PHYTOCHEMICAL CHANGES IN VEGETABLES DURING POST-HARVEST STORAGE AND PROCESSING, AND IMPLICATIONS FOR CONSUMER BENEFITS

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PHYTOCHEMICAL CHANGES IN VEGETABLES DURING POST-HARVEST STORAGE AND PROCESSING, AND IMPLICATIONS FOR CONSUMER BENEFITS

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Editorial: Phytochemical changes in vegetables during post-harvest storage and processing, and implications for consumer benefits

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Editorial on the Research Topic
Phytochemical changes in vegetables during post-harvest storage and processing, and implications for consumer benefits

The reduction of post-harvest losses is crucial to improving food and nutrition security by increasing food system efficiency and reducing production costs. Global food loss and waste at the retail and consumer level will be reduced by half by 2030 under Sustainable Development Goal 12.3. It also calls for reducing food losses along production and supply chains, including post-harvest losses. Developing countries suffer post-harvest losses of 15–30% due to quality standards set by retailers. The implementation of appropriate post-harvest technologies, such as cold chain management, modified atmosphere packaging, controlled atmosphere storage, and post-harvest treatments, helps in reducing post-harvest losses. For off-season use, seasonal vegetables are often preserved through post-harvest processes such as drying, dehydrating, pickling and fermentation. Processing, storage, and cooking affect phytochemicals (flavonoids, phenolic acids, anthocyanins, glucosinolates, carotenoids, and tocopherols) as well as health benefits. In fruit and vegetables, phytochemicals, otherwise known as non-nutritive compounds, enhance health due to their vital function in biological mechanisms. Growing knowledge of the chemo-preventive properties of fruits and vegetables has led to discussions about how to incorporate them into diets with modified recipes or include their functional ingredients to prevent non-communicable

diseases. However, there is limited information available on the effect of post-harvest storage and processing on health-promoting phytochemicals.

Aiming to reduce food losses, increase food affordability, and improve nutrition, especially for those in need, is the goal of implementing appropriate postharvest technologies. The Research Topic aims to explore how post-harvest storage and processing techniques affect health-promoting phytochemicals in exotic and underutilized vegetables, and how this influences consumers' gut microbiome and gut health. A key element in sustaining food and nutritional security at the rural level is the identification of postharvest preservation technologies for underutilized or indigenous or traditional fruits and vegetables. Promoting sweet potato roots and leaves or other traditional vegetables and fruits as part of the diet could contribute significantly to dietary diversity.

Sixteen research articles from imminent researchers in this field are included in this Research Topic on *Phytochemical Changes in Vegetables During Post-Harvest Storage and Processing*. There are several main topics that were researched and/or reviewed, including changes in phenolic compounds and biological activities of pumpkin leaves (*Cucurbita moschata* Duchesne ex Poir.) during blanching, drying of Butternut squash (*Cucurbita moschata* Duchesne ex Poir.), steaming and drying of *Cistanche deserticola* and cooking of Sunflowers (*Helianthus annuus* L.) sprouts. The application of steam blanching to leafy vegetables in plain water was recommended to improve the antioxidant capacity for rural communities. Freeze-drying is recommended to obtain leaf powders rich in functional compounds and bioactive properties for use as functional ingredients for commercial markets. Moreover, an article on long-term frozen storage of baby mustard (*Brassica juncea* var. gemmifera) or modified atmospheric packaging enabled the retention of health-promoting substances and well suited for marketing. Additionally, ascorbic acid and antioxidative activity of lamb lettuce (*Valerianella locusta*) "Vit" salad were affected by refrigerator storage. Different cooking models affected the composition of Crocetin glycosides in saffron plants (*Crocus sativus* L.) and gardenia fruits (*Gardenia jasminoides* Ellis). Furthermore, traditional chayote leaves fermented with pineapple fruit smoothies demonstrated higher antioxidant capacity during dialysis *in vitro* digestion and the fermented product is suitable for rural communities. Furthermore, fermented Date palm fruits (*Phoenix dactilyfera* L.) are an excellent source of folates for consumers in the Middle Eastern regions. An edible herbal, *Cornus officinalis*, was shown to be effective against fibrosis after high-pressure wine-steaming (HPWS).

This special issue aims to provide more information on post-harvest processing and storage of phytochemicals and functional compounds, as well as recommendations for adapting appropriate processing and cooking methods to ensure their biological activity for consumers.

Author contributions

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

Conflict of interest

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Changes in Phenolic Metabolites and Biological Activities of Pumpkin Leaves (*Cucurbita moschata* Duchesne ex Poir.) During Blanching

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Pumpkin leaves (*Cucurbita moschata* Duchesne ex Poir.) are popularly consumed in Sub-Saharan Africa and Asia. Blanching the leaves before drying is a method of preservation during off-season. In this study, different blanching treatments and media are used to test the changes in non-targeted phenolic compounds, antioxidant capacity (FRAP and ABTS activity), *in vitro* α-glucosidase activity and cell cytotoxicity of pumpkin leaves. Steam blanching in plain water led to the highest retention of total phenolic content and reduced the loss of quercetin 3-glucoside 7-rhamnoside (Rutin), kaempferol 7-neohesperidoside, isoorientin 2″-O-rhamnoside, isorhamnetin-3-O-rutinoside, quercetin 3-galactoside, coumaroyl glucaric acid, isorhamnetin-3-galactoside-6″-rhamnoside, 2-caffeoylisocitric acid, quercetin 3-galactoside 7-rhamnoside by (3.04%), (7.37%), (10.65%), (10.97%), (14.88%), (16.1%), (16.73%), (18.88%), and (23.15%), respectively, and coumaroyl isocitrate increased by 14.92%. Candidate markers, 2-O-caffeoylglucaric acid, 2-(E)-O-feruloyl-D-galactaric acid, quercetin 3-galactoside 7-rhamnoside (rutin) and unidentified compounds [(M-H) 677.28 and at RT 21.78] were responsible for the separation of the steam blanched samples in plain water from the other blanching treatments. Steam blanching in plain water increased the antioxidant capacity (FRAP and ABTS activity). There were no cytotoxic effect or inhibitory effect of α-glucosidase activity detected in the raw or blanched pumpkin leaves. Thus, this study recommends steam blanching in plain water for African cuisine, and confirms it is safe to consume pumpkin leaves frequently.

Keywords: leafy vegetable, phytochemicals, antioxidant activity, post-harvest processing, bioactivity

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INTRODUCTION

Pumpkins (*Cucurbita moschata* Duchesne ex Poir.) belong to the *Cucurbitaceae* family. Although pumpkins are indigenous to Mexico and Central America, due to their naturalization they are regarded as indigenous vegetables in the African region (1). Considered a healthy and functional vegetable, the consumption of pumpkin leaves is widespread in Korea, as well as in the Pacific Islands, India, and Bangladesh. Although pumpkins leaves play an important role in uplifting

household food security among rural people in Sub-Saharan Africa (2), limited information is available on their functional properties. In addition, Cucurbita maxima Duchesne are commonly used for traditional medicinal treatments (3).

Cucurbita moschata leaves are large alternate, simple, ovate-cordate- sub-orbicular dark green leaves with a width of 20–25 cm and length of 25–30 cm (4). One cup portion (39 g) of pumpkin leaves contain 15 mg of calcium (Ca), 170 mg of potassium (K), 41 mg of phosphorus (P), 0.87 mg of iron (Fe), and 14 μg of folate. Compared to a cup portion of pumpkin leaves, lettuce leaves contain lower amount of Ca (14.04 mg), Fe (0.34 mg), K (75.66 mg), and P (11.31 mg) (5). In the Southern African region, pumpkin leaves are eaten as part of a maize-based diet as a relish, or the leaves and the tender shoots are included in soups or stews (6). In Korean cuisine, the pumpkin leaves are used to wrap a piece of meat filling (pork or nay meat) ("ssam") (7).

Traditional vegetables are boiled mostly to improve their taste before consumption. Scientifically, blanching destroys the enzymes such as chlorophyllase and peroxidase, and prevents the loss of green color (8, 9). Traditionally, these vegetables are preserved in Africa during off seasons by adopting solar drying or shade drying; the vegetables are first blanched before being drying (10). Mkandawire and Masamba (10) showed that the vitamin C in the blanched vegetables improved when lemon juice in water was the blanching medium. Mkandawire and Masamba (10) used lemon juice as a blanching medium because the poor rural communities cannot afford commercial food additives. Raw pumpkin leaves showed stronger DPPH-, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)-radical scavenging activities, and ferric reducing antioxidant power (FRAP) compared to the fruit and the seeds (11). Polyphenols are antioxidants, known as antihypoglycemic agents, proven to lower the fasting blood sugar levels and the risk of type 2 diabetes (11). Dietary phenolic compounds inhibit the carbohydrate digestive enzymes, α-amylase and β-glucosidase (12) and reduce the re-absorption of glucose in the intestine. Type 2 diabetes is on the rise in Africa and is projected to increase to 41.5 million by 2035, and people between the ages of 40–59 are more vulnerable for type 2 diabetes (13).

No information is available on the changes in phenolic compounds present in pumpkin leaves during blanching or using lemon juice as a blanching medium the biological effects thereof. There is published research information available regarding different cooking methods on the antioxidant activity of some European vegetables (14), whilst the information on traditional vegetables is limited for consumers. Therefore, this study aims to provide the suitable blanching medium and the method that can minimize the loss of phenolic compounds for the commercial development of value-added functional plant-based diet products. The objective of this study was to investigate the effects of blanching methods such as (i) hot water dipping at 95°C in 5 or 10% lemon juice as blanching medium plain water as control, or (ii) steaming for 5 min using lemon juice solutions (5 or 10%) and plain water as the control on changes in phenolic metabolite and antioxidant properties in pumpkin leaves.

MATERIALS AND METHODS

Chemicals

Methanol, acetic acid, formic acid, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), hydrogen peroxide, disodium hydrogen phosphate heptahydrate; sodium dihydrogen phosphate monohydrate, enzyme porcine pancreatic solution, hydrochloric acid, ρ-nitrophenyl-α-D-glucoside solution, sodium carbonate, trolox, 2,2′-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic Acid), potassium persulfate, TPTZ [2,4,6-tris(2-pyridyl)-1,3,5-triazine], iron(III) chloride analytical standards (gallic, ferulic, vanillic, p-coumaric, elargic, gallic, 2.4 hydroxybenzoic, pyrogallol, protocatechuic, syringic acids, chlorogenic acid, catechin, epicatechin) at >95%, purity were purchased from Sigma Aldrich, Johannesburg, South Africa.

Sample Preparation

Pumpkin leaves were selectively harvested at suitable maturity from the smallholders farming scheme, Tshiombo in Vhembe District, Limpopo Province. After harvest, leaves free from decay or damage or insect infestation were selected for the experiment. After washing in running tap water, transportation of the leaves to the laboratory was within 6 h, in cooler boxes at 10°C, after which they were stored at 5°C in the cold room for 24 h prior to processing. Following this, 50 g of leaves were subjected to different blanching treatments given below.

Blanching Pre-treatment

There were 3 blanching treatments, (i) dipping in a 95°C water bath [thermostatically regulated water bath (PolyScience, Illinois, United States of America)], in plain water (control) or 5 or 10% lemon juice solutions (ii) steaming in a stainless-steel steamer pot for 5 min in plain water, or 5 or 10% lemon juice solutions (15).

After the blanching treatments, samples were cooled rapidly on ice to stop further biochemical reactions. Thereafter, the samples were freeze dried for untargeted and targeted phenolic metabolite analysis, *in vitro* antioxidant activity, α-glucosidase and cell cytotoxicity assay. Raw snap-frozen pumpkin leaves acted as a control, and each blanching treatment included 10 replicates for each analysis.

Changes in Phenolic Metabolite Profile

Phenolic untargeted metabolite profile was identified and quantified using a Waters Ultra-Performance Liquid Chromatograph (UPLC), fitted with a Waters Acquity Photodiode Array Detector (PDA) and linked to a Synapt G2 quadrupole time of flight mass spectrometer (Waters, Milford, MA, USA), as described by Managa et al. (15) and Ndou et al. (16), without any modifications. Phenolic compounds were extracted from freeze dried pumpkin leaves (50 mg) that underwent different blanching treatments by ultrasonication in 70% aqueous ethanol. Phenolic compounds from pumpkin leaves that underwent different blanching treatments were extracted using ultrasonication of 50 mg freeze-dried samples in 70% aqueous ethanol. Concentrations of the phenolic compounds were determined using the reference calibrants catechin (LOD 1.414333, LOQ 4.286), epicatechin (LOD

5.105, LOQ 15.469), and rutin (LOD 3,294; LOQ 9.981), to quantify compounds based on the areas of their extracted mass chromatograms. The respective calibration curves are given in **Supplementary Figure 1**.

The LOD and LOQ values for TargetLynx software processed the obtained data, as described previously by Managa et al. (15) and Ndou et al. (16), and the concentration of phenolic compounds was expressed as mg kg^{-1}.

In vitro Total Antioxidant Capacities

The Ferric reducing antioxidant power (FRAP) assay was carried out according to the method of Managa et al. (15) for traditional leafy vegetables. Pumpkin leaf samples (0.2 g) were homogenized in 2 mL sodium acetate buffer (pH 3.6), and 15 μL of the leaf exact was used in this assay. FRAP assay was determined by mixing the leaf extract with 220 μl FRAP reagent solution [10 mmol L^{-1} TPTZ [2,4,6-tris(2-pyridyl)-1,3,5-triazine] acidified with concentrated HCl, and 20 mmol l^{-1} $FeCl_3$]. Antioxidant power was expressed as mg of Trolox equivalent antioxidant content (TEAC) 100 g^{-1} FW.

2,2′-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) used for traditional vegetables by Managa et al. (15), was used for pumpkin leaves without any modifications. To prepare the $ABTS^+$ stock solution, 7.4 mM $ABTS^+$ solution and 2.6 mM potassium persulfate solution were mixed together (15) and held in darkness for 16 h at 25°C, then diluted with 0.1 mM phosphate buffer (pH 7.0) to obtain an absorbance at 734 nm (1.1 ± 0.002 units). The sample extract (15 μL), $ABTS^+$ stock solution (285 μL), was mixed and held in darkness at 25°C for 2 h, then the absorbance was measured at 734 nm. Calibration curves were constructed using Trolox as the standard, and the antioxidant activity (ABTS assay) was expressed as μmg of TEAC g^{-1} FW.

In vitro α-Glucosidase Inhibitory Activity

In vitro α-glucosidase inhibitory activity was determined according to the method described by Sagbo et al. (17) without any modifications using pumpkin leaf extract (5 μL). Pumpkin leaf extract was prepared at concentrations of 50–250 μg mL^{-1} mixed with 20 μL α-glucosidase solution (50 μg mL^{-1}) in a 96-well-plate. Thereafter, 60 μL of potassium phosphate buffer (pH 6.8; 67 mM) was pipetted into the mixture in 96-well-plate and held at 35°C for 5 min, then 10 μL of 10 mM ρ-nitrophenyl-α-D-glucoside solution (PNPGLUC) was pipetted and held at 35°C for 20 min and finally, 25 μL of 100 mM Na_2CO_3 was pipetted and the absorbance was measured at 405 nm using a micro-plate reader (CLARIOstar Plus BMG Labtec, Lasec, Cape Town, South Africa). The absorbance was measured for both the leaf extracts, acarbose and the blank control (without α-glucosidase). The IC_{50} value (i.e., the concentration of pumpkin leaf extracts from different blanching treatment and media that resulted in 50% inhibition of maximal activity) was determined.

Cell Cytotoxicity Using MTT Assay

Cell toxicity was measured using the method described by Moloto et al. (18) using (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) cytotoxicity assay and C2C12 myoblast cell (skeletal muscle) line, without any modifications. Cells with cell density of 1×10^5 cells mL^{-1} were cultured in a 96-well cell culture plate and thereafter treated with different concentrations (0.25–1.0 mg mL^{-1}) of the pumpkin leaf extracts (extracted by ultrasonication of 50 mg freeze-dried samples using 70% aqueous ethanol) and incubated at 37°C for 24 h. Thereafter, an aliquot of 20 μL of 5 mg mL^{-1} MTT was pipetted into each well and incubated at 37°C for an additional 4 h to allow the conversion of MTT to the colored formazan. The untreated cells were included as control; H_2O_2 (0.25–2.5%) was used as positive control. Cell cytotoxicity was read at 570 nm using a microtitre-plate multimode detector (Promega-Glomax Multi-detection system, Madison, WI 53711, USA), using the formula below; the blank well-included only the medium.

$$\% \text{ Viable cells } = \frac{abs\ sample - abs\ blank}{dabs\ control - abs\ blank}\ x100$$

Statistical Analysis

A completely randomized design was adopted with 10 replicates per blanching treatment and the experiments repeated twice. A factorial analysis experiment was performed with different blanching treatments and the type of blanching media on phenolic compounds and *in vitro* antioxidant activity Two-way analysis of variance (ANOVA) was used to test the significant differences between the different blanching treatment and blanching media. Means were compared among the treatments by the least significant difference (LSD) test, with $p < 0.05$, using the Genstat statistical program for Windows, 13th Edition (2010) (VSN International Hempstead, UK). The UPLC-QTOF/MS data were used for unsupervised principal component analysis (PCA). Thereafter, supervised Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) determined the compounds (candidate markers) responsible for the observed separation between the treatments and blanching media, as described previously by Managa et al. (15).

RESULTS AND DISCUSSION

Changes in Non-targeted Phenolic Metabolites in Pumpkin Leaves During Blanching

Table 1 reveals the tentative identification of 24 phenolic compounds in pumpkin leaves using the UPLC-QTOF/MS. The majority (83.3%) of the phenolic compounds identified were glycosylated and the remaining portion were tetracarboxylic acids and derivatives (16.7%). Of the glycosylated phenolic compounds, flavonoid O-glycosides were the most abundant, constituting 41.7%, and simple phenolic glycosides made up 12.5% of the total phenolic compounds identified. The remaining glycosylated compounds identified were glucuronic acid derivatives, hydroxycinnamic acid glycosides and isoflavonoid O- glycosides, which constituted 12.5, 8.3, and 4.2% of the phenolic compounds identified, respectively.

Based on UPLC–QTOF/MS analysis, the ESI MS of peak 1 showed an $[M\text{-}H]^-$ ion at *m/z* 315.0657. In the MS/MS spectrum, a base peak ion was observed at *m/z* 108[M-H-162-45] due to subsequent loss of a sugar moiety and the cleavage of a

TABLE 1 | Tentative peak assignment of the phenolic metabolites present in pumpkin leaves subjected to different blanching treatments using different types of blanching medium.

Compound	Retention time (min)	$[M-H]^-$	M-H formula	Error (ppm)	MSE fragments	UV	Tentative identification
1	8.03	315.0720	$C_{13}H_{15}O_9$	0.32	108.0212 151.9972	314	Gentesic acid 5-O-glucoside
2	8.12	371.0605	$C_{15}H_{15}O_{11}$	4.04	191.0259 209.0361	323	2-O-caffeoylglucaric acid
3	8.78	299.0762	$C_{13}H_{15}O_8$	3.24	137.0240 93.0339	326	Pseudolaroside A
4	9.24	369.0478	$C_{15}H_{13}O_{11}$	4.06	179.0393 188.9615 191.0090	326	2-O-Caffeoylhydroxycitric acid
5	9.56	385.0776	$C_{16}H_{17}O_{11}$	0.00	85.0291 385.1417	320	2-(E)-O-feruloyl-D-galactaric acid isomer
6	10.08	285.0606	$C_{12}H_{13}O_8$	3.5	108.02245	293	Diphenol glucuronide
7	10.23	341.0874	$C_{15}H_{17}O_9$	1.17	135.0494 179.0366 221.0372 281.0751	331	1-O-Caffeoylglucose
8	10.69	355.0667	$C_{15}H_{15}O_{10}$	1.12	163.0501 209.0332	314	Coumaroyl glucaric acid
9	11.15	385.0772	$C_{16}H_{17}O_{11}$	1.04	85.02256 385.0714	320	2-(E)-O-feruloyl-D-galactaric acid isomer
10	12.03	325.0927	$C_{15}H_{17}O_8$	0.62	146.0301 179.0382 119.0482	324	1-O-p-Coumaroyl-beta-D-glucose
11	12.95	593.1508	$C_{27}H_{29}O_{15}$	0.67	269.0809	324	Luteolin 7-neohesperidoside
12	13.45	353.0502	$C_{15}H_{13}O_{10}$	3.40	191.0261	331	2-caffeoylisocitric acid
13	15.52	447.0930	$C_{21}H_{19}O_{11}$	0.67	147.0478 299.7440	270	Quercitrin
14	15.63	337.0552	$C_{15}H_{13}O_9$	3.86	119.0431 191.0261	314	Coumaroyl isocitrate
15	16.67	769.2119	$C_{23}H_{45}O_{28}$	−2.08	300.0241 315.0345	330	7-Methylquercetin-3-Galactoside-6″-Rhamnoside-3‴-Rhamnoside
16	16.74	367.0648	$C_{16}H_{15}O_{10}$	6.27	173.0066 111.0049	329	Feruloyl isocitrate
17	16.80	609.1441	$C_{27}H_{29}O_{16}$	3.28	147.0174 151.0100 162.9927 179.0179 300.0256 272.5696	255	Quercetin 3-galactoside 7-rhamnoside
18	17.00	609.1444	$C_{27}H_{29}O_{16}$	2.79	151.0011 178.9964 273.0332 300.0256	255	Quercetin 3-glucoside 7-rhamnoside (Rutin)
19	17.27	431.0986	$C_{21}H_{19}O_{10}$	−0.70	77.0398 93.0344	269	Genistin
20	17.96	593.1526	$C_{27}H_{29}O_{15}$	−2.36	228.0345 256.0335 285.0390	264	kaempferol 7-neohesperidoside
21	18.59	593.1513	$C_{27}H_{29}O_{15}$	−0.17	120.0294 447.0653 473.1074	265	Isoorientin 2″-O-rhamnoside
22	18.83	623.1610	$C_{28}H_{31}O_{16}$	1.77	299.0212 315.0538	265	Isorhamnetin-3-Galactoside-6″-Rhamnoside
23	19.03	623.1600	$C_{28}H_{31}O_{16}$	3.37	299.0239 315.0662	255	Isorhamnetin-3-O-rutinoside
24	19.93	503.1177	$C_{24}H_{23}O_{12}$	3.58	177.0522 285.05307 315.5189	293	Pectolinarigenin 7-(6″-methylglucuronide)

carboxyl unit. Furthermore, a secondary peak was observed at *m/z* 152 [M-H-162]- due to loss of hexoside. Peak 1 was tentatively identified as gentisic acid 5-O-glucoside (18). In the first order mass spectrum of peak 2, an $[M-H]^-$ ion was observed at *m/z* 371.0605. The MSE spectrum showed a base peak ion at *m/z* 209 $[M-H-162]^-$ due to loss of a caffeoyl group. A secondary fragment, likely due to subsequent loss of a caffeoyl group, and a water molecule showing the presence of hydroxyl groups 191.0 $[M-C_9H_7O_3- H_2O]^-$ was observed at *m/z* 191[M-H-162-18] (19, 20). The peak was tentatively identified as 2-O-caffeoylglucaric acid. The presence of glucaric acid was evident due to the presence of the product ion at *m/z* 209.0, as similarly reported by Abukutsa-Onyango (21) and Mathias and de Oliveira (22). The first order mass spectrum of peak 3 showed an $[M-H]^-$ ion at *m/z* 299.0762. In the second order mass spectrum, a base peak ion was observed at *m/z* 137[M-H-162]$^-$ due to loss of a glycosyl unit. A secondary peak was observed at *m/z* 93[M-H-162-44]$^-$ due to subsequent cleavage of a glycosyl unit from the molecular ion and loss of the carbon dioxide from the aglycone. The loss of carbon dioxide was indicative of the presence of a carboxylic acid group in the aglycone (23). The tentative identification of peak 3 was as pseudolaroside A. In the ESI MS spectrum, peak 4 exhibited an $[M-H]^-$ ion at 369.0404. The second order mass spectrum of this peak had fragments at *m/z* 179[M-H-191] due to loss of the hydroxycitric acid moiety, *m/z* 191[M-H-179] due to loss of the caffeic acid unit, 189 [M-H-207-18] due to subsequent loss of the caffeoyl moiety and a water molecule indicating the presence of hydroxyl groups. Therefore, Peak 4 was tentatively identified as 2-O-caffeoylhydroxycitric acid. Peak 6 exhibited an $[M-H]^-$ ion at *m/z* 285.0606, in the MS/MS spectrum. A peak was observed at *m/z* 108[M-H-176] due to loss of a glucuronyl moiety (24), thus peak 6 was tentatively identified as diphenyl glucuronide.

Peak 7 exhibited a molecular ion at 341.0784 $[M-H]^-$ in the MS/MS spectrum; peaks were observed at *m/z* 179[M-H-162] due to loss of a caffeoyl unit, m/z 135[M-H-179-28] due to subsequent loss of a glucose unit followed by cleavage of a carbon monoxide unit. Peak 7 was tentatively identified as 1-O-caffeoylglucose. In the ESI MS spectrum, peak 8 exhibited an $[M-H]^-$ ion at *m/z* 355.0683. The second order mass spectrum showed peaks at *m/z* 209 and *m/z* 163. The peak at *m/z* 209[M-H-146] was due to loss of the coumaroyl moiety and the peak observed at *m/z* 163[M-H-192]$^-$ was the result of the loss of the glucoyl moiety. This fragmentation pattern was used to tentatively identify the presence of coumaroyl glucaric acid. The ESI MS spectrum of peak 9 showed an $[M-H]^-$ ion at *m/z* 385.0807. In the MS/MS spectrum a peak was observed at *m/z* 209[M-H-176]$^-$ due to loss of the feruloyl group and in the MS^2 at 146[M-H-176-18-15] due to loss of a water molecule showing the presence of a hydroxyl group and loss of a methyl group reflecting methylation of the aglycone. The peak was tentatively identified as 2-(E)-O-feruloyl-D-galactaric acid. In the first order mass spectrum, peak 10 showed a molecular ion at *m/z* 325.0927. In MSE spectrum of peaks observed at *m/z* 179, *m/z*119,04816 and *m/z*146. The peak at *m/z* 179.0382[M-H-146]$^-$ was due to loss of a coumaroyl residue, the peak observed at *m/z* 146.0301[M-H-179]$^-$ was due to loss of a glucose unit from the parent ion, and the peak observed at *m/z* 119.04816[M-H-179-28] due to subsequent loss of a coumaroyl residue followed by cleavage of a carbon monoxide unit. Peak 10 was tentatively identified as 1-O-p-coumaroyl-beta-D-glucose. The second order spectrum of peak 11 had peaks at *m/z* 269[M-H-146-179]$^-$ due to subsequent loss of a rhamnosyl fragment and a glycose unit. The peak was tentatively identified as coumaroyl isocitrate. The MS/MS spectrum of peak 12 showed peaks at *m/z* 191[M-H-162]$^-$ due to cleavage of the caffeoyl moiety; peak 12 was tentatively identified as 2-caffeoylisocitriic acid. In the ESI-MS/MS spectrum of peak 13 fragments were identified at *m/z* 147[M-H-301] from the cleavage of the aglycone from the parent ion. The peak at *m/z* 300[M-H-146] is the quercetin aglycone, which was due to loss of the rhamnose moiety. Consequently, peak 13 was tentatively identified as quercetrin, which is a glycoside of quercetin (quercetin-3-O-rhamnoside). The MS^2 spectrum of peak 14 showed a peak at *m/z* 191[M-H-146]$^-$ indicating the loss of the coumaroyl moiety, and another fragment was observed at *m/z* 119[M-H-191-28] from the loss of carbon monoxide from the coumaroyl moiety. Peak 14 was tentatively identified as coumaroyl isocitrate. The second order mass spectrum of peak 15 exhibited a peak at *m/z* 315[M-H-162-146-146] due to subsequent cleavage of the parent ion losing a hexose and two rhamnosyl fragments. A secondary peak characteristic of quercetin derivatives was observed at *m/z* 300, due to loss of a methyl group from the peak at *m/z* 315, thus revealing the presence of a methyl group attached to the quercetin moiety. Peak 15 was thus tentatively identified as 7-methylquercetin-3-galactoside-6″-rhamnoside-3‴-rhamnoside (xanthorhamnin). In the MS/MS spectrum, peak 16 had product ions at *m/z* 173[M-H-193] due to loss of a ferulic acid unit and *m/z* 111. This compound was tentatively identified as feruloyl isocitrate (25). Although the same fragmentation pattern was observed, another fragment at *m/z* 154 was not observed in this study. The MSE spectrum of peak 17 showed a base peak ion, which is the quercetin fragment at *m/z* 300.0256 [M-H-162-146] due to cleavage of the parent ion releasing the galactose and the rhamnose moieties. The fragment ion at *m/z* 300/301 is characteristic of all quercetin derivatives. The fragment observed at *m/z* 147[M-H-162-300] is the cleaved rhamnose moiety and the fragment observed at *m/z* 162[M-H-147-300] is the galactoside moiety. Other secondary fragments observed were mainly due to cleavage of the quercetin moiety and present in most quercetin derivatives; these were observed at *m/z* 273 due to loss of carbon monoxide from quercetin aglycone. Another secondary fragment observed at *m/z* 179 was a result of retrocyclization following fission on the C ring of quercetin (26). The fragment observed at *m/z* 151 was due to the loss of carbon monoxide from the *m/z* 179 fragment. Based on this fragmentation pattern, peak 17 was tentatively identified as quercetin 3-galactoside 7-rhamnoside. The MSE spectrum of peak 18 showed a base peak ion at *m/z* 300[M-H-146-162]$^-$ due to subsequent loss of the rhamnose and glycosyl units from the parent ion, revealing that the compound was di-glycosylated. Secondary fragments were observed mainly due

TABLE 2 | Influence of different blanching treatments and media on different phenolic components of pumpkin leaves.

	Raw pumpkin leaves	Water bath blanching–Plain water	Loss %	Steam blanching–Plain water	Percentage loss %	Water bath blanching –5% lemon juice	Percentage loss %	Water bath blanching –10% lemon juice	Percentage loss %	Steam blanching –5% lemon juice	Percentage loss %	Steam blanching 10% lemon juice	Percentage loss %
mg kg^{-1}													
2-O-Caffeoylglucaric acid	89.5 ± 6.4*^{a}	9.7 ± 1.2^{d}	89.16	30.6 ± 2.6^{bc}	20.72	20.6 ± 3.8^{c}	76.98	34.4 ± 1.4^{bc}	61.56	37.5 ± 2.6^{bc}	58.1	44.8 ± 2.1^{b}	49.94
Coumaroyl glucaric acid	55.3 ± 3.2^{a}	3.1 ± 0.6^{c}	94.39	45.4 ± 2.0^{a}	17.90	5.5 ± 0.1c	90.05	9.3 ± 0.6^{c}	83.18	31.0 ± 3.1^{b}	43.94	27.6 ± 1.6^{b}	50.09
2-(E)-O-feruloyl-D-galactaric acid	52.1 ± 3.2^{a}	9.7 ± 0.3^{b}	81.3	43.7 ± 2.6^{a}	16.1	9.3 ± 0.1b	82.14	13.9 ± 1.5^{b}	73.32	42.0 ± 2.9^{a}	19.38	46.0 ± 3.4^{a}	11
2-caffeoylisocitric acid	38.6 ± 1.9^{b}	3.9 ± 1.2^{d}	89.89	72.6 ± 1.0^{a}	18.88	11.6 ± 1.0^{cd}	69.94	15.9 ± 0.8^{c}	58.80	21.4 ± 1.0^{c}	44.55	25.8 ± 3.5^{bc}	33.16
Coumaroyl isocitrate	47.9 ± 2.8^{b}	6.9 ± 1.2^{c}	85.59	56.3 ± 2.5^{a}	−14.92	8.2 ± 0.6c	82.88	9.6 ± 1.6^{c}	79.95	43.8 ± 3.3^{b}	8.55	42.9 ± 2.6^{b}	10.43
Quercetin 3-galactoside 7-rhamnoside	149.9 ± 3.7^{b}	59.3 ± 3.1^{d}	60.44	115.2 ± 1.1^{b}	23.15	52.1 ± 1.6^{d}	65.24	242.9 ± 3.1^{a}	30.97	86.5 ± 6.0^{c}	42.29	92.6 ± 1.8^{c}	38.22
Quercetin 3-glucoside 7-rhamnoside (Rutin)	351.9 ± 2.9^{a}	222.3 ± 2.2^{c}	36.82	341.2 ± 2.7^{a}	3.04	189.4 ± 1.6^{c}	46.17	57.2 ± 2.5^{d}	26.34	267.9 ± 1.2^{b}	23.87	271.1 ± 2.1^{b}	22.96
Quercetin 3-galactoside	112.2 ± 2.7^{a}	42.4 ± 1.7^{d}	62.21	95.5 ± 1.1^{b}	14.88	41.9 ± 1.2^{d}	62.66	42.4 ± 3.0^{d}	62.65	58.5 ± 2.3^{c}	47.86	54.9 ± 1.3^{cd}	51.06
Kaempferol 7-neohesperidoside	123.4 ± 1.4^{a}	34.8 ± 3.7^{d}	71.79	114.3 ± 2.7^{a}	7.37	37.7 ± 2.3^{cd}	69.36	55.2 ± 2.6^{c}	55.26	60.9 ± 2.7^{bc}	50.64	75.2 ± 4.6^{b}	39.05
Isoorientin 2″-O-rhamnoside	182.1 ± 2.7^{a}	41.2 ± 2.5^{e}	77.37	162.7 ± 3.2^{b}	10.65	42.0 ± 15.4^{e}	76.93	63.8 ± 2.9^{d}	64.96	89.0 ± 3.3^{c}	51.12	90.6 ± 1.4^{c}	50.24
Isorhamnetin-3-galactoside-6″-rhamnoside	80.1 ± 2.1^{a}	18.1 ± 3.7^{d}	77.4	66.7 ± 9.7^{b}	1.7	28.6 ± 0.8^{cd}	3.2	29.9 ± 1.9^{c}	62.67	36.8 ± 2.0^{c}	54.05	34.9 ± 1.7^{c}	56.42
Isorhamnetin-3-O-rutinoside	174.1 ± 1.7^{a}	70.0 ± 2.3^{d}	59.79	155.0 ± 2.4^{b}	10.97	83.7 ± 2.2^{d}	51.92	84.2 ± 1.2^{d}	51.63	110.1 ± 1.7^{c}	36.76	100.0 ± 2.6^{c}	42.56
Total phenolic compounds	1,457.1 ± 4.1^{a}	521.4 ± 3.4^{d}		1,299.2 ± 2.5^{b}		530.6 ± 3.1^{d}		658.7 ± 2.8^{d}		885.4 ± 2.9^{c}		906.44 ± 1.7^{c}	

Rows with similar alphabetic letter are not significantly different at $p < 0.05$ according to Fisher's LSD test.

**Standard deviation (n = 3, cumulated sample of 10 makes 1 n replicate).*

to fragmentation of the quercetin moiety at *m/z* 273[M-H-308-28] due to subsequent loss of the diglycosyl unit and release of a carbon monoxide from the quercetin aglycone. Another at *m/z* 179 was a result of retro cyclization following fission on the C ring of quercetin. Fernández-Poyatos (19) observed a similar fragmentation pattern for rutin. Peak 18 was tentatively identified as quercetin 3-glucoside 7-rhamnoside (rutin). In the MS/MS spectrum, peak 19 exhibited a peak at *m/z* 93[M-H-179] due to cleavage of a chromone moiety. Another fragment observed at *m/z* 77 [M-H-179-18] was attributed to subsequent loss of the chromone moiety and cleavage of a water molecule, revealing the presence of a hydroxyl group on a benzene ring. As a result, peak 19 was tentatively identified as genistin (7 hydroxyisoflavone). The ESI-MS/MS spectrum of peak 20 exhibited peaks at *m/z* 285[M-H-146-162]$^-$ due to subsequent loss of a rhamnosyl and a glucosyl unit from the parent ion. The fragments observed at *m/z* 256 and *m/z* 228 were due to fragmentation of the kaempferol moiety (27). The ESI MS spectrum of peak 21 had an [M-H]$^-$ ion observed at *m/z* 593.1512. In the second order spectrum, the base peak ion observed at *m/z* 473.1074[M-H-120] and the peak observed at *m/z* 120[M-H-473]$^-$ were attributed to intra-glycoside fragmentation of glucose linked to the carbon atom of flavone in the isoorientin moiety. Secondary fragments were observed at *m/z* 447[M-H-146] due to the loss of the rhamnose moiety (22). Peak 21 was thus tentatively identified as Isoorientin 2″-O-rhamnoside. In the MS/MS spectrum of peak 22, a base peak ion was observed at *m/z* 315.0538[M-H-308]$^-$; Lei et al. (28) demostrated a similar fragmentation pattern. The loss of 308 indicated the compound was di-glycosylated having a hexose and a deoxyhexose with the ion at 315 being the aglycone ion. Furthermore, the secondary peak observed at *m/z* 299[M-H-308-15]$^-$ was attributed to the loss of a methyl group, revealing the aglycone moiety was methylated. Peak 22 was tentatively identified as isorhamnetin-3-galactoside-6″-rhamnoside. Peak 23 exhibited a similar fragmentation pattern to that of peak 22, revealing the compounds belonged to the same class. The second order spectrum of peak 23 showed a base peak ion at *m/z* 315[M-H-146-162]$^-$ due to subsequent loss of a deoxyhexose and a hexose sugar, with the base peak ion being the aglycone. Secondary fragments were also observed at m/z 299[M-H-308-15] due to cleavage of a methyl group from the aglycone moiety showing the presence of a methyl group in the aglycone. Peak 23 was tentatively identified as isorhamnetin-3-O-rutinoside and similar pattern of fragmentation was identified in the study as Lei et al. (28). The MSE spectrum of peak 24 had a peak at *m/z* 315[M-H-191] from cleavage of the parent ion to produce the glycone and the aglycone moiety. A peak observed at *m/z* 177 was due to demethylation of the glycone moiety. Another peak ion observed at *m/z* 285 was identified as a fragment of methylated naringenin. Thus, peak 24 was tentatively identified as pectolinarigenin 7-(6″-methylglucuronide). The MS spectra of the compounds listed in Table are given in **Supplementary Table 1**.

Table 2 illustrates the changes in different untargeted phenolic compounds and total phenolic compounds during different blanching treatments using plain water or lemon juice as the blanching medium. Overall, the total phenolic compounds were highest (1,457.1 mg kg^{-1}) in raw pumpkin leaves compared to cooked samples. This illustrates that during the process of cooking, polyphenols were broken down from the leaf tissue; a similar observation was reported by Gunathilake et al. (29). Water bath blanched leaves in plain water or 5 or 10% lemon juice, showed 521.4, 530.6, 658.7 mg kg^{-1} total phenolic compounds, respectively, indicating the loss of total phenolic compounds. Steam blanching in 5 or 10% lemon juice showed 885.4 mg kg^{-1}, 906.4 mg kg^{-1} of total phenols, respectively, which was higher than the levels observed during water bath blanching. Compared to all blanching treatments, steam blanching in plain water helped to retain the total phenolic content to 1,299.2 mg kg^{-1}. Steaming in plain water showed the lowest 10.83% loss of total phenolic compounds compared to other cooking methods with reference to the raw leaves. In contrast, the following steamed traditional vegetables, *Sesbania grandiflora* ("kathurumurunga"), *Passiflora edulis* ("passion fruit"), and *Olax Zeylanica* ("mella") leaves, showed significant loss of polyphenols compared to their raw leaves (29). Turkmen et al. (30) reported that blanching in hot water reduced the polyphenolic content by only 12–26% in spinach, swamp cabbage, kale, shallots, and cabbage; however, Kao et al. (31) showed a significant increase in polyphenols in Thai basil and sweet potato leaves, during boiling for 1–5 min.

It is also evident from our study that the type of blanching treatment used, compared to the type of blanching medium, profoundly influenced the retention of total phenolic compounds in pumpkin leaves. Compared to all 12 phenolic compounds listed in **Table 2**, quercetin 3-glucoside 7-rhamnoside (Rutin) showed the least amount of loss during water bath (36.82%) and steam blanching (3.04%) in plain water and water bath blanching in lemon juice (5%) (46.17%) or (10%) (26.34%). The observed loss of rutin during blanching can be attributed due to its reduced sensitivity to thermal treatment due to the presence of glucose in the molecular structure (32). The loss of 2-(E)-O-feruloyl-D-galactaric acid during steam blanching with different blanching media (lime juice or plain water) varied insignificantly, between 11.7 and 19.38%. Galactaric esters of ferulic acid [2-(E)-O-feruloyl-D-galactaric acid] are highly soluble due its polar nature, and its loss is reduced to 16.1% in steam blanched leaves in plain water. In addition, the acidic pH could have retained the stability of the molecule and prevented further degradation of this compound to free phenolic acid (ferulic acid) (33) during steam blanching in 5% and 10% lime juice.

The concentration of coumaroyl glucaric acid ester and coumaroyl isocitrate was 55.3 and 47.9 mg kg^{-1} in raw pumpkin leaves, respectively. It is interesting to note that coumaroyl glucaric acid was reduced by 17.90%, whilst coumaroyl isocitrate gained by −14.92% after steam blanching in plain water. This was probably because coumaroyl glucaric acid ester underwent hydrolysis and the hydroxyl-cinnamic acid (*p*-coumaric acids) could have formed conjugates via esterification between isocitric acid (34). Similarly, concentrations of 2-O-caffeoylglucaric acid and 2-caffeoylisocitric acid in raw pumpkin leaf was 89.5 and 38.6 mg kg^{-1}, respectively. After steam blanching in plain water, the concentration of caffeoylisocitric acid increased to

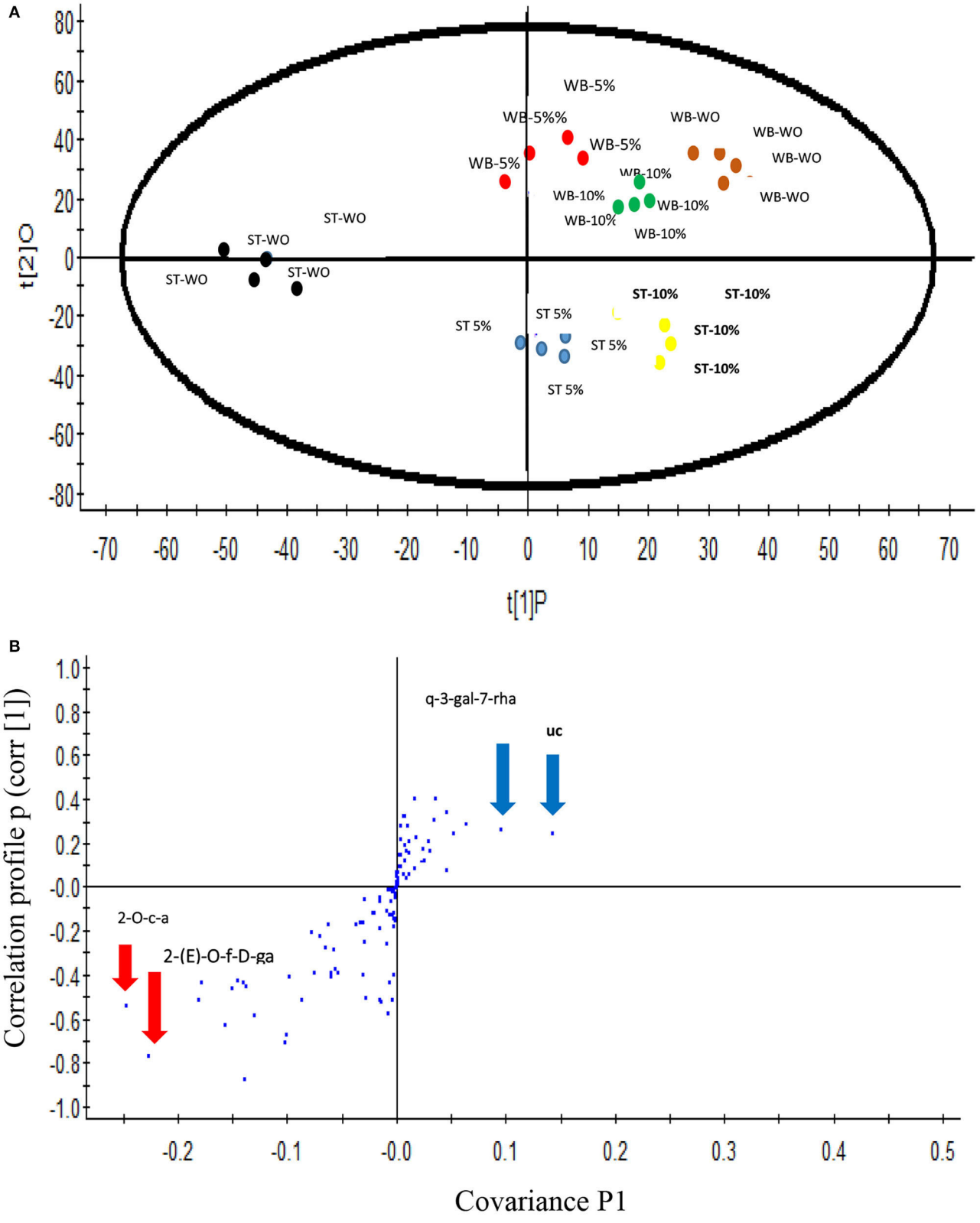

FIGURE 1 | (A) Score plot of Principal Component analysis (unsupervised) based on UPLC–Q-TOF/MS spectra of the blanched pumpkin leaves. ST WO-Steam blanching in plain water at 95°C for 5 min; WB-WO water bath blanching in plain water; WB- 5%–Water bath blanching in 5% Lemon water at 95°C for 5 min; WB-10% -Water bath blanching in 5% Lemon water at 95°C for 5 min. **(B)** Score plot of orthogonal partial least squares discriminant analysis of UPLC–Q-TOF/MS spectra

(Continued)

FIGURE 1 | of steam blanched pumpkin leaves in plain water and other samples underwent water bath blanching in plain water or 5 or 10% lemon water. Each sample set includes 4 replicates. 2-O-caffeoylglucaric acid (2-O-c-a), 2-(E)-O-feruloyl-D-galactaric acid [2-(E)-O-f-D-ga], quercetin 3-galactoside 7-rhamnoside (q-3-gal-7-rha), unidentified compound (uc) [(M-H) 677.28 and at RT 21.78].

72.6 mg kg^{-1} and 2-O-caffeoylglucaric acid was reduced to 30.6 mg kg^{-1} (**Table 2**). Kaempferol 7-neohesperidoside reduced loss by 7.37% during steam blanching in plain water, and the concentrations of this compound reduced non-significantly compared to the raw leaves, possibly because acylation may have provided increased resistance to heat treatment (35). Conversely, 10.65% of isoorientin 2"-O-rhamnoside, the c-glycosyl flavonoids was lost during steam blanching in plain water, whilst steam blanching in 5 or 10% lemon juice resulted in ∼50% loss. Likewise, steaming pumpkin leaves in plain water led to 10.97% loss of isorhamnetin-3-O-rutinoside, whereas steaming in 5 or 10% lemon water (acidic pH) resulted in greater loss by 36.76 and 42.56% respectively. Phenolic compounds have different thermal stability according to their molecular structure (32, 35, 36) and their degradation depends on the temperature, pH and duration (time) (27, 35, 37, 38). For the stability of flavonoids in a food matrix, the hydroxyl group in position 3 of the C ring, with glycosylation and sugar moiety, was reported to block its degradation during thermal treatment (32). Steamed celery or parsley leaves in plain water showed significantly greater amounts of flavonoid glycosides (38). Therefore, the blanching treatments were optimized in this study to minimize the flavonoid derivatives in pumpkin leaves that may enhance the bioavailability and efficacy.

