and reactive oxygen species (ROS) measurement, differentiated THP-1 were seeded on 96-well plates at a density 2×10^4 cells per well. After 24 h supernatants were removed from cells and cells were carefully washed, treated with alantolactone for 30 min and then infected with *S. aureus* for further 1 h. Generation of superoxide and total reactive oxygen species production in the real-time in live cells were quantified using a Cellular ROS/Superoxide kit. Detection Assays were performed according to the manufacturer's instructions. Fluorescence was measured using BioTek Synergy 4 Plate Reader (CA, USA) at Ex = 488 nm, Em = 520 nm and Ex = 550 nm, Em = 610 nm for the detection of ROS and superoxide, respectively. Results were expressed as percentage of fluorescence intensity normalized to the fluorescence of the positive control (*S. aureus*-infected cells).

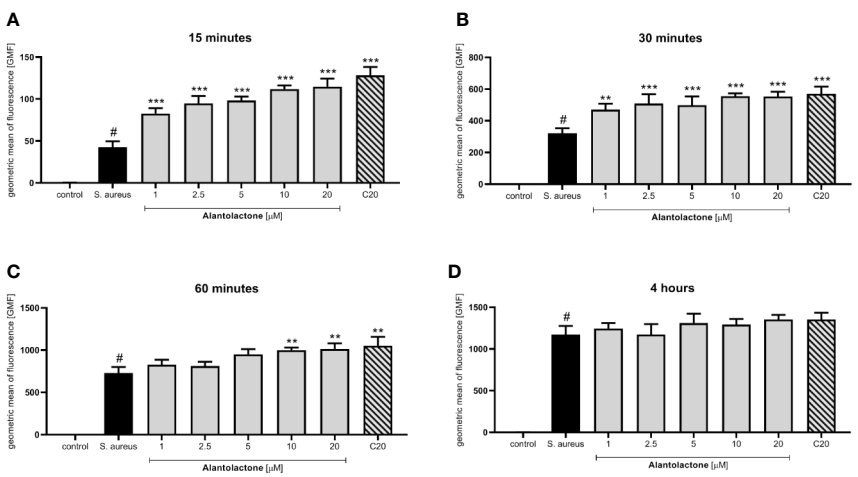

FIGURE 2 | The influence of alantolactone treatment on *S. aureus* uptake by THP-1 cells. Data show geometric mean of fluorescence (GMF) phagocytosed heat-killed *S. aureus* by THP-1 cells at different time-points: **(A)** 15 min after infection, **(B)** 30 min after infection, **(C)** 60 min after infection and **(D)** 4 hours after infection. Each experimental set was compared to *S. aureus*-stimulated control.Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus stimulated control, # statistically significant (p < 0.001) versus non-stimulated control (ANOVA and Dunnett's post hoc test); control-non-stimulated control; S. aureus - *S.aureus*-stimulated control.

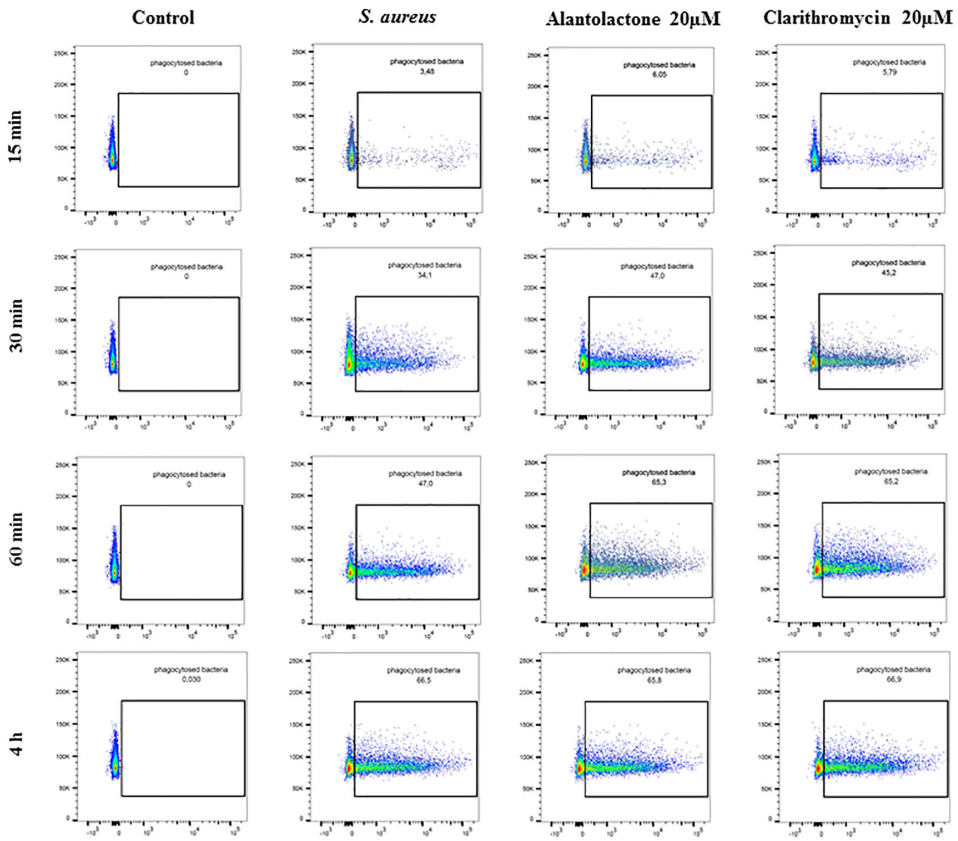

FIGURE 3 | The influence of alantolactone on S. aureus uptake by THP-1 cells at different time-points (15, 30, 60 min, and 4 h). Data show representative cytograms obtained at different time-points (15, 30, 60 min, and 4 h).

S. aureus Killing Assay

No Pre-Treated Differentiated THP-1 Cells

Differentiated THP-1 cells, seeded on 12-well plates as described above, were inoculated with *S. aureus* (MOI 1:1) for 1 h at 37°C, then washed with PBS twice, treated with 10 μg/mL lysostaphin for 30 min, washed with PBS twice and then treated for 24 h at 37°C with 0.5 μg/mL lysostaphin in the presence or absence of alantolactone or clarithromycin, as indicated. We serially diluted the suspension and plated 20 μL on TYE agar plates. Bacteria were counted after incubation at 37°C for 24 h and the amount was calculated as CFU (colony forming unit).

Pre-Treated Differentiated THP-1 Cells

Differentiated THP-1 cells were seeded as described above, they were pre-incubated with alantolactone or clarithromycin for 24 h at 37°C, then inoculated with *S. aureus* (MOI 1:1) for 12 h at 37°C, then washed with PBS twice, treated with 10 μg/mL lysostaphin for 30 min, washed with PBS twice and then treated for 24 h at 37°C with 0.5 μg/mL lysostaphin in the presence or absence of alantolactone or clarithromycin, as indicated. We serially diluted the suspension and plated 20 μL on TYE agar plates. Bacteria were counted after incubation at 37°C for 24 h and the amount was calculated as CFU (colony forming unit).

Pro- and Anti-Inflammatory Cytokine Production

Differentiated THP-1 cells were seeded on 12-well plates and infected with *S. aureus* (MOI 1:1). After 1 h, cells were treated with alantolactone or clarithromycin and cultured for 24 h. Subsequently, medium was collected and the amount of released cytokines was measured using the OptEIA™ Set (BD Biosciences, USA), which contains the components necessary to develop enzyme-linked immunosorbent assays (ELISA). The results were expressed as a percentage of the released protein compared to the *S. aureus*-stimulated control.

p65 NF-κB Concentration

Differentiated THP-1 cells were seeded on 12-well plates and infected with *S. aureus* (MOI 1:1). After 1 h, cells were treated with alantolactone or clarithromycin for 24 h. Cells were washed with ice-cold PBS, suspended in hypotonic buffer (20 mM HEPES, 5 mM NaF, 10 μM Na_2MoO_4, 0.1 mM EDTA) and left on ice for 15 min. Next, cells were centrifuged and supernatant (cytoplasmic fraction) was removed. The nuclear pellets were suspended in complete lysis buffer supplemented with protease inhibitor cocktail and left on ice for 30 min. Pellets were centrifuged for 10 min 2500 RPM and stored at -80°C for

further analysis. The protein concentration was determined by using Bradford-based assay, following manufacturer's protocol.

10 μg of nuclear fraction was incubated in 96-well plates pre-coated with immobilized oligonucleotide containing a consensus binding site for p65, following the manufacturer's instructions (Active Motif, CA, USA). The nuclear fractions were incubated with the primary antibody (1:1000 dilution) for 1 h, washed 3 times with wash buffer provided by manufacturer and incubated with HRP-conjugated antibody (1:1000 dilution) for 1 h. After incubation, plate was washed 3 times, reaction was stopped by adding stop solution and concentration of NF-κB p65 was measured spectrophotometrically at 450 nm.

Statistical Analysis

The results were expressed as the mean ± SEM from three independent experiments assayed in triplicates. All analyses were performed using Statistica 13.1 software. GraphPad Prism (version 5.01) was used to plot data. The statistical significance of the differences between means was established by ANOVA with Dunnett's *post hoc* test. P values below 0.05 were considered statistically significant.

RESULTS

Alantolactone Suppresses *S. aureus* Growth

To validate the direct impact of alantolactone on *S. aureus* viability, growth inhibition zone (GIZ) was measured. The GIZ was visible for *S. aureus* treated with alantolactone and clarithromycin tested at 20 μM. GIZ was estimated to be 14 mm for alantolactone and 23 mm for clarithromycin. Minimal inhibitory concentration (MIC) was detectable only for clarithromycin and was estimated as 0.25 μg/mL (~ 0.36 μM). Obtained results were comparable with reference values present at EUCAST (The European Committee on Antimicrobial Susceptibility Testing).

Cytotoxicity

To evaluate cytotoxic effect of alantolactone, DAPI staining was performed. Alantolactone in concentrations up to 20 μM did not influence the viability of differentiated THP-1 cells after 24 h incubation (**Figure 1B**). At 20 μM, we noticed 13.67 ± 4.93% DAPI-positive cells, which expressed % of cells with damaged cellular membrane (dead and/or necrotic) (**Figure 1**) The IC_{50} concentration against tested cells may be estimated to be 38 μM. Clarithromycin tested at 20 μM induced 10,33 ± 2,52% DAPI-positive cells.

Alantolactone Stimulates *S. aureus* Uptake

The canonical immune response of macrophages to bacterial infection is phagocytosis. The effect of alantolactone on *S. aureus* uptake was most pronounced at the beginning of the treatment (approximately 2 times higher uptake compared to *S. aureus* treated cells at 15 min) (**Figures 2**, **3**, **4** and **Figure S3**). The number of uptaken bacteria was alantolactone dose- dependent. After 1 h, the dynamics of phagocytosis changed and macrophages started to accumulate more bacteria than they were able to phagocyte (overaccumulation).

Alantolactone Treatment Leads to Phagosome Acidification

Phagosomal pH of THP-1 cells was measured in the response to heat-killed *S. aureus* suspended in sodium azide. THP-1 cells were incubated with buffer solutions for the pH adjustment from 4 to 8 for intracellular calibration. We confirmed that treatment with alantolactone (up to 5 μM) strongly stimulates phagosomal acidification ($p < 0.01$) (**Figure 5A**). Similarly to uptake results, the level of acidification was dose-dependent. The pH of the highest alantolactone concentrations (10 and 20 μM) were comparable to pH of clarithromycin tested at 20 μM.

Alantolactone Treatment Intensifies Phago-Lysosome Fusion

Matured and acidified phagosomes fuse with lysosomes forming phagolysosomes. To exclude non-specific quenching of fluorescence, analysis was performed with *S. aureus* suspended in sodium azide, as described above. *S. aureus* infection increased the phago-lysosomes fusion (**Figure 5B**). Alantolactone at the highest concentrations (10 and 20 μM) stimulated phago-lysosomal fusion by about 30%.

Alantolactone Treatment Decreases Superoxide and Reactive Oxygen Species (ROS) Production

Stimulation of macrophages with *S. aureus* resulted in increased ROS production by these cells (**Figures 6A, B**). Alantolactone treatment resulted in the reduction of the oxidative stress. The drug in dose above 2.5 μM attenuated ROS production equally to clarithromycin at 20 μM. Summarizing, the suppression of ROS production by alantolactone may maintain phagocytic properties of macrophages (especially if the infection is at an early stage).

Alantolactone Treatment Enhances Phagocytosis Efficiency

The evaluation of phagocytosis efficiency was performed on THP-1 cells infected with *S. aureus*, as described above. The measurement of CFU value was performed 24 h after starting treatment (**Figures 7A, B**). Obtained results clearly indicate that alantolactone significantly decreased CFU value at 12 h after the treatment onset, and the effect was maintained at the later time points (18 and 24 h after treatment). Clarithromycin significantly decreased CFU value, and biological effect was detectable earlier than for alantolactone (6 h after starting treatment).

Alantolactone Modulates Cytokine Production and p65 NF-κB Concentration

The stimulation with *S. aureus* resulted in a statistically significant induction of cytokines and chemokines release. Alantolactone treatment resulted in a suppression of selected pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and INF-α) production (**Figure 8**). For concentrations above 5 μM, production of

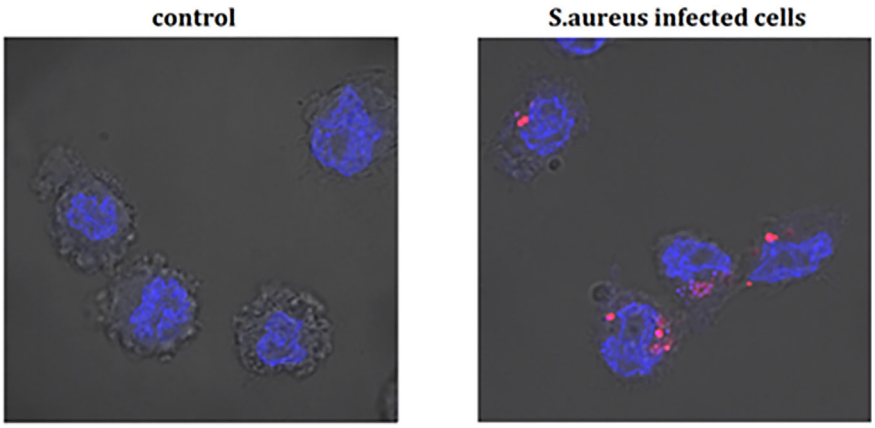

FIGURE 4 | The infection of THP-1 cells by *S. aureus*. Data show photographed controls (not infected cells) and *S. aureus* infected cells (15 min after infection). The blue marker—DAPI-labelled nucleus, the pink marker—Alexa 647 labelled-*S. aureus*.

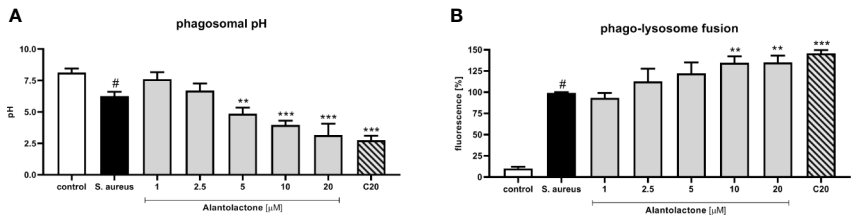

FIGURE 5 | The influence of alantolactone on **(A)** phagosomal pH and **(B)** phago-lysosome fusion. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus stimulated control (ANOVA and Dunnett's *post hoc* test); control—non-stimulated control; *S. aureus*—*S.aureus*-stimulated control. # statistically significant (p < 0.001) versus non-stimulated control.

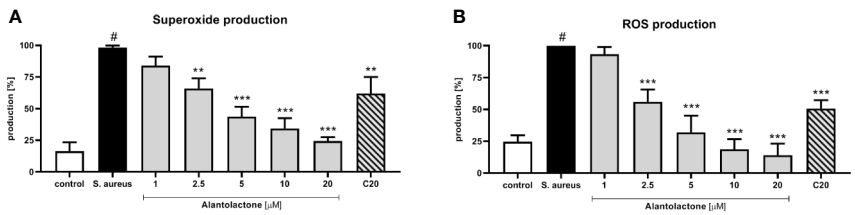

FIGURE 6 | The influence of alantolactone on **(A)** superoxide and **(B)** ROS production by *S.aureus*-stimulated THP-1 cells. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus stimulated control (ANOVA and Dunnett's *post hoc* test); control—non-stimulated control; *S. aureus*—*S.aureus*-stimulated control. # statistically significant (p < 0.001) versus non-stimulated control.

mentioned cytokines was suppressed by alantolactone in dose-dependent manner. At 1 μM (the lowest tested concentration) alantolactone was able to suppress TNF-α and IL-6 with significance $p < 0.001$ for TNF-α and $p < 0.05$ for IL-6, respectively (**Figures 8A, C**). Importantly, our observations indicated that alantolactone tested at the concentration > 5 μM stimulated IL-12 production (**Figure 8G**). Alantolactone also stimulated the production of anti-inflammatory cytokines IL-10, but only at the highest concentration (20 μM); moreover, it increased TGF-β release at the concentration > 2.5 μM (**Figures 8E, F**).

Clarithromycin at the concentration of 20 μM suppressed TNF-α, IL-1β, IL-6, and INF-α production ($p < 0.001$) and IL-8 production ($p < 0.01$) (in comparison to stimulated control 100% of release). Additionally, clarithromycin enhanced production of IL-10 and TGF-β (**Figures 8E, F**), and observed effect was comparable to alantolactone tested at 20 μM. These observations indicate the potential anti-inflammatory properties of clarithromycin (mainly known as drug with strong anti-microbial properties).

Evaluation of p65 NF-κB concentration explains the molecular mechanism responsible for previously observed cytokine concentration changes. Our data show that alantolactone at the

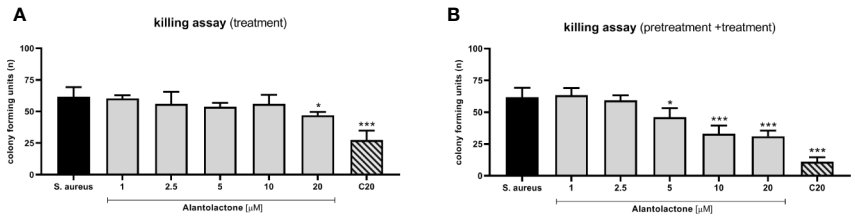

FIGURE 7 | The effect of alantolactone on *S. aureus* phagocytosis efficiency performed by **(A)** THP-1 cells treated with alantolactone after infection **(B)** and THP-1 cells pretreated before infection and treated after infection. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus stimulated control (ANOVA and Dunnett's *post hoc* test); *S. aureus*—*S. aureus*-stimulated control.

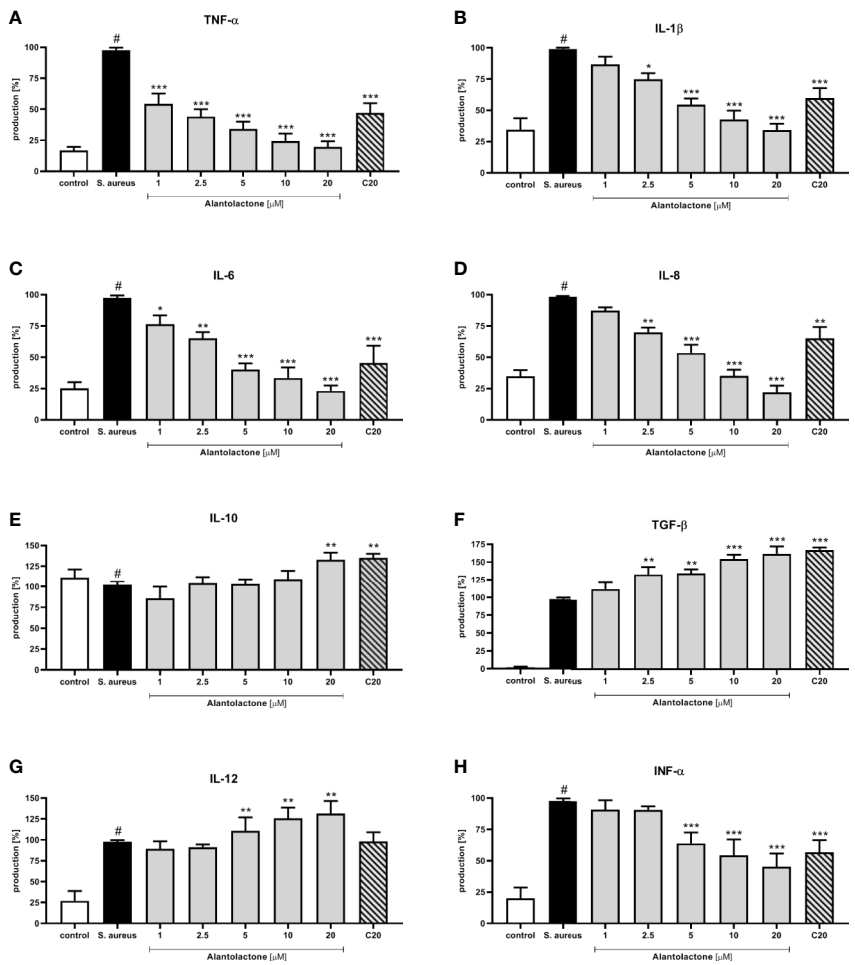

FIGURE 8 | The influence of alantolactone on the pro- and anti-inflammatory cytokines production by THP-1 cells **(A–H)** Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus stimulated control (ANOVA and Dunnett's *post hoc* test); control—non-stimulated control; *S. aureus*—*S. aureus*-stimulated control. # statistically significant (p < 0.001) versus non-stimulated control.

concentration above 5 μM significantly decreased p65 production (p < 0.001) (**Figure 9**). Similarly, clarithromycin tested at 20 μM significantly inhibited p65 production, and final effect was comparable to alantolactone tested at 5 μM.

A graphical summary of our results is shown in **Figure 10**.

DISCUSSION

The immunomodulatory characteristics of plant-based therapeutics have gathered the attention of researchers. Innovative technologies and the excessive research on immunomodulatory natural

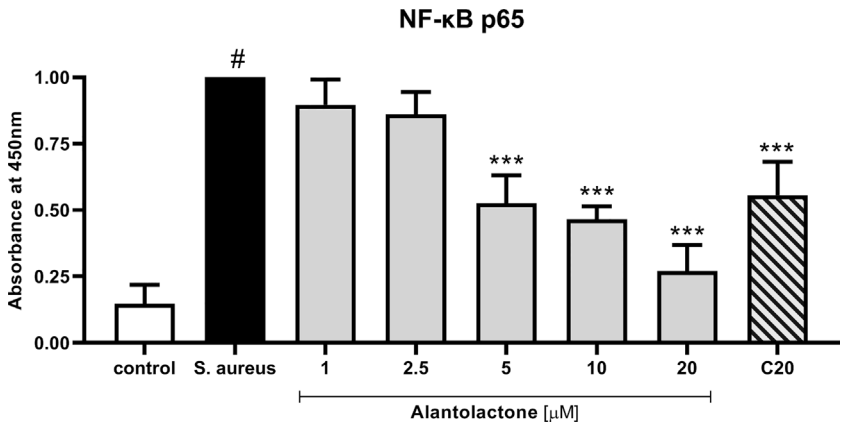

FIGURE 9 | The influence of alantolactone on p65 NF-κB concentration after *S. aureus* infection. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus stimulated control (ANOVA and Dunnett's *post hoc* test); control—non-stimulated control; *S. aureus*—*S. aureus*-stimulated control. # statistically significant (p < 0.001) versus non-stimulated control.

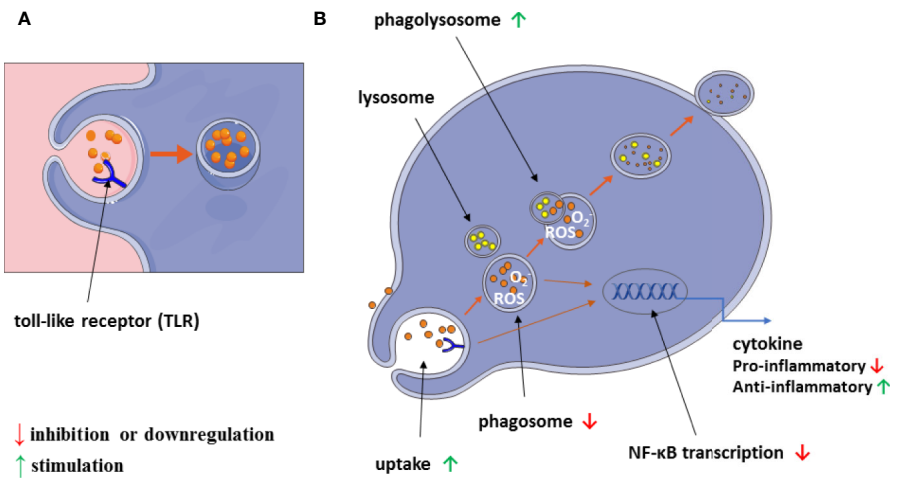

FIGURE 10 | Scheme of alantolactone influence on macrophage immunomodulatory and inflammatory properties **(A, B)**.

products, plants, their extracts, and their active moieties with immunomodulatory potential, have provided valuable entities to develop novel immunomodulatory agents to supplement the present therapies. The immunomodulatory properties of phagocytes can be enhanced pharmacologically (Xu et al., 1996).

Many plant-derived medications (plant extracts, compounds) show immunomodulatory, anti-inflammatory and anti-microbial properties, thus can be considered as promising and novel anti-*S. aureus* compounds. An example of plant with documented anti-*S. aureus* activity is *Inula helenium L.* (Asteraceae) (O'Shea et al., 2009). Root of *I. helenium* was used in the traditional medicine to treat bacteria-induced respiratory tract infections (Gierlikowska et al., 2020). *I. helenium* extract relieves symptoms of bronchial and throat infection, bronchitis, catarrh and colds (Ivancheva and Stantcheva, 2000; Leporatti and Ivancheva, 2003; Jarić et al., 2015). It also has an antitussive effect and aids coughing up of mucus (Seca et al., 2014; Shikov et al., 2014; Pranskuniene et al., 2018).

The phytochemical analysis clearly indicated alantolactone as a dominant compound in the plant material (alantolactone, 52.4%) (Bourrel et al., 1993). According to the records of the European and China Pharmacopoeia (Wang et al., 2017), alantolactone possesses a wide range of biological properties, such as anti-inflammatory, antibacterial, antifungal and immunomodulatory activities. Chemically, alantolactone belongs to sesquiterpene lactones including the α-methylene-γ-lactone group, which may potentially predispose to delayed hypersensitivity (Dupuis et al., 1980; Warshaw and Zug, 1996), but also exerts strong anti-inflammatory effects by interaction with NF-κB transcription factor (Siedle et al., 2004). Chun et al. reported the anti-inflammatory properties of alantolactone and proved that alantolactone at 10 μM suppresses inducible nitric oxide and cyclooxygenase 2 (COX-2) expression by down-

regulating mitogen-activated protein kinase (MAPK), NF-κB and activator protein 1 (AP-1) in LPS-stimulated RAW 264.7 cells (Chun et al., 2012). Lim et al. (2015) reported that alantolacone at 5 μM down-regulates STAT1 signaling in TNF-α and IFN-γ-stimulated cells. The STATs regulate various aspects of growth, survival, and differentiation in cells (Dai et al., 2006). Some studies demonstrated that STAT proteins are involved in the development and function of the immune system and play a role in maintaining immune tolerance (Dai et al., 2008). Chun et al. (2015) highlighted the ability of alantolactone to inhibit STAT3, which makes this compound a potential therapeutic agent against breast cancer.

Although alantolactone has well-documented anti-inflammatory properties, it was unknown how it regulates immunomodulatory functions of phagocytes. To answer this question, we used a standard THP-1 cell line, infected with *S. aureus* and then treated with alantolactone. Our main findings explain how alantolactone influences molecular mechanisms involved in phagocytosis.

We confirmed that at low micromolar concentrations (1–20 μM) alantolactone intensifies uptake of *S. aureus*. The effect was the most pronounced especially at the beginning of the observation (approximately 2 times higher uptake compared to *S. aureus* treated cells at 15 min). The prolonged infection time (over 1 h) may lead to overaccumulation of *S. aureus* inside of macrophages and impairment of phagocytic properties of macrophages. The literature confirms the paralysis of the phagocytosis in the prolonged infection. According to Jubrail et al. (2016) at low MOI (closely related to the beginning of infection) macrophages can kill almost all ingested *S. aureus*, but the capacity to up-regulate killing is decreased as the bacterial load grows. Moreover, some intracellular bacteria may develop the potential to survive inside macrophages (Ibarra and Steele-Mortimer, 2009; Pumerantz et al., 2011). Thus, the pharmacological enhancement of uptake of *S. aureus* at the early time point (15 min) may significantly accelerate redirecting pathogens to phagolysosomes to remove them successfully.

Additionally, we observed that alantolactone treatment leads to acidification of phagosomes. In macrophages, in which phagosome acidification is impaired, the response to *S. aureus* can be significantly limited (Ip et al., 2010). Taken together, these observations delineate the inter-dependence of phagocytosis with pH of phagosomes and suggest that therapeutics augmenting functions and biochemical properties of phagosomes may be useful in increasing host response to *S. aureus*.

It is worth emphasizing that the phagosomal pH changes in response to both dead (heat-killed, as we tested) (Renna et al., 2011) and alive bacteria (Jubrail et al., 2016). However, for scientific purposes heat-killed are recommended. Accurate measurement of phagosomal pH is challenging, mainly due to the myeloperoxidase (MPO) production by phagocytes (Sugiyama et al., 2001). The respiratory burst produces H_2O_2, which reacts with MPO and chloride anions form hypochlorous acid (Takeshita et al., 2006). The chlorination of compounds results in a quenching of fluorescence as well as a shift in their spectral properties, giving a false indication of acidification (Hurst et al., 1984). This unwanted effect can be avoided by inhibiting MPO directly with sodium azide (Nauseef et al., 1983), as we did (see Materials and Methods: *Staphylococcus aureus* culture).

Our next finding was that alantolactone stimulates phagolysosome formation/fusion. The phagolysosome formation is crucial for further intracellular pathogen killing and successful clearance (Jordao et al., 2008; Clarke and Weiser, 2011). Thus, pharmacological stimulation of this process (by alantolactone or other compound/s) may potentially enhance the phagocytosis efficiency. The obtained finding is strengthened by the knowledge, that *S.aureus* possesses diverse mechanisms, allowing avoidance of destruction in phagolysosomes (Staali et al., 2006).

Although alantolactone inhibits ROS production inside the phagosomes and phagolysosomes, it does not decrease the final phagocytosis efficiency. ROS functions are clearly concentration-dependent (Radak et al., 2013). Low amounts of ROS are essential for activation of molecular pathways responsible for intracellular microbial killing (Dupré-Crochet et al., 2013). However, the excessive amounts of ROS lead to oxidative stress and damage of phagocytic functions of immune cells, as well as surrounding host's tissues. The antioxidants of immune cells play a pivotal role in the protection against oxidative stress and therefore preserving their adequate functions. Thus, decrease of ROS amounts during bacterial infection may potentially protect phagocytes, with their phagocytic functions, and other host's tissues from dysfunction (Chakraborty et al., 2012).

Signal transduction by ROS often takes place on a subcellular scale over periods of seconds or minutes, thus in our experimental variant (1 h after infection) decrease of ROS is consistent with previous observations (Wu et al., 2012) and prognoses the appropriate function of ROS. Thus, attenuation of over-production of oxygen species may increase phagocytosis and in consequence decrease bacterial viability.

When macrophages are exposed to bacterial infection, they secrete a wide spectrum of pro- and anti-inflammatory cytokines. Cytokines are multi-functional signaling molecules, which play a crucial role during infection and inflammation progression. Cytokines modulate phagocytic functions of immune cells, thus the appropriate cytokine concentration may enhance pathogen eradication and decrease morbidity and/or mortality (Hübel et al., 2002). Recent data indicate that the low amounts of TNF-α and IL-1β increase phagocytes chemotaxis, enable intracellular phagosome maturation and stimulate phagolysosome formation, necessary for final intracellular killing (Kaufmann and Dorhoi, 2016). Also, TNF-α, IL-1β, IL-6, IL-8 may activate oxidative and non-oxidative metabolic responses of immune cells to pathogens (Seider et al., 2011). Wahl et al. documented enhancement of phagolysosome degradative properties by INFs and TGF-β (Wahl et al., 2004). It is known that cytokines promote adequate immune response, but prolonged cytokines production by leukocytes and damaged tissues may exacerbate inflammation, leading to cytokine storm. The side effects arise from excessive leukocyte chemotaxis and over-stimulation of their pro-inflammatory and immunomodulatory functions.

Our findings indicate that alantolactone significantly modulates cytokines production *via* p65 NF-κB suppression.

Based on our experiments performed on a simplified *in vitro* model, we can speculate that alantolactone at early phase of infection may potentially decrease chemotaxis of leukocytes, but, on the other hand, phagocytes are very sensitive to cytokines released to bloodstream, thus even small amounts of secreted cytokines may activate adequate immune response. Stimulation of IL-10 and TGF-β during development of inflammation is beneficial for homeostasis, as both cytokines stimulate macrophages migration and clearance of apoptotic neutrophils, pathogens and damaged tissues, contributing to the effective counterbalance of inflammation.

Comparing the activity of alantolactone with clarithromycin, it is worth to emphasize the promising competitiveness of phytotherapy. The searching of a new biological properties of plant-derived compounds has been experiencing a renaissance in recent years (Bocanegra-García et al., 2009). The differences between phagocytosis modulated by clarithromycin- and alantolactone-treated cells can be explained by the fact, that clarithromycin can be accumulated in phagocytic cells and possesses significant intracellular bactericidal activity for a long time. In contrast to clarithromycin, alantolactone is poorly absorbed by the cells (Zhou et al., 2018). Thus, it is more probable, that the observed effect for alantolactone is a consequence of modulation of the phagocytic functions of the cells, not of the direct effect of compound on bacteria.

Sesquiterpene lactones are potent NF-κB inhibitors, which determines their anti-inflammatory properties, but so far, they are not classified as immunomodulators. Till now, there was no data regarding modulation of phagocytosis by sesquiterpene lactones. Among known plant-derived compounds, some were identified as potential immunomodulators, including flavonoids, alkaloids, diterpenoids, polysaccharides and glycosides. Examples of plant-derived compounds, which have exhibited potent effects on cellular and humoral immune functions in pre-clinical investigations, are curcumin, resveratrol, quercetin, colchicine, capsaicin, andrographolide, genistein and artemisinin (Jantan et al., 2015).

To conclude, it becomes obvious that alantolactone exerts immunomodulatory and anti-inflammatory effect *via* multiple pathways. Alantolactone enhances phagocytosis, one of the main components of innate immune response, and simultaneously modulates p65 NF-κB activation affecting pro- and anti-inflammatory cytokine production involved in phagolysosome formation. We postulate that such additive pharmacodynamic effects can be beneficial for the patients with the *S. aureus* infections.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

BG conceived the study and obtained financial support. BG and WG performed the biological experiments, evaluated data and drafted the manuscript. UD critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.01339/full#supplementary-material

REFERENCES

Aberdein, J., Cole, J., Bewley, M., Marriott, H., and Dockrell, D. (2013). Alveolar macrophages in pulmonary host defence–the unrecognized role of apoptosis as a mechanism of intracellular bacterial killing. *Clin. Exp. Immunol.* 174 (2), 193–202.

Aderem, A. (2003). Phagocytosis and the inflammatory response. *J. Infect. Dis.* 187 (Suppl 2), S340–S345. doi: 10.1086/374747

Bocanegra-Garcia, V., Del Rayo Camacho-Corona, M., Ramírez-Cabrera, M., Rivera, G., and Garza-González, E. (2009). The bioactivity of plant extracts against representative bacterial pathogens of the lower respiratory tract. *BMC Res. Notes* 2, 95. doi: 10.1186/1756-0500-2-95

Bourrel, C., Vilarem, G., and Perineau, F. (1993). Chemical analysis, bacteriostatic and fungistatic properties of the essential oil of elecampane (Inula helenium L.). *J. Essent. Oil Res.* 5 (4), 411–417.

Cervin, A., Wallwork, B., Mackay-Sim, A., Coman, W. B., and Greiff, L. (2009). Effects of long-term clarithromycin treatment on lavage-fluid markers of inflammation in chronic rhinosinusitis. *Clin. Physiol. Funct. Imaging* 29 (2), 136–142. doi: 10.1111/j.1475-097X.2008.00848.x

Chakraborty, S. P., Mahapatra, S. K., and Roy, S. (2012). In vitro time-dependent vancomycin-resistant Staphylococcus aureus-induced free radical generation and status of antioxidant enzymes in murine peritoneal macrophage. *Toxicol. Mech. Methods* 22 (1), 9–22. doi: 10.3109/15376516.2011.583296

Chun, J., Choi, R. J., Khan, S., Lee, D.-S., Kim, Y.-C., Nam, Y.-J., et al. (2012). Alantolactone suppresses inducible nitric oxide synthase and cyclooxygenase-2 expression by down-regulating NF-κB, MAPK and AP-1 via the MyD88 signaling pathway in LPS-activated RAW 264.7 cells. *Int. Immunopharmacol.* 14 (4), 375–383. doi: 10.1016/j.intimp.2012.08.011

Chun, J., Li, R.-J., Cheng, M.-S., and Kim, Y. S. (2015). Alantolactone selectively suppresses STAT3 activation and exhibits potent anticancer activity in MDA-MB-231 cells. *Cancer Lett.* 357 (1), 393–403. doi: 10.1016/j.canlet.2014.11.049

Cicchese, J. M., Evans, S., Hult, C., Joslyn, L. R., Wessler, T., Millar, J. A., et al. (2018). Dynamic balance of pro- and anti-inflammatory signals controls disease and limits pathology. *Immunol. Rev.* 285 (1), 147–167. doi: 10.1111/imr.12671

Clarke, T. B., and Weiser, J. N. (2011). Intracellular sensors of extracellular bacteria. *Immunol. Rev.* 243 (1), 9–25. doi: 10.1111/j.1600-065X.2011.01039.x

Clemens, D. L., and Horwitz, M. A. (1995). Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J. Exp. Med.* 181 (1), 257–270. doi: 10.1084/jem.181.1.257

Dai, X., Sayama, K., Yamasaki, K., Tohyama, M., Shirakata, Y., Hanakawa, Y., et al. (2006). SOCS1-negative feedback of STAT1 activation is a key pathway in the dsRNA-induced innate immune response of human keratinocytes. *J. Invest. Dermatol.* 126 (7), 1574–1581. doi: 10.1038/sj.jid.5700294

Dai, X., Sayama, K., Tohyama, M., Shirakata, Y., Yang, L., Hirakawa, S., et al. (2008). The NF-kappaB, p38 MAPK and STAT1 pathways differentially regulate the dsRNA-mediated innate immune responses of epidermal keratinocytes. *Int. Immunol.* 20 (7), 901–909. doi: 10.1093/intimm/dxn048

Di, A., Brown, M. E., Deriy, L. V., Li, C., Szeto, F. L., Chen, Y., et al. (2006). CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nat. Cell Biol.* 8 (9), 933–944. doi: 10.1038/ncb1456

Dupré-Crochet, S., Erard, M., and Nüβe, O. (2013). ROS production in phagocytes: why, when, and where? *J. Leukoc. Biol.* 94 (4), 657–670. doi: 10.1189/jlb.1012544

Dupuis, G., Benezra, C., Schlewer, G., and Stampf, J. L. (1980). Allergic contact dermatitis to alpha-methylene-gamma-butyrolactones. Preparation of alantolactone-protein conjugates and induction of contact sensitivity in the guinea pig by an alantolactone-skin protein conjugate. *Mol. Immunol.* 17 (8), 1045–1051. doi: 10.1016/0161-5890(80)90099-1

Fairn, G. D., and Grinstein, S. (2012). How nascent phagosomes mature to become phagolysosomes. *Trends Immunol.* 33 (8), 397–405. doi: 10.1016/j.it.2012.03.003

Fietta, A., Merlini, C., and Gialdroni Grassi, G. (1997). Inhibition of intracellular growth of Staphylococcus aureus by exposure of infected human monocytes to clarithromycin and azithromycin. *J. Chemother.* 9 (1), 17–22. doi: 10.1179/joc.1997.9.1.17

Flannagan, R. S., Jaumouillé, V., and Grinstein, S. (2012). The cell biology of phagocytosis. *Annu. Rev. Pathol.* 7, 61–98. doi: 10.1146/annurev-pathol-011811-132445

Flannagan, R. S., Heit, B., and Heinrichs, D. E. (2015). Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of Staphylococcus aureus. *Pathog. (Basel Switzerland)* 4 (4), 826–868. doi: 10.3390/pathogens4040826

Francis, D., and Kuyyalil, S. (2018). Immunogenicity and protective efficacy of recombinant alkaline shock protein 23 from Staphylococcus aureus in a murine model. *Cent. Eur. J. Immunol.* 43 (4), 371–377. doi: 10.5114/ceji.2018.81348

Gierlikowska, B., Gierlikowski, W., Bekier, K., Skalicka-Woźniak, K., Czerwińska, M. E., and Kiss, A. K. (2020). Inula helenium and Grindelia squarrosa as a source of compounds with anti-inflammatory activity in human neutrophils and cultured human respiratory epithelium. *J. Ethnopharmacol.* 249, 112311. doi: 10.1016/j.jep.2019.112311

Gonzalez, B. E., Martinez-Aguilar, G., Hulten, K. G., Hammerman, W. A., Coss-Bu, J., Avalos-Mishaan, A., et al. (2005). Severe Staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant Staphylococcus aureus. *Pediatrics* 115 (3), 642–648. doi: 10.1542/peds.2004-2300

Hancock, R. E., Nijnik, A., and Philpott, D. J. (2012). Modulating immunity as a therapy for bacterial infections. *Nat. Rev. Microbiol.* 10 (4), 243–254. doi: 10.1038/nrmicro2745

Hayden, M. S., West, A. P., and Ghosh, S. (2006). NF-kappaB and the immune response. *Oncogene* 25 (51), 6758–6780. doi: 10.1038/sj.onc.1209943

Hübel, K., Dale, D. C., and Liles, W. C. (2002). Therapeutic use of cytokines to modulate phagocyte function for the treatment of infectious diseases: current status of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and interferon-gamma. *J. Infect. Dis.* 185 (10), 1490–1501. doi: 10.1086/340221

Hurst, J. K., Albrich, J. M., Green, T. R., Rosen, H., and Klebanoff, S. (1984). Myeloperoxidase-dependent fluorescein chlorination by stimulated neutrophils. *J. Biol. Chem.* 259 (8), 4812–4821.

Ibarra, J. A., and Steele-Mortimer, O. (2009). Salmonella–the ultimate insider. Salmonella virulence factors that modulate intracellular survival. *Cell Microbiol.* 11 (11), 1579–1586. doi: 10.1111/j.1462-5822.2009.01368.x

Ip, W. K., Sokolovska, A., Charriere, G. M., Boyer, L., Dejardin, S., Cappillino, M. P., et al. (2010). Phagocytosis and phagosome acidification are required for pathogen processing and MyD88-dependent responses to Staphylococcus aureus. *J. Immunol.* 184 (12), 7071–7081. doi: 10.4049/jimmunol.1000110

Ivancheva, S., and Stantcheva, B. (2000). Ethnobotanical inventory of medicinal plants in Bulgaria. *J. Ethnopharmacol.* 69 (2), 165–172. doi: 10.1016/s0378-8741(99)00129-4

Jantan, I., Ahmad, W., and Bukhari, S. N. (2015). Plant-derived immunomodulators: an insight on their preclinical evaluation and clinical trials. *Front. Plant Sci.* 6, 655. doi: 10.3389/fpls.2015.00655

Jarić, S., Mačukanović-Jocić, M., Djurdjević, L., Mitrović, M., Kostić, O., Karadžić, B., et al. (2015). An ethnobotanical survey of traditionally used plants on Suva planina mountain (south-eastern Serbia). *J. Ethnopharmacol.* 175, 93–108. doi: 10.1016/j.jep.2015.09.002

Jordao, L., Bleck, C. K., Mayorga, L., Griffiths, G., and Anes, E. (2008). On the killing of mycobacteria by macrophages. *Cell Microbiol.* 10 (2), 529–548. doi: 10.1111/j.1462-5822.2007.01067.x

Jubrail, J., Morris, P., Bewley, M. A., Stoneham, S., Johnston, S. A., Foster, S. J., et al. (2016). Inability to sustain intraphagolysosomal killing of Staphylococcus aureus predisposes to bacterial persistence in macrophages. *Cell Microbiol.* 18 (1), 80–96. doi: 10.1111/cmi.12485

Kaufmann, S. H. E., and Dorhoi, A. (2016). Molecular Determinants in Phagocyte-Bacteria Interactions. *Immunity* 44 (3), 476–491. doi: 10.1016/j.immuni.2016.02.014

Kim, M., Song, K., and Kim, Y. S. (2017). Alantolactone Improves Prolonged Exposure of Interleukin-6-Induced Skeletal Muscle Inflammation Associated Glucose Intolerance and Insulin Resistance. *Front. Pharmacol.* 8, 405. doi: 10.3389/fphar.2017.00405

Labandeira-Rey, M., Couzon, F., Boisset, S., Brown, E. L., Bes, M., Benito, Y., et al. (2007). Staphylococcus aureus Panton-Valentine leukocidin causes necrotizing pneumonia. *Science* 315 (5815), 1130–1133. doi: 10.1126/science.1137165

Leporatti, M. L., and Ivancheva, S. (2003). Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy. *J. Ethnopharmacol.* 87 (2-3), 123–142. doi: 10.1016/s0378-8741(03)00047-3

Lim, H. S., Jin, S. E., Kim, O. S., Shin, H. K., and Jeong, S. J. (2015). Alantolactone from Saussurea lappa Exerts Antiinflammatory Effects by Inhibiting Chemokine Production and STAT1 Phosphorylation in TNF-α and IFN-γ-induced in HaCaT cells. *Phytother. Res.* 29 (7), 1088–1096. doi: 10.1002/ptr.5354

Mediavilla, J. R., Chen, L., Mathema, B., and Kreiswirth, B. N. (2012). Global epidemiology of community-associated methicillin resistant Staphylococcus aureus (CA-MRSA). *Curr. Opin. Microbiol.* 15 (5), 588–595. doi: 10.1016/j.mib.2012.08.003

Nauseef, W. M., Metcalf, J. A., and Root, R. K. (1983). Role of myeloperoxidase in the respiratory burst of human neutrophils. *Blood* 61 (3), 483–492.

Nimmo, G. R. (2012). USA300 abroad: global spread of a virulent strain of community-associated methicillin-resistant Staphylococcus aureus. *Clin. Microbiol. Infect.* 18 (8), 725–734. doi: 10.1111/j.1469-0691.2012.03822.x

O'Shea, S., Lucey, B., and Cotter, L. (2009). In vitro activity of Inula helenium against clinical Staphylococcus aureus strains including MRSA. *Br. J. BioMed. Sci.* 66 (4), 186–189. doi: 10.1080/09674845.2009.11730271

Pitt, A., Mayorga, L. S., Stahl, P. D., and Schwartz, A. L. (1992). Alterations in the protein composition of maturing phagosomes. *J. Clin. Invest.* 90 (5), 1978–1983. doi: 10.1172/jci116077

Pospíšil, P., Prasad, A., and Rác, M. (2019). Mechanism of the Formation of Electronically Excited Species by Oxidative Metabolic Processes: Role of Reactive Oxygen Species. *Biomolecules* 9 (7), 258. doi: 10.3390/biom9070258

Pranskuniene, Z., Dauliute, R., Pranskunas, A., and Bernatoniene, J. (2018). Ethnopharmaceutical knowledge in Samogitia region of Lithuania: where old traditions overlap with modern medicine. *J. Ethnobiol. Ethnomed.* 14 (1), 70. doi: 10.1186/s13002-018-0268-x

Pumerantz, A., Muppidi, K., Agnihotri, S., Guerra, C., Venketaraman, V., Wang, J., et al. (2011). Preparation of liposomal vancomycin and intracellular killing of meticillin-resistant Staphylococcus aureus (MRSA). *Int. J. Antimicrob. Agents* 37 (2), 140–144. doi: 10.1016/j.ijantimicag.2010.10.011

Radak, Z., Zhao, Z., Koltai, E., Ohno, H., and Atalay, M. (2013). Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. *Antioxid. Redox Signal* 18 (10), 1208–1246. doi: 10.1089/ars.2011.4498

Renna, M., Schaffner, C., Brown, K., Shang, S., Tamayo, M. H., Hegyi, K., et al. (2011). Azithromycin blocks autophagy and may predispose cystic fibrosis patients to mycobacterial infection. *J. Clin. Invest.* 121 (9), 3554–3563. doi: 10.1172/jci46095

Saavedra-Lozano, J., Mejías, A., Ahmad, N., Peromingo, E., Ardura, M. I., Guillen, S., et al. (2008). Changing trends in acute osteomyelitis in children: impact of methicillin-resistant Staphylococcus aureus infections. *J. Pediatr. Orthop.* 28 (5), 569–575. doi: 10.1097/BPO.0b013e31817bb816

Schorey, J. S., and Cooper, A. M. (2003). Macrophage signalling upon mycobacterial infection: the MAP kinases lead the way. *Cell Microbiol.* 5 (3), 133–142. doi: 10.1046/j.1462-5822.2003.00263.x

Seca, A. M., Grigore, A., Pinto, D. C., and Silva, A. M. (2014). The genus Inula and their metabolites: from ethnopharmacological to medicinal uses. *J. Ethnopharmacol.* 154 (2), 286–310. doi: 10.1016/j.jep.2014.04.010

Seider, K., Brunke, S., Schild, L., Jablonowski, N., Wilson, D., Majer, O., et al. (2011). The facultative intracellular pathogen Candida glabrata subverts macrophage cytokine production and phagolysosome maturation. *J. Immunol.* 187 (6), 3072–3086. doi: 10.4049/jimmunol.1003730

Shikov, A. N., Pozharitskaya, O. N., Makarov, V. G., Wagner, H., Verpoorte, R., and Heinrich, M. (2014). Medicinal plants of the Russian Pharmacopoeia; their history and applications. *J. Ethnopharmacol.* 154 (3), 481–536. doi: 10.1016/j.jep.2014.04.007

Siedle, B., García-Piñeres, A. J., Murillo, R., Schulte-Mönting, J., Castro, V., Rüngeler, P., et al. (2004). Quantitative structure-activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-kappaB. *J. Med. Chem.* 47 (24), 6042–6054. doi: 10.1021/jm049937r

Simpson, J. L., Powell, H., Boyle, M. J., Scott, R. J., and Gibson, P. G. (2008). Clarithromycin targets neutrophilic airway inflammation in refractory asthma. *Am. J. Respir. Crit. Care Med.* 177 (2), 148–155. doi: 10.1164/rccm.200707-1134OC

Spaan, A. N., Surewaard, B. G., Nijland, R., and van Strijp, J. A. (2013). Neutrophils versus Staphylococcus aureus: a biological tug of war. *Annu. Rev. Microbiol.* 67, 629–650. doi: 10.1146/annurev-micro-092412-155746

Staali, L., Bauer, S., Mörgelin, M., Björck, L., and Tapper, H. (2006). Streptococcus pyogenes bacteria modulate membrane traffic in human neutrophils and selectively inhibit azurophilic granule fusion with phagosomes. *Cell Microbiol.* 8 (4), 690–703. doi: 10.1111/j.1462-5822.2005.00662.x

Stojanović-Radić, Z., Comić, L., Radulović, N., Blagojević, P., Denić, M., Miltojević, A., et al. (2012). Antistaphylococcal activity of Inula helenium L. root essential oil: eudesmane sesquiterpene lactones induce cell membrane damage. *Eur. J. Clin. Microbiol. Infect. Dis.* 31 (6), 1015–1025. doi: 10.1007/s10096-011-1400-1

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., et al. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* 263 (5147), 678–681. doi: 10.1126/science.8303277

Sugiyama, S., Okada, Y., Sukhova, G. K., Virmani, R., Heinecke, J. W., and Libby, P. (2001). Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am. J. Pathol.* 158 (3), 879–891. doi: 10.1016/s0002-9440(10)64036-9

Sur, L. M., Sur, G., Samasca, G., and Lupan, I. (2019). Investigations of cellular immunity in juvenile idiopathic arthritis. *Cent. Eur. J. Immunol.* 44 (1), 92–96. doi: 10.5114/ceji.2019.83615

Takeshita, J., Byun, J., Nhan, T. Q., Pritchard, D. K., Pennathur, S., Schwartz, S. M., et al. (2006). Myeloperoxidase generates 5-chlorouracil in human atherosclerotic tissue: a potential pathway for somatic mutagenesis by macrophages. *J. Biol. Chem.* 281 (6), 3096–3104. doi: 10.1074/jbc.M509236200

Tauber, A. I. (2003). Metchnikoff and the phagocytosis theory. *Nat. Rev. Mol. Cell Biol.* 4 (11), 897–901. doi: 10.1038/nrm1244

Ullrich, H. J., Beatty, W. L., and Russell, D. G. (1999). Direct delivery of procathepsin D to phagosomes: implications for phagosome biogenesis and parasitism by Mycobacterium. *Eur. J. Cell Biol.* 78 (10), 739–748. doi: 10.1016/s0171-9335(99)80042-9

Wahl, S. M., Swisher, J., McCartney-Francis, N., and Chen, W. (2004). TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J. Leukoc. Biol.* 76 (1), 15–24. doi: 10.1189/jlb.1103539

Wang, X., Yu, Z., Wang, C., Cheng, W., Tian, X., Huo, X., et al. (2017). Alantolactone, a natural sesquiterpene lactone, has potent antitumor activity against glioblastoma by targeting IKKβ kinase activity and interrupting NF-κB/COX-2-mediated signaling cascades. *J. Exp. Clin. Cancer Res.* 36 (1), 93. doi: 10.1186/s13046-017-0563-8

Warshaw, E. M., and Zug, K. A. (1996). Sesquiterpene lactone allergy. *Am. J. Contact Dermat.* 7 (1), 1–23. doi: 10.1016/s1046-199x(96)90028-7

Watts, J., Shryock, T., Apley, M., Bade, D., Brown, S., Gray, J., et al. (2008). *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standard.* (Clinical Lab Standards Institute).

Wu, Y., Zhai, H., Wang, Y., Li, L., Wu, J., Wang, F., et al. (2012). Aspirin-triggered lipoxin A_4 attenuates lipopolysaccharide-induced intracellular ROS in BV2 microglia cells by inhibiting the function of NADPH oxidase. *Neurochem. Res.* 37 (8), 1690–1696. doi: 10.1007/s11064-012-0776-3

Xu, G., Fujita, J., Negayama, K., Yuube, K., Hojo, S., Yamaji, Y., et al. (1996). Effect of macrolide antibiotics on macrophage functions. *Microbiol. Immunol.* 40 (7), 473–479. doi: 10.1111/j.1348-0421.1996.tb01097.x

Zhai, W., Wu, F., Zhang, Y., Fu, Y., and Liu, Z. (2019). The Immune Escape Mechanisms of Mycobacterium Tuberculosis. *Int. J. Mol. Sci.* 20 (2), 1–18. doi: 10.3390/ijms20020340

Zhou, B., Ye, J., Yang, N., Chen, L., Zhuo, Z., Mao, L., et al. (2018). Metabolism and pharmacokinetics of alantolactone and isoalantolactone in rats: Thiol conjugation as a potential metabolic pathway. *J. Chromatogr. B. Analyt. Technol. BioMed. Life Sci.* 1072, 370–378. doi: 10.1016/j.jchromb.2017.11.039

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Targeting Oxidative Stress Reduction and Inhibition of HDAC1, MECP2, and NF-kB Pathways in Rats With Experimentally Induced Hyperglycemia by Administration of *Thymus marshallianus* Willd. Extracts

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The effects of two lyophilized extracts obtained from the aerial parts of *Thymus marschallianus* Willd. and harvested from wild flora (TMW) and obtained from culture (TMC) were evaluated in Wistar rats with experimentally induced hyperglycemia. The hyperglycemia was induced by streptozotocin (STZ) administration and the obtained results were evaluated in comparison for TMW and TMC. The polyphenolic composition of extracts was evaluated by spectrophotometrical and LC-MS methods. *In vitro* antioxidant capacity assays (DPPH, FRAP, EPR) were performed in order to preliminary establish the ability of tested samples to protect against free radical induced damage. Afterwards, the effects of these extracts were assessed *in vivo* on rats with experimental-induced hyperglycemia. Oxidative stress biomarkers (e.g. malondialdehyde—MDA), phosphorylated transcription factor subunit of nuclear kappaB (NF-kB) p65, methyl CpG binding protein (MECP) 2 and histone deacetylase 1 (HDAC1) expressions in hippocampus and frontal lobe were assessed. Open Field Test (OFT) and Elevated Plus Maze (EPM) were conducted on tested animals. Malondialdehyde (MDA) levels and HDAC1and MeCP2 expressions increased significantly in hippocampus ($p<0.05$) and frontal lobe ($p<0.001$) of diabetes group compared to the control group in parallel with decreasing of GSH/GSSG ratio. TMW and TMC administration reduced blood glucose levels and diminished lipid peroxidation, HDAC1 expression and enhanced antioxidant capacity in frontal lobe. TMW improved central locomotion of rats, increased phospho-

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NFkB p65 and diminished MECP2 expressions in hippocampus. Both tested samples exerted a beneficial effect by increasing the antioxidant defense. Our findings indicate that the administration of these extracts might represent a good option in the treatment of diabetes and its complications.

Keywords: *Thymus marschallianus*, polyphenols, HDAC1, MECP2, NF-kB, oxidative stress, experimentally induced hyperglycemia, antioxidant

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease, characterized by hyperglycemia, due either to impairment of insulin sensitivity, insulin secretion or both. It is a major problem for public health and is responsible for a variety of complications such as an increased risk of cardiovascular disorders, nephropathy, retinopathy, neuropathy and cognitive dysfunction (World Health Organization, 2016). According to the International Diabetes Federation, in 2019, a total of 463 million people was estimated to be living with diabetes, namely 9.3% of the global adult population (20–79 years), a number that is expected to increase in the future (International Diabetes Federation, 2019; Saeedi et al., 2019). Elderly adults (≥ 60–65 years old) are at a higher risk of macrovascular complications and common geriatric syndromes, such as visual and cognitive impairment, depression, urinary incontinence and pain, as compared to younger people with diabetes (Chentli et al., 2015). Cognitive functions, such as attention, memory, learning, motor speed, visuoconstruction, somatosensory examination, motor strength, and executive functions are negatively influenced by poor glycemic control. Even though the exact link between diabetes mellitus and cognitive impairment is not fully elucidated, some authors have revealed that hyperglycemia, vascular disease (Fusco et al., 2019; Impellizzeri et al., 2019), hypoglycemia, insulin resistance, and amyloid deposition can be associated with cognitive dysfunction, especially in elderly people (Kodl and Seaquist, 2008). The efficiency of insulin and antihyperglycemic agents (e.g. biguanides, glinides and sulfonylureas) is well known. However, their administration may lead to numerous side effects (impairment of gastrointestinal function, hypoglycemia, or liver dysfunction) (Nozaki et al., 2017). Considering the possible side effects of classical medication and the hypothesis that hyperglycemia is associated with the development of diabetic complications, the need of an alternative therapy that may exert hypoglycemic activity, is a good strategy for the management of this disease (Kodl and Seaquist, 2008; Nozaki et al., 2017).

Scientific evidence maintains that hyperglycemia can lead to reactive oxygen species (ROS) production, in diabetic patients, through mitochondrial respiratory chain enzymes, xanthine oxidases, lipoxygenases, cyclooxygenases, nitric oxide synthases, and peroxidases (Balaban et al., 2005; Volpe et al., 2018). Moreover, clinical and experimental studies indicate that high sugar levels alter some signaling pathways, such as diacylglycerol, the activation of protein kinase C (PKC) and NADPH-oxidase system, thus producing oxidative stress, the formation of advanced glycation end products (AGEs), the secretion of the pro-inflammatory cytokines and cellular death (Nogueira-Machado and Chaves, 2008; Volpe et al., 2018). Additionally, it is proved that the NFkB, known for its role in inflammation, may be activated by hyperglycemia. There is scientific evidence that sustain the involvement of NFkB in synaptic plasticity, learning and memory, also mentioning that spontaneous synaptic transmission, short- and long-term synaptic plasticity, learning and memory-related behaviors are regulated by the MeCP and HDAC activity (Patel and Santani, 2009; Kavalali et al., 2011; Snow and Albensi, 2016). Moreover, the free radicals including superoxide anion from mitochondria can amplify the metabolic damage by the activation of sorbitol-aldose reductase pathways (polyol pathway) and hexosamine pathways and by the inactivation of two protective enzymes: endothelial nitric oxide synthase (NOS) and prostacyclin synthase. This further promotes inflammation resulting in a vicious cycle where hyperglycemia stimulates inflammation. ROS can produce the damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance (Balaban et al., 2005; Chentli et al., 2015; Volpe et al., 2018).

Due to their ability of scavenging ROS, the antioxidants can be used as adjuvant therapy in DM, to diminish the damages induced by oxidative stress. Among the most well-known antioxidants are medicinal plants, which exhibit their antioxidant capacity due to their chemical composition, especially to their abundance in polyphenols, which have proven along time efficient antioxidant properties (Nozaki et al., 2017).

Thymus (thyme) is one of the most important genus belonging to the Lamiaceae family (Niculae et al., 2019). This family is known worldwide for its traditional uses, in the treatment of different pathologies, being also used since ancient times for their importance as aromatic plants (Nabavi et al., 2015), that are assigned to their composition in essential oils (Tohidi et al., 2019) and phenolic compounds (Raudone et al., 2017). *Thymus marschallianus* Willd. (TM) is a species belonging to the *Thymus* genus which presents numerous chemovarieties: thymol, carvacrol, thymol/carvacrol, geraniol and carveole, that are related to the composition of its essential oils (Salehi et al., 2019). Besides the important number of volatile compounds, the species is also known for its composition in phenolic compounds, which are also responsible for its biological activity (Raudone et al., 2017). Among these compounds, the most important appears to be rosmarinic acid (RA), which is found in large amounts in the flowering aerial parts of the species

(Niculae et al., 2019). This phenolic acid is responsible for numerous protective biological activities (Nunes et al., 2017), including the antidiabetic potential and antioxidant capacity (Nunes et al., 2017; Ngo et al., 2018; Nadeem et al., 2019). The two biological effect are even more important as they can can be highly connected in mechanisms. In this context, the evaluation of the protective activity against the hyperglycemic stress of the *T. marschallianus* Willd. species appears to be important, especially as it can be further exploited in the preparation of phytomedicines having important activity in the treatment of diabetes mellitus and its related complications.

Taken all these into consideration, the objective of this study was to evaluate the effect of two *T. marschallianus* Willd. lyophilized extracts, obtained from two different samples, harvested from culture (TMC) and from spontaneous flora (TMW) on ambulatory activity and brain oxidative stress biomarkers on diabetic rats. The phenolic composition of lyophilized extracts was assessed by spectrophotometrical and LC-MS methods, while *in vitro* preliminary assays were carried out in order to assess the antioxidant capacity of tested samples. In addition, phospho-NFkB p65 subunit of transcription factor NFkB, histopathological changes; HDAC1 and MeCP2 expressions in the hippocampus and frontal lobe in rats with STZ experimental-induced hyperglycemia were investigated.

MATERIALS AND METHODS

Reagents

2-thiobarbituric acid and EDTA-Na_2 were obtained from Merck KGaA (Darmstadt, Germany), absolute ethanol and n-butanol were purchased from Chimopar (Bucharest, Romania). o-Phthaldehyde and Bradford reagent were obtained from Sigma–Aldrich Chemicals GmbH (Germany). Antibodies against HDAC1 (Cat# sc-56683, RRID : AB_783697), MeCP2 (Cat# sc-5755, RRID : AB_648930), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and secondary HRP-linked antibodies were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. The test for quantification of phospho-NFkB p65 (Ser536) InstantOne™ ELISA was bought from Blue Gene, China. Glucose levels were measured by using a kit supplied by Diagnosticum Rt (Hungary). Chlorogenic acid, p-coumaric acid, caffeic acid, rosmarinic acid, rutin, apigenin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetin, fisetin were purchased from Sigma Aldrich (St. Louis, USA). Ferulic acid, sinapic acid, gentisic acid, gallic acid, patuletin, luteolin from Roth (Karlsruhe, Germany) and cichoric acid, caftaric acid were obtained from Dalton (Toronto, Canada). HPLC grade methanol, ethanol and all reagents for spectrophotometric assays were purchased from Merck (Germany). All chemicals and reagents were of high-grade purity.

Phytochemical Analysis of Samples

Plant Material

The flowering aerial parts of *T. marschallianus* Willd. were harvested in June 2016 from the spontaneous flora of North Eastern Moldavia Flora, Cricova surroundings (voucher No. 978, from which sample TMW was obtained). The cultured species was obtained from a culture initiated in the Experimental Fields of the Botanical Garden of the Moldavian Science Academy (voucher No. 979, from which sample TMC was obtained). The taxonomic identification of the species was performed by Nina Ciocârlan, PhD, from the Botanical Garden of the Moldavian Science Academy, where voucher specimens are deposited in the Herbarium (Niculae et al., 2019).

Preparation of the Lyophilized Extracts

The plant material was air dried at room temperature for 3 days and ground to a fine powder (300 μm). Extracts were prepared by maceration of the vegetal powder with 70% ethanol for 24 h, at room temperature. After filtration, the extracts were centrifuged at 4500 rpm for 15 min, and the supernatant was recovered and subjected to evaporation of the ethanol under a vacuum at 40°C, using a rotary evaporator. Afterwards, the extracts were transferred in glass vials and freeze-dried in a lab scale VirTis Advantage Plus freeze-drier (SP Scientific, Gardiner, USA) and lyophilized. The vials were placed on the freeze-dryer shelf and cooled to −50°C at a rate of 1°C/min. The temperature was kept constant for 10 h for the complete solidification of the extract. The primary drying was performed at −30°C for 48 h and vacuum of 0.2 mbar, followed by secondary drying at 20°C for 4 h at 0.2 mbar. After lyophilization, samples were stored at room temperature. For the HPLC determinations, TMC and TMW lyophilized extracts were dissolved in EtOH 70% at a concentration of 10 mg/mL. All assays were performed in triplicate (Olah et al., 2016).

Identification and Quantification of Polyphenolic Compounds

The phytochemical profile of lyophilized extracts was qualitatively and quantitatively analyzed by two LC-MS/MS methods assessing individual polyphenolic compounds.

The first LC-MS/MS method was used for the identification of 18 phenolic compounds such as caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, sinapic acid, hyperoside, isoquercitrin, rutin, myricetin, fisetin, quercitrin, quercetin, patuletin, luteolin, kaempferol, and apigenin. The analytical method used a Zorbax SB-C18 chromatographic column for separation (100 mm x 3.0 mm i.d., 3.5 μm), while the mobile phase consisted of a mixture of methanol: 0.1% acetic acid (v/v). Compounds were eluted in a linear gradient, starting with 5% methanol and ending at 42% methanol at 35 min; for the next 3 min, isocratic elution was used, with 42% methanol; column was rebalanced in the next 7 min with 5% methanol. The flow rate was set at 1 mL/min, while the column temperature was set at 48°C. Injection volume was 5 μL. Detection of compounds was performed on UV and MS mode. The UV wavelength was set at 330 nm until 17 min (for the detection of polyphenolic acids), then at 370 nm until 38 min (to detect flavonoids and their aglycones). The MS system used an electrospray ion source in the negative mode (capillary +3000 V, nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min and dry gas temperature 360°C. The obtained chromatographic data were processed using ChemStation and DataAnalysis software from

Agilent, USA. Identified polyphenols were quantified based on their peak areas and compared with a calibration curve of their corresponding references. Results were expressed as micrograms of polyphenolic compounds per gram of lyophilized extracts (Hanganu et al., 2016b; Ielciu et al., 2017; Ielciu et al., 2018).

Six polyphenols were detected and quantified by a second LC-MS method: epicatechin, catechin, syringic acid, gallic acid, protocatechuic acid, and vanillic acid. The same chromatographic column as in the first method was used. The mobile phase consisted of a mixture of methanol (A): 0.1% acetic acid (v/v) (B) with a binary gradient (0 min: 3% A; 0–3 min: 8% A; 3–8.5 min: 20% A; 8.5–10 min 20% A and finally a rebalance of the column with 3% A). The flow rate was set at 1 mL/min and the injection volume was 5 μL. Compounds were also identified by comparison of their retention times and the MS spectra with those of corresponding references, analyzed in the same chromatographic conditions (Rusu et al., 2018).

Quantification of Total Polyphenols, Flavonoids and Phenolic Acids Content

The total phenolic content (TPC) was spectrophotometrically determined by a method using the Folin-Ciocalteau reagent, according to the European Pharmacopoeia. 2.0 mL of each sample were mixed with 1.0 mL of Folin-Ciocalteu reagent, 10.0 mL of distilled water and the mixture was diluted to 25.0 mL with a 290 g/L solution of sodium carbonate. The absorbance was measured at 760 nm after 30 min. The calibration curve was made using as reference a calibration curve plotted using gallic acid. Gallic acid concentrations that were used were set at 0.02, 0.04, 0.06, 0.08, and 0.10 mg/mL and prepared in a mixture of methanol and water (50:50, v/v). TPC values were calculated using the calibration curve of gallic acid graph (R^2 = 0.9953). Results were expressed as mg of gallic acid equivalents (GAE)/g of dry weight plant material (d.w) (Benedec et al., 2013a; Benedec et al., 2016b; Ielciu et al., 2019).

The quantitative determination of flavonoids (TFC) was performed by the spectrophotometric method using aluminum chloride. 5.0 mL of each sample were mixed with 5.0 mL of sodium acetate 100 g/L, 3.0 mL of aluminum chloride 25 g/L, and diluted to 25 mL by methanol in a calibrated flask. The absorbance was measured at 430 nm. Total flavonoids content values were determined using an equation obtained from calibration curve of the rutin graph (R^2 = 0.9996). Results were expressed as mg of rutin equivalents (RE)/g d.w. (Benedec et al., 2013a; Benedec et al., 2016b; Ielciu et al., 2017).

The quantitative determination of phenolic acids (TPA) was analyzed spectrophotometrically, in a method according to the 10th Edition of the Romanian Pharmacopoeia (*Cynarae folium* monograph), using Arnows reagent (10.0 g sodium nitrite and 10.0 sodium molybdate in 100 mL distilled water) and the results were expressed as mg of rosmarinic acid equivalents (RAE)/g of dry plant material, calculated using a rosmarinic acid calibration curve graph (R^2 = 0.9985). All experiments were performed in triplicate. Results were expressed as mg of rosmarinic acid equivalents (RAE)/g d.w. (Benedec et al., 2013a; Benedec et al., 2016b; Ielciu et al., 2019).

Antioxidant Activity Tests

DPPH Radical Scavenging Assay

The antioxidant potential of *T. marschallianus* Willd. samples were quantified using the stable DPPH radical method. The DPPH radical solution (0.1g/L) in methanol was prepared and 4.0 mL of this solution was added to 4.0 mL of extract solution (or standard) in methanol at different concentrations (10–50 μg/mL). After 30 min of incubation at 40°C in a thermostatic bath, the decrease in the absorbance (n = 3) was measured at 517 nm. The antiradical activity (three replicates per treatment) was expressed as IC_{50} (μg/mL), the concentration of vegetal material required to cause a 50% DPPH inhibition (Benedec et al., 2016a; Hanganu et al., 2016a; Ielciu et al., 2019).

FRAP Assay

This method evaluated the reduction of the iron, which is reduced from the ferric ion to the ferrous ion in a complex of iron with the radical 2,4,6-tripyridyl-s-triazine (TPTZ). The reduction of this ion is assessed by measuring the absorbance at 593 nm and the color change from green to yellow or blue. The FRAP reagent consists in a mixture of 2.5 mL 10 mM TPTZ solution in 40 mM hydrochloric acid to which 2.5 mL 20 mM ferric chloride solution and 25 mL acetate buffer at pH = 3.6 is added. At 0.4 mL of diluted sample, 6 mL the FRAP reagent was added and absorbance was measured. Blank consisted in the similar mixture without the sample. Results are expressed as mM Trolox equivalents/g dry weight vegetal material (d.w.), using a calibration curve (R^2 = 0.989) constructed with 10-40 mg/L Trolox standard (Ielciu et al., 2018; Ielciu et al., 2019).

Electron Paramagnetic Resonance (EPR) Spectroscopy Method

EPR measurements were performed on a Bruker ELEXYS E-580 spectrometer with continuous wave at X band (~9.4 GHz, modulation amplitude, 1 G, microwave power, 9.6 mM, center field 3514 and sweep field 100 G, room temperature). A DPPH solution (4.5 mM) was mixed with 10 μL samples in liquid form and transferred in EPR quartz capillaries to record the EPR spectra at different time intervals. The rate of reaction between antioxidant samples and DPPH radical was expressed by integral intensity (I) and was obtained through double integration of experimental spectra using XEPR Bruker software (Mocan et al., 2014; Hanganu et al., 2016b).

Animals and Experimental Design

Experimental procedures were approved by the Animal Ethics Board on animal welfare of the "Iuliu Haţieganu" University and by the Direction for Veterinary Surveillance and Food Safety according to the Directive 2010/63/EU on the protection of animals used for scientific purposes (Authorization No. 29/16.01.2017). Adult male Wistar rats (n = 36) were used under standard laboratory conditions, housed in a 12 h light–12 h dark cycle at room temperature (24 ± 2°C). The rats had free access to a standard normocaloric pellet diet (VRF1) and received water *ad libitum*. To evaluate the effects of TMC and TMW on brain oxidative stress biomarkers and ambulatory activity in rats with

experimental-induced hyperglycemia, the animals were divided into 4 groups of 9 rats (**Figure 1**).

The group which received only carboxymethyl cellulose (CMC) 2% served as a control group (CMC). The animals from group 2 were given CMC for 14 days and streptozotocin (STZ) on the 15th day (CMC + STZ). The other two groups of animals received: TMW respectively TMC in CMC for 14 days and STZ on the 15th day, (CMC + STZ + TMW; CMC + STZ + TMC). TMW and TMC were administered orally 200 mg/kg body weight (b.w.), dissolved in 0.5 mL CMC and STZ in a dose of 30 mg/kg b.w. was administered intraperitoneally in the 15th day (Opris et al., 2017). Fasting glycemia levels were assessed three times, on the 16th, 17th and 18th day of the experiment and DM was considered induced when blood glucose levels reached over 250 mg/dL. Subsequently, these two extracts and CMC were administrated for the next 14 days, between day 19 and 32. On the 33rd day, OFT and EPM were conducted.

In literature, various doses of STZ can be used in order to experimentally induce diabetes mellitus, such as a single moderate dose, a single large dose or multiple low doses (Deeds et al., 2011; Damasceno et al., 2014; Qian et al., 2015; Nandini and Naik, 2019). Thus, based on the literature data (Qian et al., 2015) and on our previous experience (Opris et al., 2017), we chose 30 mg/kg b.w of STZ to induce hyperglycemia.

Twenty-four hours after the last behavioral test, under anesthesia with an intraperitoneal injection of ketamine/xylazine cocktail (90 mg/kg b.w. ketamine and 10 mg/kg b.w. xylazine), all animals were euthanized. The frontal lobe from 4 rats in each group was harvested for histopathological investigations. From the other 5 rats in the group, the hippocampus and frontal lobe were collected for oxidative stress assays, ELISA test and western blotting analyzes. Oxidative stress biomarkers in hippocampus and frontal lobe homogenates (malondialdehyde—MDA, glutathione reduced (GSH)/glutathione oxidized (GSSG) ratio and phosphorylated NFkB p65 in hippocampus and frontal lobe samples were also assessed. MECP2 and HDAC1 expressions in the rats' brains were analyzed by Western Blot.

Behavioral Testing

On day 33 of the study, two different tests (OFT and EPM) were used to assess the general locomotor activity and emotionality of the rodents. The animals` activity was quantified by a visual tracking system (Smart Basic Software version 3.0 Panlab Harvard Apparatus) using specific mazes for rats (Ugo Basil Animal Mazes for Video-Tracking).

OFT

The animals were freely allowed to explore an open field arena (100 × 100 × 40 cm) for 5 min. The total travelled distance and the total number of entered squares served as an index of general locomotor activity. Increases in central locomotion (number of entries and travelled distance in the center) or in time spent in the central part of the device (time spent in the center/total time) can be considered as anxiolytic-like behavior (Gamberini et al., 2015).

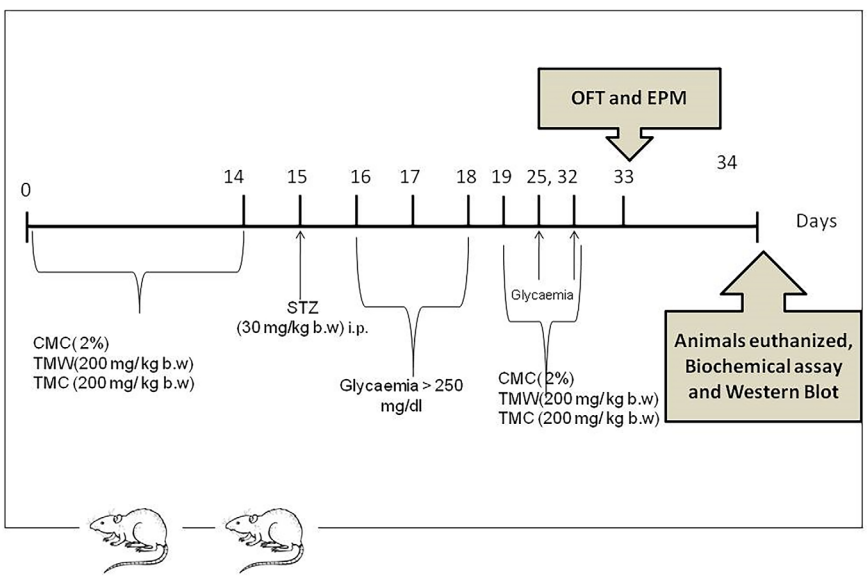

FIGURE 1 | Experimental design: Four groups of 9 adult rats each were included in the study. One group received carboxymethyl cellulose (CMC) 2% for 14 days while TMW (group 3) and TMC (group 4), were orally administered, (200 mg/kg b.w.), dissolved in 0.5 mL CMC, for 14 days prior to DM induction. On the 15th day, the animals from the three groups received one dose of STZ (30 mg/kg b.w.) administered intraperitoneally, in order to induce DM. Subsequently, CMC and the two extracts, dissolved in 0.5 mL CMC, were administrated for the next 14 days, between day 19 and 32. The results were compared with those of a control group treated with CMC without STZ (CMC). In this group CMC was administered in the same dose from day zero to day 32. On the 33rd day, OFT and EPM were conducted. Twenty-four hours later, samples from the hippocampus and frontal lobe were collected for biochemical, ELISA test, western blotting analyzes and the frontal lobe for histopathological investigations.

EPM

The plus-shaped maze consists of two open (10×50 cm) and two closed (10×50×40 cm) arms that are 60 cm elevated above the ground level. Although EPM is considered the gold standard for the evaluation of anxiety in the basic research, it also measures the motor activity. High open arms travelled distance, open arms number of entries and time ratio (open arms/total time) are considered relevant parameters of low anxiety-like behavior, whereas, total and closed arms travelled distance, total and closed arms entries are seen as an index of general locomotion in EPM. Between tasks, the mazes were cleaned with 70% ethanol to remove residual odor (Walf and Frye, 2007).

Assessment of Glycemia

On the 16th day of the study, retroorbital blood samples were collected to determine the glycemia levels, after one dose of STZ (30 mg/kg b.w.) administration. Rats with a glucose concentration exceeding 250 mg/dL, in the next 3 days after STZ injections, were considered diabetic. Later on, sugar blood levels were monitored on the 25th and 32nd days of the experiment.

Biochemical Investigations of Oxidative Stress and Antioxidant Activity

For the oxidative stress evaluation, we measured malondialdehyde (MDA) as a marker of lipid peroxidation and GSH/GSSG ratio as an antioxidant parameter from the frontal lobe and hippocampus. The MDA levels in the hippocampus and frontal lobe were determined by spectrofluorimetry, using the 2-thiobarbituric acid method. The values were expressed as nmol/mL and nmoles/mg of protein (Conti et al., 1991). GSH and GSSG were measured fluorimetrically using o-phtalaldehyde in the two tissue homogenates. The values were expressed as GSH/GSSG ratios (Hu, 1994).

Evaluation of HDAC1 and MeCP2 Expressions and Phosphorylated NFkB p65

HDAC1 and MeCP2 quantifications were performed by the Western Blot technique. Lysates (20 μg protein/lane) were separated by electrophoresis on 8% SDS PAGE gels under reducing conditions, then transferred to the polyvinylidenedifluoride membranes (BioRad), using V3 Western Workflow^{TM} (BioRad). Blots were then blocked and incubated with antibodies against NFkB, phospho-NFkB HDAC1 (catalogue number SAB4503697) and MeCP2 (catalogue number M7443) (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:500 and corresponding secondary HRP-linked antibodies (1:1500) (Santa Cruz Biotechnology). Proteins were visualized and detected using the Supersignal West Femto Chemiluminiscent substrate (Thermo Fisher Scientific, Rockford IL, USA) and a Gel Doc Imaging system equipped with an XRS camera and Quantity One analysis software (Biorad). GAPDH was used as a protein loading control. The phosphorylated NFkB p65 in the hippocampus and frontal lobe were measured by the ELISA technique (Blue Gene, China) following the manufacturer's instructions. The results were expressed as OD/mg protein (Baldea et al., 2013).

Histological Investigation of the Brain

Frontal lobe slices were harvested for histological investigation. Brain samples were fixed in 10% neutral buffered formalin, then embedded in paraffin in order to produce 5 μm thick sections which were stained with hematoxylin-eosin (HE) for light microscopy (Optika B-383LD2 microscope).

Statistical Analysis

All statistical analyses were conducted using ANOVA GraphPad Prism software, version 6.0 (GraphPad, San Diego, California, USA) and SPSS v.11.5 for Windows. The results were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used, followed by Tukey's *post hoc* test, to determine statistical significance among the four groups. A p value lower than 0.05 was considered statistically significant. Results are expressed as mean ± SD. For the extracts, differences between means were assessed by a Student–Newman–Keuls test ($p \leq 0.05$). Phytochemical assays were performed in triplicate. Each treatment contained three independent replicates and the experiments were performed twice.

RESULTS

Phytochemical Characterization of TMW and TMC Lyophilized Extracts by HPLC-MS

The compound which was found in the highest amounts, both in the cultured sample and in the spontaneous one, is rosmarinic acid (RA), which appears therefore as a phenolic marker of the species. From the two tested samples, the one harvested from culture (TMC) appears to have a larger amount of RA. Another phenolic acid found in the composition of the species is ferulic acid, also having larger amounts in the cultured sample (TMC). Regarding the polyphenols, important amounts of aglycones and flavonoids were found, luteolin being the major one found in higher amounts in the cultured sample. Another polyphenolic compound identified in significant amounts in the lyophilized extracts was apigenin, having the opposite profile for the two tested samples, namely higher amounts in the spontaneous sample. Not least, kaempferol was found in larger amounts in TMW than in TMC (**Table 1**). Other compounds as catechin and protocatechuic acid, were also found in larger amounts in TMC than in TMW, while for syringic acid and vanilic acid, the amounts were reversed for the two samples (**Table 2**).

Content of Total Polyphenols, Flavonoids and Phenolic Acid

Large amounts of total polyphenols, flavonoids and phenolic acids were determined for both samples. Small differences between the tested samples were noticed, except the content of flavonoids which appears to be almost double in the cultured sample (28.983 ± 0.32mg RE/g for TMC and 16.692 ± 0.51mg RE/g for TMW). The obtained amounts for this species are superior to the ones obtained from species belonging to other Lamiaceae species as *Ocimum basilicum* L. (Benedec et al., 2012)

TABLE 1 | Polyphenolic compounds analyzed by the LC-MS/MS method (I) in the lyophilized extracts.

Polyphenolic compounds	MonitoredIon (m/z)	Retention time (min)	Concentrations (µg/g lyophilized extract)	
			TMW	TMC
Gentisic acid	153	3.69 ± 0.04	<0.02	<0.02
Caffeic acid	179	5.60 ± 0.04	<0.02	<0.02
Chlorogenic acid	353	6.43 ± 0.05	<0.02	<0.02
p-Coumaric acid	163	9.48 ± 0.08	<0.02	<0.02
Ferulic acid	193	12.8 ± 0.10	64.86 ± 0.17*	142.01 ± 0.23*
Isoquercitrin	463	20.29 ± 0.10	<0.02	<0.02
Rutin	609	20.76 ± 0.15	<0.02	<0.02
Rosmarinic acid	360	20.8 ± 0.16	8542.14 ± 2.10*	11740.38 ± 3.5*
Quercitrin	447	23.64 ± 0.13	<0.02	<0.02
Quercetin	301	27.55 ± 0.15	27.59 ± 0.09	<0.02
Luteolin	285	29.10 ± 0.19	640.28 ± 1.09*	1105.57 ± 2.04*
Kaempferol	285	32.48 ± 0.17	66.23 ± 0.19*	59.38 ± 0.14*
Apigenin	279	33.10 ± 0.15	405.21 ± 1.01*	342.73 ± 0.91*

*Values represent the mean ± standard deviations (n = 3), *p < 0.001.*

TABLE 2 | Polyphenolic compounds analyzed by the LC-MS method (II) in the lyophilized extracts.

Polyphenolic compounds	MonitoredIon (m/z)	Retention time (min)	Concentrations (µg/g lyophilized extract)	
			TMW	TMC
Catechin	289	6.0 ± 0.09	6.54 ± 0.02*	9.65 ± 0.04*
Syringic acid	197	8.4 ± 0.09	103.14 ± 0.19*	54.07 ± 0.09*
Protocatequic acid	153	2.8 ± 0.05	206.78 ± 0.41*	480.692 ± 0.83*
Vanilic acid	167	6.7 ± 0.07	270.65 ± 0.84*	67.27 ± 0.12*

*Values represent the mean ± standard deviations (n = 3), *p < 0.001.*

or *Rosmarinus officinalis* L. (Olah et al., 2016). *Origanum vulgare* L. and *Mentha* sp. showed superior results for TPC than *T. marschallianus* Willd., but inferior for TFC and TPA (Benedec et al., 2013b; Oniga et al., 2018).

Antioxidant Activity

The antioxidant capacity of these extracts was determined by several methods, testing the behavior of these samples towards various radicals generated *in vitro*: DPPH bleaching assay, the ferric reducing antioxidant power assay (FRAP) and the electron paramagnetic resonance assay (EPR) (**Table 4**).

The DPPH scavenging ability of the TMC was 1.5 times higher than that of TMW (IC_{50} = 81.2 ± 1.3 µg/mL and IC_{50} = 121.458 ± 1.21 µg/mL, respectively) (**Table 4**). This is in good connection with the TPC values listed in **Table 3**. Compared to the reference compounds, quercetin (IC_{50} = 5.40 ± 0.32 µg/mL) and BHT (IC_{50} = 15.6 ± 0.44 µg/mL), the extracts showed lower antioxidant capacity. Other studies also cite the antioxidant activity of *Thymus* species by this type of assay, but this biological activity is attributed to the essential oils (Jianu et al., 2015; Mancini et al., 2015; Petrović et al., 2017). For species belonging to the Lamiaceae family, results obtained in the same evaluation method range between 35.03 ± 1.57 and 135.89 ± 3.10 µg/mL (Benedec et al., 2015).

TABLE 3 | The content of total polyphenols, flavonoids and phenolic acids for *T. marschallianus* Willd. Extracts.

Samples	TMC	TMW
TPC (mg GAE/g d.w.)	61.993 ± 0.31**	59.890 ± 0.42**
TFC (mg RE/g d.w.)	28.983 ± 0.32*	16.692 ± 0.51*
TPA (mg RAE/g d.w.)	26.512 ± 0.31**	25.484 ± 0.23**

*Each value represents the mean ± standard deviations of three independent measurements, *p < 0.001, **p < 0.05.*
GAE, Gallic acid equivalents; RE, Rutin equivalents, RAE, Rosmarinic acid equivalents.

The FRAP assay shows values of 233.11 ± 3.5 µM Trolox Eq/g d.w for the TMW extract and of 296.15 ± 4.7 µM Trolox Eq/g d.w for the TMC extract, indicating a superior potential for the latter, in accordance with the TPC.

Regarding the EPR method, the values of the integral intensity of these samples were compared with the DPPH radical standard, which were mixed. The rate of reaction between the antioxidant compounds of the extracts and DPPH radical was monitored using normalized double integrated residual EPR signal, correlated with the number of paramagnetic species. It is therefore observed that the integral intensity of DPPH in mixture with samples decreases, especially compared with the DPPH solution without samples. Decrease of the integral intensity for the tested samples can be observed, representing by the oxido-reduction rate of the DPPH radical. Comparing the obtained rates for both samples, it is clear that TMC has a higher antioxidant capacity than TMW. Values of the integral intensity of both samples are represented in **Table 4** compared with DPPH (Mocan et al., 2014).

All of the obtained results in these assays are in correlation with the content of polyphenols, flavonoids and phenolic acids

TABLE 4 | Antioxidant evaluation of *T. marschallianus* Willd.

Samples	TMC	TMW	Quercetin	BHT	DPPH
DPPH (IC_{50} µg/mL)	81.2 ± 1.32*	121.458 ± 1.21*	5.40 ± 0.32	15.6 ± 0.44	–
FRAP (µM Trolox Eq/g d.w.)	296.15 ± 4.73*	233.11 ± 3.53*	–	–	–
EPR (I)	218.04 ± 15.95*	341.50 ± 8.49*	–	–	797.01 ± 43.64

*Each value represents the mean ± standard deviations of three independent measurements; I = integral intensity, *p < 0.001.*

and also with the amounts of the compounds quantified by the HPLC method. These results provide important directions on possible mechanisms of actions of these samples, taking into consideration the different systems that were used, which test the behavior of these compounds towards various radicals. These assays represented the premises that allowed to further test the effects of oxidative stress in the model of STZ-induced hyperglycemia in rats.

Behavioral Studies

The effect of extracts on locomotion of rats, tested in OFT is illustrated in **Figure 2**. The OFT is used to assay general locomotor activity levels and anxiety in rodents in scientific studies (Walf and Frye, 2007; Anchan et al., 2014; Kushwah et al., 2016). Our results showed that TMW and TMC ($p<0.001$) significantly diminished the total travelled distance and travelled distance in periphery.

The effect of these two extracts on the locomotion of rats, tested in EPM is illustrated in **Figure 3**. Even though, EPM was developed to measure anxiety-related behavior, this test is used to assay general locomotor activity levels (number of entries into the open arms and total arms entries), as well (Walf and Frye, 2007).

In the EPM, the extracts administration improved general locomotion, but without any statistical significance ($p>0.05$). TMW and TMC treated rats significantly made more entries in the EPM test as compared to the STZ group ($p< 0.05$). The influence of the administration of extracts on the emotionality, tested in OFT and EPM, was exemplified in **Figure 4**. Regarding

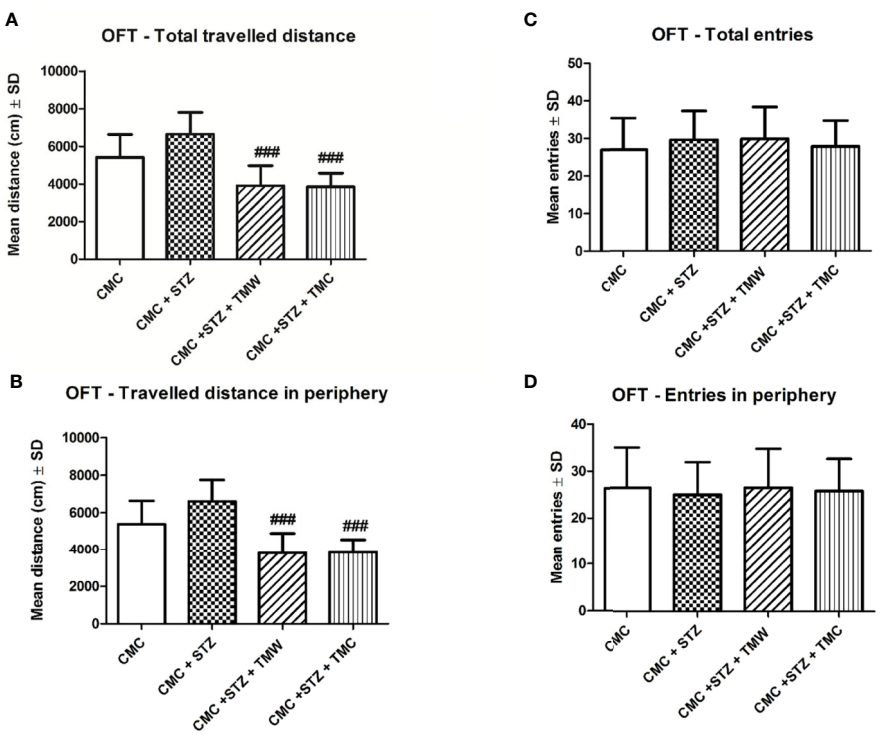

FIGURE 2 | The effects of two extracts on the total **(A)** and peripheral **(B)** travelled distance and total **(C)** and peripheral **(D)** number of entries in the open field test (OFT). TMW and TMC ($p<0.001$) significantly diminished the total travelled distance **(A)** and the travelled distance in periphery **(B)** as compared to the CMC+STZ group. Twenty-eight days of extracts treatment in comparison to STZ administration, slightly improved the locomotor activity (number of entries in periphery), but without a statistical significance ($p > 0.05$) **(D)**. Each group consisted of 9 rats. Results are expressed as mean ± SD; $p < 0.05$ as compared to CMC+STZ. The $^{###}$ means $p<0.001$ between CMC+STZ versus treated groups (CMC+STZ+TMW and CMC+STZ+TMC).

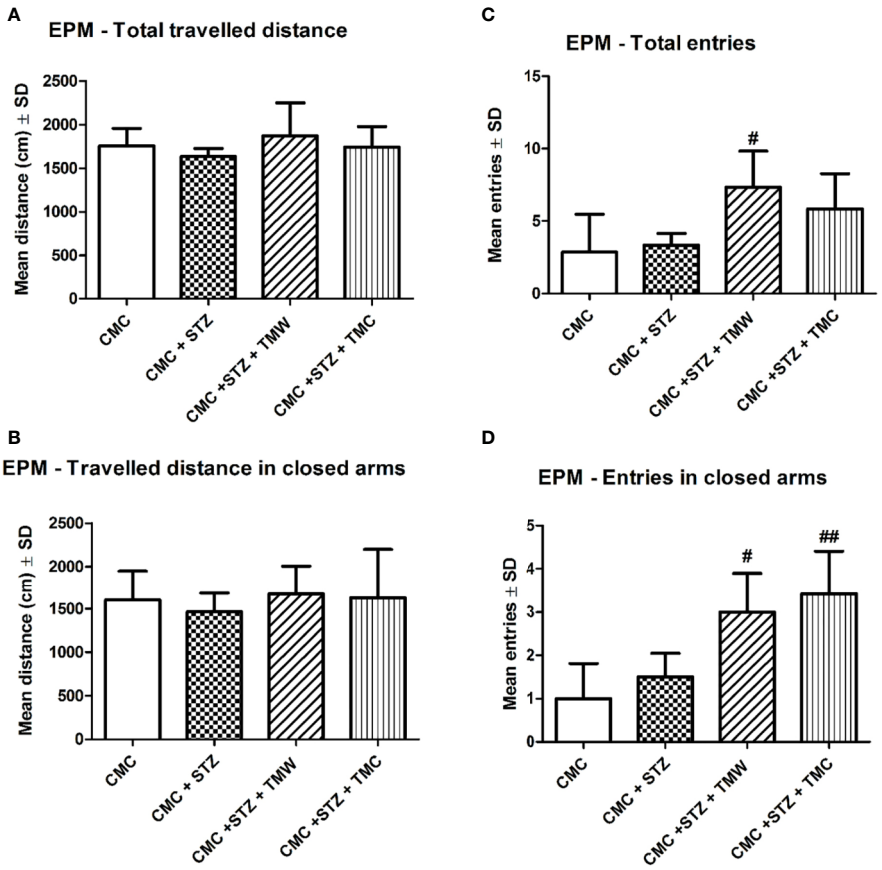

FIGURE 3 | The effects of extracts on the total **(A)** and peripheral **(B)** travelled distance and the total **(C)** and peripheral **(D)** number of entries in the elevated plus maze (EPM). In the EPM, the natural compound administration improved general locomotion, but without any statistical significance ($p > 0.05$) **(A, B)**. TMW **(C, D)** and TMC **(D)** treated rats significantly made more entries in the EPM test as compared to the CMC+STZ group ($p < 0.05$). Each group consisted of 9 rats. Results are expressed as mean ± SD; $^{\#}p < 0.05$ as compared to CMC+STZ. The $^{\#\#}$ means $p<0.01$ between CMC+STZ versus treated groups (CMC+STZ+TMW and CMC+STZ+TMC).

the emotionality in OFT, the TMW treated rats travelled significantly greater distance ($p<0.05$), made more entries ($p<0.001$), and spent more time ($p <0.001$) (C), in the central part of the OFT arena as compared to STZ. TMW administration increased the central travelled distance ($p<0.01$), the number of entries ($p<0.05$), and the central time spent ($p< 0.05$), in the open filed arena as compared to the TMC group.

EPM has been validated to assess the anxiety-like behavior in rats, based on the natural aversion of rodents for open spaces of the elevated maze (Wang et al., 2014). Thus, higher travelled distance, more entries and more time spent in the open arms of the EPM test apparatus, during a 5 min test session, is indicative of low anxiety-like behavior (Walf and Frye, 2007).

In EPM, the TMW group exhibited a significantly higher travelled distance and made more entries in the open arms, both as compared to the CMC+STZ and CMC+STZ+TMC group ($p<0.001$). Moreover, the TMW group tended to increase the time spent in the open arms of the EPM, but without statistical significance ($p>0.05$).

Assessment of Glycemia

The effects of administration of extracts on glycemia levels in the blood of the rats are exemplified in **Figure 5**. On the 25th and 32nd days of the experiment, glycemia levels were measured in the blood of the rats. On both days, glycemia displayed higher levels in the CMC+STZ group as compared to the CMC group ($p<0.001$). On the 25th day, both TMW and TMC decreased glycemia values ($p<0.001$), while on the 32nd day, TMW was the only one to exert beneficial effects ($p<0.001$). Significantly higher levels of glycemia were recorded in the CMC+STZ+TMC group as compared to the CMC+STZ+TMW group ($p<0.001$) (**Figure 5**), which is possibly due to higher content of total polyphenols in the composition.

Oxidative Stress and Antioxidant Activity Assessment in the Hippocampus and Frontal Lobe

The effects of extracts administration on oxidative stress markers in different areas of the rats' brains are found in **Figure 6**.

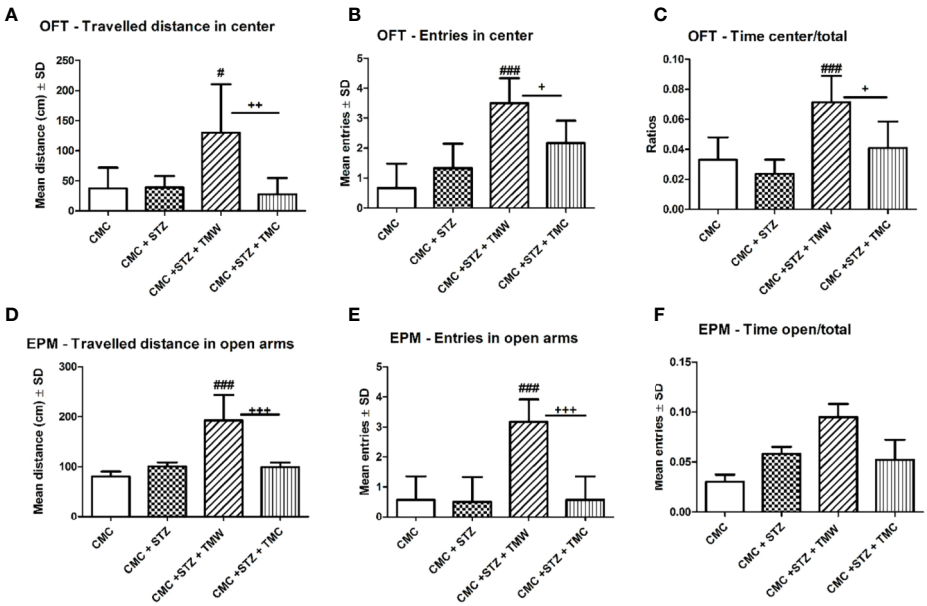

FIGURE 4 | The effects of extracts on emotionality in the open field test (OFT) **(A–C)** and in the elevated plus maze (EPM) **(D–F)**. The TMW treated rats travelled significantly greater distance ($p < 0.05$) **(A)**, made more entries ($p < 0.001$) **(B)**, and spent more time ($p < 0.001$) **(C)**, in the central part of the OFT arena as compared to CMC+STZ. TMW administration increased the central travelled distance ($p < 0.01$) **(A)**, the number of entries ($p < 0.05$) **(B)**, and the central time spent ($p<0.05$) **(C)**, in the open filed arena as compared to the CMC+STZ+TMC group. In EPM, TMW administration enhanced the travelled distance and the entries made in the open arms, both as compared to the CMC+STZ and CMC+STZ+ TMC group **(D, E)** ($p < 0.001$). TMW group tended to increase the time spent in the open arms of the EPM, but without statistical significance ($p > 0.05$). Each group consisted of 9 rats. Results are expressed as mean ± SD; $^{\#}p < 0.05$ as compared to CMC+STZ; $^{+}p < 0.05$ as compared to CMC +STZ+TMW. The $^{\#\#\#}$ means $p<0.001$ between CMC+STZ versus treated groups (CMC+STZ+TMW and CMC+STZ+ TMC). $^{++}p<0.01$ and $^{+++}p<0.001$ between CMC+STZ+TMW and CMC+STZ+TMC.

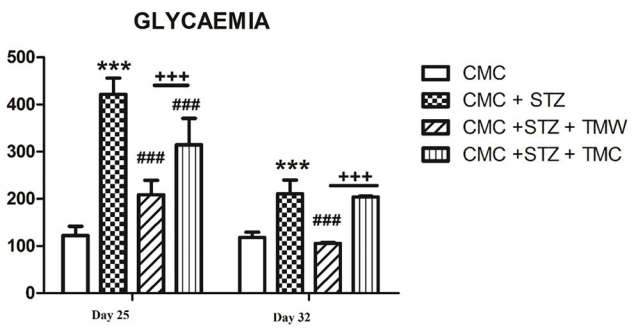

FIGURE 5 | The effects of the extracts on glycemia levels in the blood of rats. Both on 25th and 32nd days of the experiment, glycemia displayed higher levels in the CMC+STZ group as compared to the CMC group ($p<0.001$). On the 25th day, both TMW and TMC decreased glycemia values ($p<0.001$), while on the 32nd day, TMW was the only one to exert beneficial effects ($p<0.001$). Significantly higher levels of glycemia were recorded in the CMC+STZ+TMC group as compared to the CMC+STZ +TMW group ($p<0.001$). Each group consisted of 9 rats. Results are expressed as mean ± SD; $^{***}p<0.001$ as compared to CMC; $^{\#\#\#}p<0.001$ as compared to CMC+STZ; $^{+++}p<0.001$ as compared to CMC+STZ +TMW.

Evaluation of Phosphorylated NFkB p65, HDAC1, and MeCP2 Expressions

The effects of extracts on the phosphorylated NFkB p65 subunit, in the brain of the rats are illustrated in **Figure 7**. The phosphorylation of p65 NFkB at Ser 536 allows the nuclear localization of the transcriptionally active complex and transactivation of several downstream genes mediated by NFkB. Therefore, the quantification of active form of p65NFkB subunit is an indirect measure of NFkB activation and function. Phospho-NFkB p65 increased in the hippocampus of the CMC+STZ+TMW treated group as compared to CMC+STZ ($p<0.05$). In the frontal lobe, the phospho-NFkB p65 were down-regulated both by TMW and TMC, but the differences were not statistically significant ($p>0.05$). The levels of the phospho-NFkB p65 decreased 1.28 times in TMW groups as compared to CMC and 1.21 times as compared to STZ. The levels of the phospho-NFkB p65 decreased 1.79 times in TMC group as compared to CMC and 1.69 times as compared to STZ.

The influence of the administration of extracts on the expression of HDAC1 and MeCP2 expressions in the brain is shown in **Figure 8**.

STZ in CMC significantly stimulated the HDAC1 and MeCP2 levels both in the hippocampus and the frontal lobe as compared to the control group ($p<0.001$). TMW administration significantly lowered the HDAC1 value in the frontal lobe

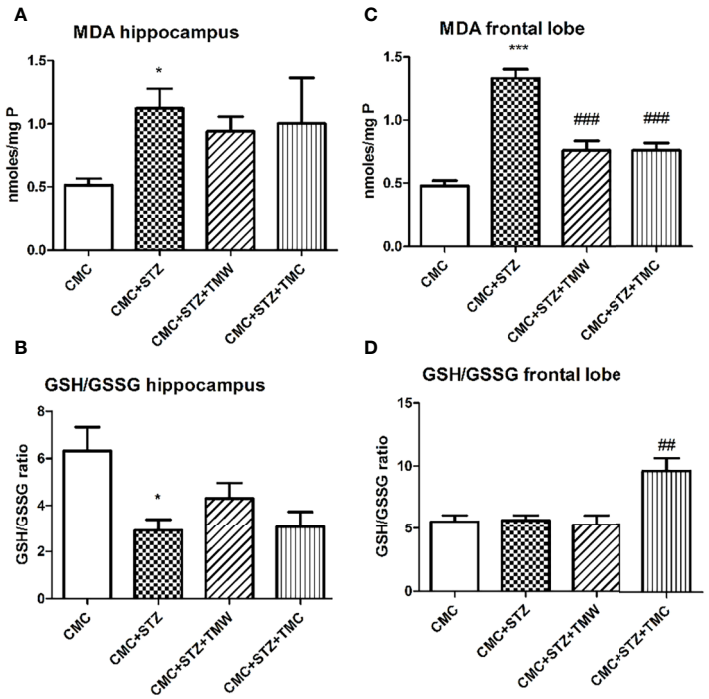

FIGURE 6 | The effects of two extracts on malondialdehyde (MDA) levels **(A, C)** and antioxidant defense (GSH/GSSG ratio), **(B, D)** in the hippocampus and frontal lobe of adult rats. MDA displayed higher levels in the hippocampus and frontal lobe of the CMC+STZ group ($p < 0.05$, **A**; $p < 0.001$, **C**). Both TMW ($p < 0.001$) and TMC ($p < 0.001$) diminished the MDA levels in the frontal lobe **(C);** GSH/GSSG ratio was significantly lower in hippocampus ($p<0.05$) **(B)** of the CMC+STZ treated group. TMC administration significantly increased the GSH/GSSG ratio in the frontal lobe of the treated rats compared to the untreated animals ($p < 0.01$) **(D)**. Each group consisted of 5 rats. Results are expressed as mean ± SD; *p<0.01 and ***p<0.001 as compared to CMC; $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 as compared to CMC +STZ.

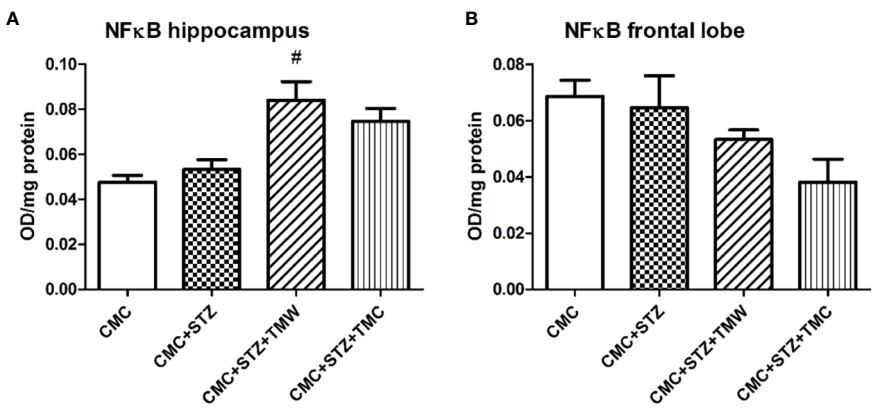

FIGURE 7 | The effects of the extracts on NFkB levels in the brains of the rats **(A, B)**. NFkB recorded elevated levels in the hippocampus of the CMC+STZ+TMW treated group as compared to CMC+STZ ($p < 0.05$) **(A)**. In the frontal lobe, the NFkB levels were down-regulated both by TMW and TMC, but the differences were not statistically significant ($p > 0.05$) **(B)**. Each group consisted of 5 rats. Results are expressed as mean ± SD; $^{\#}p < 0.05$ as compared to CMC+STZ.

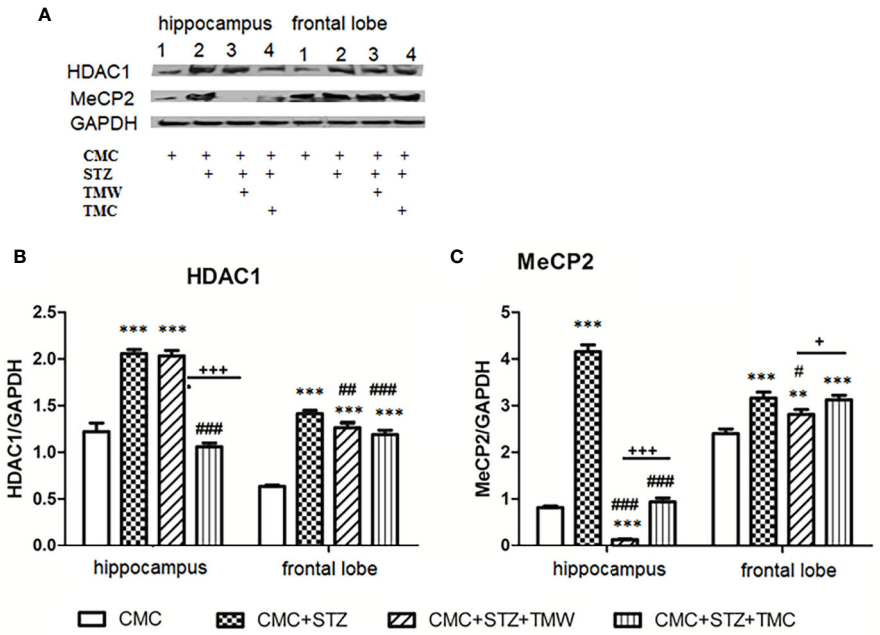

FIGURE 8 | The effects of the administration of extracts on the expression of HDAC1 and MeCP2 in the brain **(A–C).** Expression of HDAC1 and MeCP2 were analyzed by western blot (WB). The image analysis of western blot bands was completed by densitometry; the results were normalized to GAPDH. WB images, 1 - 4 hippocampus, (1 = CMC; 2 = CMC + STZ, **3** = CMC + STZ + TMW; 4 = CMC + STZ + TMW) 1 – 4 frontal lobe, (**1** = CMC; 2 = CMC + STZ, 3 = CMC + STZ + TMW; 4 = CMC + STZ + TMW); (n = 3). Each group consisted of 3 samples. Results are expressed as mean ± SD; **p<0.01 and ***p<0.001 as compared to CMC; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 as compared to CMC+STZ; $^{+}$p<0.05 and $^{+++}$p<0.001 as compared to CMC+STZ+TMW.

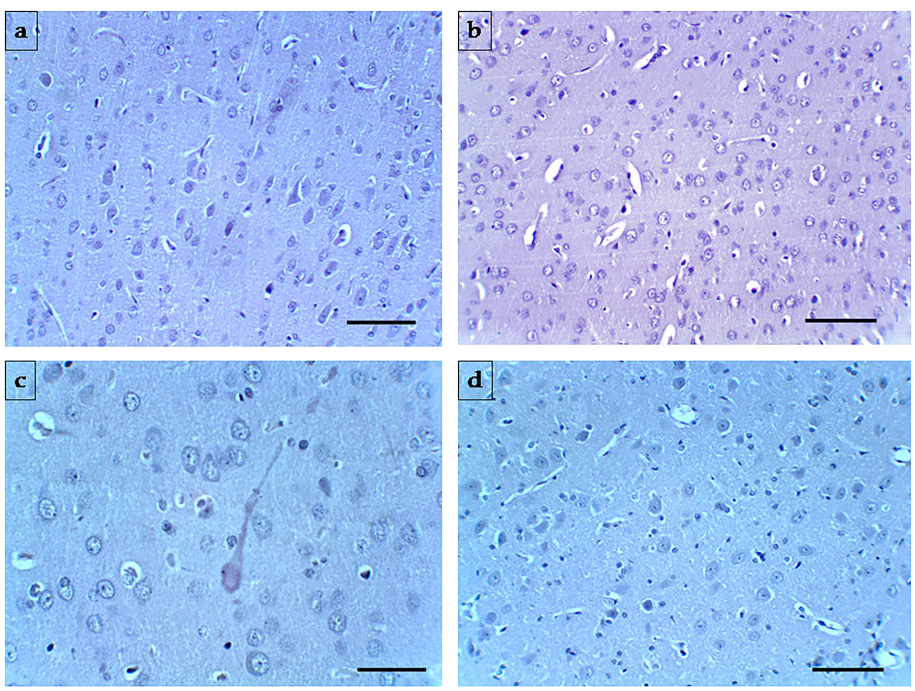

FIGURE 9 | Representative photomicrographs of the frontal cortex of the four experimental groups. **(A)** (CMC), **(B)** (CMC+STZ), **(C)** (CMC+STZ+TMW), **(D)** (CMC +STZ+TMC) prove the histological features of the frontal cortex Magnification: ×200. H&E staining. Scale bar = 20 μm.

($p<0.01$) (**Figure 8B**) and MeCP2 both in the hippocampus ($p<0.001$) and the frontal lobe ($p<0.05$) as compared to STZ in CMC (**Figure 8C**). TMC decreased the expressions of HDAC1 in the hippocampus and the frontal lobe ($p<0.001$) (**Figure 8B**) and of the MeCP2 in the hippocampus ($p<0.001$).

The effects of extracts administration on the micromorphology of the frontal cortex are illustrated in **Figure 9**. The tissue sections evaluated by morphometry displayed rare perineuronal edema in the frontal cortex of CMC group. Frequent perineuronal edema, shrinked neurons along with axonal demyelination was observed in the CMC+STZ treated group. TMW and TMC administration induced rare perineuronal edema, a few shrinked neurons along with rare axonal demyelination in the frontal lobe.

DISCUSSION

Herbal medicines are used for the treatment and prevention of various diseases starting from ancient times. Among the disorders that were treated with natural remedies DM is also included (Petrovska, 2012; Choudhury et al., 2018). Numerous natural products have shown their efficiency in reducing blood glucose levels, regulating insulin secretion and insulin sensitivity in the cells and reducing triglycerides and cholesterol levels. Therefore, many patients consider complementary and alternative medicinal (CAM) therapies over conventional ones, due to their lower costs and less side effects, medicinal plants still being used with great interest in the modern era (Choudhury et al., 2018).

In the present study, we have chosen a chemical model to induce experimental diabetes in animals, which is based on STZ administration. STZ (2-deoxy-2-(3-(methyl-3- nitrosoureido)-D-glucopyranose), synthesized by *Streptomycetes achromogenes*, has been widely used for its diabetogenic properties in rodents, either in a single or multiple dose injection. STZ causes DNA alkylation while entering the pancreatic β cells *via* the GLUT2 glucose transporter, which promotes the activation of poly ADP-ribosylation that further leads to cellular NAD^+ and ATP reduction and subsequent inhibition of insulin synthesis and secretion. Moreover, it is well known that STZ – induced DNA damage may be also related to nitric oxide (NO) delivery, a molecule released during STZ cellular metabolism (Takasu et al., 1991; Bedoya et al., 1996; Szkudelski, 2001; Deeds et al., 2011). Results of our research provided evidence that STZ (30 mg/kg b.w.) administration increased the oxidative stress parameters in the brain of the hyperglycemic rats. Thus, we observed enhanced MDA levels in the hippocampus and the frontal lobe, and reduced GSH/GSSG ratio in the hippocampus. These observations were consistent with studies that reported high lipid peroxidation, enhanced protein carbonyl content, as well as altered enzymatic activity (decreased glutathione peroxidase, superoxide dismutase, catalase), and reduced glutathione levels in hypothalamus, hippocampus and frontal cortex lysate of STZ - treated rats (Parihar et al., 2016).

There is scientific evidence that diabetes mellitus may consistently be associated with reduced cognitive performance, especially in elderly people. The exact link between diabetes mellitus and cognitive deficits is not fully elucidated, but some authors have revealed that insulin and the insulin-like growth factor (IGF) regulate neuronal and glial cell activities (e.g. growth, survival, metabolism, gene expression, protein synthesis, cytoskeletal assembly, synapse formation, neurotransmitter function, and plasticity), which are highly needed to promote cognitive function (Kodl and Seaquist, 2008; De la Monte, 2014). Conversely, brain insulin/IGF resistance induces oxidative stress, neuroinflammation, impaired cell survival, mitochondrial dysfunction, dysregulated lipid metabolism, and endoplasmic reticulum (ER) stress, thus propagating a mild neurodegenerative process, similar to Alzheimer's disease (AD) (Tong and De La Monte, 2009; De la Monte, 2014).

Some authors revealed that polyphenol administration may prevent neurodegeneration, inhibit inflammations and reduce age – related cognitive decline by scavenging free radicals, activating various signaling pathways, modulating gene expression, down-regulating NFkB and nuclear factor erythroid 2-related factor 2 (Nrf2) or inhibiting the release of cytokines (IL-1β, TNF-α) (Spencer et al., 2009; González-Gallego et al., 2010; Vauzour, 2012; Vauzour, 2017). In the CNS, NFkB transcription factors may play an important regulatory role in physiological processes, such as neurogenesis, neuritogenesis and synaptic plasticity which is related to learning and memory. Moreover, Nrf2 and NFkB are considered to regulate cellular responses to oxidative stress and inflammation, both being activated by similar stimuli. Based on scientific evidence, the absence of Nrf2 can exacerbate NFkB activity leading to increased cytokine production, whereas NFkB can modulate Nrf2 transcription and activity, having both positive and negative effects on the target gene expression (Wardyn et al., 2015). Thus, the functional cross-talk between the Nrf2 and NFkB pathways may lead to the development of improved therapeutic strategies that may regulate the NRF2-NFkB interplay response under both physiological and pathological conditions (Sivandzade et al., 2019).

The comparative effects of two lyophilized extracts of *T. marshallianus*, harvested from the wild flora (TMW) and from the culture (TMC) on STZ – induced hyperglycemia were studied in this experimental model due to the background of the *Thymus* species present in traditional medicine, but moreover, as large amounts of rosmarinic acid (RA) were found in the composition of the tested samples (**Table 1**). In the context of the well-known protective activities of this compound (Nunes et al., 2017; Nadeem et al., 2019), the promising potential of this species proved to be significant and therefore the present study aimed to highlight its potential medicinal properties.

The results of the present study evidenced that administration of both TMW and TMC diminished lipid peroxidation, whereas TMC improved the antioxidant activity in the frontal lobe of the STZ – treated rats. These observations were consistent with the literature. Umeno et al. (2016) mentioned that polyphenols may exert an antioxidant effect by Nrf2 pathway activation and by antioxidative proteins expression (e.g. HMOX-1). Among the polyphenols, RA is mentioned by scientific literature to decrease

serum MDA levels and improve CAT, SOD, GSH, GPx, tumor necrosis factor-alpha (TNF)- α and interleukin-6 (IL-6). The antioxidant mechanism of the extracts can also be related to this compound, known as the most potent scavenger of ROS, RNS and peroxynitrite identified among polyphenols (Nadeem et al., 2019).

Based on the quantitative determinations of polyphenols by HPLC, tested samples revealed a high content of RA, which is proven to be the major compound found in the composition of the species. Together with the RA, significant amounts of flavonoids were identified. Taken into consideration the background of these compounds (Bamosa et al., 2010; Maithili Karpaga Selvi et al., 2015), they may be involved in the glucose-metabolism pathways, such as, glucose absorption, regulation of glucose production in the liver or insulin tissue sensitiveness. Considering that hyperglycemia induces oxidative stress and increased levels of inflammatory mediators and apoptotic proteins associated with diabetes mellitus, these extracts might be considered for both antioxidant and hypoglycemic effects.

The results showed that rats injected with STZ showed elevated plasma glucose levels, an observation also reported by other authors (Yassa and Tohamy, 2014), whereas blood glucose levels were significantly reduced in diabetic rodent groups treated with natural compounds. Hence, our data were in agreement with previous studies that have shown the hypoglycemic efficacy of polyphenolic compounds (Chen et al., 2015).

Regarding the behavioral effects of TMW in our study, there was a decrease in the general activity, revealed by significantly lower scores in the peripheral and total travelled distance, assessed in OFT. However, based on the EPM test, administration of TMW improved the general locomotion (increased zone transition number and entries in closed arms) and TMC increased the entries in the closed arms. Additionally, TMW demonstrated an anxiolytic-like effect in OFT as it enhanced the travelled distance, the entries made and the time spent in the central part of the arena. Further, the TMW treated rats made more entries and travelled a higher distance in the open arms of the EPM as compared to the STZ treated group.

In addition, we evaluated the HDAC 1 and MeCP2 expressions in the hippocampus and frontal lobe by western blot. Histone deacetylases, (HDACs), a family of four classes enzymes, divided into zinc (*class I:* HDACs 1, 2, 3 and 8; *class II:* HDACs 4, 5, 6, 7, 9 and 10; *class IV*: HDAC 11) and nicotinamide adenine dinucleotide - dependent groups (*class III* HDACs: sirtuins), play a major role in the normal cellular brain activities by regulating, gene expression, survival or proliferation of the cells (Reddy et al., 2018; Gatla et al., 2019). Class I HDACs, expressed in various mammalian cells and tissues, have been intensively studied as histone modifiers and transcriptional repressors (Jaworska et al., 2015). Moreover, the importance of both HDAC1 and 2 in the development of CNS, as *hdac 1* mutation induced neuron and glia failure cell formation in the hindbrain, loss of segmental organization of postmitotic neurons and glia cells and deficit in the branching of motor neurons in zebrafish (Pillai et al., 2004; MacDonald and Roskams, 2008). It also seems that the deletion of both HDAC 1 and 2 may impair the normal development of the mouse brain (e.g. cortical, hippocampal and cerebellar structures) during the embryonic period, leading to neuronal death (Haberland et al., 2009; Montgomery et al., 2009).

HDAC 1 showed significant expression in glial and neural progenitor cells during brain development, and in glial cells in adult brain, whereas HDAC 2 was expressed in progenitors and neurons (Broide et al., 2007; MacDonald and Roskams, 2008). HDACs, by histone deacetylation, remove acetyl groups from N-terminal tails of histone proteins, being involved in the epigenetic modifications that stabilize the local chromatin architecture and thus decrease gene expression and protein function (Jaworska et al., 2015; Khurana and Dlugos, 2017; Dresselhaus et al., 2018).

Moreover, histone acetylation is involved in the memory formation. Thus, the high levels of acetylated hippocampal histones in mice and the efficiency of HDAC inhibitors (e.g. trichostatin A, TSA, VPA, vorinostat), that may facilitate the learning process in wild-type mice, as well as in neurodegeneration (Guan et al., 2009; Ricobaraza et al., 2012; Peixoto and Abel, 2013; Lu et al., 2015). At the same time, Groh et al. (2013) mentioned that polyphenols, such as epigallocatechin-3-gallate and genistein potently diminished the activity of HDAC in intact colon carcinoma cells and demonstrated that the modulation of HDAC activity is associated with the suppression of HDAC1. In our study, one dose of STZ increased the HDAC1 and MeCP2 expressions both in the hippocampus and the frontal lobe. TMW administration significantly inhibited the expression of HDAC1 in the frontal lobe and MeCP2, both in the hippocampus and the frontal lobe. TMC downregulated the expressions of HDAC1 in the hippocampus and frontal lobe and of MeCP2 in the hippocampus, this being in agreement with the above-mentioned data.

MeCP2, a member of the methyl-CG-binding domain (MBD) family of proteins, may repress transcription in a histone deacetylase (HDAC)-dependent process (e.g. binding of MeCP2 to Sin3A-HDAC complexes), and in a HDAC-independent manner (e.g. inhibiting of the assembly of transcription preinitiation complexes and MeCP2 functioning as a global methyl-CG-specific, histone deacetylase independent repressor) (Wan, 2001; Fuks et al., 2003; Clouaire and Stancheva, 2008; Theisen et al., 2013). However, scientific data sustained that MeCP2 can induce either repression or activation of gene transcription, processes that highly depend on the cellular and molecular context (Hansen et al., 2010; Theisen et al., 2013).

Despite the significant use of HDAC inhibitory agents (HDACi), such as valproic acid (VPA) in nervous system disorders (e.g. epilepsy), the HDACs expression in the central nervous system remains still unclear (MacDonald and Roskams, 2008; Khurana and Dlugos, 2017). Decreased HDAC2 protein expression in the nucleus accumbens was also associated with both depression in humans and chronic stress-like behavior in mice (Massart et al., 2012). Additionally, it has been reported that MeCP2 overexpression in mice neurons may be associated with anxiety, impaired memory and learning, abnormal motor coordination and abnormal hippocampal synaptic plasticity due to a HDAC repressed–transcription–mechanism. Moreover, previous evidence sustains that HDAC co-repressor proteins may negatively regulate NFκB transcriptional activity (Ashburner and Westerheide, 2001).

As NFkB modulates ROS levels and the expression of proinflammatory factors (interleukin (IL)-1, intercellular adhesion molecule 1, tumor necrosis factor α (TNF α), therefore, it can be considered to initiate the inflammatory cascade (Wang et al., 2019). Previous studies also revealed that HDACi inhibits NFkB transcription, thus repressing inflammatory response (Lu et al., 2015). Additionally, it was suggested that high blood glucose and oxidative stress levels may lead to neuroinflammation *via* NFκB signaling (Richa et al., 2017). Thus, we also evaluated the active form of p65 subunit, phospho-NFkB p65 expressions in the hippocampus and frontal lobe. In our study, both TMW and TMC tended to decrease the phospho-NFkB p65 in the frontal lobe and consequently the NFkB transcriptional activity. Conversely, in the hippocampus, with regard to administration of extracts, our results were contradictory with the above-mentioned data. These observations are consistent with the literature. Several authors reported that polyphenols administration reduced the expression of NF-κB in in different brain areas (e.g. hippocampus, striatum and frontal cortex) and cell types (astrocytes and microglia) (Vauzour, 2012; Sarubbo et al., 2017; Jha et al., 2019).

It is known that the transcription factor NFκB may exhibit cellular context-dependent function in the nervous system (Kaltschmidt and Kaltschmidt, 2009). NF-κB is involved in multiple physiological and pathological processes, (e.g. neurotransmission and neuroprotection, inflammation, immunity, apoptosis, cell proliferation, differentiation and survival) and can be activated by various factors: inflammatory cytokines, antigen receptor engagement, UV- or γ-irradiation, ischemia and hyperosmotic shock or oxidative stress (Meffert et al., 2003; Hayden et al., 2006; Oeckinghaus and Ghosh, 2009). In unstimulated cells, IκB kinase (IKK) complex can induce nuclear translocation of inactive NFκB and transcription of target genes. The constitutive active form of NFkB can be found in mature B cells, macrophages, neurons, vascular smooth muscle cells and tumoral cells (Oeckinghaus and Ghosh, 2009).

Although significant progress has been made in the understanding of the NFkB activation mechanism, the functional significance of this process in the nervous system still requires further research. Several authors sustain that NFkB can either regulate cell death or survival in the nervous system, depending on the type of cell (e.g. glia vs neurons), different stimuli or molecular context. To elucidate the influence of natural products on NFkB activation and its role in the nervous system, further studies should be performed (Kaltschmidt and Kaltschmidt, 2009; Mincheva-Tasheva and Soler, 2013). Our data showed that diabetic rats had no significant degree of histological brain damage, thus sustaining biochemical and functional - related damages rather than morphological ones.

CONCLUSIONS

Our study demonstrated that TMW and TMC administration reduced blood glucose levels. TMW improved the central locomotion of the rats, both in OFT and EPM. In the frontal lobe, both extract diminished lipid peroxidation and HDAC1 expression but enhanced the antioxidant capacity. TMW administration increased the phospho-NFkB p65 and diminished MECP2 expression in the hippocampus. Our findings indicate that administration of *Thymus marschallianus* Willd. extracts (either TMW or TMC extracts) might represent a good option in diabetes-related complications, by exerting various beneficial effects *via* several/various mechanisms. Both extracts exerted a beneficial effect by increasing the antioxidant defense and improving the central locomotion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Board on animal welfare of the "Iuliu Hatieganu" University and by the Direction for Veterinary Surveillance and Food Safety.

AUTHOR CONTRIBUTIONS

All authors have contributed directly and intellectually to the study. AS-B, II, GF, IO, DB, IC, SC and DH contributed to the design of the study, writing the original draft and revising it critically. AS-B, II, LV, AOM, A-MG, VT, BM, AM, IB, DO have contributed to the experimental part of the study. All authors have read and approved the final form of the manuscript for publication.

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REFERENCES

Anchan, D., Clark, S., Pollard, K., and Vasudevan, N. (2014). GPR30 Activation Decreases Anxiety in the Open Field Test but Not in the Elevated plus Maze Test in Female Mice. *Brain Behav.* 4 (1), 51–59. doi: 10.1002/brb3.197

Ashburner, B. P., and Westerheide, S. D. (2001). The P65 (RelA) Subunit of NF-KB Interacts with the Histone Deacetylase (HDAC) Corepressors HDAC1 and HDAC2 To Negatively Regulate Gene Expression. *Mol. Cell. Biol.* 21 (20), 7065–7077. doi: 10.1128/MCB.21.20.7065

Balaban, R. S., Nemoto, S., and Finkel, T. (2005). Mitochondria, Oxidants, and Aging. *Cell* 120 (4), 483–495. doi: 10.1016/j.cell.2005.02.001

Baldea, I., Costin, G. E., Shellman, Y., Kechris, K., Olteanu, E. D., Filip, A., et al. (2013). Biphasic Pro-Melanogenic and pro-Apoptotic Effects of All-Trans-Retinoic Acid (ATRA) on Human Melanocytes: Time-Course Study. *J. Dermatol. Sci.* 72 (2), 168–176. doi: 10.1016/j.jdermsci.2013.06.004

Bamosa, A. O., Kaatabi, H., Lebda, F. M., Al Elq, A. M., and Al-Sultan, A. (2010). Effect of Nigella Sativa Seeds on the Glycemic Control of Patients with Type 2 Diabetes Mellitus. *Indian J. Physiol. Pharmacol.* 54 (4), 344–354.

Bedoya, F. J., Solano, F., and Lucas, M. (1996). N-Monomethyl-Arginine and Nicotinamide Prevent Streptozotocin-Induced Double Strand DNA Break Formation in Pancreatic Rat Islets. *Experientia* 52 (4), 344–347. doi: 10.1007/BF01919538

Benedec, D., Vlase, L., Hanganu, D., and Oniga, I. (2012). Antioxidant Potential and Polyphenolic Content of Romanian Ocimum Basilicum. *Digest J. Nanomat Biostruct.* 7 (3), 1263–1270.

Benedec, D., Vlase, L., Oniga, I., Mot, A. C., Damian, G., Hanganu, D., et al. (2013a). Polyphenolic Composition, Antioxidant and Antibacterial Activities for Two Romanian Subspecies of Achillea Distans Waldst. et Kit. Ex Willd. *Molecules* 18 (8), 8725–8739. doi: 10.3390/molecules18088725

Benedec, D., Vlase, L., Oniga, I., Mot, A. C., Silaghi-Dumitrescu, R., Hanganu, D., et al. (2013b). LC-MS Analysis and Antioxidant Activity of Phenolic Compounds from Two Indigenous Species of Mentha. Note I. *Farmacia* 61 (2), 262–267.

Benedec, D., Hanganu, D., Oniga, I., Tiperciuc, B., Olah, N.-K., Raita, O., et al. (2015). Assessment of Rosmarinic Acid Content in Six Lamiaceae Species Extracts and Their Antioxidant and Antimicrobial Potential. *Pakistan J. Pharm. Sci.* 28 (6 (Suppl.)), 2297–2303.

Benedec, D., Filip, L., Vlase, L., Bele, C., Sevastre, B., Raita, O., et al. (2016a). In Vitro Study of Antioxidant Activity and Phenolic Content of Chrysanthemum Balsamita Varieties. *Pakistan J. Pharm. Sci.* 29 (4), 1359–1364.

Benedec, D., Hanganu, D., Oniga, I., Filip, L., Bischin, C., Silaghi-Dumitrescu, R., et al. (2016b). Achillea Schurii Flowers: Chemical, Antioxidant, and Antimicrobial Investigations. *Molecules* 21 (1050), 1–12. doi: 10.3390/molecules21081050

Broide, R. S., Redwine, J. M., Aftahi, N., Young, W., Bloom, F. E., and CJ, W. (2007). Distribution of Histone Deacetylases 1–11 in the Rat Brain. *J. Mol. Neurosci.* 31 (3), 281–287. doi: 10.1385/JMN/31

Chen, J., Mangelinckx, S., Adams, A., Tao Wang, Z., Li, W. L., and Kimpe, N. De (2015). Natural Flavonoids as Potential Herbal Medication for the Treatment of Diabetes Mellitus and Its Complications. *Natural Prod. Commun.* 10 (1), 187–200. doi: 10.1177/1934578x1501000140

Chentli, F., Azzoug, S., and Mahgoun, S. (2015). Diabetes Mellitus in Elderly. *Indian J. Endocrinol. Metab.* 19 (6), 744–752. doi: 10.4103/2230-8210.167553

Choudhury, H., Pandey, M., Hua, C. K., Shi Mun, C., Koh Jing, J., Kong, L., et al. (2018). An Update on Natural Compounds in the Remedy of Diabetes Mellitus: A Systematic Review. *J. Trad. Complement. Med.* 8 (3), 361–376. doi: 10.1016/j.jtcme.2017.08.012

Clouaire, T., and Stancheva, I. (2008). Methyl-CpG Binding Proteins: Specialized Transcriptional Repressors or Structural Components of Chromatin? *Cell. Mol. Life Sci.* 65 (10), 1509–1522. doi: 10.1007/s00018-008-7324-y

Conti, M., Morand, P. C., Levillain, P., and Lemonnier, A. (1991). Improved Fluorometric Determination of Malonaldehyde. *Clin. Chem.* 37 (7), 1273–1275. doi: 10.1093/clinchem/37.7.1273

Damasceno, D. C., Netto, A. O., Iessi, I. L., Gallego, F. Q., Corvino, S. B., Dallaqua, B., et al. (2014). Streptozotocin-Induced Diabetes Models: Pathophysiological Mechanisms and Fetal Outcomes. *BioMed. Res. Int.* 2014, 1–11. doi: 10.1155/2014/819065

Deeds, M. C., Anderson, J. M., Armstrong, A. S., Gastineau, D. A., Hiddinga, H. J., Jahangir, A., et al. (2011). Single Dose Streptozotocin-Induced Diabetes: Considerations for Study Design in Islet Transplantation Models. *Lab. Anim.* 45 (3), 131–140. doi: 10.1258/la.2010.010090

De la Monte, S. M. (2014). Relationships between Diabetes and Cognitive Impairment. *Endocrinol. Metab. Clinics North America* 43 (1), 245–267. doi: 10.1016/j.ecl.2013.09.006

Dresselhaus, E., Duerr, J. M., Vincent, F., Sylvain, E. K., Beyna, M., Lanyon, L. F., et al. (2018). Class i HDAC Inhibition Is a Novel Pathway for Regulating Astrocytic ApoE Secretion. *PloS One* 13 (3), 1–20. doi: 10.1371/journal.pone.0194661

Fuks, F., Hurd, P. J., Wolf, D., Nan, X., Bird, A. P., and Kouzarides, T. (2003). The Methyl-CpG-Binding Protein MeCP2 Links DNA Methylation to Histone Methylation. *J. Biol. Chem.* 278 (6), 4035–4040. doi: 10.1074/jbc.M210256200

Fusco, R., Scuto, M., Cordaro, M., D'Amico, R., Gugliandolo, E., Siracusa, R., et al. (2019). N-Palmitoylethanolamide-Oxazoline Protects against Middle Cerebral Artery Occlusion Injury in Diabetic Rats by Regulating the SIRT1 Pathway. *Int. J. Mol. Sci.* 20 (19), 1–22. doi: 10.3390/ijms20194845

Gamberini, M. T., Rodrigues, D. S., Rodrigues, D., and Pontes, V. B. (2015). Effects of the Aqueous Extract of Pimpinella Anisum L. Seeds on Exploratory Activity and Emotional Behavior in Rats Using the Open Field and Elevated plus Maze Tests. *J. Ethnopharmacol.* 168, 45–49. doi: 10.1016/j.jep.2015.03.053

Gatla, H. R., Muniraj, N., Thevkar, P., Yavvari, S., Sukhavasi, S., and Makena, M. R. (2019). Regulation of Chemokines and Cytokines by Histone Deacetylases and an Update on Histone Decetylase Inhibitors in Human Diseases. *Int. J. Mol. Sci.* 20 (5), 1–27. doi: 10.3390/ijms20051110

González-Gallego, J., García-Mediavilla, M.V., Sánchez-Campos, S., and Tuñó, M. J. (2010). Fruit Polyphenols, Immunity and Inflammation. *Br. J. Nutr.* 104 (SUPPL.3), S15–S27. doi: 10.1017/S0007114510003910

Groh, I. A. M., Chen, C., Lüske, C., Cartus, A. T., and Esselen, M. (2013). Plant Polyphenols and Oxidative Metabolites of the Herbal Alkenylbenzene Methyleugenol Suppress Histone Deacetylase Activity in Human Colon Carcinoma Cells. *J. Nutr. Metab.* 2013, 1–11. doi: 10.1155/2013/821082

Guan, J. S., Haggarty, S. J., Giacometti, E., Dannenberg, J. H., Joseph, N., Gao, J., et al. (2009). HDAC2 Negatively Regulates Memory Formation and Synaptic Plasticity. *Nature* 459 (7243), 55–60. doi: 10.1038/nature07925

Haberland, M., Montgomery, R. L., and Olson, E. N. (2009). The Many Roles of Histone Deacetylases in Development and Physiology: Implications for Disease and Therapy. *Nat. Rev. Genet.* 10 (1), 32–42. doi: 10.1038/nrg2485

Hanganu, D., Benedec, D., Vlase, L., Popica, I., Raita, C. B. O., Gheldiu, A.-M., et al. (2016a). Polyphenolic Content and Antioxidant Activity of Chrysanthemum Parthenium Extract. *Farmacia* 64 (4), 498–501.

Hanganu, D., Filip, L., Olah, N.-K., Mocan, A., Vlase, L., Raita, O., et al. (2016b). Evaluation of Polyphenolic Profile and Antioxidant Activity for Cytisus Nigricans and Cytisus Albus. *Farmacia* 64 (6), 863–867.

Hansen, J. C., Ghosh, R. P., and Woodcock, C. L. (2010). Binding of the Rett Syndrome Protein, MeCP2, to Methylated and Unmethylated DNA and Chromatin. *IUBMB Life* 62 (10), 732–738. doi: 10.1002/iub.386

Hayden, M. S., West, A. P., and Ghosh, S. (2006). NF-KB and the Immune Response. *Oncogene* 25 (51), 6758–6780. doi: 10.1038/sj.onc.1209943

Hu, M.-L. (1994). Measurement of Protein Thiol Groups and Glutathione in Plasma By". *233* 1987), 380–385.

Ielciu, I., Vlase, L., Frédérich, M., Hanganu, D., Păltinean, R., Cieckiewicz, E., et al. (2017). Polyphenolic Profile and Biological Activities of the Leaves and Aerial Parts of Echinocystis Lobata (Michx.) Torr. et A. Gray (Cucurbitaceae). *Farmacia* 65 (2), 179–183.

Ielciu, I., Hanganu, D., Păltinean, R., Vlase, L., Frédérich, M., Gheldiu, A.-M., et al. (2018). Antioxidant Capacity and Polyphenolic Content of the Echinocystis Lobata (Michx.) Torr. et A.Gray Flowers. *Pakistan J. Pharm. Sci.* 31 (2 (Suppl)), 677–683.

Ielciu, I., Frederich, M., Hanganu, D., Angenot, L., Olah, N.-K., Ledoux, A., et al. (2019). Flavonoid Analysis and Antioxidant Activities of the Bryonia Alba L . Aerial Parts. *Antioxidants* 8 (108), 1–11. doi: 10.3390/antiox8040108

Impellizzeri, D., Peritore, A. F., Cordaro, M., Gugliandolo, E., Siracusa, R., Crupi, R., et al. (2019). The Neuroprotective Effects of Micronized PEA (PEA-m) Formulation on Diabetic Peripheral Neuropathy in Mice. *FASEB J.* 33 (10), 11364–11380. doi: 10.1096/fj.201900538R

International Diabetes Federation (2019). *IDF Diabetes Atlas. International Diabetes Federation*. Available at: www.diabetesatlas.org.

Jaworska, J., Ziemka-Nalecz, M., and Zalewska, T. (2015). Histone Deacetylases 1 and 2 Are Required for Brain Development. *Int. J. Dev. Biol.* 59 (4–6), 171–177. doi: 10.1387/ijdb.150071tz

Jha, N. K., Jha, S. K., Kar, R., Nand, P., Swati, K., and Goswami, V. K. (2019). Nuclear Factor-Kappa β as a Therapeutic Target for Alzheimer's Disease. *J. Neurochem.* 150 (2), 113–137. doi: 10.1111/jnc.14687

Jianu, C., Mihail, R., Muntean, S. G., Pop, G., Daliborca, C. V., Horhat, G., et al. (2015). Composition and Antioxidant Capacity of Essential Oils Obtained from Thymus Vulgaris, Thymus Pannonicus and Satureja Montana Grown in Western Romania. *Rev. Chimie* 66 (12), 2157–2160.

Kaltschmidt, B., and Kaltschmidt, C. (2009). NF- B in the Nervous System. *Cold Spring Harbor Perspect. Biol.* 1 (3), a001271–a001271. doi: 10.1101/cshperspect.a001271

Kavalali, E. T., Nelson, E. D., and Monteggia, L. M. (2011). Role of MeCP2, DNA Methylation, and HDACs in Regulating Synapse Function. *J. Neurodev. Disord.* 3 (3), 250–256. doi: 10.1007/s11689-011-9078-3

Khurana, A., and Dlugos, C. A. (2017). Age-Related Alterations in Histone Deacetylase Expression in Purkinje Neurons of Ethanol-Fed Rats. *Brain Res.* 1675, 8–19. doi: 10.1016/j.brainres.2017.08.026

Kodl, C. T., and Seaquist, E. R. (2008). Cognitive Dysfunction and Diabetes Mellitus. *Endoc. Rev.* 29 (4), 494–511. doi: 10.1210/er.2007-0034

Kushwah, N., Jain, V., Deep, S., Prasad, D., Singh, S. B., and Khan, N. (2016). Neuroprotective Role of Intermittent Hypobaric Hypoxia in Unpredictable Chronic Mild Stress Induced Depression in Rats. *PloS One* 11 (2), 1–20. doi: 10.1371/journal.pone.0149309

Lu, X., Wang, L., Yu, C., Yu, D., and Yu, G. (2015). Histone Acetylation Modifiers in the Pathogenesis of Alzheimer's Disease. *Front. Cell. Neurosci.* 9, 226 (June). doi: 10.3389/fncel.2015.00226

MacDonald, J. L., and Roskams, A.J. (2008). Histone Deacetylases 1 and 2 Are Expressed at Distinct Stages of Neuro-Glial Development. *Dev. Dynamics* 237 (8), 2256–2267. doi: 10.1002/dvdy.21626

Maithili Karpaga Selvi, N., Sridhar, M. G., Swaminathan, R. P., and Sripradha, R. (2015). Efficacy of Turmeric as Adjuvant Therapy in Type 2 Diabetic Patients. *Indian J. Clin. Biochem.* 30 (2), 180–186. doi: 10.1007/s12291-014-0436-2

Mancini, E., Senatore, F., Monte, D. D., Martino, L. De, Grulova, D., Scognamiglio, M., et al. (2015). Studies on Chemical Composition, Antimicrobial and Antioxidant Activities of Five Thymus Vulgaris L. Essential Oils. *Molecules* 20 (7), 12016–12028. doi: 10.3390/molecules200712016

Massart, R., Mongeau, R., and Lanfumey, L. (2012). Beyond the Monoaminergic Hypothesis: Neuroplasticity and Epigenetic Changes in a Transgenic Mouse Model of Depression. *Philos. Trans. R. Soc. B: Biol. Sci.* 367 (1601), 2485–2494. doi: 10.1098/rstb.2012.0212

Meffert, M. K., Chang, J. M., Wiltgen, B. J., Fanselow, M. S., and Baltimore, D. (2003). NF-KB Functions in Synaptic Signaling and Behavior. *Nat. Neurosci.* 6 (10), 1072–1078. doi: 10.1038/nn1110

Mincheva-Tasheva, S., and Soler, R. M. (2013). NF-KB Signaling Pathways: Role in Nervous System Physiology and Pathology. *Neuroscientist* 19 (2), 175–194. doi: 10.1177/1073858412444007

Mocan, A., Crişan, G., Vlase, L., Crişan, O., Vodnar, D. C., Raita, O., et al. (2014). Comparative Studies on Polyphenolic Composition, Antioxidant and Antimicrobial Activities of Schisandra Chinensis Leaves and Fruits. *Molecules* 19 (9), 15162–15179. doi: 10.3390/molecules190915162

Montgomery, R. L., Hsieh, J., Barbosa, A. C., Richardson, J. A., and Olson, E. N. (2009). Histone Deacetylases 1 and 2 Control the Progression of Neural Precursors to Neurons during Brain Development. *Proc. Natl. Acad. Sci. U. S. A.* 106 (19), 7876–7881. doi: 10.1073/pnas.0902750106

Nabavi, S. M., Marchese, A., Izadi, M., Curti, V., Daglia, M., and Nabavi, S. F. (2015). Plants Belonging to the Genus Thymus as Antibacterial Agents: From Farm to Pharmacy. *Food Chem.* 173, 339–347. doi: 10.1016/j.foodchem.2014.10.042

Nadeem, M., Imran, M., Gondal, T. A., Imran, A., Shahbaz, M., Amir, R. M., et al. (2019). Therapeutic Potential of Rosmarinic Acid: A Comprehensive Review. *Appl. Sci.* 9 (15), 3139. doi: 10.3390/app9153139

Nandini, H. S., and Naik, P. R. (2019). Antidiabetic, Antihyperlipidemic and Antioxidant Effect of Vincamine, in Streptozotocin-Induced Diabetic Rats. *Eur. J. Pharmacol.* 843, 233–239. doi: 10.1016/j.ejphar.2018.11.034

Ngo, Y. L., Lau, C. H., and Chua, L. S. (2018). Review on Rosmarinic Acid Extraction, Fractionation and Its Anti-Diabetic Potential. *Food Chem. Toxicol.* 121, 687–700. doi: 10.1016/j.fct.2018.09.064

Niculae, M., Hanganu, D., Oniga, I., Benedec, D., Ielciu, I., Giupana, R., et al. (2019). Phytochemical Profile and Antimicrobial Potential of Extracts Obtained from Thymus Marschallianus Willd. *Molecules* 24 (3101), 1–12. doi: 10.3390/molecules24173101

Nogueira-Machado, J. A., and Chaves, M. M. (2008). From Hyperglycemia to AGE-RAGE Interaction on the Cell Surface: A Dangerous Metabolic Route for Diabetic Patients. *Expert Opin. Ther. Targets* 12 (7), 871–882. doi: 10.1517/14728222.12.7.871

Nozaki, T., Minaguchi, J., Takehana, K., and Ueda, H. (2017). Anti-Diabetic Activities of Traditional Chinese Herbal Medicine in Streptozotocin-Induced Diabetic Rats. *Okajimas Folia Anatomica Japonica* 93 (4), 111–118. doi: 10.2535/ofaj.93.111

Nunes, S., Madureira, A. R., Campos, D., Sarmento, B., Gomes, A. M., Pintado, M., et al. (2017). Therapeutic and Nutraceutical Potential of Rosmarinic Acid—Cytoprotective Properties and Pharmacokinetic Profile. *Crit. Rev. Food Sci. Nutr.* 57 (9), 1799–1806. doi: 10.1080/10408398.2015.1006768

Oeckinghaus, A., and Ghosh, S. (2009). The NF-KB Family of Transcription Factors and Its Regulation. *Cold Spring Harbor Perspect. Biol.* 1 (4), 1–15. doi: 10.1101/cshperspect.a000034

Olah, N.-K., Osser, G., Câmpean, R. F., Furtuna, F. R., Benedec, D., Filip, L., et al. (2016). The Study of Polyphenolic Compounds Profile of Some Rosmarinus Officinalis L . Extracts. *Pakistan J. Pharm. Sci.* 29 (6 (Suppl), 2355–2361.

Oniga, I., Puşcaş, C., Silaghi-Dumitrescu, R., Olah, N.-K., Sevastre, B., Marica, R., et al. (2018). Origanum Vulgare Ssp. Vulgare: Chemical Composition and Biological Studies. *Molecules* 23 (2077), 1–14. doi: 10.3390/molecules23082077

Opris, R., Tatomir, C., Olteanu, D., Moldovan, R., Moldovan, B., David, L., et al. (2017). The Effect of Sambucus Nigra L. Extract and Phytosinthesized Gold Nanoparticles on Diabetic Rats. *Colloids Surf. B: Biointerf.* 150, 192–200. doi: 10.1016/j.colsurfb.2016.11.033

Parihar, P., Shetty, R., Ghafourifar, P., and Parihar, M. S. (2016). Increase in Oxidative Stress and Mitochondrial Impairment in Hypothalamus of Streptozotocin Treated Diabetic Rat: Antioxidative Effect of Withania Somnifera. *Cell. Mol. Biol.* 62 (1), 77–83. doi: 10.14715/cmb

Patel, S., and Santani, D. (2009). Role of NF-KB in the Pathogenesis of Diabetes and Its Associated Complications. *Pharmacol. Rep.* 61 (4), 595–603. doi: 10.1016/S1734-1140(09)70111-2

Peixoto, L., and Abel, T. (2013). The Role of Histone Acetylation in Memory Formation and Cognitive Impairments. *Neuropsychopharmacology* 38 (1), 62–76. doi: 10.1038/npp.2012.86

Petrović, S., Ušjak, L., Milenković, M., Arsenijević, J., Drobac, M., Drndarević, A., et al. (2017). Thymus Dacicus as a New Source of Antioxidant and Antimicrobial Metabolites. *J. Funct. Foods* 28, 114–121. doi: 10.1016/j.jff.2016.11.007

Petrovska, B. B. (2012). Historical Review of Medicinal Plants' Usage. *Pharmacognosy Rev.* 6 (11), 1–5. doi: 10.4103/0973-7847.95849

Pillai, R., Coverdale, L. E., Dubey, G., and Martin, C.C. (2004). Histone Deacetylase 1 (Hdac-1) Required for the Normal Formation of Craniofacial Cartilage and Pectoral Fins of the Zebrafish. *Dev. Dynamics* 231 (3), 647–654. doi: 10.1002/dvdy.20168

Qian, C., Zhu, C., Yu, W., Jiang, X., and Zhang, F. (2015). High-Fat Diet/Low-Dose Streptozotocin-Induced Type 2 Diabetes in Rats Impacts Osteogenesis and Wnt Signaling in Bone Marrow Stromal Cells. *PloS One* 10 (8), 1–15. doi: 10.1371/journal.pone.0136390

Raudone, L., Zymone, K., Raudonis, R., Vainoriene, R., Motiekaityte, V., and Janulis, V. (2017). Phenological Changes in Triterpenic and Phenolic Composition of Thymus L. Species. *Ind. Crops Prod.* 109, 445–451. doi: 10.1016/j.indcrop.2017.08.054

Reddy, D. S., Wu, X., Golub, V. M., Mohaiza Dashwood, W., and Dashwood, R. H. (2018). Measuring Histone Deacetylase Inhibition in the Brain. *Curr. Protoc. Pharmacol.* 81 (1), 1–14. doi: 10.1002/cpph.41

Richa, R., Yadawa, A. K., and Chaturvedi, C. M. (2017). Hyperglycemia and High Nitric Oxide Level Induced Oxidative Stress in the Brain and Molecular Alteration in the Neurons and Glial Cells of Laboratory Mouse, Mus Musculus. *Neurochem. Int.* 104, 64–79. doi: 10.1016/j.neuint.2016.12.008

Ricobaraza, A., Cuadrado-Tejedor, M., Marco, S., Pérez-Otaño, I., and García-Osta, A. (2012). Phenylbutyrate Rescues Dendritic Spine Loss Associated with Memory Deficits in a Mouse Model of Alzheimer Disease. *Hippocampus* 22 (5), 1040–1050. doi: 10.1002/hipo.20883

Rusu, M. E., Gheldiu, A. M., Mocan, A., Moldovan, C., Saveta Popa, D., Tomuta, I., et al. (2018). Process Optimization for Improved Phenolic Compounds Recovery from Walnut (Juglans Regia L.) Septum: Phytochemical Profile and Biological Activities. *Molecules* 23 (11), 1–28. doi: 10.3390/molecules23112814

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

Salehi, B., Abu-Darwish, M. S., Tarawneh, A. H., Cabral, C., Gadetskaya, A. V., Salgueiro, L., et al. (2019). Thymus Spp. Plants - Food Applications and Phytopharmacy Properties. *Trends Food Sci. Technol.* 85, 287–306. doi: 10.1016/j.tifs.2019.01.020

Sarubbo, F., Esteban, S., Miralles, A., and Moranta, D. (2017). Effects of Resveratrol and Other Polyphenols on Sirt1: Relevance to Brain Function During Aging. *Curr. Neuropharmacol.* 16 (2), 126–136. doi: 10.2174/1570159x15666170703113212

Sivandzade, F., Prasad, S., Bhalerao, A., and Cucullo, L. (2019). NRF2 and NF-KB Interplay in Cerebrovascular and Neurodegenerative Disorders: Molecular Mechanisms and Possible Therapeutic Approaches. *Redox Biol.* 21, 101059. doi: 10.1016/j.redox.2018.11.017

Snow, W. M., and Albensi, B. C. (2016). Neuronal Gene Targets of NF-KB and Their Dysregulation in Alzheimer's Disease. *Front. Mol. Neurosci.* 9, 118. doi: 10.3389/fnmol.2016.00118

Spencer, J. P. E., Vauzour, D., and Rendeiro, C. (2009). Flavonoids and Cognition: The Molecular Mechanisms Underlying Their Behavioural Effects. *Arch. Biochem. Biophys.* 492 (1–2), 1–9. doi: 10.1016/j.abb.2009.10.003

Szkudelski, T. (2001). The Mechanism of Alloxan and Streptozotocin Action in B Cells of the Rat Pancreas. *Physiol. Res.* 50 (6), 537–546.

Takasu, N., Komiya, I., Asawa, T., Nagasawa, Y., and Yamada, T. (1991). Streptozocin- and Alloxan-Induced H2O2 Generation and DNA Fragmentation in Pancreatic Islets: H2O2 as Mediator for DNA Fragmentation. *Diabetes* 40 (9), 1141–1145. doi: 10.2337/diab.40.9.1141

Theisen, J. W. M., Gucwa, J. S., Yusufzai, T., Khuong, M. T., and Kadonaga, J. T. (2013). Biochemical Analysis of Histone Deacetylase-Independent Transcriptional Repression by MeCP2. *J. Biol. Chem.* 288 (10), 7096–7104. doi: 10.1074/jbc.M112.438697

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, 89–99. doi: 10.1016/j.indcrop.2019.02.038

Tong, M., and De La Monte, S. M. (2009). Mechanisms of Ceramide-Mediated Neurodegeneration. *J. Alzheimer's Dis.* 16 (4), 705–714. doi: 10.3233/JAD-2009-0983

Umeno, A., Horie, M., Murotomi, K., Nakajima, Y., and Yoshida, Y. (2016). Antioxidative and Antidiabetic Effects of Natural Polyphenols and Isoflavones. *Molecules* 21 (6), 1–15. doi: 10.3390/molecules21060708

Vauzour, D. (2012). Dietary Polyphenols as Modulators of Brain Functions: Biological Actions and Molecular Mechanisms Underpinning Their Beneficial Effects. *Oxid. Med. Cell. Longevity* 2012, 1–16. doi: 10.1155/2012/914273

Vauzour, D. (2017). Polyphenols and Brain Health. *OCL* 24 (2), 1–7. doi: 10.1051/ocl/2017008

Volpe, C. M. O., Villar-Delfino, P. H., Anjos, P. M. F. D., and Nogueira-Machado, J. A. (2018). Cellular Death, Reactive Oxygen Species (ROS) and Diabetic Complications Review-Article. *Cell Death Dis.* 9 (2), 1–9. doi: 10.1038/s41419-017-0135-z

Walf, A. A., and Frye, C. A. (2007). The Use of the Elevated plus Maze as an Assay of Anxiety-Related Behavior in Rodents. *Nat. Protoc.* 2 (2), 322–328. doi: 10.1038/nprot.2007.44

Wan, M. (2001). MECP2 Truncating Mutations Cause Histone H4 Hyperacetylation in Rett Syndrome. *Hum. Mol. Genet.* 10 (10), 1085–1092. doi: 10.1093/hmg/10.10.1085

Wang, X., Feng, S. W. U., Wang, F., and Xu, S. (2014). Modeled Behavior of Neuropathic Pain with Social Defect in Rats: A Preliminary Methodology Evaluation. *Med. Sci. Monit. Basic Res.* 20, 164–169. doi: 10.12659/MSMBR.892615

Wang, F., Shang, Y., Zhang, R., Gao, X., and Zeng, Q. (2019). A SIRT1 Agonist Reduces Cognitive Decline in Type 2 Diabetic Rats through Antioxidative and Anti-Inflammatory Mechanisms. *Mol. Med. Rep.* 19 (2), 1040–1048. doi: 10.3892/mmr.2018.9699

Wardyn, J. D., Ponsford, A. H., and Sanderson, C. M. (2015). Dissecting Molecular Cross-Talk between Nrf2 and NF-KB Response Pathways. *Biochem. Soc. Trans.* 43, 621–626. doi: 10.1042/BST20150014

World Health Organization (2016). *"Global Report on Diabetes."* Vol. 978. Available at: http://www.who.int/about/licensing/.

Yassa, H. D., and Tohamy, A. F. (2014). Extract of Moringa Oleifera Leaves Ameliorates Streptozotocin-Induced Diabetes Mellitus in Adult Rats. *Acta Histochem.* 116 (5), 844–854. doi: 10.1016/j.acthis.2014.02.002

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REVIEW
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The Therapeutic Potential of the Essential Oil of *Thymbra capitata* (L.) Cav., *Origanum dictamnus* L. and *Salvia fruticosa* Mill. And a Case of Plant-Based Pharmaceutical Development

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This review performs a comprehensive assessment of the therapeutic potential of three native herbs of Crete (*Thymbra capitata* (L.) Cav., *Salvia fruticosa* Mill. and *Origanum dictamnus* L.), their phytochemical constituents, health benefits and issues relevant to their safety, within a translational context. Issues discussed comprise: 1) Ethnopharmacological uses of the three herbs, reviewed through an extensive search of the literature; 2) Systematic analysis of the major phytochemical constituents of each plant, and their medicinal properties; 3) To what extent could the existing medicinal properties be combined and produce an additive or synergistic effect; 4) Possible safety issues. We conclude with a specific example of the use of a combination of the essential oils of these plants as an effective anti-viral product and the experience gained in a case of a plant-based pharmaceutical development, by presenting the major steps and the continuum of the translational chain.

Keywords: traditional medicine, Southeastern Europe, Mediterranean, Near East, synergy, regulatory affairs, clinical trials, antiviral

INTRODUCTION

Plants are traditionally used, in different forms, for the treatment of diseases, since the Neolithic Era (Hardy, 2019). Different populations used native flora for the production of preparations, efficient in curing different diseases and conditions (Fabricant and Farnsworth, 2001). The Cretan area (KK, Crete and Karpathos floristic region) is such an example, as its evolutionary history preserved a very diverse flora, with more than 2000 indigenous species, including a high number of endemics (Dimopoulos et al., 2013). Cretan people abundantly use plants and greens in different aspects of their life. Cretan diet, a specific entity of the Mediterranean diet, uses a diversity of plants for culinary purposes (Renaud, 1995 and references herein), while a variety of plants are used as decoctions and infusions for recreational or medical purposes. We systematically analyzed the habits of a rural population in Crete and reported that use of different plant infusions, alone or in combination, resulted in a significant protection from common cold and influenza infections (Lionis et al., 1998).

Based on this study, we have focused on three of the most efficient plants (*Coridothymus capitatus*, *Salvia fruticosa* and *Origanum dictamnus*) and developed a supplement, efficient at combatting upper respiratory tract infections (Duikler et al., 2015; Anastasaki et al., 2017).

In this review, we analyze in depth the health benefits of these plants, their most prominent active compounds and their combination. In addition, we review our experience with the development of a nutraceutical product, discussing potential bottlenecks encountered during the process.

THE PLANTS

Taxonomy and Distribution

According to the Euro + Med plantbase, ***Coridothymus capitatus* (L.) Reichenb. fil**. is a dwarf-shrub. It is the only member of the monospecific genus *Coridothymus*. According to the Euro + Med plantbase (Accessed at June 2017), the name *Coridothymus capitatus* (L.) Reichenb. fil. is synonym of *Thymbra capitata* (L.) Cav., which is included in Kingdom–Plantae, Division–Tracheophyta, Subdivision–Spermatophytina, Class–Magnoliopsida, Superorder–Asteranae, Order–Lamiales, Family–Lamiaceae Lindl., Genus–*Thymbra* L.

Accepted name: Thymbra capitata (L.) Cav., Homotypic synonyms: *Coridothymus capitatus* (L.) Rchb. f., *Origanum capitatum* (L.) Kuntze, *Satureja capitata* L., *Thymus capitatus* (L.) Hoffmanns. & Link.

***Salvia fruticosa* Mill**. is also a member of the Labiatae family. The taxon is included in Kingdom–Plantae, Division–Tracheophyta, Subdivision–Spermatophytina, Class–Magnoliopsida, Superorder–Asteranae, Order–Lamiales, Family–Lamiaceae Lindl., Genus–*Salvia* L.

Accepted name: *Salvia fruticosa* Mill, Heterotypic synonyms: *Salvia baccifera* Etl., *Salvia clusii* Jacq., *Salvia cypria* Unger & Kotschy, *Salvia fruticosa* subsp. *cypria* (Unger & Kotschy) Holmboe, *Salvia fruticosa* subsp. *thomasii* (Lacaita) Brullo and al., *Salvia incarnata* Etl., *Salvia libanotica* Boiss. & Gaill., *Salvia lobryana* Azn., *Salvia marrubioides* Vahl, *Salvia ovata* F. Dietr., *Salvia sipylea* Lam., *Salvia subtriloba* Schrank, *Salvia sypilea* Lam., *Salvia thomasii* Lacaita, *Salvia triloba* L. f., *Salvia triloba* subsp. *calpeana* (Dautez & Debeaux) P. Silva, *Salvia triloba* var. *calpeana* Dautez & Debeaux, *Salvia triloba* subsp. *libanotica* (Boiss. & Gaill.) Holmboe, *Sclarea triloba*(L. f.) Raf.

***Origanum dictamnus* L.** is a local endemic of Crete, member of Labiatae family. The taxon is included in Kingdom–Plantae, Division–Tracheophyta, Subdivision–Spermatophytina, Class–Magnoliopsida, Superorder–Asteranae, Order–Lamiales, Family–Lamiaceae Lindl., Genus–*Origanum* L.

Accepted name: *Origanum dictamnus* L., Heterotypic synonyms: *Amaracus dictamnus* (L.) Benth., *Majorana dictamnus* (L.) Kostel., Heterotypic synonyms: *Amaracus tomentosus* Moench, *Origanum dictamnifolium* St.-Lag., *Origanum saxatile* Salisb.

Coridothymus capitatus is found throughout the Mediterranean region distributed in all Mediterranean countries with the exception of France. *Salvia fruticosa* is distributed in Italy, Sicily, and the eastern Balkans, including Cyprus, while *Origanum dictamnus* is local endemic of Crete (Fielding and Turland, 2005).

METHODOLOGY

In the context of this review, PubMed, Scopus and Google Scholar were used for the retrieval of the relevant bibliography. The following inclusion criteria were used for the systematic review of the three plants: articles published in English, articles concerning countries of the Mediterranean basin and also including Jordan, Syria, Iran and Iraq, as well as studies with the following keywords in their text: *Coridothymus capitatus*, *Salvia fruticosa* and *Origanum dictamnus*. The PRISMA flow diagram, presenting the results of our search, is depicted in **Figure 1**. The primary search identified 1,162 articles, plus 10 articles from other sources. After the implementation of the eligibility criteria, 26 articles were finally incorporated. Any evaluation of studies concerning the plant quality was out of scope and has not been included in this review.

Concerning the synergistic interactions of the main constituents of the essential oil (see below) the review was carried out through a literature searching of the same databases using the following keywords in all fields: "synerg* AND (carvacrol OR eucalyptol OR 1,8-cineole OR beta-Caryophyllene)." The selection of manuscripts was based on the following criteria: articles published in English and articles where only documented synergy between pure substances was reported. The primary search identified 4,101 articles, with 1,538 from PubMed, 1,910 from Google Scholar and 653 from Scopus and after the implementation of the eligibility criteria, excluding the repetitions, 25 articles were finally included in the qualitative analysis.

ETHNOPHARMACOLOGICAL USES OF THE PLANTS

Coridothymus capitatus, *Origanum dictamnus* and *Salvia fruticosa* are renown since antiquity for their pharmaceutical properties (Karousou et al., 1998). The first two are included in the Dioscorides book "*De Materia Medica*." *Salvia fruticosa* was introduced in the Iberian Peninsula for cultivation by Greeks and Phoenicians and elements of these cultivations can be discovered today in several parts of the Iberian coast (Rivera et al., 1994). *O. dictamnus* is also mentioned from the Minoan era, through the centuries from several physicians and philosophers including Asklepios, Euripides, Aristotle, Hippocrates, Theophrastus, Virgil and Galen (Vrachnakis, 2003).

Coridothymus capitatus is known in local cultures with several common names among which Spanish oregano, thyme, headed thyme, conehead thyme, agrio thymari (αγριο θυμάρι), thymari (θυμάρι), tomilho de Creta and tomilho de Dioscórides, *Salvia fruticosa* is known with the names Greek sage and faskomilo

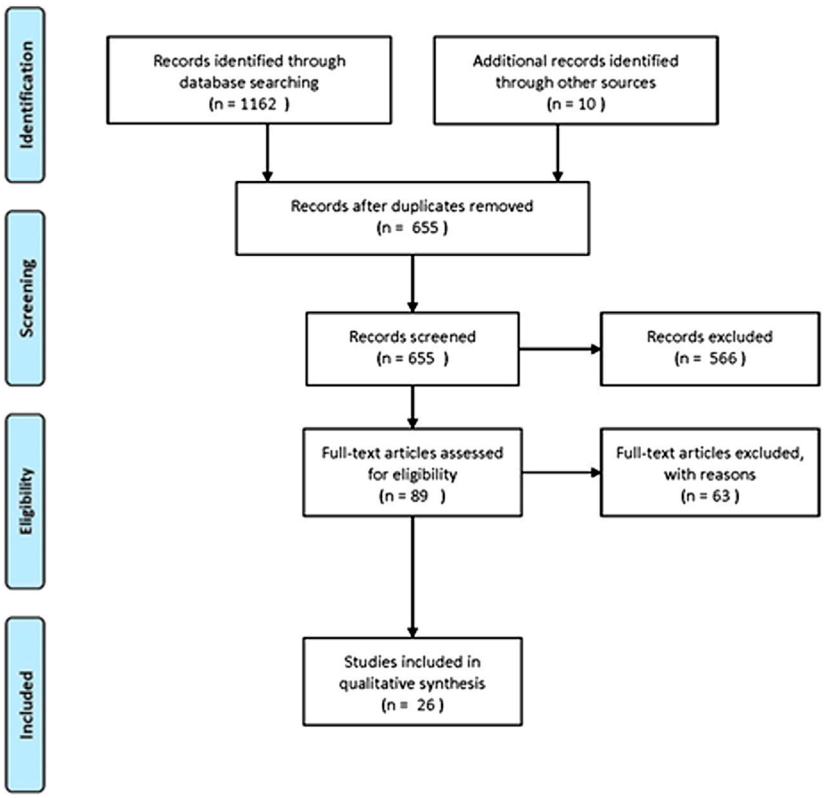

FIGURE 1 | PRISMA flow diagram for the ethnobotanical and ethnopharmacological data.

(φασκόμηλο), while *Origanum dictamnus* is known as diktamo (δίκταμο), dittany of Crete and dictamo de Creta.

The ethnobotanical and ethnopharamacological studies concerning the medical use of *Coridothymus capitatus*, *Salvia fruticosa* and *Origanum dictamnus,* as well as from studies on local herbal markets in Eastern Mediterranean region, are presented in **Tables 1–3** respectively, together with the plant part and their mode of preparation/use.

Based on this information, medicinal uses, in an ethnobotanical context, suggest that the three species have a highly antimicrobial and anti-inflammatory action. In addition, they are active against various targets and diseases, including diseases of the respiratory, digestive and urinary systems.

PHYTOCHEMICAL CONSTITUENTS

The essential oils of the three plants is produced from collected plant material, air dried in the dark, at room temperature (25°C) for 10 days. For analysis, after steam distillation, 1 ml of volatile oils were diluted with 2 ml of ether and filtered through anhydrous sodium sulfate to remove water traces and were stored at 4°C. Analysis was performed, as described previously (Duikler et al., 2015), by Gas Chromatography-Mass Spectroscopy (GC-MS, Shimadzu, QP 5050 A), with a MDN-5 column and a Quadrupole Mass Spectrometer as detector, after injection of 2 μL. The carrier gas was helium, the flow rate 0.9 ml/min. The sample was measured in a split mode procedure (1:35). For GS-MS detection an electron ionization system was used with ionization energy at 70 eV. The interested reader should refer to Duikler et al. (2015) for further analytical details.

The chemical constituents of the essential oils of the three plants, as well as their combination (1.5% v/v of pure essential oils in olive oil carrier), used in the context of upper respiratory tract infections are presented in details as Supplemental Material in Duikler et al. (2015). According to this description (see also **Figure 2**) carvacrol (52.7%) is the main constituent of the mixture, followed by eucalyptol (12.77%) and β-caryophyllene (3.41%). The compounds *p*-cymene, γ-terpinene, borneol and α-terpineol participate with concentrations 1.32, 1.17, 1.68 and 1.06% respectively, while the rest 15 compounds participate with less than 1% (**Figure 2**).

Carvacrol (5-isopropyl-2-methylphenol) is an aromatic monoterpene with molecular weight 150.2 g/mol. It is a constituent of many essential oils, especially those found in plants of the family Lamiaceae, where the *Origanum vulgare* subsp. *hirtum* (Link) (Kokkini and Vokou, 1989).

Eucalyptol (1,8-cineole) is a cyclic ether monoterpene (molecular weight 154.3 g/mol), which is found as a constituent of plant essential oils (its name is derived from its presence in essential oils of *Eucalyptus globosus*). It is also considered as a major monoterpene emitted by vegetation into the atmosphere (Guenther et al., 1994).

TABLE 1 | Medicinal uses of *Coridothymus capitatus* derived from ethnobotanical and ethnopharmacological studies.

Area	Local name	Plant part	Medicinal use	Mode of preparation/use	Literature
Israel (Golan heights and west bank region)		Foliage	Heart diseases, paralysis, diabetes, track pain and inflammation and respiratory system	An infusion of one tsp foliage in a cup of water is made and taken 1/3 times/day until improvement occurs	Said et al. (2002)
Turkey (west Anatolia)	Kekik	Herbs	For cough colds, stomachache	Internally, infusion, tea, 3 days on empty stomach	Honda et al. (1996)
Jordan (northern Badia region)	Zater faresy	Leaves	Heart and respiratory diseases, diabetes, inflammation and used as rubefacient	Infusion	Alzweiri et al. (2011)
Pelestinian area	Thyme	—	Respiratory and urinary digestive systems and inflammation	—	Ali-Shtayeh et al. (2000)
Israel	—	—	Cardiovascular system and digestive system	—	Palevirtch et al. (1986)
The levant countries	Headed thyme	—	Internal diseases, eye diseases, stomach intestine	—	Lev (2002)
Jordan	Conehead thyme	Branch	Digestive system, respiratory system	—	Lev and Amar (2002)
Cyprus	Conehead thyme	Aerial parts	Respiratory tract diseases (catarrh and common cold)	—	Lardos (2006)
Jordan (Showbak)	Thyme	Aerial parts	Antispasmodic	Syrup	Al-Qura'n (2009)
Jordan	—	Leaves	Pectoral, stomachic, and to treat urinary tract infections	Infusion	Al-Khalil (1995)
Israel (northern Israel)	—	Leaves	Heart disorders. Swelling and drops. Indigestion. Local paralysis	Tisane. Taken as needed decoction. Used as a drink for 40 days. Steam-bath. Treatment is given for a duration of a month	Dafni et al. (1984)
Spain (Barros area, Badajoz province)	—	Leaves	Disestive, tonic, carminative	—	Vázquez et al. (1997)
Cyprus	Throumpin, agrio thymari	Flowering herb, flower, leaf	Respiratory tract	Tea, chew	Lardos and Heinrich (2013)
Turkey	Ballı kekik,bal kekiği, zahter, beyazkekik	Erial parts, flowering branches, esssential oil,, fixed oil	Diabetes, analgesic, Pharyngitis, cold, flu, pleasureand medicinal tea	Drink oneteacup2–3 times a dayfor3–4 weeks/ apply 2–3 timesadayfor1–2 Weeks in, Exo,Lo, Lpw,Spc	Sargin (2015)
Egypt (Sinai peninsula)	Zaatar, Hasha	Flowering tops, leaves	Infusion as analgesic, sedative, digestive diseases, spasms, vomiting, flatulence. Liver diseases (jaundice)	—	Eissa et al. (2014)

TABLE 2 | Medicinal uses of *Salvia fruticosa* derived from ethnobotanical and ethnopharamacological studies.

Area	Local name	Plant part	Medicinal use	Mode of preparation/use	Literature
Israel (golan heights and west bank region)		Foliage	Stomach ache, intestinal gas and inflammation, diabetes and sexual weakness	An infusion of 50 g in 1 L is prepared and taken orally, 150 cc, 1–3 times/day until improvement occurs	Said et al. (2002)
Turkey (west Anatolia)	Almiya çalbasi = almiya yağı	Herbs	For flatulence and constipation for babies. For colds, cough, stomachache	Internally, volatile oil is applied on nipples before nursing to alleviate the flatulence and constipation of a baby. Infusion, as herbal tea	Honda et al. (1996)
Greece	Faskomilo, elelisfakos	Erial parts	Hypotension, diabetes, laryngitis, pharyngitis, tonsillitis, constipation, diarrhea, spasmolytic, anemia, brain stimulant, calmative, depression, common cold, arthritis, hair loss, hair tonic, stomatitis, dysmenorrhoea, stimulant	Infusion, decoction, external application (footbath, message, washings)	Hanlidou et al. (2004)
Israel	Sage	Leaf	Hemorrhages, intestinal diseases and pains	—	Lev & Amar (2000)
Jordan	Sage	Leaf	Hemorrhages, intestinal diseases and pains	—	Lev & Amar (2002)
Pelestinian area	White sage	—	Digestive system, prostate disorders, skin disorders	—	Ali-Shtayeh et al. (2000)
Israel (northern Israel)	—	Leaves	Indigestion. Coughs and colds	Tisane. Taken as needed	Dafni et al. (1984)
Jordan (northern Badia region)	Meirameieh	Foliage	Stomachache, flatulence, inflammation, diabetes and sexual weakness	Decoction	Alzweiri et al. (2011)
Cyprus	Tree lobed sage	Leaf, tip of shoot	Febrile conditions (not specified), gastro-intestinal tract disorders (tenesmus), malaria fever, absent or delayed menstruation, repellent against arachnids, insects, snakes, respiratory tract diseases (catarrh and common cold, cough), supporting treatment in pleurisy and pneumonia, wounds	External (topical applications, baths), oral	Lardos (2006)
Jordan (Showbak)	Sage, clary	Leaves stems	Sedative, for wounds healing	Syrup	Al-Qura'n (2009)
Jordan	Meriamiah	—	Astrigent and antidandruff	Lotion	Al-Khalil (1995)
Jordan (Mujib nature reserve	Meriamiah, Miramieh	Leaves	Spasm, common cold, intestinal gases	Infusion (50 g in l lof water) is made and taken orally 3 times a day. Infusion is also prepared with tea by adding 3–5 g to a cup of tea	Hudaib et al. (2008)
Cyprus	Spatzia, faskomilo	—	Hypotension, diabetes, estrogen action, diarrhea, dyspepsia, spasmolytic, anti-aging, anti-perspirant, brain stimulant, depression, and nervous tonic, aphthae, gingivitis, dysmenorrhea, antiseptic, diuretic, tonic	Decoction, infusion/oral (potion), external (Compress, washings)	Karousou & Deirmentzoglou (2011)
Jordan (Ailoun heights region)	Meriamia, Meirameieh	Leaves	Antispasmodic	Decoction	Aburjai et al. (2007)
Palestinian area	White sage	Leaves	Anti inflammatory gargle, antiseptic, antitussive, antihaemorrhoids pain, antirheumatic, anti stomach, disturbances, astringent, carminative, hypotensive	—	Ali-Shtaueh et al. (1998)
Israel (northern Israel)	—	Leaves	Indigestion goughs and colds	Tisane is taken as needed	Dafni et al. (1984)
Turkey (Marmaris, Muğla)	Adaçayı, almakeyik, almageyik	Leaves	Stomachache, flatulence, cold, tonsillitis, laxative, antipyretic	Infusion, int	Gürdal and Kültür (2013)
Palestine/west bank	—	Leaves	Diarrhea	—	Jaradat et al. (2016)

TABLE 3 | Medicinal uses of *Origanum dictamnus* derived from ethnobotanical and ethnopharmacological studies.

Area	Local name	Plant part	Medicinal use	Mode of preparation/use	References
Greece	Diktamos, erontas	Erial parts	Diabetes, liver disorders, spasmolytic, stomach ulcer, cholesterol, brain stimulant, headache, antiseptic, sanative, diuretic, dysmenorrhoea, antibacterial activities, aphrodisiac, stimulant	Infusion, external application (washings, compress)	Hanlidou et al. (2004)
Iran	Poneh kouhi	—	Dyspnea, bronchitis, allergy, depression, itch, dementia, abortifacient	Decoction	Naghibi et al. (2005)
Cyprus	Diktamo	Erial parts	Liver disorders, headache, neuralgia, cough, antiseptic, sanative, aphrodisiac, tonic	Infusion/oral (potion), external (compress)	Karousou and Deirmentzoglou (2011)
Europe	Dictamnus	—	Diabetes mellitus, obesity, disorders of lipoprotein metabolism and other lipidaemias, mood disorders, sexual dysfunction (not caused by organic disorder disease), epilepsy, acute nasopharyngitis (common cold), acute pharyngitis (unspecified), acute tonsillitis (unspecified), gingivitis and periodontal diseases, other specified disorders of teeth and supporting structures (toothache NOS), gastric ulcer, gastritis and duodenitis, diseases of liver, xerosis cutis, inflammatory polyarthropathies, pain in joint, acute nephritic syndrome, renal tubule-interstitial diseases, calculus of kidney and ureter, disorder of urinary system (unspecified), absent scanty and rare menstruation, pain and other conditions associated with female genital organs and menstrual cycle, dysmenorrhea unspecified, spontaneous abortion, long labor, cough, headache, convulsions (not elsewhere classified), multiple superficial injuries (unspecified), multiple open wounds (unspecified)	—	Martínez-Francés et al. (2015)

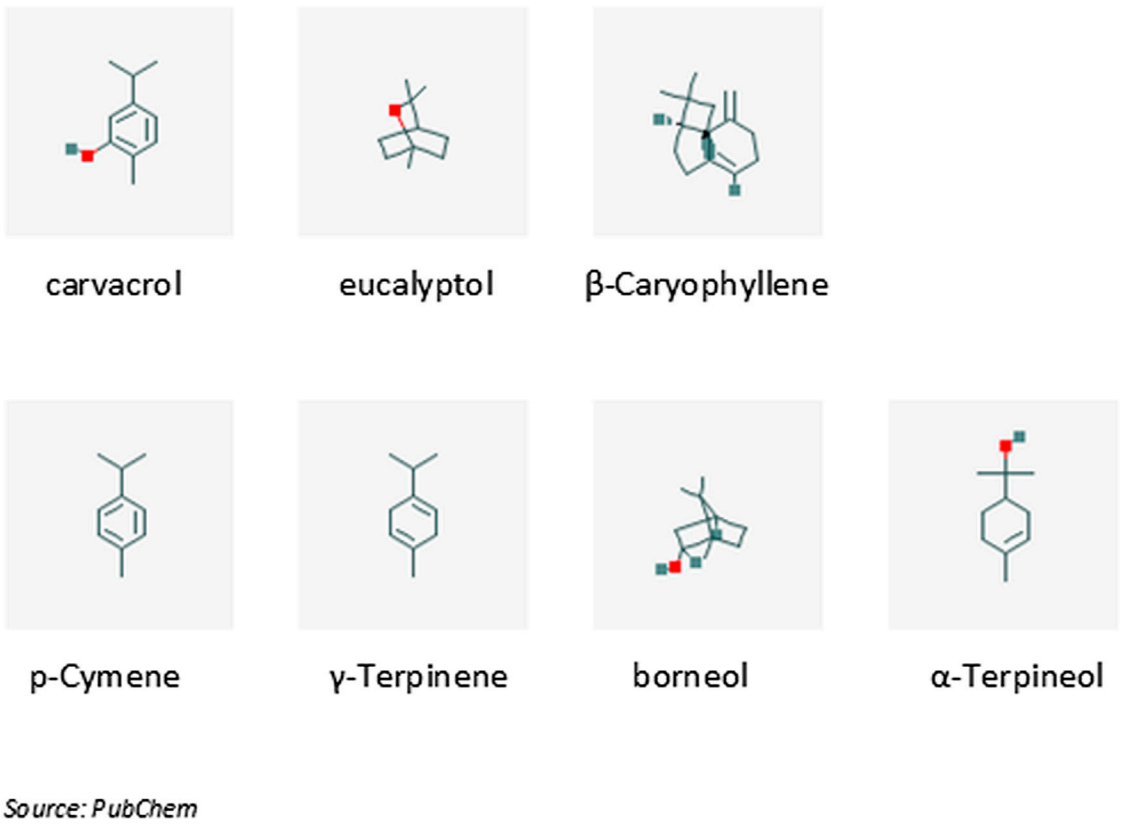

FIGURE 2 | The main phytochemical constituents contained in the essential oil of plants discussed in this review. IUPAC names: 2-methyl-5-propan-2-ylphenol (carvacrol), 1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane (eucalyptol), (1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo [7.2.0] undec-4-ene (β-Caryophyllene), 1-methyl-4-propan-2-ylbenzene (*p*-Cymene), 1-methyl-4-propan-2-ylcyclohexa-1,4-diene (γ-Terpinene), (1S,2R,4R)-1,7,7-trimethylbicyclo [2.2.1] heptan-2-ol (borneol), 2-(4-methylcyclohex-3-en-1-yl)propan-2-ol (α-Terpineol).

β-caryophyllene is a natural bicyclic sesquiterpene with molecular weight 204.4 g/mol that is a constituent of many essential oils, such as clove or rosemary oil (Bernardes et al., 2010).

Concerning the other compounds, *p*-cymene is a naturally occurring organic compound that is characterized as a hydrocarbon related to a monoterpene. Its molecular weight is 134.2 g/mol and it is a constituent of several essential oils, such as the oil of cumin (Allahghadri et al., 2010). γ-terpinene is a terpene with molecular weight 136.3 g/mol. It is a major component of essential oils made from *Citrus* fruits (Lücker et al., 2002). Borneol is a bicyclic monoterpene with molecular weight 154.3 g/mol containing exactly two rings which are fused to each other. α-Terpineol is a naturally occurring monoterpene alcohol, derived from several sources, including oil of pines. Terpineol, due to its pleasant odor (similar to lilac), is used as an ingredient in perfumes, cosmetics, and flavors (Kim and Chung, 2000).

USES OF PHYTOCHEMICAL CONSTITUENTS

Apart from the ethnobotanical and ethnopharmacological uses of plants mentioned above, several uses of the aforementioned phytochemical constituents are also documented:

Carvacrol

Carvacrol is the basic constituent of many essential oils, mainly of oregano species (Labiatae) (Kokkini and Vokou, 1989; Kirimer et al., 1995; Baydar et al., 2004; Stefanaki et al., 2016); it is characterized by its strong antioxidant properties (similar to those of vitamin E and ascorbic acid) (Mastelic et al., 2008). It is also known for its pronounced antimicrobial and antibacterial action (Suntres et al., 2015). The antimicrobial activity of several essential oils has been related to their content of carvacrol (García-Beltrán and Esteban, 2016), while, in a number of studies, the antimicrobial and the antibacterial action of the pure compound has also been investigated (Ben Arfa et al., 2006).

The antibacterial action of carvacrol extends to a variety of Gram-positive and negative bacteria (Kim et al., 1995; Rattanachaikunsopon and Phumkhachorn, 2010a; Ravishankar et al., 2010; Rivas et al., 2010). The compound acts as a transmembrane monovalent cation (hydroxyl proton for a potassium cation) exchanger (Suntres et al., 32,015). Therefore, in addition to its hydrophobic characteristics, allowing its accumulation in the membrane, the presence of free hydroxyls is essential for its antibacterial and antimicrobial activity (Ben Arfa et al., 2006).

An anti-inflammatory action of carvacrol is also documented (Landa et al., 2009; Hotta et al., 2010) and relies to the decreased

production of inflammatory mediators, including cytokines, prostaglandins, enzymes, nitric oxide (NOS) and reactive oxygen species (ROS) (da Silva Lima et al., 2013). The authors suggest that carvacrol's anti-inflammatory effects specifically induced IL-10 release, leading, subsequently, to the reduction of IL-1β and prostanoids production. In addition, it was reported that the inhibition of prostaglandin synthesis (Wagner et al., 1986) by carvacrol, is the basis of its antinociceptive activity. Additional mechanisms of antinociceptive actions of carvacrol include its agonistic activity for Transient Receptor Potential Vanilloid 3 (TRPV3), an ionic channel implicated in hyperalgesia and possibly skin sensitization (Xu et al., 2006). In addition, carvacrol activates and promptly desensitizes the specific sensor of environmental irritants TRPA1 ion channel/receptor (Xu et al., 2006).

Eucalyptol (1,8-Cineole)

1,8-cineole (cineole), also known as eucalyptol, is the principal constituent of most *Eucalyptus* oil-preparations. It is employed in drug preparations, as a percutaneous enhancer and anti-inflammatory agent, decongestant and antitussive, while in aromatherapy it is used as a skin stimulant (Santos and Rao, 2000; Yuan et al., 2006).

Juergens et al. (2004), reported that 1,8-cineol inhibited Th1/Th2-associated cytokine production by human lymphocytes and monocytes. This anti-inflammatory action resulted in a subsequent inhibition of cytokine-induced airway mucus hypersecretion.

1,8 cineole is also known for its analgesic action (Guimarães et al., 2013). Santos and Rao (2000) suggested that the potent anti-inflammatory action of the compound was associated with an outstanding peripheral analgesic effect. They also suggested that 1,8- cineole-induced analgesic effects are not associated to neuronal toxicity, while Khalil et al. (2004) documented that 1,8-cineol, in a concentration-dependent manner, acted directly on sensory nerves and blocked nerve excitability (Lima-Accioly et al., 2006; Guimarães et al., 2013), through a direct activation of TRPM8 channels, which is a specific heat/cold receptor. TRPM8 activation in sensory nerves by 1,8-cineole, produced a specific analgesic effect, in cases of peripheral nerve injury (Proudfoot et al., 2006).

β-Caryophyllene

β-caryophyllene is a widespread plant volatile compound, which is a selective ligand of the CB_2 cannabinoid receptor, specifically related to analgesia and inflammation, and devoted of psychotropic actions, mediated by CB_1 (Gertsch et al., 2008; Klauke et al., 2014). Therefore, CB_2 receptor-selective agonists, like *β*-caryophyllene, are potential analgesic drug candidates, in various types of pain (such as neuropathic pain) (Guindon and Hohmann, 2008; Kinsey et al., 2010).

p-Cymene

p-cymene is a precursor of carvacrol. It has an antimicrobial action *per se*, but it is less effective than carvacrol, when used alone (Kiskó and Roller, 2005), because *p*-cymene lacks a hydroxyl group, which is an important radical for the antimicrobial activity (Ultee et al., 1999; 2002). *p*-cymene also demonstrated an enhanced antinociceptive action in animal models of neurogenic and inflammatory pain (Guimarães et al., 2013).

γ-Terpinene

The antimicrobial activity of *γ*-terpinene is debatable, with positive (Sato et al., 2007) and negative (Sivropoulou et al., 1996) reports, against strains of Gram-positive or Gram-negative bacteria.

Borneol

Borneol is a known medicinal substance in Chinese and Indian traditional medicine. Borneol and its derivatives, possess antimicrobial (Corrêa et al., 2012), anti-inflammatory (Ehrnhöfer-Ressler et al., 2013) and antiviral activity. It also posesses a highly stimulatory action at $GABA_A$ receptors (Granger et al., 2005; Manayi et al., 2016). GABA is the predominant inhibitory transmitter in the mammalian central nervous system and stimulation of $GABA_A$ receptors produces anxiolysis, sedation, anesthesia and myorelaxation (Chebib and Johnston, 2000).

Borneol also expressed topical analgesic action. Wang et al. (2017), providing evidence for a topical analgesic efficacy in humans; TRPM8 was identified as its main molecular target. Moreover, borneol demonstrated anti-thrombotic effects, related to its anticoagulant properties (Li et al., 2008; Chen et al., 2015), possibly related to its potent modulation of the nitrite/nitrate reductase activity (Tang et al., 2009).

α-Terpineol

α-terpineol is characterized by a moderate antibacterial activity, with a broad antibacterial spectrum. It is worth mentioning that *a*-terpineol showed antibacterial activities against penicillin-resistant bacterial strains (Kotan et al., 2007).

α-terpineol exerts central and peripheral antinociceptive activity (Quintans-Júnior et al., 2011). It also inhibits NF-κB and subsequently down-regulats the expression of proinflammatory IL-1β and IL-6 cytokines (Held et al., 2007; Hassan et al., 2010), mainly explaining the compound's antinociceptive and anti-inflammatory properties (Oliveira et al., 2012).

α-terpineol also exhibited a gastroprotective action in ethanol-induced gastric ulcers (Souza et al., 2011) and beneficial effects in the cardiovascular system (Ribeiro et al., 2010). The proposed mechanism of action included endothelial NO-related vasorelaxation and activation of the NO–cGMP pathway.

REGISTERED MEDICAL APPLICATIONS

A series of clinical studies (most of them double-bind placebo-controlled trials, **Table 4**) evaluated the efficacy of 1,8-cineol (eucalyptol), in several medical issues. 1,8-cineol (eucalyptol) is a licensed medicinal product in Germany, since many years, in intestine-soluble capsules, for the treatment of acute and chronic

TABLE 4 | Clinical studies concerning the evaluation of efficacy of the main constituents of essential oil preparation from *Thymbra capitata* (L.) Cav., *Salvia fruticosa* Mill. and *Origanum dictamnus* L., in several medical conditions.

Compound	Symptome	Clinical trial	References
1,8-cineol (eucalyptol)	Anti-inflammatory activity in bronchial asthma	Double-bind placebo-controlled trial	Juergens et al. (2003)
Cineole	Acute nonpurulent rhinosinusitis	Double-blind, randomized, placebo-controlled trial	Kehrl et al. (2004)
Cineole	Acute non-purulent rhinosinusitis	Prospective, randomised, double-blinded controlled study	Tesche et al. (2008)
Cineole (eucalyptole)	Chronic obstructive pulmonary disease	Placebo-controlled double-blind trial	Worth et al. (2009)
Cineole	Asthma	Placebo-controlled, double-blind trial	Worth and Dethlefsen (2012)
Cineole	Acute bronchitis	Placebo-controlled double-blind trial	Fischer and Dethlefsen (2013)
1,8-cineol (in eucalyptus oil)	Pain and inflammatory responses after total knee replacement	Randomized clinical trial	Jun et al. (2013)
1,8-Cineole	Preoperative anxiety	Randomized clinical trial	Kim et al. (2014)

bronchitis, sinusitis, respiratory infections and rheumatoid-like joint diseases (Juergens et al., 2003; Guimarães et al., 2013).

Borneol and *p*-cymene have also been subjects of clinical studies for induced analgesia and treatment of the Fish Tapeworm disease respectively (Vartiainen, 1950; Wang et al., 2017).

SYNERGY OF PHYTOCHEMICAL CONSTITUENTS

According to a classical definition, synergy occurs when the combined effect of two or more substances is greater than the sum of the individual agents (Breitinger, 2012). When the registered effect is an add up of individual actions, the action is reported as additive. Recently, the definition of synergy has been clarified from two points of view, the pharmacodynamic (enhanced therapeutic actions on the same target) and pharmacokinetic (no direct interaction but a multi-target behavior) (Yang et al., 2014; Zhou et al., 2016). The main reason of employing combinations of active substances with synergistic interactions is to reduce the administered amount of each compound and to increase the biological activity of a preparation/mixture against a specific target. In addition, this strategy diminishes the chance of pharmaceutical resistance of the pathogenic organism (Araújo et al., 2016).

Synergistic interactions were known for specific substances long time ago. For example, Barbaste et al. (2002) proposed a "cascade" reaction from lipophilic to hydrophilic antioxidants, enhancing their biological effects, while Vardar-Ünlü et al. (2003) suggested that the crude essential oil of *Thymus pectinatus* var *pectinatus* was more effective as antioxidant (DPPH assay) than its main active components (thymol and carvacrol). Testing the antioxidant activity of thymol, carvacrol and *p*-cymene, Milos and Makota (2012) showed synergistic effects in any combination of any two of the above compounds. Moreover, *p*-cymene, the precursor of carvacrol was found to enhance the bactericidal activity of carvacrol when used in combination (Kiskó and Roller, 2005; Rattanachaikunsopon and Phumkhachorn, 2010b). Synergistic effects of *p*-cymene have also been reported in relation to thymol and γ-terpinene (Dauqan and Abdullah, 2017).

Synergistic interactions of carvacrol, eucalyptol (1,8-cineole) and β-caryophyllene are presented in **Table 5**. Synergy was also reported between carvacrol and other minor constituents, such as, *p*-cymene, carvacrol and thymol. Eucalyptol also appeared to have synergistic effects with other constituent, including camphor, terpinene-4-ol and caryophyllene oxide. Additionally, synergy was also reported between minor constituents, such as between camphor and *p*-cymene.

SAFETY ISSUES

Carvacrol, eucalyptol and β-caryophyllene, *p*-cymene, γ-terpinene, borneol and α-terpineol have been approved by the Food and Drug Administration (FDA) for food use (Code

TABLE 5 | Synergistic interactions of carvacrol, eucalyptol and β-Caryophyllene. Synergistic interactions of minor constituents of the essential oil reported in the same articles are also reported.

Substances	Biological activity	Interaction	References
Thymol/carvacrol	Antigenotoxicity	Synergy	Quintero Ruiz et al. (2017)
Carvacrol/p-cymene	Antigenotoxicity	Synergy	Quintero Ruiz et al. (2017)
Thymol/p-cymene	Antigenotoxicity	Synergy	Quintero Ruiz et al. (2017)
Carvacrol/thymol	Against pathogenic bacteria	Synergy	Kissels et al. (2017)
Carvacrol/thymol	Against rhipicephalus microplus, R. sanguineus (Acari:Ixodidae)	Synergy	Araújo et al. (2016)
Carvacrol/thymol	Cytotoxicity	Synergy	Coccimiglio et al. (2016)
Carvacrol/1,8-cineole	Inhibition of staphylococcus aureus	Synergy	Honório et al. (2015)
Thymol/carvacrol	Antibacterial – against *Brachyspira hyodysenteriae*	Synergy	Maele et al. (2016)
Thymol/carvacrol	Against dermacentor nitens (Acari: Ixodidae)	Synergy	Novato et al. (2015)
Carvacrol/20 substances	Against culex quinquefasciatus (mosquito)	Synergy	Pavela (2015)
Borneol/20 substances	Against culex quinquefasciatus (mosquito)	Synergy	Pavela (2015)
Camphor/20 substances	Against culex quinquefasciatus (mosquito)	Synergy	Pavela (2015)
Thymol/carvacrol	Against cisplatin – induced nephrotoxicity	Synergy	EL-Sayed et al. (2015)
Carvacrol/thymol	Against culex pipiens pallens (Diptera: Culicidae)	Synergy	Ma et al. (2014)
Carvacrol/thymol	Against enterococcus faecalis	Synergy	Gutiérrez-Fernández et al. (2013)
Carvacrol/thymol	Antimicrobial	Synergy	Guarda et al. (2011)
Carvacrol/p-cymene	Antimicrobial	Synergy	Custódio et al. (2011)
Thymol/p-cymene	Antimicrobial	Synergy	Custódio et al. (2011)
Thymol/carvacrol	Against *E. coli*	Synergy	Pei et al. (2009)
Carvacrol/cymene	Against listeria monocytogenes	Synergy	Periago et al. (2004)
Carvacrol/p-cymene	Antibacterial	Synergy	Burt (2004)
Thymol/carvacrol	Antioxidant	Synergy	Vardar-Unlü et al. (2003)
Carvacrol/cymene	Antimicrobial	Synergy	Ultee et al. (2000)
Thymol/carvacrol	Antibacterial	Synergy	Didry et al. (1994)
1,8-cineole/camphor	Insecticide	Synergy	Tak and Isman (2017)
Carvacrol/1,8-cineol	Against staphylococcus aureus	Synergy	Honório et al. (2015)
1,8 cineol/terpinene-4-ol	Against botrytis cinerea	Synergy	Yu et al. (2015)
1,8 cineol/camphor	Against trichoplusia ni	Synergy	Tak et al. (2016)
1,8-cineol/carvacrol	Antibacteria	Synergy	De Sousa et al. (2012)
1,8-cineol/caryophyllene oxide	Anticholinesterase	Synergy	Savelev et al. (2003)

for Federal Reguation: 21CFR172.515). Moreover, the European Commission Implementing Regulation (EU No 872/2012) of October 1, 2012, based on the evaluations of EFSA, included carvacrol, eucalyptol and β-caryophyllene, in the Union's List of Flavorings and Source Materials (**Table 6**). Their use is therefore permitted, in accordance with good agricultural and manufacturing practices and without given specific restrictions. This EU list takes also into consideration the reports of the Chemical Abstracts Service (CAS), the Joint FAO/WHO Expert Committee on Food additives (JECFA) and the Council of Europe. The rest of the constituents included in the essential oil's composition of the mixture of *Coridothymus capitatus*, *Salvia fruticosa* and *Origanum dictamnus* (Duikler et al., 2015), are also included in the same list, concerning Food supplements among others, as defined in Directive 2002/46/EC of the European Parliament and the Council, excluding food supplements for infants and young children.

However, in addition to these beneficiary compounds, the extract of the three aromatic plants includes also 0.74 and 0.52% of *cis*- and *trans*-thujone respectively (Duijker et al., 2015). Alpha and beta thujone are, according to the Regulation EC No 1334/2008 of the European Parliament and of the Council of December 15, 2008, among the substances which shall not be added as such, to food or food supplements. Maximum concentrations of thujone, naturally present in flavorings and food ingredients with flavoring properties, have been introduced. According to Regulation EC No 1334/2008, "*The maximum levels shall not apply where a compound food contains no added flavourings and the only food ingredients with flavoring properties which have been added are fresh, dried or frozen herbs and spices. After consultation with the Member States and the Authority, based on data made available by the Member States and on the newest scientific information, and taking into account the use of herbs and spices and natural flavoring preparations, the Commission, if appropriate, proposes amendments to this derogation.*" EMA/HMPC (2010) reported that exposures in the range between 3 and 7 mg/day do not pose special concerns. For higher concentrations, a case-by-case benefit/risk assessment is necessary (Lachenmeier and Uebelacker, 2010; Pelkonen et al., 2013; Sotiropoulou et al., 2016). Finally, Dettling et al. (2004) showed that a single dose of 0.28 mg/kg in men (20 mg/70 kg) and of 0.24 mg/kg (17 mg/70 kg) in women provided "borderline relevance" of adverse effects, mainly related to perturbations in driving, operating machinery, etc.

A CASE STUDY OF THE USE OF PLANT EXTRACTS AS A PHARMACEUTICAL PRODUCT: LESSONS LEARNED

The idea of using herb extracts for the treatment of upper respiratory infections was raised several years ago, when

TABLE 6 | Chemical constituents of herb essential oil preparations, which are included in the Union List of Flavourings and Source Materials (Commission Implementing Regulation (EU) No 872/2012 of October 1, 2012) based on EFSA and/or JECFA evaluations.

FL-no	Substance	CAS-number	JECFA-number	CoE-number	Restrictions of use	References
04.031	Carvacrol	499–75-2	710	2055	-	EFSA
03.001	1,8-cineol	470–82-6	1,234	182	-	EFSA
01.007	β-caryophyllene	87–44-5	1,324	2,118	-	EFSA
01.002	*p*-cymene	99–87-6	1,325	620	-	EFSA
01.020	γ-terpinene	99–85-4	1,340	11,025	-	EFSA
02.016	Borneol	507–70-0	1,385	64	-	EFSA
02.014	α-terpineol	98–55-5	366	62	-	JECFA
01.008	β-myrcene	123–35-3	1,327	2,197	—	EFSA
02.085	*cis*-sabinene hydrate	546–79-2	441	10,309	—	JECFA
02.085	*trans*-sabinene hydrate	546–79-2	441	10,309	—	JECFA
02.013	Linalool	78–70-6	356	61	—	JECFA
07.215	Camphor	464–49-3	1,395	140	There is maximum dose per day	EFSA
02.230	δ-terpineol	8,000–41-7	—	—	—	EFSA
02.072	Terpinen-4-ol	562–74-3	439	2,229	—	JECFA
04.006	Thymol	89–83-8	709	174	—	EFSA
—	δ-terpinyl acetate	—	—	—	—	—
16.043	Caryophyllene oxide	1,139–30-6	1,575	10,500	—	EFSA
08.014	n-hexadecanoic acid	57–10-3	115	14	—	JECFA

epidemiological observations revealed that people who consumed certain herbs infusions had low rates of respiratory infections and rarely suffered from common colds or influenza infections. The efficacy of the selected herbs was attributed to their antioxidant properties (Lionis et al., 1998). After years of *in vitro* and *in vivo* research, an essential oil combination was developed, based on the extracts of the three plants discussed in the present review (*Coridothymus capitatus*, *Salvia fruticosa* and *Origanum dictamnus*) in extra-virgin olive oil (Duijker et al., 2015); we showed that this combination exerted a synergistic effect against viral upper respiratory tract infections, including influenza (Anastasaki et al., 2017). Moreover, an *in vitro* study revealed that this combination exhibited a remarkable direct antiviral activity against influenza A/H1N1 viral strains, influenza B and human rhinovirus 14 (HRV14), related to a defective trafficking of influenza A Nucleoprotein (NP) (Tseliou et al., 2019). In this respect, the "one drug, one target, one disease" approach (Zhou et al., 2016) was "violated." Indeed, the combinatorial use of herbal preparations resulted in an synergistic effect, beyond the reported properties of each plant. Hence, here we address the case of the development and commercialization of a product, containing this essential oils' combination.

The scheme of the translational chain, from the step of biodiversity to the development of a commercial product, is presented in **Figure 3**. The first edge of the chain, leading from a plant extract to a final product, relies on the choice of plants, as well as the choice of secondary metabolites, whose biological activity is expected to ensure the desired health benefit (*drug design*). For example, the preparation containing the essential oils of *Thymbra capitata* (L.) Cav., *Salvia fruticosa* Mill. and *Origanum dictamnus* L. avoided plant extracts rich in alkaloids, as preliminary (Lionis et al., 1988) and clinical (Duijker et al., 2015) or laboratory evidence (Tseliou et al., 2019) documented a potent health benefit of the preparation.

Another part of the continuum is related to the implementation of a *clinical study*, which is one of the main obstacles in the translational process. In the presented case, it was valuable not only for the demonstration of an antiviral action, but also for the evaluation of the amelioration of the symptoms of the disease, due to supplementary actions of the substances involved in the combination of essential oils.

The supply of raw material is an important part of the translational chain. It is well known that several restrictions rule the natural collections (harvests) and trade of herbs and spices, especially within the framework of the EU environmental policy, as well as within the framework of the United Nations Convention on Biological Diversity. For example, 232 medicinal and aromatic plant species are listed on Appendix II of CITES, which regulates the international trade in endangered species. Worldwide, the increased needs for medicinal plants, in combination with the strengthening of the regulatory framework for their collection and trade, have caused supply constraints of medicinal plants of high ethnopharmacological interest, which introduced a serious constrain in the development and commercialization of plant-derived products (Amirkia and Heinrich, 2014). In the presented case, the plants are *cultivated* under controlled and monitored conditions, while local farmers have an important income from this agricultural activity. This activity constitutes a viable developmental axis for the local communities.

Recently, a new tool based on Ecological Niche Modeling has been developed, in order to support local farmers in the decision-making process, concerning the suitability of the area where their land is located, for cultivation of medicinal taxa of high ethnopharmacological interest (Bariotakis et al., 2019). A web-based, easy-to-use application was created in the framework of precision agriculture, where the predicted suitability scores for each area of interest can be made accessible to anyone, by the use of its GPS coordinates. So, in our case, the raw material is

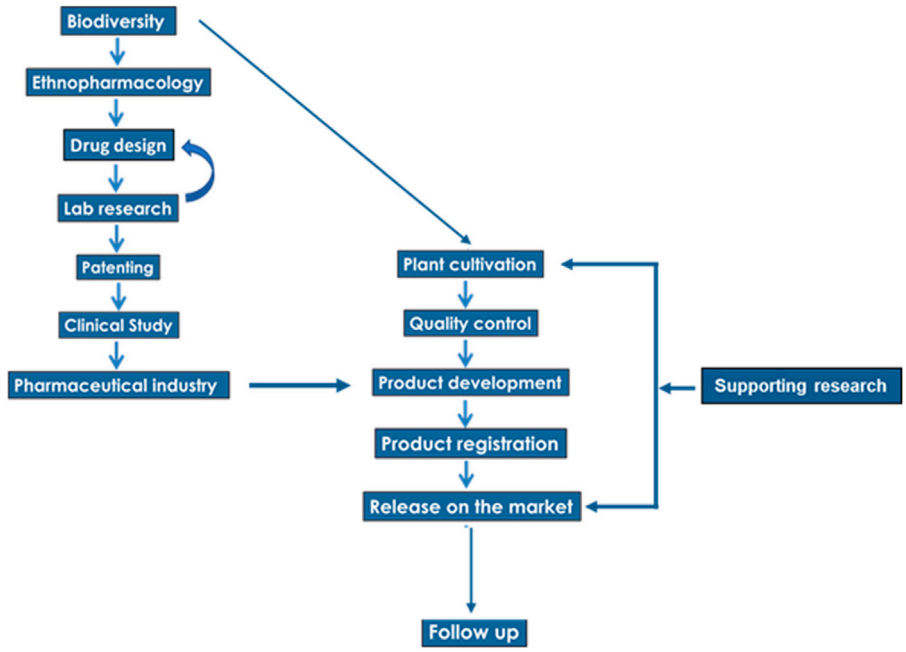

FIGURE 3 | The flow-chart of a translational chain development.

produced from organic farming, with Global GAP and precision agriculture.

Finally, it should be clear that the continuum in plant-based drug development, is not terminated at the step of release on the market. A *follow-up* of the final product, after its market release and additional supporting research is necessary. In the example case presented here, a follow up was made through a pragmatic prospective observational study (Anastasaki et al., 2017). In our opinion, this "supporting research" step is necessary as, in many cases, there are further research queries which need clarification even after the pharmaceutical evidence of an essential oil combination. In the example case, the effectiveness of the combination of essential oils in humans was documented (Duijker et al., 2015), but the mode of biological action was not understood. Limited *in vitro* data was available with ongoing research (Tseliou et al., 2019), providing a possible mechanism, at a cellular level.

In summary, two main lessons emerged from the development of a new pharmaceutical product: The first is resumed in the words "mind the gap," a well-known phrase from the London underground, as it reveals the necessity of the bridging between successional steps, or successional links of the translational chain. The second concerns the time lag in bridging some successional steps. Indeed, in practice, many subsequent successive steps are fulfilled before others, imposing several loops in the development of the final preparation, which should be treated and resolved accordingly.

However, in spite of the successful use of the three plants, further research is required, in order to decipher additional multi-target action(s), in view of supplementary beneficial effects, which need to be investigated/screened not only *in vitro* but also in preclinical and clinical studies in the context of Evidence Based Medicine (Stavrou et al., 2013). The results of this screening are expected to clarify whether the ethnopharmacological gap, between reported traditional uses in ethnobotanical studies and the tested properties, is due to a noise of data collection in ethnobotanical practice or reflects underline biological activities, which should be incorporated in the formal therapeutic practice. Moreover, there are no preclinical or clinical evidence about the exposure of sensitive groups (i.e., pregnant women, children, etc.).

A logical subsequent step might be the development a drug, instead of a dietary supplement. A number of items are required for this shift, especially: 1) the repetition of phase I/II trial, with a greater number of participants, together with a detailed pharmacokinetic study of the major active compounds; 2) the performance of a phased III trial. Phase III studies, undertaken in large numbers of patients, often in multiple centers, assess real outcomes in a variety of patients, approximating the global population of patients, who will receive the drug. Their aim is to compare new treatments with existing ones and to demonstrate long-term safety and tolerance (Hobbs and McCarthy, 2009).

Finally, taking into consideration reported variability of the plants' chemical fingerprint, at least in two of the three native Cretan herbs, which have been used for the development of the active extract (see Karousou et al., 2000; Karousou et al., 2005), it becomes clear that the biological variability in nature does not conform to the requirements for stability in the composition required by market regulations. Thus, further study is suggested concerning the interaction of environmental, chemical, genetic and epigenetic factors, for the quality assurance process. Last but not least, further studies on the cultivation and storage of the

above species are required for the standardization and quality control measures, along the whole supply chain.

AUTHOR CONTRIBUTIONS

EC, SP, CL contributed conception and design of the study, SP and EC wrote the first draft of the manuscript, CL, GS, MK and MB wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REFERENCES

Aburjai, T., Hudaib, M., Tayyem, R., Yousef, M., and Qishawi, M. (2007). Ethnopharmacological survey of medicinal herbs in Jordan, the Ajloun Heights region. *J. Ethnopharmacol.* 110, 294–304. doi:10.1016/j.jep.2006.09.031.

Al-Khalil, S. (1995). A survey of plants used in Jordanian traditional medicine. *Int. J. Pharmacogn.* 33, 317–323. doi:10.3109/13880209509065385.

Al-Qura'n, S. (2009). Ethnopharmacological survey of wild medicinal plants in Showbak, Jordan. *J. Ethnopharmacol.* 123, 45–50.

Ali-Shtayeh, M. S., Yaghmour, R. M.-R., Faidi, Y. R., Salem, K., and Al-Nuri, M. A. (1998). Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area. *J. Ethnopharmacol.* 60, 265–271. doi:10.1016/s0378-8741(97)00153-0.

Ali-Shtayeh, M. S., Yaniv, Z., and Mahajna, J. (2000). Ethnobotanical survey in the Palestinian area: a classification of the healing potential of medicinal plants. *J. Ethnopharmacol.* 73, 221–232. doi:10.1016/s0378-8741(00)00316-0.

Allahghadri, T., Rasooli, I., Owlia, P., Nadooshan, M. J., Ghazanfari, T., Taghizadeh, M., et al. (2010). Antimicrobial property, antioxidant capacity, and cytotoxicity of essential oil from cumin produced in Iran. *J. Food Sci.* 75, H54–H61. doi:10.1111/j.1750-3841.2009.01467.x.

Altmann, R. E., Campbell, E. L., and Johnston, G. A. R. (2005). (+)- and (−)-borneol: efficacious positive modulators of GABA action at human recombinant α1β2γ2L GABAA receptors. *Biochem. Pharmacol.* 69, 1101–1111. doi:10.1016/j.bcp.2005.01.002.

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, 27–35. doi:10.1016/j.jep.2011.02.007.

Amirkia, V., and Heinrich, M. (2014). Alkaloids as drug leads — a predictive structural and biodiversity-based analysis. *Phytochem. Lett.* 10, xlviii–liii. doi:10.1016/j.phytol.2014.06.015

Anastasaki, M., Bertsias, A., Pirintsos, S. A., Castanas, E., and Lionis, C. (2017). Post-market outcome of an extract of traditional Cretan herbs on upper respiratory tract infections: a pragmatic, prospective observational study. *BMC Compl. Alternative Med.* 17, 466. doi:10.1186/s12906-017-1978-7.

Andrade e Silva, H. G. (2012). *Drug synergy-mechanisms and methods of analysis, toxicity and drug testing. Rijeka*: InTech, The German University of Cairo.

Araújo, L. X., Novato, T. P. L., Zeringota, V., Maturano, R., Melo, D., Da Silva, B. C., et al. (2016). Synergism of thymol, carvacrol and eugenol in larvae of the cattle tick, Rhipicephalus microplus, and brown dog tick, Rhipicephalus sanguineus. *Med. Vet. Entomol.* 30, 377–382. doi:10.1111/mve.12181.

Ay, G., Bertsias, A., Symvoulakis, E. K., Moschandreas, J., Malliaraki, N., Derdas, S. P., et al. (2015). Reporting effectiveness of an extract of three traditional Cretan herbs on upper respiratory tract infection: results from a double-blind randomized controlled trial. *J. Ethnopharmacol.* 163, 157–166. doi:10.1016/j.jep.2015.01.030.

Bariotakis, M., Georgescu, L., Laina, D., Oikonomou, I., Ntagounakis, G., Koufaki, M.-I., et al. (2019). From wild harvest towards precision agriculture: use of ecological Niche modelling to direct potential cultivation of wild medicinal plants in Crete. *Sci. Total Environ.* 694, 133681. doi:10.1016/j.scitotenv.2019.133681.

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., and Chalier, P. (2006). Antimicrobial activity of carvacrol related to its chemical structure. *Lett. Appl. Microbiol.* 43, 149–154. doi:10.1111/j.1472-765x.2006.01938.x.

Bernardes, W. A., Lucarini, R., Tozatti, M. G., Bocalon Flauzino, L. G., Souza, M. G. M., Turatti, I. C. C., et al. (2010). Antibacterial activity of the essential oil from Rosmarinus officinalis and its major components against oral pathogens. *Z. Naturforsch. C Biosci.* 65, 588–593. doi:10.1515/znc-2010-9-1009.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. *Int. J. Food Microbiol.* 94, 223–253. doi:10.1016/j.ijfoodmicro.2004.03.022.

Carazza, J., Ribeiro, M. V., Silva, F., Rodrigues Machado, S. M., and Sousa, M. (2011). The essential oils component p-cymene induces proton leak through Fo-ATP synthase and uncoupling of mitochondrial respiration. *JEP (J. Environ. Psychol.)* 3, 69–76. doi:10.2147/jep.s16387.

Chebib, M., and Johnston, G. A. R. (2000). GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. *J. Med. Chem.* 43, 1427–1447. doi:10.1021/jm9904349.

Chen, C., Yang, F.-Q., Zhang, Q., Wang, F.-Q., Hu, Y.-J., and Xia, Z.-N. (2015). Natural products for antithrombosis, *Evid. base Compl. Alternative Med.*, 2015, 1, doi:10.1155/2015/876426.

Coccimiglio, J., Alipour, M., Jiang, Z. H., Gottardo, C., and Suntres, Z. (2016). Antioxidant, antibacterial, and cytotoxic activities of the ethanolic *Origanum vulgare* extract and its major constituents. *Oxid Med Cell Longev* 2016. doi:10.1155/2016/1404505.

Corrêa, P. R. C., Miranda, R. R. S., Duarte, L. P., Silva, G. D. F., Filho, S. A. V., Okuma, A. A., et al. (2012). Antimicrobial activity of synthetic bornyl benzoates againstTrypanosoma cruzi. *Pathog. Glob. Health* 106, 107–112.doi:10.1179/2047773212y.0000000002.

Daemon, M., Verge, S., Dumas, M., Soulet, S., Nay, B., Arnaudinaud, V., et al. (2002). Dietary antioxidants, peroxidation and cardiovascular risks. *J. Nutr. Health Aging* 6, 209–223.

Dafni, A., Yaniv, Z., and Palevitch, D. (1984). Ethnobotanical survey of medicinal plants in northern Israel. *J. Ethnopharmacol.* 10, 295–310. doi:10.1016/0378-8741(84)90017-5.

Dauqan, E. M., and Abdullah, A. (2017). Medicinal and functional values of thyme (*Thymus vulgaris* L.) herb. *J. Appl. Biol. Biotechnol.* 5, 017–022.

de Sousa, J. P., de Azerêdo, G. A., de Araújo Torres, R., da Silva Vasconcelos, M. A., da Conceição, M. L., and de Souza, E. L. (2012). Synergies of carvacrol and 1,8-cineole to inhibit bacteria associated with minimally processed vegetables. *Int. J. Food Microbiol.* 154, 145–151. doi:10.1016/j.ijfoodmicro.2011.12.026.