Moist cooking of nightshade in plain water in water bath at 95°C caused loss of different phenolic compounds (39). However, Managa et al. (39) reported lower concentrations of total phenolic compounds in raw leaves compared to the steamed blanched leaves in water or 5% lemon juice. Conversely, an opposite trend was noted in pumpkin leaves, where the raw leaves showed the highest phenolic components and total phenolic content. In contrast to our observation, in African nightshade leaves total phenolic compounds and different phenolic components increased during boiling in water (39), probably due to higher extractability of the phenolic compounds from the cellular matrix of the leaves (40). During blanching in water bath with plain water or 5 or 10% lemon juice, the different phenolic components listed in **Table 2** had probably been released into the boiling water due to the heat mediated rupture of the leaf cell wall (41). Phenolics are polar compounds highly soluble in water (42) and during steaming they are retained on the leaf surface. The structural property of the cell wall of the different vegetables determines the ability of the cell wall to withstand the thermal blanching treatment and to retain phenolic compounds within the cells (43). Due to the higher temperatures, blanching was reported to degrade the polyphenol oxidase enzymes that use the phenols as substrates during browning reaction, and steam treatments in broccoli, compared to boiling, effectively reduced the peroxidase activity (44). This could be the reason for the higher retention of total phenol compounds and different phenolic components in steamed blanched pumpkin leaves in this study, compared to the other blanching treatments.

Multivariate Analysis

Principal Component Analysis (PCA) analyses was conducted as it is difficult to interpret large quantities of generated data, so this technique helped to reduce the dimensionality of the original data set, improving the interpretation, and reducing the loss of valuable information, and therefore PCA occurred in this study, as reported by Biancolillo and Marini (45). PCA helps in data compression, while reducing the loss of information (45), in an unsupervised nature. In addition, the PCA loading scores helped to present the data, exhibiting the possible trends in the presence of clusters (45), and reveals only the group structure.

The differences in distribution of the non-targeted phenolic metabolic profiles of the pumpkin leaves that underwent different blanching treatments was demonstrated using an unsupervised (PCA) approach, using the data set obtained by the UPLC–Q-TOF/MS analysis. **Figure 1A** illustrates the PC 1 and PC 2, explaining the 38 and 16.4% of the variance and showing good statistical separation among the different blanching treatments. The PCA plot showed 3 district clusters based on the phenolic metabolites shown by different blanching treatments and media used in this study. Samples that underwent steam blanching in plain water were separated from other two which were blanched in a water bath or steam using 5 or 10% lemon juice. Also, water bath blanching and steam blanching were further separated in PCA. An Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was performed for metabolomics data to analyze the multivariate data to establish a quantitative relationship between the different blanching treatments and the phenolic compounds (46), to identify the biomarker candidates responsible for separation between the blanching treatments via a more mathematically supervised manner using the description of the algorithms. The OPLS-DA provides more information than the PCA and allows better expose separations between classes in clusters. Therefore, well-separated PCA can guide PLS classification with greater possibility of providing the information for the compounds responsible for the observed separation of the clusters (46) in this study.

The values of R^2 X and Q^2 of cross-validation in OPLS-DA score plot for pumpkin leaves were 0.972 and 0.957, respectively, and provided reliable fitness. **Figure 1B** shows the S-plot; the upper right quadrant showed the phenolic compounds that were present in pumpkin leaves at higher concentrations in steam and water blanching treatments in 5 or 10% lemon water, whilst the lower left quadrant demonstrated the compounds that were higher in concentration during steam blanching in plain water. Phenolic compounds, 2-O-caffeoylglucaric acid, 2-(E)-O-feruloyl-D-galactaric acid, quercetin 3-galactoside 7-rhamnoside (rutin) and unidentified compounds [((M-H) 677.28 and at RT 21.78)] were identified as the candidate markers responsible for the separation of the steam blanched samples in plain water from the other blanching treatments. Exact Mass/Retention Time

TABLE 3 | Exact Mass/Retention Time pairs responsible for the separation of steam blanched pumpkin leaves in plain water from the other blanching treatments.

Primary ID	Retention time	Mass	p[1]P	p(corr)[1]P	Factor of Change	Steam blanching	*Other blanching treatment
2-O-Caffeoylglucaric acid	8.12	371.0605	−0.227376	−0.769076	3.8	107.24	28.2241
2-(E)-O-Feruloyl-D-galactaric acid	9.56	385.0772	−0.138788	−0.872095	−21,2574,480.0	38.9443	−1.83203e-007
Quercetin 3-galactoside 7-rhamnoside (rutin)	17.00	609.1444	0.434539	0.671667	1.7	422.558	731.997
Unidentified compound	9.74	677.28	−0.156446	−0.631567	3.4	91.3982	26.6321

**Other blanching treatments—Steam blanching in 5 or 10% lemon juice; water bath blanching in plain water.*

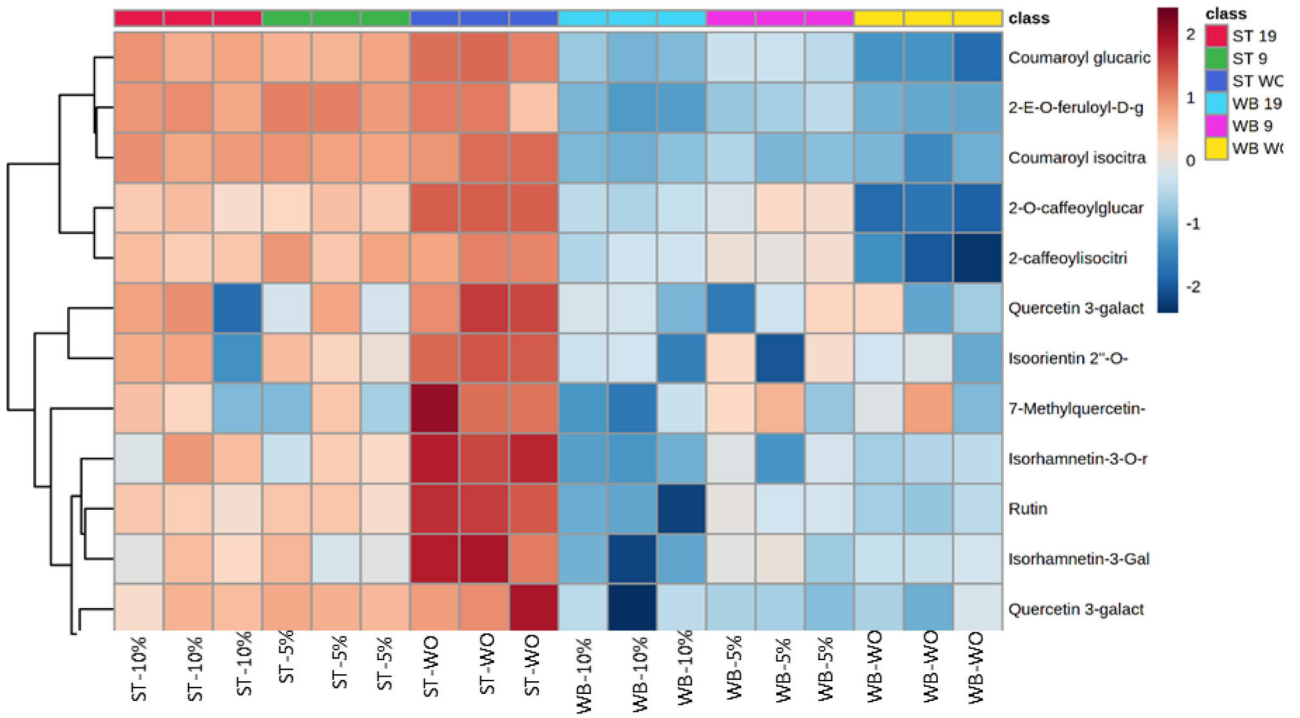

FIGURE 2 | Heat map of twelve phenolic metabolites quantified in blanched pumpkin leaves using plain water or 5 or 10% lemon water organized in hierarchical clustering.

pairs responsible for the separation of steam blanched pumpkin leaves in plain water from the other blanching treatments is given in **Table 3**. The heat map shown in **Figure 2** illustrates the intensity of different non-targeted phenolic metabolites in pumpkin leaves to the different blanching treatments. The heat map includes the clustergrams, the row data represented the concentration of phenolic metabolites relating the columns of variables the blanching treatments and the type of media used. Color block was used to express the intensity of the influence of the treatment; red box representing higher concentrations and blue box indicating the metabolites at a lower concentration. The heat map illustration clearly showed that generally, the steamed leaves contained the highest concentration of the 12 phenolic metabolites (2-O-caffeoylglucaric acid, coumaroyl glucaric acid, 2-(E)-O-feruloyl-D-galactaric acid, 2-caffeoylisocitric acid, coumaroyl isocitrate, quercetin 3-galactoside 7-rhamnoside, quercetin 3-glucoside 7-rhamnoside (rutin), quercetin 3-galactoside, isoorientin 2″-O-rhamnoside, isorhamnetin-3-galactoside-6″-rhamnoside, isorhamnetin-3-O-rutinoside) compared to the water bath blanching. Among the steam treatments, pumpkin leaves steamed in plain water showed the higher concentrations of the 12 phenolic metabolites.

In vitro Antioxidant Capacity and α-Glucosidase Activities and Cytotoxic Effect

Table 4 presents the results from *in vitro* antioxidant capacity, α-glucosidase activity, and cytotoxic effect. Antioxidant capacity (FRAP and ABTS assay) was highest in raw pumpkin leaves,

TABLE 4 | Effects of different house domestic cooking methods on *in vitro* antioxidant capacity and cytotoxic effect of blanched pumpkin leaves.

Treatments	FRAP (μmol TEAC g^{-1} FW)	ABTS (μmol TEAC g^{-1} FW	Inhibition of α-glucosidase activity IC_{50} (μg mL^{-1})	Cell viability IC_{50} (μg mL^{-1})
Raw	1.41 ± 0.29^{a}	1.52 ± 0.12^{a}	21.31 ± 0.10^{e}	46.22 ± 0.81^{b}
Water bath: plain water	0.37 ± 0.27^{e}	0.47 ± 0.20^{d}	26.54 ± 0.47^{d}	13.65 ± 0.50^{d}
Water bath: 5% lemon juice	0.91 ± 0.52^{c}	0.82 ± 0.21^{c}	28.12 ± 0.84^{c}	15.29 ± 0.34^{d}
Water bath: 10% lemon water	0.67 ± 0.41^{d}	1.38 ± 0.81^{b}	27.69 ± 0.97^{cd}	12.56 ± 0.34^{d}
Steaming: plain water	1.25 ± 0.20^{b}	1.51 ± 0.54^{a}	30.89 ± 0.11^{a}	25.62 ± 0.76^{c}
Steaming: 5% lemon juice	0.96 ± 0.73^{c}	1.34 ± 0.23^{b}	31.24 ± 0.76^{a}	25.33 ± 0.83^{c}
Steaming: 10% lemon juice	1.02 ± 0.30^{c}	1.41 ± 0.43^{b}	29.51 ± 0.50^{b}	13.08 ± 0.21^{d}
Hydrogen peroxide				70.17 ± 0.15^{a}
Acarbose			18.22 ± 0.11^{f}	

Means followed by the same letter within the column are not significantly different at $p < 0.05$ according to Fisher's LSD test.

whilst amongst the 6 blanching treatments adopted in this study, steaming in plain water showed significantly highest antioxidant capacity (FRAP and ABTS assay). The strongest antioxidant capacity (FRAP and ABTS assay) of pumpkin leaves that underwent blanching, based on FRAP (1.25 μmol TEAC g^{-1} FW) and ABTS (1.51 μmol TEAC g^{-1} FW) assay, were found in steamed blanched leaves in plain water followed by steam blanched leaves using 10% lemon water as the blanching medium. However, water bath blanched leaves using 5 or 10% lemon juice as the blanching medium showed the least antioxidant capacity. This indicated that steaming at 95°C helped to retain the antioxidant capacity of the pumpkin leaves. The observed trend in antioxidant capacity corresponds to the cumulative yield of the phenolic compounds shown in **Table 2**, and the released free phenolic compounds could have had a positive effect on the antioxidant activity in pumpkin leaves steam blanched in plain water, probably due to the destruction of the cell wall and cellular components releasing the antioxidants (30). As in our observation, steaming in water reduced the total antioxidant capacity of traditional vegetable *O. zeylanica* and Nightshade (*Solanum retroflexum* Dun) leaves compared to its raw form (29, 39). Steaming in water also preserved the antioxidant properties of green bean varieties (47). Conversely, Gunathilake et al. (29) also revealed that steamed leaves of traditional vegetable *P. edul* showed 10% higher antioxidant capacity compared to the fresh leaves. Therefore, there are discrepancies related to the preservation of antioxidant activity during different blanching and other cooking methods; blanching of Nightshade in plain water showed similar reduction in antioxidant activity as pumpkin leaves in this study (39). The highest correlation between total phenolic compounds and FRAP or ABTS activity was established by Qader et al. (48) and Augusto et al. (49).

Cytotoxic effects of leaf extracts of raw and blanched pumpkin leaves on C2C12 myoblast cell line are presented in percentage cell viability. All blanching treatments, irrespective of the type of blanching media used and the raw pumpkin leaves, showed the absence of inhibitions on cell viability at 50%, indicating the absence of toxicity, and the control (H_2O_2) showed the highest toxicity. Cytotoxic evaluation is important to screen for toxic effects, and during blanching, some of these compounds could have probably denatured or been removed (50). Raw and blanched pumpkin leaves illustrated the lowest inhibition activity with IC_{50} ranging from 21.31 to 30.89 μg mL^{-1}, indicating that the pumpkin leaves are weak inhibitors of α-glucosidase. However, the inhibitory effect of raw pumpkin leaves on α-glucosidase activity is not comparable with the commercial inhibitor acarbose, and the fruit pulp and seeds of pumpkin (*C. maxima*) reportedly had the active hypoglycemic components (50, 51).

CONCLUSION

The study indicated that the blanching methods and the type of blanching media affected the dietary phenolic compounds, and antioxidant capacity of pumpkin leaves. More specifically, steam blanching in plain water improved the retention of phenolic compounds and antioxidant capacity than all other adopted blanching methods. Use of lemon water during steam blanching is not recommended. All blanching methods and raw leaves showed the absence of cytotoxicity, and were safe for frequent consumption. This information is useful for food manufacturers and chefs, however further studies are needed to see if the leaves of other pumpkin cultivars and from different geographical regions offer health benefits.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, after the approval of the University Scientific committee.

AUTHOR CONTRIBUTIONS

FM performed the experiment, generated the data, and wrote some parts of this manuscript. TS visualized and validated the data for phenolic compounds, interpreted the chromatogram and wrote that part of the article. JS was responsible for the antidiabetic activity and data. RS provided the editorial support. DS conceptualized the research, supervised the FM and improved

the article further. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Abukutsa-Onyango M. The diversity of cultivated African leafy vegetables in three communities in Western Kenya. *Afr J Food Agric Nutr Dev.* (2007) 7:1–15.
2. Lymo MH, Nyagwegwe S, Mnkeni AP. Investigation on the effect of traditional food processing, preservation and storage methods on vegetable nutrients: a case study in Tanzania. *Plant Foods Hum Nutr.* (1991) 41:53–7. doi: 10.1007/BF02196382
3. Salehi B, Capanoglu E, Adrar N, Catalkaya G, Shahee S, Jaffer M, et al. *Cucurbits* plants: A key emphasis to its pharmacological potential. *Molecules.* (2019) 24:1854. doi: 10.3390/molecules24101854
4. Food and Agriculture Organization (FAO) of the United Nations. *Post-harvest and Processing Technologies of African Staple Foods: A Technical Compendium.* Rome: FAO (1991).
5. USDA. *Food Data Base*. Available online at: https://fdc.nal.usda.gov/fdc-app.html#food-688details/168462/nutrient (accessed January 25, 2020).
6. Lim TK. Cucurbita moschata. In: *The Encyclopedia of Earth Edible Medicinal and Non-medicinal Plants.* Heidelberg: Springer (2011) 2:266–80. doi: 10.1007/978-94-007-1764-0_41
7. Jung A. *5 Korean Ways to Eat a Pig CNN GO.* (2011). Available online at: http://travel.cnn.com/seoul/eat/5-korean-ways-eat-pig-231893/ (accessed January 25, 2020).
8. Gökmen V, Bahçeci KS, Serpen A, Acar J. Study of lipoxygenase and peroxidase as blanching indicator enzymes in peas: change of enzyme activity, ascorbic acid and chlorophylls during frozen storage. *LWT Food Sci Technol.* (2005) 38:903–8. doi: 10.1016/j.lwt.2004.06.018
9. Muftugil N. Effect of different types of blanching on the color and the ascorbic acid and chlorophyll contents of green beans. *J Food Process Preserv.* (2007) 10:69–76. doi: 10.1111/j.1745-4549.1986.tb00006.x
10. Mkandawire KTM, Masamba KG. Effect of lemon juice treatment and sun drying on vitamin C retention in three steam and water blanched indigenous vegetables over six weeks storage period. *Afr J Food Sci*. (2014) 8:316–21. doi: 10.5897/AJFS2014.1167
11. Kim MJ, Hong CO, Nam MH, Lee KW. Antioxidant effects and physiological activities of pumpkin (*Cucurbita moschata* Duch.) extract from different aerial parts. *Korean J Food Sci Technol.* (2011) 43:195–9. doi: 10.9721/KJFST.2011.43.2.195
12. Azzini E, Giacometti J, Russo GL. Antiobesity effects of anthocyanins in preclinical and clinical studies. *Oxid Med Cell Longev.* (2017) 2017:2740364. doi: 10.1155/2017/2740364
13. Zhang B, Deng Z, Ramdath DD, Tang Y, Chen PX, Liu R, et al. Phenolic profiles of 20 Canadian lentil cultivars and their contribution to antioxidant activity and inhibitory effects on α-glucosidase and pancreatic lipase. *Food Chem.* (2015) 172:862–72. doi: 10.1016/j.foodchem.2014.09.144
14. Jiménez-Monreal AM, García-Diz L, Martínez-Tomé M, Mariscal M, Murcia MA. Influence of cooking methods on antioxidant activity of vegetables. *J Food Sci.* (2009) 74:97–103. doi: 10.1111/j.1750-3841.2009.01091.x
15. Managa GM, Remize F, Garcia C, Sivakumar, D. Effect of moist cooking blanching on colour, phenolic metabolites and glucosinolate content in Chinese cabbage (*Brassica rapa* L. subsp. chinensis). *Foods.* (2019) 8:399. doi: 10.3390/foods8090399
16. Ndou A, Tinyani, PP, Slabbert RM, Sultanbawa Y, Sivakumar D. An integrated approach for harvesting natal plum (*Carissa macrocarpa*) for quality and functional compounds related to maturity stages. *Food Chem.* (2019) 293:499–510. doi: 10.1016/j.foodchem.2019.04.102
17. Sagbo IJ, van de Venter M, Koekemoer T, Bradley G. *In vitro* antidiabetic activity and mechanism of action of *Brachylaena elliptica* (Thunb.) DC. *Evid Based Complement Altern Med.* (2018) 2018:4170372. doi: 10.1155/2018/4170372
18. Moloto MR, Dao Phan AT, Shai JL, Sultanbawa Y, Sivakumar D. Comparison of phenolic compounds, carotenoids, amino acid composition, *in vitro* antioxidant and anti-diabetic activities in the leaves of seven cowpea (*Vigna unguiculata*) cultivars. *Foods.* (2020) 9:1285. doi: 10.3390/foods9091285
19. Fernández-Poyatos MDP, Ruiz-Medina A, Zengin G, Llorent-Martínez EJ. Phenolic characterization, antioxidant activity, and enzyme inhibitory properties of *Berberis thunbergii* DC. Leaves: a valuable source of phenolic acids. *Molecules.* (2019) 24:4171. doi: 10.3390/molecules24224171
20. Jusoh NHM, Subki A, Yeap SK, Yap KC, Jaganath IB. Pressurized hot water extraction of hydrosable tannins from *Phyllanthus tenellus* Roxb. *BMC Chem.* (2019) 13:134. doi: 10.1186/s13065-019-0653-0
21. Abukutsa-Onyango M. The diversity of cultivated African leafy vegetables in three communities in Western Kenya. *Afr. J. Food Agric. Nutr. Dev.* (2007) 7:1–15.
22. da Silva Mathias M, de Oliveira RR. Differentiation of the phenolic chemical profiles of *Cecropia pachystachya* and *Cecropia hololeuca*. *Phytochem Anal.* (2019) 30:73–82. doi: 10.1002/pca.2791
23. Shoko T, Maharaj V, Naidoo D, Tselanyane M, Nthambeleni R, Khorombi E, et al. Anti-aging potential of extracts from *Sclerocarya birrea* (A. rich.) Hochst and its chemical profiling by UPLC-Q-TOF-MS. *BMC Complement Altern Med.* (2018) 18:54. doi: 10.1186/s12906-018-2112-1
24. Liu H, Garrett TJ, Su Z, Khoo C, Zhao S, Gu L. Modifications of the urinary metabolome in young women after cranberry juice consumption were revealed using the UHPLC-Q-orbitrap-HRMS-based metabolomics approach. *Food Func.* (2020) 11:2466–76. doi: 10.1039/C9FO02266J
25. Manyelo TG, Sebola NA, Hassan ZM, Mabelebele M. Characterization of the phenolic compounds in different plant parts of *Amaranthus cruentus* grown under cultivated conditions. *Molecules.* (2020) 25:4273. doi: 10.3390/molecules25184273
26. Cirić A, Prosen H, Jelikić-Stankov M, Đurdević P. Evaluation of matrix effect in determination of some bioflavonoids in food samples by LC–MS/MS method. *Talanta.* (2012) 99:780–90. doi: 10.1016/j.talanta.2012.07.025
27. March RE, Miao XS. Fragmentation study of kaempferol using electrospray quadrupole time-of-flight mass spectrometry at high mass resolution. *Int J Mass Spectrom.* (2004) 231:157–67. doi: 10.1016/j.ijms.2003.10.008
28. Lei Z, Kranawetter C, Sumner BW, Huhman D, Wherritt DJ, Thomas AL, et al. Metabolomics of two pecan varieties provides insights into scab resistance. *Metabolites.* (2018) 8:56. doi: 10.3390/metabo8040056
29. Gunathilake KDPP, Ranaweera KKDS, Rupasing HPV. Effect of different cooking methods on polyphenols, carotenoids and antioxidant activities of selected edible leaves. *Antioxidants.* (2018) 7:117. doi: 10.3390/antiox7090117
30. Turkmen N, Sari F, Velioglu YS. The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chem.* (2005) 93:713–8. doi: 10.1016/j.foodchem.2004.12.038
31. Kao FJ, Chiu YS, Chiang WD. Effect of water cooking on the antioxidant capacity of carotenoid-rich vegetables in Taiwan. *J Food Drug Anal.* (2014) 22:202–9. doi: 10.1016/j.jfda.2013.09.010

32. Buchner N, Krumbein A, Rhon S, Kroh LW. Effect of thermal processing on the flavonols rutin and quercetin. *Rapid Commun Mass Spectrom.* (2006) 20:3229–35. doi: 10.1002/rcm.2720
33. Manthey JA, Grohmann K. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. *J Agric Food Chem.* (2001) 49:3268–73. doi: 10.1021/jf010011r
34. Masike K, Mhlongo MIM, Mudau S, Nobela O, Ncube EN, Tugizimana F, et al. Highlighting mass spectrometric fragmentation differences and similarities between hydroxycinnamoyl-quinic acids and hydroxycinnamoyl-isocitric acids chem. *Cent J.* (2017) 11:29. doi: 10.1186/s13065-017-0262-8
35. Wu X, Zhao Y, Haytowitz DB, Chen P, Pehrsson PR. Effects of domestic cooking on flavonoids in broccoli and calculation of retention factors. *Heliyon.* (2019) 5:e01310. doi: 10.1016/j.heliyon.2019.e01310
36. Stintzing FC, Hoffmann M, Carle R. Thermal degradation kinetics of isoflavone aglycones from soy and red clover. *Mol Nutr Food Res.* (2006) 50:373–7. doi: 10.1002/mnfr.200500187
37. Ungar Y, Osundahunsi OF, Shimoni Y. Thermal stability of genistein and daidzein and its effect on their antioxidant activity. *J Agric Food Chem.* (2003) 51:4394–9. doi: 10.1021/jf034021z
38. Cheng Y, Xu Q, Liu J, Zha C, Xue F, Zha Y. Decomposition of five phenolic compounds in high temperature water. *J Braz Chem Soc.* (2014) 25:2102–7. doi: 10.5935/0103-5053.20140201
39. Managa GM, Mpai S, Remize F, Garcia C, Sivakumar D. Impact of moist cooking methods on colour, anti-nutritive compounds and phenolic metabolites in African nightshade (*Solanum retroflexum* Dun.). *Food Chem.* (2020) 325:126805. doi: 10.1016/j.foodchem.2020.126805
40. Schweiggert U, Schieber A, Carle R. Effects of blanching and storage on capsaicinoid stability and peroxidase activity of hot chili peppers (*Capsicum frutescens* L.). *Innov Food Sci Emerg Technol.* (2006) 7:217–44. doi: 10.1016/j.ifset.2006.03.003
41. Lutz M, Henríquez C, Escobar M. Chemical composition and antioxidant properties of mature and baby artichokes (*Cynara scolymus* L.), raw and cooked. *J Food Compost Anal.* (2011) 24:49–54. doi: 10.1016/j.jfca.2010.06.001
42. Ahmed FA, Ali RFM. Bioactive compounds and antioxidant activity of fresh and processed white cauliflower. *Biomed Res Int.* (2013) 2013:367819. doi: 10.1155/2013/367819
43. Burns J, Fraser PD, Bramley PM. Identification and quantification of carotenoids, tocopherols and chlorophylls in commonly consumed fruits and vegetables. *Phytochemistry.* (2003) 62:939–47. doi: 10.1016/S0031-9422(02)00710-0
44. Severini C, Giuliani R, De Filippis A, Derossi A, De Pilli T. Influence of different blanching methods on color ascorbic acid and phenolics content of broccoli. *J Food Sci Tech.* (2016) 53:501–10. doi: 10.1007/s13197-015-1878-0
45. Biancolillo A, Marini F. Chemometric methods for spectroscopy-based pharmaceutical analysis. *Front Chem.* (2018) 6:576. doi: 10.3389/fchem.2018.00576
46. Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). *J Chemom.* (2002) 16:119–28. doi: 10.1002/cem.695
47. Preti R, Rapa M, Vinci G. Effect of steaming and boiling on the antioxidant properties and biogenic amines content in green bean (*Phaseolus vulgaris*) varieties of different colours. *J. Food Qual.* (2017) 2017:5329070. doi: 10.1155/2017/5329070
48. Qader SW, Abdulla MA, Chua LS, Najim N, Zain MM, Hamda S. Antioxidant, total phenolic content and cytotoxicity evaluation of selected Malaysian plants. *Molecules.* (2011) 16:3433–43. doi: 10.3390/molecules16043433
49. Augusto TR, Scheuermann ES, Alencar SM, d'Arce MABR, Costa de Camargo A. Phenolic compounds and antioxidant activity of hydroalcoholic extracts of wild and cultivated murtilla (*Ugni molinae* Turcz.). *Food Sci Technol.* (2014) 34:667–73. doi: 10.1590/1678-457X.6393
50. Abifarin TO, Otunola GA, Afolayan AJ. Cytotoxicity evaluation and anti-inflammatory potentials of *Cucumis africanus* L. f. leaves. *Med Plants.* (2020) 12:48–52. doi: 10.5958/0975-6892.2020.00008.8
51. Adams GG, Imran S, Wang S, Mohammad A, Kok S, Gray DA. The hypoglycaemic effect of pumpkins as anti-diabetic and functional medicines. *Food Res Int.* (2011) 44:862–7. doi: 10.1016/j.foodres.2011.03.016

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

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Effect of Long-Term Frozen Storage on Health-Promoting Compounds and Antioxidant Capacity in Baby Mustard

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This study investigated the effects of blanching and subsequent long-term frozen storage on the retention of health-promoting compounds and antioxidant capacity in frozen lateral buds of baby mustard. Results showed that all glucosinolates were well preserved during frozen storage, and 72.48% of total glucosinolate content was retained in the unblanched treatment group after 8 months, as were chlorophylls, carotenoids, ascorbic acid, total phenolics, soluble sugars, soluble proteins, and antioxidant capacity. The loss of nutritional qualities mainly occurred in the 1st month of frozen storage, and nutritional qualities in the unblanched treatment group were significantly better than those in the blanched treatment group during frozen storage. Blanching before freezing reduced contents of high-content glucosinolates and ascorbic acid, as well as antioxidant capacity levels. Therefore, we recommend using long-term frozen storage to preserve the quality of baby mustard to achieve annual supply, and freezing without blanching.

Keywords: baby mustard, long-term frozen storage, blanching, glucosinolates, antioxidants

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INTRODUCTION

Vegetables are good sources of biologically active components and play an important role in reducing obesity and various diseases (1, 2). Epidemiological studies have provided convincing evidence that the consumption of *Brassica* vegetables is associated with a reduced risk of various cancers and cardiovascular diseases. These health-promoting and anti-carcinogenic properties have been primarily attributed to the high contents of glucosinolates present in *Brassica* vegetables (3–5). Baby mustard (*Brassica juncea* var. *gemmifera*) is a variant of stem mustard and belongs to the *Brassica* genus. Aside from glucosinolates, baby mustard is also a rich source of carotenoids, ascorbic acid, and various phenolics (5, 6), which are also considered important antioxidants because of their activity against free radicals, carcinogenesis, and cardiovascular diseases, as well as in stimulating the human immune system (1, 7, 8). However, the nutritional quality of baby mustard depends on not only the bioactive molecule content when harvested, but also the changes that occur during postharvest handling and storage conditions (1).

Baby mustard is mainly produced in Southwest China and is a seasonal vegetable with a supply period of ∼4 months, usually lasting from November to February. It is not available during the other 8 months and is a highly perishable vegetable with a shelf life of on days before it is unsafe or undesirable for consumption (5, 6). Once it is harvested, baby mustard begins to undergo higher

rates of respiration, resulting in moisture loss, browning of the epidermis, and quality deterioration (6). These characteristics of baby mustard cause difficulties in its distribution and marketing as fresh produce. Therefore, the production and supply chain of baby mustard needs suitable post-harvest techniques to extend its shelf life and preserve health-promoting compounds.

Many ways of handling vegetables post-harvest have been investigated to extend shelf life and preserve health-promoting compounds, among which frozen storage is one of the most popular and effective processing approaches to maintain post-harvest quality and nutritional properties for extended periods, because at low temperature, deteriorative reactions are reduced to minimal rates (9, 10). According to previous reports, long-term frozen storage is an excellent technology to preserve the post-harvest quality of broccoli florets (11, 12). Likewise, Volden et al. (13) found that frozen cauliflower can serve as an excellent supply of important health-related compounds, even after storage for 1 year. However, in some instances the contents of health-promoting phytochemicals and antioxidant capacity in vegetables are altered during frozen storage (11). In addition, vegetables often require blanching prior to frozen storage in order to inactivate enzymes (14), although some vegetables are frozen without blanching because blanching significantly reduces the nutritional qualities of frozen products (1, 15, 16). Until now, limited information has been available about the retention of nutritional qualities in baby mustard during long-term frozen storage and the blanching process. The current study was, therefore, conducted to investigate the effects of long-term frozen storage and blanching before freezing on contents of health-promoting compounds and the antioxidant capacity of baby mustard lateral buds, and in order to determine whether these methods are a satisfactory way to achieve annual supply of baby mustard.

MATERIALS AND METHODS

Plant Materials

Baby mustard (*Brassica juncea* var. *gemmifera* cv. Linjiang-Ercai) for this study was provided by a local farm in Chengdu City, China. Heads with uniform size and absence of external damage were selected and harvested in the morning and transported to the laboratory immediately. Healthy lateral buds, the main edible parts of baby mustard, were cut off, washed in tap water, dried on blotting paper, and then cut into thin slices of ∼3 mm. The lateral buds were assigned randomly to "unblanched" and "blanched" treatment groups.

Blanching and Storage Treatments

The blanched treatment group of bud slices was randomly divided into four samples that were separately blanched at 96°C for 30 s and then immediately cooled in cold water (3°C for 90 s), drained, and blotted dry using paper towels. Similarly, the unblanched treatment group of bud slices were also randomly divided into four samples and each sample was divided into six portions (300–350 g each) that were packed into transparent polypropylene containers with lids, and then stored in a domestic deep chest freezer at −20°C. The test lasted 8 months and the baby mustard was sampled after 0, 1, 2, 4, 6, and 8 months of storage. The samples were then lyophilized in a freeze-dryer and stored at −20°C for further analysis.

Quality Assessment

Glucosinolate Composition and Contents

Glucosinolates were extracted and analyzed as previously described (17). Freeze-dried samples (100 mg) were boiled in 5 mL water for 10 min. The supernatant was collected after centrifugation, and the residues were washed once with water, centrifuged and then combined with the previous extract. The aqueous extract was applied to a DEAE-Sephadex A-25 column (Sigma Chemical Co., Saint Louis, USA). The glucosinolates were converted into their desulpho analogs by overnight treatment with 100 μL of 0.1% aryl sulphatase (Sigma Chemical Co., Saint Louis, USA), and the desulphoglucosinolates were eluted with 1 mL water. High performance liquid chromatography (HPLC) analysis of desulphoglucosinolates was carried out using an Agilent 1260 HPLC instrument equipped with a variable wavelength detector (VWD) detector (Agilent Technologies, Inc., Palo Alto, USA). Samples were separated at 30°C on a Waters Spherisorb C18 column (250 mm × 4.6 mm i.d.; 5 μm particle size) using acetonitrile and water at a flow rate of 1.0 mL min^{-1}. Absorbance was detected at 226 nm. Glucosinolates were quantified by using *ortho*-Nitrophenyl β-D-galactopyranoside (Sigma Chemical Co., Saint Louis, USA) as the internal standard and considering the response factor of each glucosinolate.

Chlorophyll and Carotenoid Contents

The contents of chlorophyll and carotenoid were determined using the method of Sun et al. (6). Two hundred mg powder of lateral bud were ground and extracted with 25 mL acetone. The samples were sonicated for 20 min, and centrifuged at 4,000 g at room temperature (20 ± 2°C) for 5 min. The supernatant was filtered through 0.22 μm nylon syringe filters and analyzed by HPLC. HPLC analysis of chlorophylls and carotenoids were carried out using an Agilent 1260 instrument with a VWD detector (Agilent Technologies, Inc., Palo Alto, USA). Samples (10 μL) were separated at 30°C on a Waters C18 column (150 mm × 3.9 mm i.d.; 4 μm particle size) using isopropanol and 80% acetonitrile-water at a flow rate of 0.5 mL min^{-1}. Absorbances were detected at 448 and 428 nm. Chlorophylls (a and b) and carotenoids (neoxanthin, violaxanthin, lutein, and β-carotene) were quantified according to the respective standard calibration curves, and their standards were obtained from Solarbio Science and Technology Co., Ltd. (Beijing, China).

Ascorbic Acid Content

Ascorbic acid content was determined according to the previous report (17). Fifty mg of sample powder was extracted with 5 mL 1.0% oxalic acid, subsequently centrifuged 5 min at 4,000 g. Each sample was filtered through a 0.45 μm cellulose acetate filter. HPLC analysis of ascorbic acid was carried out using an Agilent 1260 instrument with a VWD detector (Agilent Technologies, Inc., Palo Alto, USA). Samples were separated on a Waters Spherisorb C18 column (150 mm × 4.6 mm i.d.; 5 μm particle size), using a solvent of 0.1% oxalic acid at a flow rate of

1.0 mL min^{-1}. The amount of ascorbic acid was calculated from absorbance values at 243 nm, using authentic ascorbic acid (Sangon Biotech Co., Ltd., shanghai, China) as a standard.

Total Phenolics Content

Total phenolics were homogenized for 1 min and extracted with 10 mL of 50% ethanol, and then incubated at room temperature (20 ± 2°C) for 24 h in the dark. The suspension was centrifuged at 4,000 g for 5 min at room temperature. The supernatant was used for the measurements of total phenolics content and antioxidant activity. The supernatant was mixed with Folin-Ciocalteu reagent, after 3 min, saturated sodium carbonate was added. The absorbance was measured at 760 nm with a UV-1800 spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China) as previously described (17). Gallic acid (Sangon Biotech Co., Ltd., shanghai, China) was used as a standard and the results were expressed as mg gallic acid equivalent g^{-1} dry weight.

Ferric Reducing Antioxidant Power (FRAP)

FRAP assay was performed according to the previous report (17). The extracted samples were added to the FRAP working solution incubated at 37°C and vortexed. The absorbance was then recorded at 593 nm using a UV-1800 spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China) after the mixture had been incubated in at 37°C for 10 min. FRAP values were calculated based on $FeSO_4{\cdot}7H_2O$ standard curves and expressed as μmol g^{-1} dry weight.

2,2-Azinobis (3-Ethyl-Benzothiazoline-6-Sulfonic Acid) (ABTS) Assay

ABTS antioxidant activity was performed according to the previous report (17). An aliquot of 300 μL of each extracted sample was added to 3 mL of $ABTS^+$ solution. The absorbance was measured spectrophotometrically at 734 nm after exactly 2 h. The percentage inhibition was calculated according to the formula: % inhibition = $[(A_{control}\text{-}A_{sample})/A_{control}] \times 100\%$.

Soluble Sugar Composition and Contents

Soluble sugars, including fructose, glucose, and sucrose, were extracted and analyzed as previously described with some modification (6). Freeze-dried samples (100 mg) were added to 5 mL of distilled water and homogenized for 1 min. The mixture was then extracted in a water bath at 80°C for 30 min. The supernatant was collected after centrifugation at 8,000 g at room temperature (20 ± 2°C) for 5 min, and filtered through 0.45 μm cellulose acetate filter, and then analyzed by HPLC using an Agilent 1260 instrument equipped with a refractive index detector (Agilent Technologies, Inc., Palo Alto, USA). Samples were separated at 35°C on an Agilent ZORBAX carbohydrate column (250 mm × 4.6 mm i.d.; 5 μm particle size) using 80% acetonitrile at a flow rate of 1.0 mL min^{-1}.

Soluble Proteins Content

The soluble proteins content was determined using the method of Bradford (18). Fifty milligrams of freeze-dried powdered material was soaked in 10 mL of distilled water. The solution was stirred for 30 s using a vortex mixer, after which it was allowed to settle for 30 min. The solution was then centrifuged for 5 min at 4,000 g and 1 mL transferred to a polypropylene tube. Subsequently, Coomassie brilliant blue G-250 was combined with 1 mL of supernatant. The absorbance was measured at 595 nm within 20 min after the reaction.

Statistical Analysis

All assays were performed in quadruplicate. Statistical analysis was performed using the SPSS package program version 18 (SPSS Inc., Chicago, IL, USA). Data were analyzed using two-way analysis of variance. Principal component analysis (PCA) was performed in SIMCA-P 11.5 Demo software (Umetrics, Sweden) with unit variance (UV)-scaling to decipher the relationships among samples (17). A time-related trajectory analysis based on two-dimensional PCA map was applied to visualize the temporal alterations of postharvest quality changes under different photoperiod treatments (6).

RESULTS

Glucosinolates

The composition and contents of glucosinolate in the lateral buds of baby mustard were measured, including three aliphatic glucosinolates (sinigrin, gluconapin, and progoitrin) and four indole glucosinolates (glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, and 4-hydroxy glucobrassicin) (**Figure 1** and **Supplementary Figure 1**). The most abundant glucosinolate was sinigrin, accounting for 94.65 and 90.84% of total aliphatic and total glucosinolate contents, respectively, followed by gluconapin. The predominant indole glucosinolates were glucobrassicin and 4-methoxyglucobrassicin.

After blanching, the contents of sinigrin, gluconapin, total aliphatic glucosinolate, and total glucosinolate decreased by 20.34, 25.59, 20.22, and 19.09%, respectively compared with unblanched buds. On the other hand, the contents of progoitrin and indole glucosinolates (except for glucobrassicin) slightly increased, while glucobrassicin content remained stable after blanching (**Figure 1** and **Supplementary Figure 1**).

During long-term frozen storage, the contents of individual and total aliphatic glucosinolates (except for progoitrin) in the unblanched treatment group showed a slow, wavelike decline, while the contents in the blanched treatment group decreased rapidly in the 1st month and then remained stable (**Figure 1**). Due to the large proportion of sinigrin, the trend of total glucosinolate content was similar to that of sinigrin (**Supplementary Figure 1**). The contents of individual and total indole glucosinolates, as well as progoitrin, decreased slightly after a sharp decline in the 1st month of frozen storage regardless of blanching status (**Figure 1** and **Supplementary Figure 1**). Moreover, the contents of most glucosinolates in the unblanched treatment group were significantly higher than those in the blanched treatment group during frozen storage. For example, contents of sinigrin and total glucosinolate in the unblanched treatment group were 1.76- and 1.75-fold higher than those in the blanched treatment group after 8 months, respectively. Overall, however, at the end of storage, the total glucosinolate contents in unblanched and blanched treatment groups had decreased by 27.52 and 58.70% compared with respective initial levels,

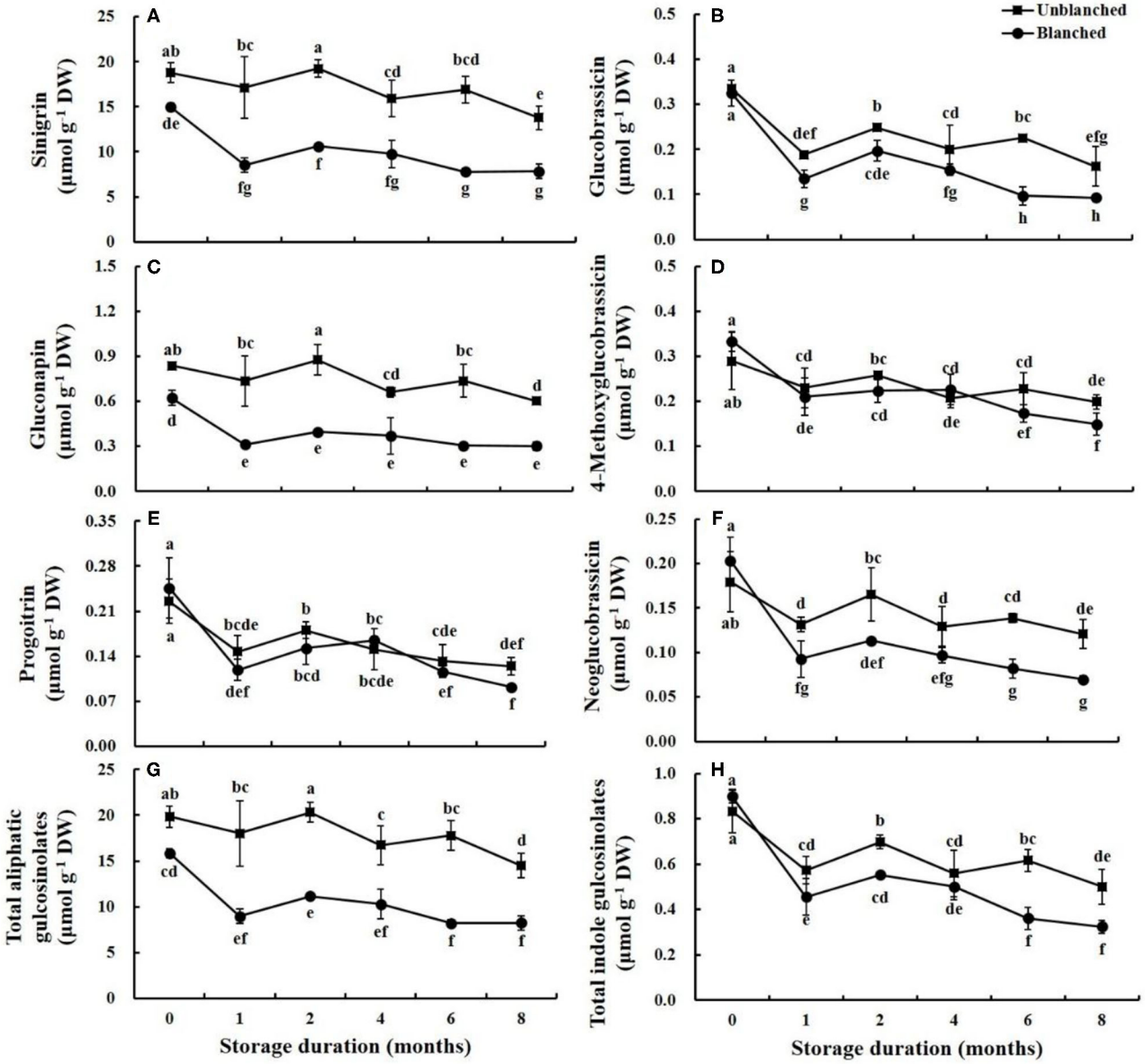

FIGURE 1 | Evolution trend of predominant glucosinolate content in baby mustard lateral buds during frozen storage at −20°C. **(A)** sinigrin; **(B)** glucobrassicin; **(C)** gluconapin; **(D)** 4-methoxyglucobrassicin; **(E)** progoitrin; **(F)** neoglucobrassicin; **(G)** total aliphatic gulcosinolates; **(H)** total indole gulcosinolates. Unblanched represents no blanching before freezing; Blanched represents blanching before freezing. Values not sharing the same letter are significantly different at $p < 0.05$.

suggesting that long-term frozen storage could be a good way to preserve the glucosinolates in frozen baby mustard.

Chlorophylls and Carotenoids

There were two chlorophylls (chlorophyll a and chlorophyll b) and four carotenoids (neoxanthin, violaxanthin, lutein, and β-carotene) detected in the baby mustard buds (**Figure 2**). Blanching before freezing did not have a significant effect on the chlorophyll and carotenoid contents in the buds. During long-term frozen storage, contents of chlorophyll and carotenoid in lateral buds in the unblanched treatment group remained stable, whereas contents in the blanched treatment group decreased rapidly in the 1st month and then remained stable, and the total chlorophyll and carotenoid contents in the blanched treatment group decreased by 54.03 and 16.32% at 8 months, respectively. Most of the individual and total chlorophyll and carotenoid contents in the unblanched treatment group were significantly higher than those in the blanched treatment group during frozen storage, and contents of total chlorophylls and carotenoids in unblanched buds were 2.15- and 1.14-fold those in blanched buds at the end of storage, respectively. In general, the chlorophyll and carotenoid contents of lateral buds were effectively preserved by long-term frozen storage, especially in the unblanched treatment group.