Dettling, A., Grass, H., Schuff, A., Skopp, G., Strohbeck-Kuehner, P., and Haffner, H. T. (2004). Absinthe: attention performance and mood under the influence of thujone. *J. Stud. Alcohol* 65, 573–581. doi:10.15288/jsa.2004.65.573.

Didry, N., Dubreuil, L., and Pinkas, M. (1994). Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. *Pharm. Acta Helv.* 69, 25–28. doi:10.1016/0031-6865(94)90027-2.

Dimopoulos, P., Raus, T., Bergmeier, E., Constantinidis, T., Iatrou, G., Kokkini, S., et al. (2013). "An annotated checklist. – Berlin Englera 31," in *Vascular plants of Greece: botanischer Garten und Botanisches Museum Berlin-Dahlem*. Athens: Hellenic Botanical Society.

Eissa, T. A. F., Palomino, O. M., Carretero, M. E., and Gómez-Serranillos, M. P. (2014). Ethnopharmacological study of medicinal plants used in the treatment of CNS disorders in Sinai Peninsula, Egypt. *J. Ethnopharmacol.* 151, 317–332. doi:10.1016/j.jep.2013.10.041.

El-Sayed, E. M., Abd-Allah, A. R., Mansour, A. M., and El-Arabey, A. A. (2015). Thymol and carvacrol prevent cisplatin-induced nephrotoxicity by abrogation of oxidative stress, inflammation, and apoptosis in rats. *J. Biochem. Mol. Toxicol.* 29, 165–172. doi:10.1002/jbt.21681.

EMA/HMPC (2010). Public statement on the use of herbal medicinal products containing thujone. EMA/HMPC/732886/2010.

Euro+Med (2019). Euro+Med PlantBase - the information resource for Euro-Mediterranean plant diversity. Available at: http://ww2.bgbm.org/EuroPlusMed/.Published on the Internet (Accessed June 2017).

Fabricant, D. S., and Farnsworth, N. R. (2001). The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect.* 109 (Suppl. 1), 69–75. doi:10.2307/3434847.

Fielding, J., and Turland, N. (2005). Flowers of Crete. Editor: B. Mathiew (London, England: Kev Royal Botanic Gardens).

Fischer, J., and Dethlefsen, U. (2013). Efficacy of cineole in patients suffering from acute bronchitis: a placebo-controlled double-blind trial. *Cough* 9, 25. doi:10.1186/1745-9974-9-25.

García-Beltrán, J. M., and Esteban, M. A. (2016). Properties and applications of plants of Origanum sp. *Genus. SM Journal of Biology* 2, 1006.

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J.-Z., Xie, X.-Q., et al. (2008). Beta-caryophyllene is a dietary cannabinoid. *Proc. Natl. Acad. Sci. U. S. A.* 105, 9099–9104. doi:10.1073/pnas.0803601105.

Guarda, A., Rubilar, J. F., Miltz, J., and Galotto, M. J. (2011). The antimicrobial activity of microencapsulated thymol and carvacrol. *Int. J. Food Microbiol.* 146, 144–150. doi:10.1016/j.ijfoodmicro.2011.02.011.

Guenther, A., Zimmerman, P., and Wildermuth, M. (1994). Natural volatile organic compound emission rate estimates for U.S. woodland landscapes. *Atmos. Environ.* 28, 1197–1210. doi:10.1016/1352-2310(94)90297-6.

Guimarães, A. G., Quintans, J. S. S., and Quintans-Júnior, L. J. (2013). Monoterpenes with analgesic activity-A systematic review. *Phytother Res.* 27, 1–15. doi:10.1002/ptr.4686.

Guindon, J., and Hohmann, A. G. (2008). Cannabinoid CB2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. *Br. J. Pharmacol.* 153, 319–334. doi:10.1038/sj.bjp.0707531.

Gürdal, B., and Kültür, Ş. (2013). An ethnobotanical study of medicinal plants in Marmaris (Muğla, Turkey). *J. Ethnopharmacol.* 146, 113–126.doi:10.1016/j.jep.2012.12.012.

Gutiérrez-Fernández, J., García-Armesto, M. R., Álvarez-Alonso, R., Del Valle, P., de Arriaga, D., and Rúa, J. (2013). Antimicrobial activity of binary combinations of natural and synthetic phenolic antioxidants against *Enterococcus faecalis*. *J. Dairy Sci.* 96, 4912–4920. doi:10.3168/jds.2013-6643.

Hanlidou, E., Karousou, R., Kleftoyanni, V., and Kokkini, S. (2004). The herbal market of Thessaloniki (N Greece) and its relation to the ethnobotanical tradition. *J. Ethnopharmacol.* 91, 281–299. doi:10.1016/j.jep.2004.01.007.

Hardy, K. (2019). Paleomedicine and the use of plant secondary compounds in the Paleolithic and Early Neolithic. *Evol. Anthropol.* 28, 60–71. doi:10.1002/evan.21763.

Hassan, S. B., Gali-Muhtasib, H., Göransson, H., and Larsson, R. (2010). Alpha terpineol: a potential anticancer agent which acts through suppressing NF-kappaB signalling. *Anticancer Res.* 30, 1911–1919.

Held, S., Schieberle, P., and Somoza, V. (2007). Characterization of α-terpineol as an anti-inflammatory component of orange juice by *in Vitro* studies using oral buccal cells. *J. Agric. Food Chem.* 55, 8040–8046. doi:10.1021/jf071691m.

Hobbs, M., and McCarthy, M. W. (2009). *"Clinical trials,"* in *Oxford American handbook of clinical pharmacy*. Editors M. W. McCarthy and D. R. Kockler (New York: Oxford University Press), 115–126.

Honda, G., Yeşilada, E., Tabata, M., Sezik, E., Fujita, T., Takeda, Y., et al. (1996). Traditional medicine in Turkey VI. Folk medicine in west Anatolia: Afyon, Kütahya, Denizli, Muğla, Aydin provinces. *J. Ethnopharmacol.* 53, 75–87. doi:10.1016/s0378-8741(96)01426-2.

Hotta, M., Nakata, R., Katsukawa, M., Hori, K., Takahashi, S., and Inoue, H. (2010). Carvacrol, a component of thyme oil, activates PPARα and γ and suppresses COX-2 expression. *J. Lipid Res.* 51, 132–139. doi:10.1194/jlr.m900255-jlr200.

Hudaib, M., Mohammad, M., Bustanji, Y., Tayyem, R., Yousef, M., Abuirjeie, M., et al. (2008). Ethnopharmacological survey of medicinal plants in Jordan, Mujib Nature Reserve and surrounding area. *J. Ethnopharmacol.* 120, 63–71. doi:10.1016/j.jep.2008.07.031.

Jaradat, N. A., Ayesh, O. I., and Anderson, C. (2016). Ethnopharmacological survey about medicinal plants utilized by herbalists and traditional practitioner healers for treatments of diarrhea in the West Bank/Palestine. *J. Ethnopharmacol.* 182, 57–66. doi:10.1016/j.jep.2016.02.013.

Juergens, U. R., Dethlefsen, U., Steinkamp, G., Gillissen, A., Repges, R., and Vetter, H. (2003). Anti-inflammatory activity of 1.8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial. *Respir. Med.* 97, 250–256. doi:10.1053/rmed.2003.1432.

Juergens, U. R., Engelen, T., Racké, K., Stöber, M., Gillissen, A., and Vetter, H. (2004). Inhibitory activity of 1,8-cineol (eucalyptol) on cytokine production in cultured human lymphocytes and monocytes. *Pulm. Pharmacol. Therapeut.* 17, 281–287. doi:10.1016/j.pupt.2004.06.002.

Jun, Y. S., Kang, P., Min, S. S., Lee, J.-M., Kim, H.-K., and Seol, G. H. (2013). Effect of Eucalyptus oil inhalation on pain and inflammatory responses after total knee replacement: a randomized clinical trial, *Evid. base Compl. Alternative Med.*, 2013, 1, doi:10.1155/2013/502727.

Karousou, R., and Deirmentzoglou, S. (2011). The herbal market of Cyprus: traditional links and cultural exchanges. *J. Ethnopharmacol.* 133, 191–203. doi:10.1016/j.jep.2010.09.034.

Karousou, R., Hanlidou, E., and Kokkini, S. (2000). "The Sage plants of Greece: distribution and infraspecific variation," in*The Sage*. Editor S. E. Kintzios (Singapore: Harwood Academic Publishers), 27–46.

Karousou, R., Koureas, D. N., and Kokkini, S. (2005). Essential oil composition is related to the natural habitats: *Coridothymus capitatus* and *Satureja thymbra* in NATURA 2000 sites of Crete. *Phytochemistry* 66, 2668–2673. doi:10.1016/j.phytochem.2005.09.020.

Karousou, R., Vokou, D., and Kokkini, S. (1998). Variation ofSalvia fruticosaEssential oils on the island of Crete (Greece). *Plant Biol.* 111, 250–254. doi:10.1111/j.1438-8677.1998.tb00705.x.

Kehrl, W., Sonnemann, U., and Dethlefsen, U. (2004). Therapy for acute nonpurulent rhinosinusitis with cineole: results of a double-blind, randomized, placebo-controlled trial. *Laryngoscope* 114, 738–742.doi:10.1097/00005537-200404000-00027.

Kim, J. M., Marshall, M., Cornell, J. A., Iii, J. F. P., III, and Wei, C. I. (1995). Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. *J. Food Sci.* 60, 1364–1368. doi:10.1111/j.1365-2621.1995.tb04592.x.

Kim, K.-Y., and Chung, H.-J. (2000). Flavor compounds of pine sprout tea and pine needle tea. *J. Agric. Food Chem.* 48, 1269–1272.doi:10.1021/jf9900229.

Kim, K. Y., Seo, H. J., Min, S. S., Park, M., and Seol, G. H. (2014). A randomized clinical trial. The effect of 1.8-cineole inhalation on preoperative anxiety. *Evidence-Based Complementary Altern. Med.* 2014. doi:10.1155/2014/820126

Kinsey, S. G., Long, J. Z., Cravatt, B. F., and Lichtman, A. H. (2010). Fatty acid amide hydrolase and monoacylglycerol lipase inhibitors produce anti-allodynic effects in mice through distinct cannabinoid receptor mechanisms. *J. Pain* 11, 1420–1428. doi:10.1016/j.jpain.2010.04.001.

Kirimer, N., Başer, K. H. C., and Tümen, G. (1995). Carvacrol-rich plants in Turkey. *Chem. Nat. Compd.* 31, 37–41. doi:10.1007/bf01167568.

Kiskó, G., and Roller, S. (2005). Carvacrol and p-cymene inactivate *Escherichia coli* O157: H7 in apple juice. *BMC Microbiol.* 5, 36. doi:10.1186/1471-2180-5-36.

Kissels, W., Wu, X., and Santos, R. R. (2017). Short communication: interaction of the isomers carvacrol and thymol with the antibiotics doxycycline and tilmicosin: in vitro effects against pathogenic bacteria commonly found in the respiratory tract of calves. *J. Dairy Sci.* 100, 970–974. doi:10.3168/jds.2016-11536.

Klauke, A.-L., Racz, I., Pradier, B., Markert, A., Zimmer, A. M., Gertsch, J., et al. (2014). The cannabinoid CB2 receptor-selective phytocannabinoid beta-

caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. *Eur. Neuropsychopharmacol* 24, 608–620. doi:10.1016/j.euroneuro.2013.10.008.

Kokkini, S., and Vokou, D. (1989). Carvacrol-rich plants in Greece. *Flavour Fragrance J.* 4, 1–7.doi:10.1002/ffj.2730040102.

Kotan, R., Kordali, S., and Cakir, A. (2007). Screening of antibacterial activities of twenty-one oxygenated monoterpenes. *Z. Naturforsch. C Biosci.* 62, 507–513. doi:10.1515/znc-2007-7-808.

Lachenmeier, D. W., and Uebelacker, M. (2010). Risk assessment of thujone in foods and medicines containing sage and wormwood – evidence for a need of regulatory changes? *Regul. Toxicol. Pharmacol.* 58, 437–443. doi:10.1016/j.yrtph.2010.08.012.

Landa, P., Kokoska, L., Pribylova, M., Vanek, T., and Marsik, P. (2009). *In vitro* anti-inflammatory activity of carvacrol: inhibitory effect on COX-2 catalyzed prostaglandin E2 biosynthesisb. *Arch Pharm. Res. (Seoul)* 32, 75–78. doi:10.1007/s12272-009-1120-6.

Lardos, A., and Heinrich, M. (2013). Continuity and change in medicinal plant use: the example of monasteries on Cyprus and historical iatrosophia texts. *J. Ethnopharmacol.* 150, 202–214. doi:10.1016/j.jep.2013.08.026.

Lardos, A. (2006). The botanical materia medica of the Iatrosophikon-A collection of prescriptions from a monastery in Cyprus. *J. Ethnopharmacol.* 104, 387–406. doi:10.1016/j.jep.2005.12.035.

Lev, E., and Amar, Z. (2000). Ethnopharmacological survey of traditional drugs sold in Israel at the end of the 20th century. *J. Ethnopharmacol.* 72, 191–205. doi:10.1016/s0378-8741(00)00230-0.

Lev, E., and Amar, Z. (2002). Ethnopharmacological survey of traditional drugs sold in the Kingdom of Jordan. *J. Ethnopharmacol.* 82, 131–145. doi:10.1016/s0378-8741(02)00182-4.

Lev, E. (2002). Reconstructed materia medica of the Medieval and Ottoman al-Sham. *J. Ethnopharmacol.* 80, 167–179.doi:10.1016/s0378-8741(02)00029-6.

Li, Y.-H., Sun, X.-P., Zhang, Y.-Q., and Wang, N.-S. (2008). The antithrombotic effect of borneol related to its anticoagulant property. *Am. J. Chin. Med.* 36, 719–727. doi:10.1142/s0192415x08006181.

Lima, M. d. S., Quintans-Júnior, L. J., de Santana, W. A., Martins Kaneto, C., Pereira Soares, M. B., and Villarreal, C. F. (2013). Anti-inflammatory effects of carvacrol: evidence for a key role of interleukin-10. *Eur. J. Pharmacol.* 699, 112–117. doi:10.1016/j.ejphar.2012.11.040.

Lima-Accioly, P. M., Lavor-Porto, P. R., Cavalcante, F. S., Magalhães, P. J. C., Lahlou, S., and Morais, S. M., et al. (2006). Essential oil of Croton nepetaefolius and its main constituent, 1,8-cineole, block excitability of rat sciatic nerve in vitro. *Clin. Exp. Pharmacol. Physiol.* 33, 1158–1163. doi:10.1111/j.1440-1681.2006.04494.x

Lionis, C., Faresjö, Å., Skoula, M., Kapsokefalou, M., and Faresjö, T. (1998). Antioxidant effects of herbs in Crete. *Lancet* 352, 1987–1988. doi:10.1016/s0140-6736(05)61333-5.

Lücker, J., El Tamer, M. K., Schwab, W., Verstappen, F. W. A., van der Plas, L. H. W., Bouwmeester, H. J., et al. (2002). Monoterpene biosynthesis in lemon (Citrus limon). *FEBS J.* 269, 3160–3171.doi:10.1046/j.1432-1033.2002.02985.x.

Ma, W.-B., Feng, J.-T., Jiang, Z.-L., Wu, H., Ma, Z.-Q., and Zhang, X. (2014). Fumigant activity of eleven essential oil compounds and their selected binary mixtures against *Culex pipiens pallens* (Diptera: Culicidae). *Parasitol. Res.* 113, 3631–3637. doi:10.1007/s00436-014-4028-0.

Maele, L. V., Heyndrickx, M., De Pauw, N., Verlinden, M., Haesebrouck, F., Martel, A., et al. (2016). *In vitro* susceptibility of *Brachyspira hyodysenteriae* to organic acids and essential oil components. *J. Vet. Med. Sci.* 78, 325–328.

Manayi, A., Nabavi, S. M., Daglia, M., and Jafari, S. (2016). Natural terpenoids as a promising source for modulation of GABAergic system and treatment of neurological diseases. *Pharmacol. Rep.* 68, 671–679. doi:10.1016/j.pharep.2016.03.014.

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.

Mastelic, J., Jerkovic, I., Blazevic, I., Poljak-Blaži, M., Borović, S., Ivančić-Baće, I., et al. (2008). Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. *J. Agric. Food Chem.* 56, 3989–3996.

Milos, M., and Makota, D. (2012). Investigation of antioxidant synergisms and antagonisms among thymol, carvacrol, thymoquinone and p-cymene in a model system using the Briggs-Rauscher oscillating reaction. *Food Chem.* 131, 296–299. doi:10.1016/j.foodchem.2011.08.042.

Naghibi, F., Mosaddegh, M., Mohammadi Motamed, M., and Ghorbani, A. (2005). Labiatae family in folk medicine in Iran: from ethnobotany to pharmacology. *Iran. J. Pharm. Res. (IJPR)* 4, 63–79.

Novato, T. P. L., Araújo, L. X., de Monteiro, C. M. O., Maturano, R., Senra, T. d. O. S., da Silva Matos, R., et al. (2015). Evaluation of the combined effect of thymol, carvacrol and (E)-cinnamaldehyde on Amblyomma sculptum (Acari: ixodidae) and Dermacentor nitens (Acari: ixodidae) larvae. *Vet. Parasitol.* 212, 331–335. doi:10.1016/j.vetpar.2015.08.021.

Oliveira, M. G., Marques, R. B., Santana, M. F., Santos, A. B., Brito, F. A., Barreto, E. O., et al. (2012). α-Terpineol reduces mechanical hypernociception and inflammatory response. *Basic & Clin. Pharm. & Toxicol.* 111, 120–125. doi:10.1111/j.1742-7843.2012.00875.x

Palevitch, D., Yaniv, Z., Dafni, A., and Simon, J. E. (1986). "Medicinal plants of Israel: an ethnobotanical survey," in *Herbs, spices and medicinal plants*. Editor L. A. Craker (Phoenix, AZ: Oryx Press), 281–345.

Pavela, R. (2015). Acute toxicity and synergistic and antagonistic effects of the aromatic compounds of some essential oils against *Culex quinquefasciatus* Say larvae. *Parasitol. Res.* 114, 3835–3853. doi:10.1007/s00436-015-4614-9.

Pei, R. S., Zhou, F., Ji, B. P., and Xu, J. (2009). Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. *J. Food Sci.* 74, 379–383. doi:10.1111/j.1750-3841.2009.01287.x.

Pelkonen, O., Abass, K., and Wiesner, J. (2013). Thujone and thujone-containing herbal medicinal and botanical products: toxicological assessment. *Regul. Toxicol. Pharmacol.* 65, 100–107. doi:10.1016/j.yrtph.2012.11.002.

Periago, P. M., Delgado, B., Fernández, P. S., and Palop, A. (2004). Use of carvacrol and cymene to control growth and viability of Listeria monocytogenes cells and predictions of survivors using frequency distribution functions. *J. Food Protect.* 67, 1408–1416. doi:10.4315/0362-028x-67.7.1408.

Proudfoot, C. J., Garry, E. M., Cottrell, D. F., Rosie, R., Anderson, H., Robertson, D. C., et al. (2006). Analgesia mediated by the TRPM8 cold receptor in chronic neuropathic pain. *Curr. Biol.* 16, 1591–1605. doi:10.1016/j.cub.2006.07.061.

Quintans-Júnior, L. J., Oliveira, M. G. B., Santana, M. F., Santana, M. T., Guimarães, A. G., Siqueira, J. S., et al. (2011) α-Terpineol reduces nociceptive behavior in mice. *Pharm. Biol.* 49, 583–586. doi:10.3109/13880209.2010.529616

Rattanachaikunsopon, P., and Phumkhachorn, P. (2010b). Assessment of synergistic efficacy of carvacrol and cymene against *Edwardsiella tarda in vitro* and in Tilapia (*Oreochromis niloticus*). *Afr. J. Microbiol. Res.* 4, 420–425.

Rattanachaikunsopon, P., and Phumkhachorn, P. (2010a). Assessment of factors influencing antimicrobial activity of carvacrol and cymene against *Vibrio cholerae* in food. *J. Biosci. Bioeng.* 110, 614–619. doi:10.1016/j.jbiosc.2010.06.010.

Ravishankar, S., Zhu, L., Reyna-Granados, J., Law, B., Joens, L., and Friedman, M. (2010). Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. *J. Food Protect.* 73, 234–240. doi:10.4315/0362-028x-73.2.234.

Renaud, S. (1995). *Le régime santé*. Paris, France: Editions Odile Jacob.

Ribeiro, T. P., Porto, D. L., Menezes, C. P., Antunes, A. A., Silva, D. F., De Sousa, D. P., et al. (2010). Unravelling the cardiovascular effects induced by alpha-terpineol: a role for the nitric oxide-cGMP pathway. *Clin. Exp. Pharmacol. Physiol.* 37, 811–816. doi:10.1111/j.1440-1681.2010.05383.x.

Rivera, D., Obon, C., and Cano, F. (1994). The botany, history and traditional uses of three-lobed sage (Salvia fruticosa miller) (Labiatae). *Econ. Bot.* 48, 190–195. doi:10.1007/bf02908216.

Said, O., Khalil, K., Fulder, S., and Azaizeh, H. (2002). Ethnopharmacological survey of medicinal herbs in Israel, the Golan heights and the west bank region. *J. Ethnopharmacol.* 83, 251–265. doi:10.1016/s0378-8741(02)00253-2.

Santos, F. A., and Rao, V. S. N. (2000). Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. *Phytother Res.* 14, 240–244. doi:10.1002/1099-1573(200006)14:4<240::aid-ptr573>3.0.co;2-x.

Sargin, S. A. (2015). Ethnobotanical survey of medicinal plants in Bozyazı district of Mersin, Turkey. *J. Ethnopharmacol.* 173, 105–126.

Sato, K., Krist, S., and Buchbauer, G. (2007). Antimicrobial effect of vapours of geraniol, (R)-(-)-linalool, terpineol,γ-terpinene and 1,8-cineole on airborne microbes using an airwasher. *Flavour Fragrance J.* 22, 435–437. doi:10.1002/ffj.1818.

Savelev, S., Okello, E., Perry, N. S. L., Wilkins, R. M., and Perry, E. K. (2003). Synergistic and antagonistic interactions of anticholinesterase terpenoids in *Salvia lavandulaefolia* essential oil. *Pharmacol. Biochem. Behav.* 75, 661–668. doi:10.1016/s0091-3057(03)00125-4.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., and Arsenakis, M. (1996). Antimicrobial and cytotoxic activities ofOriganumEssential oils. *J. Agric. Food Chem.* 44, 1202–1205. doi:10.1021/jf950540t.

Song, H., Schacher, C., and Dethlefsen, U. (2009). Concomitant therapy with Cineole (Eucalyptole) reduces exacerbations in COPD: a placebo-controlled double-blind trial. *Respir. Res.* 10, 69. doi:10.1186/1465-9921-10-69.

Sotiropoulou, N.-S., Kokkini, M., P.Megremi, S.-F., Daferera, D., Skotti, E., Kimbaris, A., et al. (2016). Determination of A- and B-thujone in wormwood and sage infusions of Greek flora and estimation of their average toxicity. *Curr. Res. Nutr. Food Sci.* 4, 152–160. doi:10.12944/crnfsj.4.special-issue-october.21.

Souma, H., Sağdiç, O., Özkan, G., and Karadoğan, T. (2004). Antibacterial activity and composition of essential oils from Origanum, Thymbra and Satureja species with commercial importance in Turkey. *Food Contr.* 15, 169–172. doi:10.1016/s0956-7135(03)00028-8.

Stavrou, A., Challoumas, D., and Dimitrakakis, G. (2013). Archibald Cochrane (1909-1988): the father of evidence-based medicine. *Interact. Cardiovasc. Thorac. Surg.* 18, 121–124. doi:10.1093/icvts/ivt451.

Stefanaki, A., Cook, C. M., Lanaras, T., and Kokkini, S. (2016). The Oregano plants of Chios Island (Greece): essential oils of *Origanum onites* L. growing wild in different habitats. *Ind. Crop. Prod.* 82, 107–113. doi:10.1016/j.indcrop.2015.11.086.

Suntres, Z. E., Coccimiglio, J., and Alipour, M. (2015). The bioactivity and toxicological actions of carvacrol. *Crit. Rev. Food Sci. Nutr.* 55, 304–318. doi:10.1080/10408398.2011.653458.

Tak, J.-H., Jovel, E., and Isman, M. B. (2016). Comparative and synergistic activity of Rosmarinus officinalis L. essential oil constituents against the larvae and an ovarian cell line of the cabbage looper, Trichoplusia ni (Lepidoptera: noctuidae). *Pest Manag. Sci.* 72, 474–480. doi:10.1002/ps.4010.

Tak, J. H., and Isman, M. B. (2017). Penetration-enhancement underlies synergy of plant essential oil terpenoids as insecticides in the cabbage looper, Trichoplusia ni. *Sci. Rep.* 7, 42432. doi:10.1038/srep42432.

Takaishi, V. G., Bezerra, J., Souza, G. T., Carvalho, R. J., Gomes-Neto, N. J., Figueiredo, R. C., et al. (2015). Inhibition of *Staphylococcus aureus* cocktail using the synergies of oregano and rosemary essential oils or carvacrol and 1, 8-cineole. *Front. Microbiol.* 6, 1223. doi:10.3389/fmicb.2015.01223.

Tang, Y., Garg, H., Geng, Y.-J., and Bryan, N. S. (2009). Nitric oxide bioactivity of traditional Chinese medicines used for cardiovascular indications. *Free Radic. Biol. Med.* 47, 835–840. doi:10.1016/j.freeradbiomed.2009.06.024.

Tarantilis, R. H. L., Cardoso, M. S. P., Menezes, C. T., Silva, J. P., De Sousa, D. P., and Batista, J. S. (2011) .Gastroprotective activity of α-terpineol in two experimental models of gastric ulcer in rats. *Daru: J. Faculty Phar.* 19, 277.

Tesche, S., Metternich, F., Sonnemann, U., Engelke, J.-C., and Dethlefsen, U. (2008). The value of herbal medicines in the treatment of acute non-purulent rhinosinusitis. *Eur. Arch. Oto-Rhino-Laryngol.* 265, 1355. doi:10.1007/s00405-008-0683-z.

Tseliou, M., Pirintsos, S. A., Lionis, C., Castanas, E., and Sourvinos, G. (2019). Antiviral effect of an essential oil combination derived from three aromatic plants (*Coridothymus capitatus* (L.) Rchb. f., *Origanum dictamnus* L. and *Salvia fruticosa* Mill.) against viruses causing infections of the upper respiratory tract. *J. Herb. Med.* 17-18, 100288. doi:10.1016/j.hermed.2019.100288.

Ultee, A., Bennik, M. H. J., and Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *AEM* 68, 1561–1568. doi:10.1128/aem.68.4.1561-1568.2002.

Ultee, A., Kets, E. P. W., and Smid, E. J. (1999). Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* 65, 4606–4610. doi:10.1128/aem.65.10.4606-4610.1999.

Ultee, A., Slump, R. A., Steging, G., and Smid, E. J. (2000). Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. *J. Food Protect.* 63, 620–624. doi:10.4315/0362-028x-63.5.620.

Vardar-Ünlü, G., Candan, F., Sökmen, A., Daferera, D., Polissiou, M., Sökmen, M., et al. (2003). Antimicrobial and Antioxidant Activity of the Essential Oil and Methanol Extracts ofThymus pectinatusFisch. et Mey. Var.pectinatus(Lamiaceae). *J. Agric. Food Chem.* 51, 63–67. doi:10.1021/jf025753e.

Vartiainen, O. (1950). The anthelmintic effects of thymol and p-cymene; a pharmacological and clinical study, with special consideration of the fish tapeworm disease. *Ann. Med. Intern. Fenn.* 39, 1–87.

Vázquez, F. M., Suarez, M. A., and Pérez, A. (1997). Medicinal plants used in the Barros area, Badajoz province (Spain). *J. Ethnopharmacol.* 55, 81–85. doi:10.1016/s0378-8741(96)01491-2.

Vrachnakis, T. (2003). Trichomes of *Origanum dictamnus* L. (Labiatae). *Phyton* 43, 109–133.

Wagner, H., and Wierer, R. (1986). In vitro-Hemmung der Prostaglandin-Biosynthese durch etherische Öle und phenolische Verbindungen. *Planta Med.* 52, 184–187. doi:10.1055/s-2007-969117.

Wang, S., Zhang, D., Hu, J., Jia, Q., Xu, W., Su, D., et al. (2017). A clinical and mechanistic study of topical borneol-induced analgesia. *EMBO Mol. Med.* 9, 802–815. doi:10.15252/emmm.201607300.

Worth, H., and Dethlefsen, U. (2012). Patients with asthma benefit from concomitant therapy with cineole: a placebo-controlled, double-blind trial. *J. Asthma* 49, 849–853.doi:10.3109/02770903.2012.717657.

Xu, H., Delling, M., Jun, J. C., and Clapham, D. E. (2006). Oregano, thyme and clove-derived flavors and skin sensitizers activate specific TRP channels. *Nat. Neurosci.* 9, 628–635. doi:10.1038/nn1692.

Yang, Y., Zhang, Z., Li, S., Ye, X., Li, X., and He, K. (2014). Synergy effects of herb extracts: pharmacokinetics and pharmacodynamic basis. *Fitoterapia* 92, 133–147. doi:10.1016/j.fitote.2013.10.010.

Yu, D., Wang, J., Shao, X., Xu, F., and Wang, H. (2015). Antifungal modes of action of tea tree oil and its two characteristic components againstBotrytis cinerea. *J. Appl. Microbiol.* 119, 1253–1262. doi:10.1111/jam.12939.

Yuan, G., Wahlqvist, M., He, G., Yang, M., and Li, D. (2006). Natural products and anti-inflammatory activity. *Asia Pac. J. Clin. Nutr.* 15, 143.

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.

Conflict of Interest: SP, CL, and EC are inventors of a patent (WO2010GB01836 20,100,929) on the use of the three examined plants for combating upper respiratory tract infections.

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

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Grindelia squarrosa Extract and Grindelic Acid Modulate Pro-inflammatory Functions of Respiratory Epithelium and Human Macrophages

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Aim of the study: Both nasal and bronchial epithelial cells have evolved sophisticated mechanisms involved in cellular response to bacterial infection. Recognition of pathogens by TLR receptors activate the NF-κB transcription factor, and lead to production of wide spectrum of cytokines (TNF-α, IL-1β, IL-6 and IL-8). Released by epithelium proinflammatory cytokines intensify migration of macrophages to damaged tissues and modulate their pro-inflammatory functions. Based on traditional use of *G. squarrosa* aerial parts we hypothesized that successful treatment of cold-related diseases may arise from modulation of the pro-inflammatory functions of respiratory epithelium and human monocytes/macrophages. The biological activity of *G. squarrosa* extract and grindelic acid were compared with clarithromycin and budesonide used as positive controls.

Methods: The expression of surface receptors (TLR-4, IL-10) and expression of adhesive molecules (ICAM-1, VCAM-1, E-selectin) was analyzed with flow cytometry. The macrophage attachment to the epithelial cells was assessed fluorimetrically. The p65 NF-κB concentration and cytokine production was measured spectrophotometrically using enzyme-linked immunosorbent assay. Antibacterial activity was examined by the standard disc-diffusion method and serial dilution method according to CLSI guidelines.

Results: *G. squarrosa* extract and grindelic acid had no antimicrobial effect. However, we noticed significant modulation of pro-inflammatory functions of LPS-stimulated nasal and bronchial epithelium. *G. squarrosa* extract treatment resulted in decrease of TLR-4 expression and p65 NF-κB concentration and inhibition of cytokines synthesis (IL-8, TNF-α, IL-1β and IL-6) in both cellular models. Additionally, *G. squarrosa* extract slightly modulated ICAM-1 expression affecting on attachment of macrophages to epithelium. Only *G. squarrosa* extract was able to stimulate the anti-inflammatory functions of

macrophages by inducing TGF-β release and IL-10 receptor surface expression. Grindelic acid, identified as a dominant compound in the plant extract, modulated pro-inflammatory functions of epithelium and macrophages slightly.

Conclusion: The obtained results support traditional use of *Grindelia squarrosa* preparations for a treatment cold-associated diseases symptoms. In our opinion, the observed biological effect of extract may be a consequence of synergistic effect of all compounds present in the extract.

Keywords: Grindelia squarrosa, grindelic acid, inflammation, cold syndrome, respiratory epithelium, macrophages

INTRODUCTION

Common cold is one of the most common occurring acute illnesses in humans. In a large-scale survey, 25% of all those questioned in the United Kingdom had suffered from 3 to 6 colds during the previous year and 73% reported having a common cold at least once during this period. The similar tendency is observed in Germany and United States. The acute episode of a cold is starting with viral infection and may be accompanied by bacterial superinfection. The most common bacterial isolates from people with cold syndromes include Gram-positive strains, such as *Streptococcus pneumoniae* responsible for pharyngitis, bronchitis or pneumonia (Klugman and Feldman, 2001); *Staphylococcus aureus* responsible for pharyngitis and epiglottitis, *Hemophilus influenzae*, which occupy a similar microenvironment within the nasopharynx as *Streptococcus pneumoniae* (Erwin and Smith, 2007), and other opportunistic organisms as *Escherichia coli* (Mizgerd, 2008).

Common cold treatment is mostly directed towards relief of symptoms. The infection results in development of full-symptomatic inflammation and overproduction of cytokines. Currently accepted cold treatment strategies focus on suppression of inflammation by anti-inflammatory drugs. Treatment may be enhanced by inclusion of antibiotic therapy. However, using antibiotics bring benefits only for the treatment of secondary bacterial infections. The valuable approach in the cold treatment may be strengthen immune system in order to prevent the uncontrolled progression of inflammation, but also protection of the respiratory epithelium against excessive damage.

Because airway epithelial cells are a border line between external and internal environment, they are particularly vulnerable to damage. Epithelial cells express a wide spectrum of receptors, such as Toll-like receptors. TLRs identify pathogen-associated molecular patterns (PAMPs), and initiate the appropriate cellular response. Among known TLRs, TLR-4 is the main LPS receptor (Zusso et al., 2019). Binding the LPS results in the activation of downstream mediators, including the transcription factor nuclear factor NF-κB, which stimulates the production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) (Spiegler et al., 2020). Released cytokines intensify migration of macrophages to the site of infection. The released cytokines modulate adhesion molecules expression (ICAM-1, VCAM-1 and E-selectin) on the respiratory epithelium, thus mediate direct emigration of macrophages across the endothelial and epithelial barriers that separate the bloodstream from the pulmonary air spaces (Martin et al., 1992; Martin, 2000; Puneet et al., 2005). The infiltrated macrophages are activated and released wide spectrum of inflammatory mediators: reactive oxygen species (ROS), cytokines (TNF-α, IL-6, TGF-β), and modulate expression of membrane receptors such as IL-10 (Michalak et al., 2018).

According to World Health Organization (WHO) guidelines, over 90% of the population of developed and developing countries, uses the wealth of phytomedicine. *Grindelia squarrosa* well-known in South America, Asia and Europe is still sold as complex preparations (liquid extracts, syrups) for treating cough and bronchitis (Strzelecka, 2000). According to European Medicines Agency (EMA) there are various types of preparations: tincture or herbal tea (**Table 1**). The toxicity of this plant to humans is negligible, what intensified the traditional use of the herb (Goetz, 2005).

The *Grindelia* is valuable source of biologically active compounds (Capasso et al., 2003). The aerial parts of *G. squarrosa* are a rich source of diterpenes. It contains grindelic, 6-oxygrindelic, 18-hydroxy-6-oxygrindelic, 7-alpha-oxodihydrogrindelic and 8-alpha-oxodihydrogrindelic acids. As dominant diterpene was identified grindelic acid, which may consist of 20–60% of grindelia resin and up to 6% of dry plant material (Johnson, 1887; Efferth and Koch, 2011; Nowak and Rychlińska, 2012; Veres et al., 2014). The plant also contains flavonoids, phenolic acids, an essential oils, polyacetylenes and saponins (Veres et al., 2014).

Although *G. squarrosa* extracts are present at pharmaceutical market, there is still lack of studies fully explaining their anti-microbial and anti-inflammatory properties. In present study, we have evaluated influence of *Grindelia squarrosa* extract on pro-inflammatory functions of LPS-stimulated nasal and bronchial epithelial cells and macrophages. We highlight new anti-inflammatory properties of the extract, and explain how *G. squarrsa* extract modulates the epithelium response to LPS.

MATERIALS AND METHODS

Chemicals

Normal Human Bronchial Epithelial Cells (NHBE) and Bronchial Epithelial Cell Growth Medium were obtained from Lonza (Switzerland). Primary Human Nasal Epithelial Cells (HNEpC) and Airway Epithelial Cell Growth Medium were

TABLE 1 | The forms of *G. squarrosa* extract administration.

Form	Administration	Part	References
Infusion	3 g in 150 ml of boiling water. Daily dose: up to 3 times daily	Aerial parts, flowers, leaves	Kalktenbach and Schimmer (1991), Ferreres et al. (2014)
Tincture	0.5–1 ml 3 times daily. Daily dose: 1.5–3 ml	Aerial parts, flowers	Kalktenbach and Schimmer (1991)
Liquid extract	0.6–1.2 ml 3 times daily. Daily dose: 1.8–3.6 ml	Aerial parts, flowers, leaves	Kalktenbach and Schimmer (1991), McFarlane (2017)

obtained from Promocell (Germany). Grindelic acid (purity ≥95% (LC/MS-ELSD)), clarithromycin (purity ≥95% (HPLC)), budesonide (purity ≥98% (HPLC)), LPS (from *Escherichia coli* 0,111:B4), Ficoll Hypaque gradient, GM-CSF, HEPES buffer, L-glutamine, RPMI 1640 medium, Fetal bovine serum (FBS), Cell Dissociation Solution (non-enzymatic), Accutase™, propidium iodide and Calcein-AM were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). TLR-4-(PE)-conjugate (cat no. HTA125) was obtained from eBioscience. Human Quantikine ELISA Kits were purchased from R&D System (Minneapolis, USA). Anti-Human CD54-(-APC) (cat no. 559771), Anti-Human CD106-(-PE) (cat no. 555647), Anti-Human CD62E-(-PE) (cat no. 551145) were purchased from BD Pharmingen.

Plant Material and Phytochemical Analysis

The plant material *Grindelia squarrosa* (Pursh) Dunal (http://www.theplantlist.org/tpl1.1/record/gcc-87994) was collected in July 2018 in Lublin Medicinal Plant Garden, Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin (51°15′22″N, 22°33′51″E) were the voucher specimen is deposited (27a/13).

The air-dried plant material (5 g) was extracted with 60% ethanol (v/v, 1:10) in a water bath (70 °C) for 1 h three times. Then, combined extracts were filtered, the ethanol was evaporated, and the water residue was lyophilized. The phytochemical characterization was performed using UHPLC-DAD-ESI-MS^n analysis (UHPLC-3000 RS system, Dionex, Germany) with DAD detection and an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik GmbH, Bremen, Germany). Separation was performed on a Zorbax SB-C_{18} column (150 × 2.1 mm, 1.9 µm) (Agilent, Santa Clara, CA, United States). The mobile phase consisted of 0.1% HCOOH in water (A) and 0.1% HCOOH in MeCN (B) using the following gradient: 0–60 min 5–100% B. The LC eluate was introduced into the ESI interface and compounds were analyzed in negative ion mode (for phenolic acids and flavonoids) and with positive ion mode (for diterpenes) with the following settings: nebulizer pressure of 40 psi; drying gas flow rate of 9 L/min; nitrogen gas temperature of 300 °C; and a capillary voltage of 4.5 kV. The mass scan ranged from 100 to 2,200 m/z. UV spectra were recorded in the range of 190–350 nm. The characterization of substances in the extract was performed by comparing the retention times and spectra (UV, MS, MS^n) with published data.

Isolation of grindelic acid (GA). The plant material (50 g) was extracted three times with 60% ethanol (1:20) at 70 °C for 2 h each time. The ethanol from combined extracts was evaporated under reduced pressure, and the aqueous residue was then extracted four times with ethyl acetate (150 ml). The ethyl acetate residue (5.2 g) was subjected to Sephadex LH-20 column chromatography (80 × 2.5 cm) and eluted 70% MeOH to obtain four main fractions of 0.236 g (F1), 0.805 g (F2), 1.062 g (F3) and 0.480 g (F4), respectively. Fraction F4, containing GA, based on TLC analysis with comparison with standard, was separated on a Silica gel column (55 × 2.5 cm; 100–200 µm) with $CHCL_3$-MeOH gradient (100:0 → 90:10) in seven steps. Ten fractions of 25 ml each were pooled into six main fractions (F4_1-F4_6) based on their TLC profiles. From fraction F4_1 (0.22 g) GA (152 mg), was isolated using Silica gel column (55 × 2.5 cm; 0.63 µm) and $CHCL_3$ as eluent. The structure of GA was confirmed by their ^1H and ^{13}C NMR spectra, which were compared with reference data (Reta et al., 2013).

Preparation of Solutions of Extract and Compounds for Bioassays

For all experiments *G. squarrosa* extract and isolated grindelic acid were used as described above. Extract was dissolved in DMSO (10 mg/ml stock solution) and tested at a concentration range 25–100 µL. Grindelic acid was dissolved in DMSO (10 mM stock solution) and tested at a concentration range of 10–50 µM. The final concentration of DMSO was adjusted to 0.1% (v/v) in culture media.

Antimicrobial Properties of *G. squarrosa* Extract and Grindelic Acid

Both Gram-positive (*Streptococcus pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 25923 ATCC 29213) and Gram-negative (*Haemophilus influenzae* ATCC 49247) bacteria strains were tested in this study.

Sensitivity of selected bacteria strains was examined by the standard disc-diffusion method according to CLSI (previously NCCLS) guidelines (Watts, 2008). The results (diameter of the growth inhibition zone) were read after 18 h of incubation at 35 °C. Minimal Inhibitory Concentrations (MIC) were evaluated by the two fold serial microdilution method (in 96-well microtiter plates) using Mueller-Hinton Broth medium (Beckton Dickinson) according to CLSI guidelines (Bandi and Kompella, 2001; Watts, 2008).

Primary Human Nasal Epithelial Cells (HNEpC) Culture and Stimulation

HNEpC cells (passage fourth to sixth), were seeded on 12-well plates, 1.5×10^5 cells per well in Airway Epithelial Cell Growth Medium supplemented with 6% Fetal Bovine Serum. The confluent cells were treated with extracts/compounds and then used for experiments.

Normal Human Bronchial Epithelial (NHBE) Culture and Stimulation

NHBE cells (passage fourth to sixth), were seeded on 12-well plates, 1×10^5 cells per well in Bronchial Epithelial Cell Growth Medium. The confluent cells were treated with extracts/compounds and then used for experiments.

Isolation of Human Monocytes and Stimulation

Monocytes were isolated immediately after collection using a Ficoll Hypaque gradient (Dudek et al., 2017). The mononuclear cell band was removed by aspiration, and cells were suspended in RPMI 1640 medium with 2 mM L-glutamine, 10 mM HEPES, antibiotics and autologous serum (5%). To promote the adherence of monocytes/macrophages, the peripheral blood mononuclear cell suspension was placed in 12-well tissue culture plates (2×10^6 per well) and incubated for 2 h at 37 °C under humidified 5% CO_2 air. After the incubation, nonadherent cells were removed, and adherent cells were cultured with RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (FBS, 10%) and granulocyte macrophage-colony stimulating factor (GM-CSF, 10 ng/ml) for 7 days to induce differentiation to macrophages. The medium and autologous serum were replaced every 2 days.

Determination of Cytotoxicity by PI-Staining

Cytotoxicity of the tested extract and compounds was determined by PI-staining. Cells were treated with the compounds for 24 h, centrifuged (1,500 RPM; 5 min; 4 °C) and re-suspended in 300 µL of PBS. 5 µL of PI (50 µg/ml) solution was added to the cell suspensions and incubated 10 min in the dark. Cytotoxic effects of the extract and compounds were measured by BD FACS LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, United States) by recording 10,000 events per sample and analyzed with FlowJo V10 software. Cells that displayed high permeability to PI were expressed as percentage of PI(+) cells.

Expression of TLR-4 Receptor on Nasal and Bronchial Epithelium

Influence of the tested extract and compounds on the TLR-4 receptor expression was determined by flow cytometry measurement. After 1 h extract- or compounds pretreatment cells were stimulated with LPS for 15 min, and centrifuged (1500 RPM; 10 min; 4 °C). After removing supernatants, cells were re-suspended in 300 µL PBS and labelled with monoclonal antibodies against TLR-4 (eBioscience, United States). The TLR-4 expression was measured by BD FACS LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, United States) by recording 10,000 cells per sample and analyzed with FlowJo V10 software.

TNF-α, IL-1β, IL-6 and IL-8 Production

Epithelial cells were pretreated with the *G. squarrosa* extract (at concentration range 25–100 µg/ml) or grindelic acid (at 10–50 µM) for 1 h at 37 °C, and then they were stimulated with LPS (1 µg/ml) for 24 h. Afterwards, cells were centrifuged (1500 RPM; 10 min; 4 °C) and collected supernatants. The total amount of released cytokines was measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (BD Biosciences, United States). The results were expressed as percentages of the released agents from samples compared to the LPS-stimulated control.

p65 NF-κB Concentration in Nasal and Bronchial Epithelium

Epithelial cells were pretreated with the *G. squarrosa* extract (at concentration range 25–100 µg/ml) or grindelic acid (at 10–50 µM) for 40 min at 37 °C, and then they were stimulated with LPS (1 µg/ml) for 20 min. The protein isolation was provided as we describe previously (Gierlikowska et al., 2020b). Cells were washed with ice-cold PBS, suspended in hypotonic buffer (5 mM NaF, 0.1 mM EDTA, 20 mM HEPES, 10 µM Na_2MoO_4), and then centrifuged. The nuclear pellets were lysed with freshly-prepared buffer supplemented with protease inhibitor cocktail and left on ice for 30 min. Pellets were centrifuged for 15 min 2.500 RPM and kept at −80 °C for further analysis.

The protein concentration was measured with Bradford-based method following manufacturer's protocol. 10 µg of nuclear fraction was transferred on 96-well plate provided by manufacturer. Then, primary antibody (1:1,000 dilution) was added on plate for 1 h, then washed 3 times and incubated with HRP-conjugated secondary antibody (1:1,000 dilution) for additional 1 h. After incubation, plate was washed 3 times and reaction was stopped by adding developing solution and stop solution, respectively. The concentration of NF-κB p65 was measured spectrophotometrically at 450 nm.

Expression of ICAM, VCAM and E-Selectin on Respiratory Surface

Cells were pre-incubated with extract or compounds for 1 h and then stimulated with LPS (1,000 ng/ml, for 24 h). Cell monolayers were washed with PBS and suspended in 200 µL of accutase. After 10 min incubation at 37 °C, the cells were harvested, centrifuged (1500 RPM; 10 min; 4 °C) and suspended in 300 µL of PBS. Cells were labelled with 5 µL of anti-human CD54 (ICAM-1), anti-human CD106 (VCAM-1) or anti-human CD62E (E-selectin).

The expression of adhesive molecules was analyzed by BD FACS LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, United States) by recording 10,000 cells per sample and analyzed with FlowJo V10 software. The effect on the surface expression of adhesion molecules was evaluated based on a software-generated marker histogram M1 for LPS stimulated cells.

The Attachment of Macrophages to Epithelial Cells

Epithelial cells were pretreated with extract or compounds for 1 h and then stimulated with LPS (1,000 ng/ml, for 24 h). Cell

monolayers were washed, suspended in 200 µL of RPMI 1640 medium and added 100 µL of 1 mM calcein-AM labelled macrophages (1×10^6) as described previously (Michalak et al., 2018). Before calcein labelling, macrophages were stimulated with LPS (100 ng/ml) for an 30 min, and then incubated with epithelial cells for additional 30 min. The cell monolayers with attached macrophages were lysed with 0.1% Triton X-100, and fluorescence was measured in a microplate reader at 485 nm excitation and 520 nm emission.

Expression of IL-10 Receptor on the Macrophage Surface

Macrophages were stimulated with LPS (100 ng/ml for 1 h) and treated with the extract or compounds for 24 h. Subsequently, cells were centrifuged (13,000 RPM, 4 °C, 1 min), suspended in 300 µL of PBS and labelled with antibodies against IL-10. The IL-10 expression was measured by BD FACS LSR flow cytometer (BD Biosciences, San Jose, CA, United States) by recording 10,000 cells per sample and analyzed with CellQuest Pro software. The results were expressed as percentages of cells expressing IL-10 receptor in comparison to control cells stimulated by LPS.

TGF-β, IL-6 and TNF-α Production by Macrophages

Macrophages were cultured in 12-well plates in the presence of LPS (100 ng/ml, 1 h) and then treated with tested extracts/compounds for 24 h at 37 °C with 5% CO_2. After 24 h, cells were harvested and centrifuged (2,000 RPM; 10 min; 4 °C). The amounts of released cytokines were measured by ELISA following the manufacturer's instructions (BD Biosciences, United States). The effect on cytokine production was calculated by comparing the percentages of the released agent to the control cells, which were stimulated but were not exposed to the test compounds.

Statistical Analysis

The results were expressed as the mean ± SEM from three independent experiments assayed in triplicates. All analyses were performed using Statistica 13.1 software. GraphPad Prism (version 5.01) was used to plot data. The statistical significance of the differences between means was established by ANOVA with Dunnett's *post hoc* test. p values below 0.05 were considered statistically significant.

RESULTS

G. squarrosa Extract Phytochemical Characterization and Grindelic Acid Isolation

The phytochemical analysis of *G. squarrosa* extract was performed using a UHPLC-DAD-ESI-MS^n method. We confirmed the presence of polyphenolic compounds which are characteristic for Astearaceae family such as caffeoyl quinic acid and dicaffeoyl quinic acids, as well as luteolin and apigenin derivatives (**Figure 1**; **Table 2**). As for *Grindelia* genus the presence of diterpenes is distinctive, we were focused on this group of compounds. However, as grindelic acid do not possess chromophore groups, its detection based on UHPLC-DAD-ESI-MS^n was less sensitive. In order to confirm the presence of grindelic acid, we isolated this compound from the extract using ethyl acetate partition and column chromatography (**Supplementary Figure S1**). We confirmed the purity and structure of isolated GA using NMR spectroscopy (**Supplementary Figure S2**).

G. squarrosa Extract and Grindelic Acid did Not Show Anti-microbial Activity

The *G. squarrosa* extract (tested at 25–100 µg/ml) and grindelic acid (10–50 µM) did not inhibit bacteria growth. We were not able to detect growth inhibition zone (GIZ, mm) and minimal inhibitory concentration (MIC, µg/mL). The growth inhibition zone was observed only for gram g-positive (*S. pneumoniae* and *S. aureus*) treated with clarithromycin. Obtained results were comparable with reference values present at EUCAST (The European Committee on Antimicrobial Susceptibility Testing) (Bandi and Kompella, 2001).

G. squarrosa Extract Decrease the TLR-4 Expression

Stimulation with LPS resulted in a significant increase of TLR-4 expression (**Figure 2**). Incubation of LPS-stimulated nasal and bronchial epithelial cells with *G. squarrosa* extract resulted in reduction of TLR-4 expression in the presence of all tested concentrations. The obtained results were comparable to budesonide, tested at 50 µM. Budesonide suppressed the TLR-4 expression on nasal epithelial cells to 63.2% and to 48.5% on bronchial epithelial cells ($p < 0.001$). Clarithromycin, as well as grindelic acid affect TLR-4 expression slightly. The tested concentrations, did not damage the cellular membrane integrity of nasal and bronchial epithelial cells as presented in **Supplementary Table S1** and **Figure S3**.

G. squarrosa Extract Modulates Cytokine Production and 65 NF-κb Concentration

Lipopolysaccharide (LPS) stimulation of human nasal and bronchial epithelial cells resulted in the production wide range of cytokines: TNF-α, IL-1β, IL-6 and IL-8 (leukocyte chemotactic factors). *G. squarrosa* extract as well as grindelic acid were tested at concentration range of 25–100 µg/ml, and 10–50 µM, respectively. IL-1β and TNF-α, called early-released proinflammatory cytokines, were significantly suppressed by *G. squarrosa* extract in both epithelial models ($p < 0.001$; **Figures 3A–D**). Budesonide, well known corticosteroid with strong anti-inflammatory activity, suppressed IL-1β and TNF-α production, similarly like extract tested at 50 µg/ml ($p < 0.001$). Additionally, *G. squarrosa* extract was more active

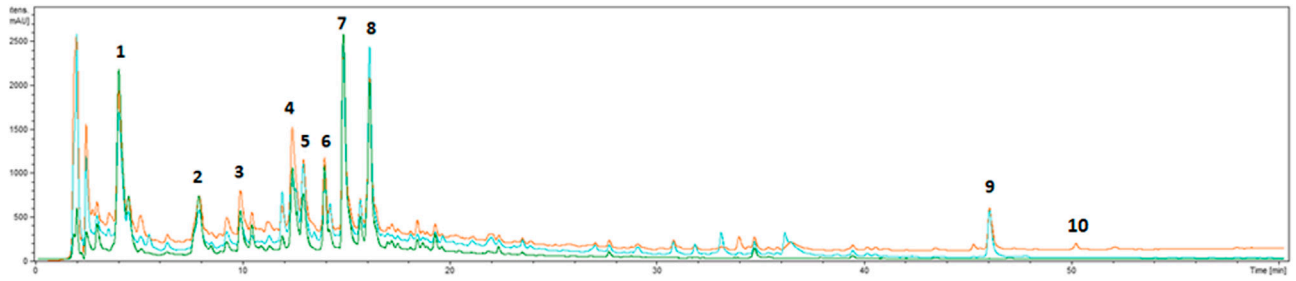

FIGURE 1 | UHPLC-DAD chromatograms of extract from *Grindelia squarrosa* at 220 nm (orange line), 254 nm (blue line) and 325 nm (green line).

TABLE 2 | Retention time, UV and MS data of the compounds present in extract from *Grindelia squarrosa*.

	Compound	Retention time [min]	UV [nm]	$[M-H]^-$ m/z	Fragment ion(s)	References
1	Caffeoyl quinic acid	4.0	325	353	191	Ferreres et al. (2014)
2	Feruloyl quinic acid	7.9	325	367	193	Ferreres et al. (2014)
3	Luteolin hexurosyl-pentoside	9.8	330	593	461, 417, 285	Ferreres et al. (2014)
4	Luteolin hexuronide	12.5	334	461	461, 285	Ferreres et al. (2014)
5	Apigenin hexurosyl-pentoside	12.9	334	577	445, 269	Ferreres et al. (2014)
6	Dicaffeoyl quinic acid (I)	14.0	325	515	353, 191, 179, 173	Ferreres et al. (2014)
7	Dicaffeoyl quinic acid (II)	14.9	325	515	353, 191, 179	Ferreres et al. (2014)
8	Dicaffeoyl quinic acid (III)	16.1	325	515	353, 191, 179, 173, 135	Ferreres et al. (2014)
9	6-Oxogrindelic acid	46.0	230	335[a]	321[a]	Mahmoud et al. (2000)
10	Grindelic acid[b]	50.1	220	321[a]	303[a]	Timmermann et al. (1985)

[a] $[M + H]^+$;
[b] *isolated in this study.*

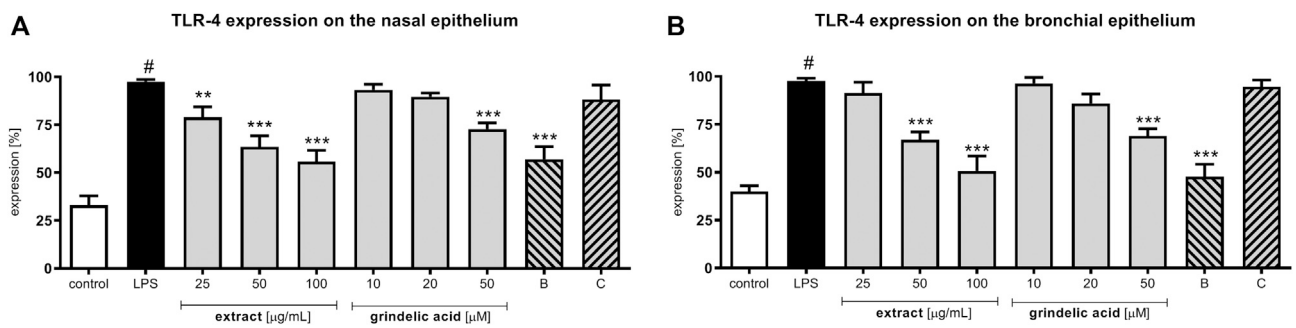

FIGURE 2 | Effects of *G. squarrosa* extract and compounds on the TLR-4 expression on the LPS-stimulated: **(A)** nasal epithelial cells (HNEpC) and **(B)** bronchial epithelial cells (NHBE). Data is expressed as the mean ± SEM; three independent experiments assayed in triplicates. Statistical significance $^{\#}p < 0.01$ compared to the non-stimulated control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to stimulated control (+LPS). Control—non-stimulated cells, LPS—LPS-stimulated cells, B—budesonide tested at 50 µM, C—clarithromycin tested at 50 µM.

than clarithromycin, which served as a second positive control ($p < 0.05$). The activity of grindelic acid was significant only in the highest concentration 50 µM ($p < 0.05$) in both epithelial models.

IL-6 and IL-8 were chosen for assessment as late-released proinflammatory cytokines. *G. squarrosa* extract at 25–100 µg/ml significantly inhibited IL-8 production in both epithelial models ($p < 0.001$) in dose dependent manner. The effect of *G. squarrosa* on IL-6 production was significant only up to 50 µg/ml (**Figures 3E–H**). The biological activity of budesonide was significant ($p < 0.001$) in both epithelial models. However, clarithromycin did not influence on IL-8 and IL-6 production.

Evaluation of p65 NF-κB concentration explains the molecular mechanism responsible for previously observed cytokine concentration changes. Our data show that *G. squarrosa* extract at the concentration range 25–100 µg/ml significantly decreased p65 production ($p < 0.001$) (**Figures 4A,B**). Similarly, budesonide tested at 50 µM significantly inhibited p65 production, and final effect was comparable to plant extract tested at 100 µg/ml.

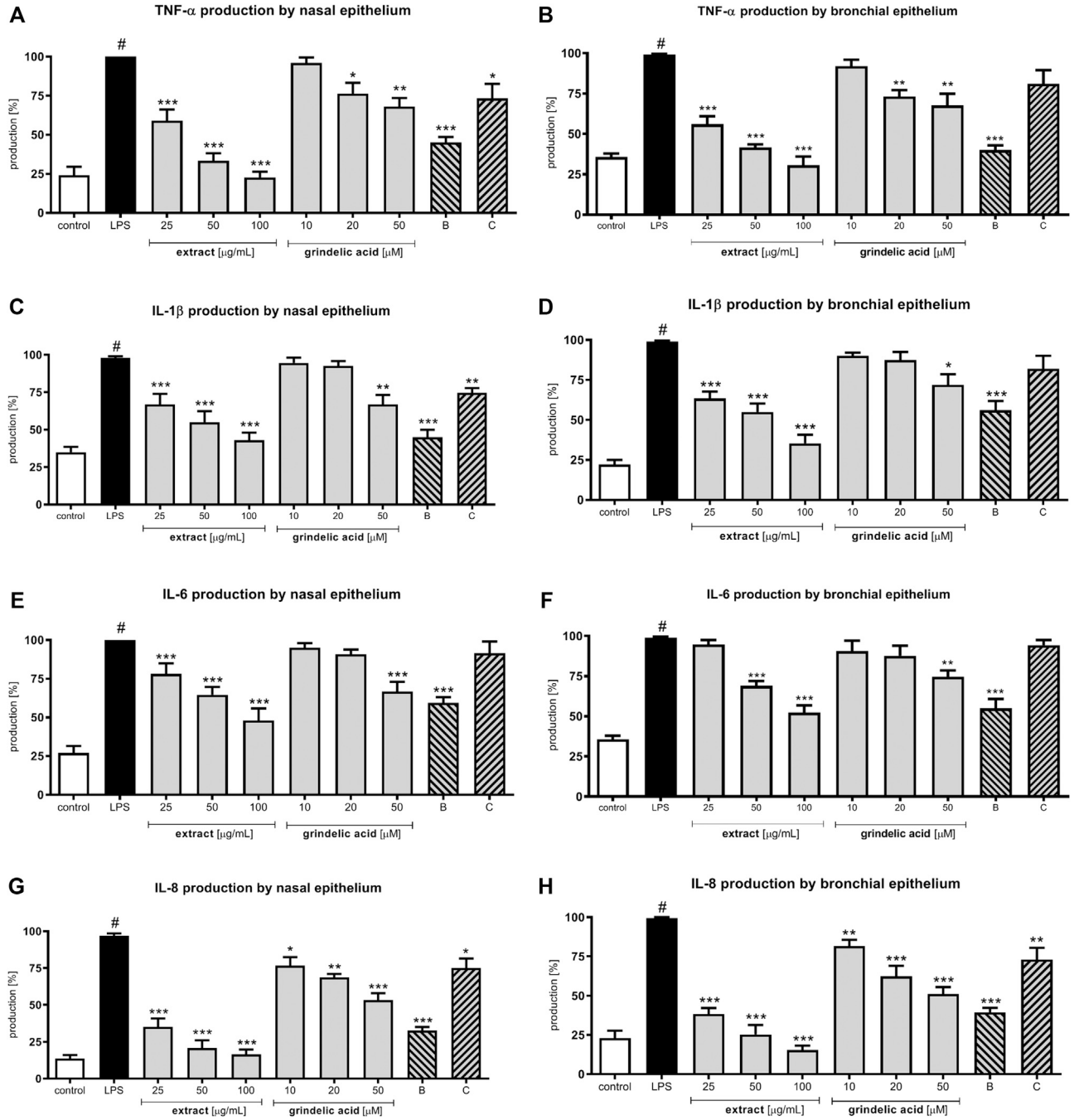

FIGURE 3 | Effects of *G. squarrosa* extract and tested compounds on chemo- and cytokines production by LPS-stimulated nasal and bronchial epithelial cells: TNF-α secretion by **(A)** nasal epithelial cells (HNEpC) and **(B)** bronchial epithelial cells (NHBE); IL-1β secretion by **(C)** nasal epithelial cells (HNEpC) and **(D)** bronchial epithelial cells (NHBE); IL-6 secretion by **(E)** nasal epithelial cells (HNEpC) and **(F)** bronchial epithelial cells (NHBE); IL-8 secretion by **(G)** nasal epithelial cells (HNEpC) and **(H)** bronchial epithelial cells (NHBE).

TABLE 3 | Effects of *G. squarrosa* extract and tested compounds on the adhesive molecules expression (ICAM-1, VCAM-1 and E-selectin) located on the nasal and bronchial epithelial cells. Data is expressed as the mean ± SEM; three independent experiments assayed in triplicates. Statistical significance [a]$p < 0.01$ compared to the non-stimulated control; [b]$p < 0.05$, [c]$p < 0.01$, [d]$p < 0.001$ compared to stimulated control (+LPS).

	ICAM-1 expression [%]		VCAM-1 expression [%]		E-selectin expression [%]	
	Nasal	Bronchial	Nasal	Bronchial	Nasal	Bronchial
Control	34.2 ± 3.6	28.4 ± 1.3	26.4 ± 7.2	30.2 ± 5.5	24.1 ± 3.9	19.3 ± 6.2
Stimulation (+LPS)	100 ± 2.1	100 ± 4.5	100 ± 5.1	100 ± 3.9	100 ± 2.8	100 ± 5.9
G. squarrosa extract + LPS						
25 µg/ml	94.3 ± 5.9	99.3 ± 3.7	89.6 ± 5.3	95.2 ± 3.4	90.9 ± 6.1	97.7 ± 9.4
50 µg/ml	88.3 ± 2.9	90.5 ± 4.2	94.2 ± 2.8	90.3 ± 6.1	94.2 ± 3.1	93.2 ± 1.8
100 µg/ml	75.3 ± 3.4[b]	78.2 ± 5.3[b]	92.5 ± 6.1	88.4 ± 7.9	93.8 ± 4.1	96.2 ± 3.9
Grindelic acid + LPS						
10 µM	89.5 ± 6.9	90.5 ± 8.2	97.4 ± 5.3	98.8 ± 2.4	96.4 ± 2.5	99.3 ± 2.8
20 µM	88.9 ± 9.8	92.1 ± 4.2	95.2 ± 4.6	97.2 ± 1.8	99.4 ± 3.7	93.2 ± 3.7
50 µM	90.4 ± 2.7	91.6 ± 3.7	89.6 ± 4.6	93.2 ± 4.1	91.3 ± 9.2	89.3 ± 7.9
Budesonide 50 µM	79.4 ± 4.9[b]	80.5 ± 3.8[b]	92.5 ± 9.1	90.3 ± 7.1	86.4 ± 6.9	89.5 ± 8.3
Clarithromycin 50 µM	95.3 ± 3.9	92.8 ± 6.2	94.3 ± 5.7	96.3 ± 3.6	99.4 ± 1.8	91.4 ± 6.7

[a]p < *0.01 vs. not stimulated cells;*
[b]p < *0.05 vs. LPS-stimulated cells;*
[c]p < *0.01 vs. LPS-stimulated cells;*
[d]p < *0.001 vs. LPS-stimulated cells.*

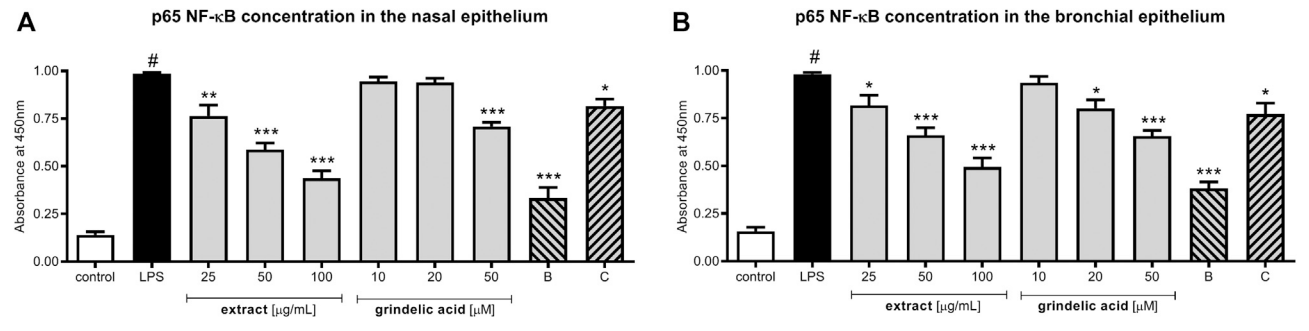

FIGURE 4 | The influence of *G. squarrosa* extract and compounds on p65 NF-κB concentration after LPS stimulation of nasal epithelial cells (HNEpC) and **(B)** bronchial epithelial cells (NHBE).

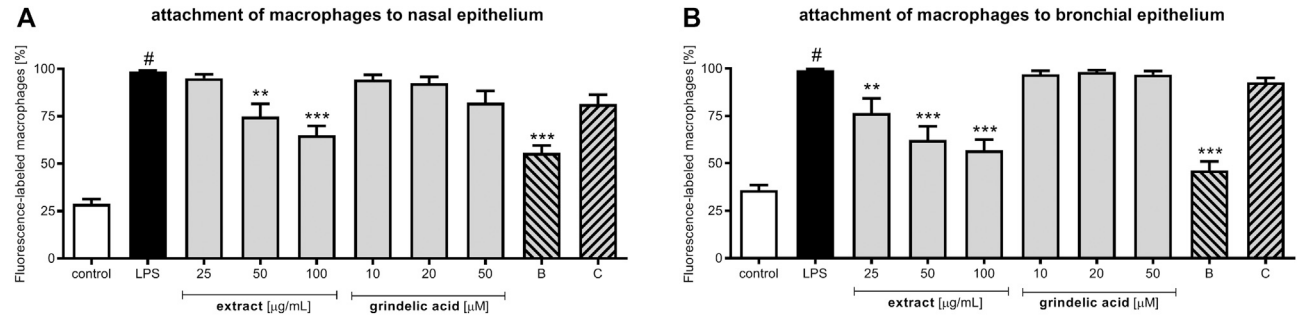

FIGURE 5 | The influence of *G. squarrosa* extract and compounds on adhesion of macrophages to nasal epithelial cells (HNEpC) and bronchial epithelial cells (NHBE).

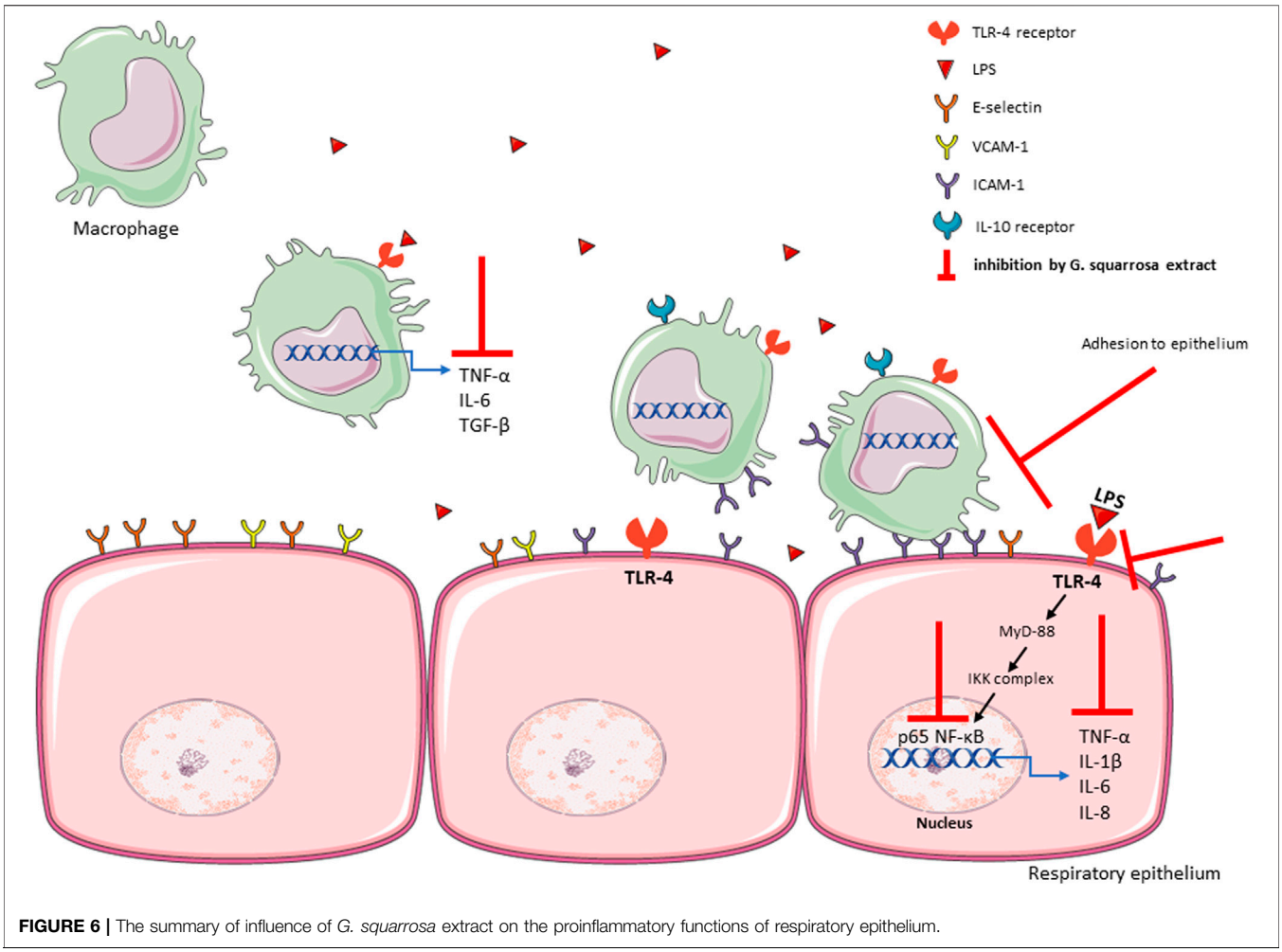

FIGURE 6 | The summary of influence of *G. squarrosa* extract on the proinflammatory functions of respiratory epithelium.

TABLE 4 | Effects of *G. squarrosa* extract and tested compounds on pro- and anti-inflammatory functions of LPS-stimulated monocytes/macrophages: IL-10 receptor expression on the surface of stimulated macrophages; TGF-β secretion by stimulated macrophages [pg/mL]; IL-6 secretion by stimulated macrophages [%]; TNF-α secretion by stimulated macrophages [%]. Data is expressed as the mean ± SEM; three independent experiments assayed in triplicates. Statistical significance $^{a}p < 0.01$ compared to the non-stimulated control; $^{b}p < 0.05$, $^{c}p < 0.01$, $^{d}p < 0.001$ compared to stimulated control (+LPS).

	Surface IL-10 receptor expression [%]	TGF-β release [pg/mL]	IL-6 release [%]	TNF-α release [%]
Control	100.2 ± 21.6	33.7 ± 7.9[a]	34.4 ± 4.2	24.3 ± 5.4
Stimulation (+LPS)	46.4 ± 15.5[a]	8.5 ± 4.8[a]	100.0 ± 2.1[a]	100.4 ± 3.3[a]
G. squarrosa extract + LPS				
25 μg/ml	257.4 ± 61.8[d]	37.5 ± 2.1	64.3 ± 6.2[d]	58.5 ± 2.5[d]
50 μg/ml	476.6 ± 62.4[d]	43.6 ± 4.8	60.2 ± 4.4[d]	55.3 ± 3.8[d]
100 μg/ml	904.3 ± 96.4[d]	58.4 ± 4.4[c]	52.5 ± 2.2[d]	45.2 ± 2.8[d]
Grindelic acid + LPS				
10 μM	13.8 ± 3.6	n.d	87.9 ± 4.7	90.4 ± 3.3
20 μM	16.5 ± 5.5	n.d	84.4 ± 8.2	85.3 ± 2.7
50 μM	18.1 ± 6.2	35.3 ± 9.8	74.3 ± 3.5[c]	76.6 ± 0.9
100 μg/ml				
Budesonide 50 μM	50.0 ± 5.6	40.3 ± 2.1	56.4 ± 3.8[d]	65.9 ± 1.6[d]
Clarithromycin 50 μM	64.4 ± 4.7	n.d	68.4 ± 6.6[c]	78.6 ± 2.1[b]

n.d., not detectable.
[a]p < *0.01 vs. not stimulated cells;*
[b]p < *0.05 vs. LPS-stimulated cells;*
[c]p < *0.01 vs. LPS-stimulated cells;*
[d]p < *0.001 vs. LPS-stimulated cells.*

Interestingly, decrease of p65 concentration was observed also for grindelic acid and clarithromycin, both tested at 50 μM.

G. squarrosa Extract Treatment Reduces Macrophage Adhesion to Epithelium

Stimulation with LPS also results in adhesive molecules expression changes (ICAM-1, VCAM-1 and E-selectin), these then direct the emigration of macrophages across the endothelial to epithelial cells. Mentioned adhesive molecules play a different roles depending on the cells where they are located. On the endothelial cells ICAM-1, VCAM-1 and E-selectin precisely coordinate migration, rolling and diapedesis of leukocytes, however, on the epithelial cells they strictly coordinate adhesion leukocytes to epithelium.

The biological effect of *G. squarrosa* treatment was significant only for ICAM-1 expression (**Table 3**). Comparing to LPS-stimulated control (100%) the plant extract tested at the highest concentration (100 μg/ml) decreased ICAM-1 concentration to 75.3 ± 3.4% in the nasal epithelium, and to 78.2 ± 5.3% in the bronchial epithelium. Similarly, budesonide, tested at 50 μM decreased ICAM-1 expression to 79.4 ± 4.9% and 80.5 ± 3.8%, respectively. Expression of VCAM-1 and E-selectin did not change after treatment.

Continuing, we evaluated influence of extract and compounds on the adhesion of macrophages to both epithelial models (**Figure 5**). As described in the Material and Methods epithelial cells were previously pre-incubated with extract or compounds, then stimulated with LPS and co-incubated with LPS-stimulated macrophages. Because bacterial infection lead to modulation of pro-inflammatory functions both respiratory epithelium as well as leukocytes, the stimulation with LPS of both models seems to be justified. The proposed cellular models reflect the cellular response to bacterial infection properly.

It is worth to emphasize, that bacterial infection resulting very often with inflammation may lead to excessive activation macrophages. Macrophages are very sensitive for a pro-inflammatory cytokine presence in the bloodstream. Very low concentrations of TNF-α or IL-8 significantly stimulate their chemotaxis to damaged tissues. Because they response to recognized chemoattractant immediately (they play a critical role in nonspecific defense (innate immunity)). Thus, there is a risk of their uncontrolled migration and over-accumulation, what increases the need of pharmacological control their adhesion to epithelium.

Our observations, indicate that *G. squarrosa* extract tested at 50 and 100 μg/ml decreased adhesion of macrophages to nasal epithelium to 74.8 ± 6.7% and to 65.0 ± 8.4%, respectively (**Figure 5A**). Budesonide, widely used for a treatment of inflammation-related diseases, decreased adhesion to 55.7 ± 5.7%. Similar tendency we observed for the bronchial epithelial cells co-cultured with macrophages (**Figure 5B**). *G. squarrosa* extract affected the macrophage adhesion to bronchial epithelium much stronger than to nasal epithelium. Tested at concentrations 25, 50, and 100 μg/ml decreased the adhesion to 76.5 ± 9.1, 62.3 ± 7.7, and 56.8 ± 5.1%. Comparing the strong anti-inflammatory activity of budesonide (decreased adhesion of macrophages to bronchial cells to 46.3 ± 8.5%) with anti-inflammatory properties of tested *G. squarrosa* extract and its lack of cytotoxic properties (**Supplementary Table S1**) our findings proved competitiveness of phytotherapy.

G. squarrosa Extract and Grindelic Acid Modulate Pro- and Anti-inflammatory Functions of LPS-Stimulated Macrophages

The *G. squarrosa* extract (at 25–100 μg/ml) significantly upregulated IL-10 receptor expression. Similar effect was observed for grindelic acid tested at 50 μM ($p < 0.05$) (**Table 4**). The extract and grindelic acid significantly promote anti-inflammatory functions of macrophages through stimulation of TGF-β production ($p < 0.001$). *G. squarrosa* extract treatment also resulted in suppression of pro-inflammatory cytokines: TNF-α and IL-6 in dose dependent manner ($p < 0.001$ for all tested concentrations). Budesonide, well known anti-inflammatory drug capable of suppressing cytokine release, at 50 μM inhibited TNF-α and IL-6 to 45.4 and 56.5%, respectively. The activity of clarithromycin was evident but more modest. The tested extract, compounds and solvent (DMSO) were not toxic for mononuclear, as shown by assessing their influence on monocyte/macrophages membrane integrity at the tested concentrations (**Supplementary Table S1**).

DISCUSSION

Aerial parts of *Grindelia* were traditionally used to treat cold-related diseases. Importantly, as liquid extracts and syrups they are still widely distributed at Polish and German markets (Gierlikowska et al., 2020a). The commercial spread of extract for cough and bronchitis treatment increased the need of understanding the biological mechanism of action.

Although the phytochemical composition of *Grindelia squarrosa* is well-known (Veres et al., 2014; Nowak et al., 2019) the biological activity is still not explained. In our previous study we documented anti-inflammatory properties of *G. squarrosa* extract tested on human neutrophilic model (Gierlikowska et al., 2020a). Now, we expand the study and present how *G. squarrosa* regulates pro-inflammatory functions of respiratory epithelium and macrophages, and how modulates their mutual interactions. Our main findings explain how *G. squarrosa* extract modulates molecular mechanisms involved in epithelial response to LPS stimulation, and how it regulates migration and adhesion of macrophages to respiratory epithelium.

The acute episode of a cold starts with virus infection and may be accompanied by bacterial superinfection. *Grindelia* species were shown to exert antimicrobial activity, especially against gram negative *E. coli* (Hassan et al., 2014). According to Hassan et al. (Hassan et al., 2014) the extract of *G. squarrosa* flowers showed inhibition diameters in excess of 19.3 mm of *A. caviae*, *M. luteus* and *P. alvei*. Similar results were obtained by Jacobs et al. (Jacobs et al., 2010). Using different extracts solvents (CCl_4 and 1-BuOH) they confirmed anti-microbial

properties of *G. squarrosa*: CCL_4-extract inhibited *E. coli*, *S. aureus* and *C. albicans* growth, whereas 1-BuOH-extract inhibited *S. aureus*, *P. aeruginosa* and *K. pneumoniae* (Jacobs et al., 2010). In the present study we tested anti-microbial activity of 60%-EtOH extract, and we did not observe any anti-microbial activity. Only clarithromycin, used as positive control, significantly inhibited growth of selected gram positive: *S. aureus* and *S. pneumoniae* (growth inhibition zone, GIZ: 30–34 mm). The water-ethanol extraction, as we performed in present study, reflects the way in which market preparations are obtained. Comparing our results with experiments performed by Hassan (Hassan et al., 2014) and Jacobs (Jacobs et al., 2010) we could hypothesize that different solvents may determine differences in the phytochemical composition and affect on their anti-microbial properties.

The pathogenic infection results in development of full-symptomatic inflammation (Roxas and Jurenka, 2007). Bacterial infection starts with interaction of pathogens with Toll-like receptors located on epithelial cells. To response to gram negative bacteria/LPS, TLR-4 expression increases. To our knowledge, there is no data showing the influence of *G. squarrosa* extract and/or grindelic acid on TLR-4 expression. Using two cellular models (nasal and bronchial epithelium) we observed significant decrease of TLR-4 expression after treatment. Modest activity of grindelic acid may suggest, that anti-inflammatory activity of the extract is not determined be grindelic acid (being the major diterpene), but it is a synergistic effect of all compounds present in the extract.

When nasal and bronchial epithelial cells are exposed to bacterial infection, they secrete a wide spectrum of pro-inflammatory cytokines in a precisely defined sequence (Broekman et al., 2016). At the beginning of infection, low amounts of "early-inflammatory cytokines"—IL-1β and TNF-α are released. Both cytokines intensify production of the "late-inflammatory cytokines"—IL-6 and IL-8 by the respiratory epithelial cells (Broekman et al., 2016), and stimulate chemotaxis of macrophages. It is known that cytokines promote adequate immune response, but prolonged cytokines production by stimulated respiratory epithelial cells may exacerbate inflammation. The side effects arise from excessive macrophage chemotaxis and prolonged stimulation of their pro-inflammatory functions.

Our next finding indicates that *G. squarrosa* extract significantly inhibited IL-1β, TNF-α, IL-6, and IL-8 production by epithelial cells *via* p65 NF-κB suppression. Our observations are coincident with observations performed by La et al. (La et al., 2010). La and al. presented anti-inflammatory properties of *Grindelia robusta* Nutt. tested on monocyte-derived macrophages (U937, human monoblastic leukemia cell line) (La et al., 2010). The authors showed that *G. robusta* extract tested at concentration range 25–100 μg/ml inhibited dose-dependently the secretion of IL-6, MCP-1 and, to a lesser extent, PGE2 and TNF-α. Such inhibition was also observed for MMP-1, -3, -7, -8, -9, and -13 production. The ability of *G. robusta* extract to reduce the LPS-induced secretion of inflammatory mediators and MMPs was associated with a reduction of nuclear factor-kappa B (NF-kB) p65 activation (La et al., 2010). Despite differences in chemical composition of *G. squarrosa* and *G. robusta* it is worth emphasizing that both extracts present ability to suppression of pro-inflammatory cytokine production (La et al., 2010; Gierlikowska et al., 2020a).

Secretion of proinflammatory chemokines and cytokines (interleukins IL-8, IL-1β and TNF-α) induces inflammatory and immune responses (Liu et al., 2016). In respiratory epithelium these effects are associated with the activation of mitogen-activated protein kinases (MAPKs), i.e., p38 kinase, p42/44 extracellular signal-regulated kinase (ERK), and c-Jun NH_2-terminal kinases (JNKs), and are regulated by two major transcription factors families, i.e., NF-κB and AP-1 (Liu et al., 2016; Yang et al., 2017; Zhang et al., 2018). In the available literature there is no data regarding the influence of *G. squarrosa* extract and grindelic acid on MAPK pathway and AP-1. However, based on results obtained for p65 NF-κB we could hypothesize that *G. squarrosa* may modulate p65 NF-κB *via* inhibition of MAPKs activity. The activation of AP-1 is also mediated by MAPKs thus verification of mentioned hypothesis may highlight new targets for a cold-related diseases treatment.

Based on our experiments performed on a two independent epithelial models (nasal and bronchial epithelium), we can speculate that *G. squarrosa*, through strong inhibition of chemoattractant production (IL-1β, TNF-α, IL-6, and IL-8), may potentially limit activation, and migration macrophages to damaged epithelial cells. However, macrophages are very sensitive to cytokines released to bloodstream, thus even small amounts of secreted cytokines may activate adequate immune response.

The migration of macrophages to damaged epithelium is strictly dependent on TNF-α, IL-β, and IL-8 concentration in the bloodstream (Rosseau et al., 2000). Released by damaged epithelium pro-inflammatory cytokines increase expression of VCAM-1, E-selectin, and ICAM-1 (Turner et al., 2011). On the contrary to VCAM-1 and E-selectin, ICAM-1 appears on the epithelium later and is located only at the site of inflammation.

In our study, we tested influence of *G. squarrosa* on the expression of mentioned above adhesive molecules, however, we did not observe influence of extract on VCAM-1 and E-selectin expression. At the highest concentration (100 μg/ml) *G. squarrosa* extract slightly inhibited only ICAM-1 expression. The comparable effect was noticed for budesonide tested at 50 μM. The reduction of ICAM-1 expression may be related with inhibition of cytokine production. As therapeutic target for ICAM-1 suppression were identified IL-8 and TNF-α (Kwon et al., 2011; Kim et al., 2013; Gao et al., 2018). Why extract modulates only adhesion molecule responsible for attachment of leukocytes to epithelium, also remains to be elucidated.

Although, *G. squarrosa* treatment did not affect on epithelial adhesive molecules expression directly, it suppressed the macrophage adhesion to epithelium. At concentration above 50 μg/mL *G squarrosa* significantly inhibited the adhesion of macrophages to both epithelial models. The lowest tested concentration (25 μg/ml) decreased adhesion of macrophages only to bronchial epithelium.

The differences between an affect of *G. squarrosa* treatment on percentage of adhered of macrophages to nasal and bronchial epithelium may be partially explained by epithelial cells anatomical location, predispose to infection/damage and ability to regeneration. The nasal cavity is constantly inhabited by pathogens and is more exposed to mechanical damage (Man et al., 2017). The lower respiratory tract is sterile, thus bronchial epithelial cells must have developed mechanisms that protect not only against pathogens, but also against excessive accumulation of phagocytes. Resseau et al. (Rosseau et al., 2000) explained the stricted regulation of emigration into the alveolar compartment by the difficulties with successful clearance of macrophages from the lung parenchyma. Thus, the observed stronger inhibition of macrophage adhesion to bronchial epithelium then nasal epithelium may be a result of an additive effect of *G. squarrosa* treatment and physiological response of bronchial cell to interactions with macrophages.

At the end, we confirmed that *G. squarrosa* extract stimulate anti-inflammatory functions of macrophages by increase of TGF-β production and IL-10 expression what accelerates the removal of pathogens and damaged tissues (Aggarwal et al., 2014). *G. squarrosa* also reduced concentration of pro-inflammatory cytokines (IL-6 and TNF-α) released by macrophages, successfully reducing inflammation.

In our opinion the biological activity of grindelic acid was a type of cell-dependent. At the highest tested concentration (50 μM) grindelic acid selectively modulated respiratory epithelium functions (TLR-4 expression, cytokine production and p65 NF-κB concentration). Interestingly, grindelic acid did not affect adhesive molecules expression and pro-inflammatory functions of macrophages. According to Leiva-Juárez et al. (Leiva-Juárez et al., 2018) the highly plastic epithelial barrier responds to detected threats *via* modulation of drug accumulation, paracellular flux, intercellular communications, mucin production, and periciliary fluid composition. Epithelial stimulation induces production of cytokines that recruit and sculpt leukocyte-mediated responses, and promotes epithelial generation of antimicrobial effector molecules that are directly microbicidal. Thus epithelium can alternately enhance tolerance to pathogens, preventing tissue damage through induced inhibitory signals and through potential accumulation of compound may attenuate of injurious leukocyte responses (Leiva-Juárez et al., 2018).

Summarizing, our results explain how *G. squarrosa* modulates pro-inflammatory functions of nasal, bronchial epithelium and macrophages, and their mutual interactions (**Figure 6**). We confirmed anti-inflammatory properties of *G. squarrosa* and influence on modulation of leukocyte adhesion to nasal and bronchial epithelial cells. The obtained results allow to better understand the therapeutic efficacy of *G. squarrosa* raw material.

CONCLUSION

The present study justified the traditional use of *Grindelia squarrosa* aerial parts for treatment of cold-related diseases. The wide-spread use of *G. squarrosa* in the traditional medicine and effectiveness during cold-related diseases treatment may be explained by modulation of pro-inflammatory functions respiratory epithelium and macrophages.

The observed suppression of inflammation was a result of "cascade suppressed pro-inflammatory cell response." The *G. squarrosa* suppressed TLR-4 expression, decreased p65 NF-κB concentration and resulted in suppression of TNF-α, IL-1β, IL-6, and IL-8 production. Moreover, we noticed stimulation of anti-inflammatory functions of macrophages and inhibition of their adhesion to nasal and bronchial epithelium. Our observations suggest the high therapeutic potential of *G. squarrosa* extract during symptomatic treatment of cold-related diseases.

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 Committee on Bioethics at the Medical University of Warsaw. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BG designed the study and supervised the work. BG and AK selected plant material and obtained financial support (FW25/PM3/18). DK and AK performed phytochemical analysis. BG, AF, WG, and JS planned and performed the biological experiments and carried out data analysis. BG wrote the manuscript. UD critically reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.534111/full#supplementary-material.

REFERENCES

Aggarwal, N. R., Tsushima, K., Eto, Y., Tripathi, A., Mandke, P., Mock, J. R., et al. (2014). Immunological priming requires regulatory T cells and IL-10-producing macrophages to accelerate resolution from severe lung inflammation. *J. Immunol.* 192 (9), 4453–4464. doi:10.4049/jimmunol.1400146

Bandi, N., and Kompella, U. B. (2001). Budesonide reduces vascular endothelial growth factor secretion and expression in airway (Calu-1) and alveolar (A549) epithelial cells. *Eur. J. Pharmacol.* 425 (2), 109–116. doi:10.1016/s0014-2999(01)01192-x

Broekman, W., Amatngalim, G. D., de Mooij-Eijk, Y., Oostendorp, J., Roelofs, H., Taube, C., et al. (2016). TNF-α and IL-1β-activated human mesenchymal stromal cells increase airway epithelial wound healing *in vitro* via activation of the epidermal growth factor receptor. *Respir. Res.* 17, 3. doi:10.1186/s12931-015-0316-1

Capasso, F., Gaginella, T. S., Grandolini, G., and Izzo, A. A. (2003). *Phytotherapy: a quick reference to herbal medicine*. Berlin, Germany: Springer Science and Business Media.

Dudek, M. K., Michalak, B., Woźniak, M., Czerwińska, M. E., Filipek, A., Granica, S., et al. (2017). Hydroxycinnamoyl derivatives and secoiridoid glycoside derivatives from Syringa vulgaris flowers and their effects on the pro-inflammatory responses of human neutrophils. *Fitoterapia*. 121, 194–205. doi:10.1016/j.fitote.2017.07.008

Efferth, T., and Koch, E. (2011). Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. *Curr. Drug Targets* 12 (1), 122–132. doi:10.2174/138945011793591626

Erwin, A. L., and Smith, A. L. (2007). Nontypeable Haemophilus influenzae: understanding virulence and commensal behavior. *Trends Microbiol.* 15 (8), 355–362. doi:10.1016/j.tim.2007.06.004

Ferreres, F., Grosso, C., Gil-Izquierdo, A., Valentão, P., Azevedo, C., and Andrade, P. B. (2014). HPLC-DAD-ESI/MS(n) analysis of phenolic compounds for quality control of Grindelia robusta Nutt. and bioactivities. *J. Pharmaceut. Biomed. Anal.* 94, 163–172. doi:10.1016/j.jpba.2014.01.046

Gao, H., Zhang, Q., Chen, J., Cooper, D. K. C., Hara, H., Chen, P., et al. (2018). Porcine IL-6, IL-1β, and TNF-α regulate the expression of pro-inflammatory-related genes and tissue factor in human umbilical vein endothelial cells. *Xenotransplantation* 25 (5), e12408. doi:10.1111/xen.12408

Gierlikowska, B., Gierlikowski, W., Bekier, K., Skalicka-Woźniak, K., Czerwińska, M. E., and Kiss, A. K. (2020a). Inula helenium and Grindelia squarrosa as a source of compounds with anti-inflammatory activity in human neutrophils and cultured human respiratory epithelium. *J. Ethnopharmacol.* 249, 112311. doi:10.1016/j.jep.2019.112311

Gierlikowska, B., Gierlikowski, W., and Demkow, U. (2020b). Alantolactone enhances the phagocytic properties of human macrophages and modulates their proinflammatory functions. *Front. Pharmacol.* 11, 1339. doi:10.3389/fphar.2020.01339

Goetz, P. (2005). Plantes médicinales d'Amérique du Nord: apport à la phytothérapie française du traitement des affections des voies respiratoires. *Phytothérapie*. 3 (1), 19–27. doi:10.1007/s10298-005-0060-7

Hassan, H. M., Jiang, Z.-H., Asmussen, C., McDonald, E., and Qin, W. (2014). Antibacterial activity of northern Ontario medicinal plant extracts. *Can. J. Plant Sci.* 94 (2), 417–424. doi:10.4141/cjps2013-258

Jacobs, J., Dixson, J., Gabel, M., Bergmann, D., DeCory, J., and Geffre, C. (2010). Antimicrobial screening on fourteen traditional American Indian medicinal plants. *Proc. S. D. Acad. Sci.* 89, 85–93.

Johnson, L. N. (1887). Grindelia squarrosa. *Science* 10 (242), 152. doi:10.1126/science.ns-10.242.152-a

Kalktenbach, G., and Schimmer, O. (1991). Volatile constituents of the herbs of grindelia robusta and grindelia squarrosa. *Planta Med.* 57 (S2), A82. doi:10.1080/10412905.1993.9698182

Kim, K. H., Park, J. K., Choi, Y. W., Kim, Y. H., Lee, E. N., Lee, J. R., et al. (2013). Hexane extract of aged black garlic reduces cell proliferation and attenuates the expression of ICAM-1 and VCAM-1 in TNF-α-activated human endometrial stromal cells. *Int. J. Mol. Med.* 32 (1), 67–78. doi:10.3892/ijmm.2013.1362

Klugman, K. P., and Feldman, C. (2001). Streptococcus pneumoniae respiratory tract infections. *Curr. Opin. Infect. Dis.* 14 (2), 173–179. doi:10.1097/00001432-200104000-00011

Kwon, D. J., Bae, Y. S., Ju, S. M., Goh, A. R., Choi, S. Y., and Park, J. (2011). Casuarinin suppresses TNF-α-induced ICAM-1 expression via blockade of NF-κB activation in HaCaT cells. *Biochem. Biophys. Res. Commun.* 409 (4), 780–785. doi:10.1016/j.bbrc.2011.05.088

La, V. D., Lazzarin, F., Ricci, D., Fraternale, D., Genovese, S., Epifano, F., et al. (2010). Active principles of Grindelia robusta exert antiinflammatory properties in a macrophage model. *Phytother Res.* 24 (11), 1687–1692. doi:10.1002/ptr.3195

Leiva-Juárez, M. M., Kolls, J. K., and Evans, S. E. (2018). Lung epithelial cells: therapeutically inducible effectors of antimicrobial defense. *Mucosal Immunol.* 11 (1), 21–34. doi:10.1038/mi.2017.71

Liu, Z., Wu, H., Jiang, K., Wang, Y., Zhang, W., Chu, Q., et al. (2016). MAPK-mediated YAP activation controls mechanical-tension-induced pulmonary alveolar regeneration. *Cell Rep.* 16 (7), 1810–1819. doi:10.1016/j.celrep.2016.07.020

Mahmoud, A. A., Ahmed, A. A., Tanaka, T., and Iinuma, M. (2000). Diterpenoid acids from Grindelia nana. *J. Nat. Prod.* 63 (3), 378–380. doi:10.1021/np9904105

Man, W. H., de Steenhuijsen Piters, W. A., and Bogaert, D. (2017). The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat. Rev. Microbiol.* 15 (5), 259–270. doi:10.1038/nrmicro.2017.14

Martin, T. R., Mathison, J. C., Tobias, P. S., Letúrcq, D. J., Moriarty, A. M., Maunder, R. J., et al. (1992). Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs. *J. Clin. Invest.* 90 (6), 2209–2219. doi:10.1172/jci116106

Martin, T. R. (2000). Recognition of bacterial endotoxin in the lungs. *Am. J. Respir. Cell Mol. Biol.* 23 (2), 128–132. doi:10.1165/ajrcmb.23.2.f189

McFarlane, J. (2017). *Fractionation-assisted NMR-based metabolomics of Grindelia squarrosa*. Available at: https://core.ac.uk/download/pdf/84872767.pdf

Michalak, B., Filipek, A., Chomicki, P., Pyza, M., Woźniak, M., Żyżyńska-Granica, B., et al. (2018). Lignans from forsythia x intermedia leaves and flowers attenuate the pro-inflammatory function of leukocytes and their interaction with endothelial cells. *Front. Pharmacol.* 9, 401. doi:10.3389/fphar.2018.00401

Mizgerd, J. P. (2008). Acute lower respiratory tract infection. *N. Engl. J. Med.* 358 (7), 716–727. doi:10.1056/NEJMra074111

Nowak, S., Lisiecki, P., Tomaszczak-Nowak, A., Grudzińska, E., Olszewska, M. A., and Kicel, A. (2019). Chemical composition and antimicrobial activity of the essential oils from flowers and leaves of Grindelia integrifolia DC. *Nat. Prod. Res.* 33 (10), 1535–1540. doi:10.1080/14786419.2017.1423302

Nowak, S., and Rychlińska, I. (2012). Phenolic acids in the flowers and leaves of grindelia robusta Nutt. And grindelia squarrosa dun. (Asteraceae). *Acta Pol. Pharm.* 69 (4), 693–698.

Puneet, P., Moochhala, S., and Bhatia, M. (2005). Chemokines in acute respiratory distress syndrome. *Am. J. Physiol. Lung Cell Mol. Physiol.* 288 (1), L3–L15. doi:10.1152/ajplung.00405.2003

Reta, G. F., Chiaramello, A. I., García, C., León, L. G., Martín, V. S., Padrón, J. M., et al. (2013). Derivatives of grindelic acid: from a non-active natural diterpene to synthetic antitumor derivatives. *Eur. J. Med. Chem.* 67, 28–38. doi:10.1016/j.ejmech.2013.06.013

Rosseau, S., Selhorst, J., Wiechmann, K., Leissner, K., Maus, U., Mayer, K., et al. (2000). Monocyte migration through the alveolar epithelial barrier: adhesion molecule mechanisms and impact of chemokines. *J. Immunol.* 164 (1), 427–435. doi:10.4049/jimmunol.164.1.427

Roxas, M., and Jurenka, J. (2007). Colds and influenza: a review of diagnosis and conventional, botanical, and nutritional considerations. *Alternative Med. Rev.* 12 (1), 25–48.

Spiegler, V., Gierlikowska, B., Saenger, T., Addotey, J. N., Sendker, J., Jose, J., et al. (2020). Root extracts from ononis spinosa inhibit IL-8 release via interactions with toll-like receptor 4 and lipopolysaccharide. *Front. Pharmacol.* 11, 889. doi:10.3389/fphar.2020.00889

Strzelecka, H. (2000). *Encyklopedia zielarstwa i ziołolecznictwa*. Warsaw, Poland: Naukowe PWN.

Timmermann, B. N., Hoffmann, J. J., Jolad, S. D., and Schram, K. H. (1985). Grindelane diterpenoids from grindelia squarrosa and. *G. camporum. Phytochemistry* 24 (5), 1031–1034. doi:10.1016/S0031-9422(00)83176-3

Turner, N. A., Das, A., O'Regan, D. J., Ball, S. G., and Porter, K. E. (2011). Human cardiac fibroblasts express ICAM-1, E-selectin and CXC chemokines in response to proinflammatory cytokine stimulation. *Int. J. Biochem. Cell Biol.* 43 (10), 1450–1458. doi:10.1016/j.biocel.2011.06.008

Veres, K., Roza, O., Laczkó-Zöld, E., and Hohmann, J. (2014). Chemical composition of essential oils of Grindelia squarrosa and *G. hirsutula*. *Nat Prod Commun*. 9 (4), 573–574. doi:10.1177/1934578X1400900438

Watts, J. (2008). Clinical and laboratory standards institute performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standard. Annapolis Junction, MD: National Committee for Clinical Laboratory Standards.

Yang, H. W., Lee, S. A., Shin, J. M., Park, I. H., and Lee, H. M. (2017). Glucocorticoids ameliorate TGF-β1-mediated epithelial-to-mesenchymal transition of airway epithelium through MAPK and Snail/Slug signaling pathways. *Sci. Rep.* 7 (1), 3486. doi:10.1038/s41598-017-02358-z

Zhang, X., Wang, T., Yuan, Z. C., Dai, L. Q., Zeng, N., Wang, H., et al. (2018). Mitochondrial peptides cause proinflammatory responses in the alveolar epithelium via FPR-1, MAPKs, and AKT: a potential mechanism involved in acute lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol.* 315 (5), L775-l786. doi:10.1152/ajplung.00466.2017

Zusso, M., Lunardi, V., Franceschini, D., Pagetta, A., Lo, R., Stifani, S., et al. (2019). Ciprofloxacin and levofloxacin attenuate microglia inflammatory response via TLR4/NF-kB pathway. *J. Neuroinflammation* 16 (1), 148. doi:10.1186/s12974-019-1538-9

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REVIEW
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Artemisia dracunculus (Tarragon): A Review of Its Traditional Uses, Phytochemistry and Pharmacology

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Artemisia dracunculus L. (tarragon), Asteraceae, is a species that has long been used in traditional Asian medicine, mainly in Iran, Pakistan, Azerbaijan and India. It is known as a spice species in Asia, Europe and the Americas. The raw materials obtained from this species are herb and leaf. The presence of essential oil with a highly variable composition, as well as flavonoids, phenolic acids, coumarins and alkamides, determines the medicinal and/or spice properties of the plant. In traditional Asian medicine, this species is used, for example, in the treatment of digestive system diseases, as an analgesic, hypnotic, antiepileptic, anti-inflammatory and antipyretic agent, and as an effective remedy in the treatment of helminthiasis. Nowadays, *A. dracunculus* is the subject of professional phytochemical and pharmacological researches. Pharmacological studies have confirmed its anti-inflammatory and analgesic effects known from traditional uses; they have also proved very important new findings regarding its biological activity, such as antioxidant, immunomodulating and anti-tumour activities, as well as hepatoprotective and hypoglycaemic effects. *A. dracunculus* has long-held an established position in the food industry as a spice. And its use is growing in the cosmetics industry. Moreover, it is the subject of biotechnological research focused mainly on the development of micro-propagation protocols.

Keywords: tarragon, traditional medicine use, chemical composition, biological activity, potential medicinal value, position in cosmetology, biotechnological studies

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INTRODUCTION

Over the last few years, there has been a noticeable increase in interest in phytochemical and pharmacological studies concerning various species of the genus *Artemisia* L (Asteraceae) (Tan et al., 1998; Willcox, 2009; Koul and Taak, 2017). This interest is undoubtedly due to the fact that the Nobel Prize in Physiology or Medicine 2015 was awarded for the discovery in *Artemisia annua* (annual mugwort) of artemisinin–a sesquiterpenoid lactone, and proving its effectiveness in the treatment of malaria (Długońska, 2015; Efferth et al., 2015). Among the subjects of research is *A. dracunculus*, a species native to Siberia and Mongolia. In Europe, this species is a popular spice plant; in Asian countries (Iran, Pakistan, Azerbaijan, India), this species has long been used in traditional medicine. It has been used both in the treatment of gastrointestinal diseases and as an anesthetic, hypnotic and anti-epileptic agent. It has been

recommended as an effective treatment for inflammation, fever and helminthiasis (Aglarova et al., 2008; Bora and Sharma, 2011; Obolskiy et al., 2011).

Contemporary professional research has proven various important aspects of the biological activity of extracts from both the entire aerial part and/or leaves of this species, as well as from its essential oil. Its antibacterial, antifungal and antiprotozoal properties have been documented, together with its extremely valuable antioxidant, immunomodulatory and antineoplastic properties (Abtahi Froushani et al., 2016; Hassanzadeh et al., 2016; Bedini et al., 2017; Navarro-Salcedo et al., 2017; Mohammadi et al., 2020). These studies have also been proven to have hepatoprotective, hypoglycaemic and thyroid regulating effects (Méndez-Del Villar et al., 2016; Zarezade et al., 2018; Mohammadi et al., 2020). An antidepressant effect has also been documented (Wang et al., 2018). The anti-inflammatory and analgesic effects known from applications in traditional medicine have also been confirmed (Abtahi Froushani et al., 2016; Wang et al., 2018; Safari et al., 2019). Moreover, an examination of relevant professional research also shows that the position of *A. dracunculus* as a plant species with cosmetic properties is rising (Ribnicky et al., 2004; Yamada et al., 2011; Chaleshtori et al., 2013). According to modern research, tarragon appears not only to maintain its position as a valuable spice plant, but above all, as an important plant with potential medicinal and cosmetic properties.

The main goal of this review is to present the latest research on both the chemistry and new findings on the biological activity of *A. dracunculus*, proven by professional research. Earlier reviews by Aglarova et al. and Obolskiy et al. (Aglarova et al., 2008; Obolskiy et al., 2011) are quite generalized and don't contain the latest, detailed information on this species, which is valuable in relation to pharmacology, cosmetology and food industries. In addition, the paper encompasses all previously known information concerning its biology and chemistry as well as the traditional medicine and culinary applications of the species under consideration.

GENERAL INFORMATION ON THE SPECIES

The name *Artemisia dracunculus* is derived from the Latin word "dracunculus" meaning "a small dragon", and refers to the shape of the leaves, which resemble dragon tongues (Aglarova et al., 2008). *A. dracunculus* has numerous (about 50) Latin synonyms, including *Absinthium cernuum* Moench, *Achillea dracunculus* Hort., *Artemisia aromatica* A. Nelson, *A. cernua* Nutt., *A. crithmifolia* L., *A. dracunculiformis* Krasch., *A. dracunculina* S. Wats., *A. dracunculoides* Pursh (GBIF.org (2020); The Plant List, 2013; Catalogue of Life, 2020; Missouri Botanical Garden, 2020). The English names include tarragon, estragon, dragon sagewort, dragon wormwood, false tarragon, French tarragon, green sagewort, linear-leaved wormwood, Russian tarragon, silky wormwood, tarragon sagewort. Some of the foreign names are Estragon (Ger.), dragon, estragon (Fr.), dragoncillo, estragão (Sp.), estragão (Port.), dragon, dragon, long hao (Chin.), pelyněk kozalec (Czech.), połyń estragon (Russ.), tárkony üröm (Hung.), and vaistinis kietis (Georg.) (GBIF.org (2020); The Plant List, 2013; Catalogue of Life, 2020; Missouri Botanical Garden, 2020). The raw materials are dried *A. dracunculus* aerial parts and leaves–*Dracunculi herba* and *Dracunculi folium*, with an intense, aromatic fragrance (Food and Drug Administration, 2020).

A. dracunculus is a hairless perennial, reaching a height of up to 150 cm. Its straight stems are ribbed and have no flowers in the lower parts. The leaves are arranged alternately, sessile. The lower leaves are tripartite at the apex, while the middle and upper leaves are lanceolate. The tip of the leaf is sharp and the leaf blade margins entire. Yellow, tubular flowers are gathered in hanging, spherical capitula forming loose panicles. The fruit are achenes. The plant has strong, woody rhizomes, 0.5–1.5 cm thick, from which clusters of small roots grow (Aglarova et al., 2008; Bakova et al., 2017; Koul and Taak, 2017). *A. dracunculus* originates from areas of Siberia and Mongolia (Aglarova et al., 2008). In its natural habitats, this species can be found in Central Asia, in Mediterranean countries, in Eastern Europe and in North America. *A. dracunculus* grows in meadows in alkaline soils, in birch forests, near rivers, on mountain slopes and steppes (Aglarova et al., 2008).

A. dracunculus is a plant widely cultivated in the Americas, Asia and Europe. Two varieties are usually grown on plantations: French tarragon, otherwise known as true tarragon, and German tarragon (*Artemisia dracunculus* var. *sativa*). Russian tarragon is also found among the cultivated plants (miscellaneous varieties, including *A. dracunculus* var. *dracunculoides,* and *A. dracunculus* var. *inodora*). It is not popular, however, because of the poorer taste and less intense fragrance of the leaves (Eisenman et al., 2011; Obolskiy et al., 2011; Watson and Kennel, 2014).

A. dracunculus has low requirements in terms of cultivation site and care, but the highest yields are obtained from crops growing on moist, sandy-clay soils with an alkaline reaction. This species, depending on the cultivar, can be propagated vegetatively or from seed (Russian tarragon), or solely vegetatively from rhizome cuttings (French and German tarragon). It is also possible to use the *in vitro* micropropagation protocols developed for this species–described later in this review. In European conditions, plantations are established in april. The cuttings are placed in rows spaced at 60 cm and covered with a thin layer of soil. The first harvest of the herb takes place in dry weather in the same year, while in the following years two-to-three harvests can be gathered per year. The collected raw material is dried in drying sheds with natural air circulation or heated to 35°C. After drying, the leafy parts of the tarragon plant are separated from the hard stems (Aglarova et al., 2008; Eisenman et al., 2011; Obolskiy et al., 2011; Watson and Kennel, 2014).

The main component of the raw materials, i.e. herb and leaves, is essential oil. The composition of *A. dracunculus* essential oil depends, *inter alia*, on the location of the cultivation site, the salinity of the soil and the age of the plant. The highest concentrations of the essential oil are observed at the beginning of leaf budding and at the beginning of flowering. The main components of the essential oil are: estragole, otherwise

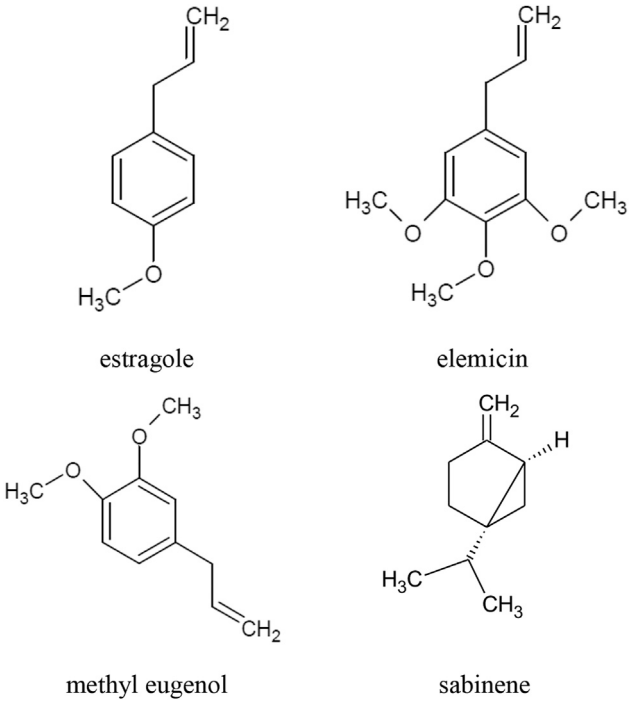

FIGURE 1 | Chemical structure of volatile compounds characteristic of the essential oil of the *A. dracunculus* herb.

known as methyl chavicol or *p*-allylanisole (40–85%), sabinene (approx. 35%), methyl eugenol (approx. 25%), and elemicin (up to 57%) (**Figure 1**). Other compounds present in the oil in concentrations greater than 10% are: terpinen-4-ol, *β*-ocimene, *cis*-ocimene, *α*-trans-ocimene, limonene and *trans*-anethole, *α*-phellandrene, *β*-phellandrene (*Z*)-artemidin, capillene (**Table 1**) (Aglarova et al., 2008; Ayoughi et al., 2011; Obolskiy et al., 2011; Tak et al., 2014; Karimi et al., 2015; Abdollahnejad et al., 2016; Hassanzadeh et al., 2016; Joshi et al., 2016; Bedini et al., 2017; Sharopov et al., 2020). Phenylpropanoids (73.5%) constitute the main group of essential oil compounds. Monoterpenoids (24.3%) and sesquiterpenoids (0.2%) are present in the oil in considerably smaller amounts (Talbi et al., 2016; Bedini et al., 2017).

The tarragon plant contains numerous coumarins, mainly herniarin (**Figure 2**), as well as coumarin, scopoletin, scoparone, dracumerin, artemidine, esculetin, esculin, and capillarin. The total amount of coumarins in the herb is over 1.0%. The biosynthesis of these compounds begins at an early stage of plant development; therefore, in three-month-old specimens, the coumarin content may even reach 1.3%. The maximum level of this group of compounds has been found in five-year-old plants (Aglarova et al., 2008; Bhutia and Valant-vetschera, 2008; Obolskiy et al., 2011; Talbi et al., 2016; Mumivand et al., 2017; Bussmann et al., 2020; Sharopov et al., 2020).

A. dracunculus has also been found to contain flavonoids, the concentration of which in wild plants varies between 0.5 and 1.9%. Under cultivation conditions, a maximum content of 4.9% can be obtained. Flavonoids typical of this species include quercetin, kaempferol, luteolin, isorhamnetin and their glycosides, naringenin, annagenin (5,6,7,8,4′-pentahydroxy-3′-methoxyflavone), pinocembrin and estragonoside C. *A. dracunculus* herb extracts have also been proven to contain phenolic acids, mainly chlorogenic acid, caffeic acid and vanillic acid. Other compounds found in the plant include alkamides (neopellitorine A, neopellitorine B, pellitorine), polyacetylenes, tannins, bitterness-imparting compounds, vitamin C, fatty acids and sterols, iodine compounds, and peroxidase (Aglarova et al., 2008; Mumivand et al., 2017; Jahani et al., 2019; Bussmann et al., 2020).

The chemical composition of *A. dracunculus* and the compounds contained in the plant's essential oil are presented in **Table 2**.

IMPORTANCE IN THE HISTORY OF MEDICINE AND PHARMACY

According to Pliny the Elder (1st c. AD), the name *A. dracunculus* L., a diminutive of the Latin word "draco" – dragon (Gr. δράκων), dracunculus–a small dragon, was given to this plant because of its serpentine rhizomes (Plinii Secundii, 1845). It was supposed to protect against snakebite when carried on one's body or imbibed as a drink, and its juice was used in ear diseases. The name *A. dracunculus* might also be a distorted version of the Arabic name for tarragon, i.e. tharchum, from which the synonyms tarchon, tarcon and torchun are derived (Rejewski, 1996).

The term "dracunculus" was often used by ancient authors, e.g. by Dioscorides (1st c. AD), to refer to another species–*Arum dracunculus* L. (Gr. Drakontaia megale, Δρακονταία μεγάλή), or *Arum maculatum* L. (Gr. Drakontaia mikre, Δρακονταία μικρή) (Pedanius, 1998). *A. dracunculus* L. was commonly called draco, e.g. the botanist and German physician Valerius Cordus (16th c.) uses the name Draco sativus. Due to the similarity of dracunculus leaves to those of flax, it was believed to grow from flax seeds embedded in a hollowed-out onion (*ex semine lini in cepe*), meaning that it did not grow naturally. Authors such as K. Gessner (Bibliotheca Universalis 1545), P. Matthiolus (Commentarii in sex libros Pedacii Dioscoridis Anazarbi de Medicina material 1570) and J. Dalechamps (Historia Generalis Plantarum 1586) did not agree with this view (Bauhin and Cherler, 1651).

P. Matthiolus (1501–1577) describes tarragon (German version Dragoncell, Dracuncellus, Dragoncellus, Dracunculus esculentus) with the following: sharp taste, warming effect, stimulating the appetite (int.), externally applied with saliva, leaves crushed, mixed with honey, smeared causes bruises to disappear (Avicenna calls it "tarcon") (Mattioli, 1586).

In the 17th-century "Herbarium" by Simon Syrennivs (1613), *A. dracunculus* L. bears the Polish name "torchun", besides Dracunculus hortensis, Dragoncellus esculentus and also Draconkraut and Dragoncello. Its leaves are described as "elongated as flax leaves", and the taste as "very peppery" or "spicy". The plant has drying, warming and stimulating properties, relieves toothache, "removes mucus from the head", stimulates digestion and has a diuretic effect. Commonly, tarragon is used in place of lettuce or in salad

TABLE 1 | Chemical composition of *A. dracunculus* essential oil.

Compounds	References
Phenylpropane derivatives	
Estragole (methylchavicol, *p*-allylanisole)	Ayoughi et al. (2011), Obolskiy et al. (2011), Obistioiu et al. (2014), Tak et al. (2014), Karimi et al. (2015), Abdollahnejad et al. (2016), Abtahi Froushani et al. (2016), Behbahani et al. (2017), Osanloo et al. (2017), Bussmann et al. (2020), Sharopov et al. (2020)
Methyl eugenol	Ayoughi et al. (2011), Obolskiy et al. (2011), Obistioiu et al. (2014), Tak et al. (2014), Karimi et al. (2015), Abdollahnejad et al. (2016), Abtahi Froushani et al. (2016), Behbahani et al. (2017), Osanloo et al. (2017), Szczepanik et al. (2018), Bussmann et al. (2020), Sharopov et al. (2020)
Elemicin	Ayoughi et al. (2011), Obolskiy et al. (2011), Szczepanik et al. (2018), Bussmann et al. (2020)
Isoelemycin	Obolskiy et al. (2011), Szczepanik et al. (2018)
Eugenol	Ayoughi et al. (2011), Osanloo et al. (2017), Bussmann et al. (2020)
Isoeugenol methyl ether	Szczepaniket al. (2018), Socaciu et al. (2020)
Asarone, isoeugenol methyl *trans*-anethole	Obolskiy et al. (2011)
(*Z*)-Anethole	Ayoughi et al. (2011), Obolskiy et al. (2011), Bussmann et al. (2020)
Prestragol	Talbi et al. (2016)
Anethole, carpacin	Abdollahnejad et al. (2016)
Dillapiole	Ayoughi et al. (2011), Behbahani et al. (2017)
3-(*p*-Methoxyphenyl)-1,2-propanediol	Güvenalp et al. (2017)
Monoterpenoids	
Sabinene	Aglarova et al. (2008), Ayoughi et al. (2011), Obolskiy et al. (2011), Karimi et al. (2015), Bedini et al. (2017), Behbahani et al. (2017), Osanloo et al. (2017), Bussmann et al. (2020), Sharopov et al. (2020), Socaciu et al. (2020)
cis allo-Ocymen, *cis allo*-ocymen hydrate, *trans*-sabinene acetate, ethyl geranyl	Obolskiy et al. (2011), Bussmann et al. (2020)
α-Fenchene, *cis*-sabinene hydrate, camphor, geranyl acetate, (*E*)-β-ionone	Sharopov et al. (2020)
γ-Terpinene	Ayoughi et al. (2011), Obolskiy et al. (2011), Bedini et al. (2017), Osanloo et al. (2017), Szczepanik et al. (2018), Sharopov et al. (2020), Socaciu et al. (2020)
α-Terpinene	Ayoughi et al. (2011), Obolskiy et al. (2011), Bedini et al. (2017), Osanloo et al. (2017), Szczepanik et al. (2018), Sharopov et al. (2020)
α-Terpineol	Aglarova et al. (2008), Bedini et al. (2017), Szczepanik et al. (2018)
4-Terpineol	Obolskiy et al. (2011), Szczepanik et al. (2018), Sharopov et al. (2020)
Terpinolene	Aglarova et al. (2008), Ayoughi et al. (2011), Obolskiy et al. (2011), Karimi et al. (2015), Bedini et al. (2017), Szczepanik et al. (2018), Bussmann et al. (2020), Sharopov et al. (2020), Socaciu et al. (2020)
α-Terpinolene	Behbahani et al. (2017), Osanloo et al. (2017)
Linalool	Ayoughi et al. (2011), Obolskiy et al. (2011), Bedini et al. (2017), Behbahani et al. (2017), Osanloo et al. (2017), Szczepanik et al. (2018), Sharopov et al. (2020)
Limonene	Aglarova et al. (2008), Ayoughi et al. (2011), Obolskiy et al. (2011), Karimi et al. (2015), Bedini et al. (2017), Behbahani et al. (2017), Osanloo et al. (2017), Szczepanik et al. (2018), Bussmann et al. (2020), Sharopov et al. (2020)
allo-Ocimene	Ayoughi et al. (2011), Obolskiy et al. (2011), Bedini et al. (2017), Behbahani et al. (2017)
trans-allo-Ocimene	Bussmann et al. (2020), Sharopov et al. (2020)
cis-β-Ocimene	Joshi et al. (2016), Sharopov et al. (2020), Socaciu et al. (2020)
trans β-Ocimene	Aglarova et al. (2008), Sharopov et al. (2020), Socaciu et al. (2020)
Citronellol	Obolskiy et al. (2011), Szczepanik et al. (2018), Bussmann et al. (2020)
Citronellol acetate, neryl acetate	Szczepanik et al. (2018), Sharopov et al. (2020)
Citronellol formate, terpineol, α-*trans*-ocimene, β-ocimene	Obolskiy et al. (2011)
(*E*)-β-*o*-cymene *p*-mentha-1,3,8-triene	Szczepanik et al. (2018), Szczepanik et al. (2018)
o-Cymene	Osanloo et al. (2017), Socaciu et al. (2020)
β-Ocimene Y, allocimene, geranial, nerol, β-elemene, tricyclen	Osanloo et al. (2017)
4-Carene, D-limonene, 1,8-cineole, *trans*-4 thujanol	Socaciu et al. (2020)
Carvone	Osanloo et al. (2017), Szczepanik et al. (2018)
Myrcene	Ayoughi et al. (2011), Obolskiy et al. (2011), Bedini et al. (2017), Osanloo et al. (2017), Sharopov et al. (2020)
Phellandrene	Osanloo et al. (2017)
α-Phellandrene	Obolskiy et al. (2011), Behbahani et al. (2017), Szczepanik et al. (2018), Vervandier-Fasseur and Latruffe (2019), Sharopov et al. (2020), Socaciu et al. (2020)
β-Phellandrene	Ayoughi et al. (2011), Obolskiy et al. (2011), Joshi et al. (2016), Sharopov et al. (2020)
α-Thujene	Osanloo et al. (2017), Szczepanik et al. (2018), Bussmann et al. (2020), Sharopov et al. (2020)

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TABLE 1 | (*Continued*) Chemical composition of *A. dracunculus* essential oil.

Compounds	References
α-Pinene	Aglarova et al. (2008), Ayoughi et al. (2011), Obolskiy et al. (2011), Karimi et al. (2015), Abdollahnejad et al. (2016), Bedini et al. (2017), Behbahani et al. (2017), Osanloo et al. (2017), Szczepanik et al. (2018), Bussmann et al. (2020), Sharopov et al. (2020)
β-Pinene	Aglarova et al. (2008), Ayoughi et al. (2011), Karimi et al. (2015), Behbahani et al. (2017), Osanloo et al. (2017), Szczepanik et al. (2018), Sharopov et al. (2020), Socaciu et al. (2020)
Camphene	Aglarova et al. (2008), Ayoughi et al. (2011), Obolskiy et al. (2011), Karimi et al. (2015), Bedini et al. (2017), Osanloo et al. (2017), Bussmann et al. (2020), Sharopov et al. (2020)
p-Cymene	Ayoughi et al. (2011), Szczepanik et al. (2018), Jahani et al. (2019), Bussmann et al. (2020), Sharopov et al. (2020)
E-β-Ocymene, *Z*-β-ocymene	Ayoughi et al. (2011), Obolskiy et al. (2011), Bedini et al. (2017), Behbahani et al. (2017)
neo-allo-Ocymene	Aglarova et al. (2008), Ayoughi et al. (2011)
Thymol	Aglarova et al. (2008), Ayoughi et al. (2011), Szczepanik et al. (2018)
β-Myrcene	Aglarova et al. (2008), Ayoughi et al (2011), Behbahani et al. (2017), Osanloo et al. (2017)
1,8-Cineol, isoterpinolene, artemisinic ketone, isobornyl acetate, pseudolimonene	Bedini et al. (2017)
2-*allo*-cimene, 2-β-pinene, endo-isofenchene, *trans*-carveol, α-fenchene	Abdollahnejad et al. (2016)
Δ3-carene	Ayoughi et al. (2011), Abdollahnejad et al. (2016), Bedini et al. (2017), Szczepanik et al. (2018)
Borneol, *E*-carvone oxide	Ayoughi et al. (2011)
β-Sesquifelandrene	Karimi et al. (2015)
Bornyl acetate	Ayoughi et al. (2011), Karimi et al. (2015), Behbahani et al. (2017), Osanloo et al. (2017), Sharopov et al. (2020)
Geraniol, *p*-pinene, *trans*-ocimene	Bussmann et al. (2020)
Myrtenal, pinocarveol	Aglarova et al. (2008)
Carvacrol, α-terpenyl acetate	Aglarova et al. (2008), Szczepanik et al. (2018)
Sesquiterpenoids	
Spatulenol	Aglarova et al. (2008), Ayoughi et al. (2011), Obolskiy et al. (2011), Karimi et al. (2015), Abdollahnejad et al. (2016), Bedini et al. (2017), Behbahani et al. (2017), Osanloo et al. (2017), Bussmann et al. (2020)
Spathunelol	Osanloo et al. (2017), Szczepanik et al. (2018), Sharopov et al. (2020)
α-Humulene	Ayoughi et al. (2011), Karimi et al. (2015), Osanloo et al. (2017), Sharopov et al. (2020)
Germacrene-D-4-ol, α-himachalene	Obolskiy et al. (2011)
Germacrene D	Obolskiy et al. (2011), Karimi et al. (2015)
Farnesane	Bussmann et al. (2020)
ar-Curcumen, caryophyllene oxide, α-bisabolol, β-bisabolen	Aglarova et al. (2008)
E-Caryophyllene	Karimi et al. (2015), Osanloo et al. (2017)
β-Caryophyllene	Bedini et al. (2017), Sharopov et al. (2020)
Caryophyllene	Szczepanik et al. (2018)
α-Cedrene	Bedini et al. (2017)
Elemene	Abdollahnejad et al. (2016)
E-β-Caryophyllene, (*E,E*)-farnesane, gleenol, α-*epi*-cadinol	Ayoughi et al. (2011)
bicyclermacren, δ-elemene	Ayoughi et al. (2011), Karimi et al. (2015)
α-Zingiberene	Karimi et al. (2015)
E,E-α-Farnesane	Karimi et al. (2015), Osanloo et al. (2017), Sharopov et al. (2020)
α-Bergamotene, acoradiene, germacrene D, *cis*-trans-α-farnesene	Osanloo et al. (2017)
α-Copaene, (*E*)-β-farnesene	Osanloo et al. (2017), Sharopov et al. (2020)
β-Sesquiphellandrene	Osanloo et al. (2017), Sharopov et al. (2020)
Γ-Elemene, *ar*-curcumene, bicyclogermacrene, δ-cadinene	Sharopov et al. (2020)
Diterpenoids	
Phytol	Ayoughi et al. (2011), Karimi et al. (2015)
Triterpenoids	
Squalene	Obolskiy et al. (2011)
Polyacetylenes	
Capillene	Aglarova et al. (2008), Chauhan et al. (2010), Verma et al. (2010), Obolskiy et al. (2011), Joshi et al. (2016)
1-Phenyl-2,4-hexadiene, 1-phenyl-2,4-hexadiene-1-one	Aglarova et al. (2008)
Isocoumarins	
3-(1-*Z*-Butenyl) isocoumarin=(*Z*)-artemidin, 2-(1-*E*-butenyl)-isocoumarin =(*E*)-artemidin	Aglarova et al. (2008), Sharopov et al. (2020)

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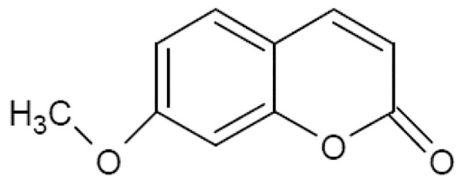
TABLE 1 | (*Continued*) Chemical composition of *A. dracunculus* essential oil.

Compounds	References
Other compounds	
Dehydro-1,8-cineole, myrysticin	Szczepanik et al. (2018), Sharopov et al. (2020)
Apiole	Szczepanik et al. (2018)
Acenaphthene	Joshi et al. (2016), Osanloo et al. (2017)
3-Methoxycinnamaldehyde, *p*-allyphenol, cyclohexylmorpholine, cinnamic acid methyl ester, cinnamyl acetate, nonadecane	Osanloo et al. (2017)
5-Phenyl-1,3-pentadiyne	Joshi et al. (2016)
Hexanal	Socaciu et al. (2020)
1,3-Oktadiene, methyl salicylate, 1-pentadecene	Sharopov et al. (2020)



herniarin

FIGURE 2 | Chemical structure of herniarin–compound characteristic for the *A. dracunculus* herb.

with other "green cabbages", or just with salt. As a spice, it restores the appetite, eaten with salad or meat (Syrennivs, 1613).

In Krzysztof Kluk's plant dictionary ("Dykcjonarz roślinny", 18th c.), the colloquial name "draganek" is given, with the information that it grows in gardens, has lanceolate leaves, tasting "very spicy and pleasant", which strengthen the stomach, "are suitable for salads and seasoning dishes; the vinegar containing these leaves can be very useful on the table" (Kluk, 1805).

In the 18th and 19th centuries, the stimulating tarragon herb was used in Europe as a spice plant rather than a medicinal plant (*Reizend, mehr in der Küche als in der Medizin angewendet*), which is confirmed by pharmacopoeias and dispensatories of that time: Dispensatorium pharmaceuticum Brunsvicense (Brunsvig 1777), P. J. Bergius, Materia Medica e Regno vegetabili (Stockholm 1782), Pharmacopoea Hispanica (Madrid 1798), Pharmacopoea Wirtemberica (Stuttgart 1798), Codex Medicamentarius sive Pharmacopoea Gallica (Paris 1818), V. L. Brera, Riccettario clinico (Padova 1825). The information given in them is as follows: *Artemisia dracunculus* L., Kaisersalat, Dragunbeifuß, Dragonkel, Estragon, Serpentine: southern European plant, grown in gardens; use is made of the herb–Herba Dracunculi esculenti sive hortensis–with thin, narrow, lanceolate, green leaves, which have a weak spicy aroma and a sharp, pungent, slightly spicy flavor (Jourdan, 1829).

The 19th-century Real-Encyclopädie der gesamten Pharmazie (1886), apart from providing a morphological description, states that *A. dracunculus* (Dragun, Bertram) is used as a spice, especially in the form of tarragon vinegar (Acetum Dracunculi) (Vulpius, 1887). The recipe for it can also be found in the Pharmaceutical Encyclopedia by L. Rządkowski: Acetum Dracunculi–Tarragon vinegar: Herbae Dracunculi recentis concisae 100, Aceti Vini 1000, Ac. Salicylici 1 – digest for eight days, squeeze in a wooden press, heat to a temperature not exceeding 100°C, filter after a few days. Pour into small bottles, tightly cork, and store lying down (Rządkowski, 1937).

APPLICATIONS IN TRADITIONAL MEDICINE AROUND THE WORLD

In traditional medicine, *A. dracunculus* is used in ailments of the digestive system, and as an appetite and digestive stimulant, especially when red meat is consumed in large quantities (Uhl and Strauss, 2000). Moreover, the *A. dracunculus* herb was used to accelerate the metabolism (Senderski, 2007). It was also used as an anesthetic for toothache, wounds and cuts (Mamedov et al., 2004). In Europe the plant's uses also included constipation, intestinal cramps, ulcers and cancer (Obolskiy et al., 2011). In Arabic cultures, the species was used in the treatment of insomnia, gingivitis, foot and mouth disease and as an agent for masking the taste of medicines, while in Central Asia, including Russia, it was used to treat irritation, allergic rashes, gastritis, dyspepsia, dermatitis, and to promote digestion and improve appetite (Mamedov et al., 2004; Sharopov et al., 2020). In Azerbaijan, *A. dracunculus* was used as an anti-epileptic drug (Alakbarov, 2001). Indian traditional medicine–Ayurveda–relates that the species is effective in the treatment of helminthiasis, intestinal smooth muscle spasms, fever of various origins, and a good tonic, an immunostimulant and to regulates the menstrual cycle (Miller and Miller, 1998; Obolskiy et al., 2011). Native people of Himachal Pradesh and Kashmir use a paste from the leaves of *A. dracunculus* in the treatment of wounds on the legs of yaks and donkeys. Moreover, they use extract of tarragon for toothache, fever, dysentery, intestinal worms and stomach ache (Joshi et al., 2016).

APPLICATIONS IN MODERN PHYTOTHERAPY AND POSITION IN GLOBAL MEDICINE

A. dracunculus is not a pharmacopeial species. The use of the species in medicine is based only on traditional medicine, but the plant has been a frequent subject of research in many centers

TABLE 2 | Chemical composition of *A. dracunculus*.

Group of compounds	Compounds	References
Flavonoids	2′,4′-Dihydroxy-4-methoxydihydrochalcone syn. DMC-2; 4-O-methyldavidigenin	Obolskiy et al. (2011), Yu et al. (2019), Majdan et al. (2020)
	Quercetin	Obolskiy et al. (2011), Mumivand et al. (2017), Jahani et al. (2019), Bussmann et al. (2020), Majdan et al. (2020)
	Kaempferol	Obolskiy et al. (2011), Bussmann et al. (2020)
	Luteolin	Obolskiy et al. (2011), Mumivand et al. (2017), Jahani et al., 2019, Bussmann et al. (2020)
	Apigenin	Mumivand et al. (2017), Majdan et al. (2020)
	Pinocembrin	Obolskiy et al. (2011), Mumivand et al. (2017), Jahani et al., 2019, Bussmann et al. (2020), Majdan et al. (2020)
	Naringenin	Obolskiy et al. (2011), Mumivand et al. (2017), Majdan et al. (2020)
	3,5,4-Trihydroxy-7,3′-dimethoxyflavone,	Obolskiy et al. (2011)
	3,5,4′-trihydroxy-7-methoxyflavone,	
	5,6,7,8,4′-pentahydroxymetoflavone,	
	5,7-dihydroxy flavone,	
	7-*O*-*β*-*D*-glycopyranoside,	
	5,7-dihydroxflavone,	
	7-*O*-*β*-*D*-glucopyranoside anangenin estragonizide	
	Davidigenin	Obolskiy et al. (2011), Yu et al. (2019), Majdan et al. (2020)
	Sacuranetine	
	Rutoside	Güvenalp et al. (2017)
	Quercetin 3-*O*-rutinoside	Ribeiro et al. (2016)
	Isoquercitrin	Majdan et al. (2020)
	Patuletin 3-*O*-malonylrobinobioside	
	Patuletin hexoside	
	Patuletin rhamnosylhexoside	
	Patuletin malonylrhamnosylhexoside	
	Vicenin	
	7,3′-Dimethyleriodictyol	Bhutia and Valant-vetschera (2008)
	7-Methyleriodictyol	
	7-Methylaringenine	
	Biocovertsetin	Bussmann et al. (2020)
	Hyperoside	
	Rutoside	
	Estroside	Aglarova et al. (2008)
	Kaempferol glycosides	
	Quercetin glycosides	
	Luteolin glycosides	
	Isorhamnetin glycosides	Aglarova et al. (2008), Majdan et al. (2020)
Coumarins	Herniarin	Aglarova et al. (2008), Güvenalp et al. (2017), Mumivand et al. (2017), Osanloo et al., 2017, Jahani et al., 2019, Aydin et al., 2020, Bussmann et al. (2020)
	3,4-Dehydroherniarin, skimmin,	Aydin et al. (2020)
	(-)-(*R*)-20-methoxydihydro-artemidine,	Obolskiy et al. (2011)
	(+)-(*R*)-(*E*)-3′-hydroxyartemidine,	
	(+)-(*S*,*R*)-epoxyartemidine,	
	4-hydroxycoumarin,	
	7,8-methylenedioxy-6-methoxycoumarin,	
	8-hydroksyartemidin artemidiol artemidynal ether,	
	7-methyl daphnetin ether methylenedaphnetin,	
	isovalerate capillarin,	
	γ,γ-dimethylallyl ether esculetin	
	6-Demethoxycapilarisine	Obolskiy et al. (2011), Majdan et al. (2020)
	Dacumerin	Bhutia and Valant-vetschera (2008), Obolskiy et al. (2011)
	Scoparon	
	Scopoletin	Obolskiy et al. (2011), Bussmann et al. (2020)
	Arethinol	Bussmann et al. (2020)
	Aridiodiol	
	Artidin	
	Isocoumarin	
	9-Hydroxyartemidine, esculetin	Aglarova et al. (2008)
	8-Hydroxycapillarin, artemidinol	Aglarova et al. (2008), Obolskiy et al. (2011)
	Esculin	
	Capillarin	
	Artemidine	Aglarova et al. (2008), Bhutia and Valant-vetschera, 2008, Obolskiy et al. (2011)
	Coumarin	Aglarova et al. (2008), Jahani et al. (2019)

(Continued on following page)

TABLE 2 | (*Continued*) Chemical composition of *A. dracunculus*.

Group of compounds	Compounds	References
Phenolic acids	(*E*) 2-Hydroxy-4-methoxycinnamic acid, 4,5-di-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid hydroxybenzoic acid, 3,5-*O*-dicaffeoylquinic acid, *p*-coumaroyl-feruloylquinic acid, *p*-coumaroyl-caffeoylquinic acid,	Obolskiy et al. (2011)
	4,5-di-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid,	Obolskiy et al. (2011), Ribeiro et al. (2016)
	Ferulic acid hexoside	Ribeiro et al. (2016), Majdan et al. (2020)
	Caffeoylquinic acid, sakuranetin	Majdan et al. (2020)
	Chicory acid	Obolskiy et al. (2011), Mumivand et al. (2017)
	Caffeic acid, chlorogenic acid	Obolskiy et al. (2011), Mumivand et al. (2017), Jahani et al. (2019), Bussmann et al. (2020), Majdan et al. (2020)
	p-Coumaric acid	Mumivand et al. (2017)
	Ferulic acid, syringic acid	Mumivand et al. (2017), Jahani et al. (2019)
	Vanillic acid	Mumivand et al. (2017), Jahani et al., 2019, Bussmann et al. (2020)
	2-Methoxycinnamic acid	Abdollahnejad et al. (2016)
Alkamides	Neopelitorin A, neopelitorin B, pelitoryin	Obolskiy et al. (2011), Majdan et al. (2020)
Sterols	Stigmasterol	Aydin et al. (2020)
Fatty acids	Myristic acid, oleic acid, palmitic acid	Obolskiy et al. (2011)
Vitamins	Vitamin C	Aglarova et al. (2008), Obolskiy et al. (2011)
Minerals	Iodine compounds	Mohammadi et al. (2020)
Enzymes	Peroxidases	Aglarova et al. (2008)
Tannins	No data	Bussmann et al. (2020)
Other compounds	1-Methoxy-4-(2-propenyl) benzene, 3,7-dimethyl-1,3,7-octatriene	Obolskiy et al. (2011)
	4-(1′,1′,2′,2′-Tetramethylpropyl)-1,2-benzenediol	Güvenalp et al. (2017)
	Phytoalexin	Talbi et al. (2016)
	Benzyl benzoate, methyl salicylate, trimethoxyallylbenzene	Aglarova et al. (2008)
	1,9,2-Octalone, 7-methoxy-1-indanone, cinnamic aldehyde, simetyloacetal, ociminon acetate, 3- methylbenzyl	Abdollahnejad et al. (2016)
	Anisaldehyde	Abdollahnejad et al. (2016), Osanloo et al. (2017)
	γ-Decalactone	Ayoughi et al. (2011), Karimi et al. (2015)
	Cuminic aldehyde, 2(3H)-furanone,5-hexyldihydro-benzen, ethanol, α-2-propenyl-methyl cinnamate	Osanloo et al. (2017)
		Karimi et al. (2015), Behbahani et al. (2017)
	(2*E*,4*E*)-1-(Piperidin-1-yl)undeca-2,4-diene-8,10-diyn-1-one, (2*E*,4*E*)-*N*-isobutyl undeca-2,4-dien-8,10-diynamide	Aydın et al. (2019), Aydin et al. (2020)

around the world, especially in Iran. New findings on the biological activity of extracts from the herb, leaves, and essential oil of this species–proven by scientific research conducted over the last 10 years–are presented below. The partially known mechanisms of action of *A. dracunculus* are presented in **Table 3**.

BIOLOGICAL ACTIVITY CONFIRMED BY SCIENTIFIC RESEARCH

Antibacterial and Antifungal Activities

Abdollahnejad et al. conducted a comparative study of the antibacterial potential of *A. dracunculus* herb oil obtained from two different methods: steam distillation and experimentally modified steam distillation. The experiment was carried out using the disk diffusion method and the microdilution method against *Staphylococcus aureus, S. epidermidis, Micrococcus luteus, Bacillus subtilis, B. cereus, Listeria monocytogenes, Streptococcus pyogenes, S. typhimurium, Escherichia coli, Klebsiella pneumoniae, Shigella flexneri, S. marcescens, Pseudomonas aeruginosa* and *Salmonella* spp. All these bacteria were found to be sensitive to the essential oil of *A. dracunculus*, with *S. epidermidis* showing the largest zone of inhibition (21.5 mm). The MIC value for Gram-positive bacteria did not differ significantly between oils obtained from the different methods, but a significantly lower MIC (minimum inhibitory concentration) value for Gram-negative bacteria was recorded for oil obtained with the modified steam distillation method (Abdollahnejad et al., 2016).

Two years later, a research team from the same facility conducted another experiment confirming the antibacterial activity of *A. dracunculus* oil against *Staphylococcus aureus, Klebsiella* spp., *Salmonella typhimurium, Staphylococcus epidermidis, Proteus* spp. and *Corynebacterium diphtheriae*. The essential oil was tested with agar well diffusion. A significant inhibitory effect on the growth of *S. aureus, Proteus* spp. and *C. diphtheriae* bacterial strains was demonstrated. The MIC value for these bacteria was determined using the essential oil at a concentration of 0.03 and 25 mg/ml (Tajbakhsh and Soleimani, 2018).

Another study evaluating the antibacterial activity of *A. dracunculus* essential oil was conducted in 2020 by Socaciu et al. The experiment was aimed at assessment of the usability

of the oil in antibacterial edible films. Bacteriostatic and bactericidal activities were evaluated with the Kirby-Bauer disk diffusion test, the minimum inhibitory concentration test (MIC) and the minimal bactericidal concentration (MBC) test. The results of the first test revealed the greatest inhibition of the growth of *Salmonella enteritidis* than *Staphylococcus aureus, Escherichia coli* and *Listeria monocytogenes.* The MIC and MBC tests displayed the highest bacteriostatic and bactericidal activity against *Escherichia coli* (5.14 MIC; 5.14 MBC) whereas in *Listeria monocytogenes* the bactericidal effect was poorer (5.14 MIC; 10.80 MBC) and lower values of MIC and MBC tests were obtained in *Salmonella enteritidis* (10.80 MIC; 10.80 MBC), and *Staphylococcus aureus* (10.80 MIC; 22.68 MBC) strains (Socaciu et al., 2020).

In 2016, conducted a study evaluating the antibacterial and antifungal activity of hydro-ethanolic extract of *A. dracunculus.* MIC and MBC tests along with the minimum fungicidal concentration test (MFC) were carried out to assess the antimicrobial activity of the extract. The experiment determined significant bactericidal activity of the extract and inhibition of the growth of *Staphylococcus aureus,* methicillin resistant (*MRSA*) *Bacillus cereus, Micrococcus flavus, Listeria monocytogenes, Pseudomonas aeruginosa,* antibiotic resistant (A.R) *Pseudomonas eruginosa, Salmonella typhimurium, Escherichia coli,* A.R. *Escherichia coli and Enterobacter cloacae* strains. *Bacillus cereus* being the most sensitive to the influence of hydro-ethanolic extract of tarragon (0.02 MIC; 0.08 MBC) followed by A.R *Pseudomonas aeruginosa* (0.04 MIC; 0.08 MBC) and *Enterobacter cloacae* (0.04 MIC; 0.08 MBC). Moreover, application of the extract in fungal colonies confirmed a notable decrement of the growth of the colonies and the fungicidal effect against *Aspergillus fumigatus, Aspergillus versicolor, Aspergillus ochraceus, Aspergillus niger, Trichoderma viride, Penicillum funiculosum, Penicillium ochrochloron, Penicillium verrucosum.* The MIC test results didn't differ significantly in different colonies. Interestingly, *Aspergillus versicolor and Aspergillus niger* had lower responses in the MFC test (0.16) compared with the remaining fungi species (0.08) (Ribeiro et al., 2016).

The effect of hydro-ethanolic extract of *A. dracunculus* leaves on *C. albicans* infection was investigated. The experiment was carried out on an animal model (mouse). The rodents were treated intraperitoneally with the plant extract in doses of 50, 100, 200 mg/kg, then they were infected with 0.2 ml of a suspension at a concentration of 10^5 colony-forming units per millilitre (CFU/ml). After sacrificing the animals, the concentration of the pathogen in liver and kidney homogenates was determined. It was found that the growth of *C. albicans* was significantly inhibited. For the maximum dose of the extract – 200 mg/kg, the concentration of the pathogen in the liver was 16.08 colony-forming units per gram of test material (CFU/g), and no traces of its presence were found in the kidneys. The amount in the control was 36.28 CFU/g and 53.31 CFU/g, respectively (Zarasvand et al., 2016).

In the other study, used the disk diffusion method, the pour-plate method and the dilution method to investigate the antibacterial and antifungal activities of a spice produced from *A. dracunculus* against strains of the bacteria: *Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, B. subtilis, Staphylococcus aureus, Streptococcus pyogenes,* and the fungi: *Aspergillus fumigatus, Penicillium expansum* and *Candida albicans*. At a concentration of 20 mg/ml, a hydro-ethanolic extract from the spice showed antimicrobial activity in the disk diffusion method. The largest zone of growth inhibition was observed for *S. pyogenes* (18 mm), and the smallest for *P. aeruginosa* (9 mm). In the pour-plate method, the extract at a concentration of 2 mg/ml was effective against *S. pyogenes, S. aureus, B. subtilis, B. cereus, C. albicans* and *A. fumigatus*. The extract showed antibacterial activity, but did not completely inhibit the growth of *E. coli* and *P. expansum*, and was ineffective against *P. vulgaris* and *P. aeruginosa*. In the microdilution method, the MIC value of the tarragon extract ranged from 2 to 32 mg/ml (Behbahani et al., 2017).

Majdan et al. investigated the antibacterial effects of aqueous extract of *A. dracunculus*. The study was conducted to evaluate the antibacterial activity of an infusion of aerial parts of tarragon against Gram-positive bacteria: *Staphylococcus aureus*, *S. epidermis, Corynebacterium diphtheriae, Enterococcus hirae* and Gram-negative bacteria: *Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris* and *Helicobacter pylori* colonies. The values of concentrations of the extracts used in the assay ranged from 0.004 to 94.000 mg/ml (mg-relates to dry extract of *A. dracunculus* and mL relates to sterile distilled water). The results demonstrated that tarragon infusion was particularly effective against *Staphylococcus aureus* (MIC 0.09 mg/ml), to a lesser extent it also impeded growth of *Staphylococcus epidermis* (MIC 0.363 mg/ml), *Corynebacterium diphtheriae* (MIC 5.9 mg/ml) colonies, *Staphylococcus aureus MRSA* (MIC 2.35 mg/ml) and *Helicobacter pylori* (MIC 11.75 mg/ml) colonies. On the contrary, minimal antimicrobial activity was displayed in *Klebsiella pneumoniae* (MIC 47 mg/mL) and *Enterococcus hirae* (MIC 23.5 mg/ml). Notably, *Escherichia coli* and *Proteus vulgaris* strains turned out to be invulnerable to the antimicrobial activity of the infusion (Majdan et al., 2020).

Antiprotozoal Activity

Iranian researchers have investigated the potential of hydro-ethanolic extract of *A. dracunculus* in the treatment of leishmaniasis. They tested the effectiveness of various concentrations of the extract (100–1000 μg/ml) by applying them to the promastigote forms of *Leishmania major* grown *in vitro*. The recorded MIC values of the extract after 24, 48 and 72 h were: 962.03, 688.36 and 585.51 μg/ml, respectively, which indicates that the plant extract can be used in the treatment of leishmaniasis (Mirzaei et al., 2016).

Antioxidant Effect

The antioxidant potential of *A. dracunculus* was assessed. For this purpose, fresh tarragon, purchased from the local market, was subjected to extraction with water and ethanol. The extract underwent a DPPH test and was used to determine total amounts of phenols and flavonoids by the spectrophotometric method. The estimated total phenolic content was 24.1 mg/g dry weight (as gallic acid eq.) and the total flavonoid content was

TABLE 3 | Pharmacological properties of *A. dracunculus*.

Activity	Mechanism of action	References
Anti-bacterial and anti-fungal	Inhibition of the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, Bacillus subtilis, Bacillus cereus, Listeria monocytogenes, Streptococcus pyogenes, Streptococcus typhimurium, Escherichia coli, Klebsiella pneumoniae, Shigella flexneri, Shigella marcescens, Pseudomonas aeruginosa* and *Salmonella* sp. under the influence of the essential oil of the *A. dracunculus* herb	Abdollahnejad et al. (2016)
	Staphylococcus aureus, Proteus spp. and *Corynebacterium diphtheriae* colony growth inhibition after application of the essential oil	Tajbakhsh and Soleimani (2018)
	essential oil of *A. dracunculus* leaves hampers the growth of *Escherichia coli, Listeria monocytogenes, Salmonella enteritidis* and *Staphylococcus aureus* strains	Socaciu et al. (2020)
	Staphylococcus aureus, Staphylococcus aureus MRSA (methicillin resistant)*, Bacillus cereus, Micrococcus flavus, Listeria monocytogenes, Pseudomonas eruginosa, A.R Pseudomonas eruginosa, Salmonella typhimurium, Escherichia coli, A.R Escherichia coli, Enterobacter cloacae* colonies growth inhibition and bactericidal effect as well as inhibition of the growth of *Aspergillus fumigatus, Aspergillus versicolor, Aspergillus ochraceus, Aspergillus niger, Trichoderma viride, Penicillum funiculosum, Penicillium ochrochloron, Penicillium verrucosum* and fungicidal activity under the influence of hydro-ethanolic extract of tarragon	Ribeiro et al. (2016)
	hydro-ethanolic extract of *A. dracunculus* leaves significantly reduces the number of colony-forming units (CFU) of *Candida albicans* in the liver and kidneys of mice	Zarasvand et al. (2016)
	Inhibition of the growth of bacteria: *Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes,* and fungi: *Aspergillus fumigatus, Penicillium expansum* and *Candida albicans*, under the influence of hydro-ethanolic herbal extract	Behbahani et al. (2017)
	Inhibition of the growth of bacteria: *Staphylococcus aureus*, *Staphylococcus epidermis, Staphylococcus aureus MRSA, Corynebacterium diphtheriae* and *Helicobacter pylori* after the application of infusion of *A. dracunculus* and minimal inhibition effect in *Enterococcus hirae, Klebsiella pneumoniae colonies*	Majdan et al. (2020)
Anti-protozoal	Inhibition of the development of the promastigote form of *Leishmania major*	Mirzaei et al. (2016)
Antioxidant	Reducing properties of the hydro-ethanolic herbal extract related to the presence of phenolic compounds and flavonoids	Behbahani et al. (2017), Ribeiro et al. (2016), Mumivand et al. (2017)
	Reduction of DPPH and ABTS in the presence of phenolic compounds	Zarezade et al. (2018)
Anti-inflammatory and analgesic	Reduction of pain sensations and reduction of xylene-induced ear edema after administration of the ethanolic herbal extract to mice	Eidi et al. (2016)
	Inhibition of ROS, IL-8 and TNF-α production in imitated inflammation	Majdan et al. (2020)
Immuno-modulating	Reduction in IL-17 and IFN-γ production and intensification of the phagocytosis process carried out by macrophages	Abtahi Froushani et al. (2016)
	Lowering of IL-17 and IL-23 levels and reduces the infiltration of leukocytes into brain cells	Safari et al. (2019)
	Increased neutrophil levels and decreased lymphocyte levels after intraperitoneal administration of the hydro-ethanolic extract from the leaves	Modaresi et al. (2018)
Anti-depressive	Increased resistance to stressful situations and reduction of stress-related levels of inflammatory cytokines	Wang et al. (2018)
	The phenolic compounds and flavonoids contained in the *A. dracunculus* herb reduce the immobility response time in mice in the writhing test and in the forced swim test	Jahani et al. (2019)
	Mild inhibition of hMAO-A and hMAO-B by extracts of *A. dracunculus*	Aydin et al. (2020)
Anti-tumor	Inhibition of proliferation of mouse lymphoma cells (L5178YD) due to the presence of polyphenols and alkamides in leaf extracts	Navarro-Salcedo et al. (2017)
Hepato-protective	Decrease in levels of alanine transaminase, aspartate transaminase, alkaline phosphatase and total bilirubin, and increase in total protein levels	Zarezade et al. (2018)
Hypo-glycaemic	Decrease in glycated hemoglobin, decrease in area under curve for insulin, decrease in total insulin secretion, decrease in systolic blood pressure, and increase in HDL-C	Méndez-Del Villar et al. (2016)
Normalizing the profile of thyroid hormones	Increase in thyroxine and triiodothyronine levels, decrease in elevated levels of thyrotropin, and increase in total antioxidant capacity, increase in glutathione, and decrease in malondialdehyde levels	Mohammadi et al. (2020)
Inhibiting the activity of carbonic anhydrase I and II	Compounds contained in herbal extracts reduce the activity of carbonic anhydrase I and II.	Aydın et al. (2019)

(Continued on following page)

TABLE 3 | (*Continued*) Pharmacological properties of *A. dracunculus*.

Activity	Mechanism of action	References
Repelling insects	Inhibition of *Calliphora vomitoria* egg laying on fresh beef, on which the essential oil of *A. dracunculus* herb was applied	Bedini et al. (2017)
	Larvacidal effect against *Anopheles stephensi* under the influence of nanoemulsion of *A. dracunculus* essential oil	Osanloo et al. (2017)

20 mg/g dry weight (as quercetin eq.). In the DPPH test, the IC_{50} value was 65.5 μg/ml, which confirms that *A. dracunculus* extracts can produce antioxidant effects (Behbahani et al., 2017).

The antioxidant activity has also been confirmed by other study where performed DPPH and ABTS tests and assessed the total phenolic content, thus verifying the activity of the hydro-ethanolic extract from the *A. dracunculus* herb. The total amount of phenols, expressed as gallic acid equivalents, was 197.22 mg/g dry weight. High activity of the extract was also demonstrated in the DPPH and ABTS tests (Zarezade et al., 2018).

The antioxidant activity of hydro-ethanolic extract of tarragon was also evaluated. First, Ribeiro et al. analyzed the phenolic content of the extract with HPLC. The results showed 147.5 mg of the phenolic content in 1g of the dried product including approximately 31.9 mg/g of flavonoids and 115.6 mg/g of phenolic acids. Then the team used different methods of assessing the antioxidant activity: the DPPH test examining radical scavenging activity, the β-carotene/linoleate test, reducing power was measured by the capacity to convert Fe^{3+} to Fe^{2+} and the TBARS assay evaluating the level of lipid peroxidation. Ribeiro et al. used ascorbic acid as a positive control. The results displayed that the extract is most effective as a reducing agent (Ribeiro et al., 2016).

In 2017, the researches from Iran and USA together also carried out DPPH tests, in addition to the ferric reducing antioxidant power (FRAP) test. For the experiment, hydro-methanolic extracts were used, which were prepared from the herb of *A. dracunculus* collected from various parts of Iran. The results showed that the antioxidant potential depended on the region from which the harvested plants originated. It was positively correlated with the concentration of compounds with phenolic and flavonoid structures. The highest reducing capacity was proven for extracts from the *A. dracunculus* herb collected in Birjand (a city in eastern Iran) – in the DPPH test the IC_{50} value was 0.039 mg/ml; in the FRAP test the extract was reduced 148.29 μmol Fe^{2+}/g dry weight. The total amount of phenols calculated as gallic acid equivalents was 96.52 mg/g dry weight, and the total amount of flavonoids calculated as rutoside equivalents was 50.4 mg/g dry weight (Mumivand et al., 2017).

Anti-inflammatory and Analgesic Effects

The Iranian research centers have investigated the antinociceptive and anti-inflammatory potential of an ethanolic extract from the *A. dracunculus* herb. The potential analgesic effect was verified in an animal model (mouse) using the hot plate test, the writhing test and the formalin test. Anti-inflammatory activity was assessed in a xylene-induced ear edema model. The study group received intraperitoneally the herbal ethanolic extract in doses of 5, 10, 50, or 100 mg/kg BW (body weight), while the control group was intraperitoneally administered a saline solution. Reduction in pain sensation was observed in all three tests. In the hot plate test, the extract administered in doses of 50 and 100 mg/kg increased the pain threshold after one hour. Interaction with opioid receptors may be responsible for the analgesic effect of the plant extract, as administration of naloxone to the animals reduced the antinociceptive effect of the extract. Anti-inflammatory activity has also been confirmed a significant reduction in ear edema was demonstrated with the extract administered in doses of 50 and 100 mg/kg (Eidi et al., 2016).

A study carried out by Majdan et al. mentioned above, evaluated the anti-inflammatory activity of aqueous extract of *A. dracunculus*. In the experiment researchers used neutrophils derived from venous peripheral blood from healthy human donors. Thereafter, to assess the secretion of cytokines after the incitement of neutrophils, an enzyme-linked immunosorbent assay (ELISA) was used. The application of aqueous extract of tarragon produced a decrement of the release of IL-8 (by 4.0 and 4.8%) and TNFα (by 7.8 and 5.2%). Moreover, ROS production was also measured. It was evaluated by microplate reader which displayed an inhibition of ROS production by 1.4% (Majdan et al., 2020).

Immunomodulatory Action

An experiment was conducted on laboratory animals (mice) to evaluate the immunomodulatory properties of an aqueous extract of *A. dracunculus* herb. The mice were immunized intraperitoneally with sheep erythrocytes then orally administered an aqueous extract of the *A. dracunculus* herb. An increase in the level of antibodies to sheep erythrocytes and a decrease in cellular immunity were documented. The treatment was also shown to reduce the production of pro-inflammatory agents–IL-17 and IFN-γ, and to increase the phagocytic potential of macrophages. The authors of the study concluded that an aqueous extract of the *A. dracunculus* herb could be a good immunomodulating agent; moreover, it was free from potentially harmful estragole and methyl eugenol (Abtahi Froushani et al., 2016).

The studies on the potential use of aqueous extract of *A. dracunculus* in the treatment of multiple sclerosis were performed, too. The experiments were carried out on mice in which autoimmune encephalomyelitis (EAE) was induced with the myelin oligodendrocyte glycoprotein. This model is an experimental animal model for multiple sclerosis. It has been proven that giving rodents aqueous extract of *A. dracunculus*

significantly alleviates the symptoms of the disease. By using the iron (III) reduction method, an increase in the antioxidant potential was verified. The use of the extract also reduced the level of inflammatory cytokines (IL-17 and IL-23) and the infiltration of leukocytes into brain cells. The results of the study indicate that the compounds contained in *A. dracunculus* can potentially be used in the treatment of multiple sclerosis (Safari et al., 2019).

Studies of Modaresi et al. determined the effect of hydro-ethanolic extract from *A. dracunculus* leaves on the hematological parameters of mice. The parameters assessed were levels of leukocytes, erythrocytes, lymphocytes, monocytes and neutrophils. It was demonstrated that intraperitoneal administration of the extract at a dose of 200 mg/kg significantly increased the level of neutrophils in the blood of rodents and reduced lymphocyte levels. There were no significant effects on the number of leukocytes, red blood cells or monocytes (Modaresi et al., 2018).

Antidepressant Effect

Ethanolic extract of *A. dracunculus* was tested for its potential to increase mental resilience. The study was conducted on mice administered orally with extract of *A. dracunculus* shoots. A model of depression with repetitive stress caused by fear of social failure was tested by leaving the rodents in a cage with an aggressive individual for 10 min and checking their tendency to avoid contact. The treatment was shown to increase resistance to depression and to reduce the level of inflammatory cytokines associated with the presence of stress (Wang et al., 2018).

An experiment conducted in 2019 has confirmed the antidepressant activity of the species. Harvested herb of *A. dracunculus* was subjected to extraction with ethanol, then the activity of the extract was assessed on animals (NMRI mice and Swiss mice), by performing the forced swim test, the writhing test, and the open-field test. The results of the study showed a reduction in immobility time in the forced swim test (for the extract dose of 400 mg/kg the immobility time was 153.6 s, and was shorter compared to the control group, in which the immobility time was 202.3 s), a reduction in immobility time in the writhing test (for the extract dose of 200 mg/kg the period of immobility was 117.2 s, and was shorter compared to the control group, in which the immobility time was 142.6 s). In the open-field test, the rodents' mobility did not change significantly, except for the trial with Swiss mice, which were administered 100 mg/kg of *A. dracunculus* herb extract.

The authors of the study associate the plant's antidepressant activity with the presence of phenolic and flavonoid compounds, such as chlorogenic acid, caffeic acid or luteolin and quercetin (Jahani et al., 2019).

In 2020 scientists from Turkey carried out an experiment evaluating the influence of *A. dracunculus* extracts on human monoamine oxidase A (hMAO-A) and monoamine oxidase-B (hMAO-B). Isoenzymes are an important factor in the development of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease as well as in depression. Inhibitors of the enzymes have displayed efficiency in the treatment of neurodegenerative diseases and they are being used in treatment of clinical depression and anxiety. The teams prepared extracts of tarragon with different solvents: ethyl acetate, acetone, methanol and water to compare the activity of various types of extracts. Moreover, pure metabolites herniarin and skimmin were also tested to verify their influence on the isoenzymes. The results determined a nonselective and lower inhibitory activity of tarragon extracts and pure metabolites on hMAO-A and hMAO-B in comparison with reference inhibitors (Selegiline and Clorgyline). The most effective of the extracts proved to be the methanol extract. Interestingly, pure metabolites had lower inhibitory activity on hMAO-A and hMAO-B compared with extracts. In this regard, the researchers suggested that there are synergistic interactions between compounds of the extract (Aydin et al., 2020).

Anti-Tumor Effect

Researchers from Mexico have assessed the effect of *A. dracunculus* leaf extract on the proliferation of mouse lymphoma L5178Y cells. Extraction of the plant material was performed with hexane, ethyl acetate, acetone, ethanol, acetonitrile and supercritical carbon dioxide ($scCO_2$). Anti-tumor activity was assessed using a tumor growth inhibition test that included measuring ascitic fluid volume and the number of tumor cells after administration of the plant extract to mice. In the control group the tumor cell count was 17.969×10^6, whereas in the group of mice receiving the acetonitrile extract from *A. dracunculus* leaves the cell count was 0.1×10^6. Oral administration of the extract obtained with supercritical carbon dioxide reduced the number of cells to 12.9×10^6, whereas intraperitoneal administration of the same extract reduced the number of cells to 0.1×10^6. The anti-tumour activity of the acetonitrile extract is likely related to the high concentration of polyphenols, and the effect of the $scCO_2$ extract is attributed to the presence of a higher concentration of alkamides in it (Navarro-Salcedo et al., 2017).

Hepatoprotective Action

The hepatoprotective activity of a hydro-alcoholic extract of the herb of *A. dracunculus* was confirmed in 2018 as part of the cooperation between three research centers in Iran. In the course of the experiment, rats were given 50, 100, or 200 mg/kg of the extract for 15 days, followed by a single dose of carbon tetrachloride. Evidence was documented of a reduction in the levels of alanine transaminase, aspartate transaminase, alkaline phosphatase and total bilirubin, as well as a total protein increase. Histopathological examination also confirmed less liver damage in the group of animals given the herbal hydro-alcoholic extract (Zarezade et al., 2018).

Hypoglycaemic Action

The effect of ethanolic extract of *A. dracunculus* herb on controlling glycaemia, insulin sensitivity and insulin secretion was tested. For this purpose, a randomized, double-blind clinical trial was conducted in 24 patients diagnosed with impaired glucose tolerance. Twice daily, the encapsulated ethanolic extract of *A. dracunculus* was administered at 1000 mg for 90 days. The documented results show a significant decrease in

systolic blood pressure (120 mm Hg in the control group, 113 mmHg in the test group), a decrease in glycosylated hemoglobin concentration (5.8% in the control group, 5.6% in the test group), a decrease in the area under the curve for insulin levels (56.136–27.426 pmol/L in the control group, 44.472 to 23.370 pmol/L in the test group), and a reduction in the insulinogenic index (0.45–0.23 in the control group, 0.35 to 0.18 in the test group). HDL cholesterol levels increased. The results of the study showed that *A. dracunculus* herb extracts may in future be used as a therapeutic agent in the treatment of impaired glucose tolerance (Méndez-Del Villar et al., 2016).

Thyroid Hormone Profile Regulation

The study assessed whether the *A. dracunculus* herb could be used in hypothyroidism. It was conducted on a group of forty-eight rats in which hypothyroidism was induced by the administration of propylthiouracil. The rodents were orally administered an aqueous extract of the herb. Samples of the animals' blood were taken during the experiment. A significant increase in the level of thyroxine and triiodothyronine was proven after the administration of 300 mg/kg of the plant extract; moreover, a decrease in the elevated level of thyrotropin was recorded in the negative control group. At a dose of 200 mg/kg, the extract increased the total antioxidant capacity (TAC) and the level of glutathione. There was also a decrease in the level of malonaldehyde, a marker of oxidative stress. The research results indicate that *A. dracunculus* aqueous extract may improve the thyroid hormone profile, but further research is needed (Mohammadi et al., 2020).

Inhibition of Carbonic Anhydrase I and II Activity

The study verified whether *A. dracunculus* herb extracts obtained with n-hexane, dichloromethane, ethanol and methanol are inhibitors of carbonic anhydrase I and II (hCA I and hCA II). In the body, these enzymes catalyze the reaction between water and carbon dioxide, which produces a proton and a bicarbonate anion. This reaction has a significant impact on the water content inside the eyeball. With excess fluid build-up, intraocular pressure rises, which can lead to the development of glaucoma. In the study, the highest activity (IC_{50} = 0.02 μg/ml for hCA I, and IC_{50} = 0.31 μg/ml for hCA II) was demonstrated for the dichloromethane extract. In order to determine the active compounds responsible for the action, the following main components were isolated from the dichloromethane extract: *trans*-anethole, stigmasterol, herniarin (2E, 4E)-N-isobutylundeca-2,4-diene-8,10-diynamide (2E, 4E)-1-(piperidin-1-yl)undeca-2,4-diene-8,10-diyn-1-one and 1-(4′-methoxyphenyl)-1,2,3-trihydroxypropane. All these compounds inhibited the activity of hCA I and hCA II. The action of 1-(4′-methoxyphenyl)-1,2,3-trihydroxypropane was more potent than that of the control acetazolamide. On the basis of their research, the authors of the study concluded that *A. dracunculus* herb extracts, like anhydrase I and II inhibitors, can reduce the accumulation of fluid inside the eyeball and thus be used in the treatment of glaucoma (Aydın et al., 2019).

Insect-Repelling Action

Italian researchers have verified that the essential oil obtained from the herb *A. dracunculus* can act as a repellent against a species of dipterous flies–*Calliphora vomitoria* (the blue bottle fly). The insect, which is a synanthropic species, is responsible for the transmission of many pathogenic microorganisms–*Salmonella typhimurium, Entamoeba coli*, and *Giardia duodenalis*. Larvae that hatch from its eggs deposited on animal or human tissue cause myiasis. The essential oil of the plant has been shown to deter *C. vomitoria* from laying eggs in fresh beef. At an oil concentration of 0.05 μL/cm^2, complete inhibition of egg laying by the insect was demonstrated (Bedini et al., 2017).

Osanloo et al. (2017) conducted a study confirming larvicidal activity of tarragon essential oil against *Anopheles stephensi*–mosquitos that are responsible for spreading malaria in the Arabian Peninsula, Indian subcontinent, Afghanistan and Iran. Chemical larvicides which are widely used to control the disease cause environmental pollution and desensitization of some species to active agents. Therefore, nanoemulsion of *A. dracunculus* essential oil was tested as a natural alternative to chemical products. The results showed that nanoemulsion consisting of 0.35% tarragon oil, 10% of Tween 20 and deionized water has a comparable larvicidal activity to chemical larvicides (Osanloo et al., 2017).

APPLICATIONS IN COSMETOLOGY

The European CosIng database allows the use of *A. dracunculus* in six forms (European Commission CosIng, 2020). The species is used in the cosmetics industry as an ingredient in skin care products, fragrances and masking agents. In cosmetology, *A. dracunculus* is used in the production of moisturizing creams, shampoos, lotions and cleansing milk. These preparations are used to care for the skin of the scalp, body and face (**Table 4**). The essential oil obtained by steam distillation is widely used as a component in perfumes (Aglarova et al., 2008). It is also used in aromatherapy during massages and baths and is added to facial masks and compresses (Hassanzadeh et al., 2016; Mumivand et al., 2017). Products containing tarragon are offered by both European and non-European cosmetics companies. Among them are brands such as the English *Lush Cosmetics*, the Italian *L'Erbolario Assenzio*, the German *AER Scent*, the French *Florame*, the Swedish *Timotei*, the Azerbaijani *Buta* and the South Korean *Missha*. As a component of women's and men's perfumes, the oil of *A. dracunculus* is very often used by prestigious fashion brands, such as the Italian *Prada, Versace, Dolce & Gabbana*, the French *Givenchy* and *Chloé*, the American *Calvin Klein* and *Tom Ford*, and many others.

APPLICATIONS IN THE FOOD INDUSTRY

The US Food and Drug Administration (FDA) states that the composition of the spice tarragon includes dried leaves and flowering tops of *A. dracunculus* (Food and Drug

Administration, 1980). Tarragon is usually used as a seasoning for meat, sauces, rice dishes, fish and marinades. It has preservative properties, so it is keenly used for pickling cabbage and cucumbers, marinating pumpkins, and for the production of tarragon mustard and herbal vinegars. It is recommended for people on a salt-free diet because it improves the taste of dishes (Kordali et al., 2005; Méndez-Del Villar et al., 2016; Çorapcı et al., 2020). It is also added to infusions, refreshing drinks, alcoholic and non-alcoholic drinks such as "Tarkhun" – a carbonated, non-alcoholic drink, the main ingredient of which are fresh *A. dracunculus* leaves. Fresh tarragon leaves can be used as side dishes or garnishes with meat dishes and in vegetable salads (Goldstein, 1999; Ribnicky et al., 2004; Aglarova et al., 2008).

The nutritional composition of tarragon comprises a high content of carbohydrates (88g/100 g dried weight), lower amounts of fructose and sucrose as well as low levels of fat with a predominance of polyunsaturated fatty acids (Ribeiro et al., 2016). Use of the species in the food industry varies from country to country. Tarragon is a important herb in French cuisine. French tarragon or estragon comes from there and is the most popular variety of the spice for use in cooking. French tarragon has a refreshing, sweet and slightly bitter fragrance. The ground parts are used whole, both fresh and dried. After drying, it is milled or crushed. However, the fresh herb is more desirable, as dried herbs quickly lose their qualities. In France, tarragon is one of the key plants used in the production of Dijon mustard, cream sauces and mayonnaize. Armenians use tarragon to season vegetables, fish and meats. In Slovenia, tarragon is used to season the pastry called 'potica'. In the United States, it is added to vinegar, tartar sauce and seafood (Goldstein, 1999; Ribnicky et al., 2004; Aglarova et al., 2008; Çorapcı et al., 2020).

The taste of *A. dracunculus* depends on the variety. French tarragon has a sweet taste and aroma similar to liquorice, with a slightly bitter aftertaste. Another description of French tarragon says that it has a herbaceous flavor similar to anise and basil. Russian tarragon is more bitter and pungent, devoid of the anise flavor (Uhl and Strauss, 2000).

Professional studies suggest that hydro-ethanolic extract of *A. dracunculus,* with its antimicrobial activity, can be considered a potential candidate for application in food as a preservative. Additionally, hydro-ethanolic extract is less toxic compared to essential oils, therefore, it has the potential to be used in the food industry. Furthermore, tarragon extract also displayed improvement in the fracture of pizza dough as well as a lower influence on dough darkening compared with ascorbic acid (Gottardi et al., 2016; Ribeiro et al., 2016).

A notable potential application of *A. dracunculus* essential oil is in edible antibacterial films used to prevention food spoilage. In 2020 a study by Socaciu and co-authors confirmed the antibacterial and antioxidant activity of tarragon oil. Moreover, the experiment also evaluated the influence of the oil on the qualities of edible antibacterial film. The results show that the application of essential oil in the film forming solution improved water solubility, protection against VIS light, puncture resistance, elasticity and transparency of the film and an increased of its moisture content. Therefore, tarragon oil can be considered an alternative to synthetic compounds in food packaging applications (Socaciu et al., 2020).

SAFETY OF USE

The FDA lists *A. dracunculus* and the oils and extracts derived from this species as safe for use (Food and Drug Administration, 2020). However, there have been reports of potential toxicity of the main components of the essential oil of the plant–estragole and methyl eugenol (Obolskiy et al., 2011). Methyl eugenol and estragole, as components of *A. dracunculus*, have both undergone extensive safety checks. Tests on animals administered estragole orally or subcutaneously have shown induction of liver tumors in mice. The appearance of tumors is associated with the formation of 1′-hydroxyestragole. This metabolite was also present in the urine of men who were given 100 μg of estragole for six months. Both compounds (estragole and 1′-hydroxyestragole) promoted abnormal DNA synthesis in rat hepatocytes, in both *in vivo* and *in vitro* tests (European Commission Health and Comsumer Protection Directorate - General, 2001).

Methyl eugenol has also been found to induce liver tumors in animal studies (rats and mice) and, additionally, the formation of neuroendocrine tumors in the glandular stomach. Neoplasms of the kidney, mammary glands and subcutaneous tissue, and mesotheliomas have also been documented in rats. High doses of methyl eugenol (minimum 30 mg/kg for 25 days) induce auto-induction of P450 cytochromes, leading to the formation of the carcinogenic 1′-hydroxymethyl eugenol. As in the case of estragole, methyl eugenol and its metabolites promote unplanned DNA synthesis; moreover, methyl eugenol forms DNA adducts (European Commission Health and Comsumer Protection Directorate-General, 2001).

After analyzing the available data, the European Food Safety Authority (EFSA) classified estragole and methyl eugenol as genotoxic and carcinogenic compounds. However, a safe threshold for their consumption has not been established. The Commission recommends limiting the use of both compounds (European Commission Health and Comsumer Protection Directorate - General, 2001).

The data of European Medicines Agency - The Committee on Herbal Medicinal Products (HMPC) from 2019 regarding the estragole use claimed that "there is the evidence of genotoxic carcinogenicity, exposure to this compound; estragole should be kept as low as practically achievable. In the evaluation of herbal medicinal products containing estragole Member States should take steps to ensure that the public are protected from exposure." HMPC recommended the acceptable intake of estragole per day for adults - 51.8 mg/kg of body weight, and for children - 1.0 μg/kg of body weight. Moreover, report concluded, that the intake of estragole from (traditional) herbal medicinal products in the population should be as low as possible (European Medicines Agency - The Committee on Herbal Medicinal Products (HMPC), 2019).

TABLE 4 | Applications of *A. dracunculus* in cosmetology as recommended by the CosIng database.

Name according to CosIng	Description	Application profile
Artemisia dracunculus flower	Flowers of the tarragon plant	Skin care agent
Artemisia dracunculus herb extract	Extract from tarragon herb	Fragrance
Artemisia dracunculus leaf/stem extract	Extract from tarragon leaves and stems	Masking agent
Artemisia dracunculus oil	Essential oil of tarragon	Fragrance, skin care agent
Artemisia dracunculus root extract	Extract from tarragon roots	Skin care agent
Artemisia dracunculus seed/*Anthemis nobilis* seed/*Hypericum androsaemum* seed extract	Extract from tarragon seeds, roman chamomile seeds, and tutsan (shrubby st. John's wort) seeds	Skin care agent

PLANT BIOTECHNOLOGY RESEARCH

Due to the extensive use of tarragon in the food industry, large losses incurred with the traditional methods of its cultivation, difficulties in vegetative reproduction, and diseases of the cultivated plants, research is being conducted on alternative, biotechnological methods of propagating *A. dracunculus*.

In 2012, scientists developed an efficient protocol for micropropagation of the French variety of *A. dracunculus*. Plant cuttings 20 mm long were cut into 1 mm long pieces. These were then used to establish cultures in Murashige & Skoog (MS) liquid media, with five apical meristems as the experimental unit. In order to propagate the plant material, the established cultures were transferred to solid MS media. The tested propagation protocols differed from one another in the concentrations of indole acetic acid (IAA) and kinetin (KIN) added to the medium. The MS medium enriched with only 0.1 mg/L each of IAA and KIN, proved to be the most effective. The length of the sprouts was 11.19 mm and the multiplication index was 1.97. Thirteen days after planting, 100% rooting was achieved. As an alternative method of propagation, the authors of the study placed the propagating pieces horizontally on a solid MS medium supplemented with 0.5 mg/L KIN and 0.5 mg/L IAA. The sprout length was 10.31 mm and the multiplication index was 1.87; good callus induction and leaf development were also demonstrated. The time required to establish the *in vitro* culture was three weeks (Fernández-Lizarazo and Mosquera-Vásquez, 2012).

Türközü et al. also undertook to develop an *A. dracunculus* micro-propagation protocol. They proved that the highest efficiency (92%) could be obtained by growing cultures in MS medium supplemented with 1.8 μM 6-benzyladenine (BA) and 0.3 μM 1-naphthylacetic acid (NAA). They also reported an adverse effect of the addition of gibberellic acid (GA_3) on microshoot development. The longest roots were obtained in the plants grown on MS medium with the addition of indolelbutyric acid (IBA) at a concentration of 0.5 mg/L (44 mm) and on ½ MS medium with 0.5 mg/L IBA (46 mm) (Türközü et al., 2014).

Another team of Ibrahim et al. has tested the performance of various explants–leaves, stems and roots of *A. dracunculus* in establishing *in vitro* cultures. Leaf explants placed on MS medium supplemented with 1.0 mg/ml of 2,4-dichlorophenoxyacetic acid (2,4-D) proved to be the best starting material for callus induction. For root and stem explants, no statistically significant effects were obtained. It was also proved that the best callus induction was caused by the addition of 0.5 mg/L BA and 0.1 mg/L NAA (75 shoots). It was also shown that the concentration of estragole in the cultured plants correlated with the type of explant and the phytohormone concentrations used. Estragole was not detected in the roots; the highest amount of it (16.7% estragole per g dry weight) was isolated from one-node cultures after the application of 0.5 mg/L 2,4-D (Ibrahim et al., 2011).

SUMMARY

A. dracunculus has been a frequent subject of research in the last few years, regarding both the chemistry and biological activity of extracts obtained from the herb and/or leaves, and the essential oil. Phytochemical tests have confirmed the presence of numerous flavonoid compounds, phenolic acids, coumarins and alkamides in the herb and leaves, as well as a very high variability of the chemical composition of the essential oil. Contemporary research on the biological activity of the above-mentioned raw materials has proven new findings in their activity–antibacterial, antifungal and antiprotozoal effects, as well as extremely valuable antioxidant, immunomodulatory and antineoplastic properties. These studies have also proven hepatoprotective, hypoglycaemic and thyroid-regulating effects. An antidepressant effect has also been documented. The anti-inflammatory and analgesic effects known from traditional medicine applications have also been confirmed.

The popularity of *A. dracunculus* in the production of cosmetics is also surprising. For this purpose, the essential oil of tarragon, extracts from the flowers, leaves, stems and seeds, as well as from the whole herb and roots are used. The essential oil is also used in aromatherapy treatments and in the production of perfumes. Cosmetics based on this species are offered by both European cosmetics companies (mainly English, German, French, Swedish) and non-European companies (mainly South Korean and Azerbaijani). The herb of the species is widely used for seasoning purposes and as an additive to alcoholic and non-alcoholic beverages. The species is the subject of research in the field of plant biotechnology, which mainly concerns the development of micropropagation protocols. The most valuable findings in the professional scientific research conducted has been the proof of new areas of the biological activity of the *A. dracunculus* herb and/or leaf extracts and essential oil–mainly their antioxidant,

immunomodulating and antineoplastic effects, as well as the hepatoprotective and hypoglycaemic effects. The species, known thus far as a spice plant, appears to be an extremely valuable medicinal and cosmetic plant.

AUTHOR CONTRIBUTIONS

Data collection: HE, JŚ, EK, PK, AR, AS; design of the study: HE; analysis and interpretation of the data: HE, EK, JŚ, PK, AR, AS, MT. Drafting the manuscript: HE, PK, AR, AS; critical revision of the manuscript: HE, AS, MT. All authors read and approved the manuscript in its final form.

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REFERENCES

Abdollahnejad, F., Kobarfard, F., Kamalinejad, M., Mehrgan, H., and Babaeian, M. (2016). Yield, chemical composition and antibacterial activity of *Artemisia dracunculus L.* essential oils obtained by two different methods. *J. Essent. Oil Bearing Plants* 19 (3), 574–581. doi:10.1080/0972060X.2014.963167

Abtahi Froushani, S. M., Zarei, L., Esmaeili Gouvarchin Ghaleh, H., and Mansori Motlagh, B. (2016). Estragole and methyl-eugenol-free extract of *Artemisia dracunculus* possesses immunomodulatory effects. *Avicenna J. Phytomedicine* 6 (5), 526–534. doi:10.22038/ajp.2016.6479

Aglarova, A. M., Zilfikarov, I. N., and Severtseva, O. V. (2008). Biological characteristics and useful properties of tarragon (*Artemisia dracunculus* L.) (review). *Pharm. Chem. J.* 42 (2), 81–86. doi:10.1007/s11094-008-0064-3

Alakbarov, F. U. (2001). Medicinal plants used in medieval Azerbaijan phytotherapy. *J. Herbal Pharmacother.* 1 (3), 35–49. doi:10.1080/j157v01n03_04

Aydin, T., Akincioglu, H., Gumustas, M., Gulcin, I., Kazaz, C., and Cakir, A. (2020). Human monoamine oxidase (hMAO) A and hMAO B inhibitors from *Artemisia dracunculus* L. herniarin and skimmin. *Z. Naturforsch. - Sect. C J. Biosci.* 75 (11–12), 459–466. doi:10.1515/znc-2019-0227

Aydın, T., Yurtvermez, B., Şentürk, M., Kazaz, C., and Çakır, A. (2019). Inhibitory effects of metabolites isolated from *Artemisia dracunculus* L. against the human carbonic anhydrase I (hCA I) and II (hCA II). *Rec. Nat. Prod.* 13 (3), 216–225. doi:10.25135/rnp.102.18.07.329

Ayoughi, F., Barzegar, M., Sahari, M. A., and Naghdibadi, H. (2011). Chemical compositions of essential oils of *Artemisia dracunculus* L. and endemic *Matricaria chamomilla* L. and an evaluation of their antioxidative effects. *J. Agric. Sci. Technol.* 13, 79–88.

Bakova, N., Logvinenko, L., and Shevchuk, O. (2017). Tarragon cultivars (*Artemisia dracunculus* L.) of the Nikita botanical gardens breeding. *B. Proc.*, 1, 445–451.

Bauhin, J., and Cherler, J. H. (1651). Historia plantarum universalis. Vol III Ebroduni. Available at: https://books.google.pl/books/about/Historia_plantarum_universalis.html?id=7ejDuAEACAAJ&redir_esc=y (Accessed November 1, 2020).

Bedini, S., Flamini, G., Cosci, F., Ascrizzi, R., Echeverria, M. C., Guidi, L., et al. (2017). *Artemisia* spp. essential oils against the disease-carrying blowfly *Calliphora vomitoria*. *Parasites Vectors* 10 (1), 1–10. doi:10.1186/s13071-017-2006-y

Behbahani, B. A., Shahidi, F., Yazdi, F. T., Mortazavi, S. A., and Mohebbi, M. (2017). Antioxidant activity and antimicrobial effect of tarragon (*Artemisia dracunculus*) extract and chemical composition of its essential oil. *Food Measure* 11 (2), 847–863. doi:10.1007/s11694-016-9456-3

Bhutia, T. D., and Valant-Vetschera, K. M. (2008). Chemodiversity of *Artemisia dracunculus* L. from Kyrgyzstan: isocoumarins, coumarins, and flavonoids from aerial parts. *Nat. Prod. Commun.* 3 (8), 1289–1292. doi:10.1177/1934578x0800300811

Bora, K. S., and Sharma, A. (2011). The GenusArtemisia: a comprehensive review. *Pharm. Biol.* 49 (1), 101–109. doi:10.3109/13880209.2010.497815

Bussmann, R. W., Batsatsashvili, K., Kikvidze, Z., Khajoei Nasab, F., Ghorbani, A., Paniagua-Zambrana, N. Y., et al. (2020). "*Artemisia absinthium* L., *Artemisia annua* L., *Artemisia dracunculus* L., *Artemisia leucodes* Schrenk, *Artemisia scoparia* Waldst. and Kit. *Artemisia vulgaris* L., *Eclipta prostrata* (L.) L," in *Asteraceae in Ethnobotany of the mountain Regions of far eastern Europe*. Editors K. Batsatsashvili, Z. Kikvidze, and R. Bussmann (Cham, Switzerland: Springer), 131–146. doi:10.1007/978-3-030-28940-9_16

Catalogue of Life (2020). Catalogue of Life. Available at: http://www.catalogueoflife.org/ (Accessed November 8, 2020).

Chaleshtori, R. S., Rokni, N., Razavilar, V., and Kopaei, M. R. (2013). The evaluation of the antibacterial and antioxidant activity of tarragon (*Artemisia dracunculus* L.) essential oil and its chemical composition. *Jundishapur J. Microbiol.* 6 (9), 1–35. Available at: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84889675506&doi=10.5812%2Fjjm.7877&partnerID=40&md5=8db9e949587e1022af0488d25336b422 (Accessed November 7, 2020).

Chauhan, R. S., Kitchlu, S., Ram, G., Kaul, M. K., and Tava, A. (2010). Chemical composition of capillene chemotype of *Artemisia dracunculus* L. from North-West Himalaya, India. *Ind. Crops Prod.* 31 (3), 546–549. doi:10.1016/j.indcrop.2010.02.005

Çorapcı, B., Köstekli, B., Eyüboğlu, A., and Kocatepe, D. (2020). The effect of different application methods of sumac (*Rhus coriaria*) and tarragon (*Artemisia dracunculus*) on some quality properties of marinated sea bream (*Sparus aurata* L., 1758). *J. Food Process. Preserv.* 44 (10), e14751. doi:10.1111/jfpp.14751

Długońska, H. (2015). The Nobel Prize 2015 in physiology or medicine for highly effective antiparasitic drugs. *Ann. Parasitol.* 61 (4), 299–301. doi:10.17420/ap6104.23

Efferth, T., Zacchino, S., Georgiev, M. I., Liu, L., Wagner, H., and Panossian, A. (2015). Nobel Prize for artemisinin brings phytotherapy into the spotlight. *Phytopharm.* 22 (13), 1–4. doi:10.1016/j.phymed.2015.10.003

Eidi, A., Oryan, S., Zaringhalam, J., and Rad, M. (2016). Antinociceptive and anti-inflammatory effects of the aerial parts of *Artemisia dracunculus* in mice. *Pharm. Biol.* 54 (3), 549–554. doi:10.3109/13880209.2015.1056312

Eisenman, S. W., Poulev, A., Struwe, L., Raskin, I., and Ribnicky, D. M. (2011). Qualitative variation of anti-diabetic compounds in different tarragon (*Artemisia dracunculus* L.) cytotypes. *Fitoterapia* 82 (7), 1062–1074. doi:10.1016/j.fitote.2011.07.003

European Commission Cosing (2020). CosIng - cosmetic database. Available at: https://ec.europa.eu/growth/sectors/cosmetics/cosing_en (Accessed November 10, 2020).

European Commission Health & Comsumer Protection Directorate - General (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methoxybenzene). *Int. J. Mod. Phys. Conf. Ser.* doi:10.1142/s2010194512007544

European Medicines Agency - The Committee on Herbal Medicinal Products (HMPC) (2019). Public statement on the use of herbal medicinal products containing estragole. Available at: https://www.ema.europa.eu/en/documents/other/second-draft-revision-1-public-statement-use-herbal-medicinal-products-containing-estragole_en.pdf (Accessed November 10, 2020).

Fernández-Lizarazo, J. C., and Mosquera-Vásquez, T. (2012). Efficient micropropagation of French tarragon (*Artemisia dracunculus* L.). *Agron. Colomb* Available at: http://www.scielo.org.co/scielo.php?pid=S0120-99652012000300004&script=sci_arttext&tlng=en (Accessed November 8, 2020).

Food and Drug Administration (2020). CPG sec 525.750 spices - definitions FDA. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-525750-spices-definitions (Accessed November 8, 2020).

Food and Drug Administration (1980). Spices - definitions. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-525750-spices-definitions.

GBIF.org (2020). GBIF Home Page. Available at: https://www.gbif.org/ (Accessed November 18, 2020).

Goldstein, D. (1999). *The Georgian feast: the vibrant culture and savory food of the Republic of Georgia*. Los Angeles, CA: University of California Press.

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

Güvenalp, Z., Özbek, H., Dursunoğlu, B., Yuca, H., Gözcü, S., Çil, Y. M., et al. (2017). α-Amylase and α-glucosidase inhibitory activities of the herbs of *Artemisia dracunculus L.* and its active constituents. *Med. Chem. Res.* 26 (12), 3209–3215. doi:10.1007/s00044-017-2014-7

Hassanzadeh, M. K., Tayarani Najaran, Z., Nasery, M., and Emami, S. A. (2016). Tarragon (*Artemisia dracunculus L.*) oils. *Essent. Oils Food Preserv., Flavor Saf.* 813–817. doi:10.1016/B978-0-12-416641-7.00092-4

Ibrahim, A. K., Ahmed, S. A., Khattab, S. E., and Sherif, F. M. E. (2011). Efficient callus induction, plant regeneration and estragole estimation in tarragon (*Artemisia dracunculus L.*). *J. Essent. Oil Res.* 23 (4), 16–20. doi:10.1080/10412905.2011.9700463

Jahani, R., Khaledyan, D., Jahani, A., Jamshidi, E., Kamalinejad, M., Khoramjouy, M., et al. (2019). Evaluation and comparison of the antidepressant-like activity of *Artemisia dracunculus* and *Stachys lavandulifolia* ethanolic extracts: an *in vivo* study. *Res. Pharm. Sci.* 14 (6), 544–553. doi:10.4103/1735-5362.272563

Joshi, R., Satyal, P., and Setzer, W. (2016). Himalayan aromatic medicinal plants: a review of their ethnopharmacology, volatile phytochemistry, and biological activities. *Medicines* 3 (1), 6. doi:10.3390/medicines3010006

Jourdan, A. J. L. (1829). Pharmacopoea universalis: oder, Uebersicht der Pharmacopöen von Amsterdam Erster Band, Weimar Available at: https://books.google.pl/books/about/Pharmacopoea_universalis.html?id=eB0SAAAAYAAJ&redir_esc=y (Accessed November 2, 2020).

Karimi, A., Hadian, J., Farzaneh, M., and Khadivi-Khub, A. (2015). Phenotypic diversity and volatile composition of Iranian *Artemisia dracunculus*. *Ind. Crops Prod.* 65, 315–323. doi:10.1016/j.indcrop.2014.12.003

Kluk, K. (1805). *Dykcyonarz roślinny*. Warszawa, Poland: Meiy Rzeczypospolitej. Available at: https://polona.pl/item/dykcyonarz-roslinny-w-ktorym-podlug-ukladu-linneusza-sa-opisane-rosliny-nietylko-kraiowe,MzM2NTk3Mw/2/#info:metadata (Accessed November 5, 2020).

Kordali, S., Kotan, R., Mavi, A., Cakir, A., Ala, A., and Yildirim, A. (2005). Determination of the chemical composition and antioxidant activity of the essential oil of *Artemisia dracunculus* and of the antifungal and antibacterial activities of Turkish *Artemisia absinthium*, *A. dracunculus*, *Artemisia santonicum*, and *Artemisia spicigera* Essential oils. *J. Agric. Food Chem.* 53 (24), 9452–9458. doi:10.1021/jf0516538

Koul, B., and Taak, P. (2017). The *Artemisia* genus: a review on traditional uses, phytochemical constituents, pharmacological properties and germplasm conservation. *J. Glycomics Lipidomics* 07 (1), 142. doi:10.4172/2153-0637.1000142

Majdan, M., Kiss, A. K., Hałasa, R., Granica, S., Osińska, E., and Czerwińska, M. E. (2020). Inhibition of neutrophil functions and antibacterial effects of tarragon (*Artemisia* dracunculus L.) infusion-phytochemical characterization. *Front. Pharmacol.* 11, 947. doi:10.3389/fphar.2020.00947

Mamedov, N., Grdner, Z., and Craker, L. E. (2004). Medicinal plants used in Central Asia for the treatment of selected skin conditions. *J. Herbs Spices Med. Plants* 11, 191–222.

Mattioli, P. (1586). Kreuterbuch: des hochgelehrten weitberuĭmbten Herrn Petri Andreae Matthioli Gruĭndtliche Erklaĭrung Aller Kreutter vnd gewechs mit Eigentlicher Beschreibung derselbigen, in *sechÃŸerley*. Frankfurt Main, Germany: Feyerabend Fischer & Dack.

Méndez-del Villar, M., Puebla-Pérez, A. M., Sánchez-Peña, M. J., González-Ortiz, L. J., Martínez-Abundis, E., and González-Ortiz, M. (2016). Effect of *Artemisia dracunculus* administration on glycemic control, insulin sensitivity, and insulin secretion in patients with impaired glucose tolerance. *J. Med. Food* 19 (5), 481–485. doi:10.1089/jmf.2016.0005

Miller, L., and Miller, B. (1998). *Ayurveda and aromatheraphy: the earth essential guide to ancient wisdom and modern healing*. New Dehli, India: Motilal Banarsidass Publisher.

Mirzaei, F., Bafghi, A. F., Mohaghegh, M. A., Jaliani, H. Z., Faridnia, R., and Kalani, H. (2016). *In vitro* anti-leishmanial activity of *Satureja hortensis* and *Artemisia dracunculus* extracts on *Leishmania major* promastigotes. *J. Parasit. Dis.* 40 (4), 1571–1574. doi:10.1007/s12639-015-0730-9

Missouri Botanical Garden (2020). *Tropicos.org*. Available at https://www.missouribotanicalgarden.org/ (Accessed November 15, 2020).

Modaresi, M., Alasvand Zarasvand, M., and Madani, M. (2018). The effects of hydro-alcoholic extract of *Artemisia dracunculus* L. (Tarragon) on hematological parameters in mice. *Jbrms* 5 (1), 10–14. doi:10.29252/jbrms.5.1.10

Mohammadi, M. M., Saeb, M., and Nazifi, S. (2020). Experimental hypothyroidism in adult male rats: the effects of *Artemisia dracunculus* aqueous extract on serum thyroid hormones, lipid profile, leptin, adiponectin, and antioxidant factors. *Comp. Clin. Pathol.* 29 (2), 485–494. doi:10.1007/s00580-019-03080-0

Mumivand, H., Babalar, M., Tabrizi, L., Craker, L. E., Shokrpour, M., and Hadian, J. (2017). Antioxidant properties and principal phenolic phytochemicals of Iranian tarragon (*Artemisia dracunculus* L.) accessions. *Hortic. Environ. Biotechnol.* 58 (4), 414–422. doi:10.1007/s13580-017-0121-5

Navarro-Salcedo, M. H., Delgado-Saucedo, J. I., Siordia-Sánchez, V. H., González-Ortiz, L. J., Castillo-Herrera, G. A., and Puebla-Pérez, A. M. (2017). *Artemisia* dracunculus extracts obtained by organic solvents and supercritical CO2 produce cytotoxic and antitumor effects in mice with L5178Y lymphoma. *J. Med. Food* 20 (11), 1076–1082. doi:10.1089/jmf.2017.0044

Obistioiu, D., Cristina, R. T., Schmerold, I., Chizzola, R., Stolze, K., Nichita, I., et al. (2014). Chemical characterization by GC-MS and *in vitro* activity against *Candida albicans* of volatile fractions prepared from *Artemisia dracunculus, Artemisia abrotanum, Artemisia absinthium* and *Artemisia vulgaris*. *Chem. Cent. J.* 8 (1), 6. doi:10.1186/1752-153X-8-6

Obolskiy, D., Pischel, I., Feistel, B., Glotov, N., and Heinrich, M. (2011). *Artemisia dracunculus* L. (tarragon): a critical review of its traditional use, chemical composition, pharmacology, and safety. *J. Agric. Food Chem.* 59 (21), 11367–11384. doi:10.1021/jf202277w

Osanloo, M., Amani, A., Sereshti, H., Abai, M. R., Esmaeili, F., and Sedaghat, M. M. (2017). Preparation and optimization nanoemulsion of Tarragon (*Artemisia dracunculus*) essential oil as effective herbal larvicide against *Anopheles stephensi*. *Ind. Crops Prod.* 109, 214–219. doi:10.1016/j.indcrop.2017.08.037

Pedanius, D. (1998). *Der wiener Dioskurides: Codex medicus graecus 1*. Editor O. Mazal. (Graz: Akademische Druck u. Verlagsanstalt).

Plinii Secundii, C. (1845). Historiae naturalis libri XXXVIII. Łukaszewicz Poznań: Drukarnia Lukasiewicza. Available at: https://polona.pl/item/k-pliniusza-starszego-historyi-naturalnej-ksiag-xxxvii-c-plinii-secundi-historiae,MTc1MjM2/2/#info:metadata (Accessed November 2, 2020).

Rejewski, M. (1996). *Pochodzenie łacińskich nazw roślin polskich: przewodnik botaniczny*. Warszawa, Poland: Książka i Wiedza.

Ribeiro, A., Barros, L., Calhelha, R. C., Carocho, M., Ćirić, A., Sokovic, M., et al. (2016). Tarragon phenolic extract as a functional ingredient for pizza dough: comparative performance with ascorbic acid (E300). *J. Funct. Foods* 26, 268–278. doi:10.1016/j.jff.2016.08.019

Ribnicky, D. M., Poulev, A., O'Neal, J., Wnorowski, G., Malek, D. E., Jäger, R., et al. (2004). Toxicological evaluation of the ethanolic extract of *Artemisia dracunculus* L. for use as a dietary supplement and in functional foods. *Food Chem. Toxicol.* 42 (4), 585–598. doi:10.1016/j.fct.2003.11.002

Rządkowski, L. (1937). Encyklopedia farmaceutyczna. *Poznań: Wydawnictwo Encyklopedii Farmaceutycznej Leon, Mikołaj Wawrzyniec Misiak i S-ka*.

Safari, H., Anani Sarab, G., and Naseri, M. (2019). *Artemisia dracunculus L.* modulates the immune system in a multiple sclerosis mouse model. *Nutr. Neurosci.* 1, 1–7. doi:10.1080/1028415X.2019.1681742

Senderski, M. E. (2007). Prawie wszystko o ziołach. Poradnik. Mateusz E. Senderski. Podkowa Leśna.

Sharopov, F. S., Salimov, A., Numonov, S., Bakri, M., Sangov, Z., Habasi, M., et al. (2020). *Chemical compositions and biological activities of essential oils-original article phytochemical study on the essential oils of tarragon* (*Artemisia dracunculus L.*) *growing in Tajikistan and its comparison with the essential oil of the species in the rest. Nat. Prod. Comm.* 15, 1–7. doi:10.1177/1934578X20977394

Socaciu, M.-I., Fogarasi, M., Semeniuc, C. A., Socaci, S. A., Rotar, M. A., Mureşan, V., et al. (2020). Formulation and characterization of antimicrobial edible films based on whey protein isolate and tarragon essential oil. *Polymers* 12 (8), 1748. doi:10.3390/POLYM12081748

Syrennivs, S. (1613). *Zielnik Herbarzem z języka łacinskiego zowią. Kraków: Drukarnia Bazylego Skalskiego*.

Szczepanik, M., Walczak, M., Zawitowska, B., Michalska-Sionkowska, M., Szumny, A., Wawrzeńczyk, C., et al. (2018). Chemical composition, antimicrobial activity and insecticidal activity against the lesser mealworm *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) of *Origanum vulgare* L. ssp. hirtum (Link) and *Artemisia dracunculus* L. essential oils. *J. Sci. Food Agric.* 98 (2), 767–774. doi:10.1002/jsfa.8524

Tajbakhsh, M., and Soleimani, N. (2018). Evaluation of the bactericidal effects of *Zingiber officinale, Aloysia citrodora* and *Artemisia dracunculus* on the survival of standard gram-positive and gram-negative bacterial strains. *Jorjani Biomed. J.* 6 (1), 22–32. doi:10.29252/jorjanibiomedj.6.1.22

Tak, I.-U.-R., Mohiuddin, D., Ganai, B. A., Chishti, M. Z., Ahmad, F., and Dar, J. S. (2014). Phytochemical studies on the extract and essential oils of *Artemisia dracunculus* L. (Tarragon). *Afr. J. Plant Sci.* 8 (1), 72–75. doi:10.5897/AJPS2013.1145

Talbi, M., Saadali, B., Boriky, D., Bennani, L., Elkouali, M. h., and Ainane, T. (2016). Two natural compounds - a benzofuran and a phenylpropane - from *Artemisia* dracunculus. *J. Asian Nat. Prod. Res.* 18 (8), 724–729. doi:10.1080/10286020.2016.1158708

Tan, R. X., Zheng, W. F., and Tang, H. Q. (1998). Biologically active substances from the genus Artemisia. *Planta Med.* 64 (4), 295–302. doi:10.1055/s-2006-957438

The Plant List (2013). The plant list. Available at: http://www.theplantlist.org/ (Accessed November 8, 2020).

Türközü, D., Yaşar, F., Ellİaltıoğlu, ş. ş., and Yıldırım, B. (2014). Researches on *in vitro* propagation of tarragon (*Artemisia dracunculus* L.). *Yüzüncü Yil Üniversitesi J. Agric. Sci.*

Uhl, S. R., and Strauss, S. (2000). *Handbook of spices, seasonings and flavorings*. Lancaster, United Kingdom: Technomic Publishing.

Verma, M. K., Anand, R., Chisti, A. M., Kitchlu, S., Chandra, S., Shawl, A. S., et al. (2010). Essential oil composition of *Artemisia dracunculus L.* (Tarragon) growing in Kashmir -India. *J. Essent. Oil Bearing Plants* 13 (3), 331–335. doi:10.1080/0972060X.2010.10643830

Vervandier-Fasseur, D., and Latruffe, N. (2019). The potential use of resveratrol for cancer prevention. *Molecules* 24 (24), 4506. doi:10.3390/molecules24244506

Vulpius, D. (1887). Real-Encyclopädie der gesammten Pharmacie. Handwörterbuch für Apotheker, Aerzte und Medicinalbeamte, herausgegeben von Prof Dr. Geissler und Prof. Dr. Möller. Verlag von Urban und Schwarzenberg. Wien und Leipzig. 1887. *Archiv der Pharmazie.* 225 (23), 1075-1077. doi:10.1002/ardp.18872252312

Wang, J., Fernández, A. E., Tiano, S., Huang, J., Floyd, E., Poulev, A., et al. (2018). An extract of *Artemisia dracunculus* L. promotes psychological resilience in a mouse model of depression. *Oxidative Med. Cell Longevity* 2018, 1. doi:10.1155/2018/7418681

Watson, B., and Kennel, E. (2014). The Herb Society of America-*Artemesia* spp. Available at: https://www.herbsociety.org/file_download/inline/d52eae8c-be89-497d-94b3-7fc8da4105f1 (Accessed November 1, 2020).

Willcox, M. (2009). Artemisia Species: from traditional medicines to modern antimalarials-and back again. *J. Altern. Complement. Med.* 15, 101–109. doi:10.1089/act.2009.1530910.1089/acm.2008.0327

Yamada, M., Nakamura, K., Watabe, T., Ohno, O., Kawagoshi, M., Maru, N., et al. (2011). Melanin biosynthesis inhibitors from Tarragon-*Artemisia dracunculus*. *Biosci. Biotechnol. Biochem.* 75 (8), 1628–1630. doi:10.1271/bbb.110306

Yu, Y., Simmler, C., Kuhn, P., Poulev, A., Raskin, I., Ribnicky, D., et al. (2019). The designer approach helps decipher the hypoglycemic bioactive principles of *Artemisia dracunculus* (Russian tarragon). *J. Nat. Prod.* 82 (12), 3321–3329. doi:10.1021/acs.jnatprod.9b00548

Zarasvand, M. A., Madani, M., and Modaresi, M. (2016). The effect of hydroalcoholic extract of *Artemisia dracunculus* L. (Tarragon) on *Candida albicans* infection in mice. *Jundishapur J. Nat. Pharm. Prod.* 11 (4), 2–5. doi:10.17795/jjnpp-29911

Zarezade, V., Moludi, J., Mostafazadeh, M., Mohammadi, M., and Veisi, A. (2018). Antioxidant and hepatoprotective effects of *Artemisia dracunculus* against CCl_4-induced hepatotoxicity in rats. *Avicenna J. Phytomedicine* 8 (1), 51–62. doi:10.22038/ajp.2017.19137.1738

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REVIEW
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Integrating Ethnobotany, Phytochemistry, and Pharmacology of *Cotinus coggygria* and *Toxicodendron vernicifluum*: What Predictions can be Made for the European Smoketree?

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The smoketree (*Cotinus coggygria*) is a historically known medicinal plant from Southeast Europe. Its ethnomedicinal use in skin and mucosal lesions is commonly accepted across countries. Other utilizations reported locally include fever reduction, cardiac diseases, hypertension, urinary diseases, cough, asthma, hemorrhoids, diabetes, numbness of arm, liver disease, and cancer. Departing from the smoketree's traditional uses, this review summarizes investigations on the phytochemistry and bioactivity of the plant. *In vitro* and *in vivo* experiments supporting wound-healing, anti-inflammatory, antibacterial, cytotoxic, antioxidative, hepatoprotective, and antidiabetic effects are presented. Metabolites from smoketree that are responsible for the main pharmacological effects of smoketree are pointed out. Furthermore, the review performs a comparison between *C. coggygria* and the lacquer tree (*Toxicodendron vernicifluum*). The latter is a comprehensively studied species used in Asian phytotherapy, with whom the European smoketree shares a consistent pool of secondary metabolites. The comparative approach aims to open new perspectives in the research of smoketree and anticipates an optimized use of C. coggygria in therapy. It also points out the relevance of a chemosystematic approach in the field of medicinal plants research.

Keywords: ***Cotinus coggygria*, *Toxicodendron vernicifluum*, common metabolites, sulfuretin, fisetin**

INTRODUCTION

Smoketree (*Cotinus coggygria* Scop., syn. *Rhus cotinus* L.) is a shrub growing wildly on sunny limestone slopes in the Balkans, Southern and Eastern Europe, the Caucasus and Central Asia (Matić et al., 2016). Aside from its natural occurrence, smoketree cultivars with colorful foliage are frequently planted in parks and gardens. Smoketree wood, distinctive through its yellow color, has been employed as a dye since ancient times. Key chemical constituents of the golden wood have been revealed in historical textiles from different parts of Europe and Asia: liturgical garments from the Athos Mountain (Valianou et al., 2009; Mantzouris et al., 2011), religious embroideries, brocaded velvets and other ethnographical fabrics from Romania (Petroviciu et al., 2014), even Chinese textiles

from Dunhuang, dating back to over thousand years (Tamburini, 2019). Aside from its importance as a dye, smoketree was used for therapeutic purposes since Antiquity. The use of the name "*Cotinus coccigria*" dates back to Theophrastus (1644) according to the treatize De Historia Plantarum, one of the most important books on the structure and use of plants. However, Linnaeus had first included the smoketree in the *Rhus* genus, under the name *Rhus cotinus* (Linnaeus, 1753). The currently used name, *Cotinus coggygria*, was conferred by (Scopoli, 1772). The genus name, "*Cotinus*", means "wild olive" and is of Greek origin ("κοτινος"), while *coggygria* stems from the Greek "κοκκυγέα" meaning smoke tree.

The knowledge of the therapeutic qualities that has been passed on from generation to generation has lead to a reservoir of information that became the subject of modern research aimed at validating these effects through *in vitro* and *in vivo* experiments. Interestingly, a closely related species, previously classified in the same genus as the European smoketree, *Rhus*, shares a significant amount of common constituents. This species, called Asian lacquer (*Toxicodendron vernicifluum* or *Rhus verniciflua*) proved to have many similar indications, but also new ones. By integrating the phytochemistry and pharmacology of these two species, the current review intends to advance and expand the practical use of the European species which has the advantage of being free of allergenic urushiols, as opposed to its Asian counterpart (Ippen, 1983; Kim et al., 2014).

TAXONOMY AND BOTANICAL ASPECTS OF *COTINUS COGGYGRIA*

The plant is a member of the Anacardiaceae family, which includes tropical representatives like mango (*Mangifera indica*) and cashew (*Anacardium occidentale*), but also species vegetating under temperate climates, for example the allergenic poison sumac (*Rhus toxicodendron*). *C. coggygria* is multi-stemmed, deciduous, averaging heights of 5–7 m. Its leaves have an ovate or obovate shape, pinnate venation and a glaucous lower face. The small flowers are grouped in large panicles, with the pedicels elongating into hairy stalks that cover the inflorescences with smoke-like puffs. This "smoked" aspect inspired several names of the plant: "smoketree", "smoke bush", and even "wig tree". The species is one of the seven of the genus *Cotinus* Mill., the others having a narrower distribution range, in North America: *C. obovatus* (native to Central and South East of the United States), *C. carranzae* and *C. chiangii* (both native to Mexico), and in Asia: *C. kanaka* (native to the southern part of the Eastern Himalayas), *C. nanus* and *C. szechuanensis* (growing in South-Central China)[1].

[1]The Plant List, ^ http://www.theplantlist.org/browse/A/Anacardiaceae/Cotinus/ (Accessed January 26, 2021) Plants of the World online, ^ http://www.plantsoftheworldonline.org/ (Accessed January 26, 2021).

TRADITIONAL USES

C. coggygria has a consistent traditional use in Europe and Asia. The Encyclopedia of Romanian Ethnobotany points to the use of the plant as both a dye and medicine, employed for the treatment of wounds and pharyngitis (Butură, 1979). An enthnobotanical survey performed in the region of Dobrogea (South-East Romania) reported on the use of the plant as a cicatrizing agent of open wounds and the treatment of gynecological disorders (Tudor and Georgescu, 2011). The application of *C. coggygria* (called "skumpina") has also been acknowledged in a survey focused on plants used by the Czech diaspora from Romanian Banat region (Vlková et al., 2015). In the local folk medicine of Southwestern Romania, several parts of the smoketree are used: young twigs and leaves boiled in water represent a remedy against sore throat, stomatitis, gingivitis and gastritis. More conspicuously, wood slices extracted in badger fat (*Meles*) were mentioned as ointment ingredients intended to treat slowly healing wounds (Antal and Ardelean, 2015). The ethnomedical use in skin and mucosal lesions is commonly accepted across countries, according to reports from Bulgaria (Nedelcheva, 2012; Koleva et al., 2015), Bosnia and Herzegovina (Redžić, 2007), Serbia (Jarić et al., 2015), Albania (Nedelcheva and Draganov 2014), and Turkey (Kültür, 2007). Other uses include fever reduction (Huang and Williams 1998; Redžić, 2007), cardiac diseases, hypertension, urinary diseases, cough, asthma, hemorrhoids, diabetes, numbness of arm (Kültür, 2007), and liver disease (Shen et al., 1991). The plant is as well mentioned in cancer treatment in Turkey (Kültür, 2007) and Bosnia and Herzegovina (Redžić, 2007). Usually, leaves are the most frequently used plant parts in traditional medicine (as infusions or fresh).

PHYTOCHEMISTRY OF *COTINUS COGGYGRIA*

Smoketree is characterized by a broad spectrum of polyphenolic secondary metabolites: tannins, various subtypes of flavonoids, phenolic acids, as well as by the presence of volatile organic compounds. Essential oil components are contained in secretory ducts associated with the phloem; ducts run through the cortex of twigs as well as the petiole and midrib of leaves (Metivier et al., 2007; Antal and Ardelean, 2015; Özcan and Yilmaz, 2020).

Tannins

These constituents are abundantly present in *C. coggygria*, a fact that relates to its taxonomic affiliation to the Anacardiaceae family–a group with economically important tanning plants like quebracho (*Schinopsis* spp.) and sumacs (*Rhus* spp.). These polyphenols are able to establish bonds with proteins, and precipitate them–a property which is at the heart of their bioactivity. Their interaction with dermal collagen fibers explains their cicatrizing effects, while the precipitation of bacterial proteins by tannins correlates with their antibacterial effect (Bruneton, 2016). Smoketree contains significant amounts of hydroysable tannins derived from gallic acid. Pentagalloyl glucose (1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose, **Figure 1**), the ester of glucose with five moieties of gallic acid has been one of the first tannins identified from the plant (Westenburg et al., 2000). The tannin content

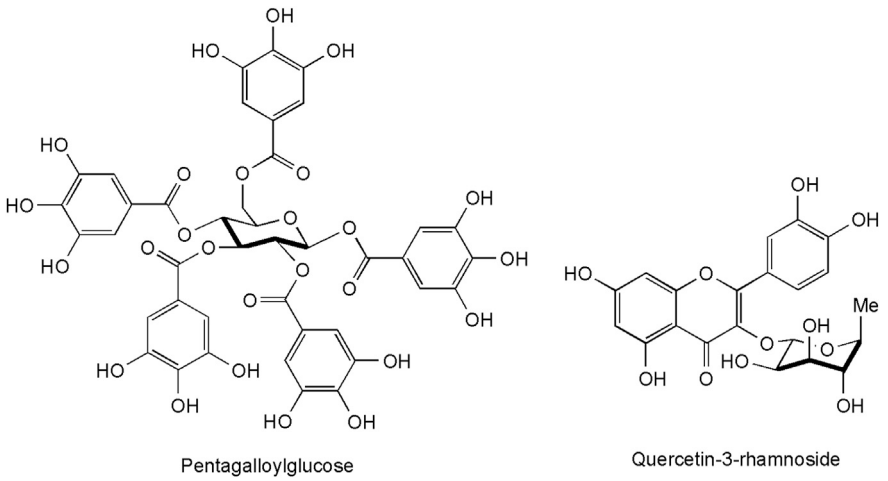

FIGURE 1 | Representative compounds from the leaves of *C. coggygria*.

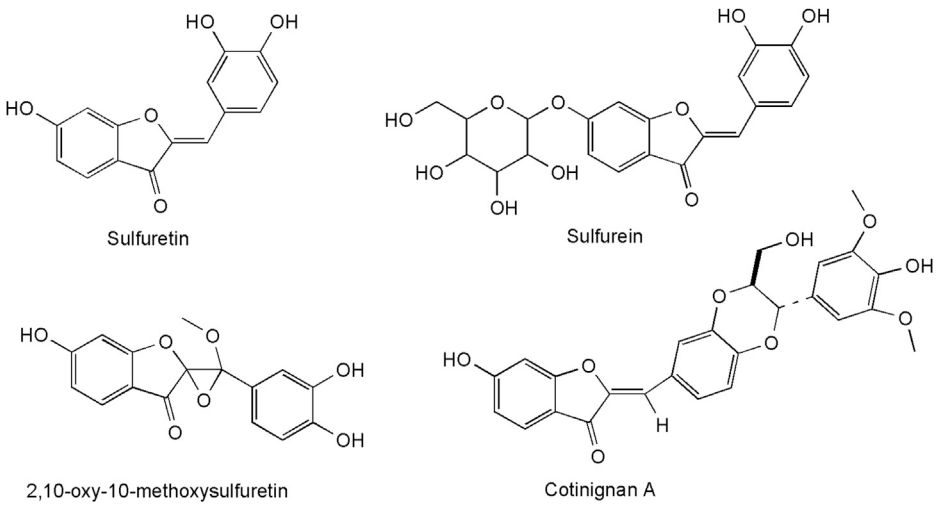

FIGURE 2 | Structure of condensed tannins from *C. coggygria*.

of leaves was reported to be highest during the flowering period, attaining 18–20% both according to earlier (Buziashvili et al., 1973) and more recent research (Šavikin et al., 2009). The most extensive data on the chemical structure of leaf tannins were reported by Rendeková and co-workers; identifications were performed upon comparison of UV and mass spectra with those from the literature or authentic standards. The presence of galloyl glucose (glucogallin), protocatechuic acid hexoside, trigalloyl hexoside, and tetragalloyl hexoside is accordingly added to the previously known hydrolysable tannins (Rendeková et al., 2016). Gallocatechin was confirmed in the leaves by (Özbek et al., 2016).

Flavonoids

Among plant species, smoketree stands out through the presence of nearly all flavonoid subclasses. These compounds have especially been investigated in stems and branches, as these parts are used for the obtainment of an orange-hued dye, containing a mixture of flavonoids.

The Heartwood

The most representative metabolite class of *C. coggygria* in the heartwood are the aurones: sulfuretin (3′,4′,6-trihydroxyaurone), its glucoside sulfurein (glycosil-7-O-sulfuretin), and its dimer disulfuretin (Westenburg et al., 2000). The latest discovery concerns two new metabolites from the plant kingdom: an aurone epoxide (2,10-oxy-10-methoxysulfuretin) and an auronolignan (**Figure 2**). In cotinignan A, the first compound of this class, the aurone sulfuretin is bridged to sinapyl alcohol by a benzodioxane ring (Novaković et al., 2019).