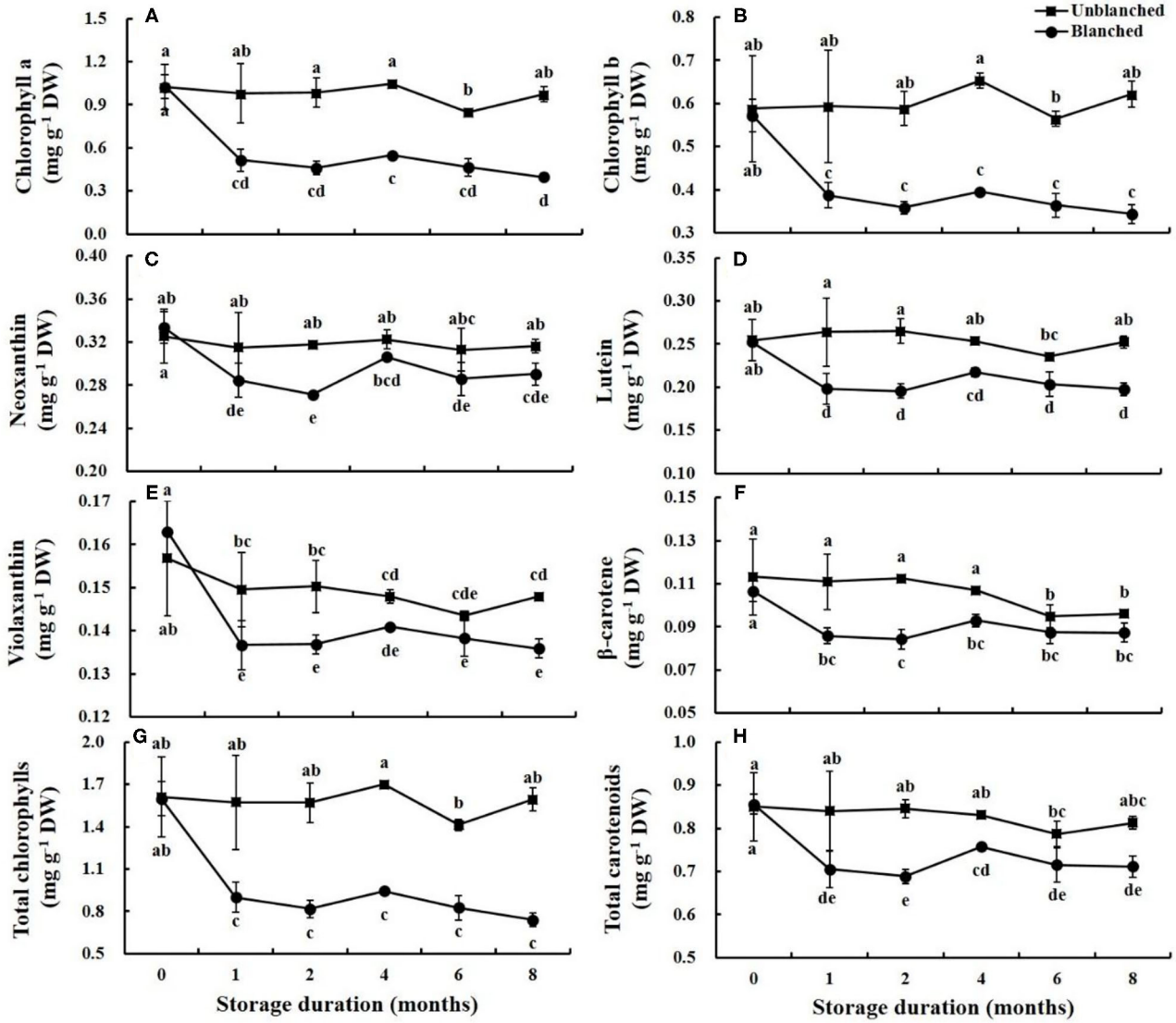

FIGURE 2 | Evolution trend of chlorophylls and carotenoids contents in baby mustard lateral buds during frozen storage at −20°C. **(A)** chlorophyll a; **(B)** chlorophyll b; **(C)** neoxanthin; **(D)** lutein; **(E)** violaxanthin; **(F)** β-carotene; **(G)** total chlorophylls; H: total carotenoids. Unblanched represents no blanching before freezing; Blanched represents blanching before freezing. Values not sharing the same letter are significantly different at $p < 0.05$.

Ascorbic Acid and Total Phenolics

Blanching before freezing significantly reduced the ascorbic acid content of lateral buds of baby mustard (by 24.23%), whereas it significantly increased their total phenolics content (by 12.76%) (**Figure 3**). During frozen storage, ascorbic acid contents in lateral buds decreased rapidly in the 1st month and then slowly went down whether the buds were blanched or not (**Figure 3A**). The total phenolics content in the blanched treatment group decreased rapidly in the 1st month and then slowly went down, while that in the unblanched treatment group remained stable in the 1st month and then slowly went down (**Figure 3B**). The ascorbic acid content in the unblanched treatment group was significantly higher than that in the blanched treatment group during storage, while total phenolics content was not significantly different between the unblanched and blanched treatment groups after 1 month. In addition, baby mustard still retained relatively high levels of ascorbic acid and total phenolics after 8 months of frozen storage, retaining 44.37% of ascorbic acid content and 73.00% of total phenolics content in the unblanched treatment group, and 34.13% of ascorbic acid content and 70.62% of total phenolics content in the blanched treatment group (**Figure 3**).

Antioxidant Capacity

We investigated antioxidant capacity using both FRAP and ABTS assays (**Figure 4**). The trends of antioxidant levels were similar to that of ascorbic acid content during frozen storage. Blanching

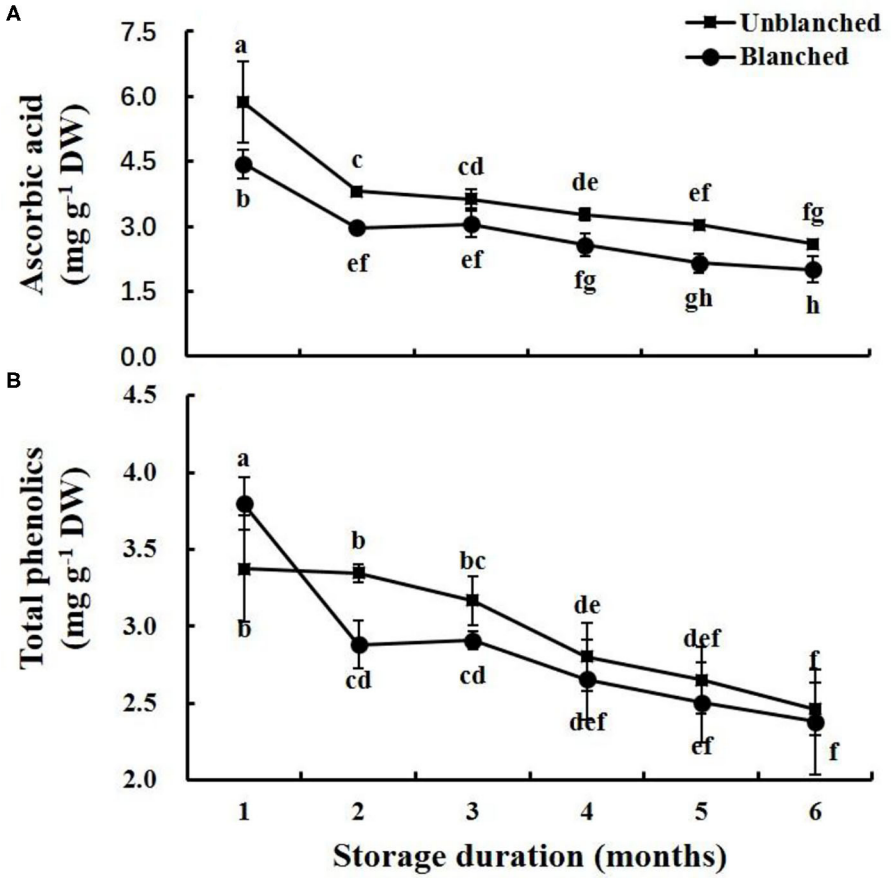

FIGURE 3 | Evolution trend of ascorbic acid and total phenolics contents in baby mustard lateral buds during frozen storage at −20°C. **(A)** ascorbic acid; **(B)** total phenolics. Unblanched represents no blanching before freezing; Blanched represents blanching before freezing. Values not sharing the same letter are significantly different at $p < 0.05$.

before freezing significantly reduced the levels detected by both FRAP and ABTS, by 9.75 and 7.39%, respectively. During long-term frozen storage, FRAP and ABTS levels in lateral buds decreased rapidly in the 1st month and then slowly went down, while the baby mustard still retained more than 50 and 70% of its antioxidant capacity as measured by FRAP and ABTS at the end of frozen storage, whether it was blanched or not. Interestingly, the FRAP level in the unblanched treatment group was significantly higher than that in the blanched treatment group during storage except for the 8th month, while the ABTS level was not significantly different between the unblanched and blanched treatment groups except for the 6th month.

Soluble Sugars

There were three kinds of soluble sugars (fructose, glucose, and sucrose) detected in lateral buds of baby mustard (**Figures 5A–C**). Blanching had no significant effect on the fructose and glucose contents, whereas it significantly increased sucrose content. During long-term frozen storage, the fructose content of lateral buds increased first and then decreased; peak fructose content appeared early and high in the blanched treatment group compared with the unblanched treatment group (**Figure 5A**). Both glucose contents and the change trends were similar between the unblanched and blanched treatment groups, first decreasing and then increasing (**Figure 5B**). The trend of sucrose content in the blanched treatment group was similar to that of glucose, while the sucrose content of the unblanched treatment group showed a wavelike enhancement (**Figure 5C**).

Soluble Proteins

Blanching before freezing significantly increased soluble protein content in lateral buds of baby mustard (by 22.55%). In long-term frozen storage, the soluble protein content in the blanched treatment group dropped dramatically (by 71.91%) in the 1st month, and then remained stable. However, soluble protein content in the unblanched treatment group remained basically stable for the first 4 months, and then increased significantly (by 42.49%) (**Figure 5D**).

A Time-Related Trajectory Analysis

A time-related trajectory analysis was performed to compare the impacts of long-term frozen storage and blanching before freezing on the retention of health-promoting phytochemicals and antioxidant capacity in baby mustard (**Figure 6**). The points

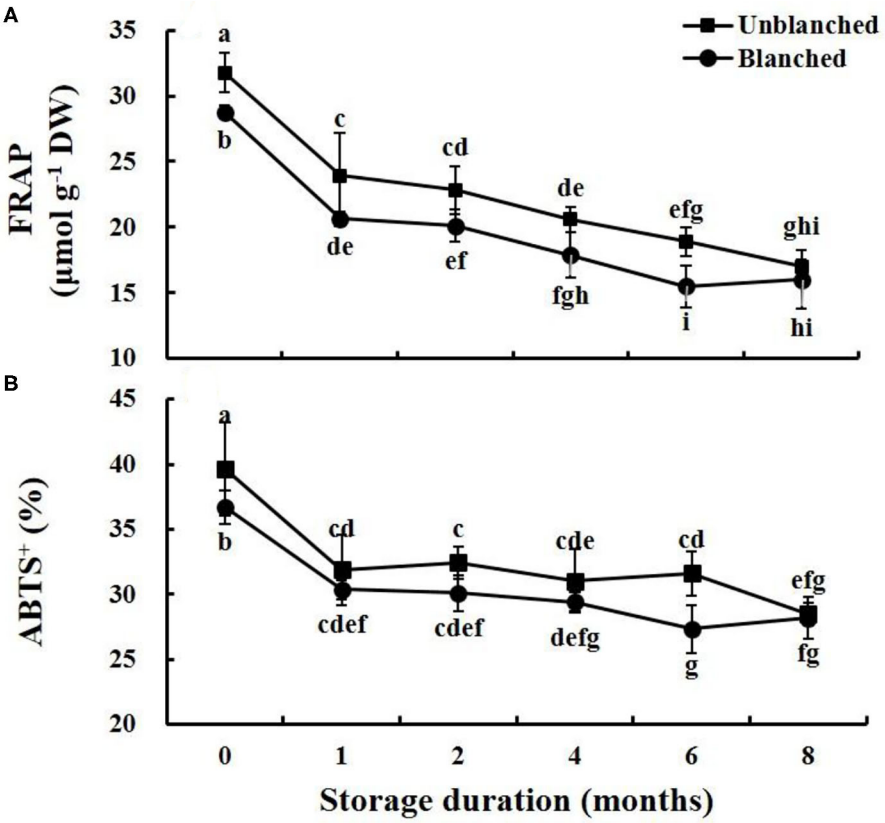

FIGURE 4 | Evolution trend of antioxidant capacity levels in baby mustard lateral buds during frozen storage at −20°C. **(A)** FRAP; **(B)** ABTS$^+$. Unblanched represents no blanching before freezing; Blanched represents blanching before freezing. Values not sharing the same letter are significantly different at $p < 0.05$.

representing different storage times and blanching status before freezing were notably separated, and the greater the distance from the origin (month 0), the higher the degree of deterioration. During long-term frozen storage, both unblanched and blanched treatment groups had the largest distance change in the 1st month and little change thereafter. In general, the unblanched treatment group had a shorter distance change than the blanched treatment group throughout storage. Thus, the loss of nutritional qualities mainly occurred in the 1st month of frozen storage, and the nutritional qualities in the unblanched treatment group were significantly better than those in the blanched treatment group during long-term frozen storage.

DISCUSSION

Glucosinolates are important bioactive compounds in baby mustard which have potential anticancer activity through inhibiting tumor cell growth (6, 19, 20). Previous studies have shown that glucosinolate content in *Brassica* vegetables (including broccoli, cauliflower, and Brussels sprouts) may be effectively maintained by long-term frozen storage (12, 15, 21). Similar results were also achieved in this study. After 8 months of long-term frozen storage, ∼50% or more of the total glucosinolate content of baby mustard was retained in both unblanched and blanched treatment groups. However, we found in a previous study that the total glucosinolate content of baby mustard was reduced by more than 80% after 6 days of storage at room temperature (20 ± 2°C) in the dark (6). This difference may be due to the inactivation of myrosinase activity caused by low temperature, which slowed down the degradation of glucosinolates to minimal rates during freezing (22). The loss of glucosinolate contents mainly occurred in the first month of frozen storage, which may be related to disruption of plant cells by ice crystals during freezing (15). However, the loss of glucosinolates in the blanched treatment group in the first month of frozen storage was greater than that in the unblanched treatment group. This is probably due to blanching leading to increased solute loss, which raises the freezing point, and therefore an increase in the amount and size of ice crystals (14). Furthermore, blanching before freezing resulted in a decline in high-content glucosinolate levels (**Figures 1A,C**), but either had no effect on low-content glucosinolates or even increased content (**Figures 1B,D–F** and **Supplementary Figure 1A**). According to previous reports, remarkable reductions in high-content glucosinolates may be explained by the dissolution of glucosinolates into water as cell lysis occurs and glucosinolates degrade during blanching (21, 23).

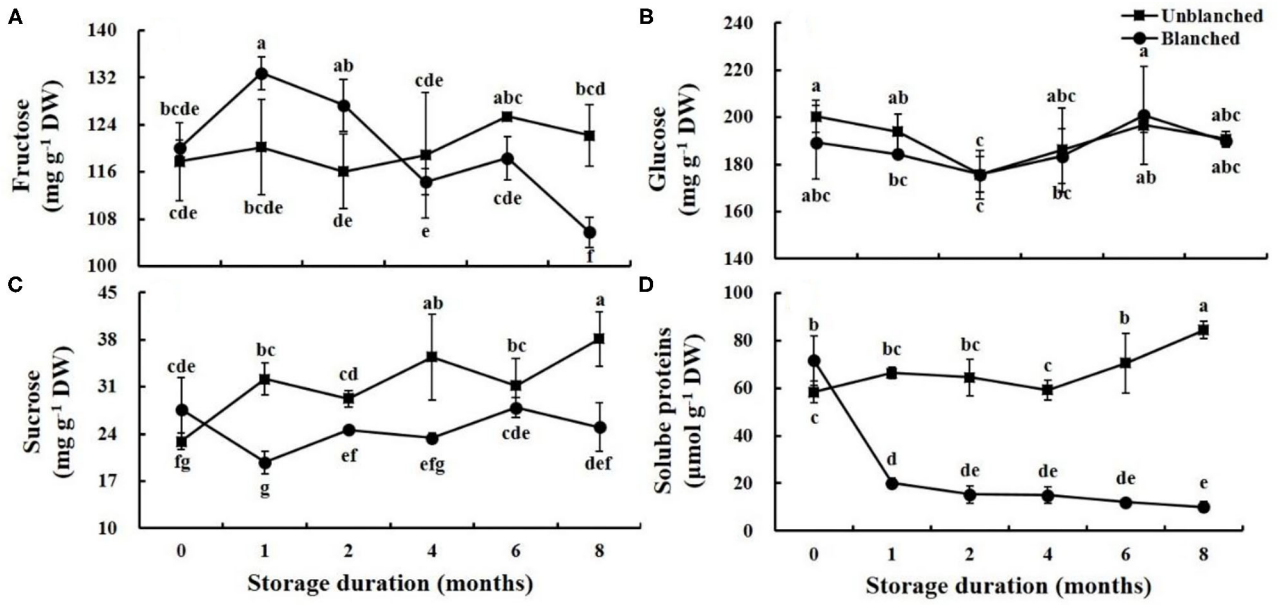

FIGURE 5 | Evolution trend of soluble sugars and soluble proteins contents in baby mustard lateral buds during frozen storage at −20°C. **(A)** fructose; **(B)** glucose; **(C)** sucrose; **(D)** soluble proteins. Unblanched represents no blanching before freezing; Blanched represents blanching before freezing. Values not sharing the same letter are significantly different at $p < 0.05$.

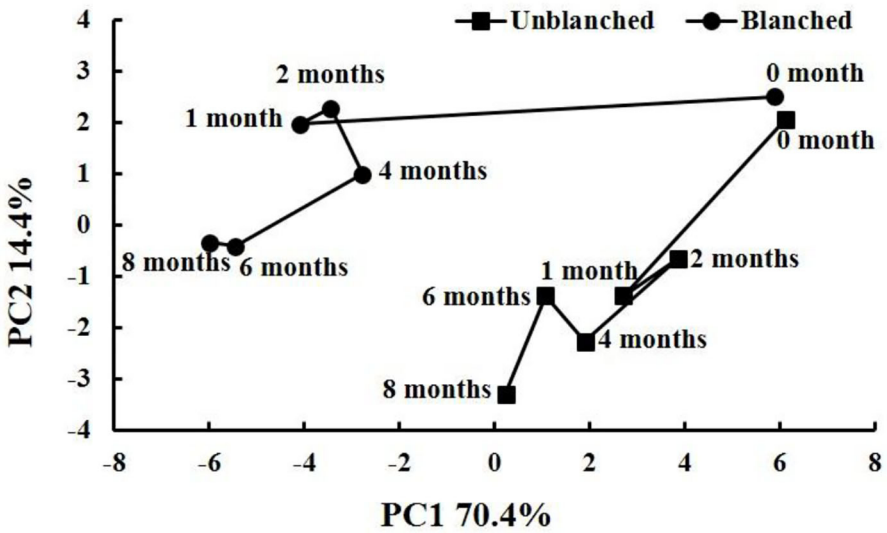

FIGURE 6 | Time-related trajectory plot showing the dynamic time-related responses of nutritional qualities in baby mustard lateral buds during frozen storage at −20°C. Unblanched represents no blanching before freezing; Blanched represents blanching before freezing.

Similar results have also been found in other *Brassica* vegetables, including broccoli florets, brussels sprouts, cauliflower, and curly kale (15, 21). On the other hand, for low-content glucosinolates, one possible explanation is that the effects of blanching through deactivation of myrosinase (15, 21, 22) and improvement of the extractability of glucosinolates (11) balanced or exceeded the effects that would normally reduce glucosinolates.

Vegetable color is an important factor in terms of quality judgment by consumers (4, 24). The color of lateral buds of baby mustard is mainly determined by chlorophylls and carotenoids (6, 17). In this study, pigment contents in the unblanched treatment group did not change significantly during frozen storage, which may be due to cell structure remaining intact without solute loss before freezing; low temperature then

substantially reduced metabolic processes and inhibited the degradation of pigments (10). The change of pigment contents in the blanched treatment group was similar to of the change in glucosinolate contents, which remained stable after a sharp decrease in the 1st month of frozen storage. This pigment loss may be due to solute loss, which could increase the freezing point and amount and the size of ice crystals (14), resulting in reduced pigment contents. However, due to inactivation of related enzyme activity during frozen storage, more than 80% of carotenoid content and approximately half of chlorophyll content remained in the blanched treatment group after 8 months of long-term frozen storage. On the whole, these findings indicate that frozen storage is an excellent way to preserve pigment contents in baby mustard. Interestingly, blanching before freezing did not have a significant effect on pigment contents, in contrast to glucosinolate contents (**Figure 1**). A similar result was also found in broccoli (21), which may be due to pigment enzyme activity being blocked during blanching (21), fat-soluble nature pigments being less prone to leaching effects (25), and even to the fact that blanching increases the extractability of pigments (11).

Antioxidants can provide electrons or hydrogen atoms, participate in the scavenging of free radicals, and prevent the accumulation of free radicals in vegetables (8, 26). Long-term frozen storage is an effective way to reserve the ascorbic acid in frozen baby mustard, and we found that retention of ascorbic acid in both unblanched and blanched treatment groups was still ∼40% after 8 months of frozen storage. This can be explained by the inactivation of oxidase at low temperature (16). Ascorbic acid is highly water-soluble and sensitive to heat, and these properties make it susceptible to blanching (27, 28), causing ascorbic acid content in baby mustard to decrease by more than one-fifth through blanching before freezing. In addition, according to the previous literature, ascorbic acid also continues to degrade during prolonged storage of frozen products (28); our results were consistent with this finding. Likewise, long-term frozen storage is also an effective way to preserve the total phenolics in frozen baby mustard. After 8 months of long-term frozen storage, over 70% of total phenolics contents were still retained in both unblanched and blanched treatment groups. Unlike ascorbic acid, total phenolics content in baby mustard significantly increases from blanching before freezing. It may be that the blanching process inactivates enzyme activity that causes the oxidation of phenolics (28), leading to the enhancement of total phenolics content after blanching. In our study, phenolic degradation still occurred in baby mustard during frozen storage, which was consistent with previous results on raspberries and blackberries, with 12 and 8% loss of total phenolics, respectively (29). Antioxidant capacity reflects the synergetic effect of multiple antioxidants (21, 30). In this study, the change trend of antioxidant capacity is similar to the change trend of ascorbic acid content, suggesting that ascorbic acid may contribute more to antioxidant capacity in baby mustard than phenolic compounds, which agrees with our previous results (6).

Soluble sugars and soluble proteins are important quality indicators for horticultural crops (17, 31). In this study, baby mustard still retained relatively high levels of soluble sugars content after 8 months of long-term frozen storage. Environmental stress during postharvest storage can induce sucrose content (32). Our results found that sucrose content in the unblanched treatment group significantly increased during frozen storage, while that in the blanched treatment group was basically stable. A possible reason is that blanching before freezing could lead to inactivation of enzymes responsible for sucrose metabolism (33), while the unblanched treatment group still had a certain level of activity of sucrose biosynthetic enzymes at low temperature (14). For soluble proteins in baby mustard, blanching before freezing significantly increased content, which may be due to the rapid production of a large number of heat shock proteins and proline in the bud cells (34, 35). Subsequently, soluble proteins content in the blanched treatment group decreased rapidly in the 1st month, which may be explained by an increase in the number and volume of ice crystals formed during the freezing process and caused by the blanching process (14).

In this study, it was observed that the main health-promoting compounds such as glucosinolates, carotenoids, and ascorbic acid, as well as antioxidant capacity in baby mustard deteriorated to varying degrees during long-term frozen storage and the blanching process, indicating that related enzymes may play an important role in regulation of these metabolites during blanching and frozen storage. Previous studies reported that freezing can inactivate peroxidase and lipoxygenase, thus improving the quality of frozen food (36), while blanching process could cause an almost complete loss of myrosinase activity, which subsequently resulted in the inhibition of glucosinolate hydrolysis (21). The effect of enzymes on postharvest storage of baby mustard is interesting, and therefore it would be included in our future research.

CONCLUSION

In summary, frozen storage successfully maintained the health-promoting compounds and antioxidant capacity in baby mustard for a long time, which could make year-round supply of baby mustard possible. We recommend against blanching baby mustard before frozen storage, in order to preserve high nutritional quality. However, further experiments are still needed to find ways to improve the quality of baby mustard during long-term frozen storage, including blanching technology improvements and application of new quick-freezing technologies.

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/s.

AUTHOR CONTRIBUTIONS

HL and BS designed the experiments. JZ, HD, PX, ZW, and ZL conducted the experiments. FZ, CZ, SH, ML, YT, and YL analyzed the data. JZ and HD wrote the manuscript. FZ and BS revised the manuscript. FZ, HL, and BS provided the financial support. All authors have read and agreed to the published version of 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/fnut.2021.665482/full#supplementary-material

Supplementary Figure 1 | Evolution trend of 4-hydroxy glucobrassicin and total glucosinolate contents in baby mustard lateral buds during frozen storage at −20°C. Unblanched represents no blanching before freezing; Blanched represents blanching before freezing. Values not sharing the same letter are significantly different at $p < 0.05$.

REFERENCES

1. Galgano F, Favati F, Caruso MC, Pietrafesa A, Natella S. The influence of processing and preservation on the retention of health-promoting compounds in broccoli. *J Food Sci*. (2007) 72:130–5. doi: 10.1111/j.1750-3841.2006.00258.x
2. Mastropasqua L, Tanzarella P, Paciolla C. Effects of postharvest light spectra on quality and health-related parameters in green *Asparagus officinalis* L. *Postharvest Biol Technol*. (2016) 112:143–51. doi: 10.1016/j.postharvbio.2015.10.010
3. Rybarczyk-Plonska A, Hagen SF, Borge GIA, Bengtsson GB, Hansen MK, Wold A. Glucosinolates in broccoli (*Brassica oleracea* L. var italica) as affected by postharvest temperature and radiation treatments. *Postharvest Biol Technol*. (2016) 116:16–25. doi: 10.1016/j.postharvbio.2015.12.010
4. Managa MG, Remize F, Garcia C, Sivakumar D. Effect of moist cooking blanching on colour, phenolic metabolites and glucosinolate content in Chinese cabbage (*Brassica rapa* L. subsp chinensis). *Foods*. (2019) 8:399. doi: 10.3390/foods8090399
5. Sun B, Lin PX, Xia PX, Di HM, Zhang JQ, Zhang CL, et al. Low-temperature storage after harvest retards the deterioration in the sensory quality, health-promoting compounds, and antioxidant capacity of baby mustard. *RSC Adv*. (2020) 10:36495–503. doi: 10.1039/D0RA07177C
6. Sun B, Di HM, Zhang JQ, Xia PX, Huang WL, Jian Y, et al. Effect of light on sensory quality, health-promoting phytochemicals and antioxidant capacity in post-harvest baby mustard. *Food Chem*. (2021) 339:128057. doi: 10.1016/j.foodchem.2020.128057
7. Nilsson J, Stegmark R, Åkesson B. Total antioxidant capacity in different pea (*Pisum sativum*) varieties after blanching and freezing. *Food Chem*. (2004) 86:501–7. doi: 10.1016/j.foodchem.2003.09.002
8. Frazie MD, Kim MJ, Ku KM. Health-promoting phytochemicals from 11 mustard cultivars at baby leaf and mature stages. *Molecules*. (2017) 22:1749. doi: 10.3390/molecules22101749
9. Kmiecik W, Lisiewska Z, Korus A. Retention of mineral constituents in frozen brassicas depending on the method of preliminary processing of the raw material and preparation of frozen products for consumption. *Eur Food Res Technol*. (2007) 224:573–9. doi: 10.1007/s00217-006-0337-6
10. Gonçalves EM, Abreu M, Brandão TRS, Silva CLM. Degradation kinetics of colour, vitamin C and drip loss in frozen broccoli (*Brassica oleracea* L. ssp Italica) during storage at isothermal and non-isothermal conditions. *Int J Refrig*. (2011) 34:2136–44. doi: 10.1016/j.ijrefrig.2011.06.006
11. Alanís-Garza PA, Becerra-Moreno A, Mora-Nieves JL, Mora-Mora JP, Jacobo-Velázquez DA. Effect of industrial freezing on the stability of chemopreventive compounds in broccoli. *Int J Food Sci Nutr*. (2015) 66:282–8. doi: 10.3109/09637486.2015.1007451
12. Miao HY, Lin JY, Zeng W, Wang MY, Yao LS, Wang QM. Main health-promoting compounds response to long-term freezer storage and different thawing methods in frozen broccoli florets. *Foods*. (2019) 8:375. doi: 10.3390/foods8090375
13. Volden J, Bengtsson GB, Wicklund T. Glucosinolates, L-ascorbic acid, total phenols, anthocyanins, antioxidant capacities and colour in cauliflower (*Brassica oleracea* L. ssp botrytis); effects of long-term freezer storage. *Food Chem*. (2009) 112:967–76. doi: 10.1016/j.foodchem.2008.07.018
14. Sman RGMVD. Impact of processing factors on quality of frozen vegetables and fruits. *Food Eng Rev*. (2020) 12:399–420. doi: 10.1007/s12393-020-09216-1
15. Cieślik E, Leszczyńska T, Filipiak-Florkiewicz A, Sikora E, Pisulewski PM. Effects of some technological processes on glucosinolate contents in cruciferous vegetables. *Food Chem*. (2007) 105:976–81. doi: 10.1016/j.foodchem.2007.04.047
16. Tosun BN, Yücecan S. Influence of commercial freezing and storage on vitamin C content of some vegetables. *Int J Food Sci Technol*. (2008) 43:316–21. doi: 10.1111/j.1365-2621.2006.01436.x
17. Sun B, Tian YX, Jiang M, Yuan Q, Chen Q, Zhang Y, et al. Variation in the main health-promoting compounds and antioxidant activity of whole and individual edible parts of baby mustard (*Brassica juncea* var. gemmifera) *RSC Adv*. (2018) 8:33845–54. doi: 10.1039/C8RA05504A
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. (1976) 72:248–54. doi: 10.1016/0003-2697(76)90527-3
19. Jin P, Yao D, Xu F, Wang HQ, Zheng YH. Effect of light on quality and bioactive compounds in postharvest broccoli florets. *Food Chem*. (2015) 172:705–9. doi: 10.1016/j.foodchem.2014.09.134
20. Wang JS, Yu HF, Zhao ZQ, Sheng XG, Shen YS, Gu HH. Natural variation of glucosinolates and their breakdown products in broccoli (*Brassica oleracea* var. italica) seeds. *J Agric Food Chem*. (2019) 67:12528–37. doi: 10.1021/acs.jafc.9b06533
21. Cai CX, Miao HY, Qian HM, Yao LS, Wang BL, Wang QM. Effects of industrial pre-freezing processing and freezing handling on glucosinolates and antioxidant attributes in broccoli florets. *Food Chem*. (2016) 210:451–6. doi: 10.1016/j.foodchem.2016.04.140
22. Dosz EB, Jeffery EH. Commercially produced frozen broccoli lacks the ability to form sulforaphane. *J Funct Foods*. (2013) 5:987–90. doi: 10.1016/j.jff.2013.01.033
23. Foo HL, Grønning LM, Goodenough L, Bones AM, Danielsen B, Whiting DA, et al. Purification and characterisation of epithiospecifier protein from *Brassica napus*: enzymic intramolecular sulphur addition within alkenyl thiohydroximates derived from alkenyl glucosinolate hydrolysis. *FEBS Lett*. (2000) 468:243–6. doi: 10.1016/S0014-5793(00)01176-5
24. Tan XY, Misran A, Daim LDJ, Ding P, Dek MSP. Effect of freezing on minimally processed durian for long term storage. *Sci Hortic*. (2020) 264:109170. doi: 10.1016/j.scienta.2019.109170
25. Hidaka T, Fukuda N, Taniguchi K, Kanzaki J. Fat-soluble components of lateral flower buds from different varieties of broccoli (*Brassica oleracea* L. var italica Plen). *Nippon Eiyo Shokuryo Gakkaishi*. (1992) 45:453–5. doi: 10.4327/jsnfs.45.453

26. Chen CK, Zhang HJ, Dong CH, Ji HP, Zhang XJ, Li L, et al. Effect of ozone treatment on the phenylpropanoid biosynthesis of postharvest strawberries. *RSC Adv*. (2019) 9:25429–38. doi: 10.1039/C9RA03988K
27. Aparicio-Cuesta MP, Garcia-Moreno C. Quality of frozen cauliflower during storage. *J Food Sci*. (1988) 53:491–3. doi: 10.1111/j.1365-2621.1988.tb07738.x
28. Rickman JC, Barrett DM, Bruhn CM. Nutritional comparison of fresh, frozen and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds. *J Sci Food Agric*. (2007) 87:930–44. doi: 10.1002/jsfa.2825
29. González EM, De Ancos B, Cano MP. Relation between bioactive compounds and free radical-scavenging capacity in berry fruits during frozen storage. *J Sci Food Agr*. (2003) 83:722–6. doi: 10.1002/jsfa.1359
30. Xiao ZL, Lester GE, Luo YG, Xie ZH, Yu LL, Wang Q. Effect of light exposure on sensorial quality, concentrations of bioactive compounds and antioxidant capacity of radish microgreens during low temperature storage. *Food Chem*. (2014) 151:472–9. doi: 10.1016/j.foodchem.2013.11.086
31. Alhamdan A, Hassan B, Alkahtani H, Abdelkarima D, Younisa M. Freezing of fresh Barhi dates for quality preservation during frozen storage. *Saudi J Biol Sci*. (2018) 25:1552–61. doi: 10.1016/j.sjbs.2016.02.003
32. Itai A, Tanahashi T. Inhibition of sucrose loss during cold storage in Japanese pear (*Pyrus pyrifolia* Nakai) by 1-MCP. *Postharvest Biol Tec*. (2008) 48:355–63. doi: 10.1016/j.postharvbio.2007.10.015
33. Wang J, Yang XH, Mujumdar AS, Wang D, Zhao JH, Fang XM, et al. Effects of various blanching methods on weight loss, enzymes inactivation, phytochemical contents, antioxidant capacity, ultrastructure and drying kinetics of red bell pepper (*Capsicum annuum* L.). *LWT Food Sci Technol*. (2017) 77:337–47. doi: 10.1016/j.lwt.2016.11.070
34. Morimoto RI. Cells in stress: transcriptional activation of heat shock genes. *Science*. (1993) 259:1409–10. doi: 10.1126/science.8451637
35. Aghdam MS, Bodbodak S. Postharvest heat treatment for mitigation of chilling injury in fruits and vegetables. *Food and Bioprocess Tech*. (2014) 7:37–53. doi: 10.1007/s11947-013-1207-4
36. Ma YK, Hu XS, Chen JL, Chen F, Wu JH, Zhao GH, et al. The effect of freezing modes and frozen storage on aroma, enzyme and micro-organism in Hami Melon. *Food Sci Technol Int*. (2007) 13:259–67. doi: 10.1177/1082013207081776

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Physicochemical Parameters and Bioaccessibility of Lactic Acid Bacteria Fermented Chayote Leaf (*Sechium edule*) and Pineapple (*Ananas comosus*) Smoothies

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In this study, popularly consumed traditional chayote leaves and locally produced pineapple fruit were used to develop a fermented smoothie using lactic acid bacteria (LAB) strains: *Lactobacillus plantarum* (*L75*), *Weissella cibaria* (*W64*), and their combination (*LW64 + 75*). The physicochemical parameters [pH, total soluble solids (TSS), and color], total phenols, and carotenoid contents of the smoothies fermented for 48 h and stored for 7 days at 4°C were compared with the unfermented (control) smoothies. Results indicated that LAB fermentation reduced the pH from 3.56 to 2.50 after 48 h (day 2) compared with the non-fermented smoothie at day 2 (pH 3.37). LAB strain *L75* significantly reduced the TSS content of the smoothies to 13.06°Bx after 2 days of fermentation. Smoothies fermented by *L75* showed overall acceptability after 7 days of storage compared with the non-fermented puree on day 0. The *LW64 + 75* significantly reduced the color change (ΔE), which was similar to the control. *L75* increased the phenolic content, and *W64* enhanced the total carotenoid content of the smoothies after 2 days of fermentation compared with other treatments. The use of an *in vitro* model simulating gastrointestinal (GI) digestion showed that fermentation with *L75* improved the total phenol recovery by 65.96% during the intestinal phase compared with the control. The dialysis phase mimicked an epithelial barrier, and 53.58% of the recovered free soluble are bioavailable from the *L75* fermented smoothies compared with the control. The antioxidant capacity of dialyzable fraction of the *L75* fermented smoothie was significantly higher than that of the control and smoothies fermented with *W64* or *LW64 + 75*.

Keywords: gastrointestinal digestion, total phenols, carotenoids, antioxidant capacity, quality

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INTRODUCTION

Several authors have reported the beneficial roles of fruits and vegetables in preventing and managing chronic diseases, such as coronary heart diseases, stroke, obesity, diabetes, and cancer (1–3). The World Health Organization (4) recommends a minimum of 400 g of fruits and vegetables, or five portions per day, excluding starchy tubers, to ensure good health.

The United States Department of Agriculture (USDA) (5) guidelines state that an individual must consume one cup (∼237 g) of raw or cooked vegetables or two cups of raw leafy greens a day. However, fruits and vegetables are highly perishable and vulnerable to post-harvest losses, especially during the supply chain, thus affecting food security (6). Efforts are being made to reduce food loss at farm gate level and to profit the local economies by introducing agro processing products.

Fruit and vegetable products are valuable sources of fibers, antioxidants, and essential fatty acids (7). Fruit and vegetable juices are generally processed through juice extraction, followed by thermal processing for microbial stabilization. The thermal processing of juice can have negative effects on vitamins, such as ascorbic acid, thiamin, and folic acid (8). However, juice extraction is a processing step that removes insoluble dietary fibers, although these exert positive health effects (9). Therefore, another possible way for consumers to obtain the nutritional benefits from fruits and vegetables with less processing is through smoothies. Smoothies are fruit only or fruit and vegetable based semi-liquid nutrient-dense products with a smooth consistency (10).

Lactic acid fermentation is one of the most economical, oldest, and natural methods of food processing and preservation that keeps or enhances the efficiency and quality of foods while improving the organoleptic qualities and nutritional properties of the product (11). The importance of fermented products benefits the local economy and the communities in developing countries (12). Lactic acid bacteria (LAB) are a group of Gram-positive bacteria, which produce lactic acid as the main product of carbohydrate fermentation. During fermentation, the decrease in pH value and production of antimicrobial compounds by LAB enables the inhibition of spoilage and pathogenic microorganisms (11). LAB fermentation improves the content of riboflavin, folate, vitamin B12, sugar polymers, aroma compounds, or low-calorie polyols (mannitol, sorbitol) in substrates (13, 14). The lactic acid fermentation modifies phenolic composition and enhances the antioxidant activity in fermented tea extracts (15). Although lactic acid fermentation improves the antioxidant components in smoothies or fruit juices, it is important to know the potential availability of antioxidant components after digestion to evaluate its benefits (16).

The numerous nutritional and health benefits of chayote vegetable to consumers have encouraged growers of its cultivation and manufacturing of products by the local industry. Chayote leaves (*Sechium edule*) are one of the Réunioneses' favorite vegetables, which are locally naturalized (17). Chayote belongs to the Cucurbitaceae family and is familiar as mirliton, choko, chouchou (Jamaica), and chuchu (Brazil). The leaves are heart-shaped, 10–25 cm wide (18). A 100 g portion of young chayote leaves contains protein (4.0 g), fat (0.4 g), carbohydrates (4.7 g), fiber (1.2 g), Ca (58 mg), P (108 mg), Fe (2.5 mg), thiamin (615 μg), riboflavin (0.08 mg), niacin (0.18 mg), and ascorbic acid (1.1 mg) (19). Chayote leaf incorporation in product development holds huge potential due to its laden nutrient and biological functions, which could help improve healthy livelihoods, reduce wastage, and enhance agricultural sustainability. In contrast, pineapple (*Ananas comosus* cv. Queen Victoria) is an exotic fruit popularly grown in the Réunion Island and with recognized sensory value; a 100 g portion of the fruit contains protein (0.5 g), sugar (9.9 g), Ca (13.00 mg), K (109 mg), Fe (0.29 mg), Na (1 mg), fiber (1.4 g), total carbohydrate (13 g), and total fat (0.1 g) (5). Pineapples contain carotenoids, and their contents vary with cultivars and range from 29 to 565 μg/100 g on fresh weight (FW) basis (20). However, during the production, there is surplus supply of pineapples at the market, and cold storage facility is limited. Therefore, fermentation of pineapples and Chayote leaves was envisaged to resolve the post-harvest loss encountered during over supply.

Fermentation of fruit and vegetable substrates with desirable microorganisms could be a strategy to improve the nutritional quality, polyphenols, and antioxidant levels of fruit and vegetable products and could help to meet the nutritional and health needs of consumers. The use of LAB as starter cultures to enrich the biological value of foods has been reported (21). Another strategy to improve the nutrient quality of fruit smoothies could be by enrichment with indigenous vegetables or fruits. Polyphenols have been reported to confer health protective effects against cardiovascular and neurodegenerative diseases in humans (22). However, polyphenols from the diet must be bioaccessible for their bioactivity after undergoing an *in vivo* gastrointestinal (GI) digestion. The *in vitro* GI digestion is a reference tool to study the bioaccessibility of the dietary polyphenols (23). In the gastric digestion phase, a low pH environment, which is typical of the stomach's conditions, helps to stabilize and enhance the release of phenolics in phenolic–protein complex compounds (24). Tagliazucchi et al. (25) showed that only 62% of the original polyphenol content of grapes were bioaccessible after GI digestion. Bouayed et al. (23) suggested that the GI tract is an "effective extractor" for polyphenols present in food matrices and the polyphenols could be made available for absorption in the intestine. The use of *in vivo* human or animal models to investigate the GI tract requires an ethical clearance, is time consuming, and is expensive; therefore, *in vitro* digestion models are used to mimic the GI tract condition of humans during transit of complex food matrix and to investigate the bioaccessibility of compounds in food (26).

In light of the above, the objectives of this study were to investigate the efficacy of selected LAB strains, as standalone or in combination, to modulate the physicochemical properties of a fermented chayote leaf–pineapple smoothie, and to evaluate the total phenol and carotenoid contents, antioxidant capacity (ferric ion reducing antioxidant power, FRAP), and the bioaccessibility of phenolic compounds of the fermented smoothie after an *in vitro* gastric and intestinal digestion.

MATERIALS AND METHODS

Chemicals

Culture media were purchased from Biokar Diagnostics (Solabia Group, Pantin, France) and Condalab (Madrid, Spain). Reagents were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and VWR chemicals (Fontenay-sous-Bois, France). Type VI-B porcine pancreatic α-amylase, type I α-glucosidase from

baker's yeast, starch, 4-nitrophenyl-β-D-glucuronide (pNPG), and voglibose for enzymatic experiments were also purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The LAB cultures *Weissella cibaria* 64 (*W64*) and *Lactobacillus plantarum* 75 (*L75*) were obtained from the Microbiology Laboratory of QualiSud, Université de La Réunion, France (27).

Plant Material and Smoothie Preparation

Pineapples (*A. comosus*) and chayote leaves (*S. edule*) were purchased from a local market in Réunion Island, France. Chayote leaves, free from dirt and damage caused by pests or decay, were selected, washed with tap water, and rinsed with distilled water. The leaves were dried on a paper towel, sliced into two halves, and homogenized with a domestic blender. Ripe pineapple fruits, free from signs of damage or decay, were peeled, cut using a knife, blended for 3 min, and then bottled in sterile glass containers. For 1.8 L of pineapple juice, 270 g of homogenized chayote leaves were added, and the mixture was blended to obtain a smoothie. The mixture was pasteurized at 82°C for 10 min using a water bath and then cooled to room temperature for 2 h.

Starter Cultures and Fermentation of Smoothie

The LAB strains used in this study, *W. cibaria* (*W64*) and *L. plantarum* (*L75*), were obtained from the Microbiology Laboratory of QualiSud, Université de La Réunion, France (27). The strains were reactivated and propagated by successive suspension in 9 ml of de Man, Rogosa, and Sharpe (MRS) broth and incubated anaerobically for 48 h at 30°C in an incubator as previously reported by Nirina et al. (28). The resulting cells were harvested by centrifuging at 12,000 × g for 5 min at 4°C, cleaned, and suspended in sterile distilled water. The concentration of cultures was determined by the turbidity method on a UV–visible Spectrophotometer (SPECTROstar Nano; BMG LABTECH GmbH, Ortenberg, Germany). The concentration of pre-cultures was adjusted to 0.05 McFarland standard concentration (1–5 × 10^8 CFU/ml) at 660 nm wavelength, and 1% inoculum was inoculated into the smoothies (29). Each strain alone and a combination were used to inoculate the smoothies. The combined starter (*W64* + *L75*) was developed at equal ratio (1:1; v/v). The smoothies were incubated at 37°C for 48 h and then stored for 7 days at 4°C. Thereafter, the fermented smoothies were stored at −20°C until analysis. The un-inoculated smoothie served as a control. The fermentation was performed in triplicate.

Microbial Enumeration

The microbial load and LAB count of the samples were evaluated at the beginning, end of fermentation, and storage through the bacteria count using the pour plating techniques (30). Briefly, successive serial dilutions of smoothie sampled at 0, 2, and 7 days were made in sterile buffered peptone water and then plated on appropriate media. For the total bacterial and fungal (yeast and mold) and surviving LAB counts, dilutions were plated on Nutrient Agar (NA), Yeast Extract Glucose Chloramphenicol Agar (YGCA), and MRS plates, respectively. The MRS plates were incubated anaerobically at 30°C for 48 h, the NA plates at 37°C for 24 h, and the YGCA plates at 27°C for 5 days. The bacterial and fungal population were enumerated and expressed as colony forming units per ml (CFU/ml) of samples. Enumeration was done using five replicate plates for each sample.

Physicochemical Properties

The physicochemical properties of the fermented and non-fermented smoothies were determined at 0 and 2 days of fermentation and 7 days of storage. The color of the fermented and non-fermented smoothies was determined using a CM-3500d spectrophotometer and analyzed using the SpectraMagic NX software (Konica Minolta, NJ, USA) to assess the effect of fermentation on the color. Measurements were made using L^*, a^*, and b^* color coordinates where L^* designates lightness, a^* is the color component from red to green, and b^* represents the component from yellow to blue (31). Total color difference (ΔE) was calculated according to Managa et al. (31), in which L_2^*, a_2^*, and b_2^* refer to the assay condition, and L_1^*, a_1^*, and b_1^* refer to the control smoothie.

The pH meter (pH2700 EUTECH Instruments, IL, USA) measured the pH of the fermented and non-fermented smoothies (10 ml). The measurement of the total soluble content of samples occurred before and after fermentation and storage, using the ATAGO PAL-3 pocket refractometer (ATAGO USA, Inc., WA, USA). The refractive index was recorded and converted to °Bx.

Total Carotenoids

Samples obtained at 0, 2, and 7 days were evaluated for total carotenoid content. Briefly, 1.5 g of samples was homogenized with 5 ml of extracting solvent (hexane/acetone/ethanol, 50:25:25; v/v/v) and centrifuged at 3,000 × g for 5 min at 5°C. Then, 1 ml of hexane was added to the supernatant, and the absorbance was measured at 450 nm using a UV-180 Shimadzu spectrophotometer (Shimadzu, Buckinghamshire, UK). External calibration with a β-carotene standard solution was used, and total carotenoid content was expressed as mg/100 g dry weight (DW).

Sensory Evaluation

The organoleptic properties of the smoothies were evaluated using the qualitative descriptive analysis method as previously described by Oliveira et al. (32) with slight modification. Seven trained panelists from a pool of healthy interested participants were used in the study, comprising both male and female participants. Participants were trained to identify the desired attributes in the smoothies prior to evaluating the test samples. The tasting was done in white light illuminated individual cubicles, and samples were presented chilled at standard room temperature. Two tasting sections were adopted in the study after the trained panelists have learnt to identify the desired attributes. The smoothies were scored using a structured scale ranging from 0 to 5 (0, absent; 1, weak; 5, strong). The reference samples used for each attribute are presented in **Table 1**. The cut-off point of 3 was set for acceptability for each attribute.

TABLE 1 | Sensory evaluation of fermented and non-fermented smoothies.

Smoothies	Color	Flavor	Consistency	Sourness	Sweetness	Overall acceptability
	Ripe Pineapple juice + 1% food grade browning	Pineapple juice (100%)	Glucose syrup solution (10%)	Sweetened yogurt	Sucrose solution (70%)	Commercial fresh fermented smoothie
Non-fermented × D0	2.67 ± 33^{ab}	2.67 ± 0.33^{a}	3.33 ± 0.67^{a}	2.33 ± 0.33^{c}	4.67 ± 0.33^{a}	1.67 ± 0.67^{c}
Non-fermented × D2	2.67 ± 33^{ab}	2.67 ± 0.33^{a}	4.33 ± 0.33^{a}	3.33 ± 0.67^{abc}	4.33 ± 0.67^{ab}	2.00 ± 0.58^{bc}
W64 × D2	3.67 ± 33^{a}	3.33 ± 0.67^{a}	4.33 ± 0.67^{a}	3.33 ± 0.67^{abc}	3.67 ± 0.33^{abc}	2.67 ± 0.33^{abc}
L75 × D2	2.33 ± 33^{bc}	3.67 ± 0.33^{a}	4.33 ± 0.33^{a}	4.33 ± 0.67^{ab}	3.00 ± 0.58^{bcd}	3.67 ± 0.33^{a}
LW64 + 7 × D2	3.67 ± 33^{a}	3.33 ± 0.67^{a}	4.67 ± 0.33^{a}	4.67 ± 0.33^{a}	2.33 ± 0.33^{cd}	2.33 ± 0.33^{abc}
Non-fermented × D7	2.67 ± 33^{ab}	2.67 ± 0.33^{a}	3.67 ± 0.33^{a}	2.67 ± 0.33^{bc}	2.67 ± 0.33^{cd}	1.67 ± 0.67^{c}
W64 × D7	2.33 ± 33^{bc}	2.67 ± 0.33^{a}	4.00 ± 0.58^{a}	2.33 ± 0.67^{c}	2.33 ± 0.56^{cd}	2.67 ± 0.33^{abc}
L75 × D7	1.33 ± 33^{c}	2.33 ± 0.33^{a}	3.33 ± 0.33^{a}	3.33 ± 0.33^{abc}	2.00 ± 0.58^{d}	3.33 ± 0.33^{ab}
LW64 + 75 × D7	2.67 ± 33^{ab}	2.67 ± 0.33^{a}	4.33 ± 0.33^{a}	3.67 ± 0.33^{abc}	0.33 ± 0.58^{e}	2.33 ± 0.33^{abc}

W64, Weissella cibaria 64; L75, Lactobacillus plantarum 75; LW64 + 75, W. cibaria 64 + L. plantarum 75; D0, day 0; D2, 2 days; D7, 7 days; non-fermented smoothies (control). Different superscript alphabets are significantly different along the columns ($p \leq 0.05$).