Other orange-colored compounds from smoketree wood are butein (trans-2′,3,4,4′-tetrahydroxychalcone) and isoliquiritigenin (trans-2′,4,4′-trihydroxychalcone), members of the chalcone subclass of flavonoids (Valianou et al., 2009).

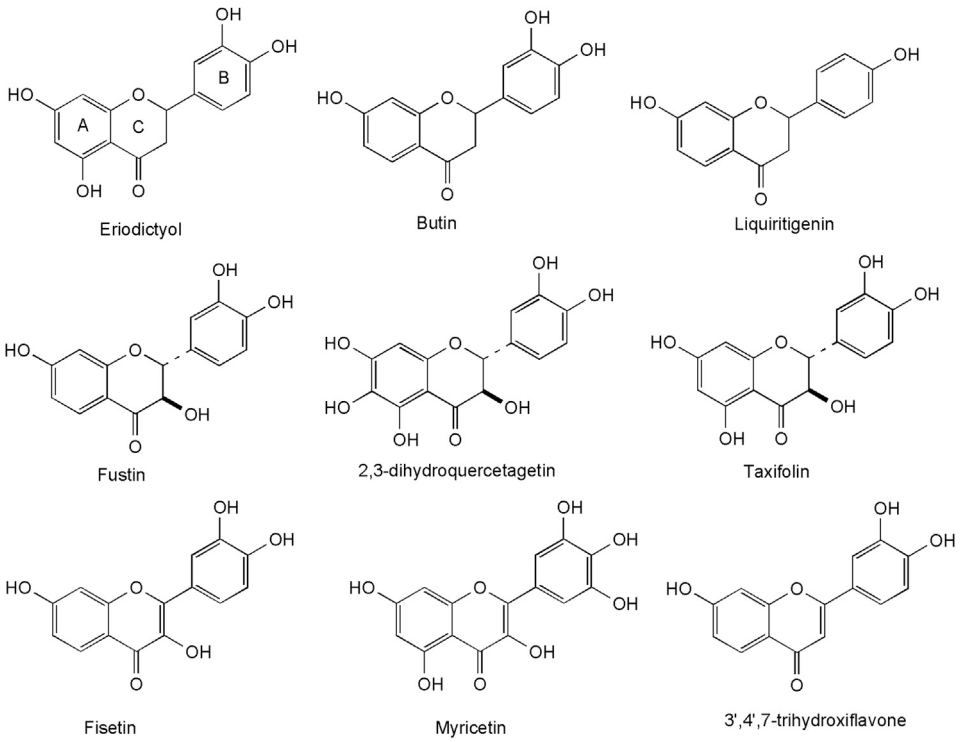

FIGURE 3 | Sulfuretin and its derivatives–typical aurones in *C. coggygria* wood.

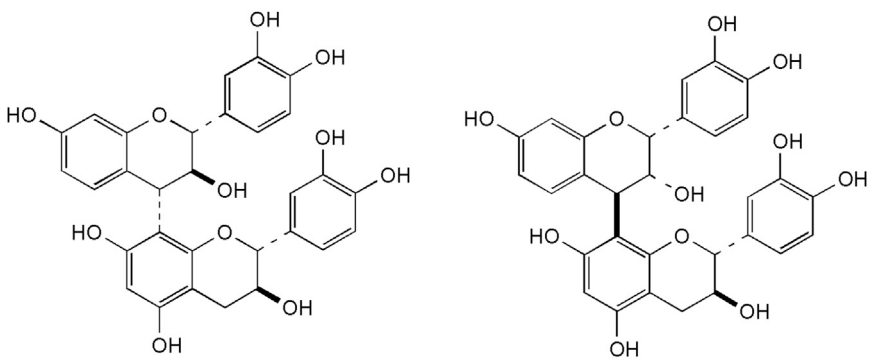

FIGURE 4 | Representative flavonoids with a 6-membered C-ring from the heartwood of *C. coggygria*, consistently displaying a hydroxy group in position 7 of the A-ring.

A high number of flavonoids have been isolated and identified in methanol extracts of the heartwood (**Figure 3**). They include the flavanones: eriodictyol, butin and its dimer (3,3″-butindimer) (Antal et al., 2010), liquiritigenin (4′,7-dihydroxyflavanone) and 4′,5,7-trihydroxyflavanone (Valianou et al., 2009), 3′,5,5′,7-tetrahydroxyflavanone (Novaković et al., 2019), the flavanonols: taxifolin and 4′,7-dihydroxyflavanol (Valianou et al., 2009), 2,3-dihydroquercetagetin (Antal et al., 2010), 2,3-trans-fustin, 3-O-methyl-2,3-trans-fustin, 3-O-galloyl-2,3-trans-fustin (Novaković et al., 2019), the flavonols: fisetin, quercetin (Antal et al., 2010) and myricetin (Matić et al., 2013), as well as the flavone: 3′,4′,7- trihydroxyflavone (Novaković et al., 2019). A

hallmark of most compounds is the presence of a hydroxy group in position 7 and the absence of a hydroxy-group in position 5 of the A-ring, considered as taxonomical markers for *C. coggygria* (Novaković et al., 2019).

Several derivatives of flavan-3-ols were isolated and structurally identified in the heartwood (Antal et al., 2010). These plant phenolics are frequent in plants, but particular attention has been given to their occurrence and biologic activity in green tea, pine bark, cacao beans, grape, cranberries and hawthorn (de la Luz Cádiz-Gurrea et al., 2014). The occurrence of polyphenolic dimers is typical for Anacardiaceae plants[2][Stevens APG website]. Two proanthocyanidins (fisetinidol-(4α→8)-(+)-catechin and epifisetinidol-(4β→8)-(+)-catechin, **Figure 4**) were reported so far from smoketree (Antal et al., 2010) They were obtained from the diethyl-ether soluble phase of *C. coggygria* crude extract, following a four-step procedure (vacuum liquid chromatography on RP-18 material, followed by gel-filtration on Sephadex LH-20, high-speed coutercurrent chromatogrphy, and a second column chromatography using Sephadex LH-20). Their structure assignment of proanthocyanidins presented a challenging case of signal duplication due to rotational isomerism (Antal et al., 2010). Dimers occur beside monomer catechin, isolated as a racemic mixture.

Other compounds. A series of phenolic acids were isolated from the heartwood of the plant: gallic acid and its methyl ether in leaves, twigs and heartwood (Westenburg et al., 2000; Antal et al., 2010), galloylshikimic acid, trigallic acid, methyl digallate, trigallate (Rendeková et al., 2016), and β-resorcylic acid (Novaković et al., 2019) in the heartwood. According to Matić and co-workers, the methanol extract of *C. coggygria* stem contains chlorogenic, caffeic, coumaric, ferulic and rosmaric acid, with the latter being the major representative of its class in the extract (Matić et al., 2013). Moreover, the heartwood also contains 3-O-β-sitosterol glucoside (Novaković et al., 2019).

Young Shoots

Young branches have a different flavonoid profile than the heartwood. The flavonoids here include kaempferol-3-O-glucoside, luteolin-7-O-glucoside, luteolin-8C-glucoside (orientin), and apigenin glycoside. Beside them, gallic acid and its derivatives were as well identified (Marčetić et al., 2013).

Leaves

As leaves were mainly considered a source of tannins, reports on the structure of leaf flavonoids are scarce. Early studies showed that these compounds are based on three main aglycons: myricetin, quercetin, and kaempferol. Sugars of flavonoid glycosides are bound in position 3 of the aglycon and are represented by D-glucose, L-rhamnose, and L-arabinose. Glycosides of the myricetin group (75–80%) are dominant over those of the quercetin group (18–23%), while kampferol glycosides are present in traces (Buziashvili et al., 1973). These early results were confirmed by recent HPLC analysis, identifying the glucosides and rhamnosides of myricetin and quercetin in crude extracts of smoketree leaves (Rendeková et al., 2016). Myricetin-3-O-rhamnoside and myricetin-3-O-galactoside were confirmed spectroscopically in the ethylacetat fraction of a leaf extract (Özbek et al., 2016). Another group of flavonoids, the anthocyanins, are responsible for the various shades of red of smoketree leaves. They include delphinidin 3-galactoside and 7-glucoside, cyanidin 3-galactoside (idaein) and 3-glucoside-7-rhamnoside as well as petunidin 3-glucoside (Tanchev and Timberlake, 1969; Iwashina, 1996).

Essential Oil Components

The volatile organic compounds from *C. coggygria* have thoroughly been investigated in plants from a variety of regions, ranging from Western Europe to the Himalayas. Due to its aromatic scent, the essential oil of the plant is used in perfumery. Bulgaria is one of the countries producing essential oil form leaves and young twigs of smoketree (Tsankova et al., 1993). The main volatile components are monoterpenes. Limonene dominates in plants from Serbia (Novaković et al., 2007) and Italy (Fraternale and Ricci, 2014). In Greek smoketree, the main components varied according to the site, with limonene being dominant in some samples and myrcene in others (Tzakou et al., 2005). Site-specific variations have as well been pointed out in Turkey, where limonene (Demirici et al., 2003), alpha-pinene (Ulukanli et al., 2014) or geranyl acetate (Bahadirli, 2020) were be the main components. Alfa-pinene is the main compound in essential oil from Bulgarian smoketree samples (Tsankova et al., 1993). Smoketree growing in the northern part of India has myrcene as main volatile constituent (Thapa et al., 2020).

PHARMACOLOGICAL DATA ON THE SMOKETREE

The long-lasting and consistent use of *C. coggygria* in traditional medicine across countries offers a sound base for its therapeutic use. However, data on the pharmacology of *C. coggygria* extracts are, for now, at a rather basic stage. Among explored properties, the anti-inflammatory effect was the first to attract the attention of researchers and to be tested experimentally (Bezruk and Lyubetskaya, 1969). Extracts obtained from both leaves and heartwood reduced inflammation in rodent models, while the water extract inhibited cyclooxygenases -1 and -2 (**Table 1**).

Wound-Healing Effect

In virtually all countries where smoketree is used in traditional phytotherapy, the wound-healing effect is the most cited one. In order to validate this effect (Aksoy et al., 2016), employed a model of excision wounds in rats with experimentally induced diabetes. The topical application of an ointment containing 5% ethanol extract from leaves demonstrated a significant wound healing effect, confirmed histologically. The elevation of hydroxyproline levels, a precursor of collagen, together with favorable antioxidative effects (elevation in glutathione and decrease in malondialdehyde levels) supported the favorable effect of the

[2]Stevens, P.F. Angiosperm Phylogeny Website. ˆ http://www.mobot.org/MOBOT/research/APweb/ (Accessed December 3, 2020)

TABLE 1 | Studies evaluating the anti-inflammatory effect of smoketree extracts and fractions.

Extract	Experimental model	Results	References
Water extract of erial parts	*In vitro* inhibition of ovine COX-1 and human recombinant COX-2	EC_{50} = 2.21 ± 0.18 mg/ml for COX-1 inhibition; EC50 = 4.10 ± 0.27 mg/ml for COX-2 inhibition; Extract had best results in comparison to that of other 8 medicinal plants	Ozsoy et al. (2017)
Heartwood/dietly ether fraction of methanol extract standardized to its content in S, B, fustin, 2,3-dihydroquercetagetin, and quercetin	Mouse ear edema (SKH1 male mice) induced with 12-O-tetradecanoyl phorbol-13-acetate; corneometric assessment	External application of 2 mg extract reduced inflammation with 50%; reduction with 26% of the skin dehydration induced by 12-O-tetradecanoyl phorbol-13-acetate	Antal et al. (2015)
Aqueous infusion from *Cotinus coggygria* leaves	Rats with the carrageenan induced paw edema	Protective effect against inflammation following the intragastric administration of the extract (10 ml/kg) for 15 days	Pavlov et al. (2014)
Young shoots/ethyl acetate fraction of acetone extract	Rats with the carrageenan induced paw edema	Doses of 50 mg/kg and 100 mg/kg reduced theoedema with 46.5 ± 18.5% and 76.7 ± 0.0%, respectively	Marčetić et al. (2013)
Total flavonoids isolated from *C. coggygria* leaves	Formalin-induced edema in mice	Oral administration of 80–160 mg/kg 2 h before and 5 h after formalin application reduced edema, decreased proliferation of cellular elements and raised capillary resistance	Bezruk and Lyubetskaya (1969)

COX: cyclooxygenase; EC50: half maximal effective concentration.

extract on wound healing. The wound healing effect is also supported by the antimicrobial effect of *C. coggygria* extracts and essential oils, comprehensively reviewed by (Matić et al., 2016). In order to seek scientific validation for the use of smoketree in the treatment of gastritis, Pavlov and co-workers evaluated the benefits of an aqueous infusion from leaves in indomethacin-induced damage of the gastric mucosa. The intragastric administration of 10 ml 2% infusion reduced the number and area of ulcerations, as well as their depth and severity. This effect was accompanied by the decrease of malondialdehyde and uric acid levels, reducing as well the activity of alkaline phosphatase (Pavlov et al., 2013a).

Hepatic Disorders

Traditionally, smoketree preparations were as well used in liver disease (Shen et al., 1991). The hepatoprotective effect were demonstrated in rodent models (Matić et al., 2011; Matić et al., 2013). Liver damage was induced by pyrogallol, and the protective effect of a relatively high dose of methanolic extract (500 mg/kg) from smoketree stem was administered either 2 or 12 h prior to the administration of pyrogallol. The effects were compared to those of myricetin, found to be the main constituent of the extract. Pretreatment with the extract, but as well with myricetin resulted in a hepatoprotective effect, reducing the elevation of serum AST, ALT, ALP and total bilirubin levels that were induced by pyrogallol. Administration of natural compounds was most efficacious when performed 12 h before pyrogallol. In quest of the mechanism of action for the observed effect, the authors explored the status of Akt or protein kinase B in the liver of rats pretreated with smoketree extract or myricetin by immunoblot analysis. Administration of the natural products, either 2 h or 12 h before the pyrogallol application increased Akt activity and phosphorylation. Moreover, an enhanced STAT3 phosphorylation was reported, enabling the adequate activation of the JAK–STAT signaling pathway, an important cascade involved in signal transduction during hepatic injury (Matić et al., 2013). Administration of *C. coggygria* extract lead to an augmentation of acute phase reactants haptoglobin and a2-macroglobulin with protective role in hepatic lesions. It improved as well markers of oxidative stress, and inhibited NF-kB (Matić et al., 2011). The hepatoprotective effect of smoketree is currently used clinically as part of a compound formula used orally in Traditional Chinese Medicine, where it is mixed with other plant extracts obtained from *Schisandra chinesis*, Chuipencao (*Sedum sarmentosum*), wolfberry (*Lycium chinense*), jujube (*Ziziphus jujuba*), gardenia (*Gardenia jasminoides*) (Hao et al., 2018; Su et al., 2018).

Pavlov et al. (2013b) investigated the toxicity of the aqueous infusion prepared from leaves (1, 2, 4%), administered intragastrically for 30 days in a concentration of 10 ml/kg. The authors did not detect pathological modifications in the organs of the treated rats, no subchronic hepatic or renal toxicity were noted. Moreover, no changes in the activity of hepatic enzymes and levels of urea, creatinine, total thyols and triacylglycerols were found. The potential toxicity was further studied for a 20% ethanol infusion; no lesions of the liver and kidney were reported (Ivanova et al., 2013).

Diabetes

There are some reports of *C. coggygria* form Turkish ethnomedicine, that describe its utilization in diabetes (Kültür, 2007). Some basic steps were performend until now to verify this indication, with regard to the inhibition of key enzymed involved in the disease. A methanol extract and its fractions (petroleum ether, dichloromethane, ethyl acetate, and n-butanol) obtained from leaves smoketree were evaluated *in vitro* with regard to their α-glucosidase and α-amylase inhibitory activities. The ethyl acetate-soluble fraction displayed the highest efficacy (IC_{50} = 0.0082 mg/ml), having an inhibitory activity roughly ten times higher than the other fractions. Alfa-amylase was not inhibited in

that experimental setting (Gözcü et al., 2016). The α-glucosidase inhibitory activity was also reported for the ethanol extract prepared from the branches of the shrub. After bioactivity-guided fractionation, 2,3,4,6-penta-*O*-galloyl-β-D-glucose was shown to have the best α-glucosidase inhibitory effect, with an IC_{50} of 0.96 mg/ml. Comparatively, the related compoung with only four gallic acid residues 1,2,3,6-tetra-O-galloyl-β-D-glucose and gallic acid had week inhibitory effects (Cha et al., 2009).

Cancer

The anti-cancer effects of *C. coggygria* have received the highest attention in the last period, since the last review on smoketree. References to this regard exist in traditional medicine (Kültür, 2007; Redžić, 2007). Some works explored the cytotoxic effect of smoketree extracts and metabolites on different cell lines, while others pointed out protective effects via antioxidant and anti-mutagenic activities (Westenburg et al., 2000; Matić et al., 2013). One of the earliest studies investigating the cytotoxic effects of this plant focused on the methanol extracts from leaves and flowers, rich in gallic acid and its derivatives. The extracts were applied on two human carcinoma cell lines (cervix–HeLa and colon–LS174). The leaf extract was more active on the HeLa cell line (IC_{50} = 19.1 ± 3.9% μg/mL), while the flower extract had a slightly better inhibition of the LS174 cell line (IC_{50} = 41.3 ± 3.9 μg/ml) (Šavikin et al., 2009).

Gospodinova and co-workers investigated the effects of an aqueous ethanolic extract from smoketree leaves, standardized in total polyphenols and flavonoids (2017 a,b,c). The cytotoxicity tests involving human breast cancer cells (MCF7) showed a significant cytotoxic activity (IC_{50} = 40.6 μg/ml) which was not dose-dependent (Gospodinova et al., 2017a). In a second study, the inhibitory effects on three additional cell lines (breast cancer T47D, cervical cancer HeLa, and ovarian cancer A2780) were explored, in comparison a non-cancerous cell line (breast epithelial cell line MCF10A). Ovarian cancer cells were the most sensitive, being inhibited at an IC_{50} = 30.8 μg/ml; a certain degree of selectivity against cancer cells were noted in comparison with normal cells (Gospodinova et al., 2017b). The research of a possible mechanism of action lead to the investigation of histone deacetylase activity in MCF7 cells following the application of the leaf extract; high levels of histone deacetylase activity is characteristic for cancer cells. The authors reported a significant reduction of HDAC5 and HDAC7 mRNA transcripts at 2 days post-treatment. A tendency to reduce HDAC3 transcriptional levels also resulted from the research (Gospodinova et al., 2017c). The last paper of this work-group focused on the cytotoxic effects against A431 cells (human squamous cell carcinoma), in comparison with normal skin cells (BJ line). The selectivity against cancer cells was observed, and the best anticancer activity was achieved by the chloroformic fraction of the extract, in comparison to that of the aqueous fraction (Gospodinova et al., 2020). Another extract obtained from leaves with ethyl acetate as solvent was applied on normal skin cells (BJ) and mammary gland epithelium cells (MCF-10A). The extract inhibited the proliferation of activated human fibroblasts expressing fibroblast activation protein α (FAP). This is a serine protease implicated in the genesis and progression of tumors (Iliev et al., 2020). In the study of (Pollio et al., 2016), ethanol extract of *C. coggygria* branches and leaves showed significant cytotoxic effects, and both cell morphology and growth were affected. The effects on the cell cycle were consistent in all cell lines (A549, MCF7, TK6 and U937), irrespective of their phenotype (adherent or in suspension). The expression profiles of distinct proteins controlling the progression of the cell cycle and cell death were altered. The percentage of cells in the G1 phase was induced by the extract (Pollio et al., 2016).

The cytotoxic effect of *C. coggygria* on gliobastoma cells (lines U87, U251, and DBTRG-05MG) were investigated by (Wang et al., 2015a; Wang et al., 2015b; Wang et al., 2016). The authors evaluated an ethanol (95%) extract from roots and stems, which contained 4% flavonoids: rutin, fisetin, quercetin, and myricetin. These flavonoids were also used as controls in the assays. The extract was able to inhibit the proliferation of the three cell lines (IC_{50} = 128.49 μg/ml for U87, 107.62 μg/ml for U251, and 93.57 μg/ml for DBTRG-05MG). It induced apoptosis via Akt inhibition, coupled with ERK protein expression. Moreover, the researchers evaluated the significance of their findings in a xenograft model glioblastoma multiforme in female BALB/c mice, reporting a significant antitumor effect. The reduction of the tumor volume after administration of 25 and 50 mg/kg extract (evaluated at 7, 14, 21, and 28 days) was in the same range as in case of the positive control, temozolomide (Wang et al., 2015a). In a subsequent study, the same extract was loaded into polyvinylpyrrolidone K-30/sodium dodecyl sulfate and polyethyleneglycol-coated liposome, in order to augment its solubility and to achieve an improved cerebral targeting. The cytotoxic mechanism of action of this preparation was studied *in vitro*. A DBTRG-05MG glioblastoma cell line was employed. Results pointed to a caspase-dependent activation of both the intrinsic and extrinsic signaling pathways of apoptosis. The proposed mechanism of the proapoptotic effect in glioblastoma cells involved the blockade of the SIRT1/p53-mediated mitochondrial pathway and of the Akt pathway (Wang et al., 2015b). The significant inhibitory effect of glioblastoma cells that the ethanol extract from roots/stem of *C. coggygria* had, was confirmed by an additional work. This study was also performed on a second extract (total flavonoids isolated from *C. coggygria* var. *cinerea*). Both extracts reduced cell proliferation and downregulated the signaling pathways PI3K/Akt and ERK (Wang et al., 2016).

The cytotoxic effects of the diethyl ether-soluble fraction obtained from smoketree heartwood were assessed before and after complexation with two cyclodextrins. Along with the extract, two of its marker compounds (the aurone sulfuretin and the chalcone butein) were as well studied. Among the four cell lines that were used in the work (HeLa, A2780, MCF7, and MDA-MB-231), the most sensitive against *C. coggygria* extract was ovarian carcinoma cell line A2780. The proliferation of these cells were inhibited in the low micromolar range. Butein had the highest activity against HeLa cells, both included in randomly methylated-β-cyclodextrin and in uncomplexed form. Sulfuretin and its complexes had a generally weaker cytotoxicity (Antal et al., 2016).

Other Effects

As for many other medicinal plants, in addition to the validation of effects known from traditional medicine, modern research aims to expand the uses of this species. In this regard, a potential field is represented by neurological disorders. Eftimov and co-workers explored the effects of an aqueous infusion from leaves (1, 2 and 4%, 10 ml/kg) on the behavior of rats, and its protective effect against lipid peroxidation (Eftimov et al., 2016). The effects were evaluated after 30 days of treatment. In the forced swim test, the timespan of immobility increased, and the effect was significant for the 2% infusion. The infusion had no sedative effect, did not induce motor dis-coordination, but reduced signs of depression. The decrease in malondialdehyde levels was not significant and the authors concluded that the positive effects on brain function were not related to an antioxidative effect (Eftimov et al., 2016).

Acetylcholine esterase inhibition is a major target for drugs combating neurodegenerative conditions like Alzheimer's disease and Parkinson's disease. A screening of various plant extracts with Ellman's reagent pointed to *C. coggygria* as having a real potential to inhibit this enzyme. The best activity was achieved by the crude methanol extract of the heartwood (IC_{50} = 89.3 μg/ml). In order to find the most active compounds, this extract was partitioned in solvents of increasing polarities (petroleum ether, diethyl ether, ethyl acetate, n-butanol, and water), with the diethyl ether fraction having the highest efficacy (IC_{50} = 25.4 μg/ml) due to its content in sulfuretin (IC_{50} = 29.9 μM). After intracerebral injection, the extract elicited an increased acetlycoline concentration (Antal et al., 2008).

Recently, *C. coggygria* extracts have been studied with regard to their potential in skin care, evaluating the effects as inhibitors of collagenase and elastase, fundamental components of the connective tissue which are responsible for the resistance and elasticity of the skin. The research also tested tyrosinase inhibitory effect, in order to identify a potential utilization as skin lightener. Departing from ethanol extracts of pedicels and leaves, an activity-guided fractionation was performed in order to point out the most active compounds. After partition, it was the ethyl-acetate soluble fraction of the pedicels that had the best effect on collagenase inhibition accompanied by a moderate inhibition of elastase and tyrosinase. The main components of the active fractions were methyl gallate, astragalin, isoquercetin, and hyperoside (Deniz et al., 2020).

COTINUS COGGYGRIA AND TOXICODENDRON VERNICIFLUUM: COMMON GROUND

Despite the research performed up to the present on *C. coggygria*, mandatory data are yet missing in the pharmacology of this species, including pharmacokinetic tests and clinical trials. Interestingly, as these data are lacking for extracts and fractions, they exist for some of the main compounds of smoketree heartwood: sulfuretin, fisetin, butein, owing mostly to the presence of these and other compounds in the Chinese lacquer (*Toxicodendron vernicifluum*, syn. *Rhus verniciflua*). In fact, *T. vernicifluum* is has a firmly established position in traditional Asian medicine. Not only have there been performed comprehensive *in vitro* and *in vivo* studies on this species, but clinical data are also available (Kim et al., 2014). Most of these studies used extracts standardized in fustin (>13.0%), fisetin (7.0%), sulfuretin, butein and other compounds (Lee et al., 2009; Lee et al., 2011). The pharmacokinetic profile of several active compounds has also been reported (Jin et al., 2015).

For *T. vernicifluum*, over 175 constituents have been isolated and described. A review of the literature on key compounds from smoketree heartwood shows that *C. coggygria* and *T. vernicifluum* share an impressive pool of secondary metabolites (**Table 2**). This situation may even change the systematics of *C. coggygria* in reassigning it back to its initial genus, *Rhus* (Novaković et al., 2019). In both species, the major components are sulfuretin and fustin (Antal et al., 2010; Li M. C. et al., 2020).

The high number of shared compounds points to possible similarities in the pharmacology of both species. Moreover, it may provide a significant support in advancing the practical use of the European species into other fields of therapeutic significance, which have already been well developed for *T. vernicifluum*. To this date, the pharmacologic activities that have been reported for this species are: anti-inflammatory, anti-oxidant, anti-cancer, anti-microbial, anti-diabetic, anti-dislipidemic, anti-platlet, anti-vasoconstrictor, as well as protective of the liver, kidneys, neurons and gastro-intestinal tract. The numerous *in vitro* and *in vivo* highlighting these effects make the object of a well-documented review by (Li W. et al., 2020). A significant drawback of scientific literature focused on the pharmacology of plant extracts, including those from *T. vernicifluum*, is represented by missing or incomplete data on the plant part, the extraction solvent(s) and key marker compounds. Representative studies investigating lacquer extracts standardized in compounds that occur in both species are listed in **Table 2**. An important advantage of *C. coggygria* over *T. vernicifluum* is the absence of allergenic urushiols (Ippen 1983; Bertrand et al., 2008), which are common in the Asian species (Li et al., 2021).

BIOACTIVITY OF COMMON SECONDARY METABOLITES

Key elements in understanding and advancing the pharmacology of smoketree come from studies investigating the metabolites that occur in the plant. Most of the available data come from research on fisetin, sulfuretin and butein, important flavonoids occuring in the woody parts of *C. coggygria* and *T. vernicifluum* (**Figure 5**,**6**).

Fisetin benefited from a special attention in recent research (Chen et al., 2020; Yan et al., 2021). This flavonol is also present in many edible plants such as strawberries, onions, apples or persimmons. The fisetin content in plants is between 2 and 160 μg/g (Kashyap et al., 2018). It was first isolated from *Rhus cotinus* and studies conducted over time showed anti-inflammatory, anticancer, antioxidant and neuroprotective effects (Grynkiewicz and Demchuk, 2019). Of particular interest is ability of fisetin to reduce senescence markers in

TABLE 2 | Secondary metabolites occuring both in the heartwoods of *Cotinus coggygria* (syn. *Rhus cotinus*) and *Toxicodendron vernicifluum* (syn. *Rhus verniciflua*).

Compound	*Cotinus coggygria*	*Toxicodendron vernicifluum*
Sulfuretin	Westenburg et al. (2000)	Li Y. et al. (2020)
Butein	Valianou et al. (2009)	Li M. C. et al. (2020)
Isoliquiritigenin (*trans*-2′,4.4′-trihydroxychalcone)	Valianou et al. (2009)	Li W. et al. (2020)
Eriodictyol	Antal et al. (2010)	Li Y. et al. (2020)
Butin	Antal et al. (2010)	Li M. C. et al. (2020)
3.3″-butindimer	Antal et al. (2010)	Li W. et al. (2020)
Liquiritigenin (4′,7-dihydroxyflavanone)	Valianou et al. (2009)	Li Y. et al. (2020)
Taxifolin	Valianou et al. (2009)	Jin et al., 2015
2,3-Dihydroquercetagetin	Antal et al. (2010)	Li M. C. et al. (2020)
2,3-trans-Fustin	Novaković et al. (2019)	Li W. et al. (2020)
3-*O*-methyl-2,3-*trans*-fustin	Novaković et al. (2019)	—
3-*O*-galloyl-2,3-*trans*-fustin	Novaković et al. (2019)	Li Y. et al. (2020)
Fisetin	Antal et al. (2010)	Li M. C. et al. (2020)
Quercetin	Antal et al. (2010)	Hashida et al., 2014
3′,4′,7- trihydroxyflavone	Novaković et al. (2019)	Kim et al., 2010
Gallic acid	Antal et al. (2010)	Kim et al., 2010
Methyl gallate	Antal et al. (2010)	Yang P.Y. et al. (2018)
β-resorcylic acid	Novaković et al. (2019)	Li W. et al. (2020)

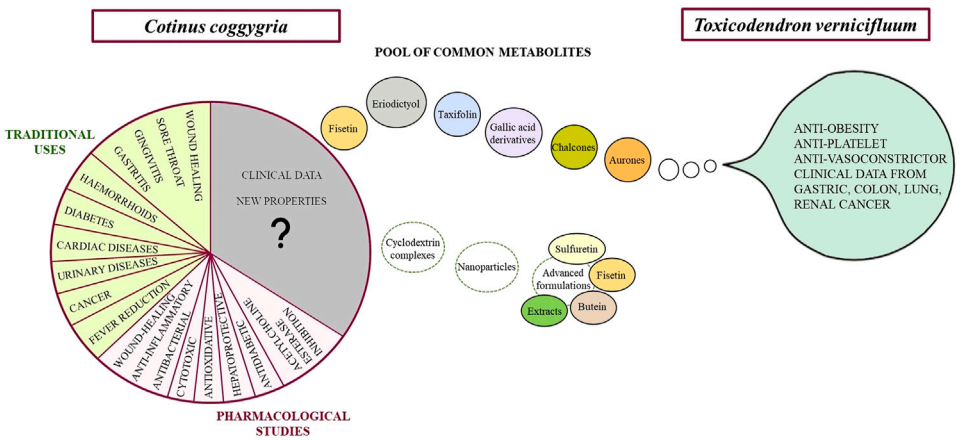

FIGURE 5 | Benefits of an interconnected approach for an optimized valorization of smoketree.

mice and human tissues. A reduction of senescent cells fraction in white adipose tissue of naturally aged C57BL/6 mice after fisetin treatment was reported (Yousefzadeh et al., 2018). Evaluation of fisetin effects in bleomycin-induced pulmonary fibrosis revealed an inhibition of alveolar epithelium cell senescence through regulation of AMPK/NF-κB signaling pathway. The anti-fibrotic activity of fisetin was therefore associated with the senolytic properties of this compound (Zhang et al., 2020). The accumulation of senescent cells has been associated with aging and with age-related dysfunctions. The characteristics of these cells have been studied and agents capable of eliminating them have been sought (Wissler Gerdes et al., 2020). Thus, senolytic agents, including fisetin, have been proposed to delay aging and reduce the severity of age-related diseases (Yousefzadeh et al., 2018).

Fisetin exerts an antiatherosclerotic effect in mice by reducing the lipid and malondialdehyde (MDA) levels and increasing the level of superoxide dismutase (SOD). The reduction of the atherosclerotic plaque was observed. Fisetin also regulates the expression of LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1) and PCSK9 (proprotein convertase subtilisin/kexin type 9) thus improving lipid metabolism (Yan et al., 2021). Association of fisetin with recombinant tissue plasminogen activator extended the therapeutic window of treatment in acute ischemic stroke. Patients receiving 100 mg fisetin daily for seven days exhibited lower levels of C-reactive protein, MMP-2 (matrix metalloproteinase-2) and MMP-9 (matrix metalloproteinase-9). Therefore, fisetin has shown potential for use as a supplement in reperfusion therapy with recombinant tissue plasminogen activator (Wang et al., 2019). A protective effect on ischemic cardiomyocytes was observed when fisetin was tested in a rat ischemia/reperfusion injury model. Fisetin reduced apoptosis in myocardial cells, prolonged coagulation and decreased Von Willebrand factor plasma levels (Long et al., 2020).

Given the association of neurodegenerative diseases with alterations in gut microbiota, the effects of fisetin as neuroprotective agent have been studied in a mice model of Parkinson's disease. Fisetin improved motor behavior and protected against dopaminergic neurodegeneration induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). It also increased the abundance of Lachnospiraceae, activity associated with its neuroprotective properties (Chen et al., 2020).

The anticancer effect of fisetin has also been studied, and the mechanisms by which it acts in cancer have been highlighted in several studies. Its effects have been evaluated in prostate cancer, colon cancer, pancreatic cancer, liver cancer, lung cancer, ovarian cancer or breast cancer cell lines (Imran et al., 2020). Fisetin inhibits the proliferation of pancreatic cancer cells PANC-1 and induces autophagy and apoptosis (Jia et al., 2019). It has been proposed as an adjuvant to 5-fluorouracil treatment in PIK3CA-mutant colorectal cancer. The association of 5-fluorouracil and fisetin resulted in a marked decrease in the viability of cancer cells. The combination determined inhibition of AKT phosphorylation and decreased PI3K (phosphatidylinositide-3-kinase) expression. A preventive effect against tumor formation was also observed for fisetin (Khan et al., 2019). Fisetin acts on oral squamous cell carcinoma by targeting PAK4 (p21-activated kinase 4). Promotion of cell apoptosis and inhibition of proliferation and migration have been reported after treatment of cancer cells with fisetin (Li W. et al., 2020). A clinical trial that included patients with colorectal cancer evaluated the effects of fisetin on inflammatory status. Administration of 100 mg fisetin per day for seven weeks, before and during chemotherapy, resulted in a decrease of hs-CRP (high-sensitivity C-reactive protein) and IL-8 (interleukin-8) levels in the intervention group, revealing the anti-inflammatory activity of this compound when used as a complementary treatment in colorectal cancer (Farsad-Naeimi et al., 2018).

Fisetin has also been studied as an anti-diabetic. In an *in vitro* model of experimental diabetes, oral administration of fisetin (10 mg/kg) reduced blood glucose and glycosylated hemoglobin levels, while the plasma insulin level was increased. This flavonoid also lead to a decrease of mRNA and expression levels of gluconeogenic genes (phosphoenolpyruvate carboxykinase, glucose-6-phosphatase) (Prasath et al., 2014). The favorable effects of gluconeogenesis inhibition in the liver by fisetin are of relevance for the antidiabetic effects of both *C. coggygria* and *T. vernicifluum*.

A recent study showed that fisetin stimulates hair growth. When applied topically in C57BL/6 mice, fisetin acts on hair growth by stimulating the transition from telogen to anagen phase, effect related to its activity on TERT (telomerase reverse transcriptase) expression in the dorsal skin cells of treated mice (Kubo et al., 2020).

Sulfuretin is a major compoud of smoketree wood, emblematic for its golden color. Among the reported effects for this compound are the anti-inflammatory, anticancer, neuroprotective and antidiabetic ones (Pariyar et al., 2017). One of the first studies reported that this aurone is active in rheumatoid arthritis (Choi et al., 2003). The research of the mechanism of the anti-inflammatory pointed to an induction of heme oxygenase-1 expression (Lee et al., 2010), inhibition of LPS-induced inducible nitric oxide synthase, inhibition of cyclooxygenase-2, and reduction of the expression of pro-inflammatory cytokines via down-regulation of NF-kappaB (Shin et al., 2010). *In vivo.* sulfuretin maintained joint integrity, a result underscored by radiologic and histopathologic evidence (Lee et al., 2012). As a potential anti-diabetic, sulfuretin inhibits aldose reductase, diminishes formation of glycation end products (Lee et al., 2008), and protects against cytokine-induced beta-cell damage in experimentally induced diabetes (Song et al., 2010). The anti-obesity effect of sulfuretin was emphasized in a study performed on obese mice. Decrease of total cholesterol and triglycerides levels, inhibition of lipid accumulation, as well as improvement of glucose metabolism were observed. Sulfuretin also prevented body weight gain (Kim et al., 2019). The anti-adipogenic activity has been associated with the properties of sulfuretin to supress adipogenic factors such as PPARγ (peroxisome proliferator-activated receptor γ), C/EBPα (CCAAT/enhancer-binding protein α) and C/EBPβ. Sulfuretin exhibited dose and time dependent proliferation-reducing effects of 3T3-L1 preadipocytes cells (Lamichhane et al., 2017).

Hepatoprotective properties have as well been reported for sulfuretin. When tested on L02 hepatic cell line, sulfuretin decreased reactive oxygen species levels and protected cells against treatment with palmitate. It acts by promoting mitophagy and the phenolic hydroxyl group was considered to be essential for the cytoprotective properties (Lu et al., 2019).

Sulfuretin showed beneficial effects in an atopic dermatitis mouse model, inhibiting IL4 production and improving several symptoms of the disease. It reduced the severity of skin lesions, the scratching frequency and decreased IgE serum levels (Jiang and Sun, 2018).

Anti-Parkinson activity was evaluated in SH-SY5Y neuroblastoma cells, revealing a protective effect of sulfuretin against the cytotoxicity induced by MPP^+ (1-methyl-4-phenyl pyridinium) neurotoxin. The neuroprotective effects are due to the effects of sulfuretin on ERK and Akt/GSK3β pathways (Pariyar et al., 2017).

Butein. Research regarding the biological properties of butein highlighted a wide range of effects, that indicated a potential of use in chronic conditions due to anti-inflammatory, antioxidant, antidiabetic, neuroprotective and anticancer effects (Padmavathi et al., 2017). The effects of this compound correlate with the medicinal properties reported in ethnopharmaceutic and pharmacological studies. Its anti-ulcerative activity was evaluated in mice with gastric ulcer induced with indomethacin and the administration of butein (10, 20, 40 mg/kg) reduced inflammatory parameters, increased PGE2 (prostaglandin E2) concentrations and reduced ulcer areas (Ugan and Un, 2020). Butein exerts preventive effects against functional β-cell damage, slowing the progression of type 1 diabetes mellitus. This effect was studied in cytokine-induced β-cell damage. The chalcone blocked cytokine-induced NO production, the expression of iNOS, the translocation of NF-κB and inhibited insulin secretion stimulation by glucose (Jeong et al., 2011). Butein has a significant anti-inflammatory effect, inhibiting the

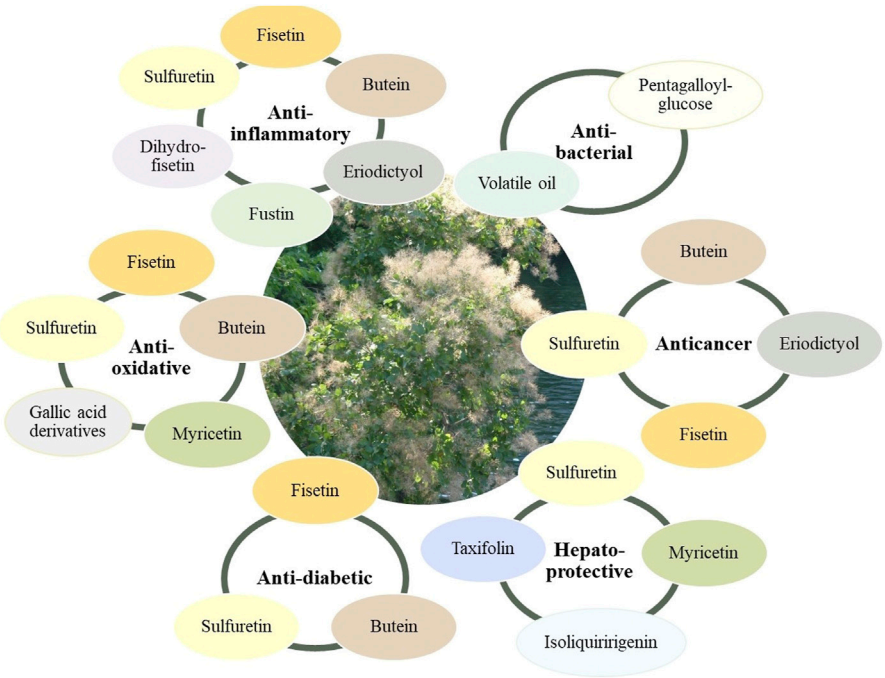

FIGURE 6 | Metabolites from smoketree that have been reported to be responsible for the main pharmacological effects of smoketree.

production of pro-inflammatory cytokines, matrix metalloproteinases, expression of enzymes involved in inflammation such as COX-2; in experimentally induced osteoarthritis it has been shown to reduce cartilage erosion and synovitis (Zheng et al., 2017). Several studies have evaluated the cytotoxic effects of butein on cell lines including human cervical cancer (C-33A, SiHa) (Yang J. et al., 2018), breast cancer MCF7 and T47D (Huang et al., 2020), non-small-cell lung cancer (Di et al., 2019), human oral squamous cancer (SAS, KB) (Bordoloi et al., 2019) and many other. The compound induces apoptosis by activating the Bax-caspase-3-PARP pathway, it induces the arrest of the cell cycle at the G2/M transition, blocks telomerase activity, reduces angiogenesis and has anti-metastatic properties (Jayasooriya et al., 2018).

The health-promoting effects of smoketree extracts are further supported by pharmacological data other secondary metabolites, including fustin, eriodictyol, isoliquiritigenin, taxifolin, myricetin and pentagalloyl-glucose. Fustin (dihydrofisetin), a major flavanonol derivative in heartwoods of *C. coggygria* and *T. vernicifluum*, has antioxidative, antiproliferative and anti-inflammatory effects (Jung et al., 2007). Fustin and an extract of *T. vernicifluum* branches standardized in 3% of this compound were tested in lipopolysaccharide (LPS)-stimulated rats and were able to prevent the elevation of interleukin-6 cytokine, as well as the expression of iNOS, and COX-2 mRNA expression (Moon et al., 2015). This flavonoid also protects against 6-hydroxydopamine-induced neuronal cell death (Park et al., 2007) and reduces experimentally induced learning impairment, due to a muscarinic M1 receptor-mediated anticholinergic effect (Jin et al., 2009).

Eriodictyol is known for anti-inflammatory, cardioprotective, neuroprotective, anti-obesity, antioxidant and anti-cancer (Islam et al., 2020) effects. This flavanone induces apoptosis of CHG-5 and U87MG glioma cell lines, thus presenting anti-tumor effects. The mechanism involved is related to the downregulation of PI3K/Akt/NF-κB signaling pathway (Li et al., 2021). Eriodictyol also targets MEK/ERK signaling pathway, exhibiting antiproliferative properties in human nasopharyngeal CNE1 cancer cells. It inhibits the migration and invasion of cancer cells and induces autophagy (Tang et al., 2020). The anti-arthritic effect of eriodictyol was shown in a study performed in a rat model of rheumatoid arthritis. Eriodictyol (20 mg/kg or 40 mg/kg) was administered orally in rats with collagen-induced rheumatoid arthritis for four weeks and it determined a reduction of paw swelling and decreased IL-6, IL-1β and TNF-α levels (Lei et al., 2020). Eriodictyol showed positive effects on dyslipidemia, insulin resistance, inflammation and hepatic steatosis when administered in diet induced C57BL/6N obese mice. A decrease in triglyceride, total cholesterol and free fatty acids levels was observed after dietary supplementation with 0.005% w/w eriodictyol for 16 weeks. Plasma glucose and plasma levels of pro-inflammatory cytokines were also reduced (Kwon and Choi, 2019).

The chalcone isoliquiritigenin is mostly known from licorice species (*Glycyrrhiza* sp.). Its anti-inflammatory, anti-oxidative, anticancer and protective (hepatoprotective, cardioprotective) effects have been reviewed by (Peng et al., 2015). Taxifolin, myricetin and quercetin are well-studied flavonoids that are already marketed as dietary supplements. They have a widespread occurrence in the Plant Kingdom and their activity

has extensively been covered by recent reviews (Andres et al., 2018; Sunil and Xu, 2019; Song et al., 2020).

Pentagalloyl glucose is a hydrolysable tannin with a high antioxidant activity due to the numerous phenolic hydroxy groups in its structure. Its properties include anti-inflammatory, anti-bacterial, antidiabetic, and anti-cancer properties. It is able to inhibit DNA replication, cause cell cyle arrest in the G1 and S phases, and elicit apoptosis (Zhang et al., 2009). This compound stands out due to its ability to bond to proteins, especially those with a high-proline content like collagens, via hydrogen bonds and hydrophobic forces. This property makes pentagalloylglucose a candidate in the treatment of vascular disease (Patnaik et al., 2019). The ability to precipitate bacterial proteins translates into an intense bacteriostatic effect, combating both Gram-positive and Gam-negative bacteria like: methicillin-resistant and quinolone-resistant *Staphylococcus aureus*, *Streptococcus mutans, Escherichia coli,* and *Pseudomonas eruginosa* (Cho et al., 2010).

PERSPECTIVES

Cotinus coggygria Scop. is a species brought in to the attention of modern pharmacological research via centuries-old traditional medicine. It is not included in European Pharmacopoeas. Most of the effects mentioned in ethnopharmacy found support after scrutiny with *in vitro* and *in vivo* experimental models: wound-healing, anti-bacterial, anti-inflammatory. The chemical composition of the plant includes three major groups of compounds–tannins, volatile organic compounds and flavonoids. With regard to the latter, smoketree showcases a distinctive profile of 5-deoxyflavonoids consisting in notable amounts of sulfuretin, fisetin and butein. Interestingly, this rare profile is shared by the lacquer tree (*Toxicodendron vernicifluum*), a species of Asian origin that is taxonomically related to smoketree. While biomedical research performed on this well-established species in Asian phytotherapy is superposing with some known health-promoting activities of the smoketree, it has a real potential to guide research and utilization toward new therapies. These chiefly include obesity, diabetes, metabolic syndrome, vascular disease and cancer treatment.

AUTHOR CONTRIBUTIONS

AD, AF, PI, and JR wrote this manuscript. AF illustrated the article. SC and DC revised the manuscript. AD presented the writing ideas and prepared the final version of the manuscript.

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REFERENCES

Aksoy, H., Sen, A., Sancar, M., Sekerler, T., Akakin, D., Bitis, L., et al. (2016). Ethanol extract of *Cotinus coggygria* leaves accelerates wound healing process in diabetic rats. *Pharm. Biol.* 54 (11), 2732–2736. doi:10.1080/13880209.2016.1181660

Andres, S., Pevny, S., Ziegenhagen, R., Bakhiya, N., Schäfer, B., Hirsch-Ernst, K. I., et al. (2018). Safety aspects of the use of quercetin as a dietary supplement. *Mol. Nutr. Food Res.* 62 (1), 1700447. doi:10.1002/mnfr.201700447

Antal, D., Ardelean, F., Andrica, F., Danciu, C., and Avram, S. (2015). Anatomical characteristics and tissue localization of various flavonoid subclasses in *Cotinus coggygria* stem and leaf cross-sections. *Planta Med.* 81 (16), PW_156. doi:10.1055/s-0035-1565780

Antal, D. S., and Ardelean, F. (2015). "*Cotinus coggygria* Scop," in *Romanian traditional medicine: results of an ethnobotanical survey performed in the South-Western part of the country. "Natural products as a source of compounds with chemopreventive and anti-inflammatory activity". 10.07.2015*. (Timisoara, Book of Abstracts), 22.

Antal, D. S., Ardelean, F., Pinzaru, I., Borcan, F., Ledeți, I., Coricovac, D., et al. (2016). Effects of cyclodextrin complexation on the anti-cancer effects of *Cotinus coggygria* extract and its constituents, butein and sulfuretin. *Rev. Chim.* 67 (8), 1618–1622.

Antal, D., Schwaiger, S., Ellmerer-Müller, E., and Stuppner, H. (2010). Cotinus coggygriaWood: novel flavanone dimer and development of an HPLC/UV/MS method for the simultaneous determination of fourteen phenolic constituents. *Planta Med.* 76 (15), 1765–1772. doi:10.1055/s-0030-1249878

Antal, D., Schwaiger, S., Hornick, A., Rollinger, J., Prast, H., and Stuppner, H. (2008). *Cotinus coggygria* heartwood: a new source of acetylcholinesterase inhibiting compounds. *Planta Med.* 74 (09), PA194. doi:10.1055/s-0028-1084192

Bahadirli, N. P. (2020). Essential oil content and composition of *Cotinus coggygria* Scop. from Hatay, Turkey. *Int. J. Agric. For. Life Sci.* 4 (1), 111–114.

Bertrand, C., Bellvert, F., Boisroux, A., Comte, G., and Taoubi, K. (2008). Urushiol congeners of *Rhus* sp. mother tincture. *Planta Med.* 74 (09), PC41. doi:10.1055/s-0028-1084559

Bezruk, P. I., and Lyubetskaya, Z. A. (1969). Antiphlogistic properties of the total flavonoids extracted from *Cotinus coggygria* leaves. *Farmakol Toksikol (Moscow)* 32 (5), 596–598.

Bordoloi, D., Monisha, J., Roy, N. K., Padmavathi, G., Banik, K., Harsha, C., et al. (2019). An investigation on the therapeutic potential of butein, a tetrahydroxychalcone against human oral squamous cell carcinoma. *Asian Pac. J. Cancer Prev.* 20 (11), 3437–3446. doi:10.31557/APJCP.2019.20.11.3437

Bruneton, J. (2016). *Pharmacognosie, phytochimie, plantes médicinales*. 5th Edn. Paris: Lavoisier Tec and Doc, 556–616.

Butură, V. (1979). Enciclopedie de etnobotanică românească. Ştiinţifică şi enciclopedică. Bucureşti.

Buziashvili, I. S., Komissarenko, N. F., and Kolesnikov, D. G. (1973). Polyphenolic compounds in the leaves of the Venetian sumac (*Rhus coriaria*) and sumac (*Cotinus coggygria*). *Fenol'nye Soedin Ikh Fiziol Svoistva. Mater. Vses Simp Fenol'nym Soedin.* 2, 159–162.

Cha, M. R., Park, J. H., Choi, Y. H., Choi, C. W., Hong, K. S., Choi, S. U., et al. (2009). Alpha-glucosidase inhibitors from the branches extract of *Cotinus coggygria*. *Korean J. Pharmacogn.* 40 (3), 229–232.

Chen, T.-J., Feng, Y., Liu, T., Wu, T.-T., Chen, Y.-J., Li, X., et al. (2020). Fisetin regulates gut microbiota and exerts neuroprotective effect on mouse model of Parkinson's disease. *Front. Neurosci.* 14, 549037. doi:10.3389/fnins.2020.549037

Cho, J.-Y., Sohn, M.-J., Lee, J., and Kim, W.-G. (2010). Isolation and identification of pentagalloylglucose with broad-spectrum antibacterial activity from *Rhus trichocarpa* Miquel. *Food Chem.* 123 (2), 501–506. doi:10.1016/j.foodchem.2010.04.072

Choi, J., Yoon, B. J., Han, Y. N., Lee, K. T., Ha, J., Jung, H. J., et al. (2003). Antirheumatoid arthritis effect of *Rhus verniciflua* and of the active component, sulfuretin. *Planta Med.* 69 (10), 899–904. doi:10.1055/s-2003-45097

de la Luz Cádiz-Gurrea, M., Fernández-Arroyo, S., and Segura-Carretero, A. (2014). Pine bark and green tea concentrated extracts: antioxidant activity and comprehensive characterization of bioactive compounds by HPLC-ESI-QTOF-MS. *Ijms* 15 (11), 20382–20402. doi:10.3390/ijms151120382

Demirci, B., Demirci, F., and Başer, K. H. C. (2003). Composition of the essential oil ofCotinus coggygria Scop. from Turkey. *Flavour Fragr. J.* 18 (1), 43–44. doi:10.1002/ffj.1149

Deniz, F. S. S., Salmas, R. E., Emerce, E., Cankaya, I. I. T., Yusufoglu, H. S., and Orhan, I. E. (2020). Evaluation of collagenase, elastase and tyrosinase inhibitory activities of *Cotinus coggygria* Scop. through *in vitro* and in silico approaches. *South Afr. J. Bot.* 132, 277–288. doi:10.1016/j.sajb.2020.05.017

Di, S., Fan, C., Ma, Z., Li, M., Guo, K., Han, D., et al. (2019). PERK/eIF-2α/CHOP pathway dependent ROS generation mediates butein-induced non-small-cell lung cancer apoptosis and G2/M phase Arrest. *Int. J. Biol. Sci.* 15 (8), 1637–1653. doi:10.7150/ijbs.33790

Eftimov, M., Pavlov, D., Nashar, M., Ivanova, D., Tzaneva, S. V., and Valcheva-Kuzmanova, S. (2016). Effects of aqueous infusion from *Cotinus coggygria* leaves on behavior and lipid peroxidation in rats. *Farmacia* 64 (1), 67–71.

Farsad-Naeimi, A., Alizadeh, M., Esfahani, A., and Darvish Aminabad, E. (2018). Effect of fisetin supplementation on inflammatory factors and matrix metalloproteinase enzymes in colorectal cancer patients. *Food Funct.* 9 (4), 2025–2031. doi:10.1039/c7fo01898c

Fraternale, D., and Ricci, D. (2014). Chemical composition and antimicrobial activity of the essential oil ofCotinus coggygriaScoop. From Italy. *J. Essent. Oil Bearing Plants* 17 (3), 366–370. doi:10.1080/0972060x.2014.895192

Gospodinova, Z., Bózsity, N., Nikolova, M., Krasteva, M., and Zupkó, I. (2017b). Antiproliferative properties against human breast, cervical and ovarian cancer cell lines, and antioxidant capacity of leaf aqueous ethanolic extract from *Cotinus coggygria* Scop. *Acta Med. Bulg.* 44 (2), 20–25. doi:10.1515/amb-2017-0014

Gospodinova, Z., Krasteva, M., and Manova, V. (2017c). Effects of *Cotinus coggygria* extract on the transcriptional levels of histone deacetylase genes in breast cancer cells *in vitro*. *Am. J. Pharm. Health Res.* 5, 60–69.

Gospodinova, Z., Nikolova, M., Vladimirova, A., and Antov, G. (2020). Assessment of *in vitro* cytotoxicity of leaf extract and fractions of *Cotinus coggygria* Scop. on human non-melanoma skin cancer cells. *MESMAP–6 Proc. Book*, 104.

Gospodinova, Z., Zupkó, I., Bózsity, N., Manova, V. I., Georgieva, M. S., Todinova, S. J., et al. (2017a). *Cotinus coggygria* Scop. leaf extract exerts high but not dose- and time-dependent *in vitro* cytotoxic activity on human breast cancer cells. *Genet. Plant Physiol.* 7, 176–183. doi:10.1515/znc-2020-0087

Gözcü, S., Yuca, H., Dursunoğlu, B., Güvenalp, Z., and Demirezer, L. Ö. (2016). "*In vitro* antidiabetic activity of *Cotinus coggygria* Scop," in *Planta med.*, 82. Rudigerstr 14, D-70469, Stuttgart, Germany: Georg Thieme Verlag KG.

Grynkiewicz, G., and Demchuk, O. M. (2019). New perspectives for fisetin. *Front. Chem.* 7, 697. doi:10.3389/fchem.2019.00697

Hao, S., Ma, J., Kong, X., Su, F., Li, J., Li, W., et al. (2018). Effects of compound *Cotinus coggygria* oral liquid on liver homogenate and liver tissue of rats. *Chin. J. Clin. Pharmacol. Ther.* 23 (6), 627. doi:10.12092/j.issn.1009-2501.2018.06.005

Hashida, K., Tabata, M., Kuroda, K., Otsuka, Y., Kubo, S., Makino, R., et al. (2014). Phenolic extractives in the trunk of *Toxicodendron vernicifluum*: chemical characteristics, contents and radial distribution. *J. Wood Sci.* 60 (2), 160–168. doi:10.1007/s10086-013-1385-8

Huang, C., Xia, X., He, J., Liu, Y., Shao, Z., Hu, T., et al. (2020). ERα is a target for butein-induced growth suppression in breast cancer. *Am. J. Cancer Res.* 10 (11), 3721–3736.

Huang, K. C., and Williams, W. M. (1998). *The pharmacology of Chinese herbs*. 2nd Edn. Berlin: Springer, 193–194.

Iliev, I., Ivanov, I., Todorova, K., and Dimitrova, M. (2020). Effects of a *Cotinus coggygria* ethyl acetate extract on two human normal cell lines. *Acta Morphol. Anthropol.* 27 (3-4), 25–29.

Imran, M., Saeed, F., Gilani, S. A., Shariati, M. A., Imran, A., Afzaal, M., et al. (2020). Fisetin: an anticancer perspective. *Food Sci. Nutr.* 9 (1), 3–16. doi:10.1002/fsn3.1872

Ippen, H. (1983). Contact allergy to Anacardiaceae. A review and case reports of poison ivy allergy in central Europe. *Derm Beruf Umwelt* 31 (5), 140–148.

Islam, A., Islam, M. S., Rahman, M. K., Uddin, M. N., and Akanda, M. R. (2020). The pharmacological and biological roles of eriodictyol. *Arch. Pharm. Res.* 43 (6), 582–592. doi:10.1007/s12272-020-01243-0

Ivanova, D. G., Pavlov, D. V., Eftimov, M., Kalchev, K., Nashar, M. A., Tzaneva, M. A., et al. (2013). Subchronic toxicity study of ethanol infusion from *Cotinus coggygria* wood in rats. *Bulg. J. Agric. Sci.* 19 (2), 182–185.

Iwashina, T. (1996). Detection and distribution of chrysanthemin and idaein in autumn leaves of plants by high performance liquid chromatography. *Ann. Tsukuba Bot. Gard.* 15, 1–8.

Jarić, S., Mačukanović-Jocić, M., Djurdjević, L., Mitrović, M., Kostić, O., Karadžić, B., et al. (2015). An ethnobotanical survey of traditionally used plants on Suva planina mountain (south-eastern Serbia). *J. Ethnopharmacol.* 175, 93–108. doi:10.1016/j.jep.2015.09.002

Jayasooriya, R. G. P. T., Molagoda, I. M. N., Park, C., Jeong, J.-W., Choi, Y. H., Moon, D.-O., et al. (2018). Molecular chemotherapeutic potential of butein: a concise review. *Food Chem. Toxicol.* 112, 1–10. doi:10.1016/j.fct.2017.12.028

Jeong, G.-S., Lee, D.-S., Song, M.-Y., Park, B.-H., Kang, D.-G., Lee, H.-S., et al. (2011). Butein from *Rhus verniciflua* protects pancreatic β cells against cytokine-induced toxicity mediated by inhibition of nitric oxide formation. *Biol. Pharm. Bull.* 34 (1), 97–102. doi:10.1248/bpb.34.97

Jia, S., Xu, X., Zhou, S., Chen, Y., Ding, G., and Cao, L. (2019). Fisetin induces autophagy in pancreatic cancer cells via endoplasmic reticulum stress- and mitochondrial stress-dependent pathways. *Cell Death Dis* 10 (2), 142. doi:10.1038/s41419-019-1366-y

Jiang, P., and Sun, H. (2018). Sulfuretin alleviates atopic dermatitis-like symptoms in mice via suppressing Th2 cell activity. *Immunol. Res.* 66 (5), 611–619. doi:10.1007/s12026-018-9025-4

Jin, C.-H., Shin, E.-J., Park, J.-B., Jang, C.-G., Li, Z., Kim, M. S., et al. (2009). Fustin flavonoid attenuates β-amyloid (1-42)-induced learning impairment. *J. Neurosci. Res.* 87 (16), 3658–3670. doi:10.1002/jnr.22159

Jin, M. J., Kim, I. S., Park, J. S., Dong, M.-S., Na, C.-S., and Yoo, H. H. (2015). Pharmacokinetic profile of eight phenolic compounds and their conjugated metabolites after oral administration of *Rhus verniciflua* extracts in rats. *J. Agric. Food Chem.* 63 (22), 5410–5416. doi:10.1021/acs.jafc.5b01724

Jung, C. H., Kim, J. H., Hong, M. H., Seog, H. M., Oh, S. H., Lee, P. J., et al. (2007). Phenolic-rich fraction from Rhus verniciflua Stokes (RVS) suppress inflammatory response via NF-κB and JNK pathway in lipopolysaccharide-induced RAW 264.7 macrophages. *J. Ethnopharmacology* 110 (3), 490–497. doi:10.1016/j.jep.2006.10.013

Kashyap, D., Sharma, A., Sak, K., Tuli, H. S., Buttar, H. S., and Bishayee, A. (2018). Fisetin: a bioactive phytochemical with potential for cancer prevention and pharmacotherapy. *Life Sci.* 194, 75–87. doi:10.1016/j.lfs.2017.12.005

Khan, N., Jajeh, F., Eberhardt, E. L., Miller, D. D., Albrecht, D. M., Van Doorn, R., et al. (2019). Fisetin and 5-fluorouracil: effective combination for PIK3CA -mutant colorectal cancer. *Int. J. Cancer* 145 (11), 3022–3032. doi:10.1002/ijc.32367

Kim, J.-S., Kwon, Y.-S., Chun, W.-J., Kim, T.-Y., Sun, J., Yu, C.-Y., et al. (2010). Rhus verniciflua Stokes flavonoid extracts have anti-oxidant, anti-microbial and α-glucosidase inhibitory effect. *Food Chem.* 120 (2), 539–543. doi:10.1016/j.foodchem.2009.10.051

Kim, J. H., Shin, Y. C., and Ko, S.-G. (2014). Integrating traditional medicine into modern inflammatory diseases care: multitargeting byRhus vernicifluaStokes. *Mediators Inflamm.* 2014, 1. doi:10.1155/2014/154561

Kim, S., Song, N.-J., Chang, S.-H., Bahn, G., Choi, Y., Rhee, D. K., et al. (2019). Sulfuretin prevents obesity and metabolic diseases in diet induced obese mice. *Biomolecules Ther.* 27 (1), 107–116. doi:10.4062/biomolther.2018.090

Koleva, V., Dragoeva, A., Nanova, Z., Koynova, T., and Dashev, G. (2015). An ethnobotanical study on current status of some medicinal plants used in Bulgaria. *Int. J. Curr. Microbiol. App. Sci.* 4 (4), 297–305.

Kubo, C., Ogawa, M., Uehara, N., and Katakura, Y. (2020). Fisetin promotes hair growth by augmenting TERT expression. *Front. Cel Dev. Biol.* 8, 566617. doi:10.3389/fcell.2020.566617

Kültür, Ş. (2007). Medicinal plants used in Kırklareli Province (Turkey). *J. Ethnopharmacology* 111 (2), 341–364. doi:10.1016/j.jep.2006.11.035

Kwon, E.-Y., and Choi, M.-S. (2019). Dietary eriodictyol alleviates adiposity, hepatic steatosis, insulin resistance, and inflammation in diet-induced obese mice. *Ijms* 20 (5), 1227. doi:10.3390/ijms20051227

Lamichhane, R., Kim, S.-G., Kang, S., Lee, K.-H., Pandeya, P. R., and Jung, H.-J. (2017). Exploration of underlying mechanism of anti-adipogenic activity of sulfuretin. *Biol. Pharm. Bull.* 40 (9), 1366–1373. doi:10.1248/bpb.b17-00049

Lee, D.-S., Jeong, G.-S., Li, B., Park, H., and Kim, Y.-C. (2010). Anti-inflammatory effects of sulfuretin from *Rhus verniciflua* Stokes via the induction of heme oxygenase-1 expression in murine macrophages. *Int. Immunopharmacology* 10 (8), 850–858. doi:10.1016/j.intimp.2010.04.019

Lee, E. H., Song, D.-G., Lee, J. Y., Pan, C.-H., Um, B. H., and Jung, S. H. (2008). Inhibitory effect of the compounds isolated from *Rhus verniciflua* on aldose reductase and advanced glycation endproducts. *Biol. Pharm. Bull.* 31 (8), 1626–1630. doi:10.1248/bpb.31.1626

Lee, K.-W., Chung, K.-S., Seo, J.-H., Yim, S.-V., Park, H.-J., Choi, J.-H., et al. (2012). Sulfuretin from heartwood of *Rhus verniciflua* triggers apoptosis through activation of Fas, Caspase-8, and the mitochondrial death pathway in HL-60 human leukemia cells. *J. Cel. Biochem.* 113 (9), 2835–2844. doi:10.1002/jcb.24158

Lee, S.-h., Choi, W.-c., and Yoon, S.-w. (2009). Impact of standardized *Rhus verniciflua* Stokes extract as complementary therapy on metastatic colorectal cancer: a Korean single-center experience. *Integr. Cancer Ther.* 8 (2), 148–152. doi:10.1177/1534735409336438

Lee, S., Kim, K., Jung, H., Lee, S., Cheon, S., Kim, S., et al. (2011). Efficacy and safety of standardized allergen-removed *Rhus verniciflua* Stokes extract in patients with advanced or metastatic pancreatic cancer: a Korean single-center experience. *Oncology* 81 (5-6), 312–318. doi:10.1159/000334695

Lei, Z., Ouyang, L., Gong, Y., Wang, Z., and Yu, B. (2020). Effect of eriodictyol on collagen-induced arthritis in rats by Akt/HIF-1α pathway. *Dddt* 14, 1633–1639. doi:10.2147/DDDT.S239662

Li, M.-C., Zhang, Y.-Q., Meng, C.-W., Gao, J.-G., Xie, C.-J., Liu, J.-Y., et al. (2021). Traditional uses, phytochemistry, and pharmacology of Toxicodendron vernicifluum (Stokes) F.A. Barkley - a review. *J. Ethnopharmacology* 267, 113476. doi:10.1016/j.jep.2020.113476

Li, M. C., Xie, C. J., Gao, J. G., Meng, C. W., He, Y. J., Liu, J. Y., et al. (2020). Chemical constituents from the heartwood of Toxicodendron vernicifluum (Stokes) F.A. Barkley. *Biochem. Syst. Ecol.* 90, 104017. doi:10.1016/j.bse.2020.104017

Li, W., Du, Q., Li, X., Zheng, X., Lv, F., Xi, X., et al. (2020). Eriodictyol inhibits proliferation, metastasis and induces apoptosis of glioma cells via PI3K/Akt/NF-κB signaling pathway. *Front. Pharmacol.* 11, 114. doi:10.3389/fphar.2020.00114

Li, Y., Jia, S., and Dai, W. (2020). Fisetin modulates human oral squamous cell carcinoma proliferation by blocking PAK4 signaling pathways. *Dddt* 14, 773–782. doi:10.2147/DDDT.S229270

Linnaeus, C. (1753). Species plantarum. Scanned version available at: https://www.biodiversitylibrary.org/item/13829#page/285/mode/1up.

Long, L., Han, X., Ma, X., Li, K., Liu, L., Dong, J., et al. (2020). Protective effects of fisetin against myocardial ischemia/reperfusion injury. *Exp. Ther. Med.* 19 (5), 3177–3188. doi:10.3892/etm.2020.8576

Lu, Y.-t., Xiao, Y.-f., Li, Y.-f., Li, J., Nan, F.-j., and Li, J.-y. (2019). Sulfuretin protects hepatic cells through regulation of ROS levels and autophagic flux. *Acta Pharmacol. Sin.* 40 (7), 908–918. doi:10.1038/s41401-018-0193-5

Mantzouris, D., Karapanagiotis, I., Valianou, L., and Panayiotou, C. (2011). HPLC-DAD-MS analysis of dyes identified in textiles from Mount Athos. *Anal. Bioanal. Chem.* 399 (9), 3065–3079. doi:10.1007/s00216-011-4665-4

Marčetić, M., Božić, D., Milenković, M., Malešević, N., Radulović, S., and Kovačević, N. (2013). Antimicrobial, antioxidant and anti-inflammatory activity of young shoots of the smoke tree, Cotinus coggygria Scop. *Phytother. Res.* 27 (11), 1658–1663. doi:10.1002/ptr.4919

Matić, S., Stanić, S., Bogojević, D., Vidaković, M., Grdović, N., Arambašić, J., et al. (2011). Extract of the plant *Cotinus coggygria* Scop. attenuates pyrogallol-induced hepatic oxidative stress in Wistar rats. *Can. J. Physiol. Pharmacol.* 89 (6), 401–411. doi:10.1139/y11-043

Matić, S., Stanić, S., Bogojević, D., Vidaković, M., Grdović, N., Dinić, S., et al. (2013). Methanol extract from the stem of *Cotinus coggygria* Scop., and its major bioactive phytochemical constituent myricetin modulate pyrogallol-induced DNA damage and liver injury. *Mutat. Res.* 755 (2), 81–89. doi:10.1016/j.mrgentox.2013.03.011

Matić, S., Stanić, S., Mihailović, M., and Bogojević, D. (2016). *Cotinus coggygria* Scop.: an overview of its chemical constituents, pharmacological and toxicological potential. *Saudi J. Biol. Sci.* 23 (4), 452–461. doi:10.1016/j.sjbs.2015.05.012

Metivier, P. S. R., Yeung, E. C., Patel, K. R., and Thorpe, T. A. (2007). *In vitro* rooting of microshoots of Cotinus coggygria Mill, a woody ornamental plant. *In Vitro Cell.Dev.Biol.-Plant* 43 (2), 119–123. doi:10.1007/s11627-007-9036-7

Moon, J. E., Shin, J.-H., Kwon, O., and Kim, J. Y. (2015). A standardized extract of *Rhus verniciflua* Stokes protects wistar rats against lipopolysaccharide-induced acute inflammation. *J. Med. Food* 18 (11), 1223–1230. doi:10.1089/jmf.2014.3411

Nedelcheva, A., and Draganov, S. (2014). "Bulgarian medical ethnobotany: the power of plants in pragmatic and poetic frames," in *Ethnobotany and biocultural diversities in the Balkans*. Editors A. Pieroni and C. Quave (New York: Springer), 45–65.

Nedelcheva, A. (2012). Medicinal plants from an old Bulgarian medical book. *J. Med. Plant Res.* 6 (12), 2324–2339. doi:10.5897/JMPR11.831

Novakovic, M., Djordjevic, I., Todorovic, N., Trifunovic, S., Andjelkovic, B., Mandic, B., et al. (2019). New aurone epoxide and auronolignan from the heartwood of *Cotinus coggygria* Scop. *Nat. Product. Res.* 33 (19), 2837–2844. doi:10.1080/14786419.2018.1508141

Novakovic, M., Vuckovic, I., Janackovic, P., Sokovic, M., Filipovic, A., Tesevic, V., et al. (2007). Chemical composition, antibacterial and antifungal activity of the essential oils of *Cotinus coggygria* from Serbia. *Jscs* 72 (11), 1045–1051. doi:10.2998/JSC0711045N10.2298/jsc0711045n

Özbek, H., Yuca, H., Dursunoğlu, B., Gözcü, S., Kazaz, C., and Güvenalp, Z. (2016). Secondary metabolites from *Cotinus coggygria* Scop. *Planta Med.* 82 (S 01), P269. doi:10.1055/s-0036-1596407

Özcan, M., and Yilmaz, S. (2020). Foliar micromorphology and anatomy of five mediterranean enclaves in artvin (Turkey). *Orman Fakültesi Dergisi.* 20 (2), 107–132. doi:10.17475/kastorman.801846

Ozsoy, N., Yilmaz-Ozden, T., Serbetci, T., Kultur, S., and Akalin, E. (2017). Antioxidant, anti-inflammatory, acetylcholinesterase and thioredoxin reductase inhibitory activities of nine selected Turkish medicinal plants. *Indian J. Tradit. Know.* 16 (4), 553–561.

Padmavathi, G., Roy, N. K., Bordoloi, D., Arfuso, F., Mishra, S., Sethi, G., et al. (2017). Butein in health and disease: a comprehensive review. *Phytomedicine* 25, 118–127. doi:10.1016/j.phymed.2016.12.002

Pariyar, R., Lamichhane, R., Jung, H., Kim, S., and Seo, J. (2017). Sulfuretin attenuates MPP+-Induced neurotoxicity through akt/gsk3β and ERK signaling pathways. *Ijms* 18 (12), 2753. doi:10.3390/ijms18122753

Park, B. C., Lee, Y. S., Park, H.-J., Kwak, M.-K., Yoo, B. K., Kim, J. Y., et al. (2007). Protective effects of fustin, a flavonoid from *Rhus verniciflua* Stokes, on 6-hydroxydopamine-induced neuronal cell death. *Exp. Mol. Med.* 39 (3), 316–326. doi:10.1038/emm.2007.35

Patnaik, S. S., Simionescu, D. T., Goergen, C. J., Hoyt, K., Sirsi, S., and Finol, E. A. (2019). Pentagalloyl glucose and its functional role in vascular health: biomechanics and drug-delivery characteristics. *Ann. Biomed. Eng.* 47 (1), 39–59. doi:10.1007/s10439-018-02145-5

Pavlov, D., Eftimov, M., Nashar, M., Tzaneva, M., Ivanova, D., and Valcheva-Kuzmanova, S. (2014). Anti-inflammatory properties of aqueous infusion from *Cotinus coggyria* leaves in rats. *Scr. Sci. Pharm.* 1, 25.

Pavlov, D., Nashar, M., Eftimov, M., Kalchev, K., Valcheva-Kuzmanova, S., Tzaneva, M., et al. (2013b). Subchronic toxicity study of aqueous infusion from *Cotinus coggygria* leaves in Wistar rats. *Compt. Rend. Acad. Bulg. Sci.* 66, 749–756. doi:10.7546/cr-2013-66-5-13101331-18

Pavlov, D. V., Ivanova, D. G., Eftimov, M., Tzaneva, M. A., Nashar, M. A., Kobakova, I., et al. (2013a). Effect of aqueous infusion from *Cotinus coggygria* leaves on indomethacin-induced gastric mucosal damage and oxidative stress in rats. *Scr. Sci. Med.* 45, 32–38.

Peng, F., Du, Q., Peng, C., Wang, N., Tang, H., Xie, X., et al. (2015). A review: the pharmacology of isoliquiritigenin. *Phytother. Res.* 29 (7), 969–977. doi:10.1002/ptr.5348

Petroviciu, I., Creţu, I., Vanden Berghe, I., Wouters, J., Medvedovici, A., and Albu, F. (2014). Flavonoid dyes detected in historical textiles from Romanian collections. *e-PS.* 11, 84–90.

Pollio, A., Zarrelli, A., Romanucci, V., Di Mauro, A., Barra, F., Pinto, G., et al. (2016). Polyphenolic profile and targeted bioactivity of methanolic extracts from Mediterranean ethnomedicinal plants on human cancer cell lines. *Molecules* 21 (4), 395. doi:10.3390/molecules21040395

Prasath, G. S., Pillai, S. I., and Subramanian, S. P. (2014). Fisetin improves glucose homeostasis through the inhibition of gluconeogenic enzymes in hepatic tissues

of streptozotocin induced diabetic rats. *Eur. J. Pharmacol.* 740, 248–254. doi:10.1016/j.ejphar.2014.06.065

Redžić, S. S. (2007). The ecological aspect of ethnobotany and ethnopharmacology of population in Bosnia and Herzegovina. *Coll. Antropol.* 31 (3), 869–890.

Rendeková, K., Fialová, S., Jánošová, L., Mučaji, P., and Slobodníková, L. (2016). The activity of *Cotinus coggygria* Scop. leaves on *Staphylococcus aureus* strains in planktonic and biofilm growth forms. *Molecules* 21 (1), 50. doi:10.3390/molecules21010050

Šavikin, K., Zdunić, G., Janković, T., Stanojković, T., Juranić, Z., and Menković, N. (2009). *In vitro* cytotoxic and antioxidative activity of *Cornus mas* and *Cotinus coggygria*. *Nat. Prod. Res.* 23 (18), 1731–1739. doi:10.1080/14786410802267650

Scopoli, G. A. (1772). Flora Carniolica exhibens plantas Carnioliae indigenas et distributas in classes, genera, species, varietates, ordine Linnaeano. Vienna: ioannis Pauli Krauss. Scanned version available at: https://books.google.ca/books?id=jl9CAAAAYAAJ.

Shen, Q., Shang, D., Ma, F., and Zhang, Z. (1991). [Pharmacological study on anti-hepatitis effect of Cotinus coggygria Scop. Syrup]. *Zhongguo Zhong Yao Za Zhi* 16 (12), 763–764.

Shin, J.-S., Park, Y. M., Choi, J.-H., Park, H.-J., Shin, M. C., Lee, Y. S., et al. (2010). Sulfuretin isolated from heartwood of Rhus verniciflua inhibits LPS-induced inducible nitric oxide synthase, cyclooxygenase-2, and pro-inflammatory cytokines expression via the down-regulation of NF-κB in RAW 264.7 murine macrophage cells. *Int. Immunopharmacology* 10 (8), 943–950. doi:10.1016/j.intimp.2010.05.007

Song, M.-Y., Jeong, G.-S., Kwon, K.-B., Ka, S.-O., Jang, H.-Y., Park, J.-W., et al. (2010). Sulfuretin protects against cytokine-induced β-cell damage and prevents streptozotocin-induced diabetes. *Exp. Mol. Med.* 42 (9), 628–638. doi:10.3858/emm.2010.42.9.062

Song, X., Tan, L., Wang, M., Ren, C., Guo, C., Yang, B., et al. (2021). Myricetin: a review of the most recent research. *Biomed. Pharmacother.* 134, 111017. doi:10.1016/j.biopha.2020.111017134

Su, F., Wen, Z., Sun, J., Hao, S., Xie, G., Li, X., et al. (2018). "Study on acute toxicity of compound coggygria oral liquid"In AIP Conference Proceedings, 2018 Apr 27 1956, 020038. Melville, NY: AIP Publishing LLC.

Sunil, C., and Xu, B. (2019). An insight into the health-promoting effects of taxifolin (dihydroquercetin). *Phytochemistry* 166, 112066. doi:10.1016/j.phytochem.2019.112066

Tamburini, D. (2019). Investigating Asian colourants in Chinese textiles from Dunhuang (7th-10th century AD) by high performance liquid chromatography tandem mass spectrometry - towards the creation of a mass spectra database. *Dyes Pigm.* 163, 454–474. doi:10.1016/j.dyepig.2018.12.025

Tanchev, S. S., and Timberlake, C. F. (1969). Anthocyanins in leaves of *Cotinus coggygria*. *Phytochemistry* 8 (12), 2367–2369. doi:10.1016/s0031-9422(00)88156-x

Tang, L., Qin, Y., Ling, K., and Wan, H. (2020). Eriodictyol inhibits the growth of CNE1 human nasopharyngeal cancer growth by targeting MEK/ERK signalling pathway, inducing cellular autophagy and inhibition of cell migration and invasion. *J. BUON.* 25 (5), 2389–2394.

Thapa, P., Prakash, O., Rawat, A., Kumar, R., Srivastava, R. M., Rawat, D. S., et al.(2020). Essential oil composition, antioxidant, anti-inflammatory, insect antifeedant and sprout suppressant activity in essential oil from aerial parts ofCotinus coggygriaScop. *J. Essent. Oil Bearing Plants* 23 (1), 65–76. doi:10.1080/0972060x.2020.1729246

Theophrastus. (1644). Eresii De historia plantarum libri decem, graece et latine. Amstelodami, typis Judoci Broers, 246 Original from the National Central Library of Florence digitalized on 30 Jan 2017; available as e-book.

Tsankova, E. T., Dyulgerov, A. S., and Milenkov, B. K. (1993). Chemical composition of the Bulgarian sumac oil. *J. Essent. Oil Res.* 5 (2), 205–207. doi:10.1080/10412905.1993.9698202

Tudor, F., and Georgescu, M. I. (2011). Ethnobotanical studies in Dobrogea, scientific papers, UASVM bucharest. *Ser. A*, Vol. LIV, 457–462.

Tzakou, O., Bazos, I., and Yannitsaros, A. (2005). Essential oils of leaves, inflorescences and infructescences of spontaneousCotinus coggygria Scop. from Greece. *Flavour Fragr. J.* 20 (5), 531–533. doi:10.1002/ffj.1456

Ugan, R. A., Un, H., and Un, H. (2020). The protective roles of butein on indomethacin induced gastric ulcer in mice. *Eurasian J. Med.* 52 (3), 265–270. doi:10.5152/eurasianjmed.2020.20022

Ulukanli, Z., Karabörklü, S., Bozok, F., Çenet, M., Öztürk, B., and Balcilar, M. (2014). Antimicrobial, insecticidal and phytotoxic activities of *Cotinus coggyria* Scop. essential oil (Anacardiaceae). *Nat. Product. Res.* 28 (23), 2150–2157. doi:10.1080/14786419.2014.928879

Valianou, L., Stathopoulou, K., Karapanagiotis, I., Magiatis, P., Pavlidou, E., Skaltsounis, A.-L., et al. (2009). Phytochemical analysis of young fustic (*Cotinus coggygria* heartwood) and identification of isolated colourants in historical textiles. *Anal. Bioanal. Chem.* 394 (3), 871–882. doi:10.1007/s00216-009-2767-z

Vlková, M., Kubátová, E., Šlechta, P., and Polesný, Z. (2015). Traditional use of plants by the disappearing Czech diaspora in Romanian Banat. *Sci. Agric. Bohem.* 46 (2), 49–56. doi:10.1515/sab-2015-0016

Wang, G., Wang, J. J., To, T. S., Zhao, H. F., and Wang, J. (2015b). Role of SIRT1-mediated mitochondrial and Akt pathways in glioblastoma cell death induced by *Cotinus coggygria* flavonoid nanoliposomes. *Int. J. Nanomedicine* 10, 5005–5023. doi:10.2147/IJN.S82282

Wang, G., Wang, J.-J., Du, L., Fei, L., and To, S.-s. T. (2016). Inhibitory kinetics and mechanism of flavonoids extracted from *Cotinus coggygria* Scop. against glioblastoma cancer. *Nutr. Cancer* 68 (8), 1357–1368. doi:10.1080/01635581.2016.1225105

Wang, G., Wang, J., Du, L., and Li, F. (2015a). Effect and mechanism of total flavonoids extracted fromCotinus coggygriaagainst glioblastoma CancerIn VitroandIn vivo. *Biomed. Res. Int.* 2015, 1. doi:10.1155/2015/856349

Wang, L., Cao, D., Wu, H., Jia, H., Yang, C., and Zhang, L. (2019). Fisetin prolongs therapy window of brain ischemic stroke using tissue plasminogen activator: a double-blind randomized placebo-controlled clinical trial. *Clin. Appl. Thromb. Hemost.* 25, 107602961987135. doi:10.1177/1076029619871359

Westenburg, H. E., Lee, K.-J., Lee, S. K., Fong, H. H. S., van Breemen, R. B., Pezzuto, J. M., et al. (2000). Activity-guided isolation of antioxidative constituents ofCotinuscoggygria. *J. Nat. Prod.* 63 (12), 1696–1698. doi:10.1021/np000292h

Wissler Gerdes, E. O., Zhu, Y., Tchkonia, T., and Kirkland, J. L. (2020). Discovery, development, and future application of senolytics: theories and predictions. *FEBS J.* 287 (12), 2418–2427. doi:10.1111/febs.15264

Yan, L., Jia, Q., Cao, H., Chen, C., Xing, S., Huang, Y., et al. (2021). Fisetin ameliorates atherosclerosis by regulating PCSK9 and LOX-1 in apoE-/-mice. *Exp. Ther. Med.* 21 (1), 1. doi:10.3892/etm.2020.9457

Yang, J., Kwon, Y. S., and Kim, M. J. (2018). Antimicrobial activity and active compounds of a *Rhus verniciflua* Stokes extract. *Z. Naturforsch. C. J. Biosci.* 73 (11-12), 457–463. doi:10.1515/znc-2018-0054

Yang, P. Y., Hu, D. N., Kao, Y. H., Lin, I. C., and Liu, F. S. (2018). Butein induces apoptotic cell death of human cervical cancer cells. *Oncol. Lett.* 16 (5), 6615–6623. doi:10.3892/ol.2018.9426

Yousefzadeh, M. J., Zhu, Y., McGowan, S. J., Angelini, L., Fuhrmann-Stroissnigg, H., Xu, M., et al. (2018). Fisetin is a senotherapeutic that extends health and lifespan. *EBioMedicine* 36, 18–28. doi:10.1016/j.ebiom.2018.09.015

Zhang, J., Li, L., Kim, S.-H., Hagerman, A. E., and Lü, J. (2009). Anti-cancer, anti-diabetic and other pharmacologic and biological activities of penta-galloyl-glucose. *Pharm. Res.* 26 (9), 2066–2080. doi:10.1007/s11095-009-9932-0

Zhang, L., Tong, X., Huang, J., Wu, M., Zhang, S., Wang, D., et al. (2020). Fisetin alleviated bleomycin-induced pulmonary fibrosis partly by rescuing alveolar epithelial cells from senescence. *Front. Pharmacol.* 11, 553690. doi:10.3389/fphar.2020.553690

Zheng, W., Zhang, H., Jin, Y., Wang, Q., Chen, L., Feng, Z., et al. (2017). Butein inhibits IL-1β-induced inflammatory response in human osteoarthritis chondrocytes and slows the progression of osteoarthritis in mice. *Int. Immunopharmacology* 42, 1–10. doi:10.1016/j.intimp.2016.11.009

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Multidirectional Effects of Tormentil Extract on Hemostasis in Experimental Diabetes

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In our previous study, we showed that ellagitannin- and procyanidin-rich tormentil extract (TE) decreased experimental arterial thrombosis in normoglycemic rats through platelet inhibition. TE also slightly increased coagulation and attenuated fibrinolysis; however, these effects did not nullify the antithrombotic effect of TE. The present study aimed to assess whether TE exerts antithrombotic activity in streptozotocin (STZ)-induced diabetes, which is characterized by pre-existing increased coagulation and impaired fibrinolysis, in *vivo* and *ex vivo* thrombosis assays. TE (100, 200, or 400 mg/kg, *p. o.*) was administered for 14 days to STZ-induced diabetic rats and mice. TE at 100 mg/kg dose decreased the thrombus area in the mice model of laser-induced thrombosis through its potent antiplatelet effect. However, TE at 200 mg/kg dose increased thrombus weight in electrically induced arterial thrombosis in rats. The prothrombotic effect could be due to increased coagulation and attenuated fibrinolysis. TE at 400 mg/kg dose also improved vascular functions, which was mainly reflected as an increase in the arterial blood flow, bleeding time prolongation, and thickening of the arterial wall. However, TE at 400 mg/kg dose did not exert antithrombotic effect. Summarizing, the present results show that TE may exert multidirectional effects on hemostasis in STZ-induced diabetic rats and mice. TE inhibited platelet activity and improved endothelial functions, but it also showed unfavorable effects by increasing the activity of the coagulation system and by inhibiting fibrinolysis. These contrasting effects could be the reason for model-specific influence of TE on the thrombotic process in STZ-induced diabetes.

Keywords: hemostasis, ellagitannins, STZ-induced diabetes, thrombosis, *Potentilla erecta*

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Abbreviation: 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin 1 α; AA, arachidonic acid; ANX V, annexin V; DiOC6(3), 3,3′-dihexyloxacarbocyanine iodide; ECLT, euglobulin clot lysis time; IBF, initial blood flow; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; NO_2^- and NO_3^-, nitrite and nitrate; PAI-1, plasminogen activator inhibitor 1; PGI_2, prostacyclin; PI, platelet procoagulant index; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PS, phosphatidylserine; *i.p.*, intraperitoneal administration (*lat. iniectio intraperitonealis*); *p.o.*, oral administration (*lat. per os*); TE, tormentil extract; TF, tissue factor; TNF-α, tumor necrosis factor α; t-PA, tissue plasminogen activator; TTO, total time to occlusion; TXA_2, thromboxane; STZ, streptozotocin.

INTRODUCTION

The present study is a continuation of our previous work wherein we showed the antithrombotic activity of *Potentilla erecta* rhizome extract in normoglycemic rats and mice (Marcinczyk et al., 2017). The genus *Potentilla* L (Rosaceae family) consists of approximately 700 species of annual and biennial plants and small shrubs. Extract from the underground parts of *Potentilla erecta* (L.) Raeusch (tormentil extract [TE]) has been used in traditional ethnomedicine for the treatment of diarrhea and mild inflammation of the oral mucosa because of its antioxidant and anti-inflammatory activity (Tomczyk and Latté, 2009; Melzig and Böttger, 2020; Augustynowicz et al., 2021). The pharmacological properties of tormentil have been discussed in several reviews and it's monographs are included in the Russian Pharmacopoeia (14th edition), State Pharmacopoeia of the Republic of Belarus, ESCOP, in the British Pharmacopoeia, or the European Pharmacopoeia (10th edition) (Shikov et al., 2021). These actions are induced by ellagitannins and procyanidins, which are rarely found together in a plant material, although the presence of ellagitannins and procyanidins in TE was confirmed by us and other researchers (Geiger et al., 1994; Mari et al., 2013; Marcinczyk et al., 2017). Ellagitannins and procyanidins are characterized by poor bioavailability after oral intake. However, their gut microbiota-synthesized metabolites have good bioavailability and are responsible for *in vivo* activity of TE. Urolithins are metabolites of ellagitannins (Piwowarski et al., 2017), while phenolic acids as well as dimeric and trimeric procyanidins are metabolites of high-molecular-weight procyanidins (Sano et al., 2003; Sánchez-Patán et al., 2012). It has been shown that procyanidins and ellagitannins exert multidirectional effects on hemostasis; however, most of these studies have been conducted *in vitro*, which makes it difficult to predict the activity of this class of compounds after oral intake. The effect of ellagitannins on platelet activity, coagulation, and fibrinolysis was mainly studied *in vitro* by using plasma (Dong et al., 1998), washed platelets (Shen et al., 2003), and chromogenic substrates (Zhang et al., 2004; Yamamoto et al., 2011). The effects of ellagitannin-rich products other than TE on hemostasis has also been studied in humans after oral intake. The consumption of pomegranate juice has been shown to decrease collagen-induced platelet aggregation (Haber et al., 2011) while blended frozen red raspberries were found to improve flow-mediated vasodilation of the brachial artery (Istas et al., 2018). The effects of procyanidins on hemostasis reported thus far include mainly increase in nitric oxide (NO) and prostacyclin (PGI_2) production (*in vitro* and *in vivo*) (Schramm et al., 2001; Byun, 2012; Novakovic et al., 2017) and decrease in platelet activity (*in vitro* and *in vivo*) (Murphy et al., 2003; Bouaziz et al., 2007; Morel et al., 2014). Furthermore, by using stasis-induced model of venous thrombosis in rats (Jiang et al., 2007) and laser-induced model of arterial thrombosis in mice, the antithrombotic effect of procyanidins after oral intake was also demonstrated (Sano et al., 2005).

In our previous study, we showed for the first time the antithrombotic activity of ellagitannin- and procyanidin-rich TE in an animal model. In our study, TE inhibited arterial thrombosis in normoglycemic rats through a mechanism dependent on platelet inhibition. The underlying mechanism of this antiplatelet effect was based on the inhibition of thromboxane (TXA_2) production in platelets, which was comparable to the effect of acetylsalicylic acid. We also showed that TE slightly enhanced fibrin formation and attenuated fibrinolysis, but these effects did not abolish the antithrombotic effect of TE (Marcinczyk et al., 2017).

Considering the multidirectional activity of TE in normoglycemic rats, the main goal of the present study was to assess whether TE exerts the antithrombotic effect in streptozotocin (STZ)-induced diabetes, i.e., in conditions with intrinsically increased fibrin formation and impaired fibrinolysis. Hyperglycemia in type 1 diabetes is a risk factor for cardiovascular diseases and thromboembolic complications (Lee et al., 2019). Enhanced coagulation, impaired fibrinolysis, and increased platelet activity expressed as an enhanced production of TXA_2 and P-selectin secretion are often observed in patients with diabetes (Wisinski and Kimple, 2016; Sobczak and Stewart, 2019). Diabetes also impairs endothelial functions and vessel wall contractility and leads to a reduction in blood flow (Lunder et al., 2018), which may affect the dynamics of thrombus formation. Furthermore, vascular complications of diabetes, such as macro- and microangiopathy, contribute to hemostasis activation (Tarnow et al., 2009; Zahran et al., 2018). Because of multidirectional hemostasis dysfunctions, the evaluation of the activity of TE, which acts on multiple targets, in diabetes seems to be justified.

In our present study, we used a model of STZ-induced diabetes (STZ-diabetes), which clinically reflects type 1 diabetes (Furman, 2015), and conducted experiments in two models of thrombosis (electrically induced and laser-induced thrombosis) that differ in the mechanism of thrombus formation. Electrically induced arterial thrombosis allows to assess thrombus weight and blood flow disturbances due to vessel-occluding thrombus formation and growth after extensive endothelium damage. Dynamics and the extent of the thrombotic process assessed in this model involve both hemodynamic conditions and hemostatic activity. Thrombus formed in this model is mainly composed of platelets and fibrin, with a minor amount of red and white blood cells (Zakrzeska et al., 2015). The model of laser-induced thrombosis combined with confocal imaging was used for the intravital observation of the thrombotic process at the site of mesenteric vein injury, where thrombus formation occurs on the exposed subendothelial matrix. Because the thrombus is composed of platelets, this model allows to assess the thrombus area and platelet activation (Marcinczyk et al., 2020). Furthermore, by using a wide range of advanced techniques, we determined the effect of TE on the components of hemostasis: platelets, coagulation system, fibrinolysis, and endothelial-dependent vascular functions. This enabled more detailed and multidimensional study of the effect of TE on the thrombotic process and hemostasis in STZ-diabetes.

MATERIALS AND METHODS

Preparation of Tormentil Extract and LC-MS Analysis

The TE extract used in the study is the same as described by Marcinczyk and co-authors (2017). It was prepared in the same extraction protocol, and can clearly correlate these two datasets. The powdered plant material (2.0 g) (batch number: 268.2020420.2020; Kawon, Gostyń, Poland) was extracted with 150 ml of 80% (v/v) methanol in an ultrasonic bath (Sonic-5, Polsonic, Poland) at a controlled temperature (40 ± 2°C) for 45 min. After solvent evaporation under reduced pressure and vacuum controlled temperature (Büchi System, Flawil, Switzerland) (temperature: 40 ± 2°C) the extract was suspended in water and subjected to lyophilization using a vacuum concentrator (Labconco, Kansas City, United States) until a constant weight of the extract was obtained (yield 0.89 g; 44.5%). Details of LC-ESI-MS analysis of the TE extract have been also described previously (Marcinczyk et al., 2017).

Animals

Male Wistar rats (weighing 260–290 g) and male C57BL6 mice (weighing 24–29 g) were used in the study. Experiments were conducted in accordance with EU Guidelines on Animal Experiments (European Directive 2010/63/EU). All procedures involving animals and their care were approved by the Local Ethical Committee on Animal Testing (Approval Nos.: 72/2018 and 73/2018).

Before conducting procedures of arterial thrombosis, laser-induced thrombosis, and assessment of P-selectin secretion and tissue factor (TF) expression, rats and mice were anesthetized. Rats were anesthetized with a single intraperitoneal injection of pentobarbital (40 mg/kg, *i. p.*, Morbital, Poland). Mice were anesthetized with a single injection of ketamine and xylazine mixture (120 mg/kg, *i. p.*, Ketamina 10%, Biowet, Poland; 12.5 mg/kg, *i. p.*, Xylapan, Biowet, Poland). After the experiments, mice were euthanized by cervical dislocation, while rats were anesthetized with pentobarbital overdose (200 mg/kg, *i. p.*).

Diabetes Induction

Diabetes was induced in rats by a single intraperitoneal injection of STZ (Sigma Aldrich, Steinheim, Germany) at the dose of 65 mg/kg. Rats from the control group (rats without diabetes, referred as VEH) were injected with an equal volume of a citrate buffer. Blood glucose level was measured on the third day after STZ injection by using an Optium Xido glucometer (Abbott, United States). Diabetes was defined as a blood glucose level of >200 mg/dl. The development of diabetes in rats occurred over a period of 5 weeks. The blood glucose level was again measured 5 weeks after STZ injection.

Diabetes was induced in mice by a single intraperitoneal injection of STZ at 200 mg/kg dose. Mice from the control group (VEH) were injected with an equal volume of a citrate buffer. The blood glucose level was measured on the third day after STZ injection. Diabetes was defined as a blood glucose level of >200 mg/dl. The blood glucose level was again measured 4 weeks after STZ injection.

TE Administration

Diabetic mice and rats received TE *per os* (*p.o.*) with an oral gastric tube, twice daily at doses of 100, 200, and 400 mg/kg in a volume of 3 ml/kg in 5% water solution of gum arabic. Rats and mice from the VEH groups (normoglycemic rats and mice) and the Diabetes groups (rats and mice with diabetes that did not receive TE) received an equal volume of a 5% water solution of gum arabic.

Primary Hemostasis Template Bleeding Time (BT) in Rats (*in vivo*)

Template bleeding time was measured according to Dejana et al. (Dejana et al., 1982) before the procedure of arterial thrombosis induction.

Electrically Induced Arterial Thrombosis in a Rat Carotid Artery (*in vivo*)

Induction of arterial thrombosis (Schumacher et al., 1993) was performed according to our modified method (Marcinczyk et al., 2017). Arterial thrombus formation was induced by electrical stimulation (1 mA, 10 min) of the right common carotid artery. Thrombus progression led to a gradual reduction in the carotid blood flow, which was monitored with a Doppler flow probe (Transonic Systems Inc., Ithaca, United States) connected to a blood flowmeter (HSE-TRANSONIC Transit Time Flowmeter, Germany). Initial blood flow (IBF) and total time to occlusion (TTO), which was defined as the time from the commencement of the stimulation to the lack of arterial blood flow due to artery occlusion by the thrombus, were measured. Fifty-five minutes after the commencement of electrical stimulation, the thrombus was removed, dried at room temperature, and weighed after 24 h. For *ex vivo* experiments, blood samples were collected from the right heart ventricle by using 3.13% sodium citrate solution (1:10, *v*/*v*) as an anticoagulant.

Histological Staining of Rat Arterial Thrombus

Fragments of the carotid artery with thrombus were acquired from three rats of the following groups: VEH, Diabetes, 100 mg/kg, 200 mg/kg, and 400 mg/kg. These fragments were immediately fixed in 10% buffered formalin and routinely embedded in paraffin. Thrombus paraffin blocks were cut into section of 4 µm thick, then stained with hematoxylin-eosin for general histological examination. An experienced histologist, blinded to the technique used, assessed the slides in terms of their overall histological quality. Histological preparations were evaluated using an Olympus BX43 light microscope (Olympus 114 Corp., Tokyo, Japan) with an Olympus DP12 digital camera (Olympus 114 Corp., Tokyo, Japan) and documented.

Confocal Microscopy Observation

In the experiments of laser-induced thrombosis, P-selectin secretion, TF expression, and visualization of fibrin net and thrombus formation on the collagen, a fixed-stage microscope Zeiss Axio Examiner Z1 (Carl Zeiss Microscopy GmbH, Germany), a confocal scanner unit (CSU-X1, Yokogawa Electric Corporation, Japan), and a W Plan-Apochromat 20×/1.0 water immersion objective (Carl Zeiss Microscopy GmbH) were used. SlideBook 6.0 (Intelligent Imaging Innovations, Inc., United States) was used to analyze the recordings and images.

(1) Laser-induced thrombosis in the mice mesenteric vein and the assessment of thrombus area and PECAM-1/thrombus ratio (*in vivo*)

Laser-induced thrombosis was performed to establish the influence of TE on thrombus formation and to assess the activity of thrombus-forming platelets. Laser-induced thrombosis was performed as described previously (Marcinczyk et al., 2020). Briefly, 5 min before mesentery vein wall damage, Alexa Fluor 647-labeled PECAM-1 antibody (Alexa Fluor 647 anti-mouse CD31 antibody, BioLegend, United States) was injected into the femoral vein. To visualize the vessel wall and phosphatidylserine (PS)-negative platelets, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 0.1 mM in 0.05 ml of the mixture of DMSO and PBS (volume ratio 1:50); Life Technologies, Molecular Probes, United States) was administered by an intramuscular injection 5 min before thrombosis induction. A midline laparotomy incision was then made, and the mesentery of the ileum was then pulled out of the abdomen and draped over a plastic mound. The mesentery vein was examined microscopically and identified. The mesentery was continuously perfused with prewarmed (37°C) PBS to prevent the vessels from drying. The mesentery vein wall was injured by a 532 nm argon ion ablation laser (Ablate™, Intelligent Imaging Innovations, Inc., United States). The induction and progression of thrombosis were recorded for 3 min. One record was divided into 25 time points. In each time point, the area of thrombus was encircled. The values of the thrombus area from each time point were added and referred to as the total thrombus area. To assess the activity of platelets in thrombus, the area of fluorescence of PECAM-1 was also measured at each time point. The area of PECAM-1 fluorescence at a particular time point was then divided by the thrombus area at that time point. The values from one record were added and referred to as the PECAM-1/thrombus ratio. One thrombus was induced in one mouse.

(2) P-selectin secretion at the site of laser injury in the mesenteric vein in mice (*in vivo*)

Five minutes before mesentery vein wall damage, Alexa Fluor 647-labeled P-selectin antibody (50 μg/kg, Alexa Fluor 647 Rat Anti-Mouse CD62 P, BD Pharmingen, United States) was injected into the femoral vein of anesthetized mice. The mesenteric vein was isolated and injured by the laser as described above. P-selectin secretion was recorded for 6 min. The record was then divided into 25 time points. The values of P-selectin fluorescence from the 25 time points were added and referred to as total P-selectin fluorescence. One P-selectin measurement was performed in one mouse.

(3) TF expression at the site of laser injury in the mesenteric artery in mice (*in vivo*)

Five minutes before mesentery arterial wall damage, Alexa Fluor 488-labeled TF antibody (35 μg/kg, CD142 Antibody, Alexa Fluor 488 conjugated, Bioss Inc., United States) was injected into the femoral vein of anesthetized mice. The mesenteric artery was isolated and injured in the same manner as that for the mesenteric vein. TF expression was recorded for 6 min. One record was divided into 25 time points. The value of TF fluorescence from each time point was multiplied by the area of its fluorescence. The resultant values from 25 time points were added and referred to as total TF fluorescence. One measurement of TF expression was performed in one mouse.

(4) Thrombus formation in a flow chamber and the assessment of platelet procoagulant index (PI) in rat blood (*ex vivo*)

Thrombus formation on collagen was performed to assess the procoagulant activity of platelets. The procoagulant index indicates the amount of irreversibly activated platelets with exposed PS which catalyzes the coagulation reaction. Thrombus formation on collagen type I fibers has been reported earlier (Misztal et al., 2019). Briefly, blood treated with the anticoagulant trisodium citrate was supplemented with Fraxiparine (5 U/mL, GlaxoSmithKline, United Kingdom) and incubated with $DiOC_6(3)$ (0.1 mM in 0.05 ml of a mixture of DMSO and PBS (volume ratio 1:50)), Life Technologies, Molecular Probes, United States) for 2 min and then supplemented with $MgCl_2$ and $CaCl_2$ (final concentration of both: 3 mM). For thrombus formation, blood was perfused through a chamber with collagen-coated surface for 4 min at the shear rate of 1000 s^{-1}. The shear rate reflected arterial circulation. The thrombus-coated area was then perfused for 3 min with HEPES buffer supplemented with Alexa Fluor 647-conjugated Annexin V (5 μg/ml) (ANX V, Alexa Fluor® 647 conjugate, Thermo Fisher Scientific, United States), which stains PS-positive platelets, and $CaCl_2$ (2 mM). The staining process was followed by washing with HEPES buffer without $CaCl_2$ and ANX V. Platelets that did not undergo irreversible activation (PS-negative platelets, aggregating platelets) were stained with $DiOC_6(3)$, which is a lipophilic dye that penetrates through the intact cell membrane. End-stage measurements for thrombus formation were performed by acquiring two-color images of thrombus composed of PS-positive platelets (labeled with ANX V) and PS-negative platelets (visualized with $DiOC_6(3)$). To determine the PI, the area of PS-positive platelets was divided by the area of PS-negative platelets.

(5) Evaluation of fibrin net density in rat plasma (*ex vivo*)

Fibrin net density in clot was evaluated as described previously (Gromotowicz-Poplawska et al., 2019). Briefly, rat blood samples were centrifuged to obtain platelet-rich plasma (PRP, 200 × *g* for

20 min) and platelet-poor plasma (PPP, centrifugation of PRP at 14,000 × *g* for 5 min). Alexa Fluor 488-labeled human fibrinogen (Fibrinogen from Human Plasma, Alexa Fluor™ 488 Conjugate, Thermo Fisher Scientific, United States; final concentration: 15 μM) was added to the samples of PRP and PPP. To induce clot formation, $CaCl_2$ (final concentration: 20 mM) was added. The samples were then incubated at 37°C for 2 h. Relative clot density was established from the images of the resultant clots. In each image, five 40 μm long straight lines were placed randomly. The number of fibrin fibers crossing each line was counted. The average of the resultant values was then referred to as the relative clot density.

Biochemical Analysis of Hemostasis in Rat Plasma (*ex vivo*)

Nitrite and nitrate (NO_2^- and NO_3^-) concentrations in rat plasma were determined by an assay using the Griess method (R and D Systems, United States). Rat plasma concentrations of active form of tissue plasminogen activator (t-PA), active form of plasminogen activator inhibitor 1 (PAI-1), plasminogen (Innovative Research, Inc., United States), 6-keto prostaglandin 1 α (6-keto $PGF_{1\alpha}$, stable metabolite of PGI_2, Cayman Chemicals, United States), and tumor necrosis factor-α (TNF-α, Abcam, United Kingdom) were measured by immunoenzymatic assays. The microplate reader ELx808 (BioTek Instruments, Inc., United States) was used in all assays.

Euglobulin Clot Lysis Time (ECLT) in Rat Plasma (*ex vivo*)

The euglobulin clot lysis time was used to determine the time of clot dissolution. The ECLT was measured according to the method of Tomczyk et al. (Tomczyk et al., 2016).

Statistical Analysis

Data were evaluated using GraphPad Prism 5. The Shapiro-Wilk test was performed to determine the normal distribution of the data. Differences between two groups were assessed using Student's *t*-test or paired samples *t*-test (for normally distributed data) or the Mann-Whitney *U* test (for non-normally distributed data). Data are expressed as mean ± SEM or median (interquartile range) of the number of determination (n). A p value of <0.05 was considered to be significant.

RESULTS

General Characteristic of the Animals

TE did not affect the body weight and blood glucose levels in rats and mice. The mean initial body weight of the rats was 282–293 g. The mean body weight of the diabetic rats (Diabetes, 100 mg/kg, 200 mg/kg, and 400 mg/kg groups) after 5 weeks of experiment was 224–232 g, whereas the mean body weight of rats from the VEH group was 390 g. The mean initial blood glucose level of the rats was 82–85 mg/dl. The mean blood glucose level of the diabetic rats (Diabetes, 100 mg/kg, 200 mg/kg, and 400 mg/kg groups) after 5 weeks of experiment was 374–388 mg/dl, whereas the mean glucose level of rats from the VEH group was 85 mg/dl. The mean initial body weight of the mice was 27–28 g. The mean body weight of the diabetic mice (Diabetes, 100 mg/kg, 200 mg/kg, and 400 mg/kg groups) after 4 weeks of experiment was 21–22 g, whereas the mean body weight of mice from the VEH group was 31 g. The mean initial blood glucose level of the mice was 127–128 mg/dl. The mean blood glucose level of the diabetic mice (Diabetes, 100 mg/kg, 200 mg/kg, and 400 mg/kg groups) after 4 weeks of experiment was 445–460 mg/dl. Based on the daily observation of the animals, no reduced water and food intake, diarrhea or apathy were observed.

Primary Hemostasis

TE prolonged BT only at the dose of 400 mg/kg (**Figure 1A**).

Electrically Induced Arterial Thrombosis in Rat Carotid Artery (*in vivo*)

(1) Dynamics of thrombus formation

TE increased the IBF and prolonged TTO at 200 and 400 mg/kg doses (**Figures 2B,C**). However, TE at 200 mg/kg dose increased thrombus weight (**Figures 2D**).

(1) Histological staining of the rat arterial thrombus

TE changed the arterial thrombus structure and arterial wall in animals from the test groups (**Figure 3**). All thrombi consisted of fibrin, platelets, trapped erythrocytes, and leukocytes in various proportions. Thinning of the middle layer of the arterial wall was observed in animals from the Diabetes group and in animals treated with TE at 100 mg/kg dose. The largest increase in the thickness of the middle layer of the arterial wall was observed in animals treated with TE at 400 mg/kg dose. The middle layer thickness in this group was similar to that observed in the VEH group. Furthermore, only single leukocytes were observed in thrombi from animals treated with TE at 400 mg/kg dose. The thrombi from the animals treated with TE at 200 mg/kg dose contained a large amount of fibrin. Many erythrocytes were entrapped in the thrombus in Diabetes animals and in animals treated with TE at 400 mg/kg dose.

Thrombus Formation in the Flow Chamber and the Assessment of PI in Rat Blood (*ex vivo*)

TE decreased PI at all the tested doses. However, this effect was less pronounced as the dose of TE increased (**Figure 4A**).

Laser-Induced Thrombosis in the Mice Mesenteric Vein and the Assessment of the Thrombus Area and PECAM-1/Thrombus Ratio (*in vivo*)

TE at only 100 mg/kg dose decreased the thrombus area (**Figure 5A**). However, at 100 and 200 mg/kg doses, TE

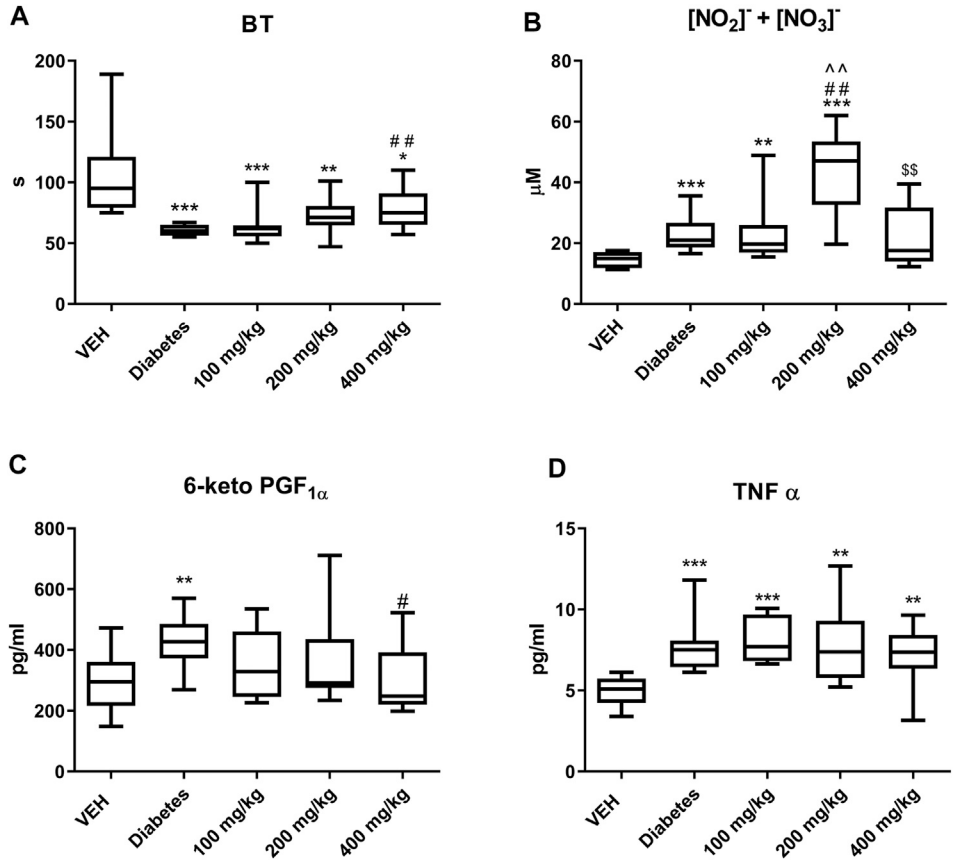

FIGURE 1 | The effect of TE on: BT **(A)**, concentration of NO_2^- and NO_3^- **(B)**, concentration of 6-keto $PGF_{1\alpha}$ **(C)**, concentration of TNF-α **(D)**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. VEH; #$p < 0.05$, ##$p < 0.01$ vs. Diabetes; ^^$p < 0.01$ vs. 100 mg/kg; $$$p < 0.01$ vs. 200 mg/kg; n = 8–11. Data are shown as median (interquartile range).

increased the PECAM-1/thrombus ratio, thus indicating reduced platelet activity (**Figure 5B**).

Assessment of P-Selectin Secretion at the Site of Laser Injury in the Mice Mesenteric Vein (*in vivo*)

TE at 100 and 400 mg/kg doses decreased P-selectin secretion at the site of laser injury of the mesenteric vein (**Figure 6B**).

Evaluation of Fibrin Net Density in Rat Plasma (*ex vivo*)

Fibrin net density was assessed in the clot formed after recalcination of PRP and PPP. The use of two different experimental environments (PPP and PRP) enabled to assess the role of platelets in the process of fibrin formation after TE treatment. TE increased the relative clot density in PRP at all the tested doses, and the largest increase was observed at 200 mg/kg (**Figure 7A**, white bars). However, the increase in the relative clot density in PPP was observed only at 100 and 200 mg/kg doses (**Figure 7A**, black bars). Similar to PRP, the most pronounced increase in the relative clot density in PPP was observed at 200 mg/kg dose.

TF Expression at the Site of Laser Injury in the Mesenteric Artery in Mice (*in vivo*)

TE at all the tested doses decreased TF fluorescence at the site of laser injury in the mesentery artery. This effect was most pronounced at 200 mg/kg dose (**Figure 8B**).

Fibrinolysis

TE prolonged ECLT at 200 and 400 mg/kg doses. This effect was most prominent at 200 mg/kg dose (**Figure 9A**). TE did not affect the concentration of plasminogen (**Figure 9B**). TE at 200 mg/kg dose increased the concentration of active form of t-PA, but its concentration was reduced at 400 mg/kg dose as compared to that at 200 mg/kg dose (**Figure 9C**). TE showed a tendency to increase the concentration of active form of PAI-1 (**Figure 9D**).

Concentrations of NO_2^- and NO_3^-

TE at 200 mg/kg dose increased the concentrations of NO_2^- and NO_3^-. However, TE at 400 mg/kg dose decreased the

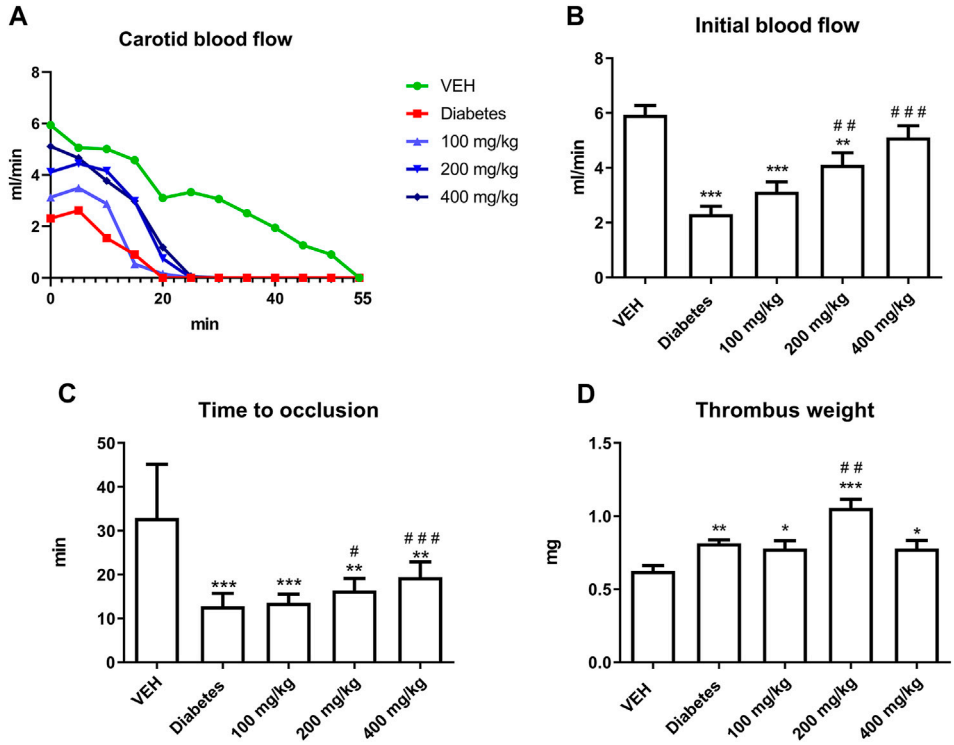

FIGURE 2 | The effect of TE on electrically induced thrombosis. Changes in the carotid blood flow in the artery of rats subjected to electrical stimulation **(A)**. The effect of TE on: IBF in the rat artery before electrical stimulation **(B)**, TTO in the rat artery **(C)**, dry thrombus weight **(D)**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. VEH; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. Diabetes; n = 8–11. Data are shown as mean ± SEM.

concentrations of NO_2^- and NO_3^- as compared to that at 200 mg/kg dose (**Figure 1B**).

Concentrations of 6-Keto $PGF_{1\alpha}$ and TNF-α

TE at 400 mg/kg dose decreased the concentration of 6-keto $PGF_{1\alpha}$ (**Figure 1C**). However, it did not affect the concentration of TNF-α (**Figure 1D**).

DISCUSSION

In the present study, we observed the multidirectional effects of TE on hemostasis in STZ-diabetic rats and mice, which were not associated with the blood glucose level. An increase in the dynamics and extent of thrombus formation in STZ-diabetes was demonstrated in the models of electrically induced and laser-induced thrombosis. Thrombosis was enhanced due to increased platelet (**Figures 4**–**6**) and coagulation activity (**Figures 7**, **8**), impaired fibrinolysis (**Figures 9C,D**), endothelial dysfunction (**Figure 1B**), reduced blood flow (**Figure 2B**), and inflammatory state (**Figures 1C,D**). *In vivo* experiments were performed in both rats and mice to make the overall effect of TE species-independent. We observed that TE in diabetic rats, unlike in normoglycemic rats (Marcinczyk et al., 2017), does not exert antithrombotic effect in the rat carotid artery. Moreover, it intensified electrically induced thrombotic process at 200 mg/kg dose, which was manifested as increased thrombus weight (**Figure 2D**). Considering the slight increase in fibrin formation in normoglycemia, we presumed that the increase in thrombus weight in STZ-diabetes could be due to enhanced coagulation. This hypothesis was confirmed in the experiment where the most pronounced fibrin net density was observed at TE dose of 200 mg/kg in clots formed in both PPP and PRP. TE at all the tested doses increased fibrin net density in PRP, while it increased the fibrin net density in PPP only at 100 and 200 mg/kg doses. This observation indicated an increase in platelet activity at 400 mg/kg dose as compared to that noted at 100 mg/kg dose and confirmed the key role of platelets in the process of fibrin formation. Increased fibrin net density was the reason for further studies on the effect of TE on coagulation. Because the TF/VIIa complex triggers coagulation *in vivo* (Smith, 2009), the next stage of the study was to determine the expression of TF in a mouse mesenteric artery. TE at all the tested doses decreased the expression of TF, and this effect was the strongest at 200 mg/kg dose (**Figure 8**). Because TE at 200 mg/kg dose caused the most dense fibrin net formation, the contribution of TF in the enhancement of coagulation was ruled out. As platelets play an essential role in the process of fibrin formation, the next stage of the study was to evaluate the influence of TE on platelet procoagulant activity. For this purpose, we used a flow chamber combined with a confocal microscopic imaging system. This model enables real-time monitoring of the process of thrombus formation on collagen fibers (*ex vivo*). Platelets in thrombus can be divided into two subpopulations:

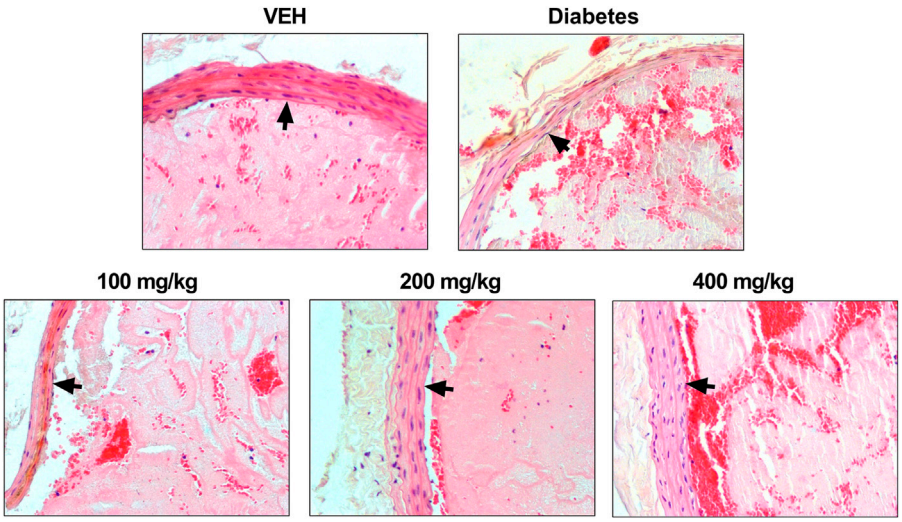

FIGURE 3 | Representative photomicrographs of the thrombi and rat carotid artery wall. Fibrin and platelet aggregates are stained pink, erythrocytes are stained red, leukocytes are stained blue, and black arrows indicate the arterial wall. Routine H and E staining, ×200 magnification.

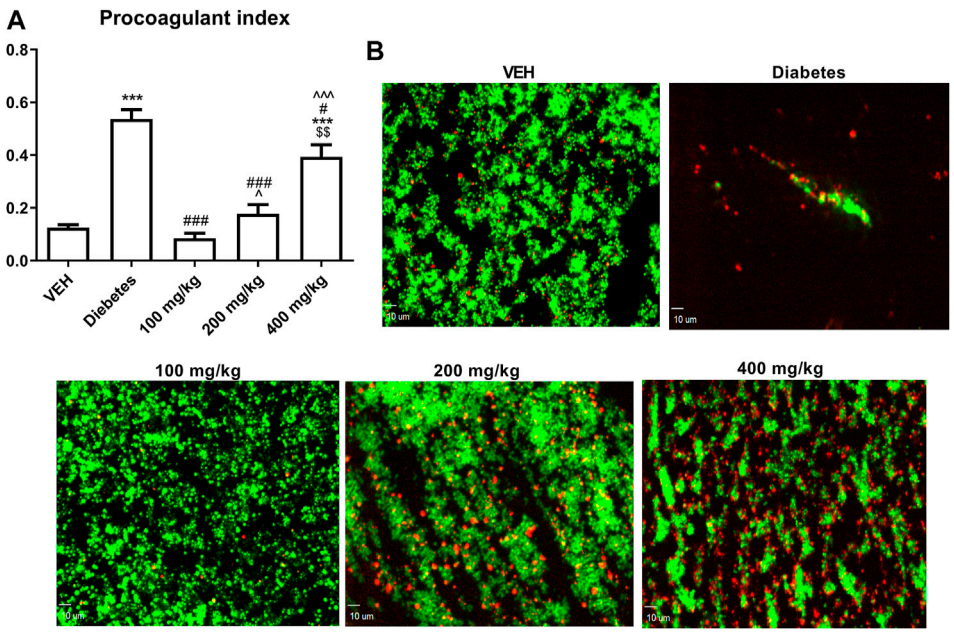

FIGURE 4 | The effect of TE on PI **(A)**. Representative confocal microscopy images of thrombus consisting of PS-negative platelets (green) and PS-positive platelets (red). Bar = 10 μm **(B)** ***$p < 0.001$ vs. VEH; #$p < 0.05$, ###$p < 0.001$ vs. Diabetes; ^$p < 0.05$, ^^^$p < 0.001$ vs. 100 mg/kg; $$$p < 0.01$ vs. 200 mg/kg; $n = 8–11$. Data are shown as mean ± SEM.

procoagulant and aggregating platelets. During sustained and potent activation, platelets change their discoidal shape to irregular shape, and the platelet cell membrane undergoes irreversible reorganization, which causes exposure of PS from the inner to the outer leaflet of the platelet plasma membrane. Because PS catalyzes the activation of coagulation factors, platelets with exposed PS are called procoagulant platelets. Aggregating platelets do not show exposure of PS (PS-negative platelets). However, because they are close to activating factors, they form pseudopods and undergo secretion (Heemskerk et al., 2013). As PS catalyzes coagulation reactions, the ratio of the area of PS-positive platelets to the area of PS-negative platelets is

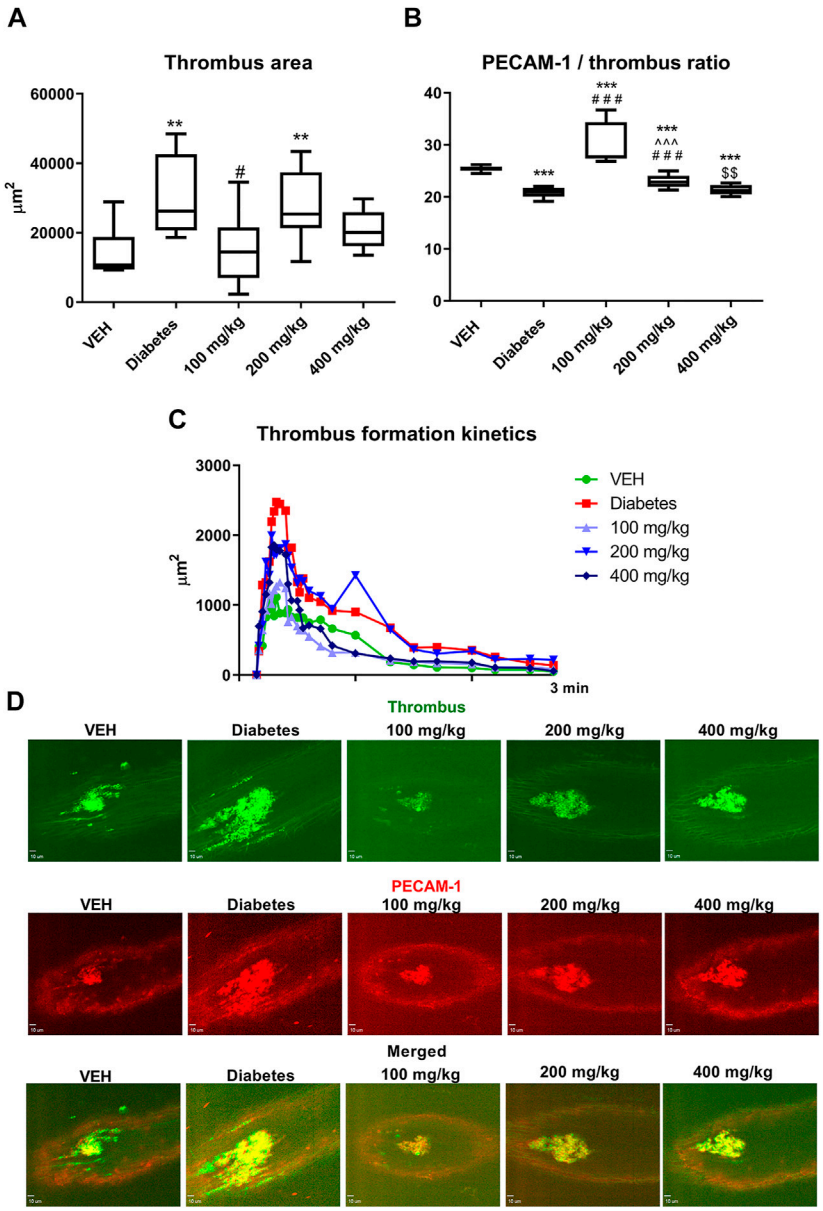

FIGURE 5 | The effect of TE on laser-induced thrombosis. The effect of TE on: thrombus area **(A)** and PECAM-1/thrombus ratio **(B)**. Kinetics of thrombus formation at the site of laser injury **(C)**. Representative confocal microscopy images of thrombus (green, top row), PECAM-1 (red, middle row), and merged channels (bottom row). Bar = 10 μm **(D)**. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. VEH; $\#p < 0.05$, $\#\#\#p < 0.001$ vs. Diabetes; $\wedge\wedge\wedge p < 0.001$ vs. 100 mg/kg; $\$\$p < 0.01$ vs. 200 mg/kg; $n = 7–10$. Data are shown as median (interquartile range).

termed as PI and reflects the extent of platelet procoagulant response. PI was reduced at all tested doses of TE; the strongest antiprocoagulant effect was observed at 100 mg/kg dose, while the weakest reduction in PI was observed at 400 mg/kg dose (**Figure 4**). Therefore, it can be assumed that the weakened antiprocoagulant effect contributed to the increased fibrin net density in clots formed in PRP at 400 mg/kg dose compared to that in the Diabetes group. However, the decreased fibrin net density at 400 mg/kg dose as compared to that at 200 mg/kg dose with and without platelets (PRP and PPP) indicates an unknown plasma-related mechanism of coagulation activation. Further experiments on the influence of TE on platelet activity were performed in a model of laser-induced thrombosis. In this model, because of the small area of the exposed subendothelial matrix, potent and sustained platelet activation does not occur, which results in thrombus composed of platelets that do not undergo irreversible activation (aggregating, PS-negative platelets) and can easily detach from the site of injury. Therefore, this model is suitable to investigate platelet activity in the aggregation state. TE only at the lowest dose (100 mg/kg) decreased the thrombus area in the model of laser-induced thrombosis (**Figure 5A**); this finding is consistent with the antiplatelet activity observed in the

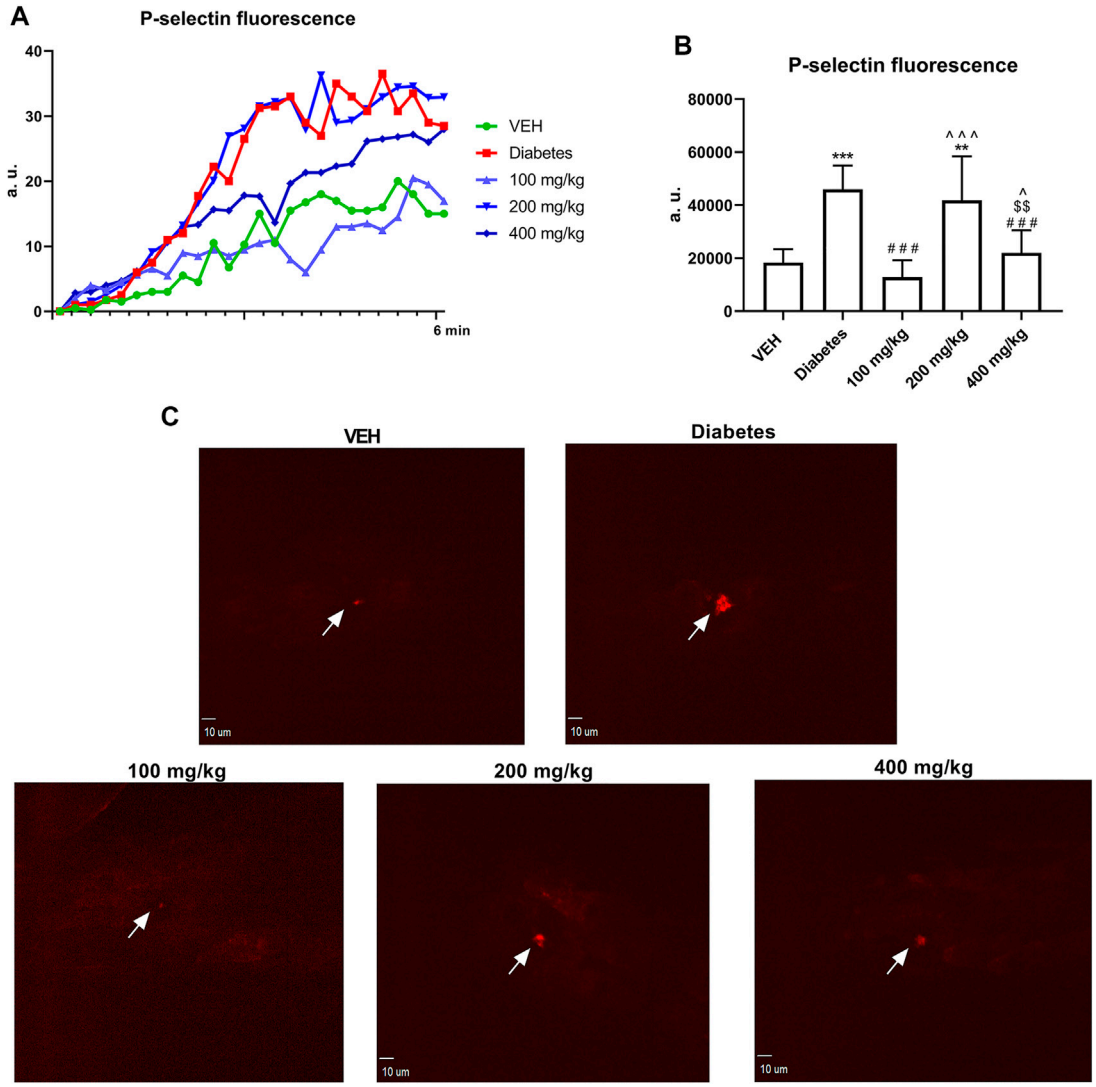

FIGURE 6 | Changes in P-selectin fluorescence at the site of injury. Fluorescence of P-selectin is presented in arbitrary units of fluorescence (a. u.) **(A)**. The effect of TE on P-selectin secretion by platelets **(B)**. Representative confocal microscopy images of P-selectin. White arrows indicate the site of injury. Bar = 10 µm **(C)**. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. VEH; ###$p < 0.001$ vs. Diabetes; ^$p < 0.05$, ^^^$p < 0.001$ vs. 100 mg/kg; $$$p < 0.01$ vs. 200 mg/kg, n = 8–9. Data are shown as mean ± SEM.

flow chamber model. The previously observed antithrombotic effect in normoglycemic rats and mice was noted only at the highest dose (400 mg/kg) and corresponded to a potent antiplatelet activity (Marcinczyk et al., 2017). Next, the assessment of the activity of aggregating platelets in thrombus was measured by the PECAM-1/thrombus ratio, which indicates the proportion of platelet-endothelial cell adhesion molecule 1 (PECAM-1) in thrombus. As PECAM-1 is considered as an antithrombotic molecule, the higher the PECAM-1/thrombus ratio, the less activated are the platelets in thrombus (Marcinczyk et al., 2020). TE increased the PECAM-1/thrombus ratio with the most pronounced effect at 100 mg/kg dose (**Figure 5B**). The antiplatelet effect of TE at 100 mg/kg dose was significant enough to translate into the antithrombotic effect. These observations indicate that TE at 100 mg/kg dose most effectively reduced platelet activity in two modes of activation: reversible, which was expressed as increased PECAM-1/thrombus ratio, and irreversible, which was expressed as decreased PI. The sections of arterial thrombi showed an increase in entrapped erythrocytes at 400 mg/kg dose (**Figure 3**). This indicates hemolysis, which leads to the enhanced incorporation of erythrocytes into thrombus (Helms et al., 2013). Furthermore, the pale red color of plasma of the 400 mg/kg group (data not shown) indicated the occurrence of hemolysis. During hemolysis, erythrocytes release platelet agonists, (e.g., ADP), which also contributes to enhanced platelet activity (Noh et al., 2010) and could partially explain the alleviation of the antiplatelet effect of TE at 400 mg/kg dose. The next stage of the study was the assessment of P-selectin secretion at the site of laser-induced mesenteric vein injury under

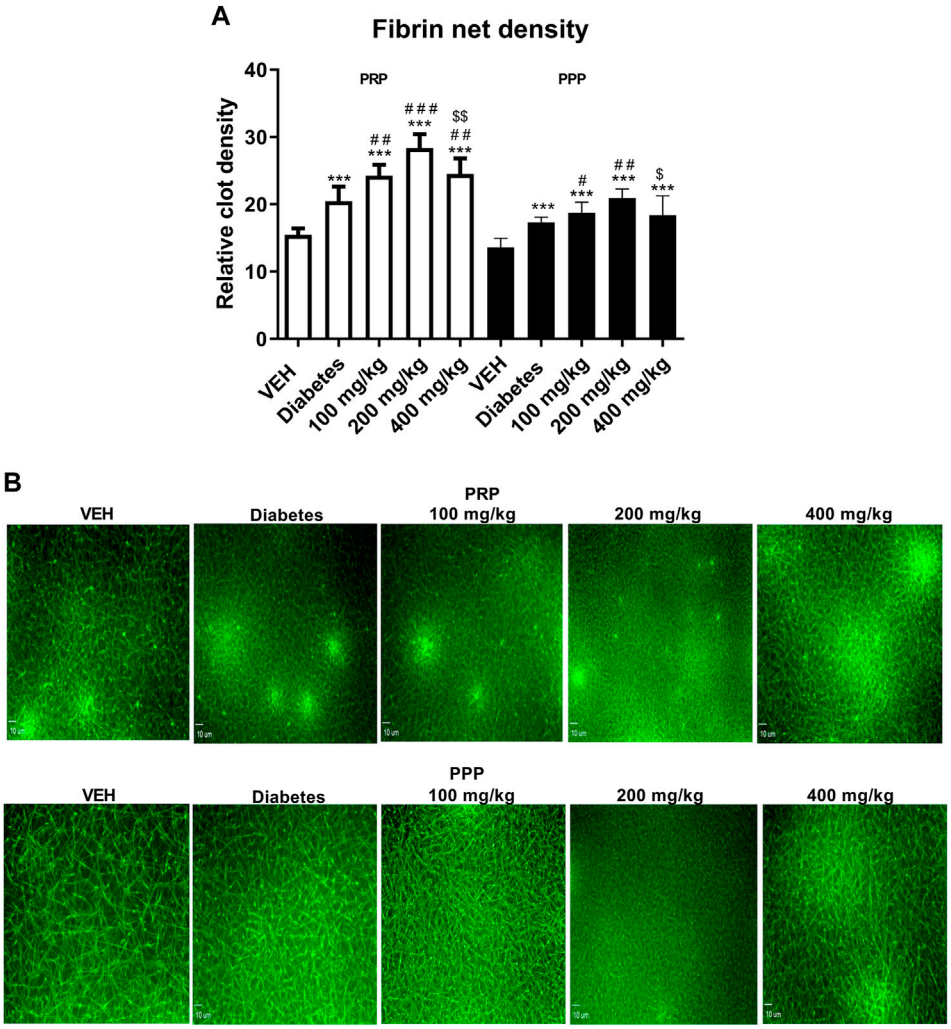

FIGURE 7 | The effect of TE on the fibrin net density in clots formed after recalcination of PRP (white bars) and PPP (black bars) **(A)**. Representative confocal microscopy images of fibrin net. Bar = 10 μm **(B)**. ***$p < 0.001$ vs. VEH; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. Diabetes; \$$p < 0.05$, \$\$$p < 0.01$ vs. 200 mg/kg; $n = 7–9$. Data are shown as mean ± SEM.

intravital conditions. P-selectin, a marker of platelet secretion, is responsible for thrombus progression and its stability, which allows direct interactions of platelets with endothelial cells and leukocytes (Prakash et al., 2017). Inhibition of P-selectin secretion was observed only at 100 and 400 mg/kg doses; this finding may suggest the decreased stability of thrombus (**Figure 6**). The curve of thrombus formation kinetics in the 100 mg/kg- and 400 mg/kg-treated groups is characterized with a high peak and the slope of the curve, which correspond to the addition of the thrombotic material shortly after vessel injury and subsequent elution of the thrombus. The curve of thrombus kinetics in the 200 mg/kg-treated group shows two peaks that correspond to two massive additions of the thrombotic material; this finding suggests increased thrombus stability as compared to that noted in the 100 and 400 mg/kg groups (**Figure 5C**). We observed that TE at 200 and 400 mg/kg doses increased the IBF in the rat carotid artery and prolonged TTO (**Figures 2B,C**); this finding indicated the contribution of the vessel wall in changes of thrombus formation kinetics. Furthermore, TE at 400 mg/kg dose also prolonged BT (**Figure 1A**), which reflects the dependence of primary hemostasis on the blood vessel response (vasoconstriction/vasodilatation) and on the activity of platelets that form a platelet plug at the site of injury (Mattix and Singh, 1999). Because of the lack of the antiplatelet effect of TE at 400 mg/kg dose in this model, the prolongation of BT indicated an improvement in vascular function. Furthermore, H and E staining revealed that TE increased the thickness of the middle layer of the artery wall (**Figure 3**), which may be an indirect effect associated with an increased blood flow (Basu et al., 2010). Some of the compounds of TE and their metabolites have potential to improve vascular functions, which may be reflected as increased blood flow observed in **Figure 2B**. Metabolites generated from ellagic acid and

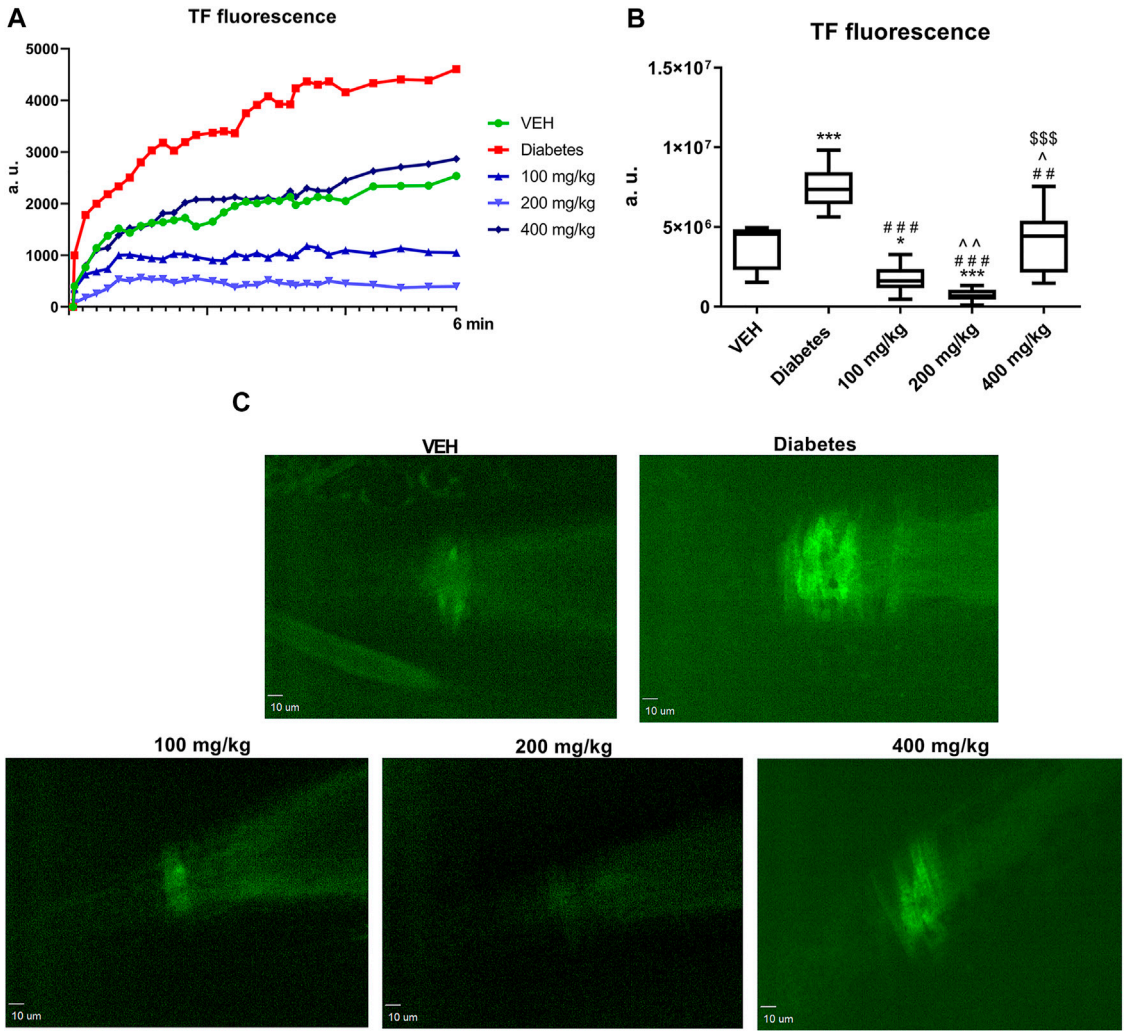

FIGURE 8 | Changes in TF fluorescence at the site of injury over time. Fluorescence of TF is presented in arbitrary units of fluorescence (a. u.) **(A)**. The effect of TE on TF expression **(B)**. Representative confocal microscopy images of TF expression. Bar = 10 μm **(C)**. *$p < 0.05$, ***$p < 0.001$ vs. VEH; ##$p < 0.01$, ###$p < 0.001$ vs. Diabetes; ^$p < 0.05$, ^^$p < 0.01$ vs. 100 mg/kg; $$$$p < 0.001$ vs. 200 mg/kg; $n = 7–9$. Data are shown as median (interquartile range).

ellagitannins by the intestinal microbiota such as urolithin A and B have been shown to enhance eNOS expression (Han et al., 2016; Spigoni et al., 2016), and the activity of procyanidin B leads to vasorelaxation of the human mammary artery (Novakovic et al., 2017). This indicated that the observed improvement in blood flow might be partially due to an endothelial-dependent mechanism. Therefore, in the next stage of the study, the concentrations of NO_2^- and NO_3^-, the stable metabolites of NO – the main vasoactive molecule released from the endothelium (Mitchell et al., 2008) – were measured. TE at 200 mg/kg dose increased the concentrations of NO_2^- and NO_3^-, which may contribute to IBF improvement and TTO prolongation (**Figures 2B,C**, respectively). Previous studies on endothelial NO release have shown that the increase in eNOS activity is linked to the decrease in TF expression (Solovey et al., 2010). Therefore, the largest decrease in TF fluorescence at 200 mg/kg dose may be due to the increased production of NO at this dose. However, the reason why NO_2^- and NO_3^- concentrations increased only at 200 mg/kg dose and not at 400 mg/kg dose remains unclear.

The next component of the prothrombotic effect of TE at 200 mg/kg dose was the attenuation of fibrinolysis, as the fibrinolytic activity of plasma measured as ECLT was most strongly inhibited at this dose (**Figure 9A**). However, TE at 200 mg/kg dose increased the concentration of the active form of t-PA (**Figure 9C**), which might be due to increase NO release (Giannarelli et al., 2007). Furthermore, compared to 200 mg/kg dose, TE at 400 mg/kg dose decreased the concentration of active form of t-PA, which may result from decreased concentration of bradykinin – one of the activators of t-PA release (Marcinczyk et al., 2017). The antifibrinolytic effect of TE could also be, to some extent, explained by an increase in the concentration of the active form of PAI-1 (**Figure 9D**). This may be due to the increased activation of platelets, which release PAI-1 during

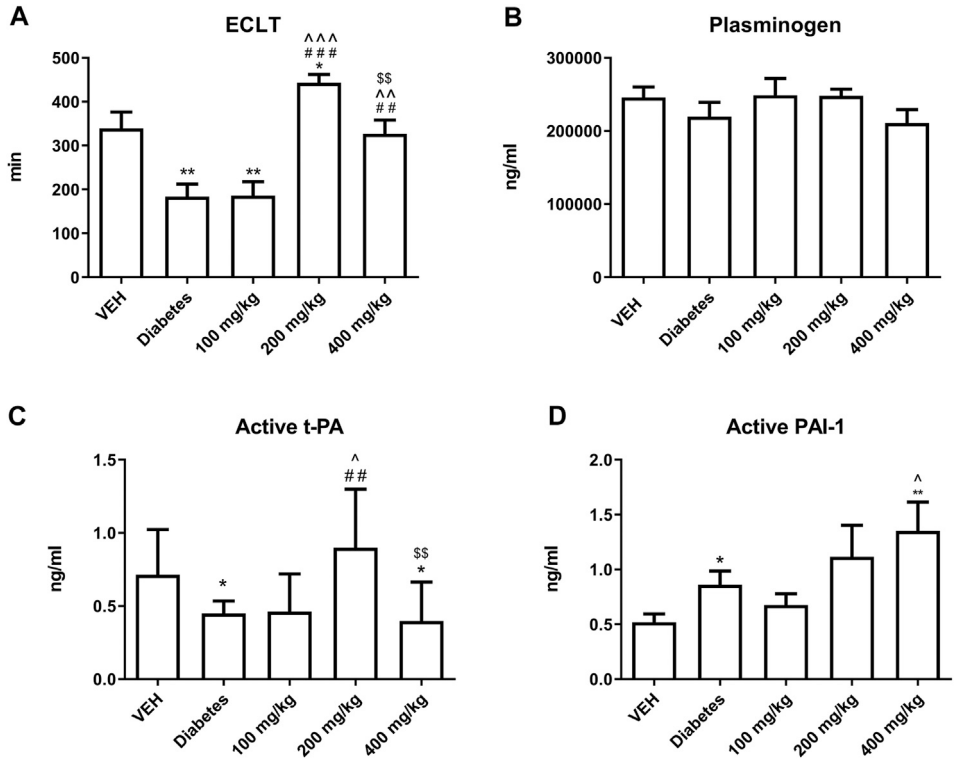

FIGURE 9 | The effect of TE on fibrinolysis. The effect of TE on: ECLT **(A)**, plasminogen concentration **(B)**, concentration of active form of t-PA **(C)**, concentration of active form of PAI-1 **(D)**. *$p < 0.05$, **$p < 0.01$ vs. VEH; ##$p < 0.01$, ###$p < 0.001$ vs. Diabetes; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs. 100 mg/kg; $$$p < 0.01$ vs. 200 mg/kg; n = 7–11. Data are shown as mean ± SEM.

the activation process (Morrow et al., 2020). Furthermore, the tendency of increasing concentration of active form of PAI-1 may be due to the decreased concentration of t-PA that binds to PAI-1, thus diminishing the measurable pool of free PAI-1 (Chandler et al., 1995).

Considering the anti-inflammatory activity of TE (Tunón et al., 1995; Wölfle et al., 2017) and the prothrombotic effect of inflammation (Esmon, 2003), the concentration of TNF-α, a commonly accepted inflammation marker, was measured. TE did not change the level of TNF-α in rat plasma (**Figure 1D**). However, no effect on TNF-α does not imply a lack of anti-inflammatory properties of TE in diabetes. The less number of leukocytes in thrombi from the 400 mg/kg-treated group than that in thrombi from the Diabetes group may indicate the anti-inflammatory activity of TE (**Figure 3**). We have also shown that TE at 400 mg/kg dose lowered the concentration of 6-keto $PGF_{1\alpha}$, thus indicating the inhibition of PGI_2 production by endothelial cells in the presence of high dose of TE (**Figure 1C**). The effect of decreased PGI_2 production may be due to the known inhibitory effect of TE or its agrimoniin-rich fraction on COX activity (Tunón et al., 1995; Hoffmann et al., 2016). Furthermore, the results of our study are consistent with those which show that the anti-inflammatory effect of TE is dependent on the inhibition of arachidonic acid (AA) metabolism. However, in those studies, the anti-inflammatory effect was demonstrated in a human keratinocyte cell line (HaCaT, *in vitro*) and in human skin (*in vivo*, applied topically) (Hoffmann et al., 2016). Thus, our study for the first time showed that after oral intake, the anti-inflammatory effect of TE could be dependent on the inhibition of AA metabolism.

In our present study, the bioavailability and plasma concentrations of specific compounds originating from TE and their metabolites were not assessed; thus, we cannot confirm which compounds or metabolites are primarily responsible for the changes observed in hemostasis. Furthermore, it is possible that the inflammatory state changed the bioavailability of specific TE components. Urolithins, which are the gut microbiota-derived metabolites of ellagitannins, undergo glucuronidation rapidly after absorption. Glucuronides of urolithins are inactive, but under inflammatory conditions, they are deconjugated by ß-glucuronidase to free urolithins (Piwowarski et al., 2017). Therefore, it can be presumed that the amount of free urolithins after the administration of equivalent doses of TE is different in normoglycemia and STZ-diabetes. Furthermore, the differences in gut microbiota in normoglycemia and diabetes (Ma et al., 2020) could lead to the formation of different metabolites of TE. In our present study, we showed for the first time that the activity of TE is dependent on the pathological condition. It is also possible that TE activity in STZ-diabetes was associated with the production of inflammatory-related TE metabolites. To summarize, we have shown that TE exerts multidirectional

effects on the activity of platelets, coagulation, fibrinolysis, and endothelial-dependent vascular functions in STZ-diabetic rats and mice. These multidirectional effects of TE on hemostasis translated into a model-dependent effect on the thrombotic process. Furthermore, the contrasting effects of TE in normoglycemia (Marcinczyk et al., 2017) and diabetes indicates that the mechanism of action of TE is related to the pathological-dependent baseline hemostatic activity.

Since the TE has been used to treat diarrhea (Tomczyk and Latté, 2009), our study may have clinical relevance in hemostasis regulation. However, considering the activation of coagulation and the inhibition of fibrinolysis by TE, the beneficial impact of ellagitannins (Haber et al., 2011; Istas et al., 2018) on hemostasis after short-term use is not so obvious. Our study's TE's doses were selected based on studies with rats and mice (Shushunov et al., 2009). Pharmacokinetics of TE in humans have not been investigated yet, but TE's doses are lower (approximately 2.5–40 mg/kg) (Subbotina et al., 2003; Huber et al., 2007). Due to the fast metabolism rate, mice and rats often require higher doses of the drug (e.g., acetylsalicylic acid) (Wientjes and Levy, 1988; Nagelschmitz et al., 2014). Nevertheless, we cannot state whether the same pharmacological effects can be expected after using the same doses of TE in humans as in rodents. Raising these issues, further studies are needed to assess the potential clinical significance of our study particularly in patients with increased risk of thromboembolic events.

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 Local Ethical Committee on Animal Testing (Approval Nos.: 72/2018 and 73/2018).

AUTHOR CONTRIBUTIONS

NM designed the research, performed experiments, analyzed the data, and wrote the manuscript. AG, IK, and JS performed experiments. TM and AG-P analyzed the results and contributed to the data statistics. MT and EC supervised the research and revised the article.

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REFERENCES

Augustynowicz, D., Latté, K. P., and Tomczyk, M. (2021). Recent Phytochemical and Pharmacological Advances in the Genus Potentilla L. Sensu Lato - an Update Covering the Period from 2009 to 2020. *J. Ethnopharmacology* 266, 113412. doi:10.1016/j.jep.2020.113412

Basu, P., Sen, U., Tyagi, N., and Tyagi, S. C. (2010). Blood Flow Interplays with Elastin: Collagen and MMP: TIMP Ratios to Maintain Healthy Vascular Structure and Function. *Vasc. Health Risk Manag.* 6, 215–228. doi:10.2147/VHRM.S9472

Bouaziz, A., Romera-Castillo, C., Salido, S., Linares-Palomino, P. J., Altarejos, J., Bartegi, A., et al. (2007). Cinnamtannin B-1 from Bay Wood Exhibits Antiapoptotic Effects in Human Platelets. *Apoptosis* 12, 489–498. doi:10.1007/s10495-006-0014-z

Byun, M.-W. (2012). Effect of Procyanidin C1 on Nitric Oxide Production and Hyperpolarization Through Ca2+-Dependent Pathway in Endothelial Cells. *J. Med. Food* 15, 1032–1037. doi:10.1089/jmf.2012.2297

Chandler, W. L., Levy, W. C., and Stratton, J. R. (1995). The Circulatory Regulation of TPA and UPA Secretion, Clearance, and Inhibition During Exercise and During the Infusion of Isoproterenol and Phenylephrine. *Circulation* 92, 2984–2994. doi:10.1161/01.CIR.92.10.2984

Dejana, E., Villa, S., and de Gaetano, G. (1982). Bleeding Time in Rats: A Comparison of Different Experimental Conditions. *Thromb. Haemost.* 48, 108–111. doi:10.1055/s-0038-1657230

Dong, H., Chen, S.-X., Kini, R. M., and Xu, H.-X. (1998). Effects of Tannins fromGeumjaponicumon the Catalytic Activity of Thrombin and Factor Xa of Blood Coagulation Cascade. *J. Nat. Prod.* 61, 1356–1360. doi:10.1021/np9801458

Esmon, C. T. (2003). Inflammation and Thrombosis. *J. Thromb. Haemost.* 1, 1343–1348. doi:10.1046/j.1538-7836.2003.00261.x

Furman, B. L. (2015). Streptozotocin-Induced Diabetic Models in Mice and Rats. *Curr. Protoc. Pharmacol.* 70 , 5.47.1–5.47.20. doi:10.1002/0471141755.ph0547s70

Geiger, C., Scholz, E., and Rimpler, H. (1994). Ellagitannins from Alchemilla xanthochloraandPotentilla Erecta*. *Planta Med.* 60, 384–385. doi:10.1055/s-2006-959510

Giannarelli, C., De Negri, F., Virdis, A., Ghiadoni, L., Cipriano, A., Magagna, A., et al. (2007). Nitric Oxide Modulates Tissue Plasminogen Activator Release in Normotensive Subjects and Hypertensive Patients. *Hypertension* 49, 878–884. doi:10.1161/01.HYP.0000260471.16113.d8

Gromotowicz-Poplawska, A., Marcinczyk, N., Misztal, T., Golaszewska, A., Aleksiejczuk, M., Rusak, T., et al. (2019). Rapid Effects of Aldosterone on Platelets, Coagulation, and Fibrinolysis Lead to Experimental Thrombosis Augmentation. *Vasc. Pharmacol.* 122-123. doi:10.1016/j.vph.2019.106598

Haber, S. L., Joy, J. K., and Largent, R. (2011). Antioxidant and Antiatherogenic Effects of Pomegranate. *Am. J. Heal. Pharm.* 68, 1302–1305. doi:10.2146/ajhp100610

Han, Q.-a., Yan, C., Wang, L., Li, G., Xu, Y., and Xia, X. (2016). Urolithin A Attenuates Ox-LDL-Induced Endothelial Dysfunction Partly by Modulating microRNA-27 and ERK/PPAR-γ Pathway. *Mol. Nutr. Food Res.* 60, 1933–1943. doi:10.1002/mnfr.201500827

Heemskerk, J. W. M., Mattheij, N. J. A., and Cosemans, J. M. E. M. (2013). Platelet-based Coagulation: Different Populations, Different Functions. *J. Thromb. Haemost.* 11, 2–16. doi:10.1111/jth.12045

Helms, C. C., Marvel, M., Zhao, W., Stahle, M., Vest, R., Kato, G. J., et al. (2013). Mechanisms of Hemolysis-Associated Platelet Activation. *J. Thromb. Haemost.* 11, 2148–2154. doi:10.1111/jth.12422

Hoffmann, J., Casetti, F., Bullerkotte, U., Haarhaus, B., Vagedes, J., Schempp, C., et al. (2016). Anti-inflammatory Effects of Agrimoniin-Enriched Fractions of Potentilla Erecta. *Molecules* 21, 792. doi:10.3390/molecules21060792

Huber, R., Ditfurth, A. V., Amann, F., Güthlin, C., Rostock, M., Trittler, R., et al. (2007). Tormentil for Active Ulcerative Colitis. *J. Clin. Gastroenterol.* 41, 834–838. doi:10.1097/MCG.0b013e31804b2173

Istas, G., Feliciano, R. P., Weber, T., Garcia-Villalba, R., Tomas-Barberan, F., Heiss, C., et al. (2018). Plasma Urolithin Metabolites Correlate with Improvements in Endothelial Function after Red Raspberry Consumption: A Double-Blind Randomized Controlled Trial. *Arch. Biochem. Biophys.* 651, 43–51. doi:10.1016/j.abb.2018.05.016

Jiang, X., Zhao, L. Z., Zhang, H. L., Zhang, J., and Wang, H. Z. (2007). [Effects of Procyanidin Oligomers on Experimental Thrombosis in Rats]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 15, 617–621.

Lee, Y.-B., Han, K., Kim, B., Lee, S.-E., Jun, J. E., Ahn, J., et al. (2019). Risk of Early Mortality and Cardiovascular Disease in Type 1 Diabetes: a Comparison with Type 2 Diabetes, a Nationwide Study. *Cardiovasc. Diabetol.* 18, 157. doi:10.1186/s12933-019-0953-7

Lunder, M., Janić, M., and Šabovič, M. (2018). Prevention of Vascular Complications in Diabetes Mellitus Patients: Focus on the Arterial Wall. *Cvp* 17, 6–15. doi:10.2174/1570161116666180206113755

Ma, Q., Li, Y., Wang, J., Li, P., Duan, Y., Dai, H., et al. (2020). Investigation of Gut Microbiome Changes in Type 1 Diabetic Mellitus Rats Based on High-Throughput Sequencing. *Biomed. Pharmacother.* 124, 109873. doi:10.1016/j.biopha.2020.109873

Marcinczyk, N., Golaszewska, A., Misztal, T., Gromotowicz-Poplawska, A., Rusak, T., and Chabielska, E. (2020). New Approaches for the Assessment of Platelet Activation Status in Thrombus under Flow Condition Using Confocal Microscopy. *Naunyn-schmiedeberg's Arch. Pharmacol.* 393, 727–738. doi:10.1007/s00210-019-01789-x

Marcinczyk, N., Jarmoc, D., Leszczynska, A., Zakrzeska, A., Kramkowski, K., Strawa, J., et al. (2017). Antithrombotic Potential of Tormentil Extract in Animal Models. *Front. Pharmacol.* 8, e534. doi:10.3389/fphar.2017.00534

Mari, A., Eletto, D., Pizza, C., Montoro, P., and Piacente, S. (2013). Integrated Mass Spectrometry Approach to Profile Proanthocyanidins Occurring in Food Supplements: Analysis of Potentilla Erecta L. Rhizomes. *Food Chem.* 141, 4171–4178. doi:10.1016/j.foodchem.2013.06.101

Mattix, H., and Singh, A. K. (1999). Is the Bleeding Time Predictive of Bleeding Prior to a Percutaneous Renal Biopsy? *Curr. Opin. Nephrol. Hypertens.* 8, 715–718. doi:10.1097/00041552-199911000-00011

Melzig, M. F., and Böttger, S. (2020). Tormentillae Rhizoma – Review for an Underestimated European Herbal Drug. *Planta Med.* 86, 1050–1057. doi:10.1055/a-1129-7214

Misztal, T., Golaszewska, A., Tomasiak-Lozowska, M. M., Iwanicka, M., Marcinczyk, N., Leszczynska, A., et al. (2019). The Myeloperoxidase Product, Hypochlorous Acid, Reduces Thrombus Formation under Flow and Attenuates Clot Retraction and Fibrinolysis in Human Blood. *Free Radic. Biol. Med.* 141, 426–437. doi:10.1016/j.freeradbiomed.2019.07.003

Mitchell, J. A., Ali, F., Bailey, L., Moreno, L., and Harrington, L. S. (2008). Role of Nitric Oxide and Prostacyclin as Vasoactive Hormones Released by the Endothelium. *Exp. Physiol.* 93, 141–147. doi:10.1113/expphysiol.2007.038588

Morel, A., Hamed, A. I., Oleszek, W., Stochmal, A., Głowacki, R., and Olas, B. (2014). Protective Action of Proanthocyanidin Fraction from Medemia Argunnuts against Oxidative/nitrative Damages of Blood Platelet and Plasma Components. *Platelets* 25, 75–80. doi:10.3109/09537104.2013.769511

Morrow, G. B., Whyte, C. S., and Mutch, N. J. (2020). Functional Plasminogen Activator Inhibitor 1 Is Retained on the Activated Platelet Membrane Following Platelet Activation. *Haematologica* 105, 2824–2833. doi:10.3324/haematol.2019.230367

Murphy, K. J., Chronopoulos, A. K., Singh, I., Francis, M. A., Moriarty, H., Pike, M. J., et al. (2003). Dietary Flavanols and Procyanidin Oligomers from Cocoa (Theobroma Cacao) Inhibit Platelet Function. *Am. J. Clin. Nutr.* 77, 1466–1473. doi:10.1093/ajcn/77.6.1466

Nagelschmitz, J., Blunck, M., Kraetzschmar, J., Ludwig, M., Wensing, G., and Hohlfeld, T. (2014). Pharmacokinetics and Pharmacodynamics of Acetylsalicylic Acid after Intravenous and Oral Administration to Healthy Volunteers. *Cpaa* 6, 51–59. doi:10.2147/CPAA.S47895

Noh, J.-Y., Lim, K.-M., Bae, O.-N., Chung, S.-M., Lee, S.-W., Joo, K.-M., et al. (2010). Procoagulant and Prothrombotic Activation of Human Erythrocytes by Phosphatidic Acid. *Am. J. Physiology-Heart Circulatory Physiol.* 299, H347–H355. doi:10.1152/ajpheart.01144.2009

Novakovic, A., Marinko, M., Jankovic, G., Stojanovic, I., Milojevic, P., Nenezic, D., et al. (2017). Endothelium-dependent Vasorelaxant Effect of Procyanidin B2 on Human Internal Mammary Artery. *Eur. J. Pharmacol.* 807, 75–81. doi:10.1016/j.ejphar.2017.04.015

Piwowarski, J. P., Stanisławska, I., Granica, S., Stefańska, J., and Kiss, A. K. (2017). Phase II Conjugates of Urolithins Isolated from Human Urine and Potential Role Ofβ-Glucuronidases in Their Disposition. *Drug Metab. Dispos.* 45, 657–665. doi:10.1124/dmd.117.075200

Prakash, P., Nayak, M. K., and Chauhan, A. K. (2017). P-selectin can promote thrombus propagation independently of Both von Willebrand factor and thrombospondin-1 in mice. *J. Thromb. Haemost.* 15, 388–394. doi:10.1111/jth.13586

Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, P. J., et al. (2012). Gut Microbial Catabolism of Grape Seed Flavan-3-Ols by Human Faecal Microbiota. Targetted Analysis of Precursor Compounds, Intermediate Metabolites and End-Products. *Food Chem.* 131, 337–347. doi:10.1016/j.foodchem.2011.08.011

Sano, A., Yamakoshi, J., Tokutake, S., Tobe, K., Kubota, Y., and Kikuchi, M. (2003). Procyanidin B1 Is Detected in Human Serum after Intake of Proanthocyanidin-Rich Grape Seed Extract. *Biosci. Biotechnol. Biochem.* 67, 1140–1143. doi:10.1271/bbb.67.1140

Sano, T., Oda, E., Yamashita, T., Naemura, A., Ijiri, Y., Yamakoshi, J., et al. (2005). Anti-thrombotic Effect of Proanthocyanidin, a Purified Ingredient of Grape Seed. *Thromb. Res.* 115, 115–121. doi:10.1016/j.thromres.2004.07.015

Schramm, D. D., Wang, J. F., Holt, R. R., Ensunsa, J. L., Gonsalves, J. L., Lazarus, S. A., et al. (2001). Chocolate Procyanidins Decrease the Leukotriene-Prostacyclin Ratio in Humans and Human Aortic Endothelial Cells. *Am. J. Clin. Nutr.* 73, 36–40. doi:10.1093/ajcn/73.1.36

Schumacher, W. A., Steinbacher, T. E., Heran, C. L., Megill, J. R., and Durham, S. K. (1993). Effects of Antithrombotic Drugs in a Rat Model of Aspirin-Insensitive Arterial Thrombosis. *Thromb. Haemost.* 69, 509–514. doi:10.1055/s-0038-1651642

Shen, Z. Q., Dong, Z. J., Peng, H., and Liu, J. K. (2003). Modulation of PAI-1 and tPA Activity and Thrombolytic Effects of Corilagin. *Planta Med.* 69, 1109–1112. doi:10.1055/s-2003-45191

Shikov, A. N., Narkevich, I. A., Flisyuk, E. V., Luzhanin, V. G., and Pozharitskaya, O. N. (2021). Medicinal Plants from the 14th Edition of the Russian Pharmacopoeia, Recent Updates. *J. Ethnopharmacology* 268, 113685. doi:10.1016/j.jep.2020.113685

Shushunov, S., Balashov, L., Kravtsova, A., Krasnogorsky, I., Latté, K. P., and Vasiliev, A. (2009). Determination of Acute Toxicity of the Aqueous Extract of Potentilla Erecta (Tormentil) Rhizomes in Rats and Mice. *J. Med. Food* 12, 1173–1176. doi:10.1089/jmf.2008.0281

Smith, S. A. (2009). The Cell-Based Model of Coagulation. *J. Vet. Emerg. Crit. Care* 19, 3–10. doi:10.1111/j.1476-4431.2009.00389.x

Sobczak, A. I. S., and Stewart, A. J. (2019). Coagulatory Defects in Type-1 and Type-2 Diabetes. *Ijms* 20, 6345. doi:10.3390/ijms20246345

Solovey, A., Kollander, R., Milbauer, L. C., Abdulla, F., Chen, Y., Kelm, R. J., et al. (2009). eNOS and NO Regulate Endothelial Tissue Factor Expressionin Vivoin the Sickle Transgenic Mouse. *Am. J. Hematol.* 85, 41-45. doi:10.1002/ajh.21582

Spigoni, V., Mena, P., Cito, M., Fantuzzi, F., Bonadonna, R., Brighenti, F., et al. (2016). Effects on Nitric Oxide Production of Urolithins, Gut-Derived Ellagitannin Metabolites, in Human Aortic Endothelial Cells. *Molecules* 21, 1009. doi:10.3390/molecules21081009

Subbotina, M. D., Timchenko, V. N., Vorobyov, M. M., Konunova, Y. S., Aleksandrovih, Y. S., and Shushunov, S. (2003). Effect of Oral Administration of Tormentil Root Extract (Potentilla Tormentilla) on Rotavirus Diarrhea in Children: a Randomized, Double Blind, Controlled Trial. *Pediatr. Infect. Dis. J.* 22, 706–711. doi:10.1097/01.inf.0000078355.29647.d0

Tarnow, I., Michelson, A. D., Barnard, M. R., Frelinger, A. L., Aasted, B., Jensen, B. R., et al. (2009). Nephropathy in Type 1 Diabetes Is Associated with Increased Circulating Activated Platelets and Platelet Hyperreactivity. *Platelets* 20, 513–519. doi:10.3109/09537100903221001

Tomczyk, M., and Latté, K. P. (2009). Potentilla-A Review of its Phytochemical and Pharmacological Profile. *J. Ethnopharmacology* 122, 184–204. doi:10.1016/j.jep.2008.12.022

Tomczyk, M., Suzuki, Y., Sano, H., Brzoska, T., Tanaka, H., and Urano, T. (2016). Bidirectional Functions of Thrombin on Fibrinolysis: Evidence of Thrombin-dependent Enhancement of Fibrinolysis provided by Spontaneous Plasma Clot Lysis. *Thromb. Res.* 143, 28–33. doi:10.1016/j.thromres.2016.04.018

Tunón, H., Olavsdotter, C., and Bohlin, L. (1995). Evaluation of Anti-inflammatory Activity of Some Swedish Medicinal Plants. Inhibition of Prostaglandin Biosynthesis and PAF-Induced Exocytosis. *J. Ethnopharmacology* 48, 61–76. doi:10.1016/0378-8741(95)01285-L

Wientjes, M. G., and Levy, G. (1988). Nonlinear Pharmacokinetics of Aspirin in Rats. *J. Pharmacol. Exp. Ther.* 245, 809–815. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3385642.

Wisinski, J. A., and Kimple, M. E. (2016). Platelet Dysfunction in Type 1 Diabetes: Stressing the Thromboxanes. *Diabetes* 65, 349–351. doi:10.2337/dbi15-0032

Wölfle, U., Hoffmann, J., Haarhaus, B., Rao Mittapalli, V., and Schempp, C. M. (2017). Anti-inflammatory and Vasoconstrictive Properties of Potentilla Erecta - A Traditional Medicinal Plant from the Northern Hemisphere. *J. Ethnopharmacology* 204, 86–94. doi:10.1016/j.jep.2017.03.058

Yamamoto, E., Nishimura, N., Okada, K., Sekido, C., Yamamichi, S., and Hasumi, K. (2011). Inhibitors of Autoactivation of Plasma Hyaluronan-Binding Protein (Factor VII Activating Protease). *Biol. Pharm. Bull.* 34, 462–470. doi:10.1248/bpb.34.462

Zahran, A. M., El-Badawy, O., Mohamad, I. L., Tamer, D. M., Abdel-Aziz, S. M., and Elsayh, K. I. (2018). Platelet Activation and Platelet-Leukocyte Aggregates in Type I Diabetes Mellitus. *Clin. Appl. Thromb. Hemost.* 24, 230S–239S. doi:10.1177/1076029618805861

Zakrzeska, A., Gromotowicz-Popławska, A., Szemraj, J., Szoka, P., Kisiel, W., Purta, T., et al. (2015). Eplerenone Reduces Arterial Thrombosis in Diabetic Rats. *J. Renin Angiotensin Aldosterone Syst.* 16, 1085–1094. doi:10.1177/1470320313515037

Zhang, Y., DeWitt, D. L., Murugesan, S., and Nair, M. G. (2004). Novel Lipid-Peroxidation- and Cyclooxygenase-Inhibitory Tannins fromPicrorhiza Kurroa Seeds. *C & B* 1, 426–441. doi:10.1002/cbdv.200490036

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Chemical Composition, Diuretic, and Antityrosinase Activity of Traditionally Used Romanian *Cerasorum stipites*

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Cherry stems (CS) represent a by-product intensively used in Eastern European countries as a traditional remedy for urinary tract disorders. Ethnopharmacological evidences sustain the use of CS as aqueous preparations (infusion and decoction), but few data were previously reported about phytochemical profile and pharmacological potential of CS hydroalcoholic extracts. In this regard, we aimed to evaluate the phenolic profile, *in vitro* antioxidant and tyrosinase inhibitory potential, and *in vivo* diuretic activity of 70% hydroethanolic cherry stems extract and cherry stems decoction (CSD). LC-DAD-ESI/MS^n analysis revealed the presence of flavonoid-type compounds as main constituents for both preparations, especially flavanones (naringenin glycosides). Antioxidant activity evaluated through DPPH, ABTS, and FRAP methods was superior for cherry stems extract, probably due to the presence of phenolic-derived compounds in higher amounts than CSD. On the other hand, tyrosinase inhibitory potential and diuretic effect exerted by CSD were stronger, highlighting that other types of hydrophilic secondary metabolites are responsible for this bioactivity. Overall, our findings indicate that CS preparations could be used as promising mild diuretic agents and encourage further investigations regarding the correlation between their chemical composition and bioactive potential.

Keywords: ***Cerasus sp.*, cherry stems, diuretic activity, polyphenols, by-products**

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INTRODUCTION

In last decades, the importance of plant-derived bioactive compounds was intensively studied, highlighting their impact on human health as modulators of metabolic pathways and processes involved in the development of different pathological conditions. Even though dietary intake can provide high amounts of plant-derived secondary metabolites, food habits and nutritional quality of our meals upset the balance between the real need and consumption of these compounds. According to Bailey (2020), more than 50% of U.S. adult population use dietary supplements; a study on E.U. food supplement market conducted by European Commission estimates that 50% of these products are based on vitamins and minerals, 43% food supplements containing other substances, and 7%

tonics and bottled nutritional drinks (classified as OTC products or marketed as functional foods) (European Advisory Services (EAS), 2007). Hence, the interest for this type of products is in a continuous and exponential increase, explaining the actual trends in the research for novel and sustainable resources for food supplements industry.

By-products resulted from the processing of different herbal resources are intensively promoted as promising sources of bioactive compounds, being recognized as cheap, eco-friendly, and sustainable alternatives for standard raw materials (Fierascu et al., 2020). Moreover, recent studies have proven that high amounts of secondary metabolites can be concentrated in plant by-products; for example, it was shown that an important fraction from total polyphenolic content of several by-products from food industry can be found as bound form, namely non-extractable polyphenols (NEP), which exerts significant antitumor, antioxidant, and hypocholesterolemiant properties (Dzah et al., 2020). Therefore, it can be assumed that one of the big goals for the future is the valorification of more and more by-products as main sources of phytochemicals with health-related properties.

Cherry stems (CS) are one of the main by-products obtained after the harvesting and processing of sweet cherries (*Prunus avium*) and sour cherries (*Prunus cerasus*). Even though these are generally recognized as a waste for food industry, the folk medicine recommends them as a traditional herbal remedy, especially for its diuretic and sedative properties; the herbal drug is known by its Latin botanical name (*Cerasorum stipites*), being used as infusion or decoction (Bastos et al., 2015; Aires et al., 2017; Švarc-Gajić et al., 2018; Nastić et al., 2020; Peixoto et al., 2020). According to the Committee on Herbal Medicinal Products (HMPC) of European Medicines Agency, CS can be used single or as herbal tea combinations with other herbal substances used in the therapeutic area for "urinary tract disorders," generically named *Species diureticae* (Länger, 2017). In Central and Eastern European countries, especially in Romania, based on ethnopharmacological recommendations, the herbal product is recognized as a popular remedy in the adjuvant treatment of kidney stones, mild urinary tract infections, edema, and hypertension. In *Encyclopedia of Romanian ethnobotany*, Butura *et al.* describe the use of CS as infusions or decoctions in intra-Carpathic areas in the treatment of kidney disorders; different traditional recipes for decoctions were reported, obtained from both stems and crushed stones from cherries or herbal mixtures including aerial parts of horsetail (*Equisetum arvense*), leaf buds of mountain pine (*Pinus mugo*), and corn stigmas (*Zea mays*) (Butura, 1979). Being one of the most popular herbal products with diuretic properties in Romanian folk medicine, pharmacies and markets sell CS-containing products conditioned as teas or capsules, recommended as adjuvant therapy in kidney stones, cystitis, or obesity (Pârvu, 2006).

Few phytochemical and pharmacological investigations were conducted on CS. Most of the studies available on this topic are from last 5 to 10 years, being focused both on qualitative and quantitative evaluation of the main constituents in the extracts and the assessment of their potential bioactivities. In a comparative study between fruits and stems focused on hydrophilic compounds profile, Bastos *et al.* showed that stems are rich in citric, malic, and oxalic acids (Bastos et al., 2015). In the same study, phenolic profile of hydroalcoholic extracts obtained from stems was evaluated; sakuranetin-5-*O*-glucoside, catechin, naringenin-7-*O*-glucoside, and aromadendrin-7-*O*-hexoside were quantified as main compounds. Other studies revealed the presence of hydroxycinnamic acids (*cis*-3-*O*-*p*-coumaroylquinic acid, chlorogenic acid, and *trans*-3-*O*-*p*-coumaroylquinic acid): quercetin and sinapic acid in high amounts (Demir et al., 2020; Peixoto et al., 2020). In this regard, corroborated with the occurrence of these compounds, several *in vitro* bioactivities were proven for CS extracts: antioxidant, antitumor, antidiabetic, and antibacterial (Bastos et al., 2015; Aires et al., 2017; Demir et al., 2020; Peixoto et al., 2020).

Except for the *in vitro* antibacterial effect on *E. coli* strains, few studies were focused on the evaluation of the real benefits of CS in the treatment of urinary tract disorders based on pharmacological investigation (Aires et al., 2017). Hence, the present study aimed to evaluate phenolic profile and pharmacological properties of two different types of extracts obtained from CS (hydroethanolic and aqueous decoction). LC-DAD-ESI/MS^n technique was employed for both qualitative and quantitative analysis of several phenolic compounds, followed by *in vitro* evaluation of antioxidant and tyrosinase inhibitory activities. *In vivo* diuretic potential of the extracts was tested using a rodent model previously established.

MATERIALS AND METHOD

Chemicals and Reagents

Acetonitrile (99.9%) was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic compound standards (chlorogenic acid, ferulic acid, naringenin, *p*-coumaric acid, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, and taxifolin) were from Extrasynthèse (Genay, France). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, United States). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated by using a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, United States). Ferric chloride; 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (97%); diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (>98%); 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazine (DPPH); 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) (≥99%); dimethyl sulfoxide (DMSO) (≥99%); phosphate buffer, mushroom tyrosinase; 3,4-dihydroxy-L-phenylalanine (L-DOPA) (≥98%); and kojic acid were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). All other reagents used, including solvents, were of analytical grade.

Plant Material

CS were purchased from a community pharmacy in Cluj-Napoca, Romania, as herbal tea conditioned *in toto* (raw material). Samples were ground to a fine powder using a laboratory mill,

sieved and immediately subjected to extraction. The quality of samples was analyzed and confirmed after organoleptic and macroscopic control by Dr. Andrei Mocan from Department of Pharmaceutical Botany, Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy (Cluj-Napoca, Romania).

Extraction Procedure

Maceration: 100 g CS powder previously weighed and transferred in an Erlenmeyer flask was mixed with 500 ml of 70% (*v:v*) ethanol, being shaken and kept at room temperature in a dark place for 72 h. After filtration, the residue was re-extracted with 500 ml of solvent in the same condition for other 6 days. The extracts were reunited in a round-bottom flask and the alcohol was evaporated under reduced pressure. The aqueous suspension obtained was further lyophilized; the dry CS extract (CSE) was being kept in a desiccator at room temperature until analysis.

Decoction: 1 L of boiling water was added over 100 g CS powder previously weighed in an Erlenmeyer flask, the mixture being maintained at 100 °C on an electric hob under continuous stirring. The aqueous extract was hot filtered, cooled at room temperature, and lyophilized, obtaining a dry CS decoction (CSD). This extract was kept in similar conditions with CSE until further analysis.

LC-DAD-ESI/MS Analysis of Phenolic Compounds

The phenolic profile was determined by LC-DAD-ESI/MS^n (Dionex Ultimate 3000 UHPLC, Thermo Scientific, San Jose, CA, United States). These compounds were separated and identified using a method previously described (Bessada et al., 2016). The obtained extracts were redissolved at a concentration of 10 mg/ml with methanol: water (80:20, *v/v*) mixture. A double online detection was performed using a DAD (280, 330, and 370 nm as preferred wavelengths) and a mass spectrometer (MS). The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, United States) equipped with an ESI source.

The identification of the phenolic compounds was performed based on their chromatographic behavior and UV-Vis and mass spectra by comparison with standard compounds, when available, and data reported in the literature giving a tentative identification. Data acquisition was carried out with Xcalibur® data system (Thermo Finnigan, San Jose, CA, United States). For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV-Vis signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The results were expressed as mg/g of extract.

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Samples redissolved in 70% ethanol with 5% DMSO at concentration of 1 mg/ml were evaluated using protocols previously described by Mocan et al. (2017). For TPC determination, samples (20 µL) were mixed with diluted Folin–Ciocalteu reagent (1:9, *v/v*) (100 µL) and shaken vigorously. After 3 min, Na_2CO_3 solution (1%) (80 µL) was added and the sample absorbance was read at 760 nm after 30 min incubation at room temperature. The total phenolic content was expressed as milligrams of gallic acid equivalents (mg GAE/g extract). In TFC assay, samples (100 µL) were mixed with 2% aluminum trichloride methanolic solution (100 µL). The sample absorbance was read at 415 nm after 10 min incubation at room temperature. Rutin was used as a reference standard and the total flavonoid content was expressed as milligrams of rutin equivalents (mg RE/g extract).

Preliminary Biochemical Assessment of Antioxidant Potential

Antioxidant Assays

The antioxidant potential of CSE and CSD was tested through three complementary methods (DPPH, ABTS, and FRAP), the protocols being previously described by Mocan et al. (2017), Babotă et al. (2018), and Rusu et al. (2018). Samples were redissolved in 70% ethanol with 5% DMSO obtaining 1 mg/ml concentration; these were further analyzed using a SPECTROstar® nano multi-detection microplate reader with 96-well plates (BMG Labtech, Ortenberg, Germany).

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay: Samples (30 µL) were mixed with a 0.004% methanol solution of DPPH (270 µL). The absorbance was read at 517 nm after 30 min incubation at room temperature in the dark. DPPH radical scavenging activity was expressed as milligrams of Trolox equivalents (mg TE/g extract).

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid) radical scavenging assay: Briefly, $ABTS^+$ was produced directly by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 12–16 in the dark at room temperature. Prior to beginning the assay, ABTS solution was diluted with methanol to an absorbance of 0.700 ± 0.02 at 734 nm. Samples (20 µL) were added to ABTS solution (10 mM and 200 µL) and mixed. The sample absorbance was read at 734 nm after 30 min incubation at room temperature. The ABTS radical scavenging activity was expressed as millimoles of Trolox equivalents (mmol TE/g extract).

FRAP (ferric reducing antioxidant power) activity assay: Samples (25 µL) were added to premixed FRAP reagent (175 µL) containing acetate buffer (0.3 M, pH 3.6); 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) (10 mM) in 40 mM HCl; and ferric chloride (20 mM) in a ratio of 10:1:1 (*v/v/v*). Then, the sample absorbance was read at 593 nm after 30 min incubation at room temperature. FRAP activity was expressed as milligrams of Trolox equivalents (mg TE/g extract).

Biological Activities Evaluation

Tyrosinase Inhibition

Sample solution (10 mg/ml, 25 µL) was mixed with tyrosinase solution (40 µL, Sigma) and phosphate buffer (100 µL, pH 6.8) in a 96-well microplate and incubated for 15 min at 25 °C. The

reaction was then initiated with the addition of L-DOPA (40 μL, Sigma). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (tyrosinase) solution. The sample and blank absorbances were read at 492 nm after 10 min incubation at 25 °C. The absorbance of the blank was subtracted from that of the sample and the tyrosinase inhibitory activity was expressed as inhibition percentage (IC_{50}) (mg/ml), calculated for each sample and kojik acid (positive control) (Uysal et al., 2017).

In vivo Studies on Diuretic Effect

Animals: Forty-eight adult male Charles River Wistar rats (Crl: WI) with a medium weight of 151 ± 8 g were obtained from the Practical Skills and Experimental Medicine Center of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. The rats were housed in polycarbonate cages (Tecniplast, Italy) and maintained under standard conditions (22 ± 2 °C, a relative humidity of 45 ± 10%, and 12:12-h light: dark cycle). The animals had free access to standard pelleted food (Cantacuzino Institute, Bucharest, Romania) and filtered water throughout the experiment, except for the day when the test substances were administered. All experimental protocols were approved by the Ethics Committee of the University (Approval No. 168/April 7, 2017) and were conducted in accordance with the EU Directive 86/609/EEC, which regulates the use of laboratory animals for scientific research.

Diuretic and saluretic effects: Diuretic and saluretic effects of CSE and CSD were tested by a method using isotonic saline solution in order to achieve an optimal hydration (Kau et al., 1984). Eight groups of Crl:WI rats ($n = 6$) were used. The negative control group of rats was treated orally by gavage only with 25 ml/kg isotonic saline solution (Braun, Germany), while the positive control group was treated orally with 10 mg/kg furosemide (Zentiva, Romania), a reference diuretic drug dissolved also in a volume of 25 ml/kg isotonic saline solution. Three groups of rats were treated orally with 125, 250, and 500 mg/kg CSE dispersed in the same volume of 25 ml/kg isotonic saline solution, respectively, while other three groups of rats received also by oral route 125, 250, and 500 mg/kg CSD dispersed in 25 ml/kg isotonic saline solution.

After the substance administrations, rats were individually placed in metabolic cages. The urine output (ml) was recorded for each animal at two time intervals: 5 and 24 h after the administration of a single dose from the tested substances (Zhang et al., 2010). Diuretic action was calculated at 24 h as the ratio of urine output in test groups to urine output in negative control group. Diuretic activity was calculated at 24 h as the ratio of urine output in test groups to urine output of the positive control (reference) group.

Additionally, the saluretic effect of CSE and CSD was investigated. The urinary concentration of sodium and potassium ions (U_{Na} and U_K) was twice determined in the collected urine samples, 5 and 24 h after the substance administration, by a potentiometric method with selective electrodes, using a VITROS 250 Chemistry System automatic analyzer (Johnson and Johnson Clinical Diagnostic), being expressed in mEq/kg (Pălținean et al., 2017). Furthermore, at 24 h, blood samples were obtained from all animals by retro-orbital sinus puncture under ketamine/xylazine anesthesia and serum concentration of sodium ions (S_{Na}) was determined by the same potentiometric method. Creatinine was spectrophotometrically determined in the serum and urine at 670 nm, with the VITROS 250 Chemistry System automatic analyzer, using a reaction which formed a triaryl imidazole leuco dye. Fractional excretion of sodium ions (FE_{Na}) was calculated with the formula

$$FE_{Na} = \frac{U_{Na} \times CR_S}{S_{Na} \times CR_U} \times 100$$

where U_{Na} is the urine concentration of sodium ions, S_{Na} is the serum concentration of sodium ions, CR_S is the serum concentration of creatinine, and CR_U the urine concentration of creatinine (Pălținean et al., 2017).

Statistical Analysis

Data were expressed as mean values ± SD and were statistically analyzed by the one-way ANOVA method. The differences between the treated groups and the negative control group were evaluated by Dunnett's t-test using GraphPad Prism six software (GraphPad Software, United States); p values <0.05 being considered statistically significant.

RESULTS AND DISCUSSION

Chemical Profile of the Extracts

Total Phenolic and Total Flavonoidic Content

Evaluation of TPC using the Folin–Ciocalteu method revealed a clear difference between the two extracts based on extraction parameters applied (**Table 1**). The 70% ethanol increased the extraction yield of phenolic compounds in CSE (TPC = 37.63 ± 2.75 mg GAE/g extract) by almost 50% in comparison with CSD (TPC = 19.11 ± 1.52 mg GAE/g extract). A similar trend was observed for TPC, the values obtained being 12.03 ± 0.72 mg QE/g extract for CSE and 5.34 ± 0.23 mg QE/g extract for CSD. Our results are in-line with those previously reported in other studies. The evaluation of TPC in a hydroalcoholic extract obtained after an optimized process by Demir et al. revealed that TPC value was 26.60 mg GAE/g extract (in comparison with predicted value, 26.1 mg GAE/g extract), optimal conditions being 35% (*v/v*) ethanol percentage, 79°C, and 119 min; for the same extract, the TFC value was 2.26 mg QE/g extract (Demir et al., 2020). On the other hand, aqueous extracts confirmed the presence of lower concentrations for both TPC and TFC - 24.31 ± 0.86 to 39.54 ± 2.10 μg GAE/mL, respectively, 15.67 ± 0.68 to 23.17 ± 1.23 μg CE (catechin equivalents)/mL for infusions (Peixoto et al., 2020). This can be correlated with low hydrophilicity of chemical components from CS and encourage the use of ethanol–water mixtures as extraction solvents in order to obtain phenolics-enriched extracts.

LC-DAD/ESI–MS^2 Analysis of Phenolic Profile

The solvents used and the extraction procedure play a significant role on the chemical profile of the extracts and their bioactive

TABLE 1 | Total phenolic and flavonoid content, DPPH and ABTS scavenging capacity, and ferric reducing ability of plasma (FRAP) of the extracts of CSE and CSD (values expressed are means ± S.D. of three parallel measurements, $p < 0.05$).

Probe ID	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	DPPH scavenging (mmol TE/g extract)	ABTS scavenging (mg TE/g extract)	FRAP (mg TE/g extract)
CSE	37.63 ± 2.75	12.03 ± 0.72	30.02 ± 0.58	107.14 ± 1.43	111.87 ± 4.14
CSD	19.11 ± 1.52	5.34 ± 0.23	14.32 ± 2.00	55.65 ± 3.62	61.07 ± 2.83

potential. In this regard, an LC-DAD/ESI–MS^2 technique was employed to evaluate the phenolic compounds present in CSE and CSD. Data of the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, and tentative identification of phenolic acid and flavonoid derivatives are presented in **Table 2**. Five major types of phenolic compounds were found in CSE and CSD: *phenolic acids* (*p*-coumaroyl (1, 5, and 6), caffeoyl (2, 3), and feruloyl 12) derivatives), *flavanones* (4, 7–9, 13–15, 18–20, and 24–27), *flavones* (10, 16, 21, and 22), *flavanols* (11), and *flavonols* (17 and 23).

With the exception of *p*-coumaroylquinic acid (compound 1), detected only in CSE–0.379 ± 0.004 mg/g, all phenolic acids were detected in both extracts, being found in higher concentrations in CSE than CSD. Compounds 1, 2, and 3 were identified as hydroxycinnamoyl derivatives, taking into account their fragmentation pattern and UV spectra at around λ_{max} = 314–330 nm. Compounds 1 [$(M–H)^-$at *m/z* of 337], 2, and 3 [$(M–H)^-$ at *m/z* of 353], all producing fragment ions with *m/z* 191, corresponding to the deprotonated quinic acid, so that they could be clearly identified as quinic acid derivatives containing a caffeic or coumaric acid units. Taking into account the following the hierarchal key developed by Clifford et al. (2003) and Clifford et al. (2006) for the identification of chlorogenic acid derivatives, these compounds were identified as 3-*p*-coumaroylquinic, 4-*O*-caffeoylquinic, and 5-*O*-caffeoylquinic acids. Compounds 5, 6 [$(M–H)^-$at *m/z* of 325], and 12 [$(M–H)^-$ at *m/z* of 355] were identified as coumaric acid hexoside and ferulic acid hexoside, all indicating the losses of a hexosyl moieties (Bastos et al., 2015; Bessada et al., 2016). The presence of phenolic acids in cherry fruits hydroalcoholic extracts was previously confirmed by Bastos et al. (2015) and Demir et al. (2020); *cis*-*p*-coumaroylquinic acid (0.56 ± 0.01 mg/g) and *trans*-*p*-coumaroylquinic acid (0.23 ± 0.02 mg/g) were reported, the amounts being comparable with our results obtained for determination of 3-*p*-coumaroylquinic acid in CSE (0.379 ± 0.004 mg/g). As we mentioned, this compound was identified only in CSE, probably due to hydroalcoholic medium, emphasizing the importance of the solvent for a better recovery of bioactive compounds.

The group of flavanones comprises the most representative compounds identified in CSE and CSD. Compounds 13, 15, and 20, showed the same pseudomolecular ion $[M–H]^-$ at *m/z* 433, thus presented a different retention time, therefore, being tentatively identified as naringenin-*O*-hexoside isomers, despite that the position and nature of the sugar could not be identified for two of the isomers, thus compound 15 was assigned as naringenin-7-*O*-glucoside, which was quantified as the main component of CSE (4.57 ± 0.09 mg/g). Compound 4 [$(M–H)^-$ at *m/z* 401], was detected in trace amounts in CSE, being tentatively identified as naringenin-*O*-pentoside (releasing one fragment at *m/z* 269, loss of a pentosyl moiety – *m/z* 132 u). To the best of our knowledge, the presence of this compound in CS extracts was not previously reported. Peaks 7–9 and 14 were attributed to four isomers forms of aromadendrin-*O*-hexoside, based on the presence of the common fragment at *m/z* 317 (aromadendrin – H)$^-$. Aromadendrin derivatives have been previously evidenced in CS hydroethanolic and water extracts (Bastos et al., 2015). Compound 22 [$(M–H)^-$at *m/z* 417] released a fragment at *m/z* 255 [$(M–162)^-$ corresponding to the loss of hexosyl moiety), being associated to a pinocembrin-7-*O*-glucoside. Compounds 24 and 27 [$(M–H)^-$ at *m/z* 447] revealed a fragmentation patter that match with either sakuranin (sakuranetin-5-*O*-glucoside) or dihydrowogonin-7-*O*-glucoside, thus it was possible to assume one of the identifications. Even though the flavanones were extracted preferentially in hydroalcoholic medium, it was observed that compounds 8, 9, and 19 were found in higher amounts in CSD than CSE.

Three isoflavones were detected, such as compounds 18 and 19, which both revealed similar characteristics and were tentatively assigned as genistein-*O*-hexoside. Nevertheless, taking into account previous findings (Bastos et al., 2015), compound 18 was identified as genistein-7-*O*-glucoside. Compound 25 [$(M–H)^-$ *m/z* 445] revealed a molecular weight 15 u higher than compounds 19 and 18, so it was assumed that it might correspond to methylgenistein-*O*-hexoside.

The presence of several flavones was also confirmed in both types of extracts. Quercetin-3-O-rutinoside (rutin), quercetin-3-O-glucose, kaempferol-3-O-rhamnoside, isorhamnetin-3-O-rhamnoside, and kaempferol-3-O-glucoside were identified in comparison with the commercial standard. Among them, rutin (quercetin-3-*O*-rutinoside) was found as the major compound (1.01 ± 0.03 mg/g in CSE and 0.404 ± 0.003 mg/g in CSD). Kaempferol-3-*O*-rhamnoside could be quantified only in CSD, while kaempferol-3-*O*-glucoside was found in both extracts. The presence of kaempferol glycosides in CS extracts was also previously reported in similar studies (Bastos et al., 2015; Aires et al., 2017; Jesus et al., 2019; Nastić et al., 2020). Only one flavonol-type compound (peak 11) was found in the extracts; it released a fragment ion at *m/z* 303 [(taxifolin – H)$^-$; loss of a hexosyl moiety, –162 u), being tentatively identified as taxifolin-3-*O*-glucoside.

Overall, LC-DAD/ESI–MS^2 analysis showed that the use of 70% ethanol as extraction solvent increased the extraction yield of phenolic compounds, CSE containing various types of phenolics

TABLE 2 | Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in CSE and CSD.

Peak	Rt (min)	$\lambda_{max\ (nm)}$	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification		*t*-Students test *p*-value
						CSE	*CSD*	
1	6.1	310	337	191 (100), 173 (15), 163 (20), 155 (5)	3-*p*-Coumarouylquinic acid[(A)]	0.379 ± 0.004	nd	–
2	6.5	322	353	191 (45), 179 (68), 173 (100), 161 (5), 135 (10)	4-*O*-Caffeoylquinic acid[(B)]	0.69 ± 0.01	0.657 ± 0.002	<0.001
3	7.2	324	353	191 (100), 179 (13), 173 (5), 161 (5), 135 (5)	5-*O*-Caffeoylquinic acid[(B)]	1.15 ± 0.01	0.290 ± 0.001	<0.001
4	7.6	287,316 sh	401	269 (100)	Naringenin-*O*-pentoside[(C)]	Tr	nd	–
5	8.6	310	325	163 (100)	*p*-Coumaric acid hexoside[(A)]	0.692 ± 0.001	0.238 ± 0.003	<0.001
6	10.0	310	325	163 (100)	*p*-Coumaric acid hexoside[(A)]	0.221 ± 0.004	0.120 ± 0.002	<0.001
7	11.2	283,320 sh	449	287 (100)	Aromadendrin-*O*-hexoside isomer 1[(C)]	1.27 ± 0.02	0.46 ± 0.01	<0.001
8	11.7	282,322 sh	449	287 (100)	Aromadendrin-*O*-hexoside isomer 2[(C)]	0.31 ± 0.01	0.37 ± 0.01	<0.001
9	12.7	283,320 sh	449	287 (100)	Aromadendrin-*O*-hexoside isomer 3[(C)]	0.019 ± 0.001	0.054 ± 0.001	<0.001
10	13.8	352	609	301 (100)	Quercetin-deoxyhexoside hexoside[(D)]	Tr	0.027 ± 0.001	–
11	14.2	284,339 sh	465	303 (100)	Taxifolin-3-*O*-glucoside[(E)]	0.47 ± 0.01	0.125 ± 0.001	<0.001
12	15.2	320	355	193 (100), 149 (5), 134 (5)	Ferulic acid hexoside[(F)]	0.133 ± 0.002	0.053 ± 0.001	<0.001
13	15.9	282,325 sh	433	271 (100)	Naringenin-*O*-hexoside isomer 1[(C)]	0.29 ± 0.01	0.139 ± 0.002	<0.001
14	16.3	283	449	287 (100)	Aromadendrin-*O*-hexoside isomer 4[(C)]	0.077 ± 0.001	0.046 ± 0.001	<0.001
15	16.9	283,325 sh	433	271 (100)	Naringenin-7-*O*-glucoside [(C)]	4.57 ± 0.09	0.71 ± 0.01	<0.001
16	17.9	353	609	301 (100)	Quercetin-3-*O*-rutinoside[(D)]	1.01 ± 0.03	0.404 ± 0.003	<0.001
17	19.1	351	463	301 (100)	Quercetin-3-*O*-glucose[(G)]	0.693 ± 0.004	0.14 ± 0.01	<0.001
18	19.9	260,330 sh	431	269 (100)	Genistein-7-*O*-glucoside[(C)]	0.0588 ± 0.002	0.0061 ± 0.0001	<0.001
19	20.4	281,330 sh	431	269 (100)	Genistein-*O*-hexoside[(C)]	Tr	0.043 ± 0.002	–
20	21.0	283,327 sh	433	271 (100)	Naringenin-*O*-hexoside isomer 2[(C)]	0.60 ± 0.01	0.078 ± 0.001	<0.001
21	21.2	340	593	285 (100)	Kaempferol-3-*O*-rhamnoside[(D)]	Tr	0.033 ± 0.001	–
22	22.2	350	623	315 (100)	Isorhamnetin-3-*O*-rhamnoside [(D)]	Tr	Tr	–
23	22.6	342	447	285 (100)	Kaempferol-3-*O*-glucoside[(G)]	0.520 ± 0.002	0.108 ± 0.003	<0.001
24	24.2	280,323 sh	447	285 (100), 270 (5)	Dihydrowogonin 7-*O*-glucoside/sakuranetin 5-*O*-glucoside[(C)]	0.44 ± 0.02	0.025 ± 0.001	<0.001
25	26.9	255,320 sh	445	283 (100), 268 (20)	Methylgenistein hexoside[(C)]	0.95 ± 0.01	0.078 ± 0.001	<0.001
26	29.9	283,320 sh	417	255 (100)	Pinocembrin-7-*O*-glucoside[(C)]	Tr	Tr	–
27	31.9	286,335 sh	447	285 (100), 270 (5)	Dihydrowogonin 7-*O*-glucoside/sakuranetin 5-*O*-glucoside[(C)]	Tr	0.0059 ± 0.0001	–
				Total phenolic compounds		**14.54 ± 0.12**	**4.205 ± 0.002**	<0.001

*Nd, not detected; tr-trace amounts. Standard calibration curves: A—*p*-coumaric acid (y = 301950x + 6,966.7, R^2 = 0.9999, LOD = 0.68 μg/ml, and LOQ = 1.61 μg/ml); B—chlorogenic acid (y = 168823x – 161,172; R^2 = 0.9999, LOD = 0.20 μg/ml, and LOQ = 0.68 μg/ml); C—naringenin (y = 18,433x + 78,903, R^2 = 0.9998, LOD = 0.17 μg/ml,, and LOQ = 0.81 μg/ml); D—quercetin-3-O-rutinoside (y = 13,343x + 76,751, R^2 = 0.9998, LOD = 0.21 μg/ml, and LOQ = 0.71 μg/ml); E—taxifolin (y = 203766x – 208,383, R^2 = 1, LOD = 0.67 μg/ml, and LOQ = 2.02 μg/ml); F—ferulic acid (y = 633126x – 185,462, R^2 = 0.999, LOD = 0.20 μg/ml, and LOQ = 1.01 μg/ml); G—quercetin-3-O-glucoside (y = 34,843x – 160,173, R^2 = 0.9998, LOD = 0.21 μg/ml, and LOQ = 0.71 μg/ml).*

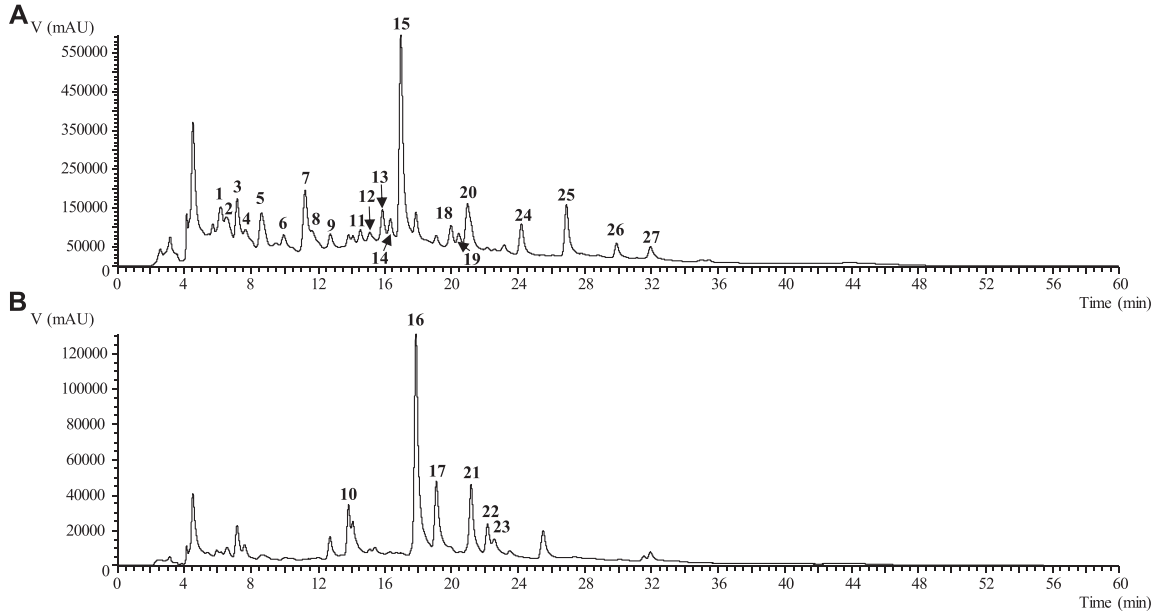

FIGURE 1 | Phenolic profile of CSE recorded at 280 nm **(A)** and 370 nm **(B)**.

in high amounts than CSD (**Figure 1**). The influence of extraction method and extraction solvent on phenolic compounds recovery from CS was previously studied by other authors. Nastić et al. showed that the use of dual solvent mixture can enhance the extraction efficiency, higher percentages of ethanol lead to a decrease in the extraction yield; it can be noticed that, in this study, CS extracts were obtained by pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) (Nastić et al., 2020). Aqueous extracts (infusion and decoction) represent a cheap and household alternative option to exploit the benefits of CS as source of phenolic compounds, based on ethnopharmacology recommendations.

Preliminary Biochemical Assessment of Antioxidant Potential

Antioxidant Assays

The free radical scavenging activity of CSE and CSD was evaluated using the DPPH and ABTS radical scavenging assays. As shown in **Table 1**, the value of DPPH radical scavenging activity for CSE was 30.02 ± 0.58 mmol TE/g extract, while for CSD decreased at 14.32 ± 2.00 mmol TE/g extract; a similar trend was also observed in ABTS and FRAP assay (107.14 ± 1.43 mg TE/g extract for CSE and 55.65 ± 3.62 mg TE/g extract for CSD, respectively, 111.87 ± 4.14 mg TE/g extract for CSE and 61.07 ± 2.83 mg TE/g extract for CSD). These variations can be explained based on the major differences observed in total and individual phenolic compounds distribution in the extracts. It was previously reported that the presence of *p*-coumaric acid and the *p*-coumaroylquinic acid derivatives increased total antioxidant capacity of 70% methanolic extracts obtained from CS (tested through ABTS assay) (Aires et al., 2017). Moreover, a comparative evaluation of DPPH scavenging activity and ferric reducing ability between infusion, decoction, and hydroalcoholic extract of CS confirmed the highest antioxidant potential for the last one correlated to the higher phenolic compounds concentration found in this type of extract (Bastos et al., 2015). A significant amount of experimental data indicate that oxidative stress may contribute not only to preexisting diseases like atherosclerosis or hypertension but it may also generate oxidative damage to renal tubular cells, reducing kidney functionality (Dennis and Witting, 2017) Thus, a potential antioxidant activity could have nephroprotective effects contributing to a normal renal homeostasis, but additional studies are necessary to clarify this relation.

Biological Activities Evaluation

Tyrosinase Inhibitory Capacity

Tyrosinase is a copper-containing enzyme responsible for the oxidation of tyrosine to L-DOPA and the hydroxylation of L-tyrosine, with an important role in melanin synthesis (a pigment that regulates skin color and plays a protective role by absorbing ultraviolet sunlight and removing reactive oxygen species from the skin), also responsible for browning of damaged fruits and vegetables (Rusu et al., 2018). Recent studies confirmed the link between tyrosinase inhibition and positive effects in several degenerative diseases (i.e., Parkinson), proving the importance of tyrosinase inhibitors as neuroprotective agents (Hubert et al., 2016; Tan et al., 2016). *In vitro* evaluations shown that tyrosinase can interfere with the activity of bradykinin and vasopressin, two hormones that modulate diuresis and blood

TABLE 3 | Enzyme inhibitory effects of the extracts of CSE and CSD (values expressed are mean ± S.D. of three parallel measurements, $p < 0.05$).

Probe ID	IC_{50} (mg/ml)
CSE	8.66 ± 1.23
CSD	3.03 ± 0.35
Kojic acid	0.05 ± 0.01

pressure through renin–angiotensin–aldosteron system (RAAS) in a pH-dependent manner (BISSET, 1962).

In this regard, the evaluation of tyrosinase inhibitory capacity of CS extracts (**Table 3**) revealed a superior potency for CSD (IC_{50} = 3.03 ± 0.35 mg/ml) in comparison with CSE (IC_{50} = 8.66 ± 1.23 mg/ml). The obtained results cannot be correlated with the presence of phenolic compounds in the extracts, but preliminary explains that some polar and hydrophilic secondary metabolites from CS could exert a strong tyrosinase inhibitory potential. It was previously reported that cherry juice (Cásedas et al., 2016) and cherry tree bark extracts (Hubert et al., 2016) have a moderate inhibitory activity on tyrosinase. To the best of our knowledge, we evaluated for the first time tyrosinase inhibitory potential of CS extracts; our results represent a start point for further assessments of chemical composition of aqueous extracts obtained from CS and encourage supplementary evaluation of the molecular mechanisms responsible for anti-tyrosinase properties of CS.

Diuretic Effect

As shown in **Figure 2**, the cherry stems extract (CSE) and cherry stems decoction (CSD) produced a dose-dependent gradual increase of the urine output, the effect being more intense at 24 h. Cherry stems decoction (CSD) at 500 mg/kg produced the most intense diuretic effect with a urine output of 6.5 ± 0.35 ml at 24 h. The reference loop diuretic, furosemide, augmented the urine output from the first hours after the oral administration, the effect increasing sharply at 5 h (7.38 ± 0.36 ml), then reaching a plateau (7.96 ± 0.37 ml), typical for a high ceiling diuretic drug. The diuretic effect of cherry stems extract (CSE) and cherry stems decoction (CSD), although inferior to furosemide, was more gradually installed, which can be of importance in a series of chronic cardiovascular diseases where a rapid diuretic effect is not desirable.

As shown in **Table 4**, the administration of cherry stems extract (CSE) and cherry stems decoction (CSD) increased the diuresis at 24 h in a dose-dependent manner. Rats treated with CSE showed a statistically significant increase of urine output at 250 and 500 mg/kg, while rats treated with CSD showed statistically significant results for all the three doses. The diuretic action ranged from 1.09 to 1.45 for the animals treated with CSE and from 1.26 to 1.56 for the animals treated with CSD. The strongest diuretic activity was observed in the animals treated with CSD at 500 mg/kg, which showed 81% from the activity of furosemide, the reference diuretic drug. Also, the cherry stems extract (CSE) and cherry stems decoction (CSD) increased the urinary excretion of Na^+ and K^+ ions (U_{Na}, U_K), the main cationic electrolytes from the urine. As shown in **Table 5** the urinary excretion of the aforementioned electrolytes produced by CSE and CSD was superior to the negative control group, and presented a similar pattern with the diuretic effect, being more intense in the 5–24 h time interval.

The most significant excretion of the tested electrolytes was produced by the 500 mg/kg dose of CSD with U_{Na} and U_K values of 3.84 ± 0.64 and 2.71 ± 0.32 mEq/kg, 24 h after the substance administration. The calculated Na^+/K^+ ratio for CSE and CSD treated groups did not show values above 10 at any moment of determination, thus indicating a lack of a potassium-sparing effect, similar with furosemide. On the contrary, both CSE and CSD produced a clear kaliuretic effect. In our experiment, the fractional excretion of sodium ions (FE_{Na}), defined as the percentage of sodium ions filtered by the kidneys and not reabsorbed, was calculated at 24 h. The fractional excretion of sodium ions (FE_{Na}) is a valuable parameter which can provide additional information on the tubular function, although the

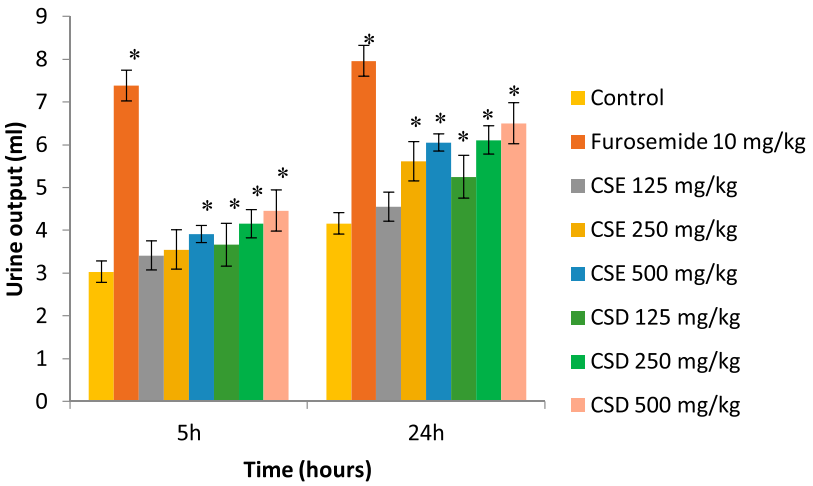

FIGURE 2 | Effect of the cherry stems extract (CSE) and cherry stems decoction (CSD) on urine output recorded at 5 and 24 h in saline-loaded Crl:WI rats (*$p < 0.05$ vs. saline control).

TABLE 4 | Parameters of the diuretic effect in saline-loaded Crl:WI rats treated with cherry stems extract (CSE) and cherry stems decoction (CSD). Values of urine output are expressed as Mean ± SD (n = 6).

Group (dose)	Urine output at 24 h (ml)	Diuretic action	Diuretic activity
Saline control	4.16 ± 0.37	–	–
Furosemide (10 mg/kg)	7.96 ± 0.37	1.91	1
CSE 125 mg/kg	4.55 ± 0.65	1.09	0.57
CSE 250 mg/kg	5.61 ± 0.38[a]	1.34	0.70
CSE 500 mg/kg	6.05 ± 0.33[a]	1.45	0.76
CSD 125 mg/kg	5.25 ± 0.32[a]	1.26	0.65
CSD 250 mg/kg	6.11 ± 0.21[a]	1.46	0.76
CSD 500 mg/kg	6.50 ± 0.35[a]	1.56	0.81

[a]$p < 0.05$ *vs. saline control.*

TABLE 5 | Effect of the cherry stems extract (CSE) and cherry stems decoction (CSD) on urinary excretion of sodium (U_{Na}) and potassium (U_K) 5 and 24 h after the substance administration, fractional excretion of sodium (FE_{Na}), and the ration Na/K in saline loaded Crl:WI rats.

Group (Dose)	U_{Na} at 5 h (mEq/kg)	U_K at 5 h (mEq/kg)	U_{Na} at 24 h (mEq/kg)	U_K at 24 h (mEq/kg)	Fe Na at 24 h (%)	Na/K at 24 h
Saline control	1.43 ± 0.31	1.13 ± 0.27	1.94 ± 0.37	1.55 ± 0.42	1.32	1.25
Furosemide (10 mg/kg)	5.59 ± 0.78*	4.91 ± 0.41*	6.32 ± 0.84*	5.31 ± 0.44*	6.81	1.19
CSE (125 mg/kg)	1.83 ± 0.35	1.57 ± 0.29	2.58 ± 0.61	1.96 ± 0.59	1.97	1.31
CSE (250 mg/kg)	2.41 ± 0.66*	1.83 ± 0.31*	2.88 ± 0.59*	2.01 ± 0.73*	2.14	1.43
CSE (500 mg/kg)	2.89 ± 0.75*	2.02 ± 0.89*	3.03 ± 0.88*	2.14 ± 0.69*	2.42	1.41
CSD (125 mg/kg)	2.15 ± 0.39	1.83 ± 0.73	2.87 ± 0.83	2.08 ± 0.68	2.05	1.37
CSD (250 mg/kg)	2.49 ± 0.44	2.12 ± 0.29	3.42 ± 0.87	2.39 ± 0.46	3.72	1.43
CSD (500 mg/kg)	3.11 ± 0.82	2.36 ± 0.55	3.84 ± 0.64	2.71 ± 0.32	4.58	1.41

Values of $U_{Na}V$ and U_KV are expressed as Mean ± SD (n = 6) ($p < 0.05$ vs. saline control).*

glomerular filtration rate and daily intake of sodium could also influence its values (Schreuder et al., 2017). As expected, the experiment showed a net increase of FE_{Na} (6.81%) for the reference diuretic drug furosemide, which specifically inhibits the sodium–potassium–chloride symporter in the thick ascending limb of the loop of Henle, and a moderate increase of FE_{Na} (2.14–2.42 and 3.72–4.58%) for CSE and CSD at doses of 250 and 500 mg/kg, also suggesting a tubular mechanism of action responsible for the diuretic effect of the tested cherry stems preparations.

The diuretic activity of powdered cherry stems (administered as capsules at an equivalent dose of 2.0 g of the plant per person) was previously evaluated in healthy human subjects, urinary volume, urinary electrolyte concentration (sodium, potassium, chloride, and calcium), and the osmolality being monitored (Hooman et al., 2009). The study revealed a mild diuretic effect for CS and a slight increase for urinary excretion of calcium, sodium, and chloride, concluding that the drug can be used as diuretic agent, but with precautious in those patients, and also in patients having any disorders associated with calcium, sodium, and/or chloride deficiency (especially in those with urolithiasis because of rising calcium excretion). A comparison between those results and our experimentally output regarding diuretic activity of CS reveals a similar trend for both type of tested CS-derived products. Our study represents the first report about diuretic and saluretic activity of the extracts obtained from CS and correlates this potential with the presence of phenolic secondary metabolites as main constituents of both hydroalcoholic and aqueous preparations.