Total Phenolic Content

The Folin–Ciocalteu assay was used to measure the total polyphenol content of the fermented and non-fermented smoothies (11). Briefly, 100 µl of samples and 15 µl of Folin–Ciocalteu reagent were mixed in a 96-well plate and incubated for 4 min at 25°C; thereafter, 60 µl of 700 mM Na_2CO_3 was added to each well, and the mixture was incubated for 1 h in the dark. The absorbance was read at 760 nm using a microplate reader (Infinite M200 PRO; Tecan, Mannedorf, Switzerland). Gallic acid was used as a standard, and the results were expressed as gallic acid equivalents (GAE) in mg/g DW.

FRAP Assay

Total antioxidant scavenging activity was determined using the method described by Llorach et al. (33). A 0.2 g sample of freeze-dried fruit puree was extracted using 2 ml of sodium acetate buffer (pH 3.6). In a microplate, 220 µl of FRAP reagent solution {10 mmol/l TPTZ [2,4,6-tris (2-pyridyl)-1,3,5-triazine]} was acidified with concentrated HCl and 20 mmol/l $FeCl_3$, followed by 15 µl of the homogenized puree samples. The absorbance was read at 593 nm with a spectrophotometer (SPECTROstar Nano; BMG LABTECH GmbH, Ortenberg, Germany). The reducing antioxidant power was reported as Trolox, expressed in µmol Trolox Equivalent Antioxidant Capacity (TEAC)/100 g DW.

In vitro Digestion of Smoothie

To evaluate the concentration of antioxidant compounds released and available for absorption from the smoothies, the simulated GI digestion method was adopted according to the Infogest nature protocols (34). All smoothie samples that were stored for 7 days at 4°C were subjected to successive gastric and pancreatic conditions. Briefly, 10 g of homogenized smoothie was mixed with simulated gastric fluid (SGF) to get a 40 ml of final volume, after the pH was adjusted to 3 with HCl and 10 ml of pepsin (2,000 U/ml) was added. The mixture was incubated at 37°C for 2 h under agitation. After the gastric phase, aliquots were collected for later analysis, and the reactions were stopped by cooling the test tubes on ice.

The intestinal phase was then divided into two successive steps: agitation step and dialysis process with tubing cellulose membrane (MWCO 10 kDa) as a simplified model of the epithelial barrier. Gastric phase sample was mixed with simulated intestinal fluid (SIF) to get an 80 ml of final volume after addition of pancreatin (100 U/ml) and bile salt (10 mM) and pH adjustment at 7. This mixture was then incubated for 2 h at 37°C. After the intestinal phase, aliquots were collected for later analysis. Dialysis bags filled with 5.5 ml NaCl (0.9%) and 5.5 ml $NaHCO_3$ (0.5 M) sealed with clips were completely immersed into the GI digested immediately after digestion. The samples with dialysis bags were then incubated for 45 min at 37°C under agitation. Aliquots were collected for later analysis at the end of the incubation time.

Statistical Analysis

The conducting of the experiment was in a completely randomized design, with three replicates per treatment and repeated twice. The software XLSTAT (Addinsoft, Paris, France) was used for all statistical analyses. Data were subjected to one-way analysis of variance (ANOVA), and significant effects of factors were detected with a Fisher's test ($p < 0.0001$). The mean scores from sensory analysis were calculated, and the significant differences were evaluated by ANOVA, followed by *post-hoc* using Duncan Multiple Range test ($p \leq 0.05$).

RESULTS AND DISCUSSION

pH, TSS, and Changes in Color

Food acidification is the primary mechanism involved in lactic acid fermentation to preserve and ensure the safety of foods by preventing the growth of spoilage and pathogenic microorganisms in fermented food. Lactic acid is a major metabolite in the homo-lactic fermentation and could serve as a preservative in fermented foods (35, 36). In this study,

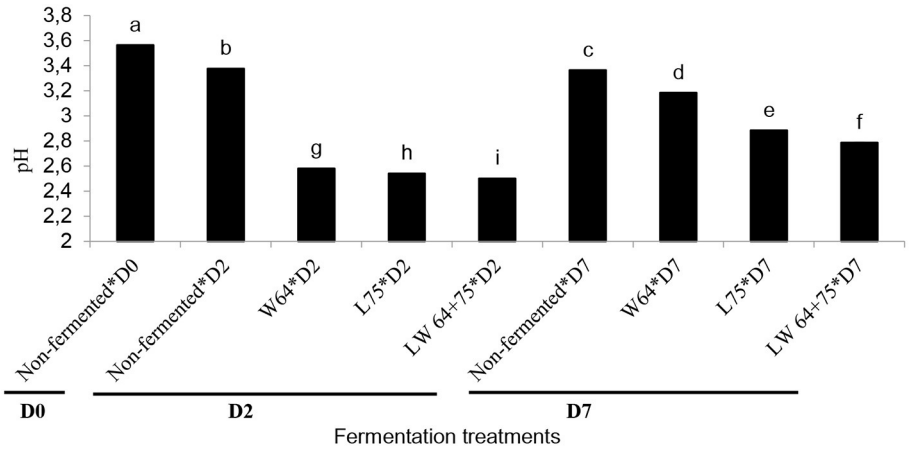

FIGURE 1 | Changes in pH value during lactic acid fermentation of smoothies. Bars with different alphabetic letters are significantly different at $p < 0.0001$ level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum 75*; *LW64 + 75, W. cibaria 64 + L. plantarum* 75; D0, day 0; D2, 2 days; D7, 7 days; non-fermented smoothies (control).

the initial pH value of the smoothie was 3.56; after 2 days of fermentation, the pH value ranged from 2.50 to 2.54, which was lower than the non-fermented smoothie (3.37). The lowest pH (2.5) was obtained in the smoothie fermented with the combination of *LW64 + 75* at 2 days (**Figure 1**). The pH values of the fermented smoothie dropped after 2 and 7 days of fermentation, thus supporting the previous report of a decrease in pH of *L. plantarum* fermented tomato juice (37). In addition, the least pH decrease (pH value of 3.18) was in the smoothie fermented with *W64*. Similar observations with a lesser decrease of pH in *W64* were reported in pineapple juice (11). However, after storage for 7 days at 4°C, the pH values of the fermented smoothies significantly increased compared with the pH values at 2 days. The smoothie fermented with combined starters (*LW64 + 75*) had the lowest pH value (2.50); this is probably due to the induced fermentation with LAB starters that could have reduced the pH during the first 2 days of fermentation, and thereafter the increased pH during storage might be due to the onset of metabolic activities of colonizing bacteria or fungi that utilize lactic acid as a carbon source, thus releasing metabolites that could alter the acidity of foods (30). The higher pH in the stored samples suggests that the LAB cultures could not survive for 7 days at the stored temperatures. Malic and citric acids are the main organic acids in pineapple (38) and could have been responsible for the acidity in the non-fermented smoothie. During the fermentation process, LAB metabolized simple sugars, such as sucrose, fructose, and glucose into organic acids, mainly lactic acid, and carbon dioxide leading to pH decrease (11).

Total soluble solids (TSS) are important quality indicators that relate to sweetness (39), often referred to as sugar index. An initial value of 14.7°Bx was obtained in the smoothies, ranging from 13.1 to 14.8°Bx in the fermented smoothies after 2 days of fermentation (**Figure 2**). As expected, the TSS content of the smoothies significantly ($p < 0.0001$) decreased during lactic acid fermentation compared with the control (**Figure 2**). However, the smoothies fermented with *W64* showed the highest TSS (14.8°Bx) after 2 days of fermentation and were not significantly different to the control at the start of fermentation, whereas the *L75* and the combination (*LW64 + 75*) significantly ($p < 0.05$) reduced the TSS of the smoothie to 13.1 and 13.7°Bx, respectively, after 2 days. A decline in TSS content during fermentation is due to the utilization of sugars in smoothies by LAB strains for metabolism, cellular growth, and bioconversion into lactic acid (40). After 7 days of storage, the TSS content of the control smoothie decreased to 13.1°Bx, and a further decrease in TSS was observed in the smoothies fermented with *W64*, *L75*, and *LW64 + 75*. The smoothies fermented with the combined starter cultures (*LW64 + 75*) showed the lowest TSS content after storage. This suggests a continuous fermentation in the fermented smoothies and the non-inoculated sample resulting in the use of sugars by fermenting and colonizing cultures, respectively, at 4°C; hence, lactic acid fermentation of pineapple–chayote smoothies can still take place at cold temperature. The TSS decrease in the smoothie fermented with *LW64 + 75* represents 18% of the initial TSS value. The decrease in TSS after fermentation and storage supports the previous assertion in this study that lactic acid fermentations proceed in the fermented smoothies after cold storage. The decrease in TSS suggests the utilization of soluble sugars by LAB in the fermented smoothies, thus corroborating the report on the decrease in TSS of *L. plantarum* fermented cashew–apple juice after 72 h of fermentation (41) and in sugarcane and beet juice (42). Most LAB follow the Embden–Meyerhof, tagatose-6-phosphate, Leloir, or phosphoketolase pathways to synthesize lactic acids and carbon dioxide from soluble sugars in substrates, thereby causing a decrease in the pH by the metabolite (43). From a nutritional point of view, the decrease in sugar content of the fermented smoothies is an advantage over the non-fermented one in managing diabetic conditions, especially when all reducing sugar contents are transformed during fermentation (44).

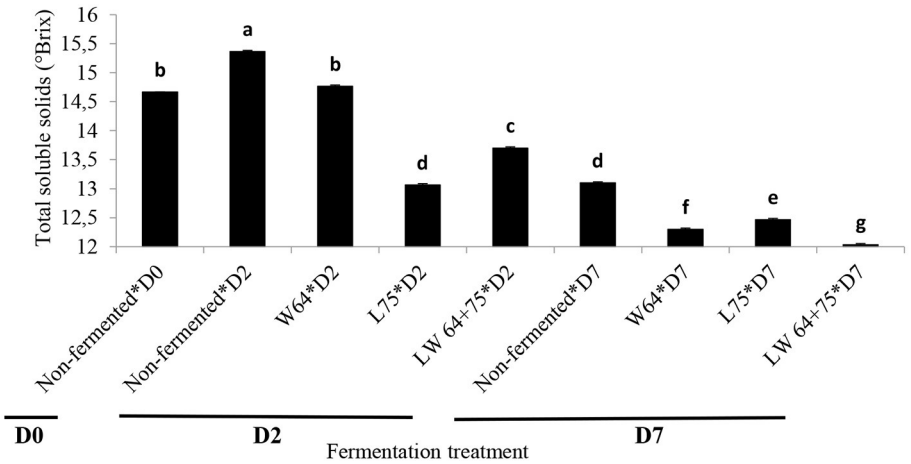

FIGURE 2 | Changes in total soluble solids during fermentation of smoothies. Bars with different alphabetic letters are significantly different at $p < 0.0001$ level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum 75*; *LW64 + 75, W. cibaria 64 + L. plantarum* 75; D0, day 0; D2, 2 days; D7, 7 days; non-fermented smoothies (control).

Color is one of the most important food parameters since it influences consumer acceptability (45). As shown in **Figure 3**, color values of the smoothie varied according to fermenting LAB strains. Non-fermented (control) smoothie color parameters showed initial values corresponding to dark brown, and the parameter values decreased with time. The combined starter culture (*LW64 + 75*) fermented smoothies showed a significantly higher L^* (luminosity) and b^* values, whereas *L75* showed the highest a^* value after 2 days of fermentation. The higher redness to greenness color attributes in the *W65* fermented smoothie could be due to the steering activity of fermenting LAB causing enzymatic oxidation during the fermentation process. However, the period of fermentation significantly ($p < 0.0001$) influenced the color parameter values for all smoothies, especially in samples fermented with *W64* (**Figure 3A**). After 7 days of storage, the lowest values were observed in the *L75* fermented smoothie. The ΔE relates to the color difference of the smoothies fermented with *W64*, *L75*, or *LW65 + 75*. The ΔE of the fermented smoothies on day 2 ranged from 1.92 to 5.60, was the lowest in the smoothies fermented with *LW65 + 75* (1.92), and was significantly comparable to the non-fermented smoothies. The *W64* fermented smoothie (5.60) had the highest ΔE. According to Wang et al. (46), a color difference equal to 2 or greater ($\Delta E \geq 2$) is regarded as a significant color change in samples. Similarly, the color difference significantly ($p < 0.0001$) increased with prolonged storage compared with the control samples at day 7 (**Figure 3B**). The continuous color change could be attributed to an extended biochemical degradation and acidity caused by the fermenting cultures in the smoothies at a lower temperature. The lower color change in the combined starter culture treatment may be due to a dominating activity of the homo-fermentative LAB (*L. plantarum*) that blocks the enzymatic degradation in the fermented smoothies. *L. plantarum* has been identified as a strong homolactic fermenter of food substrates (47). Dark colors in foods negatively affect the consumer acceptability of food products (48). Despite the color modifications during fermentation, the color change in the smoothies fermented with combined starter is still comparable to the non-fermented at 2 days of fermentation.

Impact of Fermentation on Sensory Attributes

The produced smoothies were evaluated for their organoleptic characteristics before and after fermentation and storage for 7 days. **Table 1** presents the qualitative descriptive sensory evaluation of the fermented and unfermented smoothies. The color acceptability of smoothies ranged from slightly dark color (1.33) in *L75* smoothies stored for 7 days to moderately bright yellow color associated with pineapple juice in *W64* and *L75* smoothies (3.67) at 2 days of fermentation. The fermented smoothies (*W64* and *LW64 + 75*) at 2 days of fermentation were not significantly different at $p \leq 0.05$ and also in the non-fermented samples at 0, 2, and after 7 days of storage. The lower values obtained for the fermented smoothies could be due to impaired lightness in the smoothies due to the addition of chayote leaves. The color score of samples were adjudged to be lower after storage of the smoothies. There was no significant difference in the flavor and consistency attributes of both fermented and non-fermented smoothies at 0, 2, and after storage for 7 days. However, the consistency in smoothies was adjudged as highly acceptable, whereas the flavor of the smoothies fermented for 2 days had an acceptable flavor compared with other samples. The perception of acid taste in the samples was scored the highest in *LW64 + 75* (4.67) followed by *L75* (4.33) at day 2 of fermentation and was significantly different to the non-fermented smoothies at day 0. The high perception of acid taste might be related to the high acid production by *L75*. *L. plantarum* is known to produce organic acids, with lactic

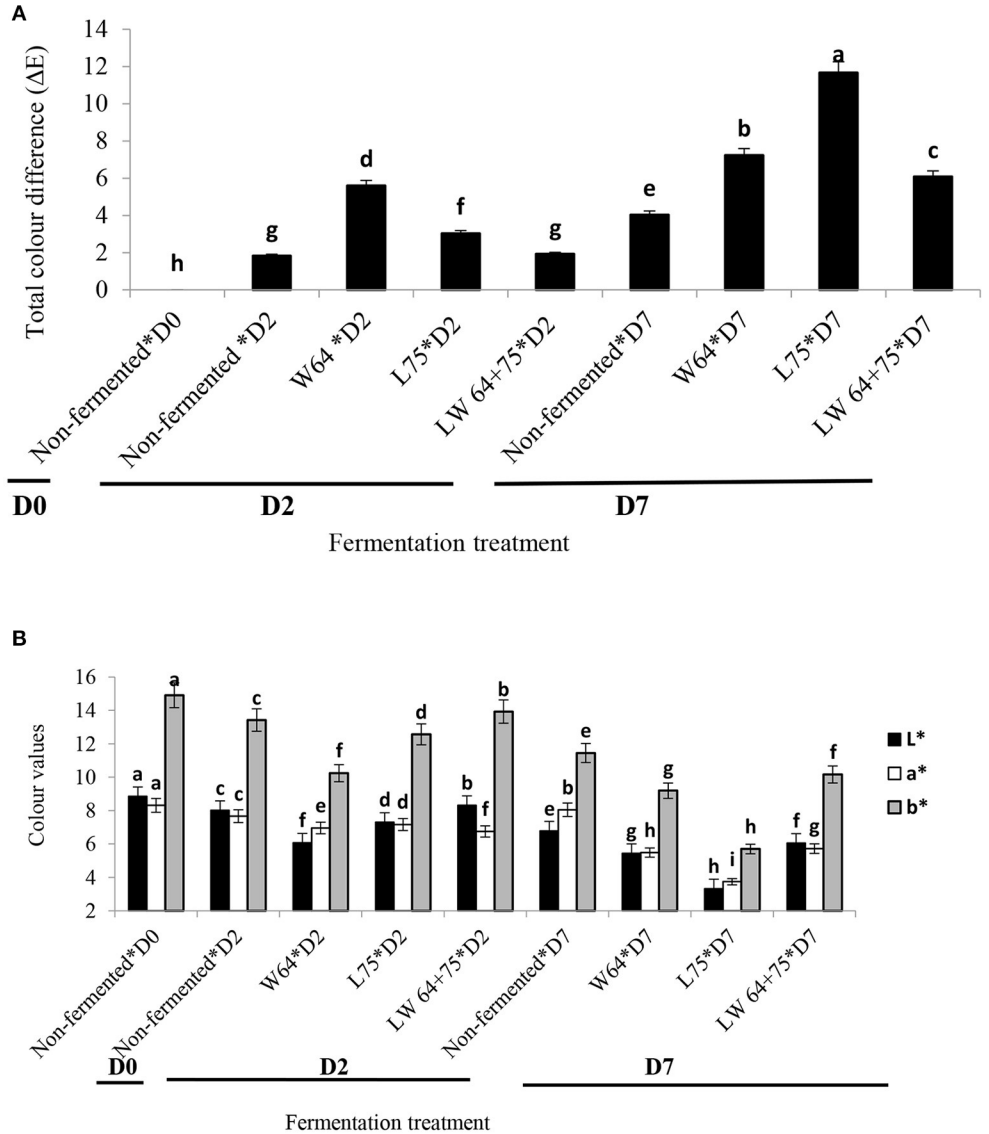

FIGURE 3 | (A) Total color difference (ΔE) of lactic acid fermented smoothies. Bars with different alphabetic letters are significantly different at $p < 0.0001$ level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum 75*; *LW64 + 75, W. cibaria 64 + L. plantarum* 75; D0, day 0; D2, 2 days; D7, 7 days; non-fermented smoothies (control); ΔE = change in color. **(B)** Color attributes (l^*, a^*, b^*) of lactic acid fermented smoothies. Bars with different alphabetic letters are significantly different at $p <$ 0.0001 level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum 75*; LW*64 + 75, W. cibaria 64 + L. plantarum* 75; D0, day 0; D2, 2 days; D7, 7 days; l^*, lightness; a^*, redness/greenish; b^*, yellowish/bluish; non-fermented smoothies (control).

acid being the major one during its biochemical degradation of sugar substrates (11); hence, the high acid taste in *LW64 + 75* could be due to a synergistic effect of acid production by the LAB strains, thus supporting the low pH obtained in this sample. The sweetness ranged from no sweetness (0.33) in *LW64 + 75* to high sweetness (4.67) in non-fermented samples at day 0. The sweetness perception reduced with fermentation and storage. The sample *L75* fermented smoothie after 2 days had the highest overall acceptability (3.67) compared with other smoothies and was significantly different to the non-fermented smoothies ($p \leq 0.05$) at 0, 2, and after 7 days of storage.

Survival of LAB in Smoothies

To assess changes in microbial quality, yeasts and mold growth were used as hygienic indicators to evaluate if the preparation was not contaminated. The microbial quality of the fermented smoothies was observed from the start of fermentation (day 0) and after 7 days (2 days of fermentation at 37°C plus 5 days of storage at 4°C) for the LAB strains, its combination, and control. From the data obtained, the yeast, mold, and aerobic bacterial population (not shown) remained below the detection level for all samples, and there were no significant differences in the treatments, thus meeting the safety of the product for

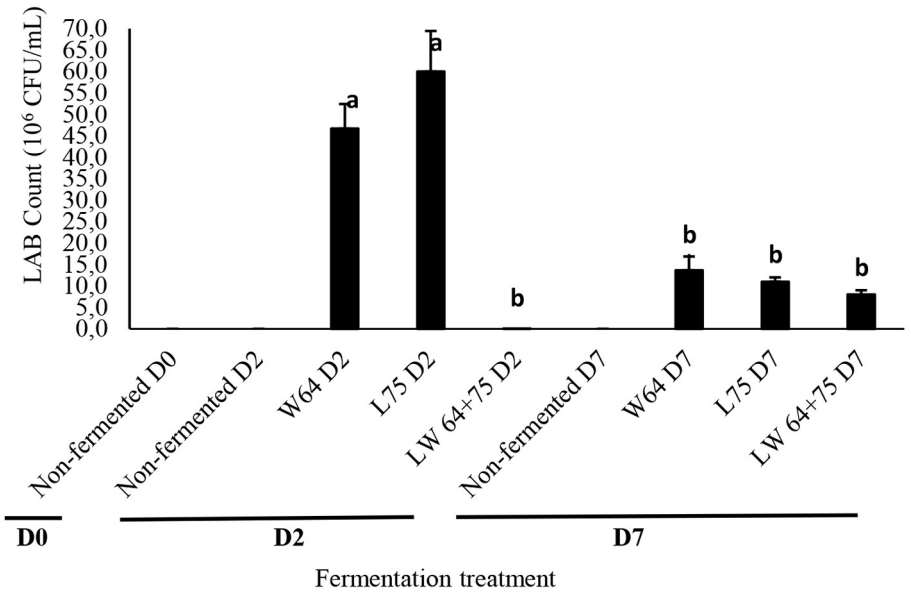

FIGURE 4 | Surviving LAB count in fermented smoothies. Bars with similar alphabetic letters are not significantly different at $p < 0.0001$ level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum 75*; *LW64 + 75, W. cibaria 64 + L. plantarum* 75; D0, day 0; D2, 2 days; D7, 7 days; non-fermented smoothies (control).

consumption. The LAB counts in the fermented and non-fermented smoothies ranged from 0.01 to 60 × 10^6 CFU/ml and were the highest in *L75* (60 × 10^6 CFU/ml) but not significantly different to *W64* at day 2 of fermentation (**Figure 4**). However, the smoothies fermented with *LW64 + 75* had lower surviving LAB at day 2 and were significantly different to *W64* and *L75*. This might be due to a competition among the inoculated starters for dominance of the fermentation, thereby limiting the proliferation of LAB cells and death of the weaker ones. There was no detectable LAB growth in the non-fermented samples. Furthermore, after storage for 7 days, the LAB counts in the fermented smoothies ranged from 1.0 to 3.21 × 10^6 CFU/ml without a significant difference between *W64*, *L75*, and *LW64 + 75* compared with the non-fermented smoothies at days 0 and 7. Low pH reportedly affects the survival of bacteria significantly in stored fruit juices (49). Hence, the higher survival of the LAB at day 2 with much lower acidity signifies the acid tolerance of *L75* and *W65* cultures, whereas the reduced LAB count at storage for 7 days signifies the non-survival of LAB at higher pH ≥3. Šeme et al. (50) had earlier reported an increase in the survival of *L. plantarum* KR6 cells in acidified medium (pH 2) and *L. plantarum* NCIMB 8826 in cranberry juice (51). The change in survival of LAB after storage for 7 days might be associated with the changes in the membrane fatty acids composition of the LAB cells, since it is important in regulating the proton permeability of the cell membranes (52).

Changes in Phenolic Content and Total Carotenoid After LAB Fermentation

Total phenolic content (TPC; 634.7 mg/L) in the non-fermented smoothie was the highest on day 2 compared with the fermented smoothies and the control samples at day 2 or 7 (**Figure 5**). However, the smoothies fermented with *L75* for 2 days showed higher concentration of TPC than the smoothies fermented with *W64* or *LW64 + 75*. LAB reportedly hold the ability to convert food matrixes into functional moieties (41). The bioconversion of aglycone from phenolic glycosides by LAB can be strain-dependent (53), based on the specific enzymes. *L. plantarum* produces different enzymes, such as β-glucosidase, lactate dehydrogenase, amylase, peptidase, decarboxylase, phenol reductase, phenolic acid decarboxylase, and proteinase (54), which aided the increase or stabilization of phenolic content in smoothies after fermentation.

Ferulic acid, caffeic acid, quercetin, myricetin, kaempferol, and chlorogenic acid have been identified in chayote leaves, with chlorogenic acid (5.80 ± 0.12 mg/100 g FW) being the most abundant phenolics (55). Similarly, according to Wen and Wrolstad (56), raw pineapple juice contains N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapylglutathione, S-sinapyl-L-cysteine, furanones, glycosides, and p-coumaric acids. The TPC reduced significantly ($p < 0.0001$) in the fermented and non-fermented smoothies after storage (day 7). At day 7 after storage, the smoothies fermented with *L75*, *W64*, and *LW64 + 75* showed 12–16% reduction in the TPCs compared with the control (day 0). The non-fermented smoothie at day 7 after storage exhibited a 9% decrease in TPC compared with day 0. The decrease in TPC of the fermented smoothie is in agreement with the report by Hashemi et al. (57) in *L. plantarum* fermented sweet lemon juice after storage. Most processing steps, such as freezing, freeze-drying, and pasteurization, cause a large degradation of phenolic compounds in fruit (58, 59). Contrarily, lactic acid fermentation is recognized as a way to preserve TPC (60, 61). The observation made in this study corroborates the previous report on the increase in TPC after fermentation. Hydrolysis of complex

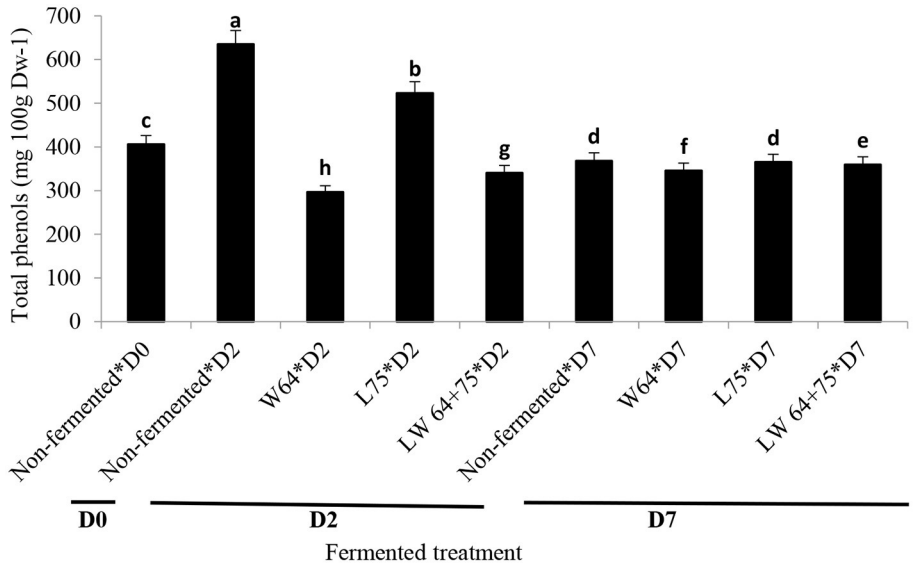

FIGURE 5 | Changes in total polyphenol content (TPC) during fermentation of smoothies. Bars with different alphabetic letters are significantly different at $p < 0.0001$ level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum 75*; *LW64 + 75, W. cibaria 64 + L. plantarum* 75; D0, day 0; D2, 2 days; D7, 7 days; DW, dry weight; non-fermented smoothies (control).

glycosides during fermentation might have contributed to the increased phenolic contents. Hydrolysis could cause a cleaving of the phenolic–sugar glycosidic bonds or decarboxylation process that could help in the release of aglycones, degradation of gallotannins to simple phenolic acids, or formation of new phenolic compounds, such as pyrogallol, which could have reacted better with the Folin–Ciocalteu reagent leading to higher values of total phenolics (53, 62).

The non-fermented smoothie (day 0) showed the highest total carotenoid contents. The effect of lactic acid fermentation on the carotenoids is highly dependent on the matrix, carotenoid composition, and LAB strain, but in general, the total carotenoid content remains stable (63). A gradual decrease of the carotenoid content of the non-fermented smoothie was observed with time of fermentation and storage. After storage, only 19% of the initial carotenoid content was present; however, on day 2 of fermentation, the decrease in total carotenoid contents of the *W64* fermented smoothie was lower than those of the *L75*, *LW64 + 75*, and control (**Figure 6**). The reduction in total carotenoid content of the smoothies after fermentation and storage has been reported in *L. plantarum* fermented sweet lemon juice (41). After 7 days of storage, the total carotenoid content of the fermented smoothie was significantly higher than that of the control at the same stage, and 29–40% of the initial carotenoid content remained. The carotenoid level in Queen Victoria pineapple is the highest among several cultivars, having 565 μg/100 g of pulp (64) and mostly composed of β-carotene. In leafy vegetables, carotenoids are mostly located in chloroplasts (64). The content of carotenoids generally decreases with processing of foods, due to isomerization, oxidation, and light degradation. However, the bioavailability of carotenoids, which is usually low in leafy vegetables, can be increased by processing steps, such as homogenization and thermal treatment (64, 65), since unbounding from complex structures and food composition, especially in lipids and fibers, plays a key role in carotenoid bioavailability.

In vitro-Simulated GI Digestion Analysis

Figure 7 illustrates the effect of GI digestion on total phenolic compounds from the fermented and non-fermented smoothies after 2 days compared with the undigested smoothies. The TPC in the non-fermented smoothies and fermented smoothies before digestion varied between 345.70 and 368.06 mg/100 g. The TPC in the gastric digesta of all fermented smoothies increased significantly ($p < 0.0001$) compared with their respective undigested sample; however, the non-fermented smoothies showed a non-significant difference ($p > 0.0001$). Moreover, the TPC in the intestinal phase was higher than that in the respective undigested samples and gastric digesta for all fermented and non-fermented smoothies, whereas the smoothies fermented by *L75* showed the highest concentration at 609.17 mg/100 g, followed by *LW64 + 75* at 521.86 mg/100 g. A significant declining trend was observed in TPC at the dialysis phase compared with the undigested samples, gastric, and intestinal digesta. The bioaccessible TPCs after GI digestion at the dialysis phase were 77.15, 67.14, 59.32, and 52.23% in the smoothies fermented by *L75*, *LW64 + 75*, *W64*, and non-fermented smoothies, respectively, in terms of the percentage recovery relative to the TPC in the respective undigested samples.

A shift from the acid gastric condition to mild alkaline at the intestinal phase was expected to reduce the levels of bioaccessible total phenols as previously described in fruit juices (23, 66). However, an opposite trend was noticed with a higher but less pronounced level of total phenols in digested apple varieties, such

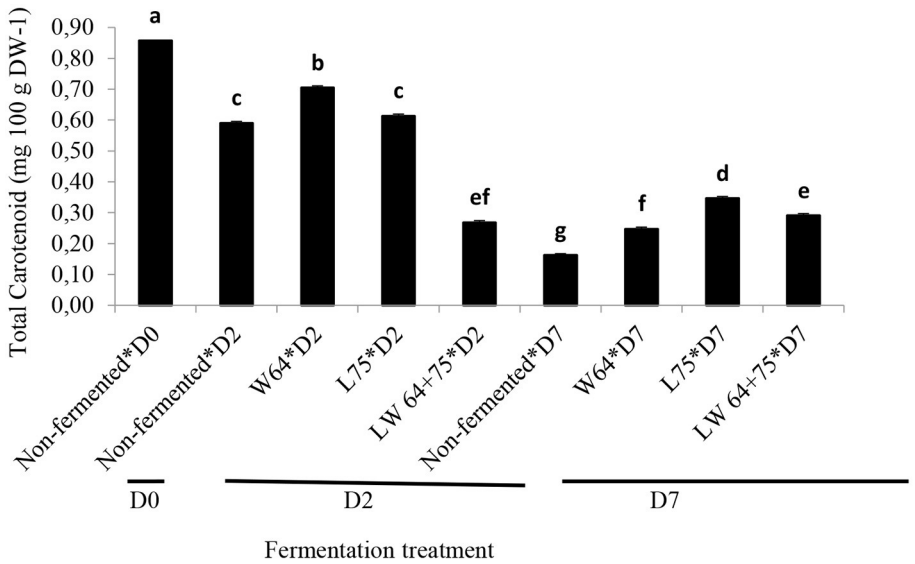

FIGURE 6 | Changes in total carotenoid content during fermentation of smoothies. Bars with different alphabetic letters are significantly different at $p < 0.0001$ level. *W64*, *Weissella cibaria* 64; *L75*, *Lactobacillus plantarum 75*; *LW64 + 75*, *W. cibaria 64 + L. plantarum* 75; D0, day 0; D2, 2 days; D7, 7 days; DW, dry weight; non-fermented smoothies (control).

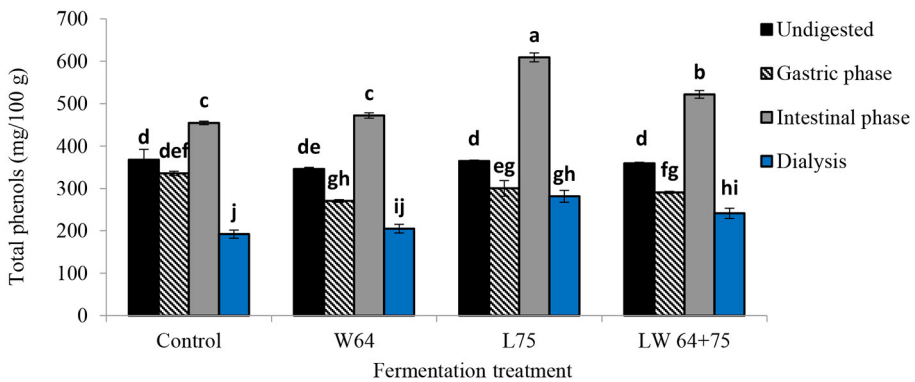

FIGURE 7 | Total polyphenol content of LAB fermented smoothies subjected to simulated *in vitro* gastrointestinal digestion. Bars with similar alphabetic letters for a specific digestive phase are not significantly different at $p < 0.001$ level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum* 75; *LW64 + 75, W. cibaria 64 + L. plantarum* 75. DW, dry weight; control, non-fermented smoothies.

as Jonaprince, Jonagold, and Golden, in the intestinal phase than in the gastric phase (23). During *in vitro* digestion of edible leaves, *Olax zeylanica* ("mella"), *Centella asiatica* ("gotukola"), and *Sesbania grandiflora* ("kathurumurunga") showed higher levels of total polyphenol content in the intestinal phase than in the gastric digesta (67). The observed increase of TPC in the intestinal smoothie digesta compared with the gastric phase with respect to the undigested sample may be due to an increased release of phenolics bound to the matrix due to the activity of the intestinal digestive enzyme (pancreatin) (23), or to the phenolic interaction with cell wall carbohydrates, such as pectin present in smoothie, obstructing the solubilization of the phenolic compounds during gastric digestion (68). Furthermore, the decrease in pH during fermentation could have increased the extractability of phenolic compounds and their stability (23), as they are more stable in more acidic conditions. Additionally, it was shown that some phenolic compounds showed specific higher bioaccessibility at the intestinal phase than at the gastric phase, such as in p-coumaric acid and quercetin from *Moringa oleifera* leaves (69). The amount of released total phenolic compound (in terms of % recovery) was high in the intestinal digesta of the smoothies fermented with *L75* compared with those fermented with *W64* (*LW75 + 64*), control (non-fermented smoothies), and the undigested smoothies, indicating the effect of LAB strain on smoothie phenolic contents.

The degree of hydrolysis of glucosides affects the bioavailability of phenolic components related to their bioactivities (53). Li et al. (70) reported a similar observation

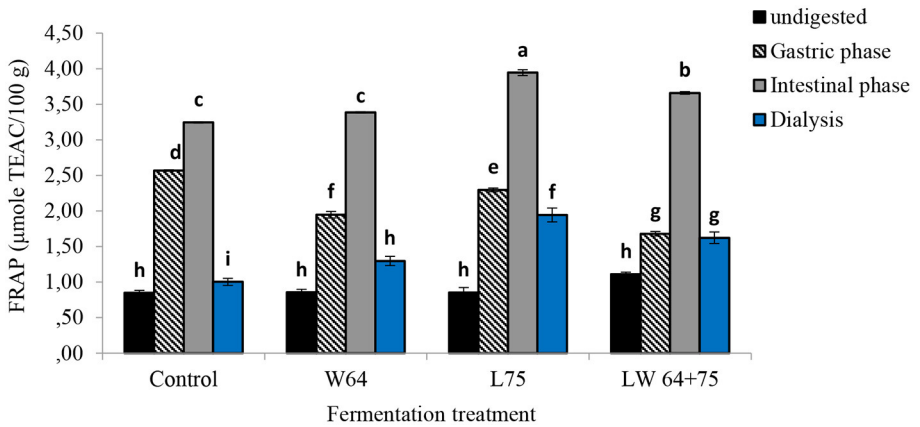

FIGURE 8 | Ferric ion reducing antioxidant power (FRAP) of LAB fermented smoothies subjected to gastrointestinal digestion and dialysis. Bars with similar alphabetic letters for a specific digestive phase are not significantly different at $p < 0.001$ level. *W64, Weissella cibaria* 64; *L75, Lactobacillus plantarum* 75; *LW64 + 75, W. cibaria 64 + L. plantarum* 75; DW, dry weight; control, non-fermented smoothies.

during *in vitro* digestion of grape marc freeze-dried powder in the presence of probiotics. The increase in total phenols in the gastric and intestinal phases can be attributed to the conversion of simple phenolic compounds due to the glycosylation or decarboxylation or the depolymerization of high-molecular-weight phenolic compounds (70, 71).

The dialyzable fraction mimics the fraction that will be available for absorption into the systematic circulation by passive diffusion, which is a provisional approach to project the degree of intestinal epithelium absorption (68). The amount of dialyzable phenolics was lowest (192.25), and 52.23% recovery was related to the original undigested sample in the unfermented smoothies. Bouayed et al. (23) reported a similar trend in different apple cultivars. Almost 77.15 and 67.14% of free soluble dialyzable polyphenols were available for passive absorption in the *L75* and *LW75 + 64* fermented smoothies, respectively. Our results suggest that although the majority of the polyphenol compounds are available in the intestinal phase, the amount accessible at the dialysis phase was low. However, fermenting the smoothies with *L75* improved the bioaccessibility of total phenols compared with the smoothies fermented by *LW64 + 75* or *W64* or non-fermented smoothies.

Antioxidant capacity (FRAP activity) in smoothies at the gastric phase was significantly higher than that in the fermented and non-fermented smoothies with respect to their original unfermented samples (**Figure 8**). Although the antioxidant capacity of the intestinal digesta of all non-fermented and fermented smoothies increased significantly compared with their respective undigested smoothie, the digesta of the smoothies fermented by *L75* showed the highest antioxidant capacity (3.94 µmol TEAC/100 g), followed by *LW64 + 75* (3.66 µmol TEAC/100 g). At the same time, the dialyzable digesta of the smoothies fermented by *L75* showed the highest antioxidant capacity (1.94 µmol TEAC/100 g) compared with undigested samples and the dialyzable digesta of the smoothies fermented by *W64* and *LW64 + 75*.

The observation confirms the increase in free soluble antioxidants responsible for antioxidant capacity compared with their corresponding non-fermented smoothie. There was a similar trend noted in the increase in total phenol and antioxidant capacity of the smoothies fermented with *L75* in the gastric and intestinal digesta, indicating positive correlations between total phenolics and total antioxidant capacity (24). At the same time, the transition from acidic (gastric) to alkaline (intestine) pH also could have played a role in boosting the antioxidant capacity of phenolics that could facilitate the deprotonation of the hydroxyl moieties in aromatic rings (23). Bouayed et al. (23) further suggested that the FRAP assay (performed at a pH of 3.6) could be more relevant to evaluate the antioxidant capacity in the gastric digesta, whereas the ABTS assay (performed at a pH of 7.4) could evaluate the intestinal digesta. Thus, factors, such as pH, interaction of phenol compounds between pectin, protein or fat or Fe and chemical structure of the phenolics (71), method of extraction for total phenols, solvents ratio, and temperature, could affect the release of free phenols and the antioxidant capacity (67–69).

CONCLUSIONS

This study demonstrated that fermentation of a smoothie composed of pineapple and chayote leaves with *L75* and *W64* increased the total phenol and carotenoid contents. The storage of the fermented smoothies at 4°C for 7 days could encourage continuous biochemical activities of fermenting LAB cultures. Fermentation with *L75* enhanced the level of free soluble antioxidant (total phenols) crossing a cellulose membrane (dialysis) and thus be potentially available for further uptake. However, further investigations on phenolic and carotenoid fractions with Caco-2 cellular models must be performed to confirm the uptake of phenolic and carotenoid components. Based on the finding of this study, the fermentation with *L75* can be recommended to local food manufacturers in Réunion Island

to improve the functional benefits and accessibilities of total phenols in chayote leaves and pineapple smoothies. Consumer's overall acceptability for marketing is higher for the smoothie composed of pineapple and chayote leaves fermented by *L75* after 7 days of storage at 4°C than for the non-fermented sample at day 0. The metabolomics profile characterization of the fermented smoothies will be necessary to gain further knowledge.

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/s.