CONCLUSION

Based on ethnopharmacological evidence, cherry stems (CS) preparations (hydroalcoholic extract and decoction) were evaluated for their phenolic profile and several bioactivities. Overall, the presence of phenolic secondary metabolites was confirmed in both extracts, the highest concentrations being found in hydroalcoholic extract. Flavonoids were the main type of phenolic compounds identified in the extracts, naringenin derivatives being quantified in high amounts. Hydroalcoholic extract exerted an important antioxidant activity, while tyrosinase inhibitory potential and diuretic activity were superior for decoction.

Our findings suggest that cherry stems are a valuable and less exploited by-product rich in phenolic secondary metabolites, with potential applications as a mild and safe diuretic agent. Even through traditional medicine recommends the use of aqueous preparations obtained from CS as adjuvant therapy in urinary tract disorders, hydroalcoholic extracts could represent an improved alternative, showing a similar diuretic potential. Nevertheless, future investigations need to be performed in

order to elucidate the intimate mechanisms responsible for CS pharmacological potential.

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 Ethics Committee of Iuliu Hatieganu, University of Medicine and Pharmacy.

AUTHOR CONTRIBUTIONS

AM, MB, OV, MD, and LB were involved in data analysis and writing the manuscript. AM and GC were involved in research funding. AM, OV, and MB were involved in experimental design. All authors participated in data analysis, correcting the draft and approving the final version of the draft. The authors declare no conflict of interests.

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REFERENCES

Aires, A., Dias, C., Carvalho, R., and Saavedra, M. J. (2017). Analysis of glycosylated flavonoids extracted from sweet-cherry stems, as antibacterial agents against pathogenic *Escherichia coli* isolates. *Acta Biochim. Pol.* 64 (2), 265–271. doi:10.18388/abp.2016_1374

Babotă, M., Mocan, A., Vlase, L., Crişan, O., Ielciu, I., Gheldiu, A., et al. (2018). Phytochemical analysis, antioxidant and antimicrobial activities of *Helichrysum arenarium* (L.) Moench. and *Antennaria dioica* (L.) Gaertn. flowers. *Molecules* 23 (2), 409. doi:10.3390/molecules23020409

Bailey, R. L. (2020). Current regulatory guidelines and resources to support research of dietary supplements in the United States. *Crit. Rev. Food Sci. Nutr.* 60 (2), 298–309. doi:10.1080/10408398.2018.1524364

Bastos, C., Barros, L., Dueñas, M., Calhelha, R. C., Queiroz, M. J. R. P., Santos-Buelga, C., et al. (2015). Chemical characterisation and bioactive properties of *Prunus avium* L.: the widely studied fruits and the unexplored stems. *Food Chem.* 173, 1045–1053. doi:10.1016/j.foodchem.2014.10.145

Bessada, S. M. F., Barreira, J. C. M., Barros, L., Ferreira, I. C. F. R., and Oliveira, M. B. P. P. (2016). Phenolic profile and antioxidant activity of *Coleostephus myconis* (L.) Rchb.f.: an underexploited and highly disseminated species. *Ind. Crops Prod.* 89, 45–51. doi:10.1016/j.indcrop.2016.04.065

Bisset, G. W. (1962). Effect OF tyrosinase preparations ON oxytocin, vasopressin and bradykinin. *Br. J. Pharmacol. Chemother.* 18 (2), 405–420. doi:10.1111/j.1476-5381.1962.tb01420.x

Butura, V. (1979). *Encyclopedia of Romanian ethnobotany*. Bucharest: Editura Ştiinţifică şi Enciclopedică.

Cásedas, G., Les, F., Gómez-Serranillos, M. P., Smith, C., and López, V. (2016). Bioactive and functional properties of sour cherry juice (*Prunus cerasus*). *Food Funct.* 7 (11), 4675–4682. doi:10.1039/c6fo01295g

Clifford, M. N., Johnston, K. L., Knight, S., and Kuhnert, N. (2003). Hierarchical scheme for LC-MSnIdentification of chlorogenic acids. *J. Agric. Food Chem.* 51 (10), 2900–2911. doi:10.1021/jf026187q

Clifford, M. N., Zheng, W., and Kuhnert, N. (2006). Profiling the chlorogenic acids of aster by HPLC–MSn. *Phytochem. Anal.* 17 (6), 384–393. doi:10.1002/pca.935

Demir, T., Akpınar, Ö., Kara, H., and Güngör, H. (2020). Cherry stem phenolic compounds: optimization of extraction conditions and *in vitro* evaluations of antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and cytotoxic activities. *J. Food Process. Preserv.* 44 (10), 11. doi:10.1111/jfpp.14804

Dennis, J. M., and Witting, P. K. (2017). Protective role for antioxidants in acute kidney disease. *Nutrients* 9 (7), 718. doi:10.3390/nu9070718

Dzah, C. S., Duan, Y., Zhang, H., Serwah Boateng, N. A., and Ma, H. (2020). Latest developments in polyphenol recovery and purification from plant by-products: a review. *Trends Food Sci. Technology* 99, 375–388. doi:10.1016/j.tifs.2020.03.003

European Advisory Services (EAS). (2007). *The use of substances with nutritional or physiological effect other than vitamins and minerals in food supplements*, Brussels, Belgium: DG SANCO.

Fierascu, R. C., Sieniawska, E., Ortan, A., Fierascu, I., and Xiao, J. (2020). Fruits by-products—a source of valuable active principles. A short review. *Front. Bioeng. Biotechnol.* 8, 319. doi:10.3389/fbioe.2020.00319

Hooman, N., Mojab, F., Nickavar, B., and Pouryousefi-Kermani, P. (2009). Diuretic effect of powdered *Cerasus avium* (cherry) tails on healthy volunteers. *Pak. J. Pharm. Sci.* 22 (4), 381–383. doi:10.3724/sp.j.1259.2011.00044

Hubert, J., Angelis, A., Aligiannis, N., Rosalia, M., Abedini, A., Bakiri, A., et al. (2016). *In vitro* dermo-cosmetic evaluation of bark extracts from common temperate trees. *Planta Med.* 82 (15), 1351–1358. doi:10.1055/s-0042-110180

Jesus, F., Gonçalves, A. C., Alves, G., and Silva, L. R. (2019). Exploring the phenolic profile, antioxidant, antidiabetic and anti-hemolytic potential of *Prunus avium* vegetal parts. *Food Res. Int.* 116, 600–610. doi:10.1016/j.foodres.2018.08.079

Kau, S. T., Keddie, J. R., and Andrews, D. (1984). A method for screening diuretic agents in the rat. *J. Pharmacol. Methods* 11 (1), 67–75. doi:10.1016/0160-5402(84)90054-8

Länger, R. (2017). Final assessment report on *Species diureticae*. 44. Available at: https://www.ema.europa.eu/en/documents/herbal-report/final-assessment-report-species-diureticae_en.pdf (Accessed September 24, 2020).

Mocan, A., Fernandes, Â., Barros, L., Crişan, G., Smiljković, M., Soković, M., et al. (2017). Chemical composition and bioactive properties of the wild mushroom *Polyporus squamosus* (Huds.) Fr: a study with samples from Romania. *Food Funct.* 9 (1), 160–170. doi:10.1039/C7FO01514C

Nastić, N., Lozano-Sánchez, J., Borrás-Linares, I., Švarc-Gajić, J., and Segura-Carretero, A. (2020). New technological approaches for recovering bioactive food constituents from sweet cherry (*Prunus avium* L.) stems. *Phytochem. Anal.* 31 (1), 119–130. doi:10.1002/pca.2872

Pârvu, C. (2006). *Universul plantelor*. 4th ed. Bucureti: Editura ASAB.

Peixoto, J., Álvarez-Rivera, G., Alves, R. C., Costa, A. S. G., Andrade, N., Moreira, A., et al. (2020). Cherry stem infusions: antioxidant potential and phenolic profile by UHPLC-ESI-QTOF-MS. *Food Funct.* 11 (4), 3471–3482. doi:10.1039/c9fo02693b

Păltinean, R., Mocan, A., Vlase, L., Gheldiu, A. M., Crişan, G., Ielciu, I., et al. (2017). Evaluation of polyphenolic content, antioxidant and diuretic activities of six Fumaria species. *Molecules* 22 (4), 639. doi:10.3390/molecules22040639

Rusu, M., Gheldiu, A.-M., Mocan, A., Moldovan, C., Popa, D.-S., Tomuta, I., et al. (2018). Process optimization for improved phenolic compounds recovery from walnut (Juglans regia L.) Septum: phytochemical profile and biological activities. *Molecules* 23 (11), 2814. doi:10.3390/molecules23112814

Schreuder, M. F., Bökenkamp, A., and van Wijk, J. A. E. (2017). Interpretation of the fractional excretion of sodium in the absence of acute kidney injury: a cross-sectional study. *Nephron* 136 (3), 221–225. doi:10.1159/000468547

Švarc-Gajić, J., Cerdà, V., Clavijo, S., Suárez, R., Mašković, P., Cvetanović, A., et al. (2018). Bioactive compounds of sweet and sour cherry stems obtained by subcritical water extraction. *J. Chem. Technol. Biotechnol.* 93 (6), 1627–1635. doi:10.1002/jctb.5532

Tan, X., Song, Y. H., Park, C., Lee, K.-W., Kim, J. Y., Kim, D. W., et al. (2016). Highly potent tyrosinase inhibitor, neorauflavane from Campylotropis hirtella and inhibitory mechanism with molecular docking. *Bioorg. Med. Chem.* 24 (2), 153–159. doi:10.1016/j.bmc.2015.11.040

Uysal, S., Zengin, G., Locatelli, M., Bahadori, M. B., Mocan, A., Bellagamba, G., et al. (2017). Cytotoxic and enzyme inhibitory potential of two Potentilla species (P. speciosa L. and P. reptans Willd.) and their chemical composition. *Front. Pharmacol.* 8, 1–11. doi:10.3389/fphar.2017.00290

Zhang, G., Zeng, X., Han, L., Wei, J.-a., and Huang, H. (2010). Diuretic activity and kidney medulla AQP1, AQP2, AQP3, V2R expression of the aqueous extract of sclerotia of *Polyporus umbellatus* Fries in normal rats. *J. Ethnopharmacol* 128 (2), 433–437. doi:10.1016/j.jep.2010.01.032

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REVIEW
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Medical Species Used in Russia for the Management of Diabetes and Related Disorders

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Background: Polyherbal mixtures called "medical species" are part of traditional and officinal medicine in Russia. This review aimed to analyze medical species used in Russia for the treatment of diabetes and related disorders. The information relevant to medical species, diabetes, and obesity was collected from local libraries, the online service E-library.ru, and Google Scholar. The prediction of the antidiabetic activity for the principal compounds identified in plants was performed using the free web resource PASS Online.

Results: We collected and analyzed information about the compositions, specificities of use, and posology of 227 medical species. The medical species represent mixtures of 2–15 plants, while the most frequently mentioned in the literature are species comprising 3–6 plants. The top 10 plants among the 158 mentioned in the literature include *Vaccinium myrtillus* L., *Phaseolus vulgaris* L., *Taraxacum campylodes* G.E. Haglund., *Urtica dioica* L., *Rosa* spp., *Hypericum* spp., *Galega officinalis* L., *Mentha* × *piperita* L., *Arctium* spp, and *Fragaria vesca* L. The leading binary combination found in medical species comprises the leaves of *V. myrtillus* and pericarp of *P. vulgaris*; leaves of *V. myrtillus* and leaves of *U. dioica*; and leaves of *V. myrtillus* and aerial parts of *G. officinalis*. In triple combinations, in addition to the above-mentioned components, the roots of *T. campylodes* are often used. These combinations can be regarded as basic mixtures. Other plants are added to improve the efficacy, treat associated disorders, improve gastrointestinal function, prevent allergic reactions, etc. Meanwhile, an increase in plants in the mixture necessitates advanced techniques for quality control. A feature of medical species in Russia is the addition of fresh juices, birch sap, seaweeds, and adaptogenic plants. Modern studies of the mechanisms of action and predicted activities of the principal compounds from medicinal plants support the rationality of polyherbal mixtures. Nevertheless, the mechanisms are not well studied and reported due to the limited number of compounds. Further investigations with calculations of synergistic or additive indices are important for strengthening the scientific fundamentals for the wider use of medical species in the therapy of diabetes. Two medical species, "Arfazetin" (7 medicinal plants) and "Myrphasinum" (12 medicinal plants), are approved for use in officinal medicine. The efficacy of these species was confirmed in several *in vivo* experiments and clinical trials.

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According to modern regulatory rules, additional experiments and clinical trials are required for more detailed investigations of the mechanisms of action and confirmation of efficacy.

Conclusion: We believe that the scientifically based utilization of rich plant resources and knowledge of Russian herbal medicine can significantly contribute to the local economy as well as to the sectors seeking natural healing products.

Keywords: polyherbal mixture, herbal medicine, obesity, blood glucose, binary combination, triple combination, mechanisms of activity, synergy

INTRODUCTION

Disorders of carbohydrate and lipid metabolism predispose individuals to diseases of the endocrine system, particularly diabetes. The rapid increase in patients with diabetes is one of the gravest and fastest-growing public health problems in the world. About 463 million people currently suffer from diabetes globally (IDF, 2019), of whom over 60 million were in European countries (Timmis et al., 2020), 34.2 million in the US (Centers for Disease Control and Prevention, 2020), and 4.5 million in Russia in 2017 (Dedov et al., 2018).

The mean cost of the development of a new drug from concept to market is considered to range from $314 million to $2.8 billion (Wouters et al., 2020). According to recent literature data, 50–70% of all the small-molecule therapeutics in clinical use today trace their origins to natural products (Newman and Cragg, 2020). Medicines derived from natural sources exhibit greater ranges of structural and physicochemical features that have been tailored through evolution for selective binding to functional macromolecules of the human body (Stratton et al., 2015). The intensive exploration of natural resources and utilization of the knowledge of traditional medicine provides an opportunity to reduce the time needed for development and keep costs reasonably low.

About four billion people around the world believe that, as "natural" products, herbal medicinal products (HMP) are "safe" or "safer" than conventional drugs and have turned to phytotherapeutics (Ekor, 2014). For centuries, Russia has been regarded a "herbophilious" society in which plants have been used as one of the primary foods and for the treatment of different diseases (Shikov et al., 2017). It is estimated that 58–60% of the population of Russia relies on HMP for the prophylaxis or treatment of different diseases (Shikov et al., 2011; Sammons et al., 2016). HMP currently makes up 20% of the Russian market for drugs (Akamova et al., 2017).

Medicinal plants have been effectively used for the treatment of diabetes in different systems of traditional as well as officinal medicine (Anzar, 2013; Shikov et al., 2014; Suzuki et al., 2017; Xiao and Luo, 2018; Okovitiy et al., 2018; Skalli et al., 2019; Salehi et al., 2019). The philosophy "one disease, one target, one drug" oversimplifies the mechanisms of disease and is becoming increasingly inefficient (Ulrich-Merzenich, 2014; Panossian et al., 2018; Shikov et al., 2018). Due to its multifactorial etiology, the holistic treatment of diabetes requires multi-pathway understanding and multi-targeting approaches. Modern network pharmacology studies emphasize the importance of the network-targeting, multicomponent therapy used in traditional Indian systems of medicine (Mukherjee et al., 2018; Banerjee et al., 2019), traditional Chinese medicine (Li et al., 2014; Xue et al., 2019), Kampo (Suzuki et al., 2017), etc.

Multicomponent or polyherbal mixtures for the treatment of diabetes are presented in different systems of Eastern traditional medicine (Namdul et al., 2001; Sato, 2004; Tong et al., 2012; Malgaonkar et al., 2016; Ghadge and Kuvalekar, 2017; Suzuki et al., 2017; Xu et al., 2019) as well as in European herbal medicine (Madić et al., 2021). Such traditional formulations include carefully selected leaves, stems, flowers, roots, seeds, sometimes minerals, and animal products. The main goal of complicated mixtures is to increase therapeutic efficacy and minimize toxicity.

Russian herbal medicine has adopted Eastern philosophy and a Western pragmatic approach. Herbal medicine in Russia is part of officinal medicine. According to the 14th edition of the State Pharmacopoeia of the Russian Federation, which became effective in 2018, an HMP has been defined as "a medicinal product manufactured or prepared from one kind of medicinal plant material or several kinds of raw materials and marketed in consumer-ready packaging form" (The State Pharmacopoeia of Russian Federation, 2018). The general monograph (OFS.1.4.1.0020.15) is devoted to polyherbal mixtures, which are defined with the specific term "medical species". A "medical species" is a formulation representing a mixture of two or more types of integral, cut, or powdered medicinal plant materials, sometimes with the addition of mineral, synthetic, plant, or animal-derived substances. Medical species are used for the preparation of aqueous decoctions/infusions, occasionally in pure form as powders, powders for insufflation or ingestion, etc. (The State Pharmacopoeia of Russian Federation, 2018). Medical species have been known in Russia for centuries and were documented in the first herbalist manuscripts (Zmeev, 1896; Shikov et al., 2021). Apparently, due to their efficacy, the medical species were adopted from traditional medicine in officinal and were monographed in the first Russian military field "Pharmacopoeia Castrensis Rossica., 1765". Thereafter, medical species were embodied in all the following pharmacopoeias of Russia.

The polyherbal mixtures used in Eastern systems of traditional medicine have gained global popularity, and several new medicinal products are being marketed in different countries. However, the potential of the medical species used in Russia

remains little known and underestimated. This review aimed to analyze the medical species used in Russia for the treatment of diabetes and related disorders and enable a better understanding of the rationality of plant combinations.

Information on medical species was collected from the Pharmacopoeias of Russia (I–VI editions), Pharmacopoeias of USSR (VII–XI editions), and online State Register of Medicinal Preparations of the Russian Federation (2021). Guided by the keywords "traditional medicine + diabetes", "phytotherapy + diabetes", "traditional medicine + obesity", and "phytotherapy + obesity", we systematically searched the literature in library catalogs, on the online service E-library.ru, and on Google Scholar. Next, the publications were screened using the keyword combination "medical species". Through this approach, 75 medical (herbalist) books were found. Some medical species were mentioned in different books. We provide several references for the same medical species in **Table 1**.

RESULTS AND DISCUSSION

For centuries, medicinal plants have been used in Russia for the management of diabetes and related disorders (Zmeev, 1896; Turova and Sapozhnikova, 1989; Minaeva, 1991; Protasenya and Vasilenko, 1992; Nazina, 2007; Chekina et al., 2010; Korsun et al., 2016; Povydysh et al., 2018). The focus of the current paper is on medical species used for the therapy of diabetes and related diseases in traditional and officinal Russian medicine.

The Characteristics of the Composition of Medical Species, Their Preparation and Posology

We collected information about the composition, specificity of preparation, and posology of 227 species (**Table 1**). The majority of the species (148) are suggested for the treatment of diabetes; 37 species are recommended for the management of obesity, and eight species are indicated for lowering blood glucose. Several species are recommended for specific cases when diabetes is accompanied by impotence in men (5 species), diabetes accompanied by liver and gallbladder diseases (3 species), diabetes accompanied by gastritis (2 species), etc. Altogether, 158 plants are mentioned in medical species, among which 96 medicinal plants are monographed in the State Pharmacopoeia of the Russian Federation and used in officinal medicine (Shikov et al., 2021). A significant proportion of the plants used in medical species are native to Russian flora.

Medical species represent mixtures of 2–15 plants. The most frequently mentioned in the literature are medical species comprising mixtures of four plants (66 species), followed by mixtures of five plants (47 species), three plants (28 species), and six plants (24 species) (**Figure 1**). According to some experts' opinions, the industrial-scale production of polyherbal mixtures with more than 10 medicinal plants is not rational (Kiseleva and Chauzova, 1999). Indeed, only 18 species among the 227 described contain over 10 plants (**Figure 1**). However, the numbers of plants in polyherbal mixtures in Ayurveda (Parasuraman et al., 2014), Kampo (Arai et al., 2020), and traditional Chinese medicine (Xutian et al., 2014) are not limited to 10. On the other hand, the species "Myrphasinum", approved as officinal medicine in Russia, includes 12 plants (**Table 1**). Although the idea of combining so many plants in one mixture is part of traditional medicine, the quality control of medical species becomes more complicated with each additional component due to challenges related to the specificity of each plant.

Medical species are prepared predominantly in form of infusions or decoctions. Infusions are common for soft plant parts such as aerial parts, leaves, and flowers. Decoctions are preferred for more hard barks, fruits, and roots. The recommended single doses vary from a tablespoon up to 200 ml and depend on the pharmacological activity of the plants in the mixture.

The Plants Most Frequently Used in Medical Species

The top 10 plants in medical species used for the therapy of diabetes and related disorders (**Table 1**) include *Vaccinium myrtillus* L. (leaves in 97, shoots in 11, and fruits in 5 species), *Phaseolus vulgaris* L. (pericarp in 65 species), *Taraxacum campylodes* G.E. Haglund. (syn. *Taraxacum officinale Wigg*) (roots in 49 and leaves in 15 species), *Urtica dioica* L. (leaves in 49 and roots in 1 species), *Rosa* spp. (fruits in 44 species), *Hypericum* spp. (aerial parts in 37 and flowers in 6 species), *Galega officinalis* L. (aerial parts in 41 species and seeds in one species), *Mentha* × *piperita* L. (leaves in 29 and aerial parts in 11 species), *Arctium* spp. (roots in 34 and leaves in 2 species), and *Fragaria vesca* L. (leaves in 26, aerial parts in 5, and roots in 1 species). Although the main plant parts used in species are the same as those recorded in the State Pharmacopoeia of the Russian Federation, multiple parts of some plants are utilized. Particularly, aerial parts, fruits, and roots (*Petroselinum crispum* (Mill.) Fuss); leaves, flowers, and roots (*Sambucus nigra* L.); and roots and leaves (*Cichorium intybus* L., *Rubus caesius* L., and *Arctium* spp.) have been used.

The Popular Combinations of Medicinal Plants and Rationality for Combination

It is believed that, in medical species, several herbs work together harmoniously to achieve an ideal therapeutic effect. Modern studies on the mechanisms of activities of individual plant extracts support the rationality of empirically composed polyherbal mixtures in traditional medicine. Furthermore, we discuss the most frequent combinations of plants used in medical species in light of their mechanisms of action. The most frequently mentioned binary combinations of plants in medical species used for the treatment of diabetes are specified in **Table 2**.

The leading binary combination noted in medical species (**Table 2**) comprises the leaves of *Vaccinium myrtillus* L. and pericarp of *Phaseolus vulgaris* L. (quoted in 40 medical species).

TABLE 1 | The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
2 plants					
2.1	*Frangula alnus* Mill. bark; *Taraxacum campylodes* G.E.Haglund. roots; (10:3)	Decoction; 1 table spoon in 200 ml of boiling water	200 ml 2–3 times a day	Obesity	Safonov (2016)
2.2	*Vaccinium myrtillus* L. leaves; *Arctium* spp.[b] roots; (1:1)	Infusion; 10 g in 200 ml of boiling water	1 table spoon 3–4 times a day before eating	Diabetes	Sokolov and Zamotaiev (1984), Matkovskaya et al. (1988), Sinyakov (1992), Sinyakov (1999), Chirkov and Seryi (1993), Efimov and Shcherbak (1993), Tarasenko et al. (1998), Dontsov and Dontsov (2000), Sokolov (2000), Blinov (2000), Podduev (2001), Dremova et al. (2003), Nazina (2006), Davydovich et al. (2008), Bogdanova and Bashkirova (2010)
2.3	*Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; (1:1)	Infusion; 1 table spoon in 200 ml of water, boil 15 min, maceration 30 min at room temp.	100 ml 3–4 times a day before eating	Diabetes	Sinyakov, (1992), Sinyakov, (1999), Efimov and Shcherbak (1993), Tarasenko et al. (1998), Podduev (2001), Smolianskii and Lifliandskii (2004), Korodetsky (2006), Davydovich et al. (2008), Balakirev (2010), Bogdanova and Bashkirova (2010)
2.4	*Inula helenium* L. roots; *Arctium lappa* L. roots; (1:1)	Infusion; 1 table spoon in 400 ml of water, boil 10 min	1 table spoon 3 times a day	Diabetes	Volynchenko (2003)
2.5	*Galega officinalis* L. aerial part; *Galega officinalis* L. seeds; (7:3)	Infusion; 1 tea spoon in 200 ml of water, boil 10 min, maceration 20–30 min,	200 ml 3 times a day 30 min before eating	Diabetes	Sinyakov (1999)
2.6	*Taraxacum campylodes* G.E.Haglund. roots; *Taraxacum campylodes* G.E.Haglund. leaves; (1:1)	Decoction; 6–10 g in 200 ml of water, boil 10 min, maceration 30 min	1 table spoon 3 times a day 30 min before eating	Diabetes	Podduev (2001)
2.7	*Taraxacum campylodes* G.E.Haglund. roots; *Mentha* × *piperita* L. leaves; (1:3)	Decoction; 4 tea spoons in 200 ml of water, boil 5–7 min, maceration 30 min	100 ml 3–4 times a day before eating	Diabetes	
3 plants					
3.1	*Urtica dioica* L. leaves; *Juniperus communis* L. fruits; *Equisetum arvense* L. aerial part; (2:3:4)	Infusion; 1 table spoon in 500 ml of boiling water	66 ml 3 times a day before eating	Obesity	Osetrov and Shreter (2001)
3.2	*Ononis spinosa* L. roots; *Taraxacum campylodes* G.E.Haglund. roots; *Frangula alnus* Mill. bark; (3:3:10)	Infusion; 3 table spoons in 600 ml of boiling water	200 ml 2–3 times a day before eating	Obesity	Lager (1991), Lager (2002), Efimov and Shcherbak (1993), Kukes (1999), Dontsov and Dontsov (2000), Bubenchikova et al. (2003)
3.3	*Tussilago farfara* L. leaves; *Betula* spp.[c] leaves; *Rubuscaesius* L. leaves; 1:1:8	Infusion; 10 g in 200 ml of boiling water	200 ml 2 times a day before eating	Obesity	Dontsov and Dontsov (2000), Sokolov (2000), Kiyanova (2005), Maznev (2005)
3.4	*Phaeophyceae* (*Cystoseira barbata* (Stackh.) C.Agardh) thallus; *Pimpinella anisum* L. fruits; *Glycyrrhiza glabra* L. roots; (2:1:1)	Decoction; 2 table spoons in 500 ml of water	100 ml 3–4 times a day	Obesity	Yordanov et al. (1972), Chirkov and Seryi (1993), Kiyanova (2005), Maznev (2005)
3.5	*Achillea millefolium* L. aerial part; *Hypericum perforatum* L. aerial part; *Phaeophyceae* (*Cystoseira barbata* (Stackh.) C.Agardh) thallus; (2:2:1)	Infusion (herbal tea); 2 table spoons in 400 ml boiling water	100 ml 3–4 times a day	Obesity	Yordanov et al. (1972), Chirkov and Seryi (1993), Efimov and Shcherbak (1993)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
3.6	*Frangula alnus* Mill. bark; *Achillea millefolium* L. aerial part; *Juniperus communis* L. fruits; (3:2:1)	Infusion; 2 table spoons in 500 ml of boiling water	200 ml 3 times a day	Obesity	Lager (1991), Lager (2002), Dontsov and Dontsov (2000), Rendiuk (2006), Safonov (2016)
3.7	*Vaccinium myrtillus* L. leaves; *Urtica dioica* L. leaves; *Sambucus nigra* L. leaves; (2:1:1)	Decoction; 1 table spoon in 200 ml boiling water	150 ml a day	Lowering of blood glucose level	Sokolov and Zamotaiev (1984), Matkovskaya et al. (1988), Sinyakov (1992), Sinyakov (1999); Efimov and Shcherbak (1993), Tarasenko et al. (1998), Kukes (1999), Blinov (2000), Dontsov and Dontsov (2000), Sokolov (2000), Bubenchikova et al. (2003), Podduev (2001), Onipko (2002), Davydovich et al. (2008), Bogdanova and Bashkirova (2010)
3.8	*Equisetum arvense* L. aerial part; *Polygonum aviculare* L. aerial part; *Fragaria vesca* L. leaves; (1:2:1)	Infusion; 1 table spoon in 400 ml hot water	400 ml a day	Lowering of blood glucose level	Sokolov and Zamotaiev (1984), Matkovskaya et al. (1988), Sinyakov (1992), Sinyakov (1999); Chirkov and Seryi (1993), Nikultseva (1994), Tarasenko et al. (1998), Trofimenko and Mogilny (1998), Kukes (1999), Dontsov and Dontsov (2000), Sokolov (2000), Bubenchikova et al. (2003), Turishchev (2000), Blinov (2000), Podduev (2001), Dremova et al. (2003), Turishchev (2005), Nazina (2006), Davydovich et al. (2008), Volynchenko (2003), Ruzhenkova (2014), Maznev (2014)
3.9	*Vaccinium myrtillus* L. leaves; *Urtica dioica* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; (1:1:1)	Infusion (herbal tea); 10 g in 200 ml of boiling water	100 ml 3 times a day before eating	Lowering of blood glucose level	Sokolov and Zamotaiev (1984), Matkovskaya et al. (1988), Sinyakov (1992), Chirkov and Seryi (1993), Efimov and Shcherbak (1993), Nikolaychuk (1997), Fedyukovich (1998), Kukes (1999), Bubenchikova et al. (2003), Turishchev (2000), Sokolov (2000), Blinov (2000), Dontsov and Dontsov (2000), Podduev (2001), Onipko (2002), Nikolaychuk and Zubitskaya (2003), Dremova et al. (2003), Brusenskaya and Kaz'min (2005), Turishchev (2005), Nazina (2006), Davydovich et al. (2008), Bogdanova and Bashkirova (2010), Pigulevskaya (2018)
3.10	*Hypericum perforatum* L. flowers; *Galega officinalis* L. aerial part; *Urtica dioica* L. leaves; (5:4:3)	Infusion; 60 g in 200 ml of boiling water	100 ml or 66 ml 4 times a day before eating	Diabetes	Brusenskaya and Kaz'min (2005)
3.11	*Hypericum perforatum* L. aerial part; *Mentha* × *piperita* L. leaves; *Vaccinium myrtillus* L. leaves; (1:1:1)	Infusion; 1 table spoon in 250 ml of boiling water	125 ml 2 times a day before eating	Diabetes	Osetrov and Shreter (2001)
3.12	*Vaccinium myrtillus* L. leaves; *Elymus repens*(L.)rhizomes; *Rubuscaesius* L. roots; (250:10:2)	Infusion; 262 g in 1,500 ml of boiling water	During the day instead of water	Diabetes	Osetrov (1993), Osetrov and Shreter (2001)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
3.13	*Vaccinium vitis-idaea* L. leaves; *Ruta graveolens* L. leaves; *Angelica archangelica* L. roots; (5:3:2)	Infusion; 1 table spoon in 200 ml of boiling water, boil 10 min, maceration 30–40 min at room temp.	100 ml 3–4 times a day 30 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Efimov and Shcherbak (1993), Tarasenko et al. (1998), Bogdanova and Bashkirova (2010)
3.14	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Valeriana officinalis* L. roots and rhizomes; *Vaccinium myrtillus* L. leaves; (1:1:2)	Infusion; 1 table spoon in 200 ml of boiling water, boil 15 min, maceration 30 min at room temp.	200 ml 3–4 times a day before eating	Diabetes	Tarasenko et al. (1998), Podduev (2001)
3.15	*Fragaria vesca* L. leaves; *Cichorium intybus* L. leaves; *Sambucus nigra* L. flowers; (2:2:1)	Infusion; 1 table spoon in 200 ml of boiling water, boil 5 min, maceration 1 h at room tempature	66 ml 3 times a day 20 min before eating	Diabetes	Efimov and Shcherbak (1993), Nikolaychuk (1997), Rendiuk (2006), Bogdanova and Bashkirova (2010)
3.16	*Vaccinium myrtillus* L. leaves; *Fragaria vesca* L. leaves; *Rubuscaesius* L. leaves; (1:1:1)	Infusion; 1 table spoon in 300 ml of boiling water, boil 3 min, maceration 10 min at room tempature	100 ml 3 times a day 20 min before eating	Diabetes	Efimov and Shcherbak (1993), Nikolaychuk (1997), Fedyukovich (1998), Nikolaychuk and Zubitskaya (2003), Dremova et al. (2003), Rendiuk (2006), Bogdanova and Bashkirova (2010), Pigulevskaya (2018)
3.17	*Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Matricaria chamomilla* L. flowers; (1:2:1)	Infusion; 40 g in 400 ml of boiling water, maceration 5–6 h at room tempature	100 ml 4 times a day 20–30 min before eating	Diabetes	Lavrenova and Lavrenov (2007)
3.18	*Arctium* spp.[b] roots; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; (1:1:1)	Infusion; 60 g in 1,000 ml of cold water, maceration 12 h at room temperature, boil 5 min, maceration 1 h	150 ml 5 times a day 1 h after eating	Diabetes	Seredin and Sokolov (1973), Lager (1991), Lager (2002), Makhlayuk (1991), Chirkov and Seryi (1993), Dmitriev et al. (1994) Nikultseva (1994), Fedyukovich (1998), Podduev (2001), Pirogov (2008), Grechanyi (2013), Melik-Gusseinov and Rekkandt (2014), Maznev (2014)
3.19	*Vaccinium myrtillus* L. leaves; *Galega officinalis* L. aerial part; *Urtica dioica* L. leaves; (1:1:1)	Infusion; 1 table spoon in 300 ml of boiling water	2 table spoons 3–4 times a day 20 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Efimov and Shcherbak (1993), Nikultseva (1994), Nikolaychuk (1997), Trofimenko and Mogilny (1998), Fedyukovich (1998), Blinov (2000), Onipko (2002), Nikolaychuk and Zubitskaya (2003), Nazina (2006), Bogdanova and Bashkirova (2010), Maznev (2014)
3.20	*Vaccinium myrtillus* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; *Galega officinalis* L. aerial part; (1:1:1)	Infusion; 1 table spoon in 300 ml of boiling water	100 ml 2–3 times a day 20 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Trofimenko and Mogilny (1998), Blinov (2000), Nazina (2006)
3.21	*Leonurus* spp.[d]leaves; *Fragaria vesca* L. leaves; *Morus alba* L. leaves; (1:2:4)	Infusion; 1 table spoon in 200 ml of boiling water	2 table spoons 3 times a day after eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Nikultseva (1994), Nikolaychuk (1997), Tarasenko et al. (1998), Trofimenko and Mogilny (1998), Fedyukovich (1998), Blinov (2000), Podduev (2001), Dremova et al. (2003), Kiyanova (2005), Nazina (2006), Davydovich et al. (2008), Bogdanova and Bashkirova (2010), Maznev (2014)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
3.22	*Vaccinium myrtillus* L. leaves; *Inula helenium* L. roots; *Polygonum aviculare* L. aerial part; (1:1:1)	Decoction; 1 table spoon in 200 ml of water	50 ml 2–3 times a day	Diabetes	Chirkov and Seryi (1993), Kukes (1999), Bubenchikova et al. (2003), Dremova et al. (2003)
3.23	*Vaccinium myrtillus* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; *Artemisia vulgaris*L., aerial part; (5:5:4)	Decoction; 1 table spoon in 300 ml of water, boil 5 min, maceration 30 min	100 ml 3–4 times a day	Diabetes	Podduev (2001)
3.24	*Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Mentha* × *piperita* L. leaves; (1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, maceration 30 min	70 ml 3 times a day before eating	Diabetes	Podduev (2001)
3.25	*Avena sativa* L. aerial part in flowering phase; *Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; (1:2:2)	Infusion; 1 table spoon in 200 ml of boiling water	200 ml 3–4 times a day before eating	Diabetes accompanied by impotence in men	Sklyarevsky and Gubanov (1989), Efimov and Shcherbak (1993), Blinov (2000); Orlova, (2001), Dremova et al. (2003), Brusenskaya and Kaz'min (2005), Kiyanova (2005), Rendiuk (2006), Davydovich et al. (2008), Bogdanova and Bashkirova (2010)
3.26	*Helichrysum arenarium* (L.) Moench. flowers; *Fagopyrum esculentum* Moench flowers and leaves; *Vaccinium myrtillus* L. leaves; (1:1:2)	Infusion; 12 g in 1,000 ml of boiling water, maceration 5–6 h at room temp., then 15 min in boil water bath	200 ml (warm) with 10 g of honey 3–4 times a day before eating	Diabetes accompanied by impotence in men	Brusenskaya and Kaz'min (2005)
3.27	*Urtica dioica* L. leaves; *Inula helenium* L. roots; Sugar; (9:1:5)	Decoction; 2 table spoons in 200 ml of water	100 ml (warm) 3 times a day before eating	Metabolism improving	Chirkov and Seryi (1993)
3.28	*Viola tricolor* L. aerial part; *Bidens tripartite* L. aerial part; *Solanum dulcamara* L. aerial part; (4:4:1)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	4 table spoon 3–4 times a day	King's evil, metabolic disorder	Chirkov and Seryi (1993)
4 plants					
4.1	*Mentha* × *piperita* L. leaves; *Foeniculum vulgare* Mill. fruits; *Matricaria chamomilla* L. flowers; *Tilia cordata* Mill. flowers; (4:3:3:3)	Infusion; 10 g in 200 ml of boiling water	200 ml 2–3 times a day	Obesity	Safonov (2016)
4.2	*Levisticum officinale* W.D.J.Koch roots; *Juniperus communis* L. fruits; *Phaeophyceae* (*Cystoseira barbata* (Stackh.) C.Agardh) thallus; *Achillea millefolium* L. aerial part; (1:1:1:1)	Decoction; 2 table spoons in 500 ml of water	132 ml or 200 ml 2–3 times a day	Obesity	Chirkov and Seryi (1993)
4.3	*Ononis spinosa* L. roots; *Persicaria hydropiper* (L.) Delarbre aerial part; *Foeniculum vulgare* Mill. fruits; *Alchemilla xanthochlora* Rothm. roots and aerial part; (6:1:1:1)	Decoction; 2 table spoons in 500 ml of water	100 ml 4 times a day before eating	Obesity	Chirkov and Seryi (1993)
4.4	*Artemisia absinthium* L., aerial part; *Salvia officinalis* L. leaves; *Rosmarinus officinalis*L.leaves;*Prunus spinosa*L. flowers; (1:1:1:1)	Infusion; 3 table spoons in 500 ml of boiling water	150 ml 3 times a day	Obesity	Osetrov and Shreter (2001)
4.5	*Frangula alnus* Mill. bark; *Taraxacum campylodes* G.E.Haglund.roots; *Petroselinum crispum* (Mill.) Fuss fruits; *Foeniculum vulgare* Mill. fruits; (3:1:1:1)	Infusion; 20 g in 400 ml of boiling water	400 ml in the morning before eating	Obesity	Dontsov and Dontsov (2000), Maznev (2005), Kiyanova (2005)
4.6	*Apium graveolens* L. leaves; *Phaseolus vulgaris* L. pericarp; *Humulus lupulus* L. fruits; *Pastinaca sativa* L. root; (4:4:3:1)	Herbal tea; 1 table spoon in 200 ml of boiling water	30 ml 6 times a day	Obesity and diabetes	Protasenya and Vasilenko (1992)
4.7	*Nasturtium officinale*R.Br.aerial part; *Morus nigra*L.leaves;*Urtica dioica* L. leaves; *Phaseolus vulgaris* L. pericarp; (1:1:1:1)	Infusion; 1 table spoon in 300 ml of boiling water	100 ml 3 times a day before eating	Diabetes	Osetrov (1993)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
4.8	*Phaseolus vulgaris* L. pericarp; *Betula* spp.d[d] leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Sinapis alba*L. seeds; (8:12:4:1)	Infusion; 1 table spoon in 300 ml of boiling water	150 ml 2 times a day	Diabetes	Osetrov (1993), Maznev (2014)
4.9	*Vaccinium myrtillus* L. leaves; *Rubus caesius* L. leaves; *Fragaria vesca* L. leaves; *Rosa majalis* Herrm. fruits; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water, maceration 30 min	100 ml 3 times a day before eating	Diabetes	Efimov and Shcherbak (1993), Nikolaychuk (1997), Fedyukovich (1998), Onipko (2002), Nikolaychuk and Zubitskaya (2003), Smolianskii and Lifliandskii (2004), Bogdanova and Bashkirova (2010)
4.10	*Juniperus communis* L. fruits; *Linum usitatissimum* L. seeds; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (1:1:1:1)	Infusion; 1 tea spoon in 200 ml of boiling water, boil 5 min, maceration 30 min	50 ml 3 times a day before eating	Diabetes	Nikolaychuk and Zubitskaya (2003), Smolianskii and Lifliandskii (2004)
4.11	*Galega officinalis* L. aerial part; *Urtica dioica* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Phaseolus vulgaris* L. pericarp; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	50 ml 3 times a day 15 min before eating	Diabetes	Dontsov and Dontsov (2000), Podduev (2001), Vinogradova et al. (2001)
4.12	*Vaccinium myrtillus* L. leaves; *Fragaria vesca* L. leaves; *Tilia cordata* Mill. flowers; *Verbascum densiflorum* Bertol. flowers; (8:5:4:3)	Infusion; 2 table spoons in 400 ml of boiling water	100–132 ml 3 times a day 30 min before eating	Diabetes	Sinyakov (1992), Efimov and Shcherbak, (1993), Tarasenko et al. (1998), Podduev (2001), Dremova et al. (2003), Bogdanova and Bashkirova (2010), Volynchenko (2003)
4.13	*Vaccinium myrtillus* L. leaves; *Urtica dioica* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Phaseolus vulgaris* L. pericarp; (1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, boil 12–15 min, maceration 30–40	66 ml 3 times a day 30 min before eating	Diabetes	Sinyakov (1992), Efimov and Shcherbak (1993), Dmitriev et al. (1994), Tarasenko et al. (1998), Kukes (1999), Sinyakov (1999), Bubenchikova et al. (2003), Bogdanova and Bashkirova (2010), Melik-Gusseinov and Rekkandt (2014)
4.14	*Equisetum arvense* L. aerial part; *Polygonum aviculare* L. aerial part; *Urtica dioica* L. leaves; *Capsella bursa-pastoris* (L.) Medik. aerial part; (1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, boil 3–5 min, maceration 30–40 min	40–50 ml 3–4 times a day 30 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999); Nikolaychuk (1997), Tarasenko et al. (1998), Podduev (2001), Nikolaychuk and Zubitskaya (2003), Davydovich et al. (2008), Maznev (2014), Pigulevskaya (2018)
4.15	*Polygonum aviculare* L. aerial part; *Equisetum arvense* L. aerial part; *Fragaria vesca* L. leaves; *Aralia elata* (Miq.) Seem roots; (7:5:5:2)	Infusion; 2 table spoons in 500 ml of boiling water, boil 3–5 min, maceration 20–30 min	40–50 ml 3–4 times a day 30 min before eating	Diabetes	Sinyakov (1992), Tarasenko et al. (1998), Podduev (2001)
4.16	*Taraxacum campylodes* G.E.Haglund. roots; *Phaseolus vulgaris* L. pericarp; *Hypericum perforatum* L. aerial part; *Vaccinium myrtillus* L. leaves; (1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, maceration 12 h in thermos	100 ml 3 times a day 30 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999); Tarasenko et al. (1998)
4.17	*Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Arctium lappa* L. roots; *Vaccinium vitis-idaea* L. leaves; (2:2:1:1)	Infusion; 1 table spoon in 200 ml of boiling water, boil 15 min, maceration 30 min	200 ml 3–4 times a day before eating	Diabetes	Tarasenko et al. (1998), Podduev (2001)
4.18	*Rubuscaesius* L. leaves;*Vaccinium vitis-idaea* L. leaves; *Primula veris* L. leaves; *Galega officinalis* L. aerial part; (3:3:2:4)	Infusion; 1 table spoon in 300 ml of boiling water, boil 3 min, maceration at room tempature	100 ml 3 times a day after eating	Diabetes	Efimov and Shcherbak (1993), Nikolaychuk (1997), Fedyukovich (1998), Dremova et al. (2003), Rendiuk (2006)
4.19	*Centaurium erythraea* Rafn aerial part; *Vaccinium myrtillus* L. leaves; *Equisetum arvense* L. aerial part; *Polygonum aviculare* L. aerial part; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water, boil 5 min, maceration at room tempature	200 ml 2–3 times a day before eating	Diabetes	Efimov and Shcherbak (1993), Nikolaychuk (1997), Fedyukovich (1998), Onipko (2002), Nikolaychuk and Zubitskaya (2003), Rendiuk (2006), Bogdanova and Bashkirova (2010)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
4.20	*Arctium lappa* L. roots; *Cichorium intybus* L. roots; *Valeriana officinalis* L. roots and rhizomes; *Rubus caesius* L. root; (2:3:3:1)	Herbal tea; 3 table spoons in 1,000 ml of boiling water	100 ml 7 times a day	Diabetes	Rendiuk (2006)
4.21	*Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Arctium lappa* L. roots; *Vaccinium vitis-idaea* L. leaves; (2:2:1:1)	Infusion; 1 table spoon in 200 ml of boiling water, boil 15 min, maceration 30 min	200 ml 3–4 times a day before eating	Diabetes	Tarasenko et al. (1998)
4.22	*Cichorium intybus* L. roots; *Plantago major* L. leaves; *Arctium lappa* L. roots; *Equisetum arvense* L. aerial part; (1:1:1:1)	Infusion; 1 table spoon in 300 ml of boiling water, boil 3 min, maceration 10 min	66 ml 3 times a day 20 min before eating	Diabetes	Efimov and Shcherbak (1993), Nikolaychuk (1997), Fedyukovich (1998), Onipko (2002), Nikolaychuk and Zubitskaya (2003), Rendiuk (2006), Bogdanova and Bashkirova (2010), Pigulevskaya (2018)
4.23	*Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water, maceration 20 min	200 ml 3–4 times a day	Diabetes	Volynchenko (2003)
4.24	*Juglans regia* L. leaves; *Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Arctium* spp.[b] roots; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of cold water, maceration 1–2 h at room tempature, boil 5–7 min	200 ml 5–6 times a day after eating	Diabetes	Volynchenko (2003)
4.25	*Vaccinium myrtillus* L. fruits; *Sambucus nigra* L. flowers; *Arctium lappa* L. roots; *Zea mays* L. corn silk; (1:1:1:1)	Decoction	1–2 table spoons 3 times a day 30 min before eating for 1–1.5 months	Diabetes	Kukes (1999)
4.26	*Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Polygonum aviculare* L. aerial part; *Arctostaphylos uva-ursi* (L.) Spreng. leaves; (1:1:1:1)	Infusion; 60 g in 300 ml of boiling water	66 ml 3 times a day	Diabetes	Lager (1991), Lager (2002), Efimov and Shcherbak (1993), Nikolaychuk (1997), Fedyukovich (1998), Nikolaychuk and Zubitskaya (2003), Bogdanova and Bashkirova (2010)
4.27	*Galega officinalis* L. aerial part; *Vaccinium myrtillus* L. leaves; *Urtica dioica* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	200 ml 3–4 times a day	Diabetes	Lager (1991), Lager, (2002), Efimov and Shcherbak (1993), Bogdanova and Bashkirova (2010)
4.28	*Phaseolus vulgaris* L. pericarp; *Galega officinalis* L. aerial part; *Betula pendula* Roth. leaves; *Vaccinium myrtillus* L. leaves;(1:1:1:1)	Infusion; 2 table spoons in 400 ml of boiling water, boil 10 min, maceration 30–40 min	100 ml 3 times a day 30 min before eating	Diabetes	Sinyakov (1999)
4.29	*Galega officinalis* L. aerial part; *Vaccinium vitis-idaea* L. leaves; *Frangula alnus* Mill. bark; *Betula pendula* Roth. Leaves; (40:40:10:10)	Infusion; 3 table spoons in 600 ml of boiling water, 15 min in boil water bath, maceration 30–40 min	130 ml 3 times a day	Diabetes	Sinyakov (1999)
4.30	*Galega officinalis* L. aerial part; *Vaccinium myrtillus* L. leaves; *Sambucus nigra* L. leaves; *Viscum album* L. aerial part; (7:7:4:2)	Infusion; 2 table spoons in 400 ml of boiling water, 15 min in boil water bath, maceration 30–40 min	50–130 ml 2–3 times a 30 min before eating	Diabetes	Sinyakov (1999)
4.31	*Vaccinium myrtillus* L. leaves; *Fragaria vesca* L. leaves; *Tilia cordata* Mill. flowers; *Verbascum densiflorum* Bertol. flowers; (8:5:4:3)	Infusion; 2 table spoons in 400 ml of boiling water, 15 min in boil water bath, maceration 30–40 min	50–130 ml 2–3 times a day 30 min before eating	Diabetes	Sinyakov (1999)
4.32	*Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; *Laurus nobilis* L. leaves; *Morus alba* L. leaves; (1:1:1:1)	Infusion; 2–3 table spoons in 500 ml of boiling water, 15 min in boil water bath, maceration 30–40 min	200 ml 3–4 times a day 30 min before eating	Diabetes	Sinyakov (1999)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
4.33	*Vaccinium vitis-idaea* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; *Urtica dioica* L. leaves; *Galega officinalis* L. aerial part; (1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, boil 5–6 min, maceration 1–2 h	100 ml 2–3 times a day 20 min before eating	Diabetes	Sinyakov (1999)
4.34	*Vaccinium myrtillus* L. leaves; *Betula pendula* Roth. leaves; *Phaseolus vulgaris* L. pericarp; *Urtica dioica* L. leaves; (60:20:10:10)	Infusion; 2 table spoons in 500 ml of boiling water, boil 10 min, maceration 1–2 h	100 ml 3 times a day 20–30 min before eating	Diabetes	Sinyakov (1999)
4.35	*Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; *Rosa* spp.[6] fruits; *Equisetum arvense* L. aerial part; (4:4:4:1)	Infusion; 65 g in 1,000 ml of water, boil 2 min, maceration 8–12 h in a dark place	100 ml 3 times a day before eating	Diabetes	Brusenskaya and Kaz'min (2005)
4.36	*Vaccinium myrtillus* L. leaves; *Hypericum* spp. [e]aerial part; *Phaseolus vulgaris* L. pericarp; *Galega officinalis* L. aerial part; (4:4:4:5)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	100 ml 2 times a day before eating	Diabetes	Efimov and Shcherbak (1993), Brusenskaya and Kaz'min (2005), Davydovich et al. (2008), Bogdanova and Bashkirova (2010)
4.37	*Avena sativa* L. aerial part; *Linum (usitatissimum* L.*)* seeds; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; (1:1:1:1)	Infusion; 3 table spoons in 600 ml of boiling water	50 ml 6–8 times a day	Diabetes	Makhlayuk (1991), Sinyakov (1992), Chirkov and Seryi (1993), Dmitriev et al. (1994), Nikultseva (1994), Tarasenko et al. (1998), Trofimenko and Mogilny, (1998), Sinyakov (1999), Podduev (2001), Volynchenko (2003), Popov (2004), Lavrenova and Lavrenov (2007), Davydovich et al. (2008), Pirogov (2008), Grechanyi (2013), Maznev (2014), Melik-Gusseinov and Rekkandt (2014)
4.38	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Galega officinalis* L. aerial part; *Vaccinium myrtillus* L. leaves; *Valeriana officinalis* L. roots; (1:1:1:1)	Infusion;1 tea spoon in 200 ml of boiling water	200 ml 3–4 times a day before eating	Diabetes	Yordanov et al. (1972), Matkovskaya et al. (1988), Lager (1991), Sinyakov (1992), Sinyakov (1999), Chirkov and Seryi (1993), Nikolaychuk (1997), Tarasenko et al. (1998), Nikolaychuk and Zubitskaya (2003), Kiyanova (2005), Davydovich et al. (2008), Maznev (2014)
4.39	*Betula pendula* Roth. leaves; *Frangula alnus* Mill. bark; *Vaccinium myrtillus* L. leaves; *Galega officinalis* L. aerial part; (1:1:4:4)	Infusion; 1 tea spoon in 200 ml of boiling water	200 ml 3–4 times a day before eating	Diabetes	Yordanov et al. (1972), Lager (1991), Larger (2002), Chirkov and Seryi (1993), Efimov and Shcherbak (1993), Dontsov and Dontsov (2000), Bogdanova and Bashkirova (2010)
4.40	*Vaccinium myrtillus* L. leaves; *Galega officinalis* L. aerial part; *Phaseolus vulgaris* L. pericarp; *Mentha* × *piperita* L. leaves; (1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water	50–66 ml 3–4 times a day 30 min before eating	Diabetes	Yordanov et al. (1972), Matkovskaya et al. (1988), Lager (1991), Lager (2002); Sinyakov (1992), Sinyakov (1999), Chirkov and Seryi (1993), Efimov and Shcherbak (1993), Nikultseva (1994), Trofimenko and Mogilny (1998), Dontsov and Dontsov (2000), Blinov (2000), Dremova et al. (2003), Maznev (2005); Nazina (2006), Davydovich et al. (2008), Maznev (2014), Pigulevskaya (2018)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
4.41	*Plantago major* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	100 ml 3–4 times a day 20 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Efimov and Shcherbak (1993), Nikolaychuk (1997), Tarasenko et al. (1998), Fedyukovich (1998), Kukes (1999), Blinov (2000), Podduev (2001), Nikolaychuk and Zubitskaya (2003), Nazina (2006), Ryzhenko (2007), Davydovich et al. (2008), Bogdanova and Bashkirova (2010), Maznev (2014)
4.42	*Capsella bursa-pastoris* (L.) Medik. aerial part; *Equisetum arvense* L. aerial part; *Polygonum aviculare* L. aerial part; *Valeriana officinalis* L. roots and rhizomes; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	1 table spoon 3–4 times a day 20–30 min before eating	Diabetes	Blinov (2000), Nazina (2006)
4.43	*Galega officinalis* L. aerial part; *Juglans regia* L. leaves; *Mentha* × *piperita* L. leaves; *Polygonum aviculare* L. aerial part ; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	66 ml 3 times a day 15–20 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Efimov and Shcherbak (1993), Nikolaychuk (1997), Tarasenko et al. (1998), Fedyukovich (1998), Blinov (2000), Onipko (2002), Nikolaychuk and Zubitskaya (2003), Dremova et al. (2003), Nazina (2006), Bogdanova and Bashkirova (2010), Maznev (2014)
4.44	*Cichorium intybus* L. leaves; *Fragaria vesca* L. leaves; *Polygonum aviculare* L. aerial part; *Taraxacum campylodes* G.E.Haglund. leaves; (4:3:2:3)	Infusion; 1 table spoon in 200 ml of boiling water	66 ml a day before eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Efimov and Shcherbak (1993), Nikultseva (1994), Nikolaychuk (1997), Tarasenko et al. (1998), Blinov (2000); Podduev (2001), Dremova et al. (2003), Nazina (2006), Korodetsky (2006), Ryzhenko (2007), Bogdanova and Bashkirova (2010)
4.45	*Mentha* × *piperita* L. leaves; *Ribes nigrum* L. leaves; *Rubus caesius* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; (1:3:2:4)	Infusion; 1 table spoon in 200 ml of boiling water	2–3 table spoons 3 times a day before eating	Diabetes	Nikultseva (1994), Nikolaychuk (1997), Sinyakov (1999), Blinov (2000), Nazina (2006), Ryzhenko (2007), Davydovich et al. (2008), Maznev (2014)
4.46	*Helichrysum arenarium* (L.) Moench flowers; *Rosa majalis* Herrm. fruits; *Vaccinium myrtillus* L. leaves; *Zea mays* L. corn silk; (1:2:5:2)	Infusion; 2 table spoons in 300 ml of boiling water, maceration 12 h in thermos	66 ml 3–4 times a day 30 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999), Efimov and Shcherbak (1993), Tarasenko et al. (1998), Fedyukovich (1998), Blinov (2000)
4.47	*Alchemilla xanthochlora* Rothm. aerial part; *Juniperus communis* L. fruits; *Linum usitatissimum* L. seeds; *Vaccinium myrtillus* L. leaves; (1:2:2:4)	Decoction; 1table spoon in 200 ml of water	200 ml 2–3 times a day	Diabetes	Yordanov et al. (1972), Matkovskaya et al. (1988); Chirkov and Seryi (1993); Efimov and Shcherbak (1993); Dremova et al. (2003); Davydovich et al. (2008); Bogdanova and Bashkirova (2010); Volynchenko(2003); Maznev (2014); Pigulevskaya (2018)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
4.48	*Galega officinalis* L. aerial part; *Mentha* × *piperita* L. leaves; *Phaseolus vulgaris* L. pericarp; *Zea mays* L. corn silk; (1:1:7:2)	Infusion; 3 table spoons in 400 ml of boiling water	100 ml 3 times a day	Diabetes	Chirkov and Seryi (1993); Efimov and Shcherbak (1993); Bogdanova and Bashkirova (2010)
4.49	*Betula* spp.[c]leaves; *Frangula alnus* Mill. bark; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (1:1:2:2)	Decoction; *Frangula* bark cut, boil 20 min in boiling water + Infusion; in 300 ml boiling water and boil 3 min	100 ml 2–3 times a day before eating	Diabetes	Ryzhenko (2007)
4.50	*Taraxacum campylodes* G.E.Haglund. leaves; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (1:1:1:1)	Infusion; 1 table spoon in 300 ml of boiling water	100 ml 2–3 times a day 20 min before eating	Diabetes	Ryzhenko, (2007)
4.51	*Avena sativa* L. aerial part & straw; *Betula pendula* Roth. leaves; *Linum usitatissimum* L. seeds; *Vaccinium myrtillus* L. leaves; (1:1:1:1)	Infusion; 3 table spoons in 600 ml of boiling water, boil 10 min, maceration 30–40 min	50 ml 6–8 times a day 20–30 min before eating	Diabetes	Podduev (2001)
4.52	*Avena sativa* L. aerial part & straw; *Equisetum arvense* L. aerial part; *Linum usitatissimum* L. seeds; *Oplopanax elatus* (Nakai) Nakai roots and rhizomes; (2:2:2:1)	Infusion; 1 table spoon in 200 ml of boiling water, boil 15 min, maceration 45 min	100 ml a day	Diabetes	Podduev (2001)
4.53	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Avena sativa* L. aerial part & straw; *Linum usitatissimum* L. seeds; *Phaseolus vulgaris* L. pericarp; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water, boil 10 min, maceration 2 h	200 ml a day	Diabetes	Podduev (2001)
4.54	*Juglans regia* L. leaves; *Mentha* × *piperita* L. leaves; *Polygonatum odoratum* (Mill.) Druce leaves; *Polygonum aviculare* L. aerial part; (3:2:2:3)	Infusion; 2 table spoons in 500 ml of boiling water, boil 2–3 min, maceration 30–40 min	100 ml 3–4 times a day 30 min before eating	Diabetes	Podduev (2001)
4.55	*Aralia elata* (Miq.) Seem roots; *Galega officinalis* L. aerial part; *Rosa majalis* Herrm. fruits; *Vaccinium myrtillus* L. fruits; (2:3:2:3)	Infusion or decoction; 10 g in 400 ml of water	66–100 ml 3 times a day	Lowering of blood glucose level	Sokolov (2000)
4.56	*Centaurium erythraea* Rafn aerial part; *Solanum tuberosum* L. juice; *Vaccinium myrtillus* L. leaves; *Viburnum opulus* L. berries juice; (1:3:4:2)	Infusion; 50 g in 1,000 ml of boiling water, maceration 10–12 h at room temp.	50–66 ml (warm) 3–4 times a day before eating	Diabetes accompanied by gastritis	Brusenskaya and Kaz'min, (2005)
4.57	*Cichorium intybus* L. roots; *Rosa majalis* Herrm. fruits; *Schisandra chinensis* (Turcz.) Baill. leaves; *Taraxacum campylodes* G.E.Haglund. roots; (3:4:1:3)	Infusion; 1 tea spoon in boiling water	2 table spoons 4 times a day before eating and 30 min before sleeping for 30 days	Diabetes accompanied by impotence in men	Brusenskaya and Kaz'min, (2005)
4.58	*Betula pendula* Roth. leaves; *Ribes nigrum* L. leaves; *Rubus caesius* L. leaves; *Trifolium pratense* L. leaves; (1:1:1:1)	Infusion; 1 table spoon in 300 ml of boiling water	100 ml with 1/2 tea spoon of honey 3 times a day before eating	Diabetes accompanied by impotence in men	Brusenskaya and Kaz'min, (2005)
4.59	*Alchemilla xanthochlora* Rothm. aerial part; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Vaccinium myrtillus* L. leaves; (1:1:1:1)	Infusion; 1 table spoon in 200 ml of water	1 table spoon 3 times a day	Diabetes accompanied by impotence in men	Brusenskaya and Kaz'min, (2005)
4.60	*Achillea millefolium* L. aerial part; *Arctium* spp.[b] roots; *Helichrysum arenarium* (L.) Moench flowers; *Hypericum* spp.[e] aerial part; (1:35:1:8)	Infusion; 2 table spoons in 300–400 ml of water	100 ml morning and evening	Diabetes accompanied by liver and gallbladder diseases	Brusenskaya and Kaz'min, (2005)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
4.61	*Crataegus* spp.[f] lowers; *Crataegus* spp.[f]fruits; *Leonurus* spp. [d]leaves; *Vaccinium myrtillus* L. leaves; (1:1:1:4)	Infusion; boiling of *Crataegus* fruits 20 min, than maceration of 30 g of mixture in boiling water 8–12 h at room tempature	100 ml 3 times a day before eating	Diabetes accompanied by angina and shortness of breath	Brusenskaya and Kaz'min, (2005)
4.62	*Asperula graveolens* M.Bieb. ex Schult. & Schult.f. aerial part; *Fragaria vesca* L. leaves; *Melissa officinalis* L. leaves; *Thymus serpyllum* L. aerial part; (1:2:1:1)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	100 ml 4–5 times a day	Metabolism improving	Chirkov and Seryi (1993)
4.63	*Betula pendula* Roth. leaves; *Prunus spinosa* L. flowers; *Sambucus nigra* L. flowers; *Urtica dioica* L. leaves; (1:1:1:1)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	200–400 ml a day, before first eating	Metabolism improving and diuretics	Chirkov and Seryi (1993)
4.64	*Frangula alnus* Mill. bark; *Glycyrrhiza glabra* L. roots; *Viola tricolor* L. aerial part; *Juglans regia* L. leaves; (1:1:4:4)	Decoction; 1 table spoon in 600 ml of water	400 ml a day	Exudative diathesis caused by metabolic disorder	Chirkov and Seryi (1993)
4.65	*Betula pendula* Roth. leaves; *Melissa officinalis* L. leaves; *Salvia officinalis* L. leaves; *Urtica dioica* L. leaves; (1:1:1:1)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	200 ml in the morning and 200 ml in the evening	Acne, in case of metabolic disorder	Chirkov and Seryi (1993)
4.66	*Betula pendula* Roth. leaves; *Frangula alnus* Mill. bark; *Linum usitatissimum* L. seeds; *Urtica dioica* L. leaves; (1:1:1:1)	Decoction; 1 table spoon in 200 ml of water	66 ml 3 times a day	Skin rash, metabolic disorder	Chirkov and Seryi (1993)
5 plants					
5.1	*Foeniculum vulgare* Mill. fruits; *Frangula alnus* Mill. bark; *Mentha* × *piperita* L. leaves; *Petroselinum crispum* (Mill.) Fuss fruits; *Taraxacum campylodes* G.E.Haglund. roots; (1:3:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water	500 ml in the morning before eating	Obesity	Yordanov et al. (1972); Lager, (1991); Sinyakov (1992); Chirkov and Seryi (1993); Efimov and Shcherbak (1993); Kukes (1999); Dontsov and Dontsov (2000); Sokolov (2000); Bubenchikova et al.(2003); Podduev (2001); Maznev (2005); Safonov (2016)
5.2	*Achillea millefolium* L. aerial part; *Frangula alnus* Mill. bark; *Juniperus communis* L. fruits; *Levisticum officinale* W.D.J.Koch roots; *Phaeophyceae* (*Cystoseira barbata* (Stackh.) C.Agardh) thallus; (10:3:1:1:3)	Decoction; 2 table spoons in 500 ml of water	100 ml 3–4 times a day	Obesity	Yordanov et al. (1972); Efimov and Shcherbak (1993); Chirkov and Seryi (1993)
5.3	*Frangula alnus* Mill. bark; *Levisticum officinale* W.D.J.Koch roots; *Ononis spinosa* L. roots; *Phaeophyceae* (*Cystoseira barbata* (Stackh.) C.Agardh) thallus; *Taraxacum campylodes* G.E.Haglund. roots; (5:1:1:2:1)	Decoction; 2 table spoons in 500 ml of water	132–200 ml 2–3 times a day	Obesity	Chirkov and Seryi (1993)
5.4	*Matricaria chamomilla* L. flowers; *Foeniculum vulgare* Mill. fruits; *Mentha* × *piperita* L. leaves; *Sambucus nigra* L. flowers; *Tilia cordata* Mill. flowers; (1:1:1:1:1)	Infusion; 10 g in 200 ml of boiling water	200 ml 2–3 times a day	Obesity	Sokolov and Zamotaiev (1984); Lager (1991); Sinyakov (1992); Efimov and Shcherbak (1993); Lager (2002); Tarasenko et al. (1998); Kukes (1999); Sokolov (2000); Dontsov and Dontsov (2000); Said-Shah (2001); Podduev (2001); Maznev (2005); Kiyanova (2005); Rendiuk (2006)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
5.5	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Frangula alnus* Mill. bark; *Linum usitatissimum* L. seeds; *Panax ginseng* C.A.Mey roots; *Taraxacum campylodes* G.E.Haglund. roots; (3:4:5:4:4)	Infusion; 10 g in 400 ml of water.	66–100 ml 3 times a day	Obesity	Sokolov (2000)
5.6	*Foeniculum vulgare* Mill. fruits; *Hypericum perforatum* L. aerial part; *Mentha* × *piperita* L. leaves; *Sambucus nigra* L. flowers *Tilia cordata* Mill. flowers; (4:4:3:3:4)	Infusion	2 table spoons 3 times a day 30 min before eating for 1–1.5 months	Obesity	Kukes (1999)
5.7	*Betula* spp. [c] leaves; *Elymus repens*(L.) Couldrhizomes; *Frangula alnus* Mill. bark; *Melissa officinalis* L. aerial part; *Taraxacum campylodes* G.E.Haglund. roots; (2:2:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, maceration 12 h in thermos	100 ml 3 times a day	Obesity	Osetrov (1993); Osetrov and Shreter (2001)
5.8	*Achillea millefolium* L. aerial part; *Carum carvi* L. fruits; *Frangula alnus* Mill. bark; *Viola tricolor* L. aerial part; *Zea mays* L. corn silk; (1:1:6:1:1)	Infusion; 4 table spoons in 800 ml of boiling water	400 ml 2 times a day	Obesity	Lager (1991), Lager (2002); Efimov and Shcherbak (1993); Kukes (1999); Dontsov and Dontsov (2000); Bubenchikova et al. (2003)
5.9	*Humulus lupulus* L. fruits; *Panax ginseng* C.A.Mey roots; *Phaseolus vulgaris* L. pericarp; *Rosa majalis* Herrm. fruits; *Sorbus aucuparia* L. fruits; (3:3:5:4:5)	Infusion; 10 g in 400 ml of water	66–100 ml 3 times a day	Lowering of blood glucose level	Sokolov (2000)
5.10	*Galega officinalis* L. aerial part; *Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; (1:1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	200 ml 3–4 times a day before eating	Diabetes (early stages)	Yordanov et al. (1972); Sinyakov (1992); Chirkov and Seryi (1993); Efimov and Shcherbak (1993); Nikultseva (1994); Dmitriev et al. (1994); Nikolaychuk (1997); Fedyukovich (1998); Nikolaychuk and Zubitskaya (2003); Ryzhenko (2007); Lavrenova and Lavrenov (2007); Davydovich et al. (2008); Bogdanova and Bashkirova (2010); Ruzhenkova (2014); Maznev (2014)
5.11	*Juglans regia* L. leaves; *Phaseolus vulgaris* L. pericarp; *Portulaca oleracea* L. leaves; *Ribes nigrum* L. leaves; *Vaccinium myrtillus* L. leaves; (2:3:2:3:2)	Herbal tea; 3 table spoons in 1,000 ml of boiling water	100 ml 7 times a day	Diabetes	Protasenya and Vasilenko (1992)
5.12	*Equisetum arvense* L. aerial part; *Hypericum* spp. [e]flowers; *Phaseolus vulgaris* L. pericarp; *Polygonum aviculare* L. aerial part; *Vaccinium myrtillus* L. leaves; (1:4:4:1:4)	Infusion; 70 g in 1,000 ml of water, boil 2 min, maceration 8–12 h in a dark place	66 ml 3–4 times a day before eating	Diabetes	Brusenskaya and Kaz'min (2005)
5.13	*Galega officinalis* L. aerial part; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Taraxacum campylodes* G.E.Haglund. leaves; *Vaccinium myrtillus* L. leaves; (4:4:3:2:5)	Infusion; 1 table spoon in 200 ml of boiling water	66–100 ml 3–4 times a day before eating	Diabetes	Brusenskaya and Kaz'min (2005)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
5.14	*Hypericum* spp. [e]flowers; *Inula helenium* L. roots; *Sambucus nigra* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; *Urtica dioica* L. leaves; (2:1:2:2:1)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	100 ml 2 times a day before eating	Diabetes	Brusenskaya and Kaz'min (2005)
5.15	*Arctium lappa* L. roots; *Galega officinalis* L. aerial part; *Oenanthe aquatica* (L.) Poir. fruits; *Polygonum aviculare* L. aerial part; *Symphytum officinale* L. root; (4:7:3:3:3)	Infusion; 2 table spoons in 500 ml of boil water	200 ml 2–3 times a day before eating	Diabetes	Yordanov et al. (1972); Efimov and Shcherbak (1993); Sinyakov (1999); Davydovich et al. (2008); Bogdanova and Bashkirova (2010)
5.16	*Galega officinalis* L. aerial part; *Taraxacum campylodes* G.E.Haglund. leaves; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (1:1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, boil 5–7 min, maceration 1–2 h at room tempature	100 ml 2–3 times a day 20 min before eating	Diabetes	Sinyakov (1992); Efimov and Shcherbak (1993); Nikultseva (1994); Nikolaychuk (1997); Tarasenko et al. (1998); Fedyukovich (1998); Blinov (2000); Podduev (2001); Nikolaychuk and Zubitskaya (2003); Nazina (2006); Korodetsky (2006); Davydovich et al. (2008); Bogdanova and Bashkirova (2010); Maznev (2014)
5.17	*Galega officinalis* L. aerial part; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	66 ml 3–4 times a day before eating	Diabetes	Matkovskaya et al. (1988); Blinov (2000); Nazina (2006); Pigulevskaya (2018)
5.18	*Betula* spp. [c]leaves; *Frangula alnus* Mill. bark; *Galega officinalis* L. aerial part; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (1:1:2:2:2)	Infusion; 7 g in 300 ml of boiling water + Decoction; *Frangula* bark cut in 300 ml of boil warter, boil 20 min	66 ml before each eating	Diabetes	Sinyakov (1992); Efimov and Shcherbak (1993); Nikultseva (1994); Nikolaychuk (1997); Tarasenko et al. (1998); Fedyukovich (1998); Blinov (2000); Podduev (2001); Nikolaychuk and Zubitskaya (2003); Nazina (2006); Davydovich et al. (2008); Bogdanova and Bashkirova (2010); Maznev (2014)
5.19	*Cichorium intybus* L. leaves; *Galega officinalis* L. aerial part; *Juglans regia* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; *Urtica dioica* L. leaves; (1:1:1:1:1:1)	Infusion; 1 table spoon in 400 ml of boiling water	2–3 table spoons 3 times a day 15–20 min before eating	Diabetes	Sinyakov (1992); Efimov and Shcherbak (1993); Nikultseva (1994); Nikolaychuk (1997); Fedyukovich (1998); Blinov (2000); Nikolaychuk and Zubitskaya (2003); Dremova et al. (2003); Nazina (2006); Ryzhenko (2007); Bogdanova and Bashkirova (2010)
5.20	*Elymus repens* (L.) Gould rhizomes; *Sambucus nigra* L. flowers; *Tilia cordata* Mill. flowers; *Tussilago farfara* L. leaves; *Verbascum densiflorum* Bertol. flowers; (1:1:1:1:1:1)	Decoction; 5 table spoons in 600 ml of water	66 ml 5–6 times a day	Diabetes	Chirkov and Seryi (1993)
5.21	*Althaea officinalis* L. roots; Centaurium erythraea Rafn aerial part;Mentha × piperita L. aerial part; *Prunusavium*(L.) L. shoots; *Zea mays* L. corn silk; (1:1:5:1:1)	90 g in 2000 ml of boiling water; evaporate to residue of 1,000 ml	150 ml in the morning before eating, than 1 table spoon every 2 h during the day	Diabetes	Osetrov (1993); Osetrov and Shreter (2001)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
5.22	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Mentha* × *piperita* L. leaves; *Ribes nigrum* L. leaves; *Rubus caesius* L. leaves; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water, maceration 30 min	100 ml 3 times a day	Diabetes	Efimov and Shcherbak (1993); Nikolaychuk (1997); Fedyukovich (1998); Smolianskii and Lifliandskii (2004); Bogdanova and Bashkirova (2010); Melik-Gusseinov and Rekkandt (2014)
5.23	*Arctium* spp.[b]roots; *Juglans regia* L. leaves; *Phaseolus vulgaris* L. pericarp; *Sambucus nigra* L. flowers or roots; *Vaccinium myrtillus* L. leaves; (1:1:1: 1:1)	Infusion; 100 g in 400 ml of boiling water, maceration 5 h	100 ml 3 times a day after eating	Diabetes	Smolianskii and Lifliandskii (2004); Pigulevskaya (2018)
5.24	*Equisetum arvense* L. aerial part; *Hypericum perforatum* L. aerial part; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; (1:1:1: 1:1)	Infusion; 1 table spoon in 200 ml of boiling water, maceration 30 min	66 ml 3 times a day before eating	Diabetes	Efimov and Shcherbak (1993); Nikolaychuk (1997); Fedyukovich (1998); Nikolaychuk and Zubitskaya (2003); Smolianskii and Lifliandskii (2004); Bogdanova and Bashkirova (2010)
5.25	*Cichorium intybus* L. roots; *Crataegus* spp.[f]fruits; *Elymus repens (L.)* Gould rhizomes; *Rosa* spp.[9]fruits; *Vaccinium myrtillus* L. fruits; (3:2:3:2:2)	Infusion; 1 table spoon in 200 ml of boiling water, boil 10 min, maceration at room tempature	100 ml 4 times a day 30 min before eating.	Diabetes	Efimov and Shcherbak (1993); Rendiuk (2006); Bogdanova and Bashkirova (2010)
5.26	*Sambucus nigra* L. flowers; *Taraxacum campylodes* G.E.Haglund. roots *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. fruits; *Vaccinium myrtillus* L. leaves; (3: 3:4:4:4)	Infusion	1–2 table spoon 3 times a day 30 min before eating for 1–1.5 months.	Diabetes	Kukes (1999)
5.27	*Arctium lappa* L. roots; *Phaseolus vulgaris* L. pericarp; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (1:1:1: 1:1)	Infusion; 20 g in 200 ml of boiling water	66 ml 3 times a day	Diabetes	Lager (1991), Lager (2002)
5.28	*Cichorium intybus* L. roots; *Hypericum perforatum* L. aerial part; *Mentha* × *piperita* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Vaccinium myrtillus* L. leaves; (1:1:1: 1:1)	Infusion; 1 table spoon in 300 ml of boiling water	66 ml 3 times a day	Diabetes	Lager (1991), Lager (2002); Efimov and Shcherbak (1993); Nikolaychuk (1997); Fedyukovich (1998); Nikolaychuk and Zubitskaya (2003); Bogdanova and Bashkirova (2010); Maznev (2014); Pigulevskaya (2018)
5.29	*Cichorium intybus* L. leaves; *Galega officinalis* L. aerial part; *Juglans regia* L. leaves; *Taraxacum campylodes* G.E.Haglund. leaves; *Urtica dioica* L. leaves; (1:1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water, boil 2–3 min, maceration 30–40 min	50 ml 3–4 times a day 15–20 min before eating	Diabetes	Sinyakov (1999)
5.30	*Galega officinalis* L. aerial part; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; (1:1:1: 1:1)	Infusion; 2 table spoons in 500 ml of boiling water, maceration 12 h in thermos	100 ml (warm) 3 times a day 30 min before eating	Diabetes	Sinyakov (1999)
5.31	*Betula pendula* Roth. leaves; *Frangula alnus* Mill. bark; *Galega officinalis* L. aerial part; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (3:2:5:5:5)	Decoction; *Frangula* bark boil for 20 min. Infusion; other part in 500 ml of boiling water, boil for 3–4 min, maceration 30 min. Mix with frangula decoction.	66–100 ml 2–3 times a day 20–30 min before eating	Diabetes	Sinyakov (1999)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
5.32	*Arctium lappa* L. roots; *Cichorium intybus* L. roots; *Linum usitatissimum* L. seeds; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; (2:2:2:7:7)	Infusion; 2–3 table spoons in 500 ml water, maceration 12 h, 15 min in boil water bath, maceration 1 h	200 ml 3–4 times a day 30 min before eating	Diabetes	Sinyakov (1999)
5.33	*Alchemilla xanthochlora* Rothm. roots and aerial part*; Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves (1:1:1:1:1)	Infusion; 1 table spoon in 200 ml of water	1 table spoon 3 times a day	Diabetes	Podduev (2001)
5.34	*Angelica archangelica* L. aerial part; *Betula sp*p. Leaves; *Frangula alnus* Mill. bark; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; (2:1:1:2:2)	Decoction; *Frangula* bark cut boil 20 min + Infusion; other part in 300 ml of boiling water, 3 min boil. Mix with frangula decoction.	70 ml 2–3 times a day before eating	Diabetes	Podduev (2001)
5.35	*Avena sativa* L. aerial part; *Fagopyrum esculentum* Moench flowers; *Linum usitatissimum* L. seeds; *Prunus laurocerasus* L. leaves; *Sambucus ebulus* L. flowers; (3:2:2:3:2)	Herbal tea; 3 table spoons in 800 ml of boiling water	50 ml 6 times a day	Diabetes. In case of severe condition of the disease course	Protasenya and Vasilenko (1992)
5.36	*Acorus calamus* L. root; *Arctium* spp.[b]leaves; *Matricaria chamomilla* L. flowers; *Frangula alnus* Mill. bark; *Vaccinium myrtillus* L. leaves; (1:3:2:1:4)	Infusion; 55 g in 1,000 ml of boiling water, maceration 10–12 h at room tempature	3 table spoons 20–30 min before eating	Diabetes accompanied by colitis and constipation	Brusenskaya and Kaz'min (2005)
5.37	*Arctium lappa* L. roots; *Cichorium intybus* L. roots; *Rubus caesius* L. root; *Valeriana officinalis* L. roots and rhizomes; *Vincetoxicum hirundinaria* Medik. roots, rhizomes, leaves and seeds; (2:3:1:3:3)	Herbal tea; 3 table spoons in 1,000 ml of boiling water	100 ml 7 times a day	Diabetes accompanied by metabolic polyarthritis, rheumatoid arthritis	Protasenya and Vasilenko (1992)
5.38	*Arctium lappa* L. roots; *Equisetum arvense* L. aerial part; *Gratiola officinalis* L. aerial part; *Orthosiphon aristatus* (Blume) Miq. leaves; *Phaseolus vulgaris* L. pericarp; (2:3:2:1:4)	Herbal tea; 3 table spoons in 1,000 ml of boiling water	70 ml 6 times a day	Diabetes accompanied by edema related to renal failure	Protasenya and Vasilenko (1992)
5.39	*Asparagus officinalis* L. rhizomes and aerial part; *Centaurium erythraea* Rafn aerial part; *Fraxinus excelsior* L. leaves *Oplopanax elatus* (Nakai) Nakai roots and rhizomes; *Plantago major* L. leaves; (2:2:3:2:3)	Herbal tea; 3 table spoons in 800 ml of boiling water	50 ml 6 times a day	Diabetes accompanied by chronic gastritis with reduced secretory function	Protasenya and Vasilenko (1992)
5.40	*Equisetum arvense* L. aerial part; *Gnaphalium uliginosum* L. aerial part; *Rosa majalis* Herrm. fruits; *Sambucus nigra* L. flowers; *Syringa vulgaris* L. buds; (3:3:2:2:2)	Herbal tea; 3 table spoons in 1,200 ml of boiling water	100 ml 6 times a day	Diabetes accompanied by hypertension and slight edema of the lower extremities	Protasenya and Vasilenko (1992)
5.41	*Centaurium erythraea* Rafn aerial part; *Cichorium intybus* L. roots; *Hypericum* spp. [e]flowers; *Juglans regia* L. leaves; *Plantago major* L. leaves; (1:2:4:1:3)	Decoction; *Cichorium* roots in 100 ml of water + Infusion; 45 g other part in 1,000 ml of boil water, maceration 3–5 h. Mix with cichorium decoction	50 ml 3 times a day before eating	Diabetes accompanied by colitis and constipation	Efimov and Shcherbak (1993); Brusenskaya and Kaz'min (2005); Davydovich et al. (2008); Bogdanova and Bashkirova (2010)
5.42	*Alnus spp. (A. incana* (L.) Moench and *A. glutinosa* (L.) Gaertn.*)* fruits; *Centaurium erythraea* Rafn aerial part; *Mentha* × *piperita* L. leaves; *Quercus* spp.[h]bark; *Vaccinium myrtillus* L. leaves; (2:1:1:4:4)	Infusion; 60 g in 1,000 ml of water, maceration 3–4 h at room tempature	50 ml 3–4 times a day before eating, for 7–10 days	Diabetes with frequent diarrhea	Brusenskaya and Kaz'min (2005)
5.43	*Betula* spp. [c]sap; *Daucus sativus*Roehl. juice; *Leonurus* spp [d]leaves; *Phaseolus vulgaris* L. pericarp; *Viburnum opulus* L. berries juice; (20:2:1:4:2)	Infusion; 40 g in 1,000 ml of boil water, maceration 3–5 h in a dark place	100 ml 4–6 times a day before eating	Diabetes accompanied by angina and shortness of breath	Brusenskaya and Kaz'min (2005)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
5.44	*Polygonum aviculare* L. aerial part *Zea mays* L. corn silk; *Hypericum* spp.[e] flowers; *Viburnum opulus* L. berries; *Arctostaphylos uva-ursi* (L.) Spreng. leaves; (1:2:2:1:2)	Infusion; 40 g in 1,000 ml of boil water, maceration 3–5 h in a dark place	100 ml 3–4 times a day after eating	Diabetes accompanied by kidney and bladder disease	Brusenskaya and Kaz'min (2005)
5.45	*Anethum graveolens* L. fruits; *Mentha* × *piperita* L. leaves; *Crataegus sanguinea* Pall. flowers; *Helichrysum arenarium* (L.) Moench. flowers; *Matricaria chamomilla L.* flowers; (3:3:2:2:2)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	100 ml 3 times a day 1 h after eating	Diabetes accompanied by chronic pancreatitis	Chirkov and Seryi (1993); Kukes (1999); Bubenchikova et al. (2003)
5.46	*Taraxacum campylodes G.E.Haglund.* roots; *Arctium lappa* L. roots; *Rubia tinctorum* L. roots; *Saponaria officinalis* L. roots or *Bidens tripartita* L. aerial part; *Glycyrrhiza glabra* L. roots; (3:3:6:6:2)	Decoction; 1 table spoon in 200 ml of water	200–400 ml a day before first eating	Exudative diathesis caused by metabolic disorder	Chirkov and Seryi, (1993)
5.47	*Humulus lupulus* L. fruits; *Menyanthes trifoliata* L. leaves; *Gentiana lutea* L. root; *Melissa officinalis* L. aerial part; *Achillea millefolium* L. aerial part; (2:3:2:4:1)	Herbal tea; 3 table spoons in 1,000 ml of boiling water	100 ml 6 times a day	Metabolic disorder with multiple skin furuncles	Protasenya and Vasilenko (1992)
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6.1	*Achillea millefolium* L. aerial part; *Carum carvi* L. fruits; *Frangula alnus* Mill. bark; *Prunus spinosa* L. flowers; *Viola tricolor* L. aerial part; *Zea mays* L. corn silk; (1:1:6:1:1:1)	Decoction; 2 table spoons in 400 ml of water	400 ml 2 times a day	Obesity	Chirkov and Seryi (1993)
6.2	*Arctium* spp.[b]roots; *Urtica dioica* L. leaves; *Avena sativa* L. aerial part; *Vaccinium myrtillus* L. leaves; *Fragaria vesca* L. leaves; *Rosa spp.* [9]fruits (3:4:4:4:3:4)	Herbal tea; 1 table spoon in 200 ml of boiling water	100 ml 2 times a day before eating	Diabetes prevention	Brusenskaya and Kaz'min (2005)
6.3	*Vaccinium myrtillus* L. leaves; *Rosa* spp. [9]fruits; *Phaseolus vulgaris* L. pericarp; *Fragaria vesca* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Achillea millefolium* L. aerial part; (4:5:4:3:2:1)	Herbal tea; 1 table spoon in 200 ml of boiling water	100 ml 2 times a day before eating	Diabetes prevention	Brusenskaya and Kaz'min (2005); Maznev (2014)
6.4	*Avena sativa* L. aerial part; *Cichorium intybus* L. roots; *Galega officinalis* L. aerial part; *Linum (usitatissimum* L.*)* seeds; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1)	Infusion; 10 g in 300 ml of boiling water, maceration 2 h	100 ml 3 times a day before eating	Insulin-dependent diabetes	Kolesova et al. (1998)
6.5	*Urtica dioica* L. leaves; *Arctium lappa* L. roots; *Linum usitatissimum* L. seeds; *Juniperus communis* L. fruits; *Taraxacum campylodes* G.E.Haglund. roots; *Vaccinium myrtillus* L. leaves; (2:2:1:1:1:3)	Infusion; 3 table spoon in 600 ml of boiling water	200 ml 2–3 times a day before eating	Diabetes	Efimov and Shcherbak (1993); Fedyukovich (1998); Dontsov and Dontsov (2000); Bogdanova and Bashkirova (2010)
6.6	*Linum usitatissimum* L. seeds; *Vaccinium vitis-idaea* L. leaves; *Inula helenium* L. roots; *Gnaphalium uliginosum* L. aerial part; *Zea mays* L. corn silk; *Matricaria chamomilla L.* flowers; (4:4:3:3:3:3)	Infusion; 3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	130–140 ml (warm) 3 times a day 20–30 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999); Tarasenko et al. (1998); Podduev (2001)
6.7	*Mentha* × *piperita* L. aerial part; *Rosa* spp. [9]fruits; *Sorbus aucuparia* L. fruits; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. shoots; (1:1:1:1:1:1)	Infusion; 6 g in 350 ml of boiling water, 10 min in boil water bath, maceration 3 h in thermos	100 ml 3 times a day before eating.	Diabetes	Vinogradova et al. (2001)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
6.8	*Arctium* spp.[b]roots; *Equisetum arvense* L. aerial part; *Fragaria vesca* L. leaves; *Mentha* × *piperita* L. aerial part; *Vaccinium myrtillus* L. shoots; *Vaccinium vitis-idaea* L. leaves; (1:1:1:1:1:1)	Infusion; 8 g in 300 ml of boiling water, boil 2 min, maceration 2 h in thermos	50–70 ml (warm) 10 min before eating	Diabetes	Vinogradova et al. (2001)
6.9	*Betula* spp. [c]leaves; *Foeniculum vulgare* Mill. fruits; *Mentha* × *piperita* L. aerial part; *Petroselinum crispum* (Mill.) Fuss aerial part; *Ribes nigrum* L. leaves; *Rosa* spp. [g]fruits; (1:1:1:1:1:1:1)	Infusion; 8 g in 300 ml of boiling water, 15 min in boil water bath, maceration 1 h in thermos	66–100 ml 3–4 times a day before eating	Diabetes	Vinogradova et al. (2001)
6.10	*Galega officinalis* L. aerial part; *Laurus nobilis* L. leaves; *Mentha* × *piperita* L. aerial part; *Phaseolus vulgaris* L. pericarp; *Sorbus aucuparia* L. fruits; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1:1)	Infusion; 8 g in 300 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	66–100 ml 3–4 times a day before eating	Diabetes	Vinogradova et al. (2001)
6.11	*Glycyrrhiza glabra* L. roots; *Hypericum* spp. [e]aerial part; *Juglans regia* L. leaves; *Phaseolus vulgaris* L. pericarp; *Syringa vulgaris* L. buds; *Vaccinium vitis-idaea* L. leaves; (1:1:1:1:1:1:1)	Infusion; 8 g in 400 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	100 ml (warm) 3 times a day10 min before eating	Diabetes	Vinogradova et al. (2001)
6.12	*Cichorium intybus* L. roots*; Elymus repens*(L.) Couldroots; *Fragaria vesca* L. leaves; *Rosa* spp. [g]leaves; *Urtica dioica* L. leaves*; Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1:1)	Infusion; 1 table spoon in 200 ml of boiling water	50 ml several times a day	Diabetes	Volynchenko (2003)
6.13	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Fragaria vesca* L. leaves; *Vaccinium vitis-idaea* L. leaves; *Rosa* spp.[g]fruits; *Vaccinium myrtillus* L. leaves; *Urtica dioica* L. leaves; (2:2:2:3:4:1)	Infusion; 70 g in 1,000 ml of water, maceration 3–5 h in a dark place	3–4 times a day after eating	Diabetes	Brusenskaya and Kaz'min (2005)
6.14	*Ribes nigrum* L. leaves; *Hypericum* spp. [e] flowers; *Sambucus nigra* L. flowers; *Urtica dioica* L. leaves; *Juglans regia* L. leaves; *Fragaria vesca* L. leaves; (4:5:4:3:4:3)	Infusion (herbal tea) 1 table spoon in 200 ml (1 glass) of boiling water	100 ml, 2 times a day; before eating	Diabetes	Brusenskaya and Kaz'min (2005)
6.15	*Vaccinium myrtillus* L. leaves; *Urtica dioica* L. leaves; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Salvia officinalis* L. leaves; *Galega officinalis* L. aerial part; (5:5:3:1:1:5)	Infusion; 1 table spoon in 500 ml of boiling water, maceration 12 h in thermos	100 ml (warm) 2–3 times a day 30 min before eating	Diabetes	Sinyakov (1992), Sinyakov (1999); Chirkov and Seryi (1993)
6.16	*Hypericum* spp. [e] aerial part; *Achillea millefolium* L. aerial part; *Plantago major* L. leaves; *Arctium* spp.[b] roots; *Centaurium erythraea* Rafn aerial part; *Matricaria chamomilla* L. flowers; (6:2:2:2:1:3)	Infusion; 80 g in 1,000 ml of boiling water, maceration 5–7 h at room temp.	66 ml 15–20 min before eating	Diabetes accompanied by colitis and constipation	Brusenskaya and Kaz'min, (2005)
6.17	*Phaseolus vulgaris* L. pericarp; *Morus* spp.[l]leaves; *Juglans regia* L. leaves; *Acorus calamus* L. root; *Frangula alnus* Mill. bark; *Ribes nigrum* L. leaves (20:5:5:5:3:15)	Infusion; 53 g in 1,000 ml of boiling water, maceration 10–12 h at room temp.	3 table spoons 20–30 min before eating	Diabetes accompanied by colitis and constipation	Brusenskaya and Kaz'min, (2005)
6.18	*Helichrysum (arenarium* (L.) Moench. flowers; *Hypericum* spp. [e]aerial part; *Polygonum aviculare* L. aerial part; *Rosa spp.* [g]fruits; *Vaccinium myrtillus* L. leaves; *Zea mays* L. corn silk; (2:2:3:2:2:2)	Infusion; 65 g in 1,000 ml of boiling water, maceration 10–12 h at room tempature	50–70 ml (warm) 3–4 times a day before eating	Diabetes accompanied by liver and gallbladder diseases	Brusenskaya and Kaz'min (2005); Bogdanova and Bashkirova (2010)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
6.19	*Vaccinium myrtillus* L. leaves; *Helichrysum (arenarium* (L.) Moench flowers; *Zea mays* L. corn silk; *Polygonum aviculare* L. aerial part; *Hypericum* spp. [e]aerial part; *Phaseolus vulgaris* L. pericarp; (4:1:2:1:2:2)	Infusion; 60 g in 1,000 ml of boiling water, maceration 10–12 h at room tempature	50–70 ml (warm) 3–4 times a day before eating	Diabetes accompanied by liver and gallbladder diseases	Brusenskaya and Kaz'min, (2005)
6.20	*Achillea millefolium* L. aerial part; *Matricaria chamomilla* L. flowers; *Hypericum* spp. [e]aerial part; *Mentha* × *piperita* L. leaves; *Quercus* spp.[h]bark; *Tanacetum vulgare* L. flowers; (30:8: 20:5:15:8)	Infusion; 86 g in 1,000 ml of boiling water, maceration 3–4 h at room tempature	50 ml 3–4 times a day before eating for 7–10 days	Diabetes with frequent diarrhea	Brusenskaya and Kaz'min, (2005)
6.21	*Betula pendula* Roth. leaves; *Foeniculum vulgare* Mill. fruits; *Frangula alnus* Mill. bark; *Glycyrrhiza glabra* L. roots; *Sambucus nigra* L. flowers; *Viola tricolor* L. aerial part; (1:1:1:1:1:1:1)	Decoction; 1 table spoon in 200 ml of boiling water	66 ml 3 times a day	Skin rash, metabolic disorder	Chirkov and Seryi, (1993)
6.22	*Arctium lappa* L. roots; *Elymus repens (L.)* Gould rhizomes; *Foeniculum vulgare* Mill. fruits; *Frangula alnus* Mill. bark; *Glycyrrhiza glabra* L. roots; *Taraxacum campylodes* G.E.Haglund. roots; (1:1:1:1:1:1:1)	Decoction; 1 table spoon in 200 ml of boiling water	200 ml (warm) in the morning before first eating	Metabolism improving	Chirkov and Seryi, (1993)
6.23	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Frangula alnus* Mill. bark; *Herniaria (glabra)* L. aerial part; *Ononis spinosa* L. roots; *Saponaria officinalis* L. roots; *Solanum dulcamara* L. aerial part; (1:1:1:1:1:1:1)	Decoction; 1 table spoon in 200 ml of boiling water	200–400 ml in the morning before first eating	Metabolism improving and diuretics	Chirkov and Seryi, (1993)
6.24	"Normavit" *Saccharina latissima* (L.) C.E.Lane, C.Mayes, Druehl & G.W.Saunders thallus; *Rosa* spp. [g]fruits; *Vaccinium vitis-idaea* L. leaves; *Leonurus* spp [d]aerial part; *Bidens (tripartita* L.*)* aerial part; *Frangula alnus* Mill. bark; (4:1:1:1:1:1:1)	Decoction; 10 g in 130 ml of boiling water	50–100 ml 3 times a day before eating for 20–30 days	Metabolism improving	Samylina et al. (2010)
7 plants					
7.1	*Betula* spp. [c]leaves; *Filipendula ulmaria*(L.) Maxim. aerial part; *Fragaria vesca* L. leaves; *Hypericum* spp. [e]aerial part; *Melissa officinalis* L. aerial part; *Prunus spinosa* L. flowers; *Rosa* spp. [g]fruits; (1:1:1:1:1:1:1:1)	Infusion; 10 g in 300 ml of boiling water, boil 5 min, maceration 2 h in thermos	100 ml 3–4 times a day 30 min before eating	Obesity	Vinogradova et al. (2001); Turishchev (2000), Turishchev (2005)
7.2	*Achillea millefolium* L. aerial part; *Matricaria chamomilla* L. flowers; *Mentha* × *piperita* L. leaves; *Ribes nigrum* L. leaves; *Sorbus aucuparia* L. fruits; *Vaccinium myrtillus* L. shoots; *Vaccinium vitis-idaea* L. leaves; (1:1:1: 1:1:1:1)	Infusion; 10 g in 300 ml of boiling water, boil 5 min, maceration 3 h in thermos	100 ml 3–4 times a day 15 min before eating	Obesity	Vinogradova et al. (2001); Turishchev (2000), Turishchev (2005)
7.3	*Tilia cordata* Mill. flowers; *Rosa majalis* Herrm. fruits; *Betula* spp. [c]leaves; *Origanum vulgare* L aerial part; *Hypericum perforatum* L. aerial part; *Calendula officinalis* L. flowers; *Ribes nigrum* L. leaves; (3:3:1:1:1:2:2)	Infusion; 2 table spoons in 2 400 ml of boiling water, maceration 8 h in thermos	100 ml 3 times a day	Obesity	Kukes (1999); Bubenchikova et al. (2003)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
7.4	*Zea mays* L. corn silk; *Frangula alnus* Mill. bark; *Cichorium intybus* L. roots; *Taraxacum campylodes* G.E.Haglund. roots; *Mentha* × *piperita* L. leaves *Petroselinum crispum* (Mill.) Fuss fruits; *Foeniculum vulgare* Mill. fruits; (5:3:3:2:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water	100 ml 4 times a day	Obesity	Lager (1991), Lager (2002)
7.5	"Arfazetin" *Vaccinium myrtillus* L. shoots; *Phaseolus vulgaris* L. pericarp; *Aralia elata* (Miq.) Seem roots; (or *Oplopanax elatus* (Nakai) Nakai roots and rhizomes); *Rosa* spp. [9]fruits; *Equisetum arvense* L. aerial part; *Hypericum perforatum* L. aerial part; *Matricaria chamomilla* L. flowers; (4:4:2:3:3:2:2)	Infusion; 10 g in 400 ml of water	66 ml 2–3 times a day before eating for 20–30 days	Lowering of blood glucose level, improving of glycogen-forming function of the liver, fortifying, anti-inflammatory	Korotkova et al. (1988); Matkovskaya et al. (1988); Efimov and Shcherbak (1993); Nikultseva (1994); Sokolov (2000); Blinov (2000); Turishchev (2000), Turishchev (2005); Mashkovskii (2002); Dremova et al. (2003); Kiyanova (2005); Nazina (2006); Ryzhenko (2007); Davydovich et al. (2008); Vichkanova et al. (2009); Ruzhenkova (2014); Maznev (2014); Letova (2019); Register Russia (2021)
7.6	*Viburnum opulus* L. berries; *Vaccinium myrtillus* L. fruits; *Galega officinalis* L. aerial part; *Vaccinium vitis-idaea* L. fresh berries (fruits); *Hypericum* spp. [e]flowers; *Fragaria vesca* L. leaves; *Arctostaphylos uva-ursi* (L.) Spreng. Leaves; (4:6:5:6:4:3:2)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	100 ml 2 times a day before eating	Diabetes	Brusenskaya and Kaz'min, (2005)
7.7	*Vaccinium myrtillus* L. leaves; *Urtica dioica* L. leaves; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Fragaria vesca* L. leaves; *Betula pendula* Roth. leaves; *Hypericum perforatum* L. aerial part; (5:2:5:2:2:2:2)	Decoction; 1 table spoon in 600 ml of water	50 ml 6 times a day	Diabetes	Chirkov and Seryi (1993); Kukes (1999); Bubenchikova et al. (2003); Dremova et al. (2003)
7.8	*Avena sativa* L. aerial part; *Fragaria vesca* L. aerial part; *Linum usitatissimum* L. seeds; *Melissa officinalis* L. aerial part; *Rosa* spp. [9]fruits; *Taraxacum campylodes* G.E.Haglund. roots; *Vaccinium vitis-idaea* L. leaves; (1:1:1:1:1:1:1:1)	Infusion; 10 g in 300 ml of boiling water, 15 min in boil water bath, maceration 1 h in thermos	66 ml 4 times a day 15 min before eating	Diabetes	Vinogradova et al. (2001)
7.9	*Fragaria vesca* L. aerial part; *Galega officinalis* L. aerial part; *Helichrysum arenarium* (L.) Moench flowers; *Laurus nobilis* L. leaves; *Levisticum officinale* W.D.J.Koch roots; *Urtica dioica* L. leaves*; Vaccinium vitis-idaea* L. leaves; (1:1:1:1:1:1:1:1)	Infusion; 10 g in 400 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	70–100 ml 3–4 times a day before eating	Diabetes	Vinogradova et al. (2001)
7.10	*Equisetum arvense* L. aerial part; *Polygonum aviculare* L. aerial part; *Fragaria vesca* L. leaves; *Astragalus dasyanthus* Pall. aerial part; *Galega officinalis* L. aerial part; *Arnica montana* L. flowers; *Plantago major* L. leaves; (4:4:4:3:3:1:3)	Infusion; 1 table spoons in 200 ml of boiling water, boil 3–5 min, maceration 10–15 min at room tempature	2 table spoons 3–4 times a day 20–30 min before eating	Diabetes	Korsun et al. (2016)
7.11	*Equisetum arvense* L. aerial part; *Vaccinium myrtillus* L. leaves; *Juglans regia* L. leaves; *Phaseolus vulgaris* L. pericarp; *Fragaria vesca* L. leaves; *Matricaria chamomilla* L. flowers; *Cichorium intybus* L. leaves; (1:3:3:4:1:1:2)	Infusion; 2 table spoons in 400 ml of boiling water, boil 15 min	66 ml 4 times a day 20–30 min before eating	Diabetes	Lavrenova and Lavrenov (2007)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
7.12	*Galega officinalis* L. aerial part; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; *Equisetum arvense* L. aerial part; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Gnaphalium uliginosum* L. aerial part; (2:2:2:1:1:1:1:1)	Infusion; 30 g in 400 ml of boiling water, boil 10 min, maceration 1 h	150 ml 4 times a day 30 min before eating	Diabetes	Lavrenova and Lavrenov (2007)
7.13	"Arfazetin E" *Vaccinium myrtillus* L. shoots; *Phaseolus vulgaris* L. pericarp; *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. roots and rhizomes; *Rosa* spp. [g]fruits; *Equisetum arvense* L. aerial part; *Hypericum perforatum* L. aerial part; *Matricaria chamomilla L.* flowers (4:4:2:3:3:2:2)	Infusion; 10 g in 400 ml of water	70–100 ml 2–3 times a day before eating for 20–30 days	Mild form of diabetes in combination with diet and exercise. Moderate diabetes in combination with oral hypoglycemic drugs or insulin	Register Russia (2021)
7.14	*Acorus calamus* L. roots; *Artemisia absinthium* L. aerial part; *Bidens tripartita* L. aerial part; *Mentha* × *piperita* L. leaves; *Origanum vulgare* L. aerial part; *Pinus sylvestris* L. buds; *Thymus serpyllum* L. aerial part; (2:3:3:3:3:3:2)	Herbal tea; 6 table spoons in 3,000 ml of boiling water	For external use, baths	Metabolic disorder with skin furuncles	Protasenya and Vasilenko (1992)
8 plants					
8.1	*Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Foeniculum vulgare* Mill. fruits; *Lavandula angustifolia* Mill. leaves; *Ononis spinosa* L. roots; *Persicaria hydropiper* L. aerial part; *Rheum palmatum* L. roots; *Rosa majalis* Herrm. fruits; *Senna alexandrina* Mill. leaves; (2:2:2:2:2:1:1:1)	Decoction; 2 table spoons in 500 ml of water	100 ml 4 times a day before eating	Obesity	Chirkov and Seryi (1993)
8.2	*Agrimonia eupatoria*L.aerial part; *Arctostaphylos uva-ursi* (L.) leaves; *Cetraria islandica* L. thallus; *Filipendula ulmaria*(L.) Maxim.aerial part;*Fumaria officinalis*L.aerial part;*Juglans regia* L. leaves; *Morus*nigra L.leaves; *Pinus silvestris* L. buds; (1:1:1:1:1:1:1:1:1)	Infusion; 2 table spoons in 500 ml of boiling water	50 ml 3 times a day before eating	Obesity	Osetrov and Shreter (2001); Korsun and Korsun (2010)
8.3	*Achillea millefolium* L. aerial part; *Matricaria chamomilla* L. flowers ; *Elymus repens* (L.) Gould rhizomes; *Equisetum arvense* L. aerial part; *Fucus vesiculosus* L. thallus; *Hypericum perforatum* L. aerial part; *Melissa officinalis* L. aerial part; *Taraxacum campylodes* G.E.Haglund. roots; (1:1:1:1:2:1:1:1)	Infusion; 1 tea spoon in 200 ml of boiling water	200 ml 2 times a day	Obesity	Osetrov (1993)
8.4	*Achillea millefolium L.,* aerial part; *Anethum graveolens* L. fruits; *Frangula alnus* Mill. bark; *Helichrysum arenarium* (L.) Moench flowers; *Orthosiphon aristatus* (Blume) Miq. shoots; *Rosa* spp. [g]fruits; *Taraxacum campylodes* G.E.Haglund. roots; *Zea mays* L. corn silk; (1:1:1:1:1:1:1:1:1)	Infusion; 15 g in 500 ml of cold water, maceration 4 h at room temp., boil 3 min, maceration 1 h in thermos	100 ml 4–5 times a day before eating	Obesity	Vinogradova et al. (2001); Turishchev (2000), Turishchev (2005)
8.5	*Tilia cordata* Mill. flowers; *Rosa* spp. [g]fruits; *Betula spp.*[c]leaves; *Origanum vulgare* L. aerial part; *Hypericum* spp. [e]aerial part; *Calendula officinalis* L. flowers; *Ribes nigrum* L. leaves; *Gnaphalium uliginosum* L. aerial part; (3:3:1:1:1:1:2:2)	Infusion; 10 g in 500 ml of boiling water, maceration 6–8 h in thermos	125 ml 3–4 times a day before eating	Obesity and diabetes mellitus	Kolesova et al. (1998)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
8.6	*Arctium* spp.[b]leaves; *Avena sativa* L. aerial part; *Juniperus communis* L. fruits; *Linum usitatissimum* L. seeds; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1:1:1)	Infusion; 8 g in 500 ml of boiling water, maceration 6–8 h in thermos	1 table spoon 6 times a day	Lowering of blood glucose level	Podduev (2001)
8.7	*Equisetum arvense* L. aerial part; *Oplopanaxelatus*(Nakai) Nakairoots and rhizomes; *Taraxacum campylodes* G.E.Haglund. roots; *Rosa* spp.[g]fruits; *Cichorium intybus* L. roots; *Linum usitatissimum* L. seeds; *Hypericum* spp. [e]aerial part; *Tilia cordata* Mill. flowers; (4:1:1:2:3:1:2:1)	Infusion (herbal tea); 1 table spoon in 200 ml of boiling water	100 ml 2 times a day before eating	Diabetes	Brusenskaya and Kaz'min (2005)
8.8	*Arctium* spp.[b] roots; *Glycyrrhiza glabra* L. roots; *Juglans regia* L. leaves; *Linum usitatissimum* L. seeds; *Rosa* spp.[g]fruits; *Sambucus nigra* L. root; *Vaccinium myrtillus* L. shoots ; *Viburnum opulus* L. shoots; (1:1:1:1:1:1:1:1:1)	Infusion; 10 g in 400 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	70–100 ml 3–4 times a day before eating	Diabetes	Vinogradova et al. (2001)
8.9	*Matricaria chamomilla* L. flowers; *Viola tricolor* L. aerial part; *Equisetum arvense* L. aerial part; *Achillea millefolium* L. aerial part; *Calendula officinalis* L. flowers; *Quercus* spp. [h]bark; *Gnaphalium uliginosum* L. aerial part; *Melilotus officinalis* (L.) Pall.aerial part; (2:1:1:2:1:2:1:1)	Infusion; 1 tea spoon in 200 ml of boiling water, 15 min in boil water bath, maceration 45 min in thermos	100 ml 2 times a day after eating	Diabetes	Korsun et al. (2016)
8.10	*Taraxacum campylodes* G.E.Haglund. leaves; *Vaccinium vitis-idaea* L. leaves; *Galega officinalis* L. aerial part; *Polygonum aviculare* L. aerial part; *Ribes nigrum* L. leaves; *Phaseolus vulgaris* L. pericarp; *Cichorium intybus* L. leaves; *Rosa* spp.[g]fruits; (1:4:4:1:2:3:2:1)	Infusion; 2 table spoons in 400 ml of boiling water, boil 8 min, maceration 2 h	100 ml 4 times a day 20–30 min before eating	Diabetes	Lavrenova and Lavrenov (2007)
9 plants					
9.1	*Matricaria chamomilla* L. flowers; *Equisetum arvense* L. aerial part; *Filipendula ulmaria*(L.) Maxim. aerial part; *Foeniculum vulgare* Mill. fruits; *Glycyrrhiza glabra* L. roots; *Hypericum* spp. [e]aerial part; *Mentha* × *piperita* L. aerial part; *Sambucus nigra* L. root; *Stachys officinalis* (L.) Trevis. aerial part; (1:1:1:1:1:1:1:1:1)	Infusion; 15 g in 500 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	100 ml 4–5 times a day 20 min before eating	Obesity	Vinogradova et al. (2001)
9.2	*Betula* spp. [c]leaves; *Foeniculum vulgare* Mill. fruits; *Fragaria vesca* L. aerial part; *Mentha* × *piperita* L. aerial part; *Petroselinum crispum* (Mill.) Fuss aerial part; *Phaeophyceae* (*Cystoseira barbata* (Stackh.) C.Agardh) thallus; *Polygonum aviculare* L. aerial part; *Rosa* spp.[g]fruits; *Urtica dioica L.* leaves; (1:1:1:1:1:1:1:1:1)	Infusion; 10 g in 300 ml of boiling water, boil 5 min, maceration 2 h in thermos	100 ml 3–4 times a day 30 min before eating	Obesity	Vinogradova et al. (2001)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
9.3	*Bidens tripartita* L. aerial part; *Matricaria chamomilla* L. flowers; *Equisetum arvense* L. aerial part; *Hypericum perforatum* L. aerial part; *Inula helenium* L. roots; *Mentha* × *piperita* L. leaves; *Oplopanaxelatus*(Nakai) Nakairoots and rhizomes;*Rosa majalis* Herrm. fruits; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1:1:2:1)	Infusion; 10 g in 400 ml of water	66 ml 3 times a day	Lowering of blood glucose level	Sokolov and Zamotaiev (1984); Lisitsyn and Molodozhnikova (1989); Lager (1991), Lager (2002); Efimov and Shcherbak (1993); Nikultseva (1994); Tarasenko et al. (1998); Sinyakov (1999); Sokolov (2000); Dontsov and Dontsov (2000); Blinov (2000); Sokolov (2000); Dremova et al. (2003); Smolianskii and Lifliandskii (2004); Davydovich et al. (2008); Bogdanova and Bashkirova (2010); Ruzhenkova (2014); Maznev (2014)
9.4	*Bidens tripartita* L. aerial part; *Matricaria chamomilla* L. flowers; *Equisetum arvense* L. aerial part; *Fragaria vesca* L. roots; *Hypericum perforatum* L. aerial part; *Inula helenium* L. roots; *Mentha* × *piperita* L. leaves; *Rosa majalis* Herrm. fruits; *Vaccinium myrtillus* L. leaves; (1:1:1:4:1:1:1:2)	Infusion; 1 table spoon in 200 ml of boiling water	66 ml 3 times a day before eating	Diabetes	Podduev (2001)
9.5	*Crataegus sanguinea* Pall. fruits; *Rosa majalis* Herrm. fruits; *Urtica dioica* L. leaves; *Leonurus quinquelobatus* Gilib. aerial part; *Linum usitatissimum* L. seeds; *Mentha* × *piperita* L. leaves; *Asparagus officinalis* L. rhizomes and aerial part; *Thymus serpyllum* L. aerial part; *Vaccinium myrtillus* L. leaves; (3:3:3:5:2:1:4:4:7)	Infusion; 2–3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	66 ml (warm) 3 times a day 20–30 min before eating	Diabetes	Ladynina and Morozova (1987), Ladynina and Morozova (1990); Blinov (2000); Sinyakov (1999); Nazina (2006); Davydovich et al. (2008); Bogdanova and Bashkirova (2010)
9.6	Carex arenaria L. rhizomes; *Cyanussegetum*Hillaerial part; *Galega officinalis* L. aerial part; *Phaseolus vulgaris* L. pericarp; *Pimpinella saxifraga*L. roots;*Salvia officinalis* L. leaves; *Sambucus nigra* L. flowers; *Taraxacum campylodes* G.E.Haglund. roots; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1:1:1:2)	Infusion; 2 table spoons in 500 ml of boiling water, 15 min in boil water bath, maceration 30–40 min	66 ml 3 times a day 30 min before eating	Diabetes	Sinyakov (1999)
9.7	*Arctium lappa* L. roots; *Capsella bursa-pastoris* (L.) Medik. aerial part; *Hypericum perforatum* L. aerial part; *Juglans regia* L. leaves; *Mentha* × *piperita* L. leaves; *Rosa majalis* Herrm. fruits; *Vaccinium myrtillus* L. leaves; *Vaccinium vitis-idaea* L. leaves; *Zea mays* L. corn silk; (6:3:3:4:1:4:4:5:5)	Infusion; 2 table spoons in 500 ml of boiling water, maceration 12 h in thermos	100 ml 3–4 times a day 30 min before eating	Diabetes	Sinyakov (1999)
9.8	*Crataegus* spp. fruits; *Fragaria vesca* L. leaves; *Hypericum spp.*[e]aerial part; *Linum usitatissimum* L. seeds; *Phaseolus vulgaris* L. pericarp; *Plantago major* L. leaves; *Ribes nigrum* L. leaves; *Rosa* spp. fruits; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1:1:1:2)	Infusion; 3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	140 ml (warm) 3 times a day 30 min before eating	Diabetes	Podduev (2001)