AUTHOR CONTRIBUTIONS

MM performed the experiment, generated the data, and wrote the first draft of this manuscript. SA validated the data, improved the write up of physicochemical, and sensory parameters related to fermentations. FR was responsible for fermentation, conceptualized the research, and visualized and validated the data for physiochemical properties responsible for *in vitro* digestion. The research collaborator and co-supervisor provided the editorial support. CG was responsible for *in vitro* digestion analysis, methodology, and training. DS conceptualized the research, supervised MM, and improved the article further. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Aderinola TA. Effects of pumpkin leaves on the chemical composition and antioxidant properties of smoothies. In: *Abstract Retrieved From Proceedings of the 4th Regional Food Science and Technology Summit (Refosts) Akure, Ondo State Nigeria* Akure (2018).
2. Andrés V, Villanueva MJ, Tenorio, MD. The effect of high-pressure processing on colour, bioactive compounds, and antioxidant activity in smoothies during refrigerated storage. *Food Chem.* (2016) 192:328–35. doi: 10.1016/j.foodchem.2015.07.031
3. Arts ICW, Hollman PCH. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr.* (2005) 81:3175–255. doi: 10.1093/ajcn/81.1.317S
4. FAO/WHO Workshop on Fruit and Vegetables for Health. In: *Report of a Joint FAO/WHO Workshop*, 1-3 September, 2004, Kobe, Japan. Available online at: https://apps.who.int/iris/bitstream/handle/10665/43143/9241592818_eng.pdf (accessed November 21, 2020).
5. USDA *Item Clusters, Percent of Consumption, and Representative Foods for Typical Choices Food Patterns*. Available online at: https://www.fns.usda.gov/usda-food-patterns (accessed November 18, 2020).
6. Porat R, Lichter A, Terry LA, Harker R, Buzby J. Postharvest losses of fruit and vegetables during retail and in consumers' homes: quantifications, causes, and means of prevention. *Postharvest Biol Technol.* (2018) 139:135–49. doi: 10.1016/j.postharvbio.2017.11.019
7. Slavin JL, Lloyd B. Health benefits of fruits and vegetables. *Adv Food Nutr.* (2012) 3:506–16. doi: 10.3945/an.112.002154
8. Reddy MB, Love M. The impact of food processing on the nutritional quality of vitamins and minerals. In: Jackson LS, Knize MG, Morgan JN, editors. *Impact of Processing on Food Safety*. *Advances in Experimental Medicine and Biology*. Vol. 459. Boston, MA: Springer. (1999) 459:99–106. doi: 10.1007/978-1-4615-4853-9_7
9. Dreher, ML. Whole fruits and fruit fiber emerging health effects. *Nutrients.* (2018) 10:1833. doi: 10.3390/nu10121833
10. Teleszko M, Wojdyło, A. Bioactive compounds vs. organoleptic assessment of 'smoothies' type products prepared from selected fruit species. *Int J Food Sci Technol.* (2014) 49:98–106. doi: 10.1111/ijfs.12280
11. Fessard A, Kapoor A, Patche J, Assemat S, Hoarau M, Bourdon E, et al. Lactic fermentation as an efficient tool to enhance the antioxidant activity of tropical fruit juices and teas. *Microorganisms.* (2017) 5:E23. doi: 10.3390/microorganisms5020023
12. Chen R, Chen W, Chen H, Zhang G, Chen, W. Comparative evaluation of the antioxidant capacities, organic acids, and volatiles of papaya juices fermented by *Lactobacillus acidophilus* and *Lactobacillus plantarum*. *J Food Qual.* (2018) 2018:90435. doi: 10.1155/2018/9490435
13. Septembre-Malaterre A, Remize F, Poucheret P. Fruits and vegetables, as a source of nutritional compounds and phytochemicals: changes in bioactive compounds during lactic fermentation. *Int Food Res J.* (2018) 104:86–99. doi: 10.1016/j.foodres.2017.09.031
14. Ghosh K, Ray M, Adak A. Role of probiotic *Lactobacillus fermentum* KKL1 in the preparation of a rice based fermented beverage. *Bioresour Technol.* (2015) 188:161–8. doi: 10.1016/j.biortech.2015.01.130
15. Zhao D, Shah NP. Lactic acid bacterial fermentation modified phenolic composition in tea extracts and enhanced their antioxidant activity and cellular uptake of phenolic compounds following in vitro digestion. *J Funct Food.* (2016) 20:182–94. doi: 10.1016/j.jff.2015.10.033
16. Ryan L, Prescott S. Stability of the antioxidant capacity of twenty-five commercially available fruit juices subjected to an *in vitro* digestion. *Int J Food Sci Technol.* (2010) 45:1191–7. doi: 10.1111/j.1365-2621.2010.02254.x
17. Deguine JP, Atiama-Nurbel T, Vanhuffel L, Cresson C. Recent advances in organic cultivation of chayote (*Sechium edule*) in Reunion Island. *Org Agric.* (2020) 10:135–43. doi: 10.1007/s13165-019-00255-5
18. *Sicyos edulis*. *Germplasm Resources Information Network (GRIN)*. Agricultural Research Service (ARS), United States Department of Agriculture (USDA). Available online at: https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomydetail?id=33882 (accessed November 21, 2020).
19. Harriman NA. Promoting the conservation and use of underutilized and neglected crops. 8. Chayote. *Sechium edule* (Jacq.) Sw. *Econ Bot.* (1998) 52:427. doi: 10.1007/BF02862078
20. Steingass CB, Vollmer K, Lux PE, Dell C, Carle R, Schweiggert RM. HPLC-DAD-APCI-MS analysis of the genuine carotenoid pattern of pineapple (*Ananas comosus* [L.] Merr.) infructescence. *Food Res Int.* (2020) 127:108709. doi: 10.1016/j.foodres.2019.108709
21. Garcia C, Guérin M, Souidi K, Remize, F. Lactic fermented fruit or vegetable juices: past, present and future. *Beverages.* (2020) 6:8. doi: 10.3390/beverages6010008
22. Cory H, Passarelli S, Szeto J, Tamez M, Mattei J. The role of polyphenols in human health and food systems: a mini review. *Front Nutr.* (2018) 5:87. doi: 10.3389/fnut.2018.00087
23. Bouayed J, Hoffmann L, Bohn T. Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: bioaccessibility and potential uptake. *Food Chem.* (2011) 28:14–21. doi: 10.1016/j.foodchem.2011.02.052
24. Domínguez-Avila JA, Wall-Medrano A, Velderrain-Rodríguez GR, Chen CYO, Salazar-López NJ Robles-Sánchez, M, et al. Gastrointestinal interactions, absorption, splanchnic metabolism and

pharmacokinetics of orally ingested phenolic compounds. *Food Func.* (2017) 8:15–38. doi: 10.1039/c6fo01475e
25. Tagliazucchi D, Verzelloni E, Bertolini D, Conte A. *In vitro* bioaccessibility and antioxidant activity of grape polyphenols. *Food Chem.* (2010) 120:599–606. doi: 10.1016/j.foodchem.2009.10.030
26. Wojtunik-Kulesza KA, Oniszczuk A, Oniszczuk T, Combrzyński M. Influence of *in vitro* digestion on composition, bioaccessibility and antioxidant activity of food polyphenols-a non-systematic review. *Nutrients.* (2020) 12:1401. doi: 10.3390/nu12051401
27. Fessard A, Remize, F. Genetic and technological characterization of lactic acid bacteria isolated from tropically grown fruits and vegetables. *Int J Food Microbiol.* (2019) 301:61–72. doi: 10.1016/J.IJFOODMICRO.2019.05.003
28. Nirina HA, Miora R, Vincent P, Abel H, Fabienne R, Louisette R. Phytochemical composition and antioxidant activity of fermented *Moringa oleifera* leaf powder. *Eur J Nutr Food Saf.* (2017) 7:77–83. doi: 10.9734/EJNFS/2017/29627
29. Koch AL. Growth measurement. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR, editors. *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology (1949). p. 248–77.
30. Cabello-Olmo M, Oneca M, Torre P, Díaz JV, Encio IJ, Barajas M, et al. Influence of storage temperature and packaging on bacteria and yeast viability in a plant-based fermented food. *Foods.* (2020) 9:302. doi: 10.3390/foods9030302
31. Managa G, Remize F, Sivakumar D. Effect of moist cooking blanching on colour, phenolic metabolites and glucosinolate content in Chinese cabbage (*Brassica rapa* L. subsp. Chinensis). *Foods.* (2019) 8:399. doi: 10.3390/foods8090399
32. Oliveira ADN, Ramos AM, Minim VPR, Chaves JBP. Sensory stability of whole mango juice: influence of temperature and storage time. *Ciência e Tecnologia de Alimentos.* (2012) 32:819–25. doi: 10.1590/S0101-20612012005000115
33. Llorach R, Tomas-Barberan FA., Ferreres F. Lettuce and chicory by-products as a source of antioxidant phenolic extracts. *J Agric Food Chem.* (2004) 52:5109–16. doi: 10.1021/jf040055a
34. Brodkorb A, Egger L, Alminger M, Alvito P, Assunção R, Ballance S, et al. INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nat Protoc.* (2019) 14:991–1014. doi: 10.1038/s41596-018-0119-1
35. König H, Unden G, Fröhlich, J. *Biology of Microorganisms on Grapes, in Must and in Wine*. Heidelberg: Springer (2009). p. 3–30.
36. Mustafa SM, Chua LS, El-Enshasy, HA, Abd Majid FA, Hanapi, et al. Effect of temperature and pH on the probiotication of *Punica granatum* juice using Lactobacillus species. *J Food Biochem.* (2019) 43:e12805. doi: 10.1111/jfbc.12805
37. Kaur S, Kau, HP, Rover, J. Fermentation of tomato juice by probiotic lactic acid bacteria. *Int J Adv Pharm Biol Chem.* (2016) 5:212–9. Available online at: http://www.ijapbc.com/files/04-06-16/16-5217.pdf (accessed November 22, 2020).
38. Saradhuldhat P, Paull, R. Pineapple organic acid metabolism and accumulation during fruit development. *Sci Hort.* (2007) 112:297–303. doi: 10.1016/j.scienta.2006.12.031
39. Magwaza LS, Opara UL. Review analytical methods for determination of sugars and sweetness of horticultural products—a review. *Sci Hort.* (2015) 184:179–92. doi: 10.1016/j.scienta.2015.01.001
40. Liu J Chan, S.H.J, Chen J, Solem C, Jensen PR. Systems biology – a guide for understanding and developing improved strains of lactic acid bacteria. *Front Microbiol.* (2019) 10:876. doi: 10.3389/fmicb.2019.00876
41. Kaprasob R, Kerdchoechuen O, Laohakunjit N, Sarkar D, Shetty K. Fermentation-based biotransformation of bioactive phenolics and volatile compounds from cashew apple juice by select lactic acid bacteria. *Process Biochem.* (2017) 59:141–9. doi: 10.1016/j.procbio.2017.05.019
42. Soibam H, Ayam VS, Chakraborty I. Preparation, and evaluation of wine from sugarcane and beet juice. *Adv Biores.* (2017) 8. doi: 10.15515/abr.0976-4585.8.4.216219
43. Ruiz Rodríguez LG, Mohamed F, Bleckwedel J, Medina RB, De Vuyst L, Hebert EM, et al. Diversity and functional properties of lactic acid bacteria isolated from wild fruits and flowers present in Northern Argentina. *Front. Microbiol.* (2019) 10:1019. doi: 10.3389/fmicb.2019.01091
44. Östman EM, Nilsson M, Helena GM, Elmståhl L, Molin G, Björck IME. On the effect of lactic acid on blood glucose and insulin responses to cereal products: mechanistic studies in healthy subjects and *in vitro. J Cereal Sci.* (2002) 36:339–46. doi: 10.1006/jcrs.2002.0469
45. Salehi F, Kashaninejad M. Modeling of moisture loss kinetics and color changes in the surface of lemon slice during the combined infrared-vacuum drying. *Inf Process Agric.* (2018) 5:516–23. doi: 10.1016/j.inpa.2018.05.006
46. Wang YJ, Liu XQ, Zhang JZ. Structural characterization and *in vitro* antitumor activity of polysaccharides from *Ziziphus jujuba* cv. *Muzao RSC Adv.* (2015) 5:7860–7. doi: 10.1039/C4RA13350A
47. Lee JY, Kim CJ, Kunz B. Identification of lactic acid bacteria isolated from kimchi and studies on their suitability for application as starter culture in the production of fermented sausages. *Meat Sci.* (2006) 72:437–45. doi: 10.1016/j.meatsci.2005.08.013
48. Granato D, de Castro IA, Piekarski FBW, Benincá B, Masson, ML. Influence of passion fruit juice on colour stability and sensory acceptability of non-sugar Yacon-based pastes. *Braz Arch Biol Technol.* (2011) 54:149–59. doi: 10.1590/S1516-89132011000100020
49. Nualkaekul S, Charalampopoulos D. Survival of *Lactobacillus plantarum* in model solutions and fruit juices. *Int J Food Microbiol.* (2011) 146:111–7. doi: 10.1016/j.ijfoodmicro.2011.01.040
50. Šeme H, Gjuračić K, Kos B, Fujs Š, Štempelj M, Petković H, et al. Acid resistance and response to pH-induced stress in two *Lactobacillus plantarum* strains with probiotic potential. *Benef Microbes.* (2015) 6:369–79. doi: 10.3920/bm2014.0069
51. Srisukchayakul P, Charalampopoulos D, Karatzas KA. Study on the effect of citric acid adaptation toward the subsequent survival of *Lactobacillus plantarum* NCIMB 8826 in low pH fruit juices during refrigerated storage. *Food Res Int.* (2018) 111:198–204. doi: 10.1016/j.foodres.2018.05.018
52. Lemos JA, Burne RA. A model of efficiency: stress tolerance by *Streptococcus mutans*. *Microbiology.* (2008) 154:3247. doi: 10.1099/mic.0.2008/023770-0
53. Shimojo Y, Ozawa Y, Toda T, Igami K., Shimizu T. Probiotic *Lactobacillus paracasei* A221 improves the functionality and bioavailability of kaempferol-glucoside in kale by its glucosidase activity. *Sci Rep.* (2018) 8:9239. doi: 10.1038/s41598-018-27532-9
54. Hur SJ, Lee SY, Kim YC, Choi I, Kim GB. Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chem.* (2014) 160:346–56. doi: 10.1016/j.foodchem.2014.03.112
55. Septembre-Malaterre A, Stanislas G, Douraguia E, Gonthier MP. Evaluation of nutritional and antioxidant properties of the tropical fruits banana, litchi, mango, papaya, passion fruit and pineapple cultivated in Réunion French Island. *Food Chem.* (2016) 212:225–33. doi: 10.1016/j.foodchem.2016.05.147
56. Wen L, Wrolstad R E. Phenolic composition of authentic pineapple juice. *J Food Sci.* (2002) 67:155–61. doi: 10.1111/j.1365-2621.2002.tb11376.x
57. Hashemi SMB, Khaneghah, AM, Barba FJ, Nemati Z, Shokofti SS, Alizadeh F. Fermented sweet lemon juice (*Citrus limetta*) using *Lactobacillus plantarum* LS5: chemical composition, antioxidant and antibacterial activities. *J Funct Foods.* (2017) 38:409–14. doi: 10.1016/j.jff.2017.09.040
58. Gil-Izquierdo A, Gil MI, Ferreres F. Effect of processing techniques at industrial scale on orange juice antioxidant beneficial health compounds *J Agric Food Chem.* (2002) 50:5107–14. doi: 10.1021/jf020162+
59. Rosales-Soto MU., Powers, JR, Alldredge JR. Effect of mixing time, freeze-drying and baking on phenolics, anthocyanins and antioxidant capacity of raspberry juice during processing of muffins. *J Sci Food Agric.* (2012) 92:1511–8. doi: 10.1002/jsfa.4735
60. Degrain A, Manhivi V, Remize F, Garcia C, Sivakumar D. Effect of lactic acid fermentation on color, phenolic compounds and antioxidant activity in African nightshade. *Microorganisms.* (2020) 8:1324. doi: 10.3390/microorganisms8091324
61. Zhou Y, Wang R, Zhang Y, Yang Y, Sun X, Zhang Q, et al. Biotransformation of phenolics metabolites the change in antioxidant activity in kiwifruit induced by *Lactobacillus plantarum* fermentation. *J Sci Food Agric.* (2020) 100:3283–90. doi: 10.1002/jsfa.10272
62. Rodrígue, H, Landete JM, de las Rivas B, Muñoz R. Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748T. *Food Chem.* (2008) 107:1393–8. doi: 10.1016/j.foodchem.2007.09.067
63. Mapelli-Brahm P, Barba FJ, Remize F, Garcia C, Fessard A, Khaneghah AM, et al. The impact of fermentation processes on the production, retention and

bioavailability of carotenoids: an overview. *Trends Food Sci. Technol.* (2020) 99:389–401. doi: 10.1016/j.tifs.2020.03.013
64. Thane C, Reddy S. Processing of fruit and vegetables: effect on carotenoids. *Nutr Food Sci.* (1997) 97:58–65. doi: 10.1108/00346659710161858
65. van Het Hof KH, West CE, Weststrate JA, Hautvast JG. Dietary factors that affect the bioavailability of carotenoids. *J Nutr.* (2000) 130:503–6. doi: 10.1093/jn/130.3.503
66. Rodríguez-Roque MJ, Rojas-Graü MA, Elez-Martínez P, Martín-Belloso O. Changes in vitamin C, phenolic, and carotenoid profiles throughout *in vitro* gastrointestinal digestion of a blended fruit juice. *J Agric Food Chem.* (2013) 61:1859–67. doi: 10.1021/jf3044204
67. Gunathilake KDPP, Ranaweera KKDS, Rupasinghe HPV. Change of phenolics, carotenoids, and antioxidant capacity following simulated gastrointestinal digestion and dialysis of selected edible green leaves. *Food Chem.* (2018) 245:371–9. doi: 10.1016/j.foodchem.2017.10.096
68. Mosele J, Macia A, Romero MP, Motilva MP. Stability and metabolism of *Arbutus unedo* bioactive compounds (phenolics and antioxidants) under *in-vitro* digestion and colonic fermentation. *Food Chem.*(2016) 201:120–30. doi: 10.1016/j.foodchem.2016.01.076
69. Caicedo-Lopez LH, Luzardo-Ocampo I, Cuellar-Nuñez M L, Campos-Vega R, Mendoza S, Loarca-Piña G. Effect of the *in vitro* gastrointestinal digestion on free-phenolic compounds and mono/oligosaccharides from *Moringa oleifera* leaves: bioaccessibility, intestinal permeability and antioxidant capacity. *Food Res Int.* (2019) 120:631–42. doi: 10.1016/j.foodres.2018.11.017
70. Li Z, Teng J, Lyu Y, Hu X, Zhao Y, Wang M. Enhanced antioxidant activity for apple juice fermented with *Lactobacillus plantarum* ATCC14917. *Molecules.* (2019) 24:51. doi: 10.3390/molecules24010051
71. Othman NB, Roblain D, Chammen N, Thonart P Hamdi M. Antioxidant phenolic compounds loss during the fermentation of Chétoui olives. *Food Chem.* (2009) 116:662–9. doi: 10.1016/j.foodchem.2009.02.084

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

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Influence of Different Types of Drying Methods on Color Properties, Phenolic Metabolites and Bioactivities of Pumpkin Leaves of var. Butternut squash (*Cucurbita moschata* Duchesne ex Poir)

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Leaves of pumpkin species var. Butternut squash (*Cucurbita moschata* Duchesne ex Poir) is a popularly consumed leafy vegetable in the Southern African region. Traditional vegetables are commonly sun-dried as a method of postharvest preservation during the off-season. However, different drying methods affect the superior quality, functional properties, and bioactivities of the final product. Therefore, in this study, var. Butternut squash (*C. moschata*) underwent different drying methods, such as freeze-, oven, sun-, solar, and microwave drying to evaluate the color properties, pigments, phenolic metabolites, *in vitro* antioxidants, and antidiabetic activities. Results indicate that freeze-drying retained the total chlorophyll content with green color by reducing the color difference *(ΔE)*, improved the concentration of different phenolic metabolites and the content of ascorbic acid, and enhanced the FRAP, ABTS activities and the inhibitory effects of α-glucosidase, and α-amylase. Freeze-dried leaves contained the highest concentrations of quercetin 3-glucoside 7-rhamnoside (rutin), quercetin 3-galactoside, isorhamnetin-3-galactoside-6″-rhamnoside, isorhamnetin-3-O-rutinoside compared with the leaves that underwent four other drying treatments and raw leaves. The OPLS-DA and the UPLC–QTOF/MS and chemometric approach showed that the peak at *m/z* 609, 1441 (quercetin 3-galactoside 7-rhamnoside) separated the freeze-dried leaves of var. Butternut squash (*C. moschata*) from the other four drying treatments. Therefore, freeze-drying is highly recommended to obtain good quality leaf powders that are rich in functional compounds and bioactive properties for use as functional ingredients.

Keywords: Cucurbitaceae, polyphenols, α-glucosidase, FRAP activity, chlorophyll

INTRODUCTION

Pumpkin species var. Butternut squash (*Cucurbita moschata* Duchesne ex Poir), belonging to the family Cucurbitaceae, is indigenous to Mexico and Central America and is considered as an indigenous vegetable in the African region due to its naturalization in Africa (1). The FAO/WHO (2) recommends a serving size of 80–100-g green leafy vegetables in four to five servings for adults. The nutritional information present in the leaves of pumpkin species *C. moschata* is given in detail in the study of Mashitoa et al. (3). The leaves of pumpkin species (*C. moschata*) are functional vegetables due to their higher phenolics, flavonoid content, and antioxidant scavenging activity (4–6). In addition, leaves of pumpkin species (*C. moschata*) contain cinnamic acid, *p*-hydroxybenzoic acid, gentisic acid, protocatechuic acid, *p*-coumaric acid, ferulic acid, and caffeic acid (4). Ko et al. (4) reported the presence of caffeic acid, *p*-coumaric acid, ferulic acid, and gentisic acid as the predominant phenolic compounds in the leaves of pumpkin species (*C. moschata*). Pumpkin leaves are highly perishable, and the lack of cold storage infrastructure in rural areas affects their marketability. Therefore, postharvest drying is a suitable way to reduce losses and increase shelf life by preventing microbial decay without adding preservatives. Currently, different types of drying systems, such as solar drying, freeze-drying, oven drying, and microwave drying, are available.

Solar drying uses renewable energy, which is cost effective and easy to operate. Bananas, pineapples, mangoes, and vegetables, such as, potatoes and carrots, are currently solar dried (7). Oven drying is a common method used but is energy inefficient (8). Conventional microwave drying adds benefits to fresh produce such as fruits that have higher sugar content and improves their quality (9). Furthermore, freeze-drying works based on the sublimation process and helps to preserve the biochemical constituents due to the temperatures involved (as low as −50 to −80°C) by stopping the changes (10).

Managa et al. (11) reported the traditionally adopted sun-drying method negatively affected the product quality due to changes in phytonutritional compounds and the lack of food safety guidelines. Besides, the demand for dried vegetable powders, known as the functional powders demand, are increasing for use as nutritional and fortifying food additives. The functional foods market size will increase to US$275.77 in 2025 due to the increasing consumer demand for healthy food (12). However, temperature and duration of drying are vital since they affect the chemical, functional properties, and quality of the dried food product; thus, the selection of the best drying method depends on quality requirement and the affordability of the drying method (13). Therefore, the objective of this study was to investigate the influence of different drying methods, such as drying in a solar cabinet dryer, a hot air oven, freeze-drying, and traditional sun-drying methods, on the changes in (i) color properties, (ii) changes in lipophilic pigments, (iii) phenolic metabolite components, (iv) ascorbic acid, and (v) antioxidant property of the leaves of var. Butternut squash pumpkin (*C. moschata*).

MATERIALS AND METHODS

Pumpkin Leaves

Leaves of var. Butternut squash (*C. moschata*) were harvested from the Tshiombo irrigation scheme in Venda, Limpopo, South Africa, in the summer of 2019. The harvesting of pumpkin leaves took place 3–4 weeks after planting, and leaves that were free from dirt, pest damage, or decay were selectively harvested and washed in tap water, as described previously by Managa et al. (11).

Chemicals

All chemicals used in this study were purchased from Sigma Aldrich, Johannesburg, South Africa.

Postharvest Drying Treatments

This study adopted four different drying methods as follows:

Solar drying, using a solar dryer erected for the Tshiombo tribal council community jointly with Scaled Impact Pty (Ltd), Johannesburg and Tshwane University of Technology, Pretoria, South Africa, as described previously by Managa et al. (11). In the solar cabinet, uniform size leaves (380 g) were placed in 20 trays (894 × 605 × 80 mm), and the warm air circulated uniformly through the trays. The air circulation was facilitated with fans fitted with the solar photovoltaic panel. The solar cabinet was set at 45–50°C and the RH to around 30–45% based on the weather conditions. Drying duration on a warm day was 48 h and was continued until the leaves obtained constant weight.

Oven drying was carried out by placing 5–6 kg of the samples of leaves of var. Butternut squash (*C. moschata*) on a stainless-steel tray (915 × 840 × 915 mm) and drying them in a conventional drying oven (95 L) with Forced Convection System (Digital series, EcoTherm, Hartkirchen, Austria) at 100°C, RH 12% to 14% until constant weight (2 days), as described previously by Managa et al. (11).

Freeze-drying, pumpkin leaves (5–6 kg) were frozen at −80°C and then freeze-dried in a vacuum freeze-drier (YK-118-50, Taiwan) between −47 and −53°C for 72 h, as described previously by Managa et al. (11).

Microwave drying was performed in a domestic digital microwave oven (Samsung 40l microwave oven, Seoul, South Korea) with 230 V, 50 Hz, a frequency of 2,450 MHz (a wavelength of 12.24 cm), and an output power of 900 W for 15 min using 25 g of a leaf sample spread uniformly on the rotating glass platform of the microwave (11).

Sun-drying was performed by placing 5–6 kg of the samples of leaves of var. Butternut squash (*C. moschata*) on the pumpkin leaves on plain white paper sheets and under direct sun for 2 days to mimic the traditional way of drying adopted by the rural communities.

Color Changes

Dried leaves of var. Butternut squash (*C. moschata*) leaves were ground into a homogeneous powder using a domestic coffee grinder for 30 s, as described by Managa et al. (11). Color coordinates, L^*, a^*, b, were recorded with a color meter (CM-700d, Konica Minolta Sensing Inc., Tokyo, Japan), as described previously by Managa et al. (11), and the total color change, ΔE,

was calculated to show the color difference between fresh (raw) and dried leaves.

Color Pigments

Leaf pigments, chlorophyll a (*Chl a*) and b (*Chl b*), total chlorophyll, and carotenoid concentrations were quantified byadopting the method described by Mampholo et al. (14) and Managa et al. (11). Both chlorophyll a (*Chl a*) and b (*Chl b*) and total chlorophyll were determined using pumpkin leaf samples (0.2 g), homogenized with 2-ml acetone and hexane 4:6 (v/v), thereafter extracted for 2 h, and centrifuged (Hermle Labortechnik GmbH, Z326 K, 2010, Wehingen, Germany) for 10 min at 4°C (9,558 × g). The resulting supernatant was decanted and a portion of the solution was measured at 470, 646, and 662 nm (Biochrom Anthos Zenyth 200 Microplate Reader; SMM Instruments, Biochrom Ltd., Johannesburg, South Africa). The *Chl a* and *Chl b* contents were determined according to Managa et al. (11) using the following equations: *Chl a* = 15.65A662 – 7.340 A646, *Chl b* = 27.05 A646 −11.21 A662. The total chlorophyll was determined from *Chl a* + *Chl b*. Total carotenoids = (1,000 A470 – 2.270 *Chl a* – 81.4 *Chlb*)/227. All pigments were expressed in mg per 100 g on a dry-weight basis.

Ascorbic Acid Content

Ascorbic acid content was quantified using 30 g of leaves of var. Butternut squash (*C. moschata*) with five replicates from each drying treatment, according to Managa et al. (11) without any modifications, adopting the 2,6-dichlorophenol-indophenol titration method. A set of 5 g of leaves was homogenized in 10 ml of 4% oxalic acid, and then the mixture was filtered; the final volume was converted to 25 ml using 4% oxalic acid and quantified in 100 g^{-1} mg on a dry-weight basis.

Changes in Phenolic Metabolite Profile and Multivariate Analysis

Predominant polyphenolic metabolites present in leaves of var. Butternut squash (*C. moschata)* were extracted and quantified using a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS), hyphenated to a Waters Aquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA), as described earlier by Mashitoa et al. (3). Briefly, the instrument was used in the negative mode electrospray ionization with a cone voltage of 15 V, a desolvation temperature of 275°C, and desolvation gas of 650 L/h, and the rest of the MS settings were optimized for best resolution and sensitivity. Data were obtained by scanning from 150–1,500 *m/z* in the resolution mode as well as in the MSE mode. Two channels of MS data were acquired in the MS mode, one at low collision energy (4 V) and the second using a collision energy ramp (40–100 V) to obtain fragmentation data. Leucine enkephalin was used as a lock mass (a reference mass) for accurate mass determination, and the instrument was calibrated with sodium formate. Separation was achieved on a Waters HSS T3, 2.1 × 100 mm, a 1.7-μm column. The injection volume was 2 μl with the mobile phases being water with 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B. The following chromatographic method was used on 100% of solvent A for 1 min, changing to 28% of solvent B over 22 min then 40% of solvent B over 50 s, and a wash of 1.5 min at 100% of solvent B was carried out. The final step was re-equilibration to initial conditions for 4 min. The flow rate was 0.3-ml min^{-1}, and the column temperature was 55°C throughout the run. Three replicate samples of 50 mg from oven drying, solar drying, microwave drying, and freeze-drying were prepared by ultrasonication in 70% aqueous ethanol. The different phenolic components were quantified, using the reference calibrants catechin (LOD 1.41, LOQ 4.29), epicatechin (LOD 5.11, LOQ 15.5), and rutin (LOD 3, 29; LOQ 9.98), based on the areas of their extracted mass chromatograms. Thereafter, the obtained data were processed using TargetLynx software, as described previously by Mashitoa et al. (3), and the concentration of phenolic compounds was expressed as mg kg^{-1}. The differences between the phenolic metabolic profiles of the different postharvest drying treatments were analyzed, using an unsupervised Principal Component Analysis (PCA) approach using the data obtained from the UPLC–Q-TOF/MS. PCA was conducted to reduce the number of variables in the data matrix in order to choose the most discriminating postharvest drying on the phenolic metabolites in the leaves of var. Butternut squash *C. moschata* (3). Therefore, UPLC data were exported as an mzXML file, aligned by Marker Lynx 4.1 in the Apex Trac™ tool for the PCA analysis. However, to explain the differences between the different kinds of postharvest drying and to identify the potential characteristic markers (compounds) responsible for discrimination between the different postharvest drying treatments, supervised Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was undertaken.

Total Antioxidant Capacities

The ferric-reducing antioxidant power (FRAP) assay was performed according to the method of Managa et al. (11) and Mashitoa et al. (3) for leaves of var. Butternut squash (*C. moschata*). As described previously by Mashitoa et al. (3), leaf samples (0.20 g) were homogenized in 2 ml of 80% aqueous methanol. A 15-μl aliquot of this leaf extract was incubated for 10 min with 220 μl of the FRAP reagent solution [10 mmol L^{-1} TPTZ [2,4,6-tris(2-pyridyl)-1,3,5-triazine]] and acidified with concentrated HCl and 20 mmol L^{-1} $FeCl_3$. The absorbance was read at 620 nm (Multiplate reader, BMG LABTECH GmbH, SpectroStar Nano, Ortenberg, Germany). Antioxidant power was expressed as TEAC μmol 100 g^{-1} on a dry-weight basis.

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was performed for leaves of var. Butternut squash (*C. moschata*) based on the method previously described by Mashitoa et al. (3) without any modifications. The ABTS cation was produced by reacting the ABTS stock solution (7 mM) with an equal volume of 4.9 mM potassium persulfate and leaving the mixture to stand in the dark at room temperature for at least 12 h before use. The ABTS+ stock solution (285 μl) was mixed with 15 μl of the 80% aqueous methanolic extract and incubated in darkness at 25°C for 2 h; subsequently, the absorbance was measured at 734 nm (BMG LABTECH GmbH, SpectroStar Nano, Ortenberg, Germany). Calibration curves were constructed using Trolox as the standard, and the

TABLE 1 | Effect of different drying treatments on color values and color change in the leaves var. Butternut squash (*C. moschata*).

Postharvest drying	*L**	*a**	*b**	Δ*E*
Raw	33.22 ± 2.46^{c}	-7.45 ± 0.99^{c}	10.61 ± 1.64^{d}	
Freeze-drying	34.46 ± 0.65^{c}	-4.06 ± 0.14^{a}	9.54 ± 0.34^{d}	3.76 ± 0.19^{d}
Microwave-drying	41.71 ± 1.75^{b}	-7.54 ± 0.20^{c}	14.52 ± 0.73^{b}	11.98 ± 0.22^{a}
Oven-drying	44.41 ± 0.14^{a}	-9.16 ± 0.07^{bc}	16.08 ± 0.08^{a}	9.83 ± 0.16^{b}
Solar-drying	41.65 ± 0.84^{b}	-7.38 ± 0.16^{c}	12.62 ± 0.39^{c}	5.70 ± 0.02^{c}
Sun-drying	42.38 ± 0.85^{a}	-8.66 ± 0.22^{b}	13.81 ± 0.37^{c}	6.13 ± 0.23^{c}

Means followed by the same letter within the column are not significantly different, $p < 0.05$, according to Fisher's LSD test.
Raw leaves data are presented on a fresh-weight basis; sun-dried leaves are included as control since they are used traditionally.

antioxidant activity (ABTS assay) was expressed μmol TEAC 100 g^{-1} on a dry-weight basis.

In vitro α-Amylase Inhibition Assay

The α-amylase inhibition assay was determined using porcine pancreatic α-amylase, as described previously by Moloto et al. (15), using an aliquot of 500 μl of the sample [leaf extract of var. Butternut squash (*C. moschata*)] and 500 μl of 0.02 M sodium phosphate buffer (pH 6.9), with 0.006 M sodium chloride containing α-amylase solution (0.5 mg ml^{-1}). The reaction mixture was incubated at 25°C for 10 min. Thereafter, 1% starch solution (500 μl) in a 0.02-M sodium phosphate buffer (pH 6.9 with 0.006-M sodium chloride) was pipetted at different time intervals and held at 25°C for 10 min, and then the reaction was stopped by adding 100 μl of KAT amylase reagent for 5 min; the absorbance was measured at 540 nm (Multiplate reader, BMG LABTECH GmbH, SpectroStar Nano, Ortenberg, Germany). Dimethylsulfoxide (100 μl) without leaf extract and commercial α-amylase inhibitor Acarbose served as controls. The enzyme inhibitory activity was expressed as the IC_{50} (μg ml^{-1}) of α-amylase inhibition.

In vitro α-Glucosidase Inhibition Assay

In vitro α-glucosidase inhibitory activity was determined according to the method described by Mashitoa et al. (3), without any modifications, using leaf extract of var. Butternut squash (*C. moschata)* (5 μl) and concentrations of 50–250 μg ml^{-1}. A 96-well plate containing 20 μl α-glucosidase solution (50 μg ml^{-1}) and 60 μl of potassium phosphate buffer (pH 6.8; 67 mM) was incubated at 35°C for 5 min; 10 μl of 10-mM ρ-nitrophenyl-α-D-glucoside solution was then added and incubated at 35°C for 20 min, and after adding 25 μl of 100 mM Na_2CO_3, the absorbance was read at 405 nm using a microplate reader (CLARIOstar Plus BMG Labtec, Lasec, Cape Town, South Africa). The leaf extracts, acarbose, and the blank control (without α-glucosidase) were included for comparison and the IC_{50} value was measured.

Statistical Analysis

The experiment design included a completely randomized design with 10 replicates per treatment (different drying methods) and repeated two times for accuracy. For UPLC–Q-TOF/MS and chemometric analysis, three replicate samples per drying treatment were included. A one-way ANOVA analyzed the significant differences between different postharvest drying treatments on different parameters at $p < 0.05$. Treatment means were separated using Fisher's protected least significant difference (LSD) *t*-test at the 5% level of significance. Data were analyzed using the statistical program GenStat for Windows (2004).

RESULTS AND DISCUSSION

Changes in Color Properties and Pigments

Table 1 shows the influence of different drying methods with the changes in color properties. Freeze-dried leaves of var. Butternut squash (*C. moschata*) showed L^* (luminosity) and b^* (that relates to yellow) values, similar to the raw leaves. The increase in a^* value (higher negative), relating to the lower intensity of green color, showed the lowest color change ($\Delta E = 3.76$) than the raw leaves. The highest color change ($\Delta E = 11.98$) was noted in leaves that underwent microwave-drying. **Table 2** shows the influence of different drying treatments on leaf pigments. Total chlorophyll content in freeze-dried leaves of var. Butternut squash (*C. moschata*) was increased by 59.24% compared with the raw leaves, followed by sun-dried (36.52%) and solar-dried (31.18%) leaves, while oven- and microwave-dried leaves showed significantly highest loss in total chlorophyll content of 16.48 and 1.11%, respectively. The higher temperatures (100°C) during oven and microwave drying could have possibly converted the chlorophyll to pheophytin, as shown in **Table 1** by the olive green color and relating to higher color change (Δ*E*). Similar to the findings in this study, freeze-drying increased the total chlorophyll content in African nightshade, Chinese cabbage (11), and *Ipomoea aquatica* forsk leaves (16).

Conversely, different drying treatments, such as solar, sun, oven, and microwave drying, significantly increased the total chlorophyll in African nightshade and Chinese cabbage leaves compared with the raw leaves (11). More than 50% of the carotenoid content was lost during all the drying processes compared with the raw leaves. Managa et al. (11) showed, in their previous studies, that freeze-drying, solar drying, and sun drying increased the carotenoid content in African nightshade and Chinese cabbage. As per the findings in this study, oven drying and microwave drying caused significant loss of chlorophyll and carotenoid contents in African nightshade and Chinese cabbage (11). The air temperature increase relates to the loss of carotenoid content in vegetables (17). Additionally, microwave

TABLE 2 | Effect of different drying treatments on chlorophylls and total carotenoids in the leaves var. Butternut squash (*C. moschata*).

Postharvest drying	Total chlorophyll (mgg^{-1})	% loss	Carotenoids (mg100g^{-1})	% loss	Ascorbic acid content (mg100g^{-1})	%loss
Raw leaves	4.49 ± 0.52^{a}		2.23 ± 0.04^{a}		62.37 ± 1.86^{a}	
Freeze-drying	7.15 ± 0.12^{a}	59.24 ± 0.04^{d}	1.01 ± 0.05^{b}	-54.71 ± 1.11^{c}	46.24 ± 0.92^{b}	25.86 ± 0.12^{e}
Microwave-drying	4.54 ± 0.13^{cd}	-1.11 ± 1.25^{b}	0.11 ± 0.01^{d}	-95.06 ± 0.45^{a}	34.41 ± 0.86^{e}	44.62 ± 1.31^{b}
Oven-drying	3.75 ± 0.30^{d}	-16.48 ± 2.18^{a}	0.08 ± 0.02^{d}	-96.41 ± 2.01^{a}	26.88 ± 186^{f}	56.90 ± 0.43^{a}
Solar-drying	5.89 ± 0.50^{c}	31.18 ± 1.33^{c}	0.47 ± 0.01^{c}	-78.92 ± 1.65^{b}	39.78 ± 1.22^{c}	36.22 ± 0.11^{d}
Sun-dying	6.13 ± 0.13^{b}	36.52 ± 0.25^{c}	0.15 ± 0.09^{d}	-93.27 ± 0.77^{a}	37.63 ± 0.44^{d}	39.67 ± 0.71^{c}

Means followed by the same letter within the column are not significantly different, $p < 0.05$, according to Fisher's LSD test.
Sun-dried leaves are included as control since they are used traditionally.

TABLE 3 | Influence of different drying treatments on different phenolic components of leaves of var. Butternut squash (*C. moschata*).

Phenolic components (mg kg^{-1})	Raw	Freeze- drying	Solar drying	Sun drying	Microwave drying	Oven drying
2-O-caffeoylglucaric acid	89.5 ± 6.4^{b}	86.3 ± 1.2^{b}	117.3 ± 5.4^{a}	$114.7 \pm 4.a$	62.0 ± 12.5^{c}	24.4 ± 3.3^{d}
Gentesic acid 5-O-glucoside	4.8 ± 0.6^{e}	48.4 ± 4.8^{c}	19.9 ± 2.4^{d}	82.8 ± 6.9^{b}	162.0 ± 12.3^{a}	11.9 ± 0.9^{d}
Coumaroyl glucaric acid	55.3 ± 3.2^{a}	29.9 ± 0.5^{b}	28.4 ± 2.6^{b}	18.7 ± 2.9^{bc}	6.9 ± 0.7^{c}	8.1 ± 1.5^{c}
2-(E)-O-feruloyl-D-galactaric acid	83.7 ± 6.3^{a}	60.4 ± 15.7^{b}	49.7 ± 1.3^{bc}	56.8 ± 7.3^{b}	37.4 ± 4.9^{c}	14.6 ± 1.9^{d}
2-caffeoylisocitric acid	38.6 ± 1.9^{c}	98.6 ± 1.9^{a}	97.8 ± 9.3^{a}	74.3 ± 4.9^{b}	67.5 ± 13.3^{b}	41.7 ± 3.0^{c}
Coumaroyl isocitrate	47.9 ± 4.8^{d}	82.2 ± 5.7^{a}	64.1 ± 0.9^{b}	45.8 ± 6.0^{d}	51.1 ± 9.5^{c}	49.4 ± 1.9^{c}
7-Methylquercetin-3-Galactoside-6"-Rhamnoside-3"'-Rhamnoside;	12.3 ± 5.6^{b}	17.0 ± 6.0^{a}	8.3 ± 1.4^{d}	15.9 ± 1.3^{a}	12.7 ± 3.3^{b}	0.9 ± 0.3^{d}
Feruloyl isocitrate	12.6 ± 2.0^{d}	44.6 ± 3.2^{a}	25.9 ± 0.4^{b}	32.4 ± 1.1^{ab}	28.8 ± 6.6^{ab}	17.0 ± 1.7^{c}
Quercetin 3-galactoside 7-rhamnoside	149.9 ± 3.7^{a}	111.8 ± 7.1^{b}	127.9 ± 2.9^{b}	63.1 ± 12.3^{c}	73.9 ± 14.8^{c}	19.6 ± 4.0^{d}
Quercetin 3-glucoside 7-rhamnoside (Rutin)	351.9 ± 12.9^{a}	354.5 ± 10.5^{a}	312.9 ± 32.4^{b}	256.8 ± 8.3^{c}	272.3 ± 23.3^{c}	94.3 ± 9.8^{d}
Quercetin 3-galactoside	112.2 ± 7.7^{a}	109.9 ± 1.5^{a}	98.7 ± 12.3^{b}	52.2 ± 3.2^{c}	49.2 ± 15.0^{c}	15.5 ± 4.2^{d}
kaempferol 7-neohesperidoside	123.4 ± 6.4^{a}	114.9 ± 5.7^{ab}	96.0 ± 6.2^{b}	77.6 ± 35.6^{c}	117.0 ± 35.2^{a}	21.1 ± 0.9^{d}
Isoorientin 2"-O-rhamnoside	182.1 ± 3.7^{a}	176.3 ± 3.4^{a}	142.0 ± 8.3^{b}	143.6 ± 10.2^{b}	177.4 ± 20.2^{a}	28.7 ± 2.6^{c}
Isorhamnetin-3-Galactoside-6"-Rhamnoside	80.1 ± 2.1^{a}	73.8 ± 9.9^{a}	28.8 ± 3.0^{c}	35.8 ± 11.2^{b}	37.2 ± 11.3^{b}	5.9 ± 0.4^{d}
Isorhamnetin-3-O-rutinoside	174.1 ± 8.7^{a}	174.3 ± 13.2^{a}	89.6 ± 14.6^{c}	125.3 ± 1.7^{b}	116.6 ± 13.0^{b}	14.0 ± 5.9^{d}
Total polyphenolic compounds	1518.4 ± 6.9^{b}	1582.9 ± 3.6^{a}	1307.3 ± 5.1^{c}	1195.8 ± 1.9^{e}	1272.0 ± 6.8^{d}	367.1 ± 3.7^{f}

drying caused a 77.50% loss of carotenoid content in parsley leaves (18). Furthermore, freeze-drying resulted in minimum chemical changes, while oven drying at 45–140°C caused rapid degradation of primary metabolites (19).

Ascorbic Acid Content

Freeze-dried leaves of var. Butternut squash (*C. moschata*) showed a 25.86% loss of ascorbic acid (vitamin C) compared with the raw leaves, while oven- and microwave-dried and sun-dried samples (**Table 2**) showed 56.90 and 44.62% loss, respectively. Besides, Gupta et al. (20) showed that ascorbic acid was better retained in freeze-dried samples than oven-dried samples. Therefore, among the tested drying methods, oven drying and microwave drying were the most unfavorable, contributing toward marked degradation of vitamin C. Managa et al. (11) reported retention of ascorbic acid in freeze-dried African nightshade and Chinese cabbage leaves compared with oven and microwave drying, as mentioned earlier. Khantoniar et al. (21). showed degradation of ascorbic acid content during microwave heating and explained that it was probably due to the power categories (90–1,000 W) during microwave drying. Furthermore, openly sun-dried cowpea leaves lost 82–86% ascorbic acid (22), whereas, in this study, sun drying caused a 39.67% loss. In this study, the solar-drying cabinet significantly reduced the loss of ascorbic acid by 36.22% in pumpkin leaves. The destruction of the cell structure during thermal drying and the contact of the oxidizing enzymes of ascorbic acid with the substrate to dehydroascorbic acid could have hydrolyzed to 2,3-diketogulonic acid and eventually polymerized to form other compounds (23, 24).

Changes in Phenolic Metabolites

Table 3 shows the identified and quantified different phenolic components using UPLC-QTOF/MS. Gentesic acid 5-O-glucoside, 2-O-caffeoylglucaric acid, 2-O-caffeoylhydroxycitric acid, 2-(E)-O-feruloyl-D-galactaric acid isomer, 1-O-caffeoylglucose, coumaroyl glucaric acid, 2-(E)-O-feruloyl-D-galactaric acid isomer, 1-O-p-coumaroyl-beta-D-glucose, luteolin 7-neohesperidoside, 2-caffeoylisocitric acid, quercitrin, coumaroyl isocitrate, 7-methylquercetin-3-galactoside-6″-rhamnoside-3″-rhamnoside, feruloyl isocitrate, quercetin 3-galactoside 7-rhamnoside, genistin,

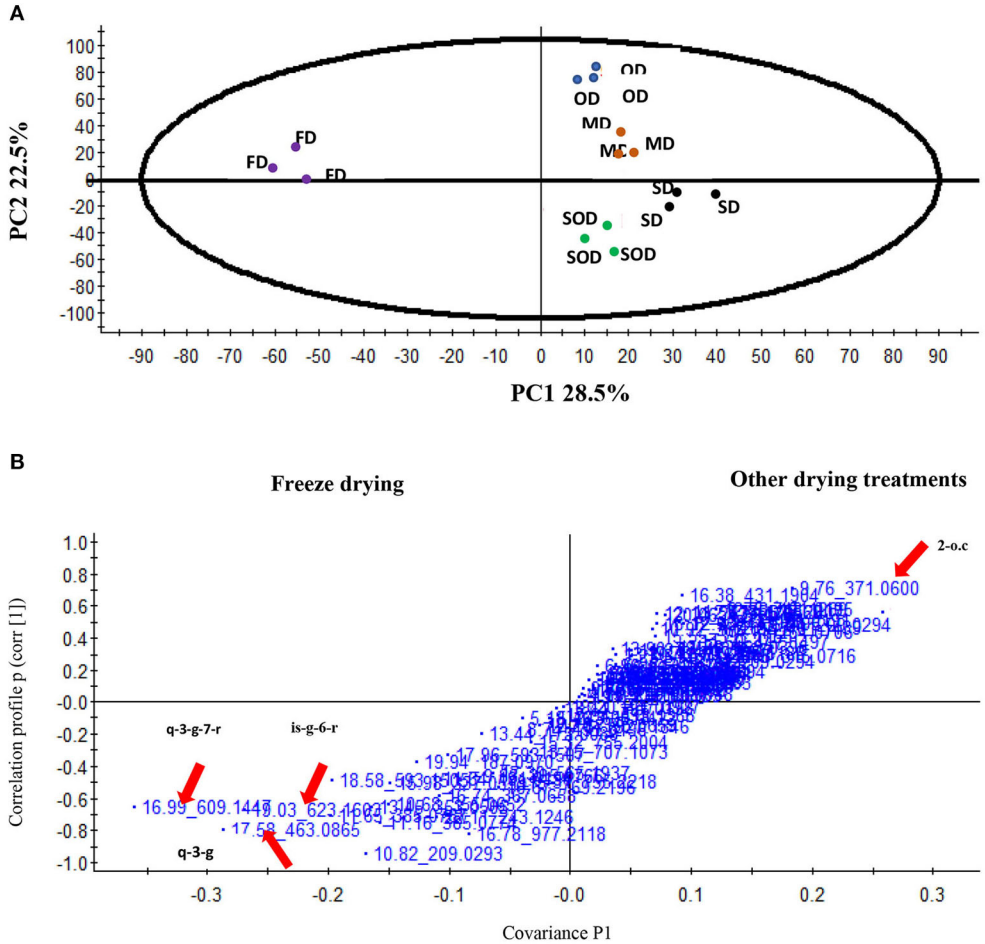

FIGURE 1 | (A) A score plot of principal component analysis (unsupervised) based on a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS), hyphenated to a Waters Aquity ultra-performance liquid chromatograph (UPLC) (UPLC–Q-TOF/MS) spectra (phenolic metabolic analysis) of the leaves of var. Butternut squash (*Cucurbita moschata)* that underwent five different postharvest drying treatments. PC1 = 28.50% and PC2 = showing 22.5% of the leaves of three different pumpkin species. Each postharvest-drying treatment included three replicate samples. OD, oven drying; MD, microwave drying; SD, sun-drying; SOD, solar drying; FD, freeze-drying. **(B)** A score plot of orthogonal partial least squares discriminant analysis of UPLC–QTOF/MS spectra of the leaves of var. Butternut squash (*Cucurbita moschata)* subjected to different postharvest drying treatments. Each sample set includes three replicates. *m/z* 371.05997, 2-O-caffeoylglucaric acid (2-o.c); *m/z* 609.1441 quercetin 3-galactoside 7-rhamnoside (q-3-g-7- r); *m/z* 463.08 quercetin 3-galactoside (q-3-g); *m/z* 623.16 isorhamnetin-3-galactoside-6″-rhamnoside. isorhamnetin-3-galactoside-6″-rhamnoside (is-g-6-r).

kaempferol 7-neohesperidoside, isoorientin 2″-O-rhamnoside, isorhamnetin-3-galactoside-6″-rhamnoside, and isorhamnetin-3-O-rutinoside, pectolinarigenin 7-(6″-methylglucuronide) were identified and quantified in the leaves of var. Butternut squash belonging to *C. moschata* species (3). The impact of different drying methods on the retention of total polyphenol content in the leaves occurred in the following order: freeze-drying > solar drying > microwave drying > sun-drying > oven drying.

Freeze-dried leaves showed the highest total polyphenols compared with the raw leaves and the other four drying treatments. Freeze-dried leaves contained the highest concentration of coumaroyl isocitrate (82.20 mg kg^{-1}), quercetin 3-glucoside 7-rhamnoside (Rutin) (354.50 mg kg^{-1}), quercetin 3-galactoside (109.90 mg kg^{-1}), isorhamnetin-3-galactoside-6″-rhamnoside (73.80 mg kg^{-1}), and isorhamnetin-3-O-rutinoside (174.30 mg kg^{-1}) compared with the raw leaves of var. Butternut squash and other four drying treatments. Freeze-drying enables the formation of ice crystals within the cell, and their rapid expulsion from the cell during freezing and the rapid removal of the ice crystals during the process help to maintain the cell structure that enables it to retain the phenolic compounds (25).

However, freeze-dried or microwave-dried leaves increased the concentration of feruloyl isocitrate, kaempferol 7-neohesperidoside, and isoorientin 2″-O-rhamnoside significantly in the leaves of var. Butternut squash (*C. moschata*) compared with all other drying treatments and raw leaves. Additionally, freeze-dried and sun-dried leaves of var. Butternut squash (*C. moschata*) contained the significantly highest concentration of 7-methylquercetin-3-galactoside-6″-rhamnoside-3‴-rhamnoside

TABLE 4 | Effect of different drying treatments on antioxidant activities and inhibitory effect on digestive enzymes in the leaves var. Butternut squash (*C. moschata*).

Treatments	FRAP (μmol TEAC 100 g^{-1})	ABTS (μmo lTEAC 100 g^{-1})	α-Glucosidase IC_{50} (μgmL^{-1})	α-Amylase IC_{50} (μgmL^{-1})
Raw	141.88 ± 0.36^{b}	23.35 ± 0.80^{c}	21.20 ± 0.21	18.11 ± 0.11^{c}
Freeze dried	383.13 ± 0.30^{a}	25.01 ± 1.1^{a}	19.14 ± 0.13^{e}	16.97 ± 0.14^{e}
Microwaved dried	233.33 ± 0.21^{de}	22.04 ± 1.2^{d}	26.09 ± 0.12^{b}	20.34 ± 0.08^{a}
Oven dried	141.41 ± 0.01^{e}	$10.18 \pm 0.1.01^{e}$	27.24 ± 0.10^{a}	23.44 ± 0.13^{a}
Solar dried	242.42 ± 0.14^{cd}	24.31 ± 1.1^{b}	22.39 ± 0.10^{d}	17.14 ± 0.04^{d}
Sun dried	248.16 ± 0.02^{c}	23.52 ± 0.90^{c}	25.20 ± 0.17^{c}	18.83 ± 0.06^{c}
			Acarbose 18.03 ± 0.11^{f}	Acarbose 19.07 ± 0.01^{b}

Means followed by the same letter within the column are not significantly different, $p<0.05$, according to Fisher's LSD test.
Sun-dried leaves are included as control since it is used traditionally.

TABLE 5 | Exact mass/retention time pairs responsible for the separation of freeze-dried pumpkin leaves from the other drying treatments.

Primary ID	Retention time	Mass	p[1]P	p(corr)[1]P	Factor of change	Freeze drying	Others
Quercetin 3-galactoside	17.58	463.08	−0.28	−0.79	1.9	279.07	537.61
Isorhamnetin-3-galactoside-6″-rhamnoside	19.03	623.16	−0.26	−0.66	2.0	417.64	207.73
Quercetin 3-galactoside 7-rhamnoside	16.99	609.14	−0.36	−0.66	2.1	541.08	263.03
2-O-caffeoylglucaric acid	6.963	371.05	0.24	0.68	1.4	1337.7	945.08

and feruloyl isocitrate compared with the other counterpart treatments and raw leaves. Oven drying negatively affected the 15 different phenolic compounds detected in the leaves of var. Butternut squash (*C. moschata*) and showed the lowest concentrations.

Multivariate Analysis

The untargeted phenolic metabolites in the leaves of var. Butternut squash (*C. moschata*) showed differences in distribution when an unsupervised (PCA) approach was used for the data obtained by the UPLC–Q-TOF/MS analysis.

The metabolite data were used to perform the PCA and discriminate the different postharvest drying treatments of var. Butternut squash (*C. moschata*) based on the greater impact of different metabolites, and PC1 accounted for 28.50% of the variance with the leaves of var. Butternut squash (*C. moschata*) that underwent freeze-drying and is positioned along with negative PC1 score values and all other drying treatments: solar, sun-, oven, and microwave drying positioned along the positive PC1 (**Figure 1A**). The phenolic metabolites that showed a greater impact on PC2 accounted for 22.50% of the variance with the leaves of var. Butternut squash (*C. moschata*) that underwent microwave drying positioned along with positive PC2 and the rest of the drying treatments along the negative PC2 (**Figure 1A**) based on the metabolites, facilitating the discrimination of the leaves of var. Butternut squash (*C. moschata*) that underwent different kinds of postharvest drying. OPLS-DA was performed as a tool for metabolite data to analyze the multivariate data to relate a quantitative relationship between leaves of var. Butternut squash (*C. moschata*), and the four different kinds of postharvest drying and the phenolic compounds helped to identify the marker candidate responsible for the separation of the four different drying treatments. **Figure 1B**, **Table 5** illustrates the S-plot, showing the marker ions *m*/*z* 609,14 (quercetin 3-galactoside 7-rhamnoside), *m*/*z* 463.08 (quercetin 3-galactoside), *m*/*z* 623.16 (isorhamnetin-3-galactoside-6″-rhamnoside) separating the freeze-dried leaves of var. Butternut squash (*C. moschata*) from the other four drying treatments, while the other four drying treatments were separated from the freeze-drying by the marker ion *m*/*z* 371,06 (2-O-caffeoylglucaric acid).

Antioxidant Activities and Inhibitory Effect of α-Amylase and α-Glucosidase

Freeze-dried leaves of var. Butternut squash (*C. moschata*) showed the highest FRAP (383.13 μmol TEAC100 g^{-1}) and ABTS (25.01 μmol TEAC100 g^{-1}) activities, while the oven-dried samples showed the lowest antioxidant activities, probably due to the lower polyphenol content, as shown in **Table 4**. Similarly, Managa et al. (11) showed higher FRAP and ABTS activities in the leaves of freeze-dried African nightshade and Chinese cabbage and the lowest antioxidant activity in oven-dried leaves. The antioxidant property of the vegetables depends on the presence and the concentrations of antioxidant components, and thermal processing at 65 or 100°C reduced the antioxidant activities in food products (26).

Freeze-drying is a nonthermal process, which probably conserved the antioxidants, such as ascorbic acid and carotenoids, of pumpkin leaves. These compounds have higher redox potential and contribute significantly toward antioxidant activities (11, 26). In addition, the presence of higher numbers of hydroxyl groups in phenolic compounds contributes more toward the antioxidant activity (26). The leaves of var. Butternut squash (*C. moschata*) contain flavonoid glycosides,

and, sometimes, the presence of mono- or diglycosidic molecules and the arrangement of hydroxyl group on the flavonoid B and C rings affect the antioxidant property. Furthermore, in the sun-, solar-, and oven-dried leaves, the thermal process could have released the antioxidants due to destruction of the cell wall and cellular components or production of antioxidants, such as Millard-derived melanoidins (27), and denaturation of polyphenol oxidase and polyphenol peroxidase enzymes could have protected the antioxidants and their activity (13). The microwave output power also plays a vital role in determining the antioxidant activity in vegetables, and the higher output power of 800 W improves the antioxidant activity (28). Phenolic antioxidants correlated well with FRAP activity (29). In this study, FRAP activity correlated strongly with quercetin 3-glucoside 7-rhamnoside (rutin) ($r = 0.80$, $p < 0.05$), coumaroyl isocitrate ($r = 0.75$, $p < 0.05$) and feruloyl isocitrate ($r = 0.73$, $p < 0.05$). Therefore, higher retention of polyphenolic compounds and antioxidant activity in freeze-dried leaves of var. Butternut squash (*C. moschata*) acts as a good natural antioxidant, a food preservative, or a dietary supplement in health-promoting foods.

Table 4 presents the inhibition of α-glucosidase and α-amylase activities. Inhibitory activities of α-glucosidase (IC_{50} 19.77 μgml^{-1}) and α-amylase (IC_{50} 16.97 μgml^{-1}) were highest in freeze-dried leaves of var. Butternut squash with sucrose substrate compared with the other four postharvest drying treatments and raw leaves. However, oven-dried leaves reduced the inhibition of α-glucosidase and α-amylase compared with the other drying treatments and raw leaves. The acarbose showed the significantly highest inhibition of α-glucosidase activity (IC_{50}18.03 μgml^{-1}) compared with the freeze-dried leaf extract samples. At the same time, acarbose showed a lower inhibitory effect on α-amylase (IC_{50} 19.07 μgml^{-1}) than the freeze-dried leaf extract.

The presence of the hydroxyl group on position 4 (ring B) of the molecular structure of quercetin 3 galactoside, isorhamnetin-3-galactoside-6″-rhamnoside, isoorientin 2″-O-rhamnoside, genistin, quecetin 3 galactoside 7-rhamnoside, kaempferol neohesperoside, isohermnetin 3-O-rutinosidem, and rutin plays a vital role in their α-glucosidase and α-amylase inhibitory effect (30, 31). Furthermore, quercetin 3 galactoside, isorhamnetin-3-galactoside-6″-rhamnoside, isoorientin 2″-O-rhamnoside have another hydroxyl group in position 7 (ring A), which also contributes to the α-glucosidase and α-amylase inhibitory effect (30). Besides, the C2 = C3 double bond in the C ring of flavonoids, such as quercetin 3 galactoside, isorhamnetin-3-galactoside 6" rhamnoside, and isoorientin 2" rhamnoside, is essential for their α-glucosidase and α-amylase inhibition activity (31). The degree of high hydroxylation of the non-flavonoid phenolic compounds, including gentesic acid, 2 caffeoylglucaric acid, 2 caffeoyl hydroxycitric acid, feruloyl isocitrate, and 1-O-caffeoyl glucose, also could have significantly enhanced their amylase inhibition activity (30). Furthermore, the synergistic effect on different phenolic compounds and their varying concentrations could have had a great impact on the degree of inhibition of these two digestive enzymes.

Although the Food and Drug Administration has approved commercial synthetic-inhibiting agents, the synthetic inhibitor caused side effects, e.g., liver disorders. Therefore, plant-based enzyme inhibitors, such as the leaf extracts of var. Butternut squash (*C. moschata*), can be regarded as safer to manage type 2 diabetics (32).

CONCLUSIONS

The presented data indicated that freeze-drying was the best postharvest drying method. Freeze-drying preserved the color properties, most phenolic metabolites, antioxidant activity, and antidiabetic activity of the leaves of var. Butternut squash (*C. moschata*). Oven-drying caused the highest reduction of different phenolic metabolites, ascorbic acid content, antioxidant activity, and inhibitory activities of α-glucosidase and α-amylase. The study also helped to identify the biomarker candidates that separated the freeze-dried pumpkin leaves from the samples that underwent other drying treatments using novel practical tools, such as UPLC-QTOF/MS and the chemometric approach. Although freeze-drying is expensive at the rural level, industrial and pilot-scale freeze dryers are available for the production of functional powders in large-scale at privatelyowned companies involved in the functional powder trade. Therefore, the findings of this study provide evidence-based information that the use of an appropriate postharvest drying method to obtain the functional powder from the leaves of var. Butternut squash (*C. moschata)* is a functional ingredient for the further development of functional foods.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FM was the first author and the Ph.D. student who performed the experiment, generated the data, and wrote some parts of this manuscript. TS visualized and validated the data for phenolic compounds, interpreted the chromatogram, and wrote that part of the article. JS was responsible for the antidiabetic activity and data. RS, the research collaborator and co-supervisor, provided editorial support. YS the research collaborator, presented the data visualization. DS the grant holder, conceptualized the research, supervised the first author, and improved the article further. 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/fnut.2021.694649/full#supplementary-material

REFERENCES

1. Mujumdar AS, Jangam SV. *Some Innovative Drying Technologies for Dehydration of foods*. Athens: ICEF (2011). p. 555–6.
2. WORLD HEALTH ORGANIZATION (WHO), FAO. (2017). *Fruit and Vegetable Health Initiative. Report*. Available online at: http://www.fao.org/3/ai6807e.pdf.
3. Mashitoa FM, Shoko T, Shai JL, Slabbert RM, Sivakumar D. Changes in phenolic metabolites and biological activities of pumpkin leaves (*Cucurbita moschata* Duchesne ex Poir.) during blanching. *Front Nutr*. (2021) 8:641939. doi: 10.3389/fnut.2021.641939
4. Ko JY, Ko MO, Kim DS, Lim SB. Enhanced production of phenolic compounds from pumpkin leaves by subcritical water hydrolysis. *Prev Nutr Food Sci.* (2016) 21:132–7. doi: 10.3746/pnf.2016.21.2.132
5. Cha YY. Experimental study on effects of *Cucurbita moschata* Duch. on antioxidation. *J Korean Med Obes Res.* (2009) 9:57–63. Available online at: https://www.koreascience.or.kr/article/JAKO200927742024236.page (accessed April 13, 2021).
6. Kim MJ, Hong CO, Nam MH, Lee KW. Antioxidant effects and physiological activities of pumpkin (*Cucurbita moschata* Duch.) extract from different aerial parts. *Korean J. Food Sci. Technol.* (2011) 43:195–9. doi: 10.9721/KJFST.2011.43.2.195
7. Jayaraman KS, Gupta DDK, Rao NB. Solar drying of vegetables. In: Mujumdar AS, Suvachittanont S, editors. *Developments in Drying,* vol. 1: *Food dehydration Bangkok*. Thailand: Kasetsart University Press (2000). p. 179–206.
8. Bhat MA, Anju B. Study on physico-chemical characteristics of pumpkin blended cake. *J Food Processing Techno*. (2013) 4:262. doi: 10.4172/2157-7110.1000262
9. Orsat V, Changrue V, Raghavan V.G.S. Microwave drying of fruits and vegetables. *Stewart Postharvest Rev.* (2006) 6:4. doi: 10.2212/spr.2006.6.4
10. Ratti C. Hot air and freeze-drying of high-value foods: a review. *J Food Eng.* (2001) 49:311–9. doi: 10.1016/S0260-8774(00)00228-4
11. Managa GM, Sultanbawa Y, Sivakumar D. Effects of different drying methods on untargeted phenolic metabolites, and antioxidant activity in Chinese cabbage (*Brassica rapa* L. subsp. chinensis) and nightshade (*Solanum retroflexum* Dun.). *Molecules.* (2020) 25:1326. doi: 10.3390/molecules25061326
12. Report link. *Functional Foods Market Size, Share & Trends Analysis Report by Ingredient, by Product, by Application and Segment Forecasts.* (2019)–(2025). Available online at: https://www.researchandmarkets.com/reports/4764576/functional-foods-market-size-share-and-trends (accessed February 02, 2021).
13. Garcia LM, Ceccanti C, Negro C, De Bellis L, Incrocci L, Pardossi A, et al. Effect of drying methods on phenolic compounds and antioxidant activity of *Urtica dioica* L. *Leaves. Horticulturae.* (2021) 7:10. doi: 10.3390/horticulturae7010010
14. Mampholo BM, Sivakumar D, Beukes M, van Rensburg JW. Effect of modified atmosphere packaging on the quality and bioactive compounds of Chinese cabbage (*Brasicca rapa* L. ssp. chinensis). *J Sci Food Agric.* (2013) 93:2008–15. doi: 10.1002/jsfa.6007
15. Moloto MR, Phan ADT, Shai JL, Sultanbawa Y, Sivakumar D. Comparison of phenolic compounds, carotenoids, amino acid composition, *in vitro* antioxidant and anti-diabetic activities in the leaves of seven cowpea (*Vigna unguiculata*) cultivars. *Foods*. (2020) 9:1285. doi: 10.3390/foods9091285
16. Shin LER, Zzaman W, Kuang YT, Bhat R. Influence of dehydration techniques on physicochemical, antioxidant and microbial qualities of *Ipomoea aquatica* forsk: an underutilized green leafy vegetable. *J. Food Process. Preserv*. (2015) 6:1118–24. doi: 10.1111/jfpp.12326
17. Eim VS, Urrea D, Rossello C, Vicente Garcia-Perez J, Femenia A, Simal S. Optimization of the drying process of carrot (*Daucus carota* v. Nantes) on the basis of quality criteria. *Dry Technol.* (2013) 31:951–62. doi: 10.1080/07373937.2012.707162
18. Kamel SM, Thabet HA, Algadi EA. Influence of drying process on the functional properties of some plants. *Chem Mater Res.* (2013) 3, 2224–3224. Available online at: https://core.ac.uk/download/pdf/234666143.pdf (accessed April 13, 2021).
19. Shonte TT, Duodu KG, de Kock JG. Effect of drying methods on chemical composition and antioxidant activity of underutilized stinging nettle leave. *Heliyon.* (2020) 5:e03938. doi: 10.1016/j.heliyon.2020.e03938
20. Gupta S, Gowri BS, Jyothi LA, Prakash J. Retention of nutrients in green leafy vegetables on dehydration. *J. Food Sci. Technol*. (2013) 50:918–25. doi: 10.1007/s13197-011-0407-z
21. Khatoniar S, Barooah MS, Das M. Effect of different drying methods on micronutrient content of selected green leafy vegetables. *Int J Curr Microbial App Sci.* (2019) 8:1317–25. doi: 10.20546/ijcmas.2019.807.156
22. Gong X, Huang X, Yang T, Wen J, Zhou, W. Effect of drying methods on physicochemical properties and antioxidant activities of okra pods. *J Food Process Pres.* (2019) 43:14277. doi: 10.1111/jfpp.14277
23. Kaur A, Kaur D, Oberoi DPS, Gill BS, Sogi DS. Effect of dehydration on physicochemical properties of mustard, mint and spinach. *J Food Process Preserv.* (2008) 32:103–16. doi: 10.1111/j.1745-4549.2007.00168.x
24. Parsons HT, Fry SC. Oxidation of dehydroascorbic acid and 2, 3-diketogulonate under plant apoplastic conditions. *Phytochemistry.* (2012) 75:41–9. doi: 10.1016/j.phytochem.2011.12.005
25. Meng Q, Fan H, Li Y, Zhang L. Effect of drying methods on physico-chemical properties and antioxidant activity of *Dendrobium officinale*. *J. Food Meas.* (2018) 12:1–10. doi: 10.1007/s11694-017-9611-5
26. De Santiago E, Dominguez M, Cid C, De Peña M. Impact of cooking process on nutritional composition and antioxidants of cactus cladodes (*Opuntia ficus-indica*). *Food Chem.* (2018) 240:1055–62. doi: 10.1016/j.foodchem.2017.08.039
27. Miranda M, Maureira H, Rodriguez K, Vega- Gálvez A. Influence of temperature on the drying kinetics, physicochemical properties, and antioxidant capacity of aloe vera (*Aloe Barbadensis* Miller) gel. *J. Food Eng.* (2009) 91:297–304. doi: 10.1016/j.jfoodeng.2008.09.007
28. Hamrouni-Sellami I, Rahali FZ, Rebey IB, Bourgou S, Limam F, Marzouk B. Total phenolics, flavonoids, and antioxidant activity of sage (*Salvia officinalis* L.) plants as affected by different drying methods. *Food Bioproc Tech.* (2013) 6:806–17. doi: 10.1007/s11947-012-0877-7
29. Medoua, G.N.; Oldewage-Theron, W.H. Effect of drying and cooking on nutritional value and antioxidant capacity of morogo (*Amaranthus hybridus*) a traditional leafy vegetable grown in South Africa. *J. Food Sci. Technol.* (2014) 51:736–42. doi: 10.1007/s13197-011-0560-4
30. Rasouli H, Hosseini-Ghazvini SM, Adibi H, Khodarahmi R. Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic compounds: a virtual screening perspective for the treatment of obesity and diabetes. *Food Funct.* (2017), 8:1942–54. doi: 10.1039/C7FO00220C
31. Li K, Yao F, Xue Q, Fan H, Yang L, Li X, Sun L, Liu Y. Inhibitory effects against α-glucosidase and α-amylase of the flavonoids-rich extract from Scutellaria baicalensis shoots and interpretation of structure–activity relationship of its eight flavonoids by a refined assign-score method. *Chem Cent J.* (2018) 12:1–11. doi: 10.1186/s13065-018-0445-y
32. Asgar AMD. Anti-diabetic potential of phenolic compounds: a review. *Int. J. Food Prop*. (2013) 16:91–103. doi: 10.1080/10942912.2011.595864

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

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Quantitative Assessment of Abiotic Stress on the Main Functional Phytochemicals and Antioxidant Capacity of Wheatgrass at Different Seedling Age

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The wheat seedlings of 6 days old were daily subjected to ultraviolet irradiation (irradiating for 5, 10, 20, 40, and 60 min/day, respectively), Polyethylene glycol 6000 (5, 10, 15, 20, 25% in 1/2 Hoagland solution, respectively), and salinity solution (10, 25, 50, 100, 200 mM in 1/2 Hoagland solution, respectively), while the control group (CK) was supplied only with the Hoagland solution. The wheatgrass was harvested regularly seven times and the total soluble polysaccharides, ascorbic acid, chlorophyll, total polyphenol, total triterpene, total flavonoid, and proanthocyanins content were tested. The antioxidant capacity was evaluated through 2,2′-azino-bis (3-ethylbenzthia-zoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, and ferric ion reducing power. Technique for order preference by similarity to ideal solution (TOPSIS) mathematical model was adopted to comprehensively assess the functional phytochemicals of the different treatments. The results showed that the accumulation patterns of phytochemicals under abiotic stress were complex and not always upregulated or downregulated. The antioxidant activity and functional phytochemicals content of wheatgrass were significantly affected by both the stress treatments and seedling age, while the latter affected the chemicals more efficiently. The top five highest functional phytochemicals were observed in the 200 mM NaCl treated group on the 21st and 27th day, 25% PEG treated group on the 24th day, 200 mM NaCl treated group on the 24th day, and the group of 40 min/day ultraviolet exposure on 27th day.

Keywords: wheat young leaves, ultraviolet stress, drought stress, salinity stress, functional compounds, comprehensive nutrition ranking

INTRODUCTION

Wheatgrass, the mature shoots of the common wheat plant (*Triticum aestivum*, Poaceae family) which has been considered the most edible grain cereal-grass crop globally (1), is used as herbal medicine and nutraceutical traditionally (2). The consumption of wheatgrass could be traced to as early as ancient Egypt of 5,000 years ago or Mesopotamian Civilization (3). The recent interests in the wheatgrass was boomed immensely by Dr. Ann Wigmore in 1970s

who developed wheatgrass juice as a part of her herbal therapeutic nutritional approach and compiled "The Wheatgrass Book" (4). Wheatgrass was demonstrated to have a wide range of health benefits under conditions, such as common cold, astriction, diabetes, kidney swelling, anemia, eczema, (5) thalassemia, and myelodysplastic syndrome (2), and possess antimutation (6), anti-inflammatory, antioxidant, immunoregulation, hemostasis, diuresis, antimicrobial, antiaging, and anti-cancer (e.g., cervical cancer and oral squamous cell carcinoma) properties (7–9).

The therapeutic properties of wheatgrass could be attributed to the rich phytochemical components, such as chlorophyll, ascorbic acid, bioflavonoids, and so on (8, 10), which varied according to the production process and growing environments (11). Similar views were shared that abiotic stress factors could affect the plant bioactive compounds and produce differences in physiological condition and nutritional value (12, 13). Literature has shown that ascorbic acid, β-carotene, carotenoids, functional phytochemicals that include phenolics, flavonoids, and antioxidant activity of leafy vegetables were augmented under abiotic stresses, such as drought (14, 15) and salinity stress (16, 17). In addition, there were reports on the upregulation of ascorbic acid under abiotic stress during rice seed priming (12), but was observed decrease in wheatgrass and soybean plants under drought stress (18). As to triterpene, it was reported to be augmented in some plants (19). In blue light treated einkorn wheatgrass and red light treated emmer wheatgrass, the phenol and flavonoid content was increased (20). Falcinelli et al. (21) revealed that NaCl treatment could markedly increase the total polyphenols content (TPC) and antioxidant activity in wheatgrass. Jaiswal et al. (22) discovered that selenium and ultraviolet-B radiation or their combination could enhance the flavonoid and phenolic content. Benincasa et al. (23) demonstrated that optimal combination of temperature and time, light modulation, and salt stress would upregulate the phytonutrients such as ascorbic acid, tocopherol, β-carotene, phenols, and flavonoids in some extent. Hence, it was presumed that the plants as refer to wheatgrass would generally produce some phytochemicals, as secondary metabolites, whose role was to help plants cope with the unfavorable environmental conditions. However, all the existing data referring to the abiotic stress effect on phytochemicals of wheatgrass, to our best knowledge, were not integrated and focused mostly on a few kinds of compounds, such as flavonoids, phenols, tocopherol, or carotenes. The previous reported cereal grasses were normally germinated 6–10 days and no more than 14 days. The information that the long-term (exceeding 14 days) accumulation of phytonutrients, such as triterpene, proanthocyanin, soluble polysaccharides, ascorbic acid, and the like under different stress density still remains scarce.

Therefore, to reveal the effect of abiotic stress on the main functional phytochemicals and antioxidant activity, the wheat seedlings were suffered from the different densities of NaCl, PEG, and ultraviolet-C radiation and tested for the ascorbic acid content (AAC), TPC, total triterpenes content (TTC), total flavonoids content (TFC), total proanthocyanins content (TPAC), and total chlorophyll content (TChl) content and the antioxidant activity through 2,2'-azino-bis (3-ethylbenzthia- zoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays and ferric ion reducing antioxidant power (FRAP) of different seedling ages.

MATERIALS AND METHODS

Material and Reagents

The wheat seeds were kindly provided by a local farmer in Lingbi County, Suzhou City, Anhui Province, China. Hoagland Nutrition reagent was purchased from Qingdao Hope Bio-Technology Co., LTD (Qingdao, China). Polyethylene glycol 6000 was bought from Wuxi Yatai Allied Chemicals Co., Ltd. Glucose was from Shanghai Zhanyun Chemical Co., Ltd (Shanghai, China). Vanillin was from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ursolic acid, Folin–Ciocalteu's phenol reagent, gallic acid, rutin hydrate, (+)-catechin, L-ascorbic acid, ABTS, DPPH were purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). (±)-6-Hydroxy 2,5,7,8-tetramethylchromane-2-Carboxylic acid (Trolox) was from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). All the reagents were of analytical grade.

Plant Materials and Abiotic Stress Treatment

Abiotic Treatment

The cultivation of the wheatgrass was executed based on the procedures proposed by the team (24). Briefly, 55 g of the thrice-washed seeds were sown evenly in a hydroponic tray sized 32.5 × 24.5 × 4.5 cm in four rectangular pieces, then were semi-immersed in deionized water for 24 h away from light to let malt. The wheat malts were supplied with 1/2 Hoagland solution and cultivated at 22 ± 1°C under 16 h-photoperiod (20 W) light irradiance. The aged 6 days seedlings were exposed to UV-C irradiation daily (40 W, Ozone free, for 5, 10, 20, 40, and 60 min correspondingly noted as UV 5, UV 10, UV 20, UV 40, and UV 60), Polyethylene glycol 6000 (5, 10, 15, 20, and 25% in 1/2 Hoagland solution correspondingly noted as PEG 5%, PEG 10%, PEG 15%, PEG 20%, and PEG 25%), and salinity solution (10, 25, 50, 100, and 200 mM in 1/2 Hoagland solution correspondingly noted as NaCl 10, NaCl 25, NaCl 50, NaCl 100, and NaCl 200), respectively, while the control group (CK) was supplied only with the Hoagland solution. All the media solutions used during the cultivation were 450 ml per tray and refreshed every 2 days. The deionized water was replenished properly on the interval of the culture matrix refreshing to restore the initial weight and keep the matrix concentration constant. The grass was sampled on 9th, 12th, 15th, 19th, 21st, 24th, and 27th day after seeding (noted as D9, D12, D15, D21, D24, and D27, respectively) and immediately frozen in liquid nitrogen (LN_2) and stored at −80°C until use.

Preparation of Wheatgrass Extracts

Grass extracts to be analyzed for TTC, TPC, TFC, TPAC, and antioxidant capacity were prepared as followings: the wheatgrass was well ground in LN_2 and 1.0 g sample of each treated group

were ultrasonic extracted thrice (80 W, 20 min) with 25 ml of 80% methanol. The three filtrates were pooled and brought to 100 ml. The extracts for total soluble polysaccharides content (TSPC) analysis were prepared as: the above pellets were again aqueously extracted thrice in boiling water-bath for 30 min and standardized to 100 ml. The methanol extracts were stored at −20°C until use while the aqueous extracts were stored at 4°C and detected within 3 days. In the preparation, 112 samples in total were yielded and tested for the seven kinds of compounds and three antioxidant activity indices. All the tests were applied in a microplate reader (Thermo Fisher Scientific, Type 1510).

Determination of TTC

The TTC was measured using the methods described by Siyuan Luo et al. (25) with slight modifications. In this method, 100 μl extracts or ursolic acid standard solutions (20–120 μg ml^{-1}) were mixed with 100 μl vanillin-acetic acid (2.5%) and 200 μl perchloric acid. After 60°C incubation for 15 min, 650 μl glacial acetic acid was added and well-mixed. In addition, 300 μl of the mixture was pipetted to a 96-well-microplate (CELLSTAR® from Greiner Bio-One GmbH) and read at 550 nm using the microplate reader after standing for 10 min. The TTC was calculated from the standard curve and expressed as milligram ursolic acid equivalents/gram (mg UAE/g) of fresh weight (FW).

Determination of TPC

The determination of TPC was performed using the Folin–Ciocalteau method previously described by Sarker and Oba (26). The extracts (100 μl) or series of standards (12.5, 25, 50, 100, 150, and 200 μg ml^{-1} gallic acid) were added. After reagent mixing and reaction, 300 μl was moved to a 96-well-plate and read at 740 nm. The results were estimated as equivalent to gallic acid standard (mg GAE/g FW).

Determination of TFC

The TFC was determined according to an assay described by Guo et al. (27). Briefly, the addition volume of the extracts or standard rutin solution (20–100 μg ml^{-1}), $NaNO_2$ solution (5%, w/v), Al $(NO_3)_3$ solution (10%, w/v), and NaOH solution (1 mol/L) were adjusted to 2.0, 0.2, 0.2, and 2.0 ml, respectively. The mixture (300 μl) was pipetted to a 96-well-plate and read at 510 nm. The amount of TFC was expressed as rutin equivalents/gram (mg RE/g FW).

Determination of TPAC

The TPAC was detected by using a method previously reported by Zribi et al. (28) which was adjusted to be feasible in the microplate reader. The volume of extracts or standard catechin solution (4–32 μg ml^{-1}), 4% vanillin–methanol (w/v), and hydrochloric acid were adjusted to 200 μl, 1.0 ml, and 0.5 ml, respectively. The mixture (300 μl) was transferred to a 96-well-plate and read at 500 nm. TPAC was presented as milligram catechin equivalent/gram fresh weight (mg CE/g FW) using the catechin calibration curve.

Determination of TSPC

The TSPC was detected based on phenol-sulfuric acid assay described by Lei Guo et al. (29), of which the reagent volume was diminished aliquot for high throughput detection. Briefly, 0.2 ml of aqueous extracts or standard glucose solution (10–60 μg ml^{-1}) was mixed with 0.2 ml 5% distilled phenol solution and 1 ml concentrated sulfuric acid. After vortex and 30 min standing at room temperature, the mixture (300 μl) was transferred and read at 490 nm. The results were obtained using a calibration curve from standard glucose solutions and expressed as milligram glucose equivalent/gram in fresh weight (mg GE/g FW).

Determination of Chlorophyll Content

The chlorophyll (such as chlorophyll a noted as Chl *a*, chlorophyll b noted as Chl *b*, and total chlorophyll noted as TChl) was extracted based on the procedure reported by Mashabela et al. (30) with modifications and detected by using the methods previously reported by Warren (31). Specifically, the wheatgrass samples were well-ground in liquid nitrogen and 1.0 g was weighed and macerated in 25 ml methanol at room temperature for 48 h. Afterward, the resulting mixtures were passed through a 0.22-μm nylon filter and diluted 1-fold. The dilutions of 200 μl were removed to a flat-bottomed 96-well-plate and read at 652 and 665 nm. The chlorophyll concentration was calculated from the following formula:

$$\text{Chl } a\ (\mu g\ mL^{-1}) = -8.0962\ A_{652,1cm} + 16.5169\ A_{665,\ 1cm}$$

$$\text{Chl } b\ (\mu g\ mL^{-1}) = 27.4405\ A_{652,1cm} - 12.1688\ A_{665,\ 1cm}$$

$$\text{TChl} = \text{Chl } a + \text{Chl } b$$

$$A_{652,1cm} = (A_{652,microplate} - \text{blank})/0.51$$

$$A_{665,1cm} = (A_{665,microplate} - \text{blank})/0.51$$

Where, $A_{652,1cm}$ and $A_{652,microplate}$ represents the absorbance at 652 nm in a spectrophotometer and a microplate reader, respectively; $A_{665,1cm}$ and $A_{665,microplate}$ represents the absorbance at 665 nm in a spectrophotometer and a microplate reader, respectively.

The results were expressed as milligram/gram fresh weight (mg/g FW).

Determination of AAC

The AAC was determined by a second-order derivative spectrometer method (32, 33). The wheatgrass samples of 2.0 g were ground and macerated in 100 ml 1.0 M HCl which was gradually added for 10 min. The extracts were passed through a 0.45-μm aqueous syringe filter and quantitatively diluted 5-fold. The peak-baseline amplitudes of the filtrates in the second-order derivation absorption spectra at 267.5 nm (detected using Type U-3900 UV/VIS spectrometer from HITACHI High-Tech Science Corporation, Tokyo, Japan) was used to construct the calibration curve of standard ascorbic acid solutions with the concentration ranging from 5 to 30 μg ml^{-1}. The results were expressed as milligram ascorbic acid/g of FW.

Antioxidant Activity

ABTS Radical Scavenging Assay

The detection of ABTS bleaching ability was conducted using the method described by Lin et al. (34) with slight adjustments. An equal volume (25 ml) of 7.4 mM ABTS solution and 2.6 mM aqueous $K_2S_2O_8$ were mixed at room temperature for at least 16 h away from light to generate $ABTS^{\bullet+}$ stock solution. Afterward, the stock solution was diluted using 5 mM pH 7.4 PBS to the

absorbance of 0.75 at 734 nm in a 96-well-plate to prepare the working solution, of which 200 μl was mixed with 40 μl of the extracts or standard Trolox solution (0.02–0.16 mM). After incubation for 6 min in the dark and vibration for 15 s, the absorbance was read. The results were calculated from the Trolox calibration curve and expressed as milligram Trolox Equivalents/gram FW (mg TE/g FW).

DPPH Radical Scavenging Assay

The DPPH radical scavenging ability was evaluated according to a previously described method (34) with minor adjustment. The solution (200 μl) of 0.15 mM DPPH was blended with 100 μl of the extracts or standard Trolox solution (0.02–0.16 mM) in a 96-well-plate. After 37°C incubation for 30 min and 15 s vibration, the absorbance was read at 517 nm vs. 80% methanol blank. The results were expressed as milligram TE/gram FW.

FRAP Assay

The FRAP assay was conducted by using the method described by Lin et al. (34).

TOPSIS Model Establishments

The TOPSIS model proposed by Hwang and Yoon (35) was used to evaluate the comprehensive nutrition value of all the treated groups. The decision matrix was established as $X = (X_{ij})_{m\times n}$, where m means the different abiotic groups, such as five treatments of PEG, five treatments of NaCl, five treatments of ultraviolet, and a CK, while n represents seven criteria that include TTC, TPC, TFC, TPAC, TSPC, TChl, and AAC. The weight of individual criterion ω_i was 1.0, 1.0, 1.0, 0.5, 1, 1.5, and 1.0, respectively.

Statistics Handling

All the tests were performed in triplicates and the results were shown as mean ± SD. Duncan's new multiple range was used for the difference analysis tests. Correlation-ship was analyzed using Pearson's correlation. A multi-factor analysis of variance (MANOVA) was conducted to examine which factor (seedling age or various abiotic treatments) dominates the corresponding phytochemicals. The principal component analysis (PCA) was also carried out. All the statistics handling were carried out using SPSS software (IBM Corp. USA, version 20.0). Asterisks indicated significant differences ($^{**}p < 0.01$, $^{*}p < 0.05$). The heatmap hierarchical clustering analysis (HHCA) of the measured functional compounds was run using TBtools software.

RESULTS AND DISCUSSION

Triterpene

The TTC of wheatgrass ranged from 1.83 ± 1.40 to 12.00 ± 2.30 mg UAE/g FW, shown in **Figure 1A**. The results of MANOVA showed that the TTC was significantly affected by abiotic treatment and seedling age, while seedling age determined dominantly the TTC other than abiotic treatment [$p < 0.01$, $F_{(\text{seedlingage})} = 76.02 > F_{(\text{abiotictreatment})} = 5.24$]. Previous data indicated that UV-B treatment could contribute to an increment of TTC in the *Adhatoda vasica* plant (36) and that water deficit could stimulate the triterpene accumulation in *Hypnum plumaeforme* (but not in *Pogonatum cirratum*) (37) or that abiotic stress-induced the accumulation of some triterpenes in *Quillaja brasiliensis* (38). However, in the present study, based on MANOVA, we found that the NaCl 10, NaCl 25, and NaCl 50 groups had a lower TTC than the CK which showed no significant differences between the other groups like UV-, PEG-, or NaCl 100 and NaCl 200- treated group ($p < 0.05$). As a kind of UV-absorbing secondary metabolites, the accumulation content of triterpene was impacted by the impairment of metabolism and the upregulation of relative genes and enzymes (36, 38). The results suggested that UV exposure affected the triterpene minorly before D24 compared to control might be explained by the balance of the impairment and upregulation or by the different interactions of individual triterpene that existed in wheatgrass because there was a report that revealed some triterpene compounds were not affected by UV (36). On D24 and D27, that the TTC was upregulated non-/significantly in UV 10–60 groups (compared with control) might be due to the loss of water. The reason that the TTC was not incremented by PEG and salt stress could be as well-attributed to the different reactions of individual triterpene contained in wheatgrass. It might be inferred that the triterpene upregulation was not the pathway for wheat to cope with unfavorable conditions.

An obvious accumulation peak was noticed on D12 and D21 (based on MANOVA, $p < 0.05$), which shared a similar changing pattern with the previous study in UV-treated barley grass where the peak appeared on D15 and D21 (24). Specifically, except for UV40 and PEG 25% dose group, the TTC of other groups increased significantly on D12. The group of NaCl 10, NaCl 50, PEG 25%, UV5, UV10, and UV20 had a second peak TTC on D21. The TTC of wheatgrass could rival some fruit, such as Chinese jujube (TTC ranged from 7.52 ∼ 16.57 mg UAE/g FW) (39).

Polyphenol

The TPC of wheatgrass in the present study varied from 1.59 ± 0.03 to 2.89 ± 0.01 mg GAE/g FW (**Figure 1B**). The MANOVA results showed that both the abiotic treatments and seedling age could affect the TPC significantly while the former worked more effectively [$p < 0.01$, $F_{(\text{seedlingage})} = 6.36 > F_{(\text{abiotictreatment})} = 5.14$]. Generally, except for the treatments of 50 mM NaCl, 5% PEG, 10% PEG, and 5-min-UV, the other treatments could induce a higher TPC than the control. The accumulation patterns of the TPC of all the groups were presented in a wavy manner and peak values appeared on D15 and D24 based on MANOVA. The TPC of the results from D9 to D12 was consistent with a previous study that no significant differences during quinoa malting were found when the seeds were treated with different wavelength light or solutions (40). Specifically, on D15 all the groups except NaCl 50 had a higher TPC than the control. On D21 and D24 except for group UV5, and on D27 except for group PEG 5% and PEG 10%, all the other groups showed higher TPC than the control, suggesting that abiotic treatment could improve or reserved the TPC under prolonged treating time. Differentiated results were found on whether the TPC increased or declined by abiotic stress (16, 40–42). Hence, it was explained that TPC generally stayed stable from D9 to D12 when the wheatgrass was struggling

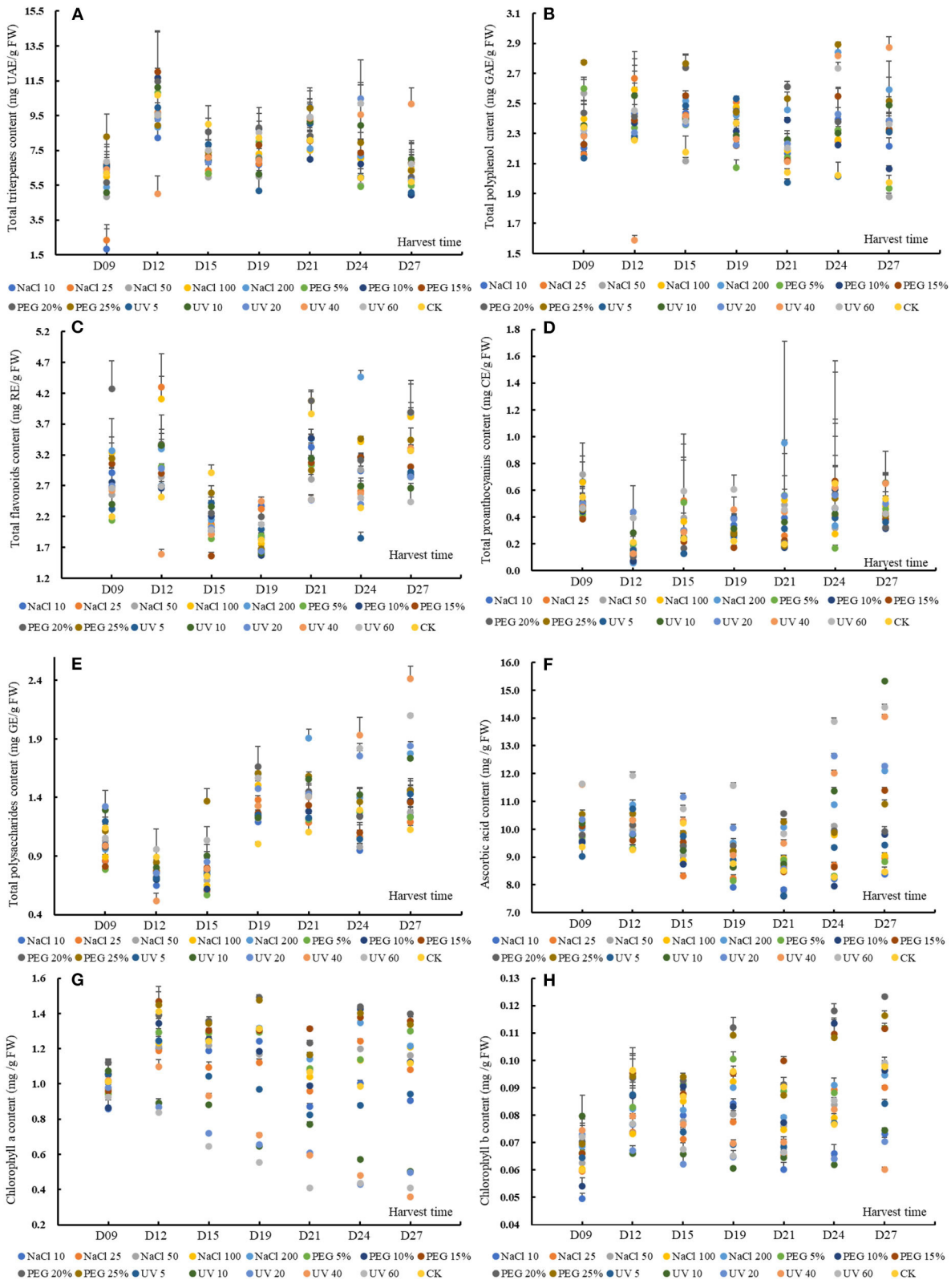

FIGURE 1 | Continued

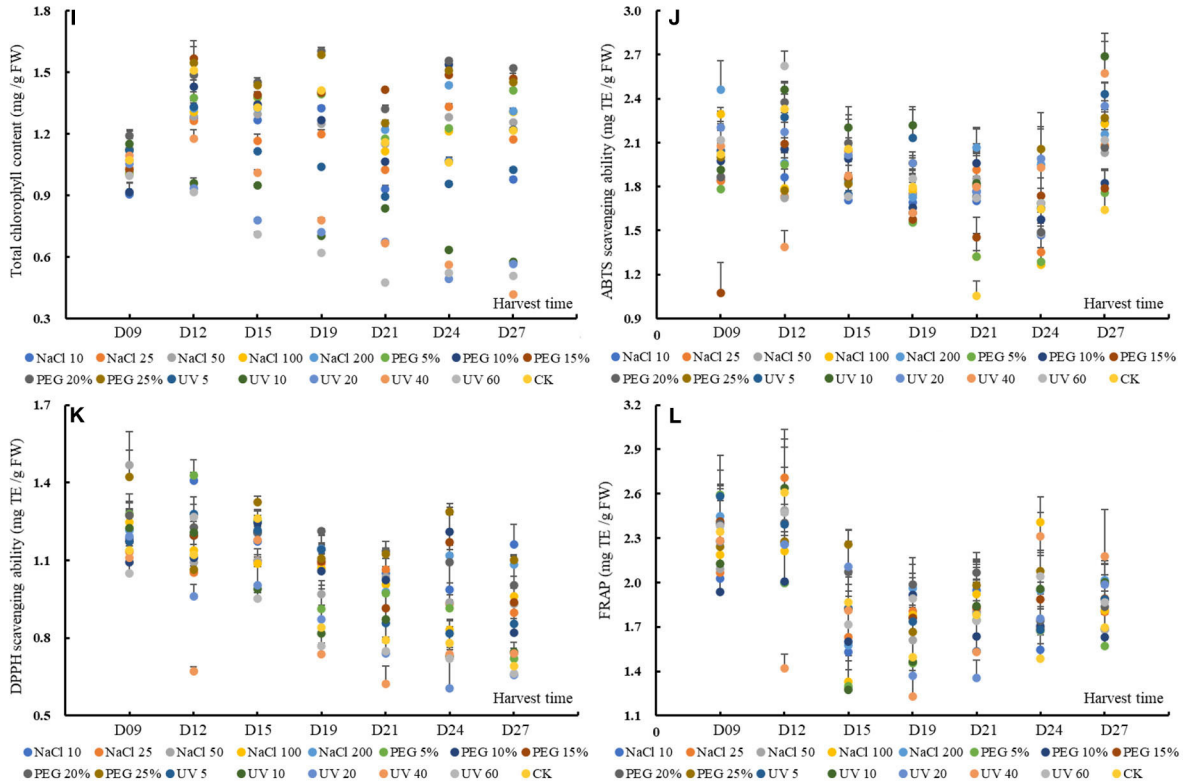

FIGURE 1 | The content or value of total triterpenes **(A)**, total polyphenols **(B)**, total flavonoids **(C)**, total proanthocyanins **(D)**, total soluble polysaccharides **(E)**, ascorbic acid **(F)**, chlorophyll a **(G)**, chlorophyll b **(H)** and total chlorophyll **(I)**, 2,2'-azino-bis (3-ethylbenzthia- zoline-6-sulfonic acid) (ABTS) **(J)**, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(K)** scavenging ability and ferric iron-reducing antioxidant power **(L)** of wheatgrass on the 9th∼27th day after seed sowing. A total of 112 samples (16 groups × 7 times of harvesting) were analyzed. The results were expressed as mean ± SD.

to adapt itself to the adverse conditions, then incremented to handle the situation. The different reactions of TPC between the previous reports and the results attributed to cultivar variations.

The TPC was much higher than some vegetables, such as sweet potatoes (43), or leafy vegetables such as amaranth (44, 45), and common fruits, e.g., apple, apricot, cherry, peach, plum (46), banana, mango, papaya, passion fruit, and so on (47).

Flavonoid

The TFC of wheatgrass in the present study ranged from 1.56 ± 0.05 to 4.47 ± 0.11 mg RE/g FW, shown in **Figure 1C**. In the present study, different intensity of UV stress or PEG-simulated drought was probed on the accumulations of flavonoid and found that the TFC were prone to be more susceptible to the seedling age rather than these abiotic treatments (including NaCl stress), on which the flavonoid value upregulated or not depended [$p < 0.01$, $F_{(\text{seedlingage})} = 71.06 > F_{(\text{abiotictreatment})} = 10.77$, data from MANOVA results]. The overall accumulation patterns of the total flavonoid varied significantly with the seedling age and three accumulation peaks appeared, respectively, on D12, D21, and D27. The MANOVA results showed that the 25 mM NaCl -, 100 mM NaCl -, 200 mM NaCl -, and 20% PEG treatment could improve the TFC. The previous studies reported that the flavonoid could be enhanced by drought treatment within 48 h in wheat leaves (48) or by violet treatment for 30 days in root culture of *Setiva rebaudiana* (49). It has been postulated that the TFC could be enhanced by abiotic stress, such as drought, salinity, and UV stress (41), however, in the results, the situation was complicated. Specifically, all the abiotic treated groups (except for PEG 5% on D9, UV40 on D12, and UV5 on D24) had non-/significantly higher TFC than the control on D9, D12, and D24. In contrast, all the treated groups (except for NaCl 100 on D21) showed a significantly lower TFC content than the control on D15 and D21. The accumulation patterns of TFC varied with the difference of treatments intensity, which could be explained by that the increase or decrease of some secondary metabolites in plants depends on the sensitivity of the plants to this type of stress condition (50). The reason for the fluctuation of TFC could be attributed to the genetic regulation mode of these compounds.

The TFC in the present study was much higher than some green vegetables, such as amaranth (41, 51), lettuce, salad spinach, mitsuba, pok choi, mizuna, komatsuna in Gifu (Japan) (52) and was in the same level of some Thai fruits, such as egg fruit, manila tamarind, otatheite apple, ivy gourd, and governor's plum (53) or tomato and lotus root (54, 55).

Proanthocyanin

The TPAC, ranging from 0.06 ± 0.02 to 0.95 ± 0.76 mg CE/g FW (**Figure 1D**), was not a remarkable functional phytochemical in wheatgrass but was still fell into the same level with rice of Sri Lanka (56), or some fruit, such as jujube (39), pomegranate (57), or even wild berries from Himalaya area (58). The overall TPAC changing patterns were found to decrease remarkably from D9 to D12 and then uprose gradually ($p < 0.05$) on basis of MANOVA. The previous studies revealed that condensed tannins (proanthocyanins) were ubiquitous in ligneous pants but almost absent in herbaceous species (59), which was in accordance with this study results. It was known from the MANOVA results that the seedling age significantly affected the TPAC ($p < 0.05$) while abiotic treatments did the little effect on the content. Saoussen et al. (60) found that salt stress could significantly increase the TPAC content of Tunisian safflower (a medicinal plant), however, in the present study results, no significant differences were noticed between the CKs and stress treatment groups ($p < 0.05$). To address specifically, UV20 and UV40 on D12, UV5, UV20, UV40, and UV60 on D19, and NaCl 100 on D21 had remarkably higher TPAC than the control. An interesting finding was that the higher TPAC compared to the control was mostly observed in UV treated groups, suggesting that certain intensity of UV stress could promote the proanthocyanins synthesis in some particular seedling age despite of the genetic restriction of this species.