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TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
10 plants					
10.1	*Anethum graveolens* L. fruits; *Matricaria chamomilla* L. flowers; *Frangula alnus* Mill. bark; *Hypericum* spp. [e]aerial part; *Juniperus communis* L. fruits; *Prunus spinosa* L. flowers; *Rosa* spp.[g]fruits; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Zea mays* L. corn silk; (1:1:1:1:1:1:1:1:1:1)	Infusion; 10 g in 300 ml of boiling water, maceration 3 h in thermos	100 ml 3–4 times a day 15 min before eating	Obesity	Vinogradova et al. (2001)
10.2	*Betula* spp. [c]leaves; *Cichorium intybus* L. roots; *Frangula alnus* Mill. bark; *Glycyrrhiza glabra* L. roots; *Mentha* × *piperita* L. aerial part; *Morus* spp.[i] leaves; *Orthosiphon aristatus* (Blume) Miq. shoots; *Petroselinum crispum* (Mill.) Fuss roots; *Taraxacum campylodes* G.E.Haglund. roots; *Zea mays* L. corn silk; (1:1:1:1:1:1:1:1:1:1)	Infusion; 15 g in 500 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	100 ml 4–5 times a day 20 min before eating	Obesity	Vinogradova et al. (2001)
10.3	*Vaccinium vitis-idaea* L. leaves; *Zea mays* L. corn silk; *Syringa vulgaris* L. buds; *Arctium lappa* L. roots; *Mentha* × *piperita* L. leaves; *Juglans regia* L. leaves; *Hypericum perforatum* L. aerial part; *Gnaphalium uliginosum* L. aerial part; *Vaccinium myrtillus* L. leaves; *Rosa majalis* Herrm. fruits; (4:4:2:5:2:3:2:2:3:1)	Infusion; 2–3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	66 ml (warm) 3 times a day,20–30 min before eating	Diabetes	Ladynina and Morozova (1987), Ladynina and Morozova (1990); Blinov, 2000; Ladynina (2005); Nazina, (2006)
10.4	*Achillea millefolium L.* aerial part; *Arctium* spp.[b] roots; *Elymus repens*(L.) Gouldrhizomes; *Fragaria vesca* L. aerial part; *Galega officinalis* L. aerial part; *Laurus nobilis* L. leaves; *Phaeophyceae* (*Cystoseira barbata* (Stackh.) C.Agardh) thallus; *Rosa* spp.[g]fruits; *Trifolium pratense* L. flowers; *Vaccinium myrtillus* L. leaves; (1:1:1:1:1:1:1:1:1:1)	Infusion; 12 g in 350 ml of boiling water, 10 min in boil water bath, maceration 2 h in thermos.	100 ml (warm) 3 times a day10 min before eating	Diabetes	Vinogradova et al. (2001)
10.5	*Arctium lappa* L. roots; *Artemisia absinthium* L. aerial part; *Bidens tripartita* L. aerial part; *Calendula officinalis* L. flowers; *Matricaria chamomilla* L. flowers; *Equisetum arvense* L. aerial part; *Gnaphalium uliginosum* L. aerial part; *Hypericum perforatum* L. aerial part; *Inula helenium* L. roots; *Salvia officinalis* L. aerial part; (1:1:1:1:1:1:1:1:1:1)	Decoction; 1–2 table spoons in 200 ml of water	70–100 ml 3 times a day 30 min before eating	Diabetes accompanied by chronic pancreatitis	Chirkov and Seryi, (1993)
11 plants					
11.1	*Achillea millefolium* L. aerial part; *Arctium* spp.[b]roots; *Cichorium intybus* L. roots; *Elymus repens*(L.) Gouldroot; *Inula helenium* L. roots; *Phaseolus vulgaris* L. pericarp; *Polygonum aviculare* L. aerial part; *Ribes nigrum L.* leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Tilia cordata* Mill. flowers; *Vaccinium myrtillus* L. leaves; (1:2:1:2:1:2:2:1:2:2:2)	Decoction; 10 g in 500 ml of water, 2 h in boil water bath	100–150 ml 3–4 times a day before eating	Insulin-dependent diabetes	Kolesova et al. (1998)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
11.2	*Betula pubescens* Ehrh. leaves; *Crataegus sanguinea* Pall. fruits; *Orthosiphon aristatus* (Blume) Miq. leaves; *Rosa majalis* Herrm. fruits; *Mentha* × *piperita* L. leaves; *Veronica officinalis* L. aerial part; *Centaurium erythraea* Rafn aerial part; *Arctium lappa* L. roots; *Leonurus quinquelobatus* Gilib. aerial part; *Glycyrrhiza glabra* L. roots; *Cichorium intybus* L. roots (2:3:2:2:2:1:5:5:3:2:4)	Infusion; 2–3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	66 ml (warm) 3 times a day 20–30 min before eating	Diabetes	Ladynina and Morozova (1987), Ladynina and Morozova (1990); Blinov (2000); Dremova et al. (2003); Ladynina (2005); Nazina (2006); Bogdanova and Bashkirova (2010); Maznev (2014)
11.3	*Vaccinium myrtillus* L. leaves; *Phaseolus vulgaris* L. pericarp; *Crataegus sanguinea* Pall. Fruits; *Hypericum perforatum* L. aerial part; *Plantago major* L. leaves; *Ribes nigrum* L. leaves; *Rosa majalis* Herrm. fruits; *Linum usitatissimum* L. seeds; *Mentha* × *piperita* L. leaves; *Fragaria vesca* L. leaves *Sambucus nigra* L. flowers; (4:3:2:2:2:2:1:1:1:1:1:1)	Infusion; 3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	130–140 ml (warm) 3 times a day 20–30 min before eating	Diabetes	Sinyakov (1992); Sinyakov (1999); Tarasenko et al. (1998); Davydovich et al. (2008)
11.4	*Artemisia absinthium* L. aerial part; *Avena sativa* L. aerial part; *Urtica dioica* L. leaves; *Arctium* spp.[b]roots; *Inula helenium* L. roots; *Alchemilla xanthochlora* Rothm. aerial part; *Taraxacum campylodes* G.E.Haglund. leaves; *Vaccinium myrtillus* L. leaves; *Gnaphalium uliginosum* L. aerial part; *Sambucus nigra* L. flowers; *Linum usitatissimum* L. seeds; (4:4:4:2:1:2:1:2:4:4:2)	Infusion; 2 table spoons in 500 ml of boiling water, maceration 12 h at room temp.	100 ml 3 times a day 15 min before eating.	Diabetes	Rendiuk (2006)
11.5	*Alchemilla xanthochlora* Rothm. aerial part; *Centaurium erythraea* Rafn aerial part; *Elymus repens*(L.) Couldrhizomes; *Gnaphalium uliginosum* L. aerial part; *Juniperus communis* L. fruits; *Mentha* × *piperita* L. aerial part; *Rumex confertus* Willd. roots; *Sorbus aucuparia* L. fruits; *Syringa vulgaris* L. buds; *Taraxacum campylodes* G.E.Haglund. roots; *Trifolium pratense* L. flowers; (1:1:1:1:1:1:1:1:1:1:1)	Infusion; 12 g in 350 ml of boiling water, 10 min in boil water bath, maceration 2 h in thermos	100 ml (warm) 3 times a day 10 min before eating	Diabetes	Vinogradova et al. (2001)
11.6	*Arctium* spp.[b] roots; *Avena sativa* L. aerial part; *Betula* spp.[c]leaves; *Galega officinalis* L. aerial part; *Glycyrrhiza glabra* L. roots; *Hypericum* spp. [e]aerial part; *Juglans regia* L. leaves; *Juniperus communis* L. fruits; *Laurus nobilis* L. leaves; *Vaccinium myrtillus* L. leaves ; *Vaccinium vitis-idaea* L. leaves; (1:1:1:1:1:1:1:1:1:1:1)	Infusion; 10 g in 400 ml of cold water, maceration 4 h, boil 3 min, maceration 2 h in thermos	100 ml 3 times a day before eating	Diabetes	Vinogradova et al. (2001)
11.7	*Betula* spp. [c]leaves; *Cyanus segetum* Hill flowers; *Foeniculum vulgare* Mill. fruits; *Laurus nobilis* L. leaves; *Linum usitatissimum* L. seeds; *Ononis spinosa* L. roots; *Petroselinum crispum* (Mill.) Fuss roots; *Phaseolus vulgaris* L. pericarp; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. roots; *Viburnum opulus* L. flowers; (1:1:1:1:1:1:1:1:1:1:1)	Infusion; 12 g in 400 ml of boiling water, 15 min in boil water bath, maceration 1.5 h in thermos	70–100 ml 3–4 times a day 10 min before eating	Diabetes	Vinogradova et al. (2001)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
11.8	*Arctium lappa* L. roots; *Centaurium erythraea* Rafn aerial part; *Cichorium intybus* L. roots; *Crataegus sanguinea* Pall. fruits; *Rosa majalis* Herrm. fruits; *Glycyrrhiza glabra* L. roots; *Leonurus quinquelobatus* Gilib. aerial part; *Orthosiphon aristatus* (Blume) Miq. leaves; *Mentha* × *piperita* L. leaves; *Betula nigra* L. leaves; *Veronica officinalis* L. aerial part; (3:3:2:2:2:2:2:1:1:1:1)	Infusion; 2–3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	130 ml (warm) 3 times a day 30 min before eating	Diabetes	Sinyakov (1999)
12 plants					
12.1	*Crataegus sanguinea* Pall. fruits; *Mentha* × *piperita L.* leaves; *Rosa majalis* Herrm. fruits; *Sambucus nigra* L. flowers; *Hypericum perforatum* L. aerial part; *Fragaria vesca* L. leaves; *Plantago major* L. leaves; *Ribes nigrum* L. leaves; *Saccharina latissima* (L.) C.E.Lane, C.Mayes, Druehl & G.W.Saunders thallus; *Linum usitatissimum* L. seeds; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. leaves; (3:2:2:2:3:1:3:3:2:2:5:7)	Infusion; 2–3 table spoons in 500 ml of boiling water, maceration 12 h in thermos	66 ml (warm) 3 times a day 20–30 min before eating	Diabetes	Ladynina and Morozova (1987), Ladynina and Morozova (1990); Lisitsyn and Molodozhnikova (1989); Lager (1991); Lager (2002); Efimov and Shcherbak (1993); Sinyakov (1999); Blinov (2000); Ladynina (2005); Nazina (2006); Bogdanova and Bashkirova (2010);
12.2	"Myrphasinum" *Vaccinium myrtillus* L. shoots; *Phaseolus vulgaris* L. pericarp; *Rosa* spp.[g]fruits; *Urtica dioica* L. leaves; *Plantago major* L. leaves; *Matricaria chamomilla* L. flowers; *Calendula officinalis* L. flowers; *Leonurus* spp [d] aerial part; *Hypericum* spp.[e] aerial part; *Achillea millefolium* L. aerial part; *Glycyrrhiza glabra* L. roots; *Inula helenium* L. roots; (2:2:1:1:1:1:1:1:1:1:1:1)	Infusion; 10 g in 400 ml of boiling water	100 ml 2–3 times a day 30 min before eating for 20–30 days	Mild forms of diabetes	Turishchev (2000); Dremova et al. (2003); Belodubrovskaya et al. (2004); Turishchev (2005); Davydovich et al. (2008); Ruzhenkova (2014)
13 plants					
13.1	*Avena sativa* L. aerial part; *Capsella bursa-pastoris* (L.) Medik. aerial part; *Cyanus segetum* Hill flowers; *Frangula alnus* Mill. bark; *Laurus nobilis* L. leaves; *Petroselinum crispum* (Mill.) Fuss aerial part; *Phaseolus vulgaris* L. pericarp; *Pimpinella anisum* L. fruits; *Rumex confertus* Willd. roots; *Syringa vulgaris* L. buds; *Taraxacum campylodes* G.E.Haglund. roots; *Tilia cordata* Mill. flowers; *Vaccinium myrtillus* L. leaves ; (1:1:1:1:1:1:1:1:1:1:1:1:1)	Infusion; 12 g in 350 ml of boiling water, 10 min in boil water bath, maceration 2 h in thermos	100 ml (warm) 3 times a day 10 min before eating	Diabetes	Vinogradova et al. (2001)
13.2	*Alchemilla xanthochlora* Rothm. leaves; *Arctostaphylos uva-ursi* (L.) Spreng. leaves; *Centaurium erythraea* Rafn aerial part; *Dioscorea spp.* root; *Helichrysum arenarium* (L.) Moench flowers; *Juniperus communis* L. fruits; *Mentha* × *piperita* L. aerial part; *Morus alba* L. leaves; *Plantago major* L. leaves; *Ribes nigrum* L. leaves; *Saccharina latissima* (L.) C.E.Lane, C.Mayes, Druehl & G.W.Saunders thallus; *Vaccinium vitis-idaea* L. leaves; *Veronica officinalis* L. aerial part; (1:1:1:1:1:1:1:1:1:1:1:1:1)	Infusion; 12 g in 350 ml of boiling water, 10 min in boil water bath, maceration 2 h in thermos	100 ml (warm) 3 times a day 10 min before eating	Diabetes	Vinogradova et al. (2001)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
13.3	*Juglans regia* L. leaves; *Laurus nobilis* L. leaves; *Morus* spp.[i]leaves; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. shoots; *Avena sativa* L. aerial part; *Fragaria vesca* L. leaves; *Hypericum* spp. [e]aerial part; *Linum usitatissimum* L. seeds; *Plantago major* L. leaves; *Taraxacum campylodes* G.E.Haglund. roots; *Urtica dioica* L. leaves; *Veronica officinalis* L. aerial part; (3:3:3:3:3:3:1:1:1:1:1:1:1:1:1)	Infusion; 15 g in 400 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	100 ml 4 times a day, 15 min before eating	Diabetes	Vinogradova et al. (2001)
14 plants					
14.1	*Achillea millefolium* L. aerial part; *Betula* spp. [c]leaves; *Matricaria chamomilla* L. flowers; *Equisetum arvense* L. aerial part; *Foeniculum vulgare* Mill. fruits; *Frangula alnus* Mill. bark; *Glycyrrhiza glabra* L. roots; *Hypericum* spp. [e]aerial part; *Juniperus communis* L. fruits; *Linum usitatissimum* L. seeds; *Melissa officinalis* L. aerial part; *Petroselinum crispum* (Mill.) Fuss roots; *Ribes nigrum* L. leaves; *Urtica dioica* L. leaves; (1:1:1:1:1:1:1:1:1:1:1:1:1:1)	Infusion; 15 g in 500 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos	100 ml 4–5 times a day 20 min before eating	Obesity	Vinogradova et al. (2001)
14.2	*Anethum graveolens* L. fruits; *Crataegus* spp.[f]fruits; *Fragaria vesca* L. leaves; *Helichrysum arenarium* (L.) Moench flowers; *Laurus nobilis* L. leaves; *Linum usitatissimum* L. seeds; *Mentha* × *piperita* L. aerial part; *Orthosiphon aristatus* (Blume) Miq. shoots; *Polygonum aviculare* L. aerial part; *Prunus spinosa* L. flowers; *Rheum palmatum* L. roots; *Rosa* spp.[g]fruits; *Salvia officinalis* L. leaves; *Sorbus aucuparia* L. fruits; (1:1:1:1:1:1:1:1:1:1:1:1:1:1)	Infusion; 10 g in 500 ml of cold water, maceration 4 h at room temp., boil 3 min, maceration 1 h in thermos	100 ml 4–5 times a day before eating	Obesity	Vinogradova et al. (2001)
14.3	*Juglans regia* L. leaves; *Plantago major* L. seeds; *Syringa vulgaris* L. buds; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. shoots; *Arctium* spp. [b]roots; *Taraxacum campylodes* G.E.Haglund. roots; *Ribes nigrum* L. leaves; *Helichrysum (arenarium* (L.) Moench flowers; *Betula* spp. [c]leaves; *Urtica dioica* L. leaves; *Hypericum* spp. [e]aerial part; *Equisetum arvense* L. aerial part; *Fragaria vesca* L. aerial part; (3:1:1:4:4:4:4:2:2:2:2:2:2:2:2)	Infusion; 15 g in 400 ml of boiling water, boil 3 min, maceration 3 h in thermos	100 ml 4 times a day 15 min before eating	Diabetes	Vinogradova et al. (2001)
14.4	*Laurus nobilis* L. leaves; *Vaccinium myrtillus* L. shoots; *Phaseolus vulgaris* L. pericarp; *Galega officinalis* L. aerial part; *Elymus repens*(L.) Couldrhizomes; *Centaurium erythraea* Rafn aerial part; *Melissa officinalis* L. aerial part; *Rosa* spp.[g]fruits; *Glycyrrhiza glabra* L. roots; *Vaccinium vitis-idaea* L. leaves; *Betula* spp. [c]leaves; *Linum usitatissimum* L. seeds; *Avena sativa* L. aerial part; *Trifolium pratense* L. flowers; (4:4:4:4:2:2:2:2:1:1:1:1:1:1:1)	Infusion; 15 g in 400 ml of boiling water, 15 min in boik water bath, maceration 2 h in thermos.	100 ml 4 times a day 15 min before eating	Diabetes	Vinogradova et al. (2001)

(Continued on following page)

TABLE 1 | (*Continued*) The list of medical species used for the management of diabetes and related disorders in Russia.

Code[a]	Plant name, part used /(proportion)	Method of preparation	Recommended dosage	Indication	Reference
15 plants					
15.1	*Arctium* spp.[b]roots; *Galega officinalis* L. aerial part; *Laurus nobilis* L. leaves; *Phaseolus vulgaris* L. pericarp; *Vaccinium myrtillus* L. shoots ; *Vaccinium vitis-idaea* L. leaves; *Betula* spp.[c]leaves; *Centaurium erythraea* Rafn aerial part; *Linum usitatissimum* L. seeds; *Rosa* spp.[g]fruits; *Syringa vulgaris* L. buds; *Melissa officinalis* L. aerial part; *Sorbus aucuparia* L. fruits *Trifolium pratense* L. flowers; *Viburnum opulus* L. flowers; (3:3:3:3:3:3:2:2:2:2: 2:1:1:1:1)	Infusion; 15 g in 400 ml of boiling water, 15 min in boil water bath, maceration 2 h in thermos.	100 ml 4 times a day 15 min before eating	Diabetes	Vinogradova et al. (2001)

[a]*Code include number of plants and species number (for example 2.1 is mean 2 plants, species 1).*
[b]*Arctium* spp. = *Arctium lappa* L., *A. tomentosum* Mill., *A. minus* (Mill.) *Bernh.*
[c]*Betula* spp. = *Betula pendula* Roth., *B. pubescens* Ehrh.
[d]*Leonurus* spp. = *Leonurus quinquelobatus* Gilib., *L. cardiaca* L.
[e]*Hypericum* spp. = *Hypericum perforatum* L., *H. maculatum* Crantz.
[f]*Crataegus* spp. = *Crataegus laevigata* (Poir.) DC. *(C. oxyacantha sensu* Pojark.*), C. korolkovii* L., Henry, *C.chlorocarpa* Lenne *et C. koch (C. altaica (Lond.) Lange), C. dahurica Koehne ex Schneid., C. monogina* Jacq., *C.alemanniensis* Cin., *C. pentagyna* Waldst. *et* Kit., *C. orientobaltica* Cin., *C. curvisepala* Lindm., *C. x curonica* Cin.,*C. x dunensis* Cin.
[g]*Rosa* spp. = *Rosa majalis* Herrm. *(R. cinnamomea* L.*); R. acicularis* Lindl.*; R. davurica* Pall.*; R. beggeriana* Schrenk.*; R. fedtschenkoana* Regel.*; R. rugosa* Thunb. et al.
[h]*Quercus* spp. = *Quercus. robur* L. and *Q. petraea* (Mattuschka) Liebl.
[i]*Morus spp. = Morus alba L., M. nigra L.*

The leaves of *Vaccinium myrtillus* were widely used in Europe for the treatment of diabetes for a long time before the discovery of insulin (Helmstädter and Schuster, 2010). The extract was considered a potent inhibitor of α-glucosidase, with an IC50 value not statistically significantly different from the IC50 of acarbose (Bljajić et al., 2017), and to decrease blood glucose (Cignarella et al., 1996) and glycated hemoglobin (Sidorova et al., 2017). An extract from the pericarp of *Phaseolus vulgaris* L. significantly decreases the levels of plasma triacylglycerol and low-density lipoprotein in the blood (Pari and Venkateswaran, 2004; Sidorova et al., 2017), lowers blood glucose and cholesterol in the blood, and inhibits α-amylase activity (Micheli et al., 2019). The normalization of lipid profiles and systemic antioxidant effects are also attributed to

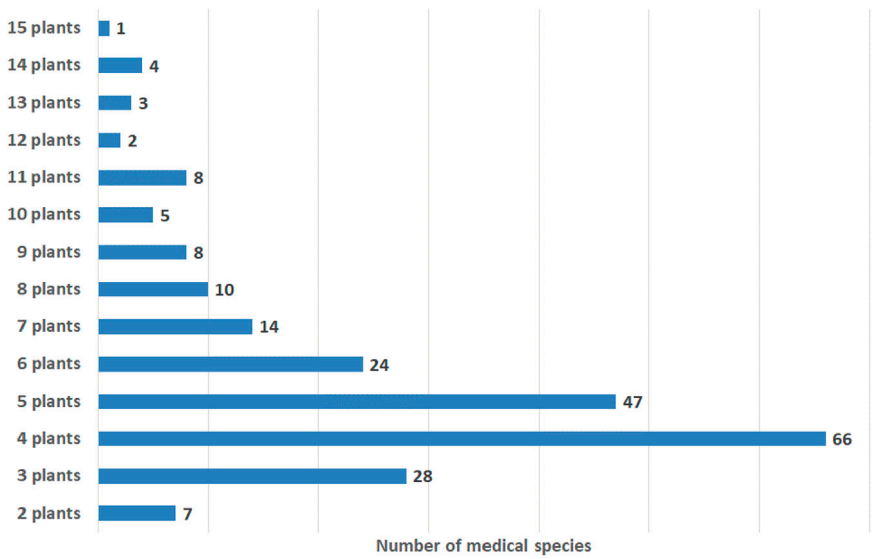

FIGURE 1 | Number of medical species according to the number of plants in the formulation.

TABLE 2 | The most frequently mentioned binary combination of plants in medical species used for the treatment of diabetes.

	***Vaccinium myrtillus* L. leaves**	***Phaseolus vulgaris* L. pericarp**	***Urtica dioica* L. leaves**	***Galega officinalis* L. aerial part**	***Taraxacum campylodes* G.E.Haglund. roots**	***Fragaria vesca* L. leaves**	***Rosa* spp. fruits**	***Arctium spp.* root**	***Vaccinium vitis-idaea* L. leaves**	***Hypericum spp.***	***Mentha* × *piperita* L. leaves**
Phaseolus vulgaris L. pericarp	40										
Urtica dioica L. leaves	26	17									
Galega officinalis L. aerial part	22	17	14								
Taraxacum campylodes G.E.Haglund. roots	21	19	19	9							
Fragaria vesca L. leaves	14	8	8	4	5						
Rosa spp. fruits	16	10	6	4	4	10					
Arctium spp. roots	15	9	5	5	2	4	8				
Vaccinium vitis-idaea L. leaves	13	8	5	10	2	4	7	8			
Hypericum spp.	15	12	8	4	7	8	9	5	4		
Mentha × *piperita* L. leaves	12	6	2	4	4	3	10	5	4	7	
Linum usitatissimum L. seeds	11	11	4	2	5	5	9	5	5	5	3

this plant by other scientists (Venkateswaran et al., 2002; Helmstädter, 2010; Almuaigel et al., 2017).

The next most popular binary combination includes leaves of *Vaccinium myrtillus* L. and leaves of *Urtica dioica* L. (noted in 26 medical species). In addition to *Vaccinium myrtillus* L., the extract from the leaves of *Urtica dioica* L. reduces glycemia, potentiates the activity of insulin, enhances the utilization of glucose (El Haouari and Rosado, 2019), protects pancreatic β-cells (Golalipour and Khori, 2007), inhibits intestinal glucose absorption (Bnouham, et al., 2003), and shows total cholesterol-lowering activity (Avci et al., 2006). Eight weeks of treatment of patients with type 2 diabetes with *Urtica dioica* extract resulted in reductions in plasma glucose, triglycerides, and liver serum glutamic-pyruvic transaminase. Meanwhile, NO and superoxide markedly increased (Behzadi et al., 2016).

The combination of the leaves of *Vaccinium myrtillus* L. and aerial parts of *Galega officinalis* L. is described for 22 medical species. Complimentarily to *Vaccinium myrtillus* L., the extract from *Galega officinalis* L. reduces blood glucose, promotes the recovery of pancreatic β-cells (Sabeva et al., 2004; Shojaee et al., 2015), increases insulin-stimulated glucose uptake, activates peroxisome proliferator-activated receptor (PPARγ) (Christensen et al., 2009), normalizes neutrophils, reduces lymphoblast numbers, and inhibits the apoptosis of lymphocytes, which prevents the development and progression of diabetic complications (Nagalievska et al., 2018). *Galega officinalis* L. is a world-renowned herbal lineage containing metformin (Bailey, 2017). It should be noted that the efficacy of the binary combination of extracts of *Vaccinium myrtillus* L. and *Galega officinalis* L. was confirmed *in vivo*. A dry extract of this combination (50 mg/kg) was intragastrically administered to rats with streptozotocin (STZ)-induced diabetes. After 21 days of treatment, histological examination evidenced the recovery of degenerative and focal necrobiotic changes in the parenchymatous structures of the liver and kidneys and their blood flow caused by STZ (Kurylo et al., 2018). In another study, the same combination of extracts was administered intragastrically to rats with STZ-induced diabetes for 28 days. After 7 days of treatment, blood glucose was decreased by 69% compared with control, while after 28 days of treatment, blood glucose was decreased by 25% compared with control. A positive effect of the combination was also observed in the oral glucose tolerance test (OGTT) (Kurylo et al., 2020). The rationality of the *Vaccinium myrtillus* L. and *Galega officinalis* L. combination was confirmed in a number of experiments by Achilov (2020). A screening study of the individual extracts (*Vaccinium myrtillus* L. (50 mg/kg) and *Galega officinalis* L. (70 mg/kg)) and a combination at 50 mg/kg showed that, in OGTT in rats, the combination of the extracts decreased glucose more effectively than the individual extracts. The strongest effect was observed at 60 min. Studies on models of epinephrine-induced hyperglycemia in rats, alloxan-induced diabetes in rats, and dithizone-induced diabetes in rabbits showed hypoglycemic activity of the combined extract at 60 mg/kg (Achilov, 2020).

TABLE 3 | Complimentary mechanisms of most often used medicinal plants in binary combination in medical species used for the treatment of diabetes.

Medicinal plant and part used	Mechanisms	References
Fragaria vesca L. leaves	Decrease of total cholesterol, triglycerides, low- and high-density lipoproteins, normalization in antioxidant system (decrease of malondialdehyde and increase of superoxide dismutase); inhibition of α-glucosidase and α-amylase enzyme activity; reduce blood glucose level.	Tassa et al. (2012), Takács et al. (2020)
Rosa spp. fruits	Reduce blood glucose level, regulate lipid metabolism by inhibiting fat accumulation (mainly visceral), decrease serum triglycerides, regeneration of pancreas β-cells, increase expression of insulin-dependent genes Gck and Ptp1b.	Ninomiya et al. (2007), Orhan et al. (2009), Taghizadeh et al. (2016), Fattahi et al. (2017), Bahrami et al. (2020)
Arctium spp. roots	Decrease of blood glucose, increase of insulin synthesis, suppression of lipid synthesis by activating 5′-adenosine monophosphate activated protein kinase, regulated the expression of sterol regulatory element-binding protein-1 and stearoyl-CoA desaturase.	Kuo et al. (2012), Ahangarpour et al. (2017), Chen et al. (2020)
Vaccinium vitis-idaea L. leaves	Decrease of blood glucose, increase of insulin synthesis, decrease of triglycerides, and high-density lipoproteins.	Barnaulov (2008), Zagayko et al. (2016)
Hypericum spp.	Reduce blood glucose level, inhibit pancreatic lipase, fat accumulation reduce hypercholesterolemia, lowered total cholesterol and low-density cholesterol, triglycerides, improved the insulin sensitivity, reduce expression of Dgat1, ColV, andLp1 genes involved in the biosynthesis of triglycerides	Arokiyaraj et al. (2011), Husain et al. (2011), Hernández-Saavedra et al. (2016), Tokgöz and Altan (2020)
Mentha × *piperita* L. leaves	Decrease of serum glucose, cholesterol, triglycerides, very low density lipoprotein, low density lipoprotein. Increase the high density lipoprotein cholesterol levels; inhibit glucosidase and tyrosinase	Barbalho et al. (2011), Bayani et al. (2017), Pavlić et al. (2021), Zeljković et al. (2021)
Linum usitatissimum L. seeds	Decrease blood glucose and polyphagia, control of lipid peroxidation (thiobarbituric acid-reactive substances) and antioxidant enzymes (glutathione peroxidase, superoxide dismutase, and catalase), inhibit glucosidase and α-amylase	Bhat et al. (2011), Bouzghaya et al. (2020)

Roots of *Taraxacum campylodes* G.E. Haglund. in combination with the leaves of *Vaccinium myrtillus* L. are contained in 21 medical species; the former in combination with the leaves of *Urtica dioica* L. are used in 19 medical species, and the same in combination with the pericarp of *Phaseolus vulgaris* L. are used in 19 medical species (**Table 2**). The leaves of *Taraxacum campylodes* G.E. Haglund. are also used in binary combinations with the leaves of *Vaccinium myrtillus* L. and leaves of *Urtica dioica* L. The *Taraxacum campylodes* G.E. Haglund. root extract inhibits adipogenesis, regulates lipid metabolism by inhibiting fat accumulation, increases lipolysis, and normalizes cholesterol and triglyceride levels (García-Carrasco et al., 2015). The leaf extract inhibited pancreatic lipase *in vitro* and *in vivo*, reduced triglyceride levels in the plasma of mice (Zhang et al., 2008), and stimulated the release of insulin in pancreatic β-cells (Hussain et al., 2004).

All the other binary combinations are used in fewer than 10% of the medical species discussed in this review. Therefore, we summarize the mechanisms of activities of the other plants cited in **Table 2** separately in **Table 3**. The above-mentioned literature data suggest that binary combinations provide additive/synergistic effects.

Notably, the binary combinations of the leaves of *Vaccinium myrtillus* L. and pericarp of *Phaseolus vulgaris* L.; leaves of *Vaccinium myrtillus* L. and roots of *Arctium* spp.; and roots of *Taraxacum campylodes* G.E. Haglund. and leaves of *Mentha* × *piperita* L. occur as self-sufficient medical species (**Table 1**).

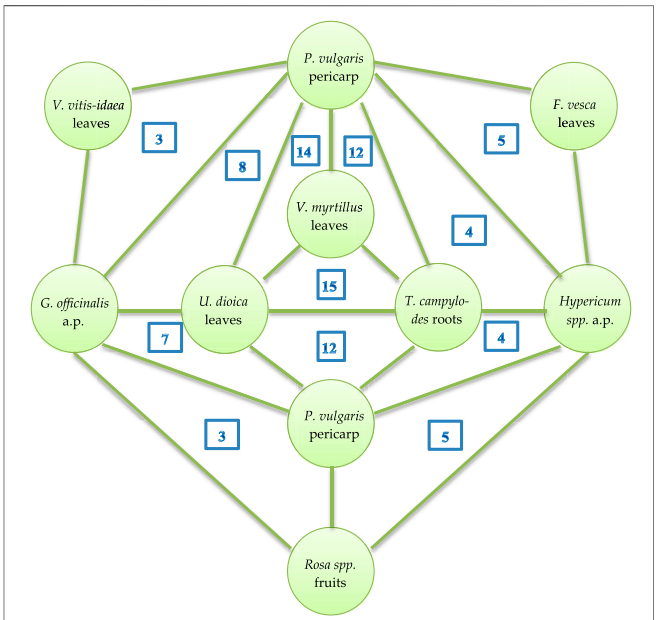

FIGURE 2 | Leading ternary plant combinations in medical species. a.p.—aerial part; the numbers inside triangles indicate how often these ternary combinations occur in medical species.

Deeper analysis of all the medical species allowed us to identify leading ternary plant combinations, which are presented in **Figure 2**. It is not surprising that

TABLE 4 | The active compounds from medicinal plants most often used in combinations in medical species and probability of predicted antidiabetic mechanisms assitiated with these compounds.

Medicinal plant/ (abbreviation)	Active compound	Mechanism of action	P_a	P_i
Vaccinium myrtillus L. (VM)	Isoorientin	Antidiabetic	0.806	0.005
	Vitexin-2"-rhamnosid	α-glucosidase inhibitor	0.854	0.001
		Antidiabetic	0.767	0.005
	Inositol	Sugar-phosphatase inhibitor	0.961	0.002
Phaseolus vulgaris L. (PV)	Isoorientin	Antidiabetic	0.806	0.005
	Myricetin	Lipid peroxidase inhibitor	0.836	0.003
		β-glucuronidase inhibitor	0.679	0.005
Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. (ES)	Syringin	Antidiabetic	0.684	0.007
		Hypolipemic	0.674	0.016
	Eleutheroside E	Sugar-phosphatase inhibitor	0.887	0.005
Hypericum perforatum L. (HP)	Hyperoside	Antidiabetic	0.661	0.008
		Lipid peroxidase inhibitor	0.976	0.002
		Sugar-phosphatase inhibitor	0.874	0.006
		α-glucosidase inhibitor	0.842	0.001
Aralia elata (Miq.) Seem (AE)	Araloside A	Antidiabetic	0.639	0.009
		Hypolipemic	0.955	0.003
		Insulin promoter	0.753	0.004
	Araloside B	Lipid peroxidase inhibitor	0.969	0.002
		Hypolipemic	0.953	0.003
		α-glucosidase inhibitor	0.932	0
	Araloside C	Hypolipemic	0.952	0.003
Fragaria vesca L. (FV)	Taxifolin-3-O-arabinofuranoside	Antidiabetic	0.617	0.011
		Lipid peroxidase inhibitor	0.95	0.002
		Antihypercholesterolemic	0.901	0.003
Urtica dioica L. (UD)	2-O-caffeoylmalic acid	Lipid metabolism regulator	0.836	0.005
	Quercetin p-coumaroyl glucoside	Lipid peroxidase inhibitor	0.978	0.002
		α-glucosidase inhibitor	0.729	0.001
Galega officinalis L. (GO)	Galegine	Sugar-phosphatase inhibitor	0.632	0.047
		Glucose oxidase inhibitor	0.691	0.025
	Phytol	Lipid metabolism regulator	0.828	0.005
		Hypolipemic	0.68	0.015
Taraxacum campylodes G.E.Haglund. (TC)	Taraxacin	β-glucuronidase inhibitor	0.619	0.011
Rosa spp. (Rsp)	Lycopene	Sugar-phosphatase inhibitor	0.794	0.017
		Lipid metabolism regulator	0.880	0.004
Arctium spp. (Asp)	Arctigenic acid	Insulin promoter	0.579	0.017
Vaccinium vitis-idaea L. (VVI)	Hydroquinone	Sugar-phosphatase inhibitor	0.906	0.004
		Inulinase inhibitor	0.690	0.004
Mentha × *piperita* L. (MP)	Menthol	Insulin promoter	0.773	0.004
		Sugar-phosphatase inhibitor	0.804	0.016
Linum usitatissimum L. (LU)	Gallic acid	Sugar-phosphatase inhibitor	0.941	0.003
		Glucan endo-1.6-beta-glucosidase inhibitor	0.933	0.002

the leaves of *Vaccinium myrtillus* L., pericarp of *Phaseolus vulgaris* L., roots of *Taraxacum campylodes* G.E. Haglund., leaves of *Urtica dioica* L., and aerial parts of *Galega officinalis* L. are principal members of the ternary combinations.

In Silico Probability of Antidiabetic Activity for Principal Compounds Identified in Selected Plants

The progress in computer science in symbiosis with modern pharmacology has led to the active implementation of computer-based prognosis for the activity of herb-derived compounds. Using an *in silico* approach, we analyzed the probability of antidiabetic activity for the principal compounds identified in the plants most often mentioned in binary and ternary combinations.

The prediction was performed using the free web resource PASS Online (Prediction of Activity Spectra for Substances). The prediction is based on an analysis of the structure and biological activity relationships for more than 300,000 organic compounds (Filimonov et al., 2014). **Table 4** includes the prediction results for the antidiabetic efficacy of active compounds from selected medicinal plants with appropriate probability values: the likelihood of the given activity being revealed (Pa) or not revealed (Pi). If Pa>0.5, the substance is very likely to exhibit the activity (Lagunin et al., 2000).

The predicted Pa values for the active compounds identified in the most frequently used combinations of plants in medical species were over 0.5 and ranged from 0.619 for compounds from *Fragaria vesca* L. up to 0.976 for compounds from *Hypericum perforatum* L. (**Table 4**). The highest Pa values were found for compounds derived from *Urtica dioica* L.,

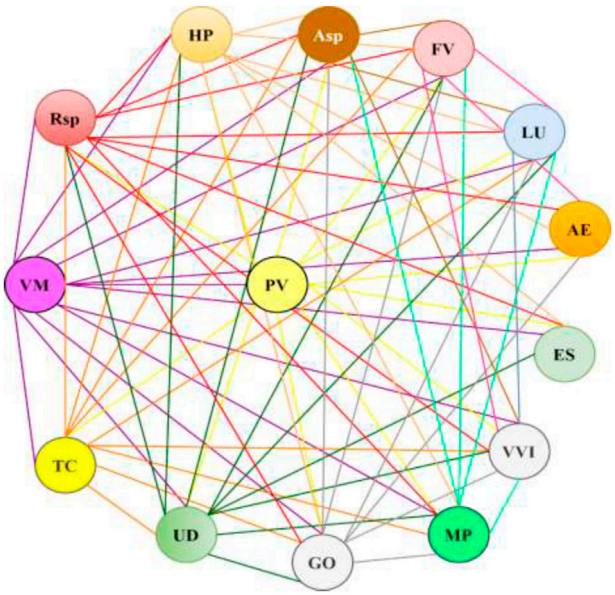

FIGURE 3 | The combinations of the medicinal plants with a high probability of antidiabetic effects in medical species. The plant abbreviations are presented in **Table 4**.

Hypericum perforatum L., *Vaccinium myrtillus* L., *Fragaria vesca* L., *Linum usitatissimum* L., and *Vaccinium vitis-idaea* L. (Pa>0.9). The diagram in **Figure 3** demonstrates crosslinks between medicinal plants with a high probability of predicted antidiabetic effects (**Table 4**) used in medical species.

The calculated data support the rationality of the traditional use of medical species for the treatment of diabetes and its complications. Nevertheless, the chemical principles responsible for the observed effects are rarely studied. Except for the success story of metformin derived from *Galega officinalis* L., no other compounds are on the market. Systematic studies of the combinatory action of different plant decoctions/infusions, as well as plant-derived compounds, are needed.

Specificity of Medical Species Used in Russia

Several plants used in Russian traditional medicines for the treatment of diabetes and its complications are widely known in other countries. The leaves of *Urtica dioica* L., pericarp of *Phaseolus vulgaris* L., leaves of *Vaccinium myrtillus* L., and leaves and roots of *Taraxacum campylodes* G.E. Haglund. are among the most frequently used components for the management of diabetes by herbalists in Croatia (Končić and Bljajić, 2019). *Phaseolus vulgaris* is a well-known antidiabetic plant in the Ayurveda and Unani medicine systems (Ganesan and Xu, 2017). It is widely used in medicine in Poland (Łabuda et al., 2017). The antidiabetic potential of *Urtica dioica* is well documented in Arabic traditional medicine (Said et al., 2008). *Galega officinalis* L. has been used for the treatment of diabetes in Bulgaria (Petkov, 1982), Italy (Leporatti and Ivancheva, 2003), and Iran (Nowbandegani et al., 2015).

Unlike in other systems of medicine, the juices of some plants (the berries of *Viburnum opulus* L., *Solanum tuberosum* L., and *Daucus sativus* Roehl. and birch sap) have been used for preparing some medical species. Interestingly, eleven medical species contain seaweeds (*Cystoseira barbata* (Stackh.) C. Agardh; *Saccharina latissima* (L.) C.E.Lane, C.Mayes, Druehl & G.W.Saunders; *Fucus vesiculosus* L.), and one species contains lichen (*Cetraria islandica* L.). It is noteworthy that several adaptogenic plants have been used in medical species. Besides the common properties of promoting the adaptability, resilience, and survival of living organisms under stress (Panossian et al., 2021), each adaptogen has some specific activity. In particular, *Oplopanax elatus* (Nakai) Nakai lowered blood glucose and increased insulin levels *in vivo* (Molokovskii et al., 2002). Glucose- and cholesterol-lowering effects, decreased glycosuria, and increased insulin levels were observed in diabetic patients after complex therapy with *Oplopanax elatus* (Nakai) Nakai (Klimakova and Kazmanm, 1962). *Aralia elata* (Miq.) Seem decreases blood glucose, inhibits insulin resistance, alleviates hyperlipidemia *in vivo* (Hwang et al., 2015), and improves blood glucose and lipid metabolism in humans (Abidov et al., 2006). The activity could be associated with aralosides (Pa, 0.639–0.969, **Table 4**). *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim. and its active compounds lowered blood glucose, increased glycogen levels, ameliorated insulin resistance, and increased insulin levels *in vivo* (Molokovskii et al., 2002; Niu et al., 2008; Ahn et al., 2013). The activity is associated with syringing and eleutheroside E (Pa, 0.684 and 0.887; **Table 4**). These adaptogens are not only used in medical species in traditional medicine but are included in the officinal medical species "Arfazetin" (**Table 1**).

Principles for Compilation of Medical Species

Due to the specific location of Russia, Russian herbal medicine has adopted the philosophy of Eastern traditional medicine and the pragmatic approach of Western medicine. One of the main principles for the compilation of the formulas used in traditional Chinese medicine is described in Shen-nong Ben-Cao Jing. An effective formula should be based on a strong monarch, accompanied by a minister, assistant, and guide, which mimics a well-organized society (Xin et al., 2014; Xutian et al., 2014). However, this principle is difficult to follow in practice, due to the multiple symptoms of diseases and polyfunctionality of medicinal plants. Therefore, many formulas of TCM contain secrets that are not always explained by rationality (Wang et al., 2021).

The philosophy and conceptualization for the compilation of medical species in Russian medicine are not well described. After a comprehensive medical examination of a patient, a Russian phytotherapeutic doctor initially prescribes a basic medical species, which includes the plants that lower blood glucose. The binary and triple combinations emphasized in this review can be regarded as basic mixtures. Diabetes is often accompanied by obesity. Therefore, the basic mixture is fortified with plants reducing hypercholesterolemia. In the case of hypertension, the

species include anti-hypertensive plants. The practical doctors also take into account the peculiarities of the gastrointestinal tracts of the patients. In this respect, medical species can include plants with astringent or laxative properties. To prevent allergic reactions, doctors recommend taking a basic mixture for a week and then continuously increasing the number of plants in the medical species one by one. Plants with antiallergic properties are sometimes included in the mixtures (Kovaleva, 1972; Ladynina and Morozova, 1987; Ladynina and Morozova, 1990).

This approach could be illustrated by the following medical species frequently cited in the literature. The species 4.39 (**Table 1**) includes a synergistic combination of *Vaccinium myrtillus* L. and *Galega officinalis* L., which effectively decreases glucose levels (Achilov, 2020; Kurylo et al., 2020). These basic plants have antidiabetic properties. *Frangula alnus* Mill. acts as a laxative, and *Betula pendula* Roth. acts as a diuretic (Belodubrovskaya et al., 2004). Another species, 4.40 (**Table 1**), besides the synergistic combination of *Vaccinium myrtillus* L. and *Galega officinalis* L., includes *Phaseolus vulgaris* L., which reduces plasma triacylglycerol and low-density lipoprotein, and lowers blood glucose and cholesterol (Pari and Venkateswaran, 2004; Sidorova et al., 2017; Micheli et al., 2019). These basic plants ensure the antidiabetic effect, while *Mentha* × *piperita* L. additionally provides anti-hypertensive, antiallergic, and spasmolytic effects (Mahendran and Rahman, 2020). The species 5.10 (**Table 1**) comprises 5 plants. The power of the basic mixture of the synergistic combination of *Vaccinium myrtillus* L. and *Galega officinalis* L. and *Phaseolus vulgaris* L. is reinforced by *Taraxacum campylodes* G.E. Haglund., which inhibits adipogenesis and fat accumulation (García-Carrasco et al., 2015). Additionally, *Urtica dioica* L. potentiates the activity of insulin and enhances the utilization of glucose (El Haouari and Rosado, 2019). We understand that the interpretation of the rationality of the above-mentioned medical species compilation took into account the results of modern research. Apparently, the architects of the discussed medical species compiled them according to their own experience and knowledge. Similar to the TCM formulas, some Russian medical species also have secrets that are yet to be deciphered. Nevertheless, the knowledge and practical experience of Russian traditional medicine were successfully utilized for the development of medical species used in officinal medicine.

Medical Species Used in Russian Officinal Medicine

In Russia, medical species are part of officinal medicine. Although medical species are available as OTC products, consultations with phytotherapeutic doctors are helpful and will lead to more effective results. Among the 227 medical species discussed in this review, only two, "Arfazetin" and "Myrphasinum", are approved for use in officinal medicine. Both species are recommended in the mild form of diabetes. The medical species "Arfazetin" was developed in the All-Union Institute of Medicinal and Aromatic Plants and was approved for medicinal use in 1986 (Ferubko et al., 2016). "Arfazetin" comprises seven medicinal plants (**Table 1**). In 1992, the composition of species was revised. The roots of *Aralia elata* (Miq.) Seem (syn. *Aralia mandshurica* Rupr. et Maxim.) or roots and rhizomes of *Oplopanax elatus* (Nakai) Nakai were excluded. Instead of these plants, the roots and rhizomes of *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim. were included in species at the same rate. A new species was named "Arfazetin-E". Both these species ("Arfazetin" and "Arfazetin-E") are now included in the State register (2021).

"Myrphasinum" was developed in 1985 by scientists from the first Moscow medical institute, named after I.M. Sechenov (Fas'kov et al., 1991), and was approved for medicinal use in 1991. The composition is complicated and includes the 12 medicinal plants (**Table 1**).

According to the regulatory requirements of the USSR/Russia, medical species are subject to preclinical and clinical evaluations of safety and efficacy.

Preclinical and Clinical Data

The efficacy of "Arfazetin" was studied in several experiments *in vivo*. Rats with alloxan-induced diabetes were administered an infusion of "Arfazetin" (10 ml/kg per day, orally) five days before alloxan injection and seven days after injection. Distilled water was administered in the control group. The blood glucose level, elevated by alloxan, was decreased in "the Arfazetin"-treated rats by 24 and 38% when compared with control on the third and seventh days, respectively, after alloxan injection. The total cholesterol, creatinine, and malondialdehyde in the blood and liver were decreased by 27, 37, 30, and 30%, respectively, compared with the control group on the seventh day after treatment. The treatment of rats with "Arfazetin" led to an increase in serum immunoreactive insulin and C-peptide after glucose load by 22 and 55%, respectively, when compared with the control group (Azhunova et al., 2001). Similar results were observed in a prolonged study. The oral administration of "Arfazetin" (5 ml/kg of infusion) to rats with alloxan-induced diabetes over 30 days (7 days before alloxan induction and 23 days after injection) resulted in decreases in blood glucose of 46 and 39%, respectively, compared with the control group on the 15th and 30th days of the experiment. On Day 30, glycogen in the liver was increased by 17% when compared with the control group (Ishankulova et al., 2013). A further study by the same group evidenced the lipid-lowering properties of "Arfazetin" (infusion, 5 ml/kg, orally). The total cholesterol in the blood decreased by 33% when compared with the negative control after 30 days of the treatment of rats with alloxan-induced diabetes. The levels of triglycerides and low- and high-density lipoproteins normalized and were equal to those in the intact group after 30 days of treatment with the infusion of "Arfazetin" (Ishankulova and Yuldasheva, 2019). In another study, the effects of "Arfazetin" on energy metabolism in rats were reported. Rats with alloxan-induced diabetes were treated with the infusion of "Arfazetin" (10 ml/kg, orally, daily) for 21 days. The control group received the same volume of distilled water. The treatment of rats with "Arfazetin" resulted in a double increase in adenosine triphosphate production in the liver when compared with control, starting from the seventh day of the experiment. The

concentration of lactic acid decreased by 1.7 fold, while the activity of pyruvate kinase increased by 1.5 fold when compared with the control group after 21 days of treatment. The authors suggest positive effects of "Arfazetin" on energy metabolism (Lemza et al., 2014). The blood glucose in rats with alloxan-induced diabetes was decreased by 3.2 fold when compared with control at 3 h after the intragastric administration of a dry extract of the medical species "Arfazetin" (1,200 ng/kg). The efficacy of the extract was equal to that of gliclazide (50 mg/kg, intragastric administration) (Kvasova, 2011).

The acute and chronic toxicity of "Arfazetin" was studied in mice after oral administration. The LD_{50} for the dry extract of "Arfazetin" was 24 g/kg (acute toxicity). No signs of toxicity were observed in the mice after 30 days of the administration of the "Arfazetin" infusion and dry extract at 1,200 mg/kg (there times a day every 4 h) (Kvasova et al., 2010).

The antidiabetic potential of the medical species "Myrphasinum" was investigated in rats. Diabetes was modulated by the subcutaneous injection of alloxan. The glucose level in the blood was increased from 5.6 mmol/L (intact group) to 9.55 mmol/L, body weights were decreased, and the rats had no appetite. The aqueous infusion of "Myrphasinum" (25 g/L) was administered to rats by an intragastric route at the dose of 5 ml/kg three times per day for two weeks. The control group was administered saline. The blood glucose in the treated group was decreased to 3.4 mol/L (vs. an increase up to 10.7 ml/L in the control group) two weeks after the beginning of treatment. The body weights and appetite were improved. Meanwhile, 25% of the animals in the control group died. The animals were observed for five extra weeks after the end of treatment with "Myrphasinum". Three weeks after the end of treatment, the blood glucose in the treated group was equal to that in the intact group (5.77 mmol/L) and was stable until the fifth week (Grinkevich et al., 1997). In another study, outbred rats with alloxan-induced diabetes were orally administered 10 ml/kg of an infusion of "Myrphasinum" 3 times a day for three weeks. The control group received the same dose of normal saline. The treatment with "Myrphasinum" resulted in a statistically significant decrease in blood glucose by 26% compared with control. Glycogen in the liver and skeletal muscles was increased by 35 and 21% respectively, when compared with control and was equal to the level in an intact group (Dzhafarova, 2013). Subsequently, the efficacy of "Myrphasinum" in outbred rats with alloxan-induced diabetes was evaluated. The rats were treated orally with 10 ml/kg of an infusion of ""Myrphasinum" 3 times a day. The control group received normal saline. Metformin (5 mg/kg, 2 times a day) served as a positive control. The administration of "Myrphasinum" for 21 days did not affect the body weights of the rats, and no signs of toxicity were observed. The level of glucose in the "Myrphasinum"-treated group was decreased by 75% compared with control (vs. 59% in the metformin group). The insulin and C-peptide levels were dramatically reduced by 3 and 3.3 fold, respectively, in rats after alloxan injection in those treated with "Myrphasinum" (vs. 1.5 and 1.7 fold increases, respectively, in the animals treated with metformin) (Jafarova and Garayev, 2013).

We have found, in the available literature, only a few publications about clinical trials with medical species. The efficacy of "Arfazetin" was studied in a group of 32 patients (18–65 years old) with types I and II diabetes mellitus. "Arfazetin" was prepared in the form of an aqueous infusion (10 g in 400 ml) and administered in warm form at the dose of 1/3 glass, three times a day, 30 min before meals, for one month. The patients with type I diabetes (12 persons) were administered "Arfazetin" in combination with an appropriate dose of insulin and diet. The five patients with a mild form of type II diabetes were administered "Arfazetin" in combination with an appropriate diet. The group of 15 patients with a moderate form of type II diabetes were administered "Arfazetin" in combination with an appropriate dose of hypoglycemic drugs and diet. In the patients with type I diabetes treated with "Arfazetin", a statistically significant decrease in blood glucose (by 38%) was registered at 11.00 pm when compared with 9.00 am of the same day. The effect was not cumulative. More pronounced results were observed in patients with type II diabetes. "Arfazetin" effectively ameliorated hyperglycemia. The doses of hypoglycemic drugs were reduced in 7 patients. In two patients, it was possible to maintain normal blood glucose levels without hypoglycemic drugs (Korotkova et al., 1988).

A "Myrphasinum" infusion was used in clinical praxis for the therapy of patients with and without diabetes decompensation. The treatment of patients with diabetes in the compensation stage resulted in statistically significant decreases in glucose by 15 and 44% in the blood and urine, respectively. Cholesterol and B-lipoproteins were decreased by 18 and 21%, respectively. The effects of "Myrphasinum" in patients with diabetes in the decompensation stage were less pronounced (Fas'kov et al., 1991). However, the data provided in the inventor's certificate are limited and lacking other details.

The comparative efficacy of "Arfazetin" and "Myrphasinum" was studied in 57 patients with diabetes (22–76 years old) in an open clinical trial. The first group (26 persons) was treated with "Arfazetin", while the second group (31 persons) received "Myrphasinum". Basic therapy includes oral hypoglycemic drugs. "Myrphasinum" was considered more effective and resulted in a statistically significant decrease in blood glucose, surpassing "Arfazetin" in efficacy (Firsova et al., 1990). However, no more details were provided in this conference paper.

CONCLUSION

In this review, we analyze the compositions and potential of medical species used in Russian traditional and officinal medicine for the treatment of diabetes and related diseases. Several species besides medicinal plants contain fresh juices from berries, birch sap, and seaweeds. Another aspect of medical species is the presence of adaptogens. The philosophy and conceptualization for the compilation of medical species in Russian medicine are not well described. We have highlighted the most common binary and triple combinations of plants exploited in medical species. These combinations can be considered base mixes. Other plants are added to the mixtures to improve the efficacy, treat associated disorders, improve gastrointestinal function, prevent allergic reactions, etc. Obviously, Russian phytotherapeutic doctors

compile polyherbal mixtures according to their own experience and knowledge. Modern studies of the mechanisms of action and predicted activities of the principal compounds from medicinal plants support the rationality of polyherbal mixtures. However, the mechanisms are not well studied and reported due to the limited number of compounds. Deeper investigations including gene expression will enable a better understanding of molecular mechanisms and targets. Although a few studies have evidenced possible additive/synergistic effects of herbal mixtures, additional investigations with calculations of synergistic or additive indices will assist in providing a scientific foundation for the wider use of medical species for the therapy of diabetes. Even though most medical species comprise mixtures of three to six plants, other species also deserve careful study. It appears to us that the species with seven or more plants have rationality that is difficult to explain and some secrets that are yet to be deciphered. On the other hand, modern good praxis rules require the identification of all the plants in medical species. An increase in plants in the mixture requires advanced techniques for quality control. Notably, two medical species approved for use in officinal medicine include 7 and 12 plants. The efficacy of these species was investigated *in vivo*. However, all the activities were proved using only one model of alloxan-induced diabetes. Clinical trials were completed in small groups, and several details are not indicated in the reports. According to modern regulatory rules, additional pharmacological experiments and clinical trials are required for more detailed investigations of the mechanisms of action and the confirmation of efficacy. We believe that the scientifically based utilization of rich plant resources and knowledge of Russian herbal medicine can significantly contribute to the local economy as well as to the sectors seeking natural healing products.

AUTHOR CONTRIBUTIONS

AS, AA, and OP designed the study. AS, AA, ON, VL and OP collected information on the medical species. AS, AA, IM and OP conducted the literature search, extracted the data, and wrote the first draft. AA and MP collected information from web resources. ON, EF and IN oversaw the research project, including checking the research work, reviewing, and interpreting the results. All authors are involved in reviewing and approval of the final manuscript.

REFERENCES

Abidov, M. T., del Rio, M. J., Ramazanov, T. Z., Klimenov, A. L., Dzhamirze, S., and Kalyuzhin, O. V. (2006). Effects of Aralia Mandshurica and Engelhardtia Chrysolepis Extracts on Some Parameters of Lipid Metabolism in Women with Nondiabetic Obesity. *Bull. Exp. Biol. Med.* 141 (3), 343–346. doi:10.1007/s10517-006-0167-3

Achilov, D. D. (2020). *Hypoglycemic Properties of Phytobiabetol Obtained Based on Plants Vaccinium Myrtillus L., Galega Oficinalis L.* Thesis of PhD dissertation. Tashkent, Uzbekistan. Available at: http://repository.tma.uz/jspui/handle/1/801.

Ahangarpour, A., Heidari, H., Oroojan, A. A., Mirzavandi, F., Nasr Esfehani, K., and Dehghan Mohammadi, Z. (2017). Antidiabetic, Hypolipidemic and Hepatoprotective Effects of Arctium Lappa Root's Hydro-Alcoholic Extract on Nicotinamide-Streptozotocin Induced Type 2 Model of Diabetes in Male Mice. *Avicenna J. Phytomed.* 7 (2), 169–179.

Ahn, J., Um, M. Y., Lee, H., Jung, C. H., Heo, S. H., and Ha, T. Y. (2013). Eleutheroside E, an Active Component of *Eleutherococcus Senticosus*, Ameliorates Insulin Resistance in Type 2 Diabetic Db/db Mice. *Evid. Based Complement. Alternat. Med.* 2013, 934183. doi:10.1155/2013/934183

Akamova, A. V., Nemyatykh, O. D., and Narkevich, I. A. (2017). Multiple View Marketing Analysis of the Russian Plant-Based Drugs Market. *Drug Dev. registration* 4 (21), 276–280.

Almuaigel, M. F., Seif, M. A., Albuali, H. W., Alharbi, O., and Alhawash, A. (2017). Hypoglycemic and Hypolipidemic Effects of Aqueous Extract of Phaseolus Vulgaris Pods in Streptozotocin-Diabetic Rats. *Biomed. Pharmacother.* 94, 742–746. doi:10.1016/j.biopha.2017.07.135

Anzar, M. A. (2013). Drugs Indicated for the Management of Ziabetus Shakri (Diabetes Mellitus) in Unani Medicine-An Overview. *Int. Jour. Pharmamedix India* 1 (3), 460–474.

Arai, Y.-C., Makino, I., Ikemoto, T., Saisu, H., Terajima, Y., and Owari, K. (2020). Kampo for the Treatment of Pain in Japan: A Review. *Pain Ther.* 9 (1), 161–170. doi:10.1007/s40122-020-00160-w

Arokiyaraj, S., Balamurugan, R., and Augustian, P. (2011). Antihyperglycemic Effect of Hypericum perforatum Ethyl Acetate Extract on Streptozotocin-Induced Diabetic Rats. *Asian Pac. J. Trop. Biomed.* 1 (5), 386–390. doi:10.1016/S2221-1691(11)60085-3

Avci, G., Kupeli, E., Eryavuz, A., Yesilada, E., and Kucukkurt, I. (2006). Antihypercholesterolaemic and Antioxidant Activity Assessment of Some Plants Used as Remedy in Turkish Folk Medicine. *J. Ethnopharmacol.* 107 (3), 418–423. doi:10.1016/j.jep.2006.03.032

Azhunova., T. A., Lemza, S. V., and Linkhoyeva, E. I. (2001). Pharmacotherapeutic Efficacy of Complex Plant Remedy in Experimental Diabetes. *Bull. East. Siberian Scientific Cent. Siberian Department Russ. Acad. Med. Sci.* 1-2 (77), 104–108.

Bahrami, G., Miraghaee, S. S., Mohammadi, B., Bahrami, M. T., Taheripak, G., Keshavarzi, S., et al. (2020). Molecular Mechanism of the Anti-diabetic Activity of an Identified Oligosaccharide from Rosa Canina. *Res. Pharm. Sci.* 15 (1), 36–47. doi:10.4103/1735-5362.278713

Bailey, C. J. (2017). Metformin: Historical Overview. *Diabetologia* 60 (9), 1566–1576. doi:10.1007/s00125-017-4318-z

Balakirev, G. V., Gubanov, I. A., Kozina, E. M., Samokhina, E. B., Taranova, I. A., Tugunova, O., et al. (2010). *Travnik. Encyclopedia of Medicinal Plants.* G.A. Nepokoichitsky (editor-in-chief). 640. Moscow: Publishing House.

Banerjee, S., Bhattacharjee, P., Kar, A., and Mukherjee, P. K. (2019). LC-MS/MS Analysis and Network Pharmacology of Trigonella Foenum-Graecum - A Plant from Ayurveda against Hyperlipidemia and Hyperglycemia with Combination Synergy. *Phytomedicine* 60, 152944. doi:10.1016/j.phymed.2019.152944

Barbalho, S. M., Damasceno, D. C., Spada, A. P. M., Silva, V. S. D., Martuchi, K. A., Oshiiwa, M., et al. (2011). Metabolic Profile of Offspring from Diabetic Wistar Rats Treated with Mentha piperita (Peppermint). *Evidence-Based Complement. Altern. Med.* 2011, 1–6. doi:10.1155/2011/430237

Barnaulov, O. D. (2008). The Comparative Estimation Influence of Drugs from Russian Flora Plants on Insuline and Glucose Blood Level in Alloxane-Diabetic Rats. *Psychopharmacol. Biol. Narcol.* 8 (3–4), 2484–2490.

Bayani, M., Ahmadi-Hamedani, M., and Jebelli Javan, A. (2017). Study of Hypoglycemic, Hypocholesterolemic and Antioxidant Activities of Iranian Mentha Spicata Leaves Aqueous Extract in Diabetic Rats. *Iran J. Pharm. Res.* 16 (Suppl. l), 75–82.

Behzadi, A. A., Kalalian-Moghaddam, H., and Ahmadi, A. H. (2016). Effects of Urtica dioica supplementation on blood lipids, hepatic enzymes and nitric oxide levels in type 2 diabetic patients: A double blind, randomized clinical trial. *Avicenna J. Phytomed.* 6 (6), 686.

Belodubrovskaya, G. A., Blinova, K. F., Vandyshev, V. V., Zhokhova, E. V., Klemper, A. V., Komarova, M. N., et al. (2004). *Medicinal plant raw materials. Pharmacognosy: textbook*. Editors G.P. Yakovlev and K. Blinova. St. Petersburg: Spezlit, 765. [in Russia].

Bhat, M., Zinjarde, S. S., Bhargava, S. Y., Kumar, A. R., and Joshi, B. N. (2011). Antidiabetic Indian Plants: a Good Source of Potent Amylase Inhibitors. *Evidence-Based Complement. Altern. Med.* 2011, 1–6. doi:10.1093/ecam/nen040

Blinov, V. A. (2000). *Medicinal Plants for Diabetes Mellitus*. Moscow: Publishing house Raduga.

Bljajić, K., Petlevski, R., Vujić, L., Čačić, A., Šoštarić, N., Jablan, J., et al. (2017). Chemical Composition, Antioxidant and α-glucosidase-inhibiting Activities of the Aqueous and Hydroethanolic Extracts of Vaccinium Myrtillus Leaves. *Molecules* 22 (5), 703. doi:10.3390/molecules22050703

Bnouham, M., Merhfour, F. Z., Ziyyat, A., Mekhfi, H., Aziz, M., and Legssyer, A. (2003). Antihyperglycemic Activity of the Aqueous Extract of Urtica Dioica. *Fitoterapia* 74 (7–8), 677–681. doi:10.1016/s0367-326x(03)00182-5

Bogdanova, O., and Bashkirova, N. A. (2010). The Big Book of Diabetics: Everything You Need to Know about Diabetes: Moscow: AST. St. Petersburg: Prime-Euroznak, 280. [In Russian].

Bouzghaya, S., Amri, M., and Homblé, F. (2020). Improvement of Diabetes Symptoms and Complications by an Aqueous Extract ofLinum usitatissimum (L.) Seeds in Alloxan-Induced Diabetic Mice. *J. Med. Food* 23 (10), 1077–1082. doi:10.1089/jmf.2019.0205

Brusenskaya, I. V., and Kaz'min, V. D. (2005). *Treatment by Traditional Medicines*. Rostov on the Don: Phoenix. [In Russian].

Bubenchikova, V. N., Bubenchikov, A. A., and Filippenko, N. G. (2003). *Home Doctor Book: Phytotherapy in Your House*. Moscow, RIPOL CLASSIC. [In Russian].

Centers for Disease Control and Prevention (2020). *National Diabetes Statistics Report, 2020*. Atlanta, GA: Centers for Disease Control and Prevention, US Department of Health and Human Services. Available at: https://www.cdc.gov/diabetes/pdfs/data/statistics/national-diabetes-statistics-report.pdf.

Chekina, N. A., Chukaev, S. A., and Nikolaev, S. M. (2010). Diabetes Mellitus: Possibilities of Pharmacotherapy Using Plant Remedies. *Vestnik Buryatskogo Gos Universiteta* 12, 71–78.

Chen, M., Xu, J., Wang, Y., Wang, Z., Guo, L., Li, X., et al. (2020). Arctium Lappa L. Polysaccharide Can Regulate Lipid Metabolism in Type 2 Diabetic Rats through the SREBP-1/SCD-1 axis. *Carbohydr. Res.* 494, 108055. doi:10.1016/j.carres.2020.108055

Chirkov, A. I., and Seryi, V. S. (1993) Medical Species (Monography). Moscow: Medicine, 287. [In Russian].

Christensen, K. B., Minet, A., Svenstrup, H., Grevsen, K., Zhang, H., Schrader, E., et al. (2009). Identification of Plant Extracts with Potential Antidiabetic Properties: Effect on Human Peroxisome Proliferator-Activated Receptor (PPAR), Adipocyte Differentiation and Insulin-Stimulated Glucose Uptake. *Phytother. Res.* 23 (9), 1316–1325. doi:10.1002/ptr.2782

Cignarella, A., Nastasi, M., Cavalli, E., and Puglisi, L. (1996). Novel Lipid-Lowering Properties of Vaccinium Myrtillus L. Leaves, a Traditional Antidiabetic Treatment, in Several Models of Rat Dyslipidaemia: a Comparison with Ciprofibrate. *Thromb. Res.* 84, 311–322. doi:10.1016/s0049-3848(96)00195-8

Davydovich, M. G., Kostarev, O. V., and Farkhutdinov, R. G. (2008). *Phytotherapy during Diabetes Mellitus*. Ufa: Gilem.

Dedov, I. I., Shestakova, M. V., Vikulova, O. K., Zheleznyakova, A. V., and Isakov, M. A. (2018). Diabetes Mellitus in Russian Federation: Prevalence, Morbidity, Mortality, Parameters of Glycaemic Control and Structure of Glucose Lowering Therapy According to the Federal Diabetes Register, Status 2017. *Diabetes mellitus* 21 (3), 144–159. doi:10.14341/DM20171S810.14341/dm9686

Dmitriev, A. I., Smolyansky, B. L., Kreknin, A. A., Mironets, T. N., and Vizgalova, I. I. (1994). Nutrition and phytotherapy for diabetes mellitus. Dnepropetrovsk: OGTIP "Dneprkniga" (40 p.) [In Russian]

Dontsov, V. V., and Dontsov, I. V. (2000). *Medicinal Plants and Bee Products. More than 400 Recipes and Healing Species for All Diseases*. Moscow: Tsentrpoligraf Publishing house, OOO "Polus". [In Russian].

Dremova, N. B., Bubenchikova, V. N., Drozdova, I. L., and Dzharu, M. (2003). *Modern Medicines and Phytoterapy in the Complex Treatment and Prevention of Diabetes Mellitus : Educational and Methodical Manual*. Kursk, Russia: Publishing house of the Kursk State Medical University. [In Russian].

Dzhafarova, R. A. (2013). Study of Action of Phytocomplex "Antidiabet" and "Mirfazin" to Sugar in Blood Plasma and Glycogen in Liver and Muscle Tissue. *Bull. Russ. Mil. Med. Acad.* 4, 172–174.

Efimov, A. S., and Shcherbak, A. V. (1993). *Phytotherapy of Obesity and Diabet*. Naukova dumka, Phoenix: Kiev. [In Russian].

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 Haouari, M., and Rosado, J. A. (2018). Phytochemical, Anti-diabetic and Cardiovascular Properties of Urtica Dioica L. (Urticaceae): A Review. *Mrmc* 19 (1), 63–71. doi:10.2174/1389557518666180924121528

Fas'kov, M. N., Nikolaev, A. V., Grinkevich, N. I., Balandina, I. A., Mamedov, L. A., Firsova, S. V., et al. (1991). Remedy for the Treatment of Diabetes. *Inventor's certificate SU 1697820* A1–12.

Fattahi, A., Niyazi, F., Shahbazi, B., Farzaei, M. H., and Bahrami, G. (2017). Antidiabetic Mechanisms of Rosa Canina Fruits. *J. Evid. Based Complement. Altern Med.* 22 (1), 127–133. doi:10.1177/2156587216655263

Fedyukovich, N. I. (1998). *Modern Hypoglycemic Drugs*. Minsk: Universitetskoye. [In Russian].

Ferubko, E. V., Trumpe, T. E., Kurmanova, E. N., Kolkhir, V. K., Sidelnikova, G. F., Sidelnikov, N. I., et al. (2016). Human Use of FGBNU VILARs Phytopreparations. *Probl. Biol. Med. Pharm. Chem.* 19 (6), 11–15.

Filimonov, D. A., Lagunin, A. A., Gloriozova, T. A., Rudik, A. V., Druzhilovskii, D. S., Pogodin, P. V., et al. (2014). Prediction of the Biological Activity Spectra of Organic Compounds Using the PASS Online Web Resource. *Chem. Heterocycl. Comp.* 50 (3), 444–457. doi:10.1007/s10593-014-1496-1

Firsova, S. V., Kalinkina, M. A., and Koroleva, T. V. (1990). *Comparative Clinical Evaluation of Antidiabetic Herbal speciesAbstracts of the All-union Scientific Conference of Young Scientists and Students Dedicated to the 225 Anniversiary of the First MMI Youth for Practical Health Care*. Moscow, 65.

Ganesan, K., and Xu, B. (2017). Polyphenol-rich Dry Common Beans (Phaseolus vulgaris L.) and Their Health Benefits. *Ijms* 18 (11), 2331. doi:10.3390/ijms18112331

García-Carrasco, B., Fernandez-Dacosta, R., Dávalos, A., Ordovás, J., and Rodriguez-Casado, A. (2015). *In Vitro* Hypolipidemic and Antioxidant Effects of Leaf and Root Extracts of *Taraxacum Officinale*. *Med. Sci.* 3 (2), 38–54. doi:10.3390/medsci3020038

Ghadge, A. A., and Kuvalekar, A. A. (2017). Controversy of Oral Hypoglycemic Agents in Type 2 Diabetes Mellitus: Novel Move towards Combination Therapies. *Diabetes Metab. Syndr. Clin. Res. Rev.* 11, S5–S13. doi:10.1016/j.dsx.2016.08.009

Golalipour, M. J., and Khori, V. (2007). The Protective Activity of Urtica Dioica Leaves on Blood Glucose Concentration and β-cells in Streptozotocin-Diabetic Rats. *Pakistan J. Biol. Sci.* 10 (8), 1200–1204. doi:10.3923/pjbs.2007.1200.1204

Grechanyi, I. A. (2013). *Complete Reference Book of Medicinal Herbs and Healing Species: 600 Plants and species. Kharkiv: Family Leisure Club*. Belgorod: Book Club "Family Leisure Club". [In Russian].

Grinkevich, N. I., Nikolaev, A. V., Firsova, S. V., Balandina, I. A., and Mamedov, L. A. (1997). The Study of Specific (Antidiabetic) Activity of Multicomponent Medical Species. *Scientific Proceedings All-union Scientific Research Institute Pharmacy*. Resource Pharmacognostic Study Medicinal Flora USSR Vol. XXV, 109–111.

Helmstädter, A. (2010). Beans and Diabetes: Phaseolus vulgaris Preparations as Antihyperglycemic Agents. *J. Med. Food* 13 (2), 251–254. doi:10.1089/jmf.2009.0002

Helmstädter, A., and Schuster, N. (2010). Vaccinium myrtillus as an antidiabetic medicinal plant–research through the ages. *Die Pharmazie* 65 (5), 315–321. doi:10.1691/ph.2010.9402

Hernández-Saavedra, D., Pérez-Ramírez, I. F., Ramos-Gómez, M., Mendoza-Díaz, S., Loarca-Piña, G., and Reynoso-Camacho, R. (2016). Phytochemical Characterization and Effect of Calendula officinalis, Hypericum perforatum, and Salvia Officinalis Infusions on Obesity-Associated Cardiovascular Risk. *Med. Chem. Res.* 25 (1), 163–172. doi:10.1007/s00044-015-1454-1

Husain, G. M., Chatterjee, S. S., Singh, P. N., and Kumar, V. (2011). Hypolipidemic and Antiobesity-like Activity of Standardised Extract of Hypericum perforatum L. In Rats. *ISRN Pharmacol.* 2011, 1–7. doi:10.5402/2011/505247

Hussain, Z., Waheed, A., Qureshi, R. A., Burdi, D. K., Verspohl, E. J., Khan, N., et al. (2004). The Effect of Medicinal Plants of Islamabad and Murree Region of Pakistan on Insulin Secretion from INS-1 Cells. *Phytother. Res.* 18 (1), 73–77. doi:10.1002/ptr.1372

Hwang, K.-A., Hwang, Y.-J., Kim, G. R., and Choe, J.-S. (2015). Extracts from Aralia Elata (Miq) Seem Alleviate Hepatosteatosis via Improving Hepatic Insulin Sensitivity. *BMC Complement. Altern. Med.* 15 (1), 1–9. doi:10.1186/s12906-015-0871-5

IDF (2019). *Diabetes Atlas*. Available at: https://www.diabetesatlas.org/en/ (Accessed March 26, 2012).

Ishankulova, B. A., and Yuldasheva, U. P. (2019). Comparative Study of Anti-diabetic Effect of Species "Marankhuch" and "Chordoru". Proceedings of the Conference "Medical Science of the XXI century – Looking towards the Future". *Dushanbe* 3, 69–70.

Ishankulova, B. A., Yuldasheva, U. P., and Urunova, M. V. (2013). Comparative Characteristics of Some Glucose-Lowering Plants and Anti-diabetic Collections of Tajikistan on Their Basis. *Avicenna Bull.* 1, 121125.

Jafarova, R. A., and Garayev, G. Sh. (2013). Comparative Pharmacological Study of the Effect of Collection of "Antidiabet" Galenical Medicines of mulberry Leaves and white and Grass clover Medicinalis on the Course of Alloxan Modeling Diabetes Mellitus. *Amea-nın Xıbırlıri*. Biologiy Aelmlıriseriyası 68, 125–130.

Kiseleva, T. L., and Chauzova, A. V. (1999). Development of Algorithm for Preparation of Medical Species Based on the Traditional Recipes. *Proceedings Book of the 3rd International Congress Actual Problems of Creation of New Medicinal Preparations of Natural Origin*. Pushkin: St-Petersburg, 211–215.

Kiyanova, I. V. (2005). *Holy apothecary. Treatment of metabolic disorders (diabet, obesity, thyroid diseases)*. Moscow: Orthodox Brotherhood of the Holy Apostle John the Theologian. [In Russian].

Klimakova, A. I., and Kazmanm, M. A. (1962). Echinopanax Tincture in Complex Therapy of Patients with Diabetes Mellitus. *Med. Preparations Plants*, 250–258.

Kolesova, V. G., Marchenko, V. A., and Syrovezhko, N. V. (1998). *Medicinal Plants: Myths and realityTraditional (Folk) Medicine in the Lens of Science*. St. Petersburg: SPCPA Publishing. [In Russian].

Končić, M. Z., and Bljajić, K. (2019).Traditional Herbal Products Used for the Management of Diabetes in Croatia: Linking Traditional Use with α-Glucosidase-Inhibitory Activity *Bioactive Food as Dietary Interventions for Diabetes*. United States: Academic Press, 647–664.

Korodetsky, A. V. (2006). Korodetskii's green Apothecary against Diabetes: Basketplant. *Stevia. Periwinkle*. St. Petersburg: Piter, 92–96. [In Russian].

Korotkova, V. D., Perelygina, A. A., Trumpe, T. E., Sokolov, S. Ia., and Lobanova, A. M. (1988). [Arfazetin in the Treatment of Diabetes Mellitus]. *Probl. Endokrinol (Mosk)* 34 (4), 25–28.

Korsun, V. F., and Korsun, E. V. (2010) *Phytotherapy: Tradition of Russian Herbalism*. Moscow: Eksmo. [In Russian].

Korsun, V. F., Trumpe, T. E., Korsun, E. V., Ershov, N. V., and Ogrenich, N. A. (2016). *Phytotherapy against Diabetes. Herbs of Life*. Moscow: ZAO Tsentrpoligraph Publishing House. [In Russian].

Kovaleva, N. G. (1972). *The Therapy with Plants*. Moscow: Medizina. [In Russian].

Kukes, V. G. (1999). *Phytotherapy with the Basics of Clinical Pharmacology*. Moscow: Medizina. [In Russian].

Kuo, D.-H., Hung, M.-C., Hung, C.-M., Liu, L.-M., Chen, F.-A., Shieh, P.-C., et al. (2012). Body Weight Management Effect of Burdock (Arctium Lappa L.) Root Is Associated with the Activation of AMP-Activated Protein Kinase in Human HepG2 Cells. *Food Chem.* 134, 1320–1326. doi:10.1016/j.foodchem.2012.03.023

Kurylo, K., Budniak, L., Volska, A., Zablotskyy, B., and Klishch, I. (2020). Influence of Phytocompositions on Dynamics of Changes in Basal Glycemia and Glycemia in Oral Glucose Tolerance Test in Rats with Streptozotocin-Nicotinamide-Induced Diabetes Mellitus Type 2. *Georgian Med. News* (300), 112–116.

Kurylo, K. I., Klishch, I. M., Nebesna, Z. M., Furdela, M. Y., Volska, A. S., and Lytvynyuk, S. O. (2018). Histological Changes in Liver and Kidneys in Experimental Type 2 Diabetes Mellitus and its Correction by Administration of Galega Officinalis L. Phytocompositions. *Womab* 14 (63), 133–136. doi:10.26724/2079-8334-2018-1-63-133-136

Kvasova, T. M., Demidova, M. A., and Amosov, V. V. (2010). Assessment of Acute and Chronic Toxicity of Dry Extract of the Collection Arfazetin. *Doctor -Aspirant* 6 (43), 158–162.

Kvasova, T. M. (2011). Effect of Arfazetin Medical Species on the Experimental Diabetes Mellitus in Rats. *Postgrad. Doctor* 5 (48), 157–162. doi:10.26.724/2079-8334-2018-1-63-133-136

Ladynina, E. A., and Morozova, R. S. (1987). *Phytotherapy*. 1st ed. Moscow: Medizina. [In Russian].

Ladynina, E. A., and Morozova, R. S. (1990). *Phytotherapy*. 2nd ed. Moscow: Medizina. [In Russian].

Ladynina, E. A. (2005). The Wisdom of Herbs. *Herbal Medicine and Homeopathy*. Moscow: AiF-Print. [In Russian].

Lager, A. A. (2002). *Herbs' Guide of Siberian Healer*. Moscow: RIPOL CLASSIC. [In Russian].

Lager, A. A. (1991). *Phytotherapy Diseases of the Digestive System*. Krasnoyarsk: Publishing house of Krasnoyarsk University. [In Russian].

Lagunin, A., Stepanchikova, A., Filimonov, D., and Poroikov, V. (2000). PASS: Prediction of Activity Spectra for Biologically Active Substances. *B*ioinformatics 16 (8), 747–748. doi:10.1093/bioinformatics/16.8.747

Lavrenova, G. V., and Lavrenov, V. K. (2007). *Complete Encyclopedia of Essential Medicinal Plants*. Donetsk: Stalker. [In Russian].

Lemza, S. V., Linkhoeva, E. G., Toropova, A. A., Azhunova, T. A., and Petrov, E. V. (2014). Influence of Plant Remedy «glucovit» on the Energy Status of Organism in Experimental Diabetes Mellitus. *Bulletin of the Buryat State University*, 12, 12–15.

Leporatti, M. L., and Ivancheva, S. (2003). Preliminary Comparative Analysis of Medicinal Plants Used in the Traditional Medicine of Bulgaria and Italy. *J. Ethnopharmacology* 87 (2–3), 123–142. doi:10.1016/s0378-8741(03)00047-3

Letova, I. (2019). *Diabetes Mellitus: The Best Recepies of Health*. Saint-Petersburg: Dilia. [In Russian].

Li, S., Fan, T.-P., Jia, W., Lu, A., and Zhang, W. (2014). Network Pharmacology in Traditional Chinese Medicine. *Evid Based. Complement. Altern. Med.* 2014, 1–2. doi:10.1155/2014/138460

Lisitsyn, A. F., and Molodozhnikova, L. M. (1989). *Phytotherapy: Eclectical Medicine: Collection of Medicinal Herbs.*VDNKh USSR Centralized pavilion "Health" Subject of Public Speaking "Green Cosmetics, Medicinal Herbs and Sensible Nutricion" LK. Arnica, Moskow: VDNKh USSR Centralized pavilion Health. [In Russian].

Madić, V., Petrović, A., Jušković, M., Jugović, D., Djordjević, L., Stojanović, G., et al. (2021). Polyherbal Mixture Ameliorates Hyperglycemia, Hyperlipidemia and Histopathological Changes of Pancreas, Kidney and Liver in a Rat Model of Type 1 Diabetes. *J. Ethnopharmacol*. 265, 113210. doi:10.1016/j.jep.2020.113210

Mahendran, G., and Rahman, L. U. (2020). Ethnomedicinal, Phytochemical and Pharmacological Updates on Peppermint (Mentha × Piperita L.)-A Review. *Phytotherapy Res.* 34 (9), 2088–2139. doi:10.1002/ptr.6664

Makhlayuk, V. P. (1991). *Medicinal Plants in Traditional Medicine*. Saratov: Volga Book Publishing House. [In Russian].

Malgaonkar, M., Shirolkar, A., Murthy, S. N., and Pawar, S. (2016). Ayurvedic Plants with Antidiabetic Potential. *Medicinal Plants-Recent Advances in Research and Development*. Germany: Springer, 439–468. doi:10.1007/978-981-10-1085-9_19

Mashkovskii, M. D. (2002). *Medicinal Agents* Vol. 1. Moscow: Novaya Volna. [In Russian].

Matkovskaya, A. N., Trumpe, T. E., and Sokolov, S. I. A. (1988). *Phytotherapy in Combination Therapy of Diabetes Mellitus: Prelection*. Moscow: Central Institute for Advanced Medical Training Order of Lenin. [In Russian].

Maznev, N. I. (2005). *Diabetes Mellitus and Obesity: Profilactics and Threatment with Herbs*. Moscow: ASS-Center. [In Russian].

Maznev, N. I. (2014). *Stop – Diabet! 500 Recepies and Useful Tips*. Moscow: AST [In Russian].

Melik-Gusseinov, V. V., and Rekkandt, S. A. (2014). *Phytotherapy. A Reference Book for Using Medicinal Plants in Traditional and Alternative Medicine (Monography)*. Volgograd: VolGMU Publishing house. [In Russian].

Micheli, L., Lucarini, E., Trallori, E., Avagliano, C., Caro, C., Russo, R., et al. (2019). Phaseolus vulgaris L. Extract: Alpha-Amylase Inhibition against Metabolic Syndrome in Mice. *Nutrients* 11 (8), 1778. doi:10.3390/nu11081778

Minaeva, V. G. (1991). *Medicinal Plants of Siberia*. Novosibirsk: Nauka. Siberian branch [In Russian].

Molokovskii, D. S., Davydov, V. V., and Khegai, M. D. (2002). Antidiabetic Activities of Adaptogenic Formulations and Extractions from Medicinal Plants. *Rastitel'nye Resursy* 38, 15–28.

Mukherjee, P. K., Banerjee, S., and Kar, A. (2018). Exploring Synergy in Ayurveda and Traditional Indian Systems of Medicine. *Synergy* 7, 30–33. doi:10.1016/j.synres.2018.10.003

Nagalievska, M., Sabadashka, M., Hachkova, H., and Sybirna, N. (2018). Galega Officinalis Extract Regulate the Diabetes Mellitus Related Violations of Proliferation, Functions and Apoptosis of Leukocytes. *BMC Complement. Altern. Med.* 18 (1), 1–13. doi:10.1186/s12906-017-2079-3

Namdul, T., Sood, A., Ramakrishnan, L., Pandey, R. M., and Moorthy, D. (2001). Efficacy of Tibetan Medicine as an Adjunct in the Treatment of Type 2 Diabetes. *Diabetes Care* 24 (1), 176–177. doi:10.2337/diacare.24.1.176

Nazina, Yu. V. (2006). *Diabetes. The Best Recipes of Traditional Medicine from A to Ya*. Moscow: OLMA media group. [In Russian].

Newman, D. J., and Cragg, G. M. (2020). Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *J. Nat. Prod.* 83 (3), 770–803. doi:10.1021/acs.jnatprod.9b01285

Nikolaychuk, L. V. (1997). *Treatment of Diabetes mellitus with herbs*. Minsk: The modern word, 256. [In Russian].

Nikolaychuk, L. V., and Zubitskaya, N. P. (2003). *A 1000 Recepies for Patients with Diabetes Mellitus*. Minsk: Interpresservis, 160. [In Russian].

Nikultseva, T. M. (1994). *Phytotherapy: Collection of Folk Unconversional Methods of Treatments*. Moscow: TWIC, 384. [In Russian].

Ninomiya, K., Matsuda, H., Kubo, M., Morikawa, T., Nishida, N., and Yoshikawa, M. (2007). Potent Anti-obese Principle from Rosa Canina: Structural Requirements and Mode of Action of Trans-tiliroside. *Bioorg. Med. Chem. Lett.* 17 (11), 3059–3064. doi:10.1016/j.bmcl.2007.03.051

Niu, H.-S., Liu, I.-M., Cheng, J.-T., Lin, C.-L., and Hsu, F.-L. (2008). Hypoglycemic Effect of Syringin from Eleutherococcus Senticosus in Streptozotocin-Induced Diabetic Rats. *Planta Med.* 74 (02), 109–113. doi:10.1055/s-2008-1034275

Okovitiy, S. V., Napalkova, S. M., Povydysh, M. N., Luzhanin, V. G., Goncharov, M. Yu., and Yakovlev, G. P. (2018). Medicinal Plants as a Source of Perspective Pharmaceutical Substances for Correcting Carbohydrate Exchange Disorders. *Pharmacy* 67 (7), 8–13. doi:10.29296/25419218-2018-07-0

Onipko, V. D. (2002). *Comfortable Life and Diabetes Mellitus*. Saint-Petersburg: Ves [In Russian].

Orhan, N., Aslan, M., Hosbas, S., and Deliorman, O. D. (2009). Antidiabetic Effect and Antioxidant Potential of Rosa Canina Fruits. *Pharmacognosy Mag.* 5 (20), 309–315. doi:10.4103/0973-1296.58151

Orlova, E. A. (2001). *Phytotherapy: Medicinal Sacrament of Nature*. Moscow: Terra – Book Club. [In Russian].

Osetrov, V. D. (1993). *Alternative Herbal Medicine*. Kiev: Naukova Dumka. [In Russian].

Osetrov, V. D., and Shreter, A. I. (2001). *Herbalist for Women. A Practical Guide to Folk and Scientific Herbal Medicine and Homeopathy*. Moscow: MSP Publishing House. [In Russian].

Panossian, A. G., Efferth, T., Shikov, A. N., Pozharitskaya, O. N., Kuchta, K., Mukherjee, P. K., et al. (2021). Evolution of the Adaptogenic Concept from Traditional Use to Medical Systems: Pharmacology of Stress- and Aging-related Diseases. *Med. Res. Rev.* 41 (1), 630–703. doi:10.1002/med.21743

Panossian, A., Seo, E.-J., and Efferth, T. (2018). Synergy Assessments of Plant Extracts Used in the Treatment of Stress and Aging-Related Disorders. *Synergy* 7, 39–48. doi:10.1016/j.synres.2018.10.001

Parasuraman, S., Thing, G., and Dhanaraj, S. (2014). Polyherbal Formulation: Concept of Ayurveda. *Phcog. Rev.* 8 (16), 73–80. doi:10.4103/0973-7847.134229

Pari, L., and Venkateswaran, S. (2004). Protective Role ofPhaseolus Vulgarison Changes in the Fatty Acid Composition in Experimental Diabetes. *J. Med. Food* 7 (2), 204–209. doi:10.1089/1096620041224120

Pavlić, B., Teslić, N., Zengin, G., Đurović, S., Rakić, D., Cvetanović, A., et al. (2021). Antioxidant and Enzyme-Inhibitory Activity of Peppermint Extracts and Essential Oils Obtained by Conventional and Emerging Extraction Techniques. *Food Chem.* 338, 127724. doi:10.1016/j.foodchem.2020.127724

Petkov, V. D. (1982). *Modern Phytotherapy*. Sofia: Medizina I. Fizkultura Publications House. [In Russian].

Pharmacopoeia Castrensis Rossica (1765). *Military Pharmacopoeia Castrensis Rossica*. 1st Edition. Petropoli: Academia Scientiarum.

Pigulevskaya, I. S. (2018). *Everything You Need to Know about Diabetes: An Essential Book for Diabetics: Types of Diabetes, Principles of Treatment, Delicious and Healthy Dishes, Proper Exercises, Traditional Medicine, Herbal Medicine*. Moscow: Centerpolygraph. [In Russian].

Pirogov, S. A. (2008). *Secrets Of the Medicinal Species. Expirience Of a Work of Folk Phytopracticer*. Barnaul: Altai printing plant. [In Russian].

Podduev, P. S. (2001). *Medicinal Herbs and Traditional Methods of Treating Diseases: A Handbook*. Bryansk: Bryansk Regional Printing House. [In Russian].

Popov, A. P. (2004). *Doctor Book of Medicinal Herbs*. Ekaterinburg: U-Faktoria. [In Russian].

Povydysh, M. N., Luzhanin, V. G., Ivkin, D. Yu., Belousov, M. V., and Yakovlev, G. P. (2018). Prospects for the Use of Phytotherapeutic Agents for Disorders of Fat and Carbohydrate Metabolism. *Drug Dev. Registration* (3), 130–135.

Protasenya, N. I., and Vasilenko, Yu. V. (1992). *Medical Species (600 Prescriptions for the Treatment of the Most Common Diseases)*. Simferopol: Tavrida. [In Russian].

Register Russia (2021).On Line State Register of Medicinal Preparations by the Ministry of Public Health and Social Development of the Russian Federation. Available at: http://grls.rosminzdrav.ru/GRLS.aspx. [In Russian] (Accessed 2021).

Rendiuk, T. D. (2006). *The way to the health. Herbal teas*. Moscow: Drofa-Plus. [In Russian].

Ruzhenkova, I. V. (2014). *Phytotherapy of Diabetes Mellitus: Medicinal Plant Treatment*. Phoenix: Rostov on the Don. [In Russian].

Ryzhenko, V. I. (2007). *Medical Species*. Moscow: Onix. [In Russian].

Sabeva, N., Petkov, N., and Ogneva, V. (2004). Effect of Galega Officinalis Extracts on Glucose Homeostasis in Streptozotocin Diabetic Mice. *Comptes Rendus-Academie Bulgare des Sci.* 57 (2), 95–98.

Safonov, N. N. (2016). *Atlas of Medicinal Plants. 900 home Recipes that Will Help You Find health Gift Editions. Beauty and Health*. Moscow: Publishing House 'E'.

Said, O., Fulder, S., Khalil, K., Azaizeh, H., Kassis, E., and Saad, B. (2008). Maintaining a Physiological Blood Glucose Level with 'Glucolevel', a Combination of Four Anti-diabetes Plants Used in the Traditional Arab Herbal Medicine. *Evidence-Based Complement. Altern. Med.* 5 (4), 421–428. doi:10.1093/ecam/nem047

Said-Shah, R. (2001). *Spices that Heal*. St. Petersburg: VES Publishing House.

Salehi, B., Ata, A., V. Anil Kumar, N., Sharopov, F., Ramírez-Alarcón, K., Ruiz-Ortega, A., et al. (2019). Antidiabetic Potential of Medicinal Plants and Their Active Components. *Biomolecules* 9 (10), 551. doi:10.3390/biom9100551

Salehi Nowbandegani, A., Kiumarcy, S., Rahmani, F., Dokouhaki, M., Khademian, S., Zarshenas, M. M., et al. (2015). Ethnopharmacological Knowledge of Shiraz and Fasa in Fars Region of Iran for Diabetes Mellitus. *J. Ethnopharmacol.* 172, 281–287. doi:10.1016/j.jep.2015.06.017

Sammons, H. M., Gubarev, M. I., Krepkova, L. V., Bortnikova, V. V., Corrick, F., Job, K. M., et al. (2016). Herbal Medicines: Challenges in the Modern World. Part 2. European Union and Russia. *Expert Rev. Clin. Pharmacol.* 9 (8), 1117–1127. doi:10.1080/17512433.2016.1189326

Samylina, I. A., Ermakova, V. A., Bobkova, N. V., and Anosova, O. G. (2010). *Pharmacognosia. T. 3:* Medicinal Plant Raw Materials, Collections. Plant Powders. Medicines Based on Ground Plant Raw Materials. Moscow: GEOTAR-Media. [In Russian].

Sato, Y. (2004). The Treatment Strategy for Diabetes Mellitus by Kampo Medicine. *Kampo Med. Nihon Toyo Igaku Zasshi* 55 (6), 737–750. doi:10.3937/KAMPOMED.55.737

Seredin, R. M., and Sokolov, S. D. (1973). *Medichinal Plants and Their Usage*. Stavropol: Stavropol's Publishing house. [In Russian].

Shikov, A. N., Narkevich, I. A., Flisyuk, E. V., Luzhanin, V. G., and Pozharitskaya, O. N. (2021). Medicinal Plants from the 14th Edition of the Russian Pharmacopoeia, Recent Updates. *J. Ethnopharmacology* 268, 113685. doi:10.1016/j.jep.2020.113685

Shikov, A. N., Pozharitskaya, O. N., Kamenev, I. Yu., and Makarov, V. G. (2011). Medicinal and Spice Plants in Russia. *Z. Arznei&Gewurzpflanzen* 16, 135–137.

Shikov, A. N., Pozharitskaya, O. N., and Makarov, V. G. (2018). Challenges in the Investigation of Combinatory Modes of Action of Nutrients and Pharmaceuticals. *Synergy* 7, 36–38. doi:10.1016/j.synres.2018.10.002

Shikov, A. N., Pozharitskaya, O. N., Makarov, V. G., Wagner, H., Verpoorte, R., and Heinrich, M. (2014). Medicinal Plants of the Russian Pharmacopoeia; Their History and Applications. *J. Ethnopharmacology* 154 (3), 481–536. doi:10.1016/j.jep.2014.04.007

Shikov, A. N., Tsitsilin, A. N., Pozharitskaya, O. N., Makarov, V. G., and Heinrich, M. (2017). Traditional and Current Food Use of Wild Plants Listed in the Russian Pharmacopoeia. *Front. Pharmacol.* 8, 841. doi:10.3389/fphar.2017.00841

Shojaee, S. S., Vahdati, A., Assaei, R., and Sepehrimanesh, M. (2015). Effect of Galega Officinalis Leaf Powder and Trigonella Foenum-Graecum Seed Powder on Blood Glucose Levels and Weight Gain in a Diabetes Mellitus Rat Model. *Comp. Clin. Pathol.* 24 (1), 145–148. doi:10.1007/s00580-013-1873-7

Sidorova, Y., Shipelin, V., Mazo, V., Zorin, S., Petrov, N., and Kochetkova, A. (2017). Hypoglycemic and Hypolipidemic Effect of Vaccinium Myrtillus L. Leaf and Phaseolus vulgaris L. Seed Coat Extracts in Diabetic Rats. *Nutrition* 41, 107–112. doi:10.1016/j.nut.2017.04.010

Sinyakov, A. F. (1992). *About shoots and roots*. Moscow: Physical education and sports, 272. [In Russian].

Sinyakov, A. F. (1999). *Herbal Treatment. A Practical Guide*. Moscow: JSC EKSMO-Press Publishing House. [In Russian].

Skalli, S., Hassikou, R., and Arahou, M. (2019). An Ethnobotanical Survey of Medicinal Plants Used for Diabetes Treatment in Rabat, Morocco. *Heliyon* 5 (3), e01421. doi:10.1016/j.heliyon.2019.e01421

Sklyarevsky, L. I. A., and Gubanov, I. A. (1989) *Medicinal Herbs in Daily Graft*. Moscow: Rosagropromizdat. [In Russian].

Smolianskii, B. L., and Lifliandskii, V. G. (2004). *Diabetes Mellitus Treatment*. Saint-Petersburg: Neva Publishing house. [In Russian].

Sokolov, S. Ya. (2000). *Phytotherapy and Phytopharmacology: A Guide for Physicians*. Moscow: Medical Information Agency. [In Russian].

Sokolov, S. Ya., and Zamotaiev, I. P. (1984). Guide to Medicinal Plants. *Phytotherapy*. Moscow: Medizina. [In Russian].

Stratton, C. F., Newman, D. J., and Tan, D. S. (2015). Cheminformatic Comparison of Approved Drugs from Natural Product versus Synthetic Origins. *Bioorg. Med. Chem. Lett.* 25 (21), 4802–4807. doi:10.1016/j.bmcl.2015.07.014

Suzuki, H., Sone, H., and Watanabe, K. (2017). Japanese Kampo Medicines for Diabetes Mellitus. *Japanese Kampo Medicines for the Treatment of Common Diseases:* Focus on Inflammation. Netherlands: Elsevier, 69–73. doi:10.1016/B978-0-12-809398-6.00008-1

Xutian, S., Tai, S., and Yuan, C. S. (2014). *Handbook of Traditional Chinese Medicine* (Singapore: World Scientific). (In 3 Volumes).

Taghizadeh, M., Rashidi, A. A., Taherian, A. A., Vakili, Z., Sajad Sajadian, M., and Ghardashi, M. (2016). Antidiabetic and Antihyperlipidemic Effects of Ethanol Extract of Rosa Canina L. Fruit on Diabetic Rats. *J. Evid. Based Complement. Altern Med.* 21 (4), NP25–NP30. doi:10.1177/2156587215612626

Takács, I., Szekeres, A., Takács, Á., Rakk, D., Mézes, M., Polyák, Á., et al. (2020). Wild Strawberry, Blackberry, and Blueberry Leaf Extracts Alleviate Starch-Induced Hyperglycemia in Prediabetic and Diabetic Mice. *Planta Med.* 86 (11), 790–799. doi:10.1055/a-1164-8152

Tarasenko, V. P., Ipatev, V. A., Shubin, V. A., Ivanina, E. V., Tarasenko, I. A., and Kholodilova, L. V. (1998). *Modern Phytotherapy*. Novosibirsk: The Science. Siberian enterprise RAS. [In Russian].

Tassa, B. D., Gogoi, G., and Das, S. (2012). A Comparative Study of the Hypolipidaemic and Antioxidant Activities of Ethanolic Extracts of Leaves of Phlogacanthus Thyrsiflorus, Oxalis Corniculata L. And Fragaria Vesca in Albino Rats. *Asian J. Pharm. Biol. Res.* 2, 12–18.

The State Pharmacopoeia of Russian Federation. (2018). Fourteenth ed. Moscow: Ministry of public health of Russian Federation. [In Russian].

Timmis, A., Townsend, N., Gale, C. P., Torbica, A., Lettino, M., Petersen, S. E., and De Smedt, D. (2020). European Society of Cardiology: Cardiovascular Disease Statistics 2019. *Eur. Heart J.* 41 (1), 12–85. doi:10.1093/eurheartj/ehz859

Tokgöz, H. B., and Altan, F. (2020). Hypericum perforatum L.: a Medicinal Plant with Potential as a Curative Agent against Obesity-Associated Complications. *Mol. Biol. Rep.* 47 (11), 8679–8686. doi:10.1007/s11033-020-05912-7

Tong, X.-L., Dong, L., Chen, L., and Zhen, Z. (2012). Treatment of Diabetes Using Traditional Chinese Medicine: Past, Present and Future. *Am. J. Chin. Med.* 40 (05), 877–886. doi:10.1142/S0192415X12500656

Trofimenko, A. G., and Mogilny, N. P. (1998). *Doctor Book*. Moscow: JSC ASTV. [In Russian].

Turishchev, S. N. (2005). *Phytotherapy for Everyone*. Moscow: OLMA-PRESS.Invest. [In Russian].

Turishchev, S. N. (2000). *Rational Phytotherapy: Herbal Therapy*. Moscow: Informpechat [In Russian].

Turova, A. D., and Sapozhnikova, E. N. (1989). *Medicinal Plants of USSR and Their Applications*. Moscow: Medizina [In Russian].

Ulrich-Merzenich, G. S. (2014). Combination Screening of Synthetic Drugs and Plant Derived Natural Products-Potential and Challenges for Drug Development. *Synergy* 1 (1), 59–69. doi:10.1016/j.synres.2014.07.011

Venkateswaran, S., Pari, L., and Saravanan, G. (2002). Effect ofPhaseolus Vulgarison Circulatory Antioxidants and Lipids in Rats with Streptozotocin-Induced Diabetes. *J. Med. Food* 5 (2), 97–103. doi:10.1089/10966200276017818

Vichkanova, S. A., Kolkhir, V. K., Sokolskaya, T. A., Voskoboinikova, I. V., and Bykov, V. A. (2009). *Medicinal Products from Plants*. Moscow: Scientific publication.ADRIS. [In Russian].

Vinogradova, T. A., GazhevVinogradov, B. N., Vinogradov, V. M., and Martynov, V. K. (2001). *Practical Phytotherapy*. Moscow: Valerie SPDEksmo-Press [In Russian].

Volynchenko, V. A. (2003). *Medical Herbal Species: 3600 Healing Recipies*. Donetsk Stalker Publishing house ACT. [In Russian].

Wang, J., Zhou, B., Hu, X., Dong, S., Hong, M., Wang, J., et al. (2021). Deciphering the Formulation Secret Underlying Chinese Huo-Clearing Herbal Drink. *Front. Pharmacol.* 12, 654699. doi:10.3389/fphar.2021.654699

Wouters, O. J., McKee, M., and Luyten, J. (2020). Estimated Research and Development Investment Needed to Bring a New Medicine to Market, 2009-2018. *Jama* 323 (9), 844–853. doi:10.1001/jama.2020.1166

Xiao, E., and Luo, L. (2018). Alternative Therapies for Diabetes: A Comparison of Western and Traditional Chinese Medicine (TCM) Approaches. *Cdr* 14 (6), 487–496. doi:10.2174/1573399813666170519103230

Xin, S., Jia, Y., and Huinan, Q. (2014). Research Progress and Prospect of Shennong Bencao Jing. *Chin. Arch. Traditional Chin. Med.* 32 (9), 2151–2154.

Xu, Y. X. Z., Xi, S., and Qian, X. (2019). Evaluating Traditional Chinese Medicine and Herbal Products for the Treatment of Gestational Diabetes Mellitus. *J. Diabetes Res.* 2019, 1–6. doi:10.1155/2019/9182595

Xue, J., Shi, Y., Li, C., and Song, H. (2019). Network Pharmacology-based Prediction of the Active Ingredients, Potential Targets, and Signaling Pathways in Compound Lian-Ge Granules for Treatment of Diabetes. *J. Cel. Biochem.* 120 (4), 6431–6440. doi:10.1002/jcb.27933

Yordanov, D., Nikolov, P., and Boychinov, A. S. P. (1972). Phytotherapy. Sofia: Medicine and Physical Education. [In Russian].

Zagayko, A. L., Chumak, O. I., Filimonenko, V. P., and Koshevoy, O. M. (2016). Investigation of the Effect of Dry Extracts from the Leaves of Cowberry and Bilberry Adding L-Arginine on Metabolic Parameters in Experimental Diabetes Type II. *Ukr. Biofarm. Ž.* 3 (44), 70–74. doi:10.24959/ubphj.16.43

Zeljković, Ć. S., Šišková, J., Komzáková, K., De Diego, N., Kaffková, K., and Tarkowski, P. (2021). Phenolic Compounds and Biological Activity of Selected Mentha Species. *Plants* 10 (3), 550. doi:10.3390/plants10030550

Zhang, J., Kang, M.-J., Kim, M.-J., Kim, M.-E., Song, J.-H., Lee, Y.-M., et al. (2008). Pancreatic Lipase Inhibitory Activity of taraxacum Officinale *In Vitro* and *In Vivo*. *Nutr. Res. Pract.* 2 (4), 200–203. doi:10.4162/nrp.2008.2.4.200

Zmeev, L. E. (1896). *Russkie Vrachebniki: A Study in the Field of Our Ancient Medical Writing*. Saint-Petersburg: Typography by Lamakov, 276. [In Russian].

Łabuda, H., Buczkowska, H., Papliński, R., and Najda, A. (2017). Secondary Metabolites of Phaseoli Pericarpium. *Acta Scientiarum Polonorum, Hortorum Cultus* 16 (6), 187–200. doi:10.24326/asphc.2017.6.17

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