Soluble Polysaccharide

The TSPC of wheatgrass in the present study ranged from 0.52 ± 0.07 to 2.41 ± 0.11 mg GE/g FW (**Figure 1E**). Some previous studies showed that the TSPC decreased significantly with the ripening or seedling age (24, 61). In this study, all the tested groups shared similar accumulation patterns of TSPC that decreased from D9 to D12 and then climbed up significantly ($p < 0.05$). Based on the MANOVA results, the TSPC was significantly affected by the seedling age and abiotic treatments, among which the seedling age influenced the content more efficiently [$p < 0.01$, $F_{(\text{seedlingage})} = 136.80 > F_{(\text{abiotictreatment})} = 13.84$]. Only 200 mM NaCl-, 20% PEG-, 25% PEG-, and UV-treatment (in exception of UV5) could enhance the TSP content ($p < 0.05$) and these results were subtly diverse with previous reports that salinity exposure enhanced the TSPC during germination and seedling period (62) or PEG limited low-molecular-mass TSPC (63). From D9 to D12, most of the abiotic groups (except UV20 and UV60) showed a lower TSPC than the control. When it was on D19, D21, and D27, all the treated groups showed a higher TSPC than the control. It could be explained that the decreased TSP from D9 to D12 was converted to monosaccharides and the latterly increased TSP was from the breakdown of wheat cell wall under the stress conditions (64), which was indirectly validated by previous reports that the activity of relative glycoside-hydrolyzing enzymes was increased under drought stress (64).

Besides, the TSPC in the current study was found to be much lower than some common fruits and vegetables (65) but still rival to medicinal-use fruit like jujube cv. *Dazao*, jujube cv. *Junzao*, and jujube cv. *Huizao* (66).

Ascorbic Acid

The AAC of the wheatgrass in the present study was ranging from 7.59 ± 0.10 to 15.32 ± 0.05 mg/g FW (**Figure 1F**). Based on MANOVA results, the AAC could be significantly affected by the seedling age and abiotic treatments [$F_{(\text{seedlingage})} = 25.39 > F_{(\text{abiotictreatment})} = 23.86$; $p < 0.01$]. The overall accumulation patterns of AAC gradually were observed to be decreased from D9 to D21, then increased from D21 to D27. Through MANOVA, 200 mM NaCl-, 20% PEG-, 25% PEG-, and UV-treatment (in exception of UV5) could significantly enhance the AAC ($p < 0.05$). An interesting founding was that the comparative higher AAC than the control was observed in intense abiotic stress groups, which was discording with previous studies that AAC declined under salt stress (30–40 days) in all the tested wheat genotypes and the decreasing magnitude augmented with salinity levels (67) and that drought decreased the AAC (68). There was a literature showing a similar result with ours that UV radiation treatment for 2 weeks elevated the AAC in Arabidopsis thaliana (69).

The different abiotic treatments showed different accumulation modes. More specifically, from D9 to D12, all the groups had a higher AAC than the control excluding UV5 on D9. In addition, on D24 and D27, most of the abiotic groups showed a higher AAC than the control except PEG 10% and NaCl 10 on D24 and NaCl 10/50 on D27. However, on D15, except groups of UV20–UV60, all other abiotic treatments induced a significantly lower AAC than the control. The literature with respect to the effects of abiotic stress on AAC mostly was of single-point sampling or no more than three times sampling throughout stressing period and concluded if the AAC was elevated or suppressed. However, the AAC response to stressors was regulated complicatedly by a series of successive biochemical reactions, activation or inhibition of relative enzymes, synthesis of other protective substances, and so on (70), hence, it was vitally important to take the growth time and stress intensity as well as cultivars into consideration.

The AAC in the results was much higher than most of the common fruits known as their high ascorbic acid value, such as strawberry, lemon, orange, kiwifruit, mandarin, mango (71), and leafy vegetables, such as amaranth (72, 73).

Chlorophyll

The Chl a content varied from 0.36 ± 0.00 to 1.49 ± 0.01 mg/g FW (**Figure 1G**), which was overwhelmingly higher than the Chl b content (0.049 ± 0.002 to 0.123 ± 0.001 mg/g FW, **Figure 1H**), which conforms with the results of leafy vegetable amaranth (74). The TChl content ranged from 0.42 ± 0.00 to 1.60 ± 0.02 mg/g FW (as shown in **Figure 1I**). The TChl content was more significantly affected by the different abiotic treatments [$F_{(\text{seedlingage})} = 21.56 < F_{(\text{abiotictreatment})} = 62.12$, $p < 0.01$] than the seedling age. PEG the 10 mM NaCl and UV treatments significantly decreased the chlorophyll content while 15∼25% treatments could markedly upregulate it compared to control ($p < 0.05$). PEG was usually used to simulate the drought stress. There are reports that the drought stress decreased the chlorophyll or have no significant effect on chlorophyll concentration and that the chlorophyll was increased in some

TABLE 1 | TOPSIS ranking results of wheatgrass phytochemicals of different seedling ages under different abiotic treatments.

Abiotic treatments	D09		D12		D15		D19		D21		D24		D27	
	R_j	Ranking	R_j	Ranking	R_j	Ranking	R_j	Ranking	R_j	Ranking	R_j	Ranking	R_j	Ranking
NaCl 10	**0.3412**	**108**	0.4327	74	0.3720	103	0.4596	56	0.4750	44	0.4235	83	0.3898	95
NaCl 25	**0.3229**	**109**	0.4963	34	0.3826	99	0.4542	62	0.4683	48	0.4711	45	0.4887	39
NaCl 50	0.4315	78	0.4570	58	0.3881	96	0.4183	87	0.5147	23	0.4362	72	0.4705	46
NaCl 100	0.4377	71	0.5033	30	0.4085	91	0.4975	32	0.5444	16	0.4892	37	0.5509	14
NaCl 200	0.4333	73	0.4616	54	0.4136	89	0.5084	27	**0.6674**	**1**	**0.6002**	**4**	**0.6169**	**2**
PEG 5%	0.3707	104	0.4889	38	0.4162	88	0.4780	43	0.5070	28	0.4200	86	0.4853	40
PEG 10%	0.3813	100	0.4903	35	0.4022	92	0.4570	59	0.4393	69	0.5502	15	0.4494	64
PEG 15%	0.3963	93	0.5257	20	0.4204	85	0.4630	51	0.5163	22	0.5549	11	0.5531	13
PEG 20%	0.4903	36	0.5198	21	0.4621	53	0.5791	6	0.5735	8	0.5532	12	0.5684	9
PEG 25%	0.5039	29	0.5111	25	0.5310	17	0.5297	18	0.5587	10	**0.6109**	**3**	0.5739	7
UV 5	0.4320	77	0.4670	49	0.3850	98	0.3874	97	0.4322	76	0.3425	107	0.4297	79
UV 10	0.4426	67	0.4644	50	0.3497	106	**0.3077**	**111**	0.4828	41	0.4413	68	0.4966	33
UV 20	0.4581	57	0.4249	82	**0.3207**	**110**	0.3765	102	0.4449	66	0.4983	31	0.4516	63
UV 40	0.4324	75	**0.3065**	**112**	0.3562	105	0.3922	94	0.4274	81	0.5288	19	**0.5834**	**5**
UV 60	0.4291	80	0.4485	65	0.3776	101	0.4546	60	0.4091	90	0.5122	24	0.4811	42
CK	0.4222	84	0.5103	26	0.4629	52	0.4544	61	0.4696	47	0.4388	70	0.4614	55

Rj represents the closeness coefficient. The higher the Rj value, the higher the comprehensive content of the phytochemicals. TOPSIS, technique for order preference by similarity to ideal solution. The bold values mean the top 5 highest or lowest comprehensive phytochemicals content.

TABLE 2 | Correlation coefficients of total polyphenols, total flavonoids, total proanthocyanins, total triterpenes, total soluble polysaccharides, ascorbic acid, total chlorophyll, ABTS and DPPH scavenging ability, and ferric iron-reducing antioxidant power of wheatgrass on the 9th∼19th harvest day[a,b].

D09	**TPC**	**TFC**	**TPAC**	**TTC**	**TSPC**	**AAC**	**TChl**	**ABTS**	**DPPH**	**FRAP**
TPC	1	0.014	0.101	0.361*	−0.053	0.118	0.302*	0.011	0.517**	0.038
TFC		1	0.099	−0.070	−0.140	0.084	−0.028	0.046	0.125	0.018
TPAC			1	−0.257	−0.012	−0.110	−0.109	0.106	0.190	−0.105
TTC				1	0.154	0.295*	0.306*	0.061	0.047	0.265
TSPC					1	0.027	0.300*	0.282	0.119	0.032
AAC						1	0.054	0.182	−0.194	0.034
TChl							1	0.100	0.311*	0.249
ABTS								1	−0.031	0.070
DPPH									1	0.022
FRAP										1
D12	**TPC**	**TFC**	**TPAC**	**TTC**	**TSPC**	**AAC**	**TChl**	**ABTS**	**DPPH**	**FRAP**
TPC	1	0.713**	0.081	0.438**	0.399**	−0.056	0.047	0.256	0.533**	0.526**
TFC		1	−0.156	0.267	0.163	−0.018	0.083	0.013	0.329*	0.416**
TPAC			1	0.070	0.357*	0.180	−0.570**	0.471**	−0.065	0.197
TTC				1	0.192	−0.213	0.247	0.469**	0.417**	0.329*
TSPC					1	0.243	−0.084	0.450**	0.255	0.552**
AAC						1	−0.340*	0.175	−0.119	0.109
TChl							1	−0.190	0.169	−0.010
ABTS								1	0.368*	0.294*
DPPH									1	0.263
FRAP										1
D15	**TPC**	**TFC**	**TPAC**	**TTC**	**TSPC**	**AAC**	**TChl**	**ABTS**	**DPPH**	**FRAP**
TPC	1	0.061	−0.131	0.216	0.389**	−0.043	0.278	−0.055	0.331*	0.445**
TFC		1	−0.169	0.474**	0.320*	0.149	0.082	0.189	0.267	0.301*
TPAC			1	−0.350*	−0.021	−0.015	−0.155	0.049	−0.199	−0.210
TTC				1	0.095	0.344*	0.009	0.029	0.263	0.388**
TSPC					1	0.444**	−0.183	−0.044	0.057	0.499**
AAC						1	−0.579**	0.002	−0.095	0.531**
TChl							1	−0.034	0.685**	−0.070
ABTS								1	0.009	0.063
DPPH									1	0.309*
FRAP										1
D19	**TPC**	**TFC**	**TPAC**	**TTC**	**TSPC**	**AAC**	**TChl**	**ABTS**	**DPPH**	**FRAP**
TPC	1	0.332*	−0.001	−0.142	0.226	−0.058	0.221	0.232	0.524**	0.419**
TFC		1	0.356*	−0.087	0.063	−0.106	−0.075	−0.239	0.032	0.038
TPAC			1	−0.148	0.189	0.528**	−0.665**	0.229	−0.415**	−0.034
TTC				1	0.119	0.200	0.196	−0.464**	−0.082	0.192
TSPC					1	0.479**	0.087	−0.043	0.230	0.321*
AAC						1	−0.445**	0.151	−0.290*	0.149
TChl							1	−0.406**	0.662**	0.360*
ABTS								1	−0.130	−0.099
DPPH									1	0.558**
FRAP										1

[a]*Correlations between the data obtained were run using a standard Pearson's correlation.* [b]***P < 0.01; *P < 0.05 (two–tailed).*

drought-tolerant wheat cultivars after 39 days of stress (75). The enhancement of TChl in young wheat leaves in the present study could be due to the activation of the enzyme in the light-dependent stage of biosynthesis (76). The TChl content was decreased non-/significantly under salinity stress from D9 to D21 but was increased on D24 compared to control. A previous study revealed that some wheat cultivars recorded a higher TChl at the tittering stage (77), which shared roughly consistent with the results to some extent. It could be inferred that the salinity stress could increment the TChl at some

TABLE 3 | Correlation coefficients of total polyphenols, total flavonoids, total proanthocyanins, total triterpenes, total soluble polysaccharides, ascorbic acid, total chlorophyll, abts and dpph scavenging ability, and ferric iron-reducing antioxidant power of wheatgrass on the 21st∼27th harvest day[a,b].

D21	**TPC**	**TFC**	**TPAC**	**TTC**	**TSPC**	**AAC**	**TChl**	**ABTS**	**DPPH**	**FRAP**
TPC	1	0.234	−0.016	−0.064	0.580**	0.617**	0.351*	0.443**	0.588**	0.331*
TFC		1	−0.132	−0.402**	−0.092	−0.014	0.596**	−0.242	0.538**	0.353*
TPAC			1	−0.111	0.365*	0.152	−0.111	0.146	−0.036	−0.077
TTC				1	−0.129	0.097	−0.390**	0.049	−0.169	−0.043
TSPC					1	0.599**	0.068	0.457**	0.099	0.113
AAC						1	0.120	0.123	0.104	0.266
TChl							1	−0.201	0.669**	0.408**
ABTS								1	0.179	−0.166
DPPH									1	0.517**
FRAP										1
D24	**TPC**	**TFC**	**TPAC**	**TTC**	**TSPC**	**AAC**	**TChl**	**ABTS**	**DPPH**	**FRAP**
TPC	1	0.522**	0.014	0.461**	0.517**	0.519**	−0.050	0.514**	0.248	0.479**
TFC		1	−0.035	−0.096	−0.096	−0.076	0.586**	0.121	0.611**	0.271
TPAC			1	0.042	0.034	0.012	−0.027	0.149	0.128	−0.178
TTC				1	0.734**	0.685**	−0.609**	0.384**	−0.366*	0.384**
TSPC					1	0.793**	−0.652**	0.469**	−0.458**	0.468**
AAC						1	−0.708**	0.417**	−0.518**	0.433**
TChl							1	−0.221	0.854**	−0.168
ABTS								1	0.084	0.088
DPPH									1	−0.076
FRAP										1
D27	**TPC**	**TFC**	**TPAC**	**TTC**	**TSPC**	**AAC**	**TChl**	**ABTS**	**DPPH**	**FRAP**
TPC	1	0.203	0.221	0.612**	0.741**	0.681**	−0.415**	0.589**	0.097	0.712**
TFC		1	−0.101	0.152	−0.177	−0.319*	0.498**	−0.079	0.492**	0.087
TPAC			1	0.415**	0.293*	0.303*	−0.403**	0.324*	−0.280	0.189
TTC				1	0.677**	0.549**	−0.378**	0.331*	−0.157	0.453**
TSPC					1	0.845**	−0.734**	0.542**	−0.345*	0.577**
AAC						1	−0.690**	0.516**	−0.367*	0.551**
TChl							1	−0.576**	0.555**	−0.343*
ABTS								1	0.044	0.498**
DPPH									1	−0.025
FRAP										1

[a]*Correlations between the data obtained were run using a standard Pearson's correlation.* [b]***P < 0.01; *P < 0.05 (two-tailed).*

particular stage and UV stress would promote the degradation of chlorophyll.

Antioxidant Capacity

The ABTS scavenging capacity of wheatgrass was from 1.05 ± 0.10 to 2.69 ± 0.16 mg TE/g FW, shown in **Figure 1J**, and was rival to some herbal plants (*Leguminosae*, *Bignoniaceae*, *Moraceae, Pluchea* family, and so on) from the Amazonian region or Indonesia (78, 79) or some common fruits and vegetables, such as lemon, onion, parsley, sweet potato, and vegetable amaranths (80–82). According to the MANOVA results, the overall changing mode of ABTS bleaching ability was found decreasing from D12 to D24 and then rising on D27. Both the seedling age and abiotic treatments could significantly affect the ABTS scavenging capacity which could attribute to the enhancement of the phytochemicals in the plant. In addition, the seedling age influenced the ABTS radical scavenging ability more efficiently than the abiotic treatments [$F_{(\text{seedlingage})} = 22.27 > F_{(\text{abiotictreatment})} = 5.63, p < 0.01$], coinciding with the variation of functional chemical content. The treated groups that could improve the ABTS scavenging abilities were groups of NaCl 200, UV5, UV10, and UV20, discording to the previous studies that 25∼100 mM NaCl reduced the ABTS scavenging ability in a short time (83). Specifically, on D21 and D27, all the groups showed a higher scavenging capacity (significantly or non-significantly) than the control.

The DPPH scavenging ability of our study ranged from 0.61 ± 0.12 to 1.47 ± 0.13 mg TE/g FW (**Figure 1K**), which was much higher than different vegetable amaranths (84, 85). The DPPH bleaching ability, on basis of MANOVA results, was significantly influenced by the seedling age and abiotic treatments, of which the former affected the DPPH radical scavenging ability more efficiently [$F_{(\text{seedlingage})} =$

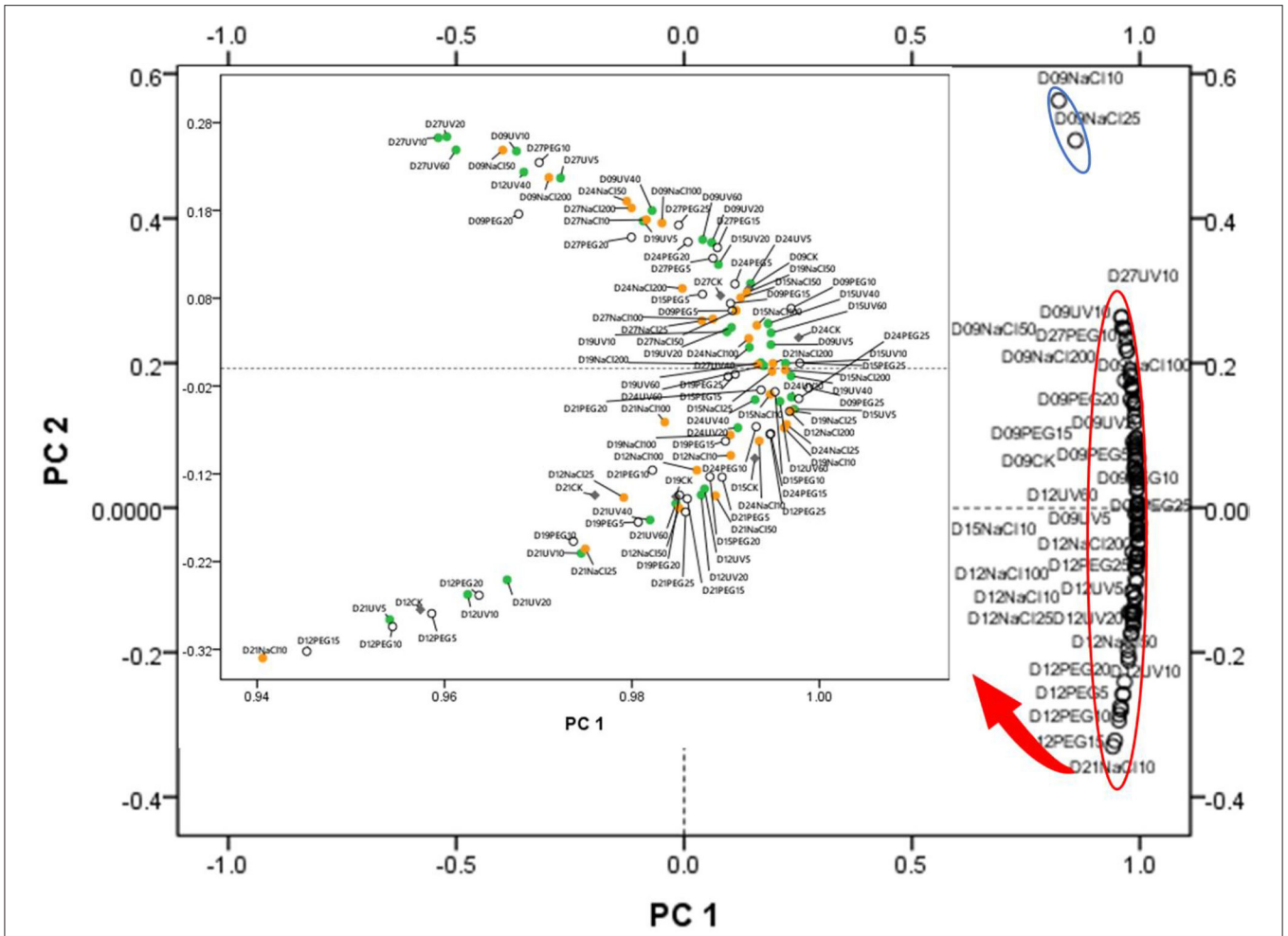

FIGURE 2 | Principal component analysis (PCA) of the abiotic stress (15 kinds of treatments and one control group) × seedling ages (from D9 to D27) interactions. PC 1 explained 96.527% of the variance which correlated with total polyphenol content (TPC), total flavonoid content (TFC), total triterpene content (TTC), ABTS, DPPH, and ferric ion reducing antioxidant power (FRAP), while PC 2 took up for 2.569% of the variance and correlated with total proanthocyanins content (TPAC), total Soluble polysaccharides content (TSPC), ascorbic acid content (AAC), and total chlorophyll content (TChl). The green dots represent the UV treatments, the orange dots represent the NaCl treatments, the blank dots mean the PEG treatments, and the gray ones mean the control group (CK).

59.87 > $F_{(abiotictreatment)}$ = 18.93, $p < 0.01$], coinciding with the variation of functional chemical content. Except for the UV10∼60 treatments, all other treatments could markedly improve the DPPH bleaching ability, discording to the previous studies that 25∼50 mM NaCl decreased the DPPH bleaching ability (83). The DPPH scavenging ability decreased significantly with the prolonging of the seedling age. Another notable finding was that NaCl and PEG treated groups rather than UV groups possessed significantly higher DPPH scavenging ability than the control, especially from D19 to D27 ($p < 0.05$).

The FRAP ranged from 1.23 ± 0.24 to 2.71 ± 0.26 mg TE/g FW (**Figure 1L**), which was comparable to common food like sweet potatoes (80) or papaya (86). Similarly, the FRAP was more significantly affected by the seedling age than abiotic treatments [$F_{(seedlingage)}$ = 39.48 > $F_{(abiotictreatment)}$ = 2.87, $p <$ 0.01]. Interestingly, though the abiotic treatments could affect the FRAP, no statistically differences were found between the abiotic treating groups and the control, which discord with former studies that abiotic stress could significantly augment the antioxidant activity, such as FRAP (87). The discordance could be explained by the varied ability of the phytochemicals to the reducing power.

TOPSIS Ranking Results

The closeness coefficient (R_j) was calculated and the ranking list was presented in **Table 1**. Through the comprehensive ranking of the functional phytochemicals in wheatgrass, the top five highest functional phytochemicals were observed under NaCl 200 on D21 and D27, PEG 25% on D24, NaCl 200 on D24, and UV40 on D27, respectively.

Correlation Analysis Results

Tables 2, **3** show the results of correlation analysis. On the 9th day, only TPC and TChl correlated positively with DPPH

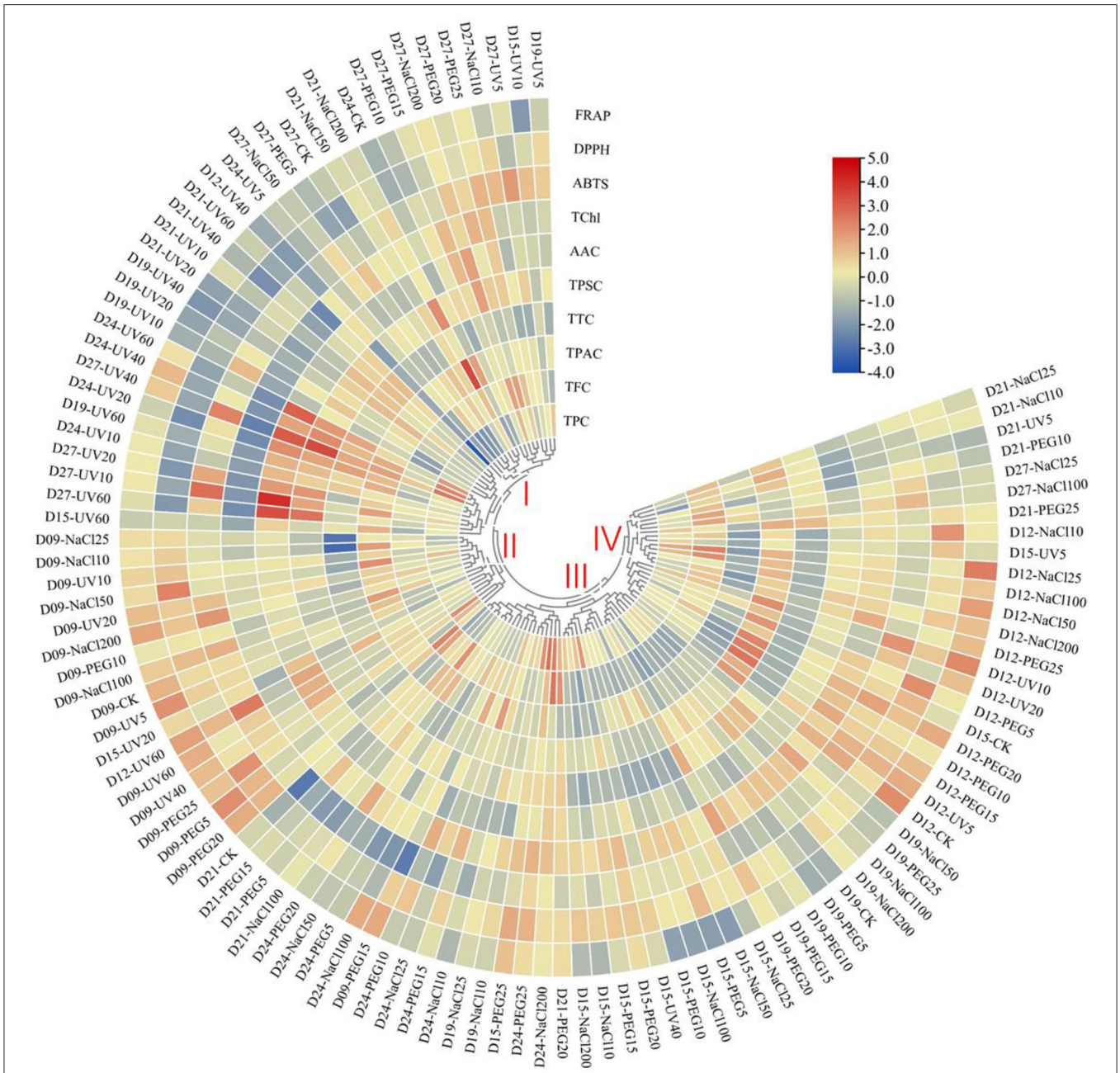

FIGURE 3 | Heatmap hierarchical clustering analysis (HHCA) of the various abiotic treatments on different seedling ages of wheatgrass. Each value of the seven main functional phytochemicals and three antioxidant activity indices of abiotic treatments are visualized in a column, and each of the treatments is represented by a row. The abundance of each phytochemical content is represented by a bar with unique color. Red means upregulation, and blue means downregulation. The heatmap is constructed by TBtools software. Distance methods are using Pearson's distance.

scavenging ability, meaning that at the early vegetative stage, the polyphenol and chlorophyll were the main antioxidants in wheatgrass ($p < 0.01$ and $p < 0.05$, respectively). On the 12th day, the TPC, TFC, and TTC were significantly or extremely significantly correlated with DPPH scavenging ability and FRAP, while TTC and TPAC correlated with ABTS bleaching capacity, suggesting that the content of these compounds started to increase and play a critical role in antioxidant activity. On the 15th day, the AAC, TTC, TFC, and TPC had a high correlation-ship with FRAP, meanwhile the TChl and TPC correlated with DPPH scavenging ability. On the 19th day, the TPC and TChl were highly related to DPPH scavenging ability and FRAP and were supposed to be the main antioxidant compounds as it was on the 21st day when TFC was included. The TTC had a negative correlation with ABTS bleaching ability ($p < 0.01$) on the 19th day.

On 24th and 27th days, it was an interesting finding that the TTC, TChl, and AAC were highly positively correlated with ABTS and FRAP scavenging ability ($p < 0.01$) but were negatively correlated with DPPH, the reason for this was unclear. TPC showed a high correlation with ABTS and FRAP, and the TFC was correlated with DPPH ($p < 0.01$).

PCA and HHCA Reveal Differences in the Main Functional Phytochemicals

Principal component analysis and HHCA were further performed to depict the differences in the seven main functional chemicals of different abiotic treatments and antioxidant ability. The PCA plot (**Figure 2**) exhibited the similarities and differences among the abiotic treated groups of different seedling ages. PC 1 explained 96.527% of the variance which correlated with TPC, TFC, TTC, ABTS, DPPH, and FRAP, while PC 2 took up for 2.569% of the variance and correlated with TPAC, TSPC, AAC, and TChl. The samples were not separated from different abiotic treatments into groups, suggesting that each abiotic treatment did not have a relatively distinct phytochemical.

In the present study, the HHCA demonstrated abiotic variations in terms of the relative content of phytochemicals and antioxidant capacity (**Figure 3**). Based on the distinct accumulation patterns, the abiotic treatments could be divided into four main clusters. The abiotic treatments in cluster I generally contributed to the decrease in the value of ABTS scavenging ability, DPPH scavenging ability, FRAP, TPC, and TChl, but increased the TSPC value. The ones in cluster II roughly contributed to the upregulation of the antioxidant capacity and TPAC. The ones in cluster III contributed to the slight upregulation of TChl and DPPH scavenging ability and slight downregulation of the value of other detected items. The abiotic treatments in cluster IV roughly contributed to the augment of antioxidant ability, TChl, TPC, and TTC value and the decrease of TPAC and TSPC.

CONCLUSION

The antioxidant activity and functional phytochemical content of wheatgrass could be significantly affected by both the stress treatments and seedling ages, while the latter affected phytochemicals except chlorophyll more efficiently. The different treatments did not always lead to an increase in the content of these functional compounds supposedly but resulted in the different accumulation patterns. Through the average Rj value (data not shown), the treatments with higher comprehensive phytochemical values were PEG 15∼25% and NaCl 100∼200. The artificial abiotic treatments would lead to some reduction in the yield though they could somehow elevate the content of the compounds, hence, taking into consideration the cost of the treatment application, it is better to harvest the wheatgrass at a particular seedling age to acquire better nutritional value.

The highest TPC, TFC, TPAC, TTC, TSPC, AAC, and TChl were 2.89 ± 0.01 mg GAE/g FW, 4.47 ± 0.11 mg RE/g FW, 0.95 ± 0.76 mg CE/g FW, 12 ± 2.3 mg UAE/g FW, 2.41 ± 0.11 mg GE/g FW, 15.32 ± 0.05 mg/g FW, 11.6 ± 0.02 mg/g FW, respectively, and were found in groups of PEG25% on D24, NaCl 200 on D24, NaCl 200 on D21, PEG 15% on D12, UV 40 on D27, UV10 on D27, and PEG20% on D19, which could serve as a theoretical basis to high yielding of the individual functional compounds. The time-dependent pathway on how the functional compounds were regulated between different stress-tolerant cultivars was complex and still not clear; hence, it needs further investigation.

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 field experiments in this research were carried out as per provisions of National Standards GB/T 27476.1-2014—Safety in testing laboratories (China) and National standards GB 19489-2008—Laboratories—General requirements for biosafety (China) issued by *General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China* and *Standardization Administration of China*.

AUTHOR CONTRIBUTIONS

BJ designed and funded the research. GG sponsored the publication fee and helped revise work of the manuscript. MR, YB, FG, WY, XX, MS, JW, and RC were responsible for the methodology, conducting, and data recording. LX helped in statistics handling. XZ and FF funded the research equally. QC drafted the manuscript and took the duty of data handling and revising the study. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Suriyavathana M, Roopavathi I. Phytochemical characterization of triticum aestivum (Wheat Grass). *J Pharmacogn Phytochem.* (2016) 5:283. Available online at: www.phytojournal.com
2. Durairaj V, Hoda M, Shakya G, Babu SPP, Rajagopalan R. Phytochemical screening and analysis of antioxidant properties of aqueous extract of wheatgrass. *Asian Pac J Trop Med.* (2014) 7:S398–404. doi: 10.1016/S1995-7645(14)60265-0
3. Murali M, Archa Raj M, Akhil S, Liji R, Kumar S, Nair A, et al. Preliminary phytochemical analysis of wheat grass leaf extracts. *Int J Pharm Sci Rev Res.* (2016) 40:307–12. Available online at: www.globalresearchonline.net
4. Kumar N, Iyer U. Impact of wheatgrass (*Triticum aestivum* L.) supplementation on atherogenic lipoproteins and menopausal symptoms in hyperlipidemic South Asian women - a randomized controlled study. *J Diet Suppl.* (2017) 14:503–13. doi: 10.1080/19390211.2016.1267063
5. Devi CB, Bains K, Kaur H. Effect of drying procedures on nutritional composition, bioactive compounds and antioxidant activity of wheatgrass (*Triticum aestivum* L). *J Food Sci Technol.* (2018) 56:491–6. doi: 10.1007/s13197-018-3473-7
6. Kulkarni SD, Tilak JC, Acharya R, Rajurkar NS, Devasagayam T, Reddy A. Evaluation of the antioxidant activity of wheatgrass (*Triticum aestivum* L.) as a function of growth under different conditions. *Phytother Res.* (2006) 20:218–27. doi: 10.1002/ptr.1838
7. Padalia S, Drabu S, Raheja I, Gupta A, Dhamija M. Multitude potential of wheatgrass juice (Green Blood): an overview. *Chronicles Young Sci.* (2010) 1:23–8. doi: 10.4103/2229-5186.79341
8. Rana S, Kamboj JK, Gandhi V. Living life the natural way–wheatgrass and health. *Funct Foods Health Dis.* (2011) 1:444–56. doi: 10.31989/ffhd.v1i11.112
9. Gore RD. Wheatgrass: green blood can help to fight cancer. *J Clin Diagn Res.* (2017) 11:ZC40–2. doi: 10.7860/JCDR/2017/26316.10057
10. Ghumman A, Singh N, Kaur A. Chemical, nutritional and phenolic composition of wheatgrass and pulse shoots. *Int J Food Sci Technol.* (2017) 52:2191–200. doi: 10.1111/ijfs.13498
11. Bar-Sela G, Cohen M, Ben-Arye E, Epelbaum R. The medical use of wheatgrass: review of the gap between basic and clinical applications. *Mini Rev Med Chem.* (2015) 15:1002–10. doi: 10.2174/1389557515121507311128 36
12. Thomas DT, Puthur JT. Amplification of abiotic stress tolerance potential in rice seedlings with a low dose of UV-B seed priming. *Funct Plant Biol.* (2019) 46:455. doi: 10.1071/FP18258
13. Złotek U, Szymanowska U, Jakubczyk A, Sikora M, Swieca M. Effect of arachidonic and jasmonic acid elicitation on the content of phenolic compounds and antioxidant and anti-inflammatory properties of wheatgrass (*Triticum aestivum* L.). *Food Chem.* (2019) 288:256–61. doi: 10.1016/j.foodchem.2019.02.124
14. Sarker U, Oba S. Drought stress enhances nutritional and bioactive compounds, phenolic acids and antioxidant capacity of Amaranthus leafy vegetable. *BMC Plant Biol.* (2018) 18:258. doi: 10.1186/s12870-018-1484-1
15. Sarker U, Oba S. Response of nutrients, minerals, antioxidant leaf pigments, vitamins, polyphenol, flavonoid and antioxidant activity in selected vegetable amaranth under four soil water content. *Food Chem.* (2018) 252:72–83. doi: 10.1016/j.foodchem.2018.01.097
16. Sarker U, Oba S. Salinity stress enhances color parameters, bioactive leaf pigments, vitamins, polyphenols, flavonoids and antioxidant activity in selected Amaranthus leafy vegetables. *J Sci Food Agric.* (2019) 99:2275–84. doi: 10.1002/jsfa.9423
17. Sarker U, Oba S. The response of salinity stress-induced A. *tricolor to* growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. *Front Plant Sci.* (2020) 11:559876. doi: 10.3389/fpls.2020.559876
18. Seminario A, Song L, Zulet A, Nguyen HT, Gonzalez EM, Larrainzar E. Drought stress causes a reduction in the biosynthesis of ascorbic acid in soybean plants. *Front Plant Sci.* (2017) 8:1042. doi: 10.3389/fpls.2017.01042
19. Takshak S, Agrawal SB. Defense potential of secondary metabolites in medicinal plants under UV-B stress. *J Photochem Photobiol B Biol.* (2019) 193:51–88. doi: 10.1016/j.jphotobiol.2019.02.002
20. Benincasa P, Tosti G, Farneselli M, Maranghi S, Bravi E, Marconi O, et al. Phenolic content and antioxidant activity of einkorn and emmer sprouts and wheatgrass obtained under different radiation wavelengths. *Ann Agric Sci.* (2020) 65:68–76. doi: 10.1016/j.aoas.2020.02.001
21. Falcinelli B, Benincasa P, Calzuola I, Gigliarelli L, Lutts S, Marsili V. Phenolic content and antioxidant activity in raw and denatured aqueous extracts from sprouts and wheatgrass of einkorn and emmer obtained under salinity. *Molecules.* (2017) 22:2132. doi: 10.3390/molecules22122132
22. Jaiswal SK, Prakash R, Skalny AV, Skalnaya MG, Grabeklis AR, Skalnaya AA, et al. Synergistic effect of selenium and UV-B radiation in enhancing antioxidant level of wheatgrass grown from selenium rich wheat. *J Food Biochem.* (2018) 42:e12577. doi: 10.1111/jfbc.12577
23. Benincasa P, Falcinelli B, Lutts S, Stagnari F, Galieni A. Sprouted grains: a comprehensive review. *Nutrients.* (2019) 11:421. doi: 10.3390/nu11020421
24. Jiang B, Geng F, Chang R, Ruan M, Bian Y, Xu L, et al. Comprehensive evaluation of the effect of ultraviolet stress on functional phytochemicals of hulless barley (Qingke) grass in different growth times at vegetative stage. *ACS Omega.* (2020) 5:31810–20. doi: 10.1021/acsomega.0c04576
25. Luo S, Zeng C, Li J, Feng S, Zhou L, Chen T, et al. Effects of ultrasonic-assisted extraction on the yield and the antioxidative potential of bergenia emeiensis triterpenes. *Molecules.* (2020) 25:4159. doi: 10.3390/molecules25184159
26. Sarker U, Oba S. Phenolic profiles and antioxidant activities in selected drought-tolerant leafy vegetable amaranth. *Sci Rep.* (2020) 10:18287. doi: 10.1038/s41598-020-71727-y
27. Guo L, Zhu WC, Liu YT, Wu Y, Zheng AQ, Liu YL, et al. Response surface optimized extraction of flavonoids from mimenghua and its antioxidant activities in vitro. *Food Sci Biotechnol.* (2013) 22:1–8. doi: 10.1007/s10068-013-0214-6
28. Zribi I, Omezzine F, Haouala R. Variation in phytochemical constituents and allelopathic potential of Nigella sativa with developmental stages. *South African J Botany.* (2014) 94:255–62. doi: 10.1016/j.sajb.2014.07.009
29. Guo L, Guo J, Zhu W, Jiang X. Optimized synchronous extraction process of tea polyphenols and polysaccharides from Huaguoshan Yunwu tea and their antioxidant activities. *Food Bioprod Process.* (2016) 100:303–10. doi: 10.1016/j.fbp.2016.08.001
30. Mashabela MN, Selahle KM, Soundy P, Crosby KM, Sivakumar D. Bioactive compounds and fruit quality of green sweet pepper grown under different colored shade netting during postharvest storage. *J Food Sci.* (2015) 80:H2612–8. doi: 10.1111/1750-3841.13103
31. Warren CR. Rapid measurement of chlorophylls with a microplate reader. *J Plant Nutr.* (2008) 31:1321–32. doi: 10.1080/01904160802135092
32. Pfendt LB, Vukašinović VL, Blagojević NZ, Radojević MP. Second order derivative spectrophotometric method for determination of vitamin C content in fruits, vegetables and fruit juices. *Europ Food Res Technol.* (2003) 217:269–72. doi: 10.1007/s00217-003-0746-8
33. Liu S, Li X, Guo Z, Zhang X, Chang X. Polyphenol content, physicochemical properties, enzymatic activity, anthocyanin profiles, and antioxidant capacity of cerasus humilis(Bge.) Sok. *Genotypes. J Food Qual.* (2018) 2018:1–13. doi: 10.1155/2018/5479565
34. Lin S, Guo H, Gong JDB, Lu M, Lu, M.-Y., et al. Phenolic profiles, β-glucan contents, and antioxidant capacities of colored Qingke (Tibetan hulless barley) cultivars. *J Cereal Sci.* (2018) 81:69–75. doi: 10.1016/j.jcs.2018.04.001
35. Hwang CL, Yoon K. *Multiple Attribute Decision Making. Methods and Applications*. Berlin Heidelberg: Springer-Verlag (1981).
36. Pandey A, Jaiswal D, Agrawal SB. Ultraviolet-B mediated biochemical and metabolic responses of a medicinal plant Adhatoda vasica Nees. at different growth stages. *J Photochem Photobiol B.* (2021) 216:112142. doi: 10.1016/j.jphotobiol.2021.112142
37. Liu BY, Lei CY, Jin JH, Li S, Zhang YS, Liu WQ. Physiological responses of two moss species to the combined stress of water deficit and elevated nitrogen deposition. *I. Secondary metabolism. Int J Plant Sci.* (2015) 176:446–57. doi: 10.1086/681023
38. de Costa F, Yendo ACA, Fleck JD, Gosmann G, Fett-Neto AG. Accumulation of a bioactive triterpene saponin fraction of Quillaja brasiliensis leaves is associated with abiotic and biotic stresses. *Plant Physiol Biochem.* (2013) 66:56–62. doi: 10.1016/j.plaphy.2013.02.003
39. Kou X, Chen Q, Li X, Li M, Kan C, Chen B, et al. Quantitative assessment of bioactive compounds and the antioxidant activity of 15 jujube cultivars. *Food Chem.* (2015) 173:1037–44. doi: 10.1016/j.foodchem.2014.10.110

40. Fischer S, Wilckens R, Jara J, Aranda M, Valdivia W, Bustamante L, et al. Protein and antioxidant composition of quinoa (Chenopodium quinoa Willd.) sprout from seeds submitted to water stress, salinity and light conditions. *Ind Crops Prod.* (2017) 107:558–64. doi: 10.1016/j.indcrop.2017.04.035
41. Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng B. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. *Molecules.* (2019) 24:2452. doi: 10.3390/molecules24132452
42. Kowalczewski PL, Radzikowska D, Ivanisova E, Szwengiel A, Kacaniova M, Sawinska Z. Influence of abiotic stress factors on the antioxidant properties and polyphenols profile composition of green barley (Hordeum vulgare L.). *Int J Mol Sci.* (2020) 21:397. doi: 10.3390/ijms21020397
43. Alam MK, Rana ZH, Islam SN. Comparison of the proximate composition, total carotenoids and total polyphenol content of nine orange-fleshed sweet potato varieties grown in Bangladesh. *Foods.* (2016) 5:64. doi: 10.3390/foods5030064
44. Sarker U, Hossain MN, Iqbal MA, Oba S. Bioactive components and radical scavenging activity in selected advance lines of salt-tolerant vegetable amaranth. *Front Nutr.* (2020) 7:587257. doi: 10.3389/fnut.2020.587257
45. Sarker U, Oba S. Nutraceuticals, phytochemicals, and radical quenching ability of selected drought-tolerant advance lines of vegetable amaranth. *BMC Plant Biol.* (2020) 20:564. doi: 10.1186/s12870-020-02780-y
46. Denev P, Lojek A, Ciz M, Kratchanova M. Antioxidant activity and polyphenol content of Bulgarian fruits. *Bulg J Agric Sci.* (2013) 19:22–27. Available online at: www.researchgate.net/publication/258447428
47. Murillo E, Britton GB, Durant AA. Antioxidant activity and polyphenol content in cultivated and wild edible fruits grown in Panama. *J Pharm Bioallied Sci.* (2012) 4:313–7. doi: 10.4103/0975-7406.103261
48. Ma D, Sun D, Wang C, Li Y, Guo T. Expression of flavonoid biosynthesis genes and accumulation of flavonoid in wheat leaves in response to drought stress. *Plant Physiol Biochem.* (2014) 80:60–6. doi: 10.1016/j.plaphy.2014.03.024
49. Idrees M, Sania B, Hafsa B, Kumari S, Khan H, Fazal H, et al. Spectral lights trigger biomass accumulation and production of antioxidant secondary metabolites in adventitious root cultures of Stevia rebaudiana (Bert.). *C R Biol.* (2018) 341:334–42. doi: 10.1016/j.crvi.2018.05.003
50. Zlotek U, Szymanowska U, Baraniak B, Karas M. Antioxidant activity of polyphenols of adzuki bean (Vigna angularis) germinated in abiotic stress conditions. *Acta Sci Pol Technol Aliment.* (2015) 14:55–63. doi: 10.17306/J.AFS.2015.1.6
51. Sarker U, Oba S. Polyphenol and flavonoid profiles and radical scavenging activity in leafy vegetable Amaranthus gangeticus. *BMC Plant Biol.* (2020) 20:499. doi: 10.1186/s12870-020-02700-0
52. Khanam UKS, Oba S, Yanase E, Murakami Y. Phenolic acids, flavonoids and total antioxidant capacity of selected leafy vegetables. *J Funct Foods.* (2012) 4:979–987. doi: 10.1016/j.jff.2012.07.006
53. Kubola J, Siriamornpun S, Meeso N. Phytochemicals, vitamin C and sugar content of Thai wild fruits. *Food Chem.* (2011) 126:972–81. doi: 10.1016/j.foodchem.2010.11.104
54. Yi Y, Sun J, Xie J, Min T, Wang LM, Wang HX. Phenolic profiles and antioxidant activity of lotus root varieties. *Molecules.* (2016) 21:863. doi: 10.3390/molecules21070863
55. Mahieddine B, Amina B, Faouzi SM, Sana B, Wided D. Effects of microwave heating on the antioxidant activities of tomato (Solanum lycopersicum). *Ann Agric Sci.* (2018) 63:135–9. doi: 10.1016/j.aoas.2018.09.001
56. Priyanthi C, Sivakanesan R. The total antioxidant capacity and the total phenolic content of rice using water as a solvent. *Int J Food Sci.* (2021) 2021:5268584. doi: 10.1155/2021/5268584
57. Yan L, Zhou X, Shi L, Shalimu D, Ma C, Liu Y. Phenolic profiles and antioxidant activities of six Chinese pomegranate (*Punica granatum* L.) cultivars. *Int J Food Propert.* (2017) 20:S94–107. doi: 10.1080/10942912.2017.1289960
58. Bahukhandi A, Barola A, Sekar KC. Antioxidant activity and polyphenolics of fragaria nubicola: a wild edible fruit species of Himalaya. *Proc Natl Acad Sci India Sect B Biol Sci.* (2019) 90:761–7. doi: 10.1007/s40011-019-01142-5
59. Furlan CM, Motta LB, Santos D, Petridis G. Tannins: what do they represent in plant life. In: Petridis GK, editor. *Tannins: Types, Foods Containing and Nutrition*. New York, NY: Nova Science Publishers, Inc (2011). p. 251–63.
60. Abdallah SB, Rabhi M, Harbaoui F, Zar-kalai F, Lachâal M, Karray-Bouraoui N. Distribution of phenolic compounds and antioxidant activity between young and old leaves of *Carthamus tinctorius* L. and their induction by salt stress. *Acta Physiol Plantarum.* (2012) 35:1161–9. doi: 10.1007/s11738-012-1155-z
61. Ninio R, Lewinsohn E, Mizrahi Y, Sitrit Y. Changes in sugars, acids, and volatiles during ripening of koubo [*Cereus peruvianus* (L.) Miller] fruits. *J Agric Food Chem.* (2003) 51:797–801. doi: 10.1021/jf020840s
62. Ahmad F, Singh A, Kamal A. Osmoprotective role of sugar in mitigating abiotic stress in plants. In: Roychoudhury A, Tripathi DK, editors. *Protective Chemical Agents in the Amelioration of Plant Abiotic Stress: Biochemical and Molecular Perspectives.* Chichester: John Wiley & Sons Ltd (2020). p. 53–70. doi: 10.1002/9781119552154.ch3
63. Le Gall H, Philippe F, Domon JM, Gillet F, Pelloux J, Rayon C. Cell wall metabolism in response to abiotic stress. *Plants.* (2015) 4:112–66. doi: 10.3390/plants4010112
64. Konno H, Yamasaki Y, Sugimoto M, Takeda K. Differential changes in cell wall matrix polysaccharides and glycoside-hydrolyzing enzymes in developing wheat seedlings differing in drought tolerance. *J Plant Physiol.* (2008) 165:745–54. doi: 10.1016/j.jplph.2007.07.007
65. Englyst H, Bingham S, Runswick S, Collinson E, Cummings J. Dietary fibre (non-starch polysaccharides) in fruit, vegetables and nuts. *J Human Nutr Dietetics.* (1988) 1:247–86. doi: 10.1111/j.1365-277X.1988.tb00197.x
66. Chen K, Fan D, Fu B, Zhou J, Li H. Comparison of physical and chemical composition of three chinese jujube (Ziziphus jujuba Mill.) cultivars cultivated in four districts of Xinjiang region in China. *Food Sci Technol.* (2019) 39:912–21. doi: 10.1590/fst.11118
67. Sairam R, Srivastava G, Agarwal S, Meena R. Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. *Biol Plant.* (2005) 49:85. doi: 10.1007/s10535-005-5091-2
68. Bartoli CG, Simontacchi M, Tambussi E, Beltrano J, Montaldi E, Puntarulo S. Drought and watering-dependent oxidative stress: effect on antioxidant content in *Triticum aestivum* L. leaves. *J Exp Botany.* (1999) 50:375–83. doi: 10.1093/jxb/50.332.375
69. Bartoli CG, Yu J, Gomez F, Fernandez L, McIntosh L, Foyer CH. Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in Arabidopsis thaliana leaves. *J Exp Bot.* (2006) 57:1621–31. doi: 10.1093/jxb/erl005
70. Badridze G. Influence of ultraviolet irradiation and acid precipitations on the content of antioxidants in wheat leaves. *Appl Ecol Environ Res.* (2015) 13:993–1013. doi: 10.15666/aeer/1304_9931013
71. Szeto YT, Tomlinson B, Benzie IF. Total antioxidant and ascorbic acid content of fresh fruits and vegetables: implications for dietary planning and food preservation. *Br J Nutr.* (2002) 87:55–9. doi: 10.1079/BJN2001483
72. Sarker U, Hossain MM, Oba S. Nutritional and antioxidant components and antioxidant capacity in green morph Amaranthus leafy vegetable. *Sci Rep.* (2020) 10:1336. doi: 10.1038/s41598-020-57687-3
73. Sarker U, Oba S, Daramy MA. Nutrients, minerals, antioxidant pigments and phytochemicals, and antioxidant capacity of the leaves of stem amaranth. *Sci Rep.* (2020) 10:3892. doi: 10.1038/s41598-020-60252-7
74. Sarker U, Islam MT, Rabbani MG, Oba S. Variability in total antioxidant capacity, antioxidant leaf pigments and foliage yield of vegetable amaranth. *J Integr Agric.* (2018) 17:1145–53. doi: 10.1016/S2095-3119(17)61778-7
75. Keyvan S. The effects of drought stress on yield, relative water content, proline, soluble carbohydrates and chlorophyll of bread wheat cultivars. *J Anim Plant Sci.* (2010) 8:1051–60. Available online at: m.elewa.org/JAPS
76. Nikolaeva M, Maevskaya S, Shugaev A, Bukhov N. Effect of drought on chlorophyll content and antioxidant enzyme activities in leaves of three wheat cultivars varying in productivity. *Russian J Plant Physiol.* (2010) 57:87–95. doi: 10.1134/S1021443710010127
77. Ghogdi EA, Izadi-Darbandi A, Borzouei A. Effects of salinity on some physiological traits in wheat (*Triticum aestivum* L.) cultivars. *Indian J Sci Technol.* (2012) 5:1901–6. doi: 10.17485/ijst/2012/v5i1.23
78. Silva EM, Souza JNS, Rogez H, Rees JF, Larondelle Y. Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. *Food Chem.* (2007) 101:1012–8. doi: 10.1016/j.foodchem.2006.02.055

79. Andarwulan N, Batari R, Sandrasari DA, Bolling B, Wijaya H. Flavonoid content and antioxidant activity of vegetables from Indonesia. *Food Chem.* (2010) 121:1231–5. doi: 10.1016/j.foodchem.2010.01.033
80. Rautenbach F, Faber M, Laurie S, Laurie R. Antioxidant capacity and antioxidant content in roots of 4 sweetpotato varieties. *J Food Sci.* (2010) 75:C400–5. doi: 10.1111/j.1750-3841.2010.01631.x
81. Romaric GB. Phenolic compounds and antioxidant activities in some fruits and vegetables from Burkina Faso. *Afr J Biotechnol.* (2011) 10:7934–41. doi: 10.5897/AJB10.2010
82. Sarker U, Oba S. Antioxidant constituents of three selected red and green color Amaranthus leafy vegetable. *Sci Rep.* (2019) 9:18233. doi: 10.1038/s41598-019-52033-8
83. Islam MZ, Park B-J, Lee Y.-T. Effect of salinity stress on bioactive compounds and antioxidant activity of wheat microgreen extract under organic cultivation conditions. *Int J Biol Macromol.* (2019) 140:631–6. doi: 10.1016/j.ijbiomac.2019.08.090
84. Sarker U, Oba S. Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected Amaranthus tricolor under salinity stress. *Sci Rep.* (2018) 8:12349. doi: 10.1038/s41598-018-30897-6
85. Sarker U, Oba S. Leaf pigmentation, its profiles and radical scavenging activity in selected Amaranthus tricolor leafy vegetables. *Sci Rep.* (2020) 10:18617. doi: 10.1038/s41598-020-66376-0
86. Addai ZR, Abdullah A, Mutalib SA. Effect of extraction solvents on the phenolic content and antioxidant properties of two papaya cultivars. *J Med Plants Res.* (2013) 7:3354–9. doi: 10.5897/JMPR2013.5116
87. Rosales MA, Cervilla LM, Sanchez-Rodriguez E, Rubio-Wilhelmi Mdel M, Blasco B, Rios JJ, et al. The effect of environmental conditions on nutritiona quality of cherry tomato fruits: evaluation of two experimental Mediterranean greenhouses. *J Sci Food Agric.* (2011) 91:152–62. doi: 10.1002/jsfa.4166

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Modified Atmosphere Packaging Maintains the Sensory and Nutritional Qualities of Post-harvest Baby Mustard During Low-Temperature Storage

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Baby mustard is a popular, yet highly perishable, *Brassica* vegetable. There is a need to develop effective methods for maintaining post-harvest qualities of baby mustard. Here, the lateral buds of baby mustard were packed in transparent polyethylene bags with no holes (M0), 6 mm in diameter holes (M1), or 12 mm in diameter holes (M2) and stored at 4°C. The effect of different modified atmosphere packaging (MAP) treatments on the sensory quality, health-promoting compounds, and antioxidant capacity was investigated by comparison with non-wrapped baby mustard. M1 and M2 delayed sensory quality deterioration and slowed declines in the content of ascorbic acid, total phenolics, and glucosinolates and antioxidant capacity during storage. M1 was most effective in prolonging the shelf life (three additional days compared with control lateral buds) and maintaining the content of glucosinolates. However, M0 accelerated the decline in the odor score, acceptability score, and ascorbic acid content and shortened the shelf life of baby mustard by more than 5 d compared with the control. These findings indicate that the effect of MAP treatment depends on the size of the holes in the bag. Based on these results, M1 was an alternative method for prolonging the shelf life and maintaining post-harvest qualities of baby mustard stored at 4°C.

Keywords: baby mustard, modified atmosphere packaging, sensory quality, antioxidant, glucosinolate, low-temperature storage

INTRODUCTION

Baby mustard (*Brassica juncea* var. *gemmifera*) is a variant of stem mustard that has become increasingly popular among consumers for its aesthetically pleasing appearance and high levels of health-promoting compounds such as glucosinolates, ascorbic acid, and phenolics (1–3). However, the lateral buds of baby mustard are perishable and susceptible to browning, dehydration, and the loss of health-promoting compounds during storage at ambient temperatures; indeed, maintaining post-harvest quality is one of the major challenges of the post-harvest processing of baby mustard (2, 3). There is thus a need to develop safe and effective methods for prolonging the shelf life of baby mustard and maintaining its sensory and nutritional qualities.

Our previous studies have shown that low temperature (4°C) storage can effectively maintain the sensory and nutritional quality of baby mustard (2). In addition, low temperature storage combined with modified atmosphere packaging (MAP) can delay senescence and reduce losses in quality during the storage of several vegetables, such as broccoli (4), lettuce (5), and fresh-cut watercress (6). MAP application can create an atmosphere of low O_2 and high CO_2, which can reduce the respiration rate of vegetables and delay senescence (7). MAP treatment can also maintain a high relative humidity (RH), which reduces water loss and helps maintain the visual quality of vegetables (4). In addition, MAP is simple and economical method, and can prevent cross-infection (5). These properties suggest that MAP could have commercial-scale applications. However, it has been reported that excessive accumulation of CO_2 in MAP can damage the cell membrane and cause physiological injuries in mushroom (8). There is thus a need to determine the MAP treatment conditions suitable for specific produce during storage.

To the best of our knowledge, the effects of MAP on post-harvest quality of baby mustard have not been investigated. The aim of the current study was to evaluate the possibilities of MAP to maintain the sensory and nutritional qualities of post-harvest baby mustard and identify the optimal MAP treatment for baby mustard during post-harvest storage at 4°C. We hope that this work can provide an alternative application for the preservation of post-harvest baby mustard from the perspective of consumers and producers.

MATERIALS AND METHODS

Plant Materials

Baby mustard (*Brassica juncea* var. *gemmifera* cv. Linjiang-Ercai), harvested early in the morning, was obtained from a local farm in Chengdu City, China, and transported to the laboratory within 2 h under ambient temperature. Fresh baby mustard with uniform size and absence of external damage was selected for experiments. Healthy lateral buds, the main edible parts of baby mustard, were removed and washed in an NaOCl solution (50 mg L^{-1}) for 3 min, rinsed with tap water for 1 min, and then dried on blotting paper.

MAP Treatment

The lateral buds were randomly assigned to four groups and stored in incubators at 4°C with a RH of 75% under continuous darkness. Approximately 300 g of baby mustard lateral buds was placed in three types of transparent polyethylene bags (18 cm × 25 cm, 80 μm thickness): (1) without holes (M0), (2) with 6 mm in diameter holes (eight holes, four holes on each side of the bag) (M1), and (3) with 12 mm in diameter holes (eight holes, four holes on each side of the bag) (M2). The O_2 and CO_2 transmission rates of polyethylene bags are 7.0×10^{-7} and 2.4×10^{-6} L m^{-2} s^{-1} at 25°C and standard pressure, respectively. As a control, lateral buds were stored without wrapping in transparent polypropylene containers without lids. Samples were taken after 0, 3, 6, 9, and 12 d. A bag of baby mustard lateral buds was collected as a repeat, and four repeats were used per sampling period. Several fresh samples were used for analyses of shelf life, sensory quality, and weight loss, and other samples were lyophilized in a freeze dryer and stored at −20°C for subsequent analyses of phytochemicals and antioxidant capacity.

Quality Assessment

Shelf Life and Sensory Quality Evaluation

Shelf life and sensory quality of the baby mustard lateral buds were assessed daily and on sampling day, respectively. They were evaluated by a six-member panel, who are engaged in fresh produce research for at least 2 years. The samples were coded with random numbers to mask the treatment identity to minimize subjectivity and to ensure test accuracy. The lateral buds were considered to have reached the end of their shelf life when they became soft, shrank, and exhibited browning (3). Sensory attributes were quantified on a scale from 5 to 1 as follows. Color was rated using 5 = bright green without defects, 3 = lighter green with a few browning spots, and 1 = yellowish lateral buds with severe browning. Odor was rated using 5 = no off-odors, 3 = slight but obvious off-odor, and 1 = strong off-odor. Texture was rated using 5 = very tight and firm, 3 = slightly soften but acceptable, and 1 = very soften. Acceptance was rated using 5 = excellent and having a freshly harvested appearance, 3 = average, and 1 = unmarketable.

Weight Loss

Weight loss was analyzed as previously reported (3). Weight loss (%) was calculated by the formula $(W_x - W_0)/W_0 \times 100$, where W_0 is the weight at 0 d, and W_x is the weight at a certain day after storage.

Sucrose, Fructose, and Glucose Content

Sucrose, fructose, and glucose were extracted and analyzed as previously described (9). Freeze-dried samples (100 mg) were added to 5 mL of distilled water and homogenized for 1 min. The mixture was then extracted in a water bath at 80°C for 30 min. The supernatant was collected after centrifugation at 8,000 *g* at room temperature for 5 min, and filtered through 0.45 μm cellulose acetate filter, and then analyzed by high performance liquid chromatography (HPLC) using an Agilent 1260 instrument equipped with a refractive index detector (Agilent Technologies, Inc., Palo Alto, USA). Samples were separated at 35°C on an Agilent ZORBAX carbohydrate column (250 × 4.6 mm i.d.; 5 μm particle size) using 80% acetonitrile at a flow rate of 1.0 mL min^{-1}. Content of sucrose, fructose, and glucose were determined using the standard curves for each sugar (Sangon Biotech Co., Ltd., shanghai, China). Results of sucrose, fructose, and glucose content were expressed as mg g^{-1} of dry weight.

Ascorbic Acid Content

Ascorbic acid content was determined according to the previous report (1). Fifty milligram of sample powder was extracted with 5 mL 1.0% oxalic acid, subsequently centrifuged 5 min at 4,000 *g*. Each sample was filtered through a 0.45 μm cellulose acetate filter. HPLC analysis of ascorbic acid was carried out using an Agilent 1260 instrument with a variable wavelength detector (VWD) detector (Agilent Technologies, Inc., Palo Alto, USA).

Sample were separated on a Waters Spherisorb C18 column (150 × 4.6 mm i.d.; 5 μm particle size), using a solvent of 0.1% oxalic acid at a flow rate of 1.0 mL min^{-1}. The amount of ascorbic acid was calculated from absorbance values at 243 nm, using authentic ascorbic acid (Sangon Biotech Co., Ltd., shanghai, China) as a standard. Result of ascorbic acid content was expressed as mg g^{-1} of dry weight.

Total Phenolics Content

Total phenolics were extracted with 10 mL of 50% ethanol and incubated at room temperature for 24 h in the dark. The suspension was centrifuged at 4,000 *g* for 5 min at room temperature. The supernatant was used for the measurements of total phenolics content and antioxidant activity. The supernatant was mixed with Folin-Ciocalteu reagent (Sangon Biotech Co., Ltd., shanghai, China), after 3 min, saturated sodium carbonate was added. The absorbance was measured at 760 nm with the spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China) as previously described (1). Gallic acid (Sangon Biotech Co., Ltd., shanghai, China) was used as a standard and the results were expressed as mg gallic acid equivalent g^{-1} dry weight.

Ferric Reducing Antioxidant Power (FRAP)

FRAP assay was performed according to the previous report (10). The extracted samples were added to the FRAP working solution incubated at 37°C and vortexed. The absorbance was then recorded at 593 nm using a spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China) after the mixture had been incubated in at 37°C for 10 min. FRAP values were calculated based on $FeSO_4{\cdot}7H_2O$ standard curves and expressed as μmol g^{-1} dry weight.

2,2-Azinobis (3-Ethyl-Benzothiazoline-6-Sulfonic Acid) (ABTS) Assay

ABTS antioxidant activity was performed according to the previous report (1). An aliquot of 300 μL of each extracted sample was added to 3 mL of $ABTS^+$ solution. The absorbance was measured spectrophotometrically (Mapada Instruments Co., Ltd., Shanghai, China) at 734 nm after exactly 2 h, and then the value was calculated.

Glucosinolate Composition and Content

Glucosinolates were extracted and analyzed as previously described (1). Freeze-dried samples (100 mg) were boiled in 5 mL water for 10 min. The supernatant was collected after centrifugation, and the residues were washed once with water, centrifuged, and then combined with the previous extract. The aqueous extract was applied to a DEAE-Sephadex A-25 column (Sigma Chemical Co., Saint Louis, USA). The glucosinolates were converted into their desulpho analogs by overnight treatment with 100 μL of 0.1% aryl sulphatase (Sigma Chemical Co., Saint Louis, USA), and the desulphoglucosinolates were eluted with 1 mL water. HPLC analysis of desulphoglucosinolates was carried out using an Agilent 1260 HPLC instrument equipped with a VWD detector (Agilent Technologies, Inc., Palo Alto, USA). Samples were separated at 30°C on a Waters Spherisorb C18 column (250 × 4.6 mm i.d.; 5 μm particle size) using acetonitrile and water at a flow rate of 1.0 mL min^{-1}. Absorbance was detected at 226 nm. Glucosinolates were quantified by using *ortho*-Nitrophenyl β-D-galactopyranoside (Sigma Chemical Co., Saint Louis, USA) as the internal standard and considering the response factor of each glucosinolate. Result of glucosinolate content was expressed as μmol g^{-1} of dry weight.

Statistical Analysis

To measure shelf life and visual quality, six replicates were prepared for each treatment. Other assays were performed in quadruplicate. Statistical analysis was performed using the SPSS package program version 18 (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way analysis of variance. Principal component analysis (PCA) was performed in SIMCA-P 11.5 Demo software (Umetrics, Sweden) with unit variance (UV)-scaling to decipher the relationships among samples (1). A time-related trajectory analysis based on two-dimensional PCA map was applied to visualize the temporal alterations of post-harvest quality changes under different MAP treatments (3).

RESULTS

Sensory Analysis

Baby mustard gradually deteriorated as lateral buds shriveled and browned on the peel during storage, and MAP treatments significantly delayed deterioration (**Figure 1**, **Supplementary Figure 1**). M1 and M2 significantly ($P < 0.05$) prolonged the shelf life of baby mustard after harvest (**Figure 2A**). The longest shelf life, which was observed in M1, was three additional days compared with the control. However, M0 shortened the shelf life of baby mustard after harvest by more than 5 d compared with the control.

Weight loss is one of the key sensory characteristics for evaluating fresh vegetable quality. Weight loss increased during storage time. Weight loss in the control was the most dramatic, which decreased by 28.3% at 6 d. MAP treatments significantly ($P < 0.05$) suppressed weight loss. The weight loss in all MAP treatments was <10% at 12 d (**Figure 2B**).

Throughout the entire storage period, the sensory parameter scores of both MAP-treated and control baby mustard decreased gradually. Compared with the control, higher color and texture scores of lateral buds were observed under MAP treatments, and no significant differences were observed between the different MAP treatments (**Figures 2C,E**). The odor score of M0 decreased rapidly during storage, and the odor score of the control, M1, and M2 slightly decreased during storage (**Figure 2D**). Compared with the control, M0 accelerated the decrease in acceptance scores; however, M1 and M2 delayed the decline in acceptance scores (**Figure 2F**).

Soluble Sugars

Sucrose, fructose, and glucose were identified in baby mustard, and glucose was the most abundant (**Figure 3**). The content of sucrose slightly decreased early in the storage period and increased later in the storage period in the control, M1, and M2. However, the increase in sucrose in M1 was significantly ($P < 0.05$) slower than the increase in the control in the late storage

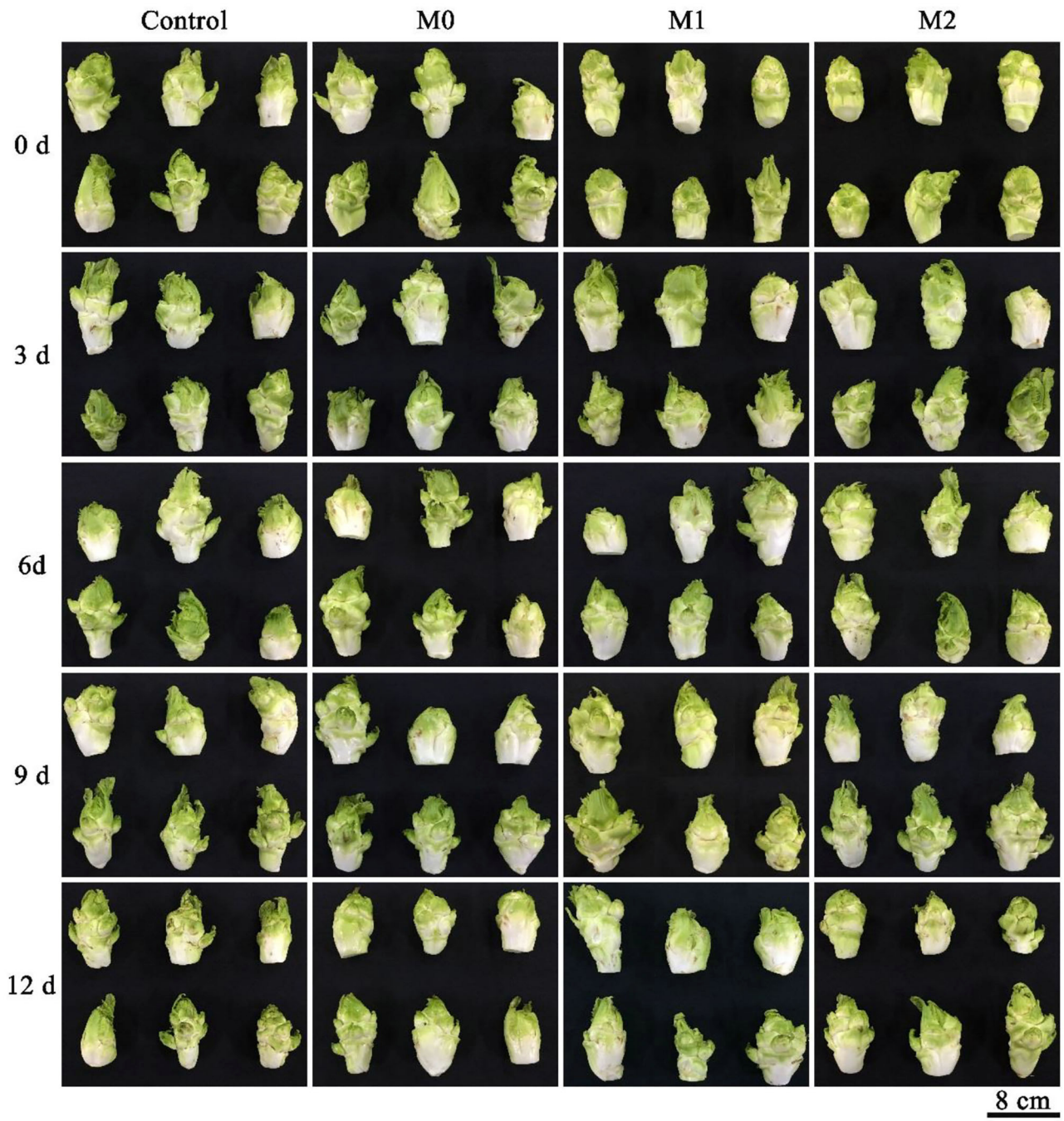

FIGURE 1 | Appearance of different baby mustard lateral buds during storage at 4°C under different MAP treatments. M0 indicates packaging with no holes; M1 indicates packaging with 6 mm in diameter holes; and M2 indicates packaging with 12 mm in diameter holes. Scale bar = 8 cm.

period, and the content of sucrose was 19.9% lower in M1 than in the control at the end of storage. The content of sucrose gradually decreased in M0 and was significantly ($P < 0.05$) lower in M0 than in the other treatments throughout storage (**Figure 3A**). The fructose content decreased during storage in the MAP treatments and the control, and the change in the fructose content was the smallest in M1 (**Figure 3B**). The glucose content first decreased and then increased during storage, and the change in M1 was the smallest (**Figure 3C**).

Ascorbic Acid and Total Phenolics

The ascorbic acid content in the control and M0 gradually increased during the first 6 d of storage but decreased sharply after 9 and 6 d, respectively; at the end of storage, the ascorbic

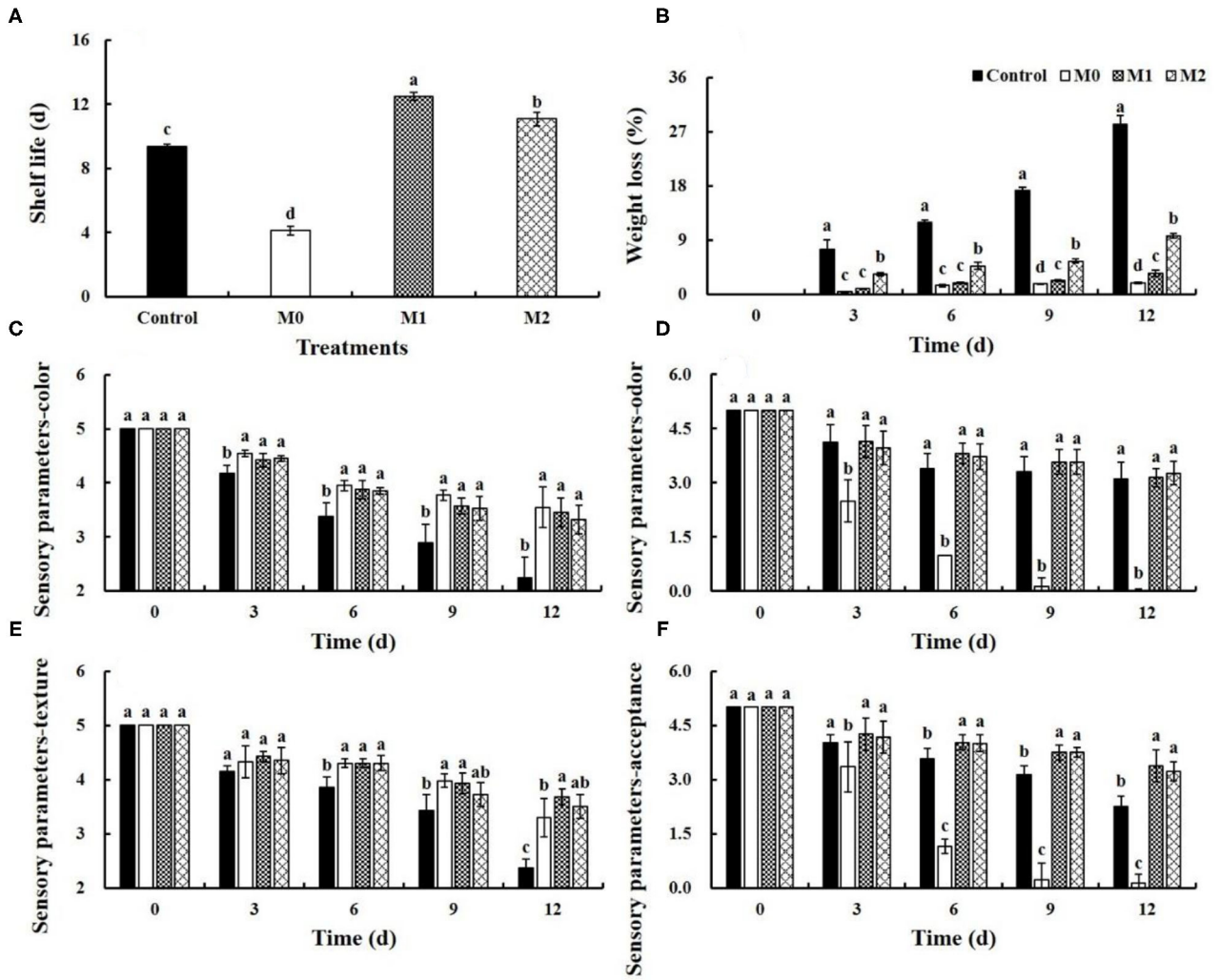

FIGURE 2 | Shelf life **(A)**, weight loss **(B)**, and sensory parameters **(C–F)** of different baby mustard lateral buds during storage at 4°C under different MAP treatments. Sensory parameters include color, odor, texture, and acceptance of lateral buds. Different letters in **(A)** indicate statistically significant differences among treatments ($P < 0.05$), and different letters in **(B–F)** indicate statistically significant differences among treatments for each storage day ($P < 0.05$). M0 indicates packaging with no holes; M1 indicates packaging with 6 mm in diameter holes; M2 indicates packaging with 12 mm in diameter holes.

acid content in the control and M0 decreased by 68.6 and 81.8%, respectively. The ascorbic acid content in M1 and M2 treatments was significantly ($P < 0.05$) higher than that in control and M0 treatment at the end of storage. The ascorbic acid content in M1 and M2 increased by 24.1 and 31.7%, respectively, over the entire storage period (**Figure 4A**). The total phenolics content in the control increased early during storage and decreased rapidly after 9 d of storage by 39.5%. The total phenolics content increased in M0, M1, and M2 during storage by 14.0, 23.1, and 25.2%, respectively, over the entire storage period. However, there is no significant ($P < 0.05$) difference between MAP treatments (**Figure 4B**). Overall, MAP treatments, especially M1 and M2, promoted the accumulation of ascorbic acid and phenolics in baby mustard.

Antioxidant Capacity

The FRAP level in the control increased in the early stage of storage and decreased rapidly after 9 d of storage by 56.2%; however, in M0, M1, and M2, the FRAP level increased by 1.9, 7.9, and 14.8%, respectively, over the entire storage period (**Figure 4C**). The ABTS levels in the control and MAP treatments were relatively stable in the first 9 d of storage. After 9 d of storage, the ABTS levels in the control decreased significantly (down by 47.7%), and that in M1 and M2 decreased slightly, and there were no significant differences between MAP treatments (**Figure 4D**). In short, MAP treatment was beneficial for maintaining the antioxidant capacity, and antioxidant capacity decreased in the control after 9 d of storage.

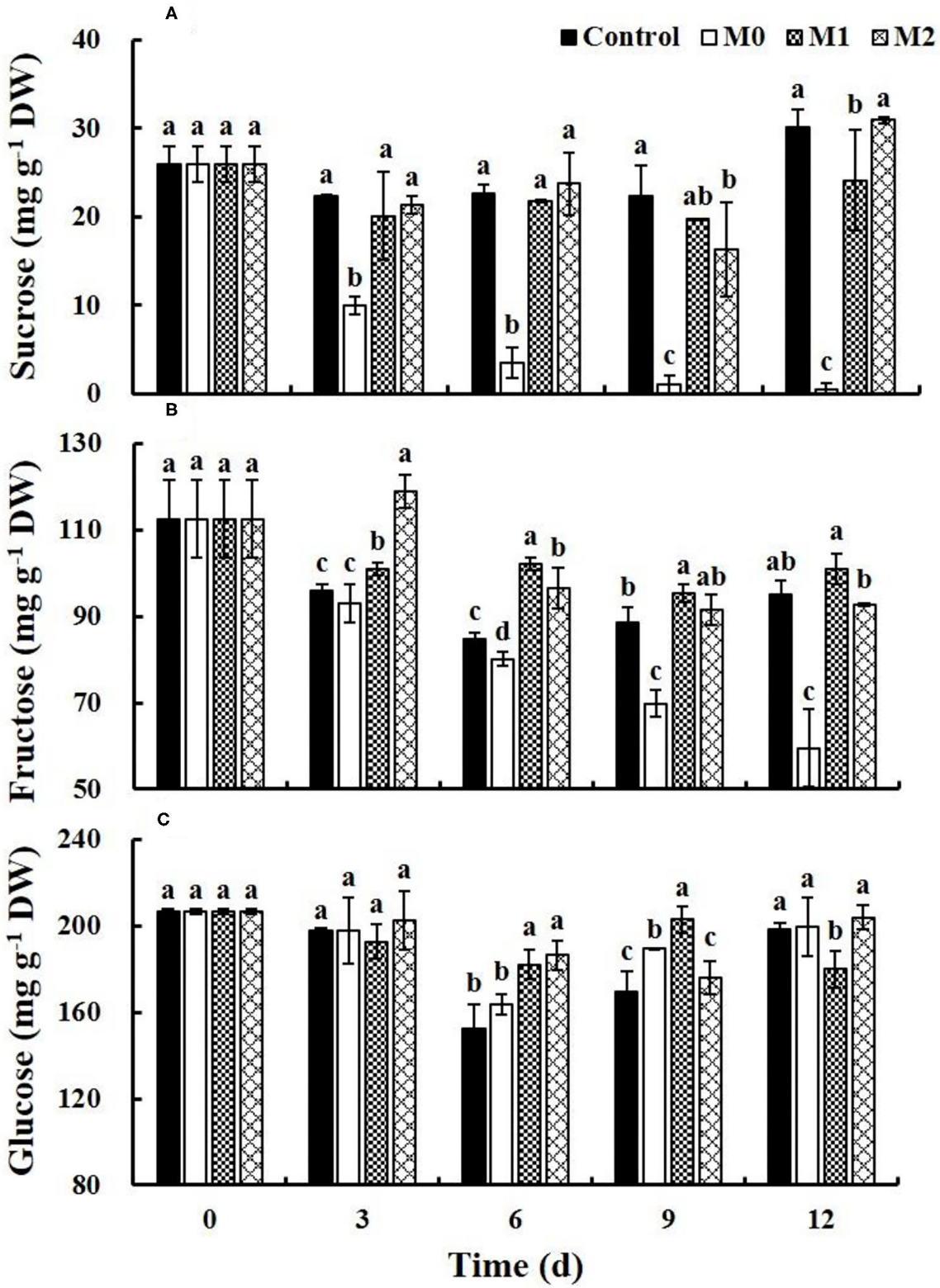

FIGURE 3 | Content of sucrose **(A)**, fructose **(B)**, and glucose **(C)** of different baby mustard lateral buds during storage at 4°C under different MAP treatments. Different letters in the figure indicate statistically significant differences among treatments for each storage day ($P < 0.05$). M0 indicates packaging with no holes; M1 indicates packaging with 6 mm in diameter holes; M2 indicates packaging with 12 mm in diameter holes.

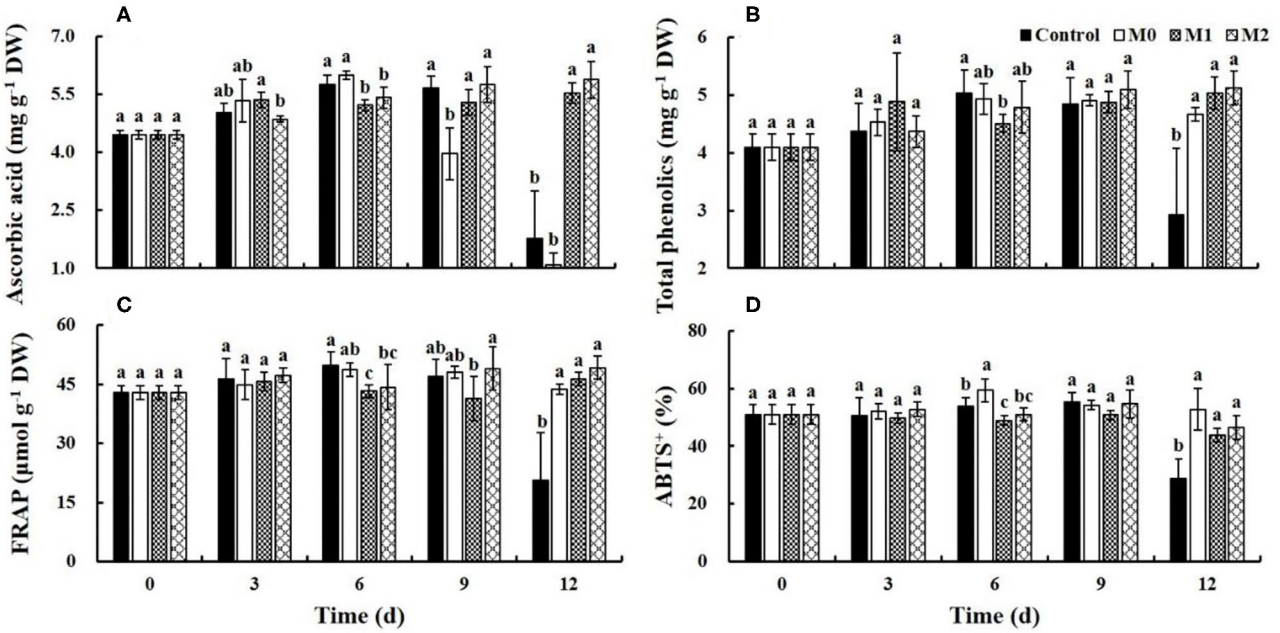

FIGURE 4 | The main antioxidants content and antioxidant capacity level of different baby mustard lateral buds during storage at 4°C under different MAP treatments. **(A)** Ascorbic acid content; **(B)** total phenolics content; (C) FRAP level; **(D)** $ABTS^+$ level. Different letters in the figure indicate statistically significant differences among treatments for each storage day ($P < 0.05$). M0 indicates packaging with no holes; M1 indicates packaging with 6 mm in diameter holes; M2 indicates packaging with 12 mm in diameter holes.

Glucosinolates

Three aliphatic and four indole glucosinolates were identified by HPLC in the lateral buds of baby mustard (**Figure 5**). The content of aliphatic glucosinolates in the control slightly decreased during the first 3 d of storage and remained basically unchanged thereafter, with the exception of gluconapin. The content of aliphatic glucosinolates in M1 increased after 3 d of storage and was significantly higher in M1 than in the control at the end of the storage period; the content of sinigrin, gluconapin, progoitrin, and total aliphatic glucosinolates was 1.3-, 2.2-, 1.5-, and 1.3-fold higher in M1 than in the control, respectively (**Figures 5A,C,E,G**). The indole glucosinolate content in the control increased during the first 6 d of storage and then decreased, with the exception of 4-hydroxyglucobrassicin. The indole glucosinolate content in M1 also increased during the first 6 d of storage but remained relatively unchanged thereafter. At the end of storage, the content of glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin, and total indole glucosinolates was 2.1-, 1.9-, 2.7-, 2.8-, and 1.4-fold higher in M1 than in the control, respectively (**Figures 5B,D,F,H,J**). Because of the large proportion of sinigrin, the change in the content of total glucosinolates was similar to the change in sinigrin during storage (**Figure 5I**). Furthermore, the most of glucosinolates content in M0 and M2 treatments was also significantly ($P < 0.05$) higher than that in the control, but significantly ($P < 0.05$) lower than that in M1 treatment. These results indicated that MAP treatment could effectively preserve the content of glucosinolates, and M1 was the most effective.

Time-Related Trajectory Analysis

The points representing different storage times and treatments were notably separated, and longer distances between points correspond to greater deterioration in quality over a certain storage period. The quality of baby mustard in the control decreased rapidly and continuously during the entire storage period; that in M0 and M1 decreased mainly in the first 6 d of storage; and that in M2 decreased mainly in the first 9 d of storage. In general, the total length of the fold line was shortest in M1, which indicated that the deterioration in the quality of baby mustard in M1 was the lowest (**Figure 6**).

DISCUSSION

Sensory Quality

In this study, M1 and M2 extended the shelf life and delayed weight loss and declines in sensory parameter scores in baby mustard during post-harvest storage compared with the control (**Figures 1**, **2**). The greater visual quality under M1 and M2 likely results from the delay in the senescence of fresh produce associated with decreased respiration rates (5, 11). A study of lettuce indicated that fresh lettuce consumes O_2 and produces CO_2 when packed, which reduces its respiratory rate, extends its shelf life, and maintains

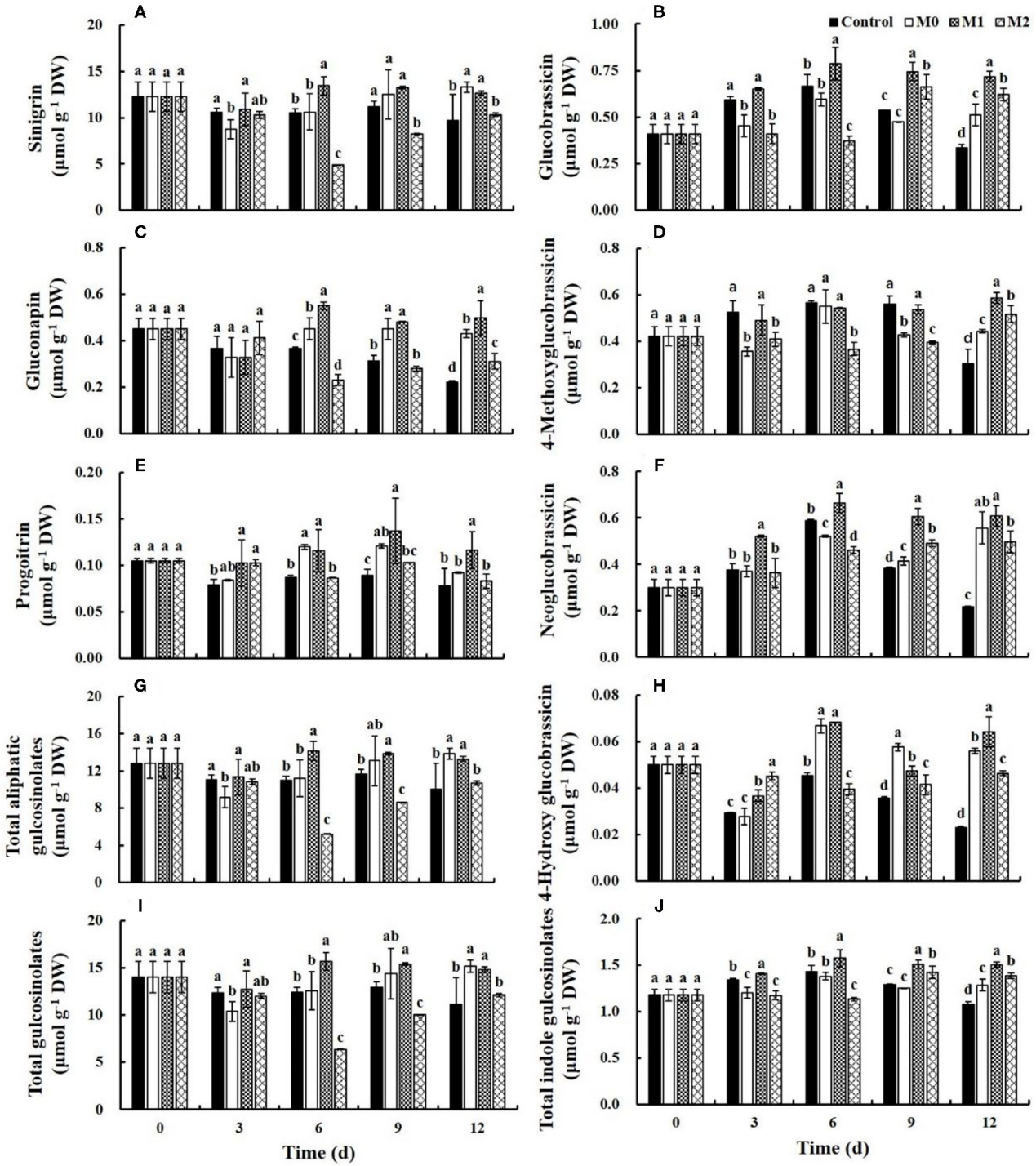

FIGURE 5 | Glucosinolates content of different baby mustard lateral buds during storage at 4°C under different MAP treatments. **(A)** sinigrin; **(B)** glucobrassicin; **(C)** gluconapin; **(D)** 4-methoxyglucobrassicin; **(E)** progoitrin; **(F)** neoglucobrassicin; **(G)** total aliphatic gulcosinolates; **(H)** 4-hydroxy glucobrassicin; **(I)** total gulcosinolates; **(J)** total indole gulcosinolates. Different letters in the figure indicate statistically significant differences among treatments for each storage day ($P < 0.05$). M0 indicates packaging with no holes; M1 indicates packaging with 6 mm in diameter holes; M2 indicates packaging with 12 mm in diameter holes.

its visual quality (5). Similar findings have also been obtained for broccoli florets (4), *Toona sinensis* (12), and watercress (6).

The weight loss of MAP treated baby mustard was reduced (**Figure 2B**), which may be due to the ability of the plastic film to restrict the diffusion of water vapor, which increases the

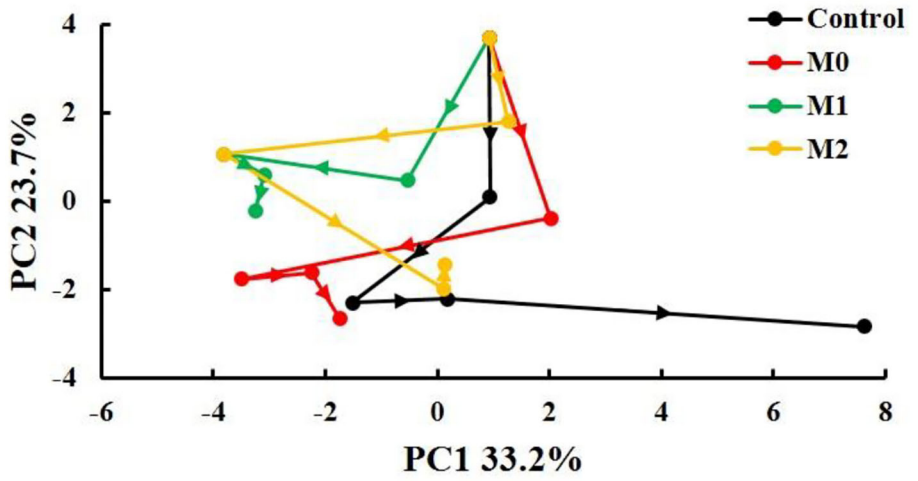

FIGURE 6 | Time-related trajectory plot showing the dynamic time-related responses of sensory and nutritional qualities of different baby mustard lateral buds during storage at 4°C under different MAP treatments. M0 indicates packaging with no holes; M1 indicates packaging with 6 mm in diameter holes; M2 indicates packaging with 12 mm in diameter holes.

water vapor pressure and relative humidity inside the package (4, 12). A shriveled appearance is associated with weight loss (5). Thus, the difference in weight loss between the control and the MAP-treated baby mustard may partially explains why the appearance of the MAP-treated baby mustard was superior to that of the control.

M0 shortened the shelf life and promoted declines in odor and acceptance scores in baby mustard during storage compared with the control (**Figure 2**). This may stem from the fact that M0 increased the production of acetaldehyde and ethanol by anaerobic metabolism because of respiratory consumption in a tightly sealed environment, producing an off-odor (4). Similar observations have also been made in broccoli florets (13). Thus, the post-harvest preservation effect of MAP may depend on the size of the punched hole in the film (4).

Soluble Sugars

Sugars are an essential source of energy for extending the post-harvest life of perishable horticultural commodities (14). MAP treatment can create a low O_2 and high CO_2 atmosphere. Along with the decrease of O_2 concentration and the increase of CO_2 concentration when packed, the aerobic respiration of vegetables will decrease (7). However, when the O_2 concentration is too low in the bag, baby mustard may produce anaerobic respiration (5). Anaerobic respiration provides less energy than aerobic respiration, and consumes more respiratory substrates in life activities, which accelerates the senescence of vegetables (15). The content of sucrose and fructose decreased rapidly during M0 (**Figures 3A,B**). This may be explained by the too low oxygen concentration caused by respiratory consumption in a tightly sealed environment, resulting in anaerobic respiration, thereby accelerating sugar consumption (15). The content of fructose and glucose in M1 changed less compared with other treatments (**Figures 3B,C**). This may due that the aerobic respiration of baby mustard is inhibited in M1 treatments (7). Meanwhile, the existence of holes ensures that there is a certain gas exchange between the microenvironment in the bag and the external environment to avoid the occurrence of anaerobic respiration. Therefore, compared with other treatments, M1 is most conducive to reducing respiration, which was beneficial to inhibit fructose and glucose degradation.

Sucrose content can be increased by environmental stress during post-harvest storage (16), and this is supported by our previous studies (2, 3). In this study, the increase in the sucrose content was slower in M1 than in the control in the late storage period (**Figure 3A**). This may be explained by the reduction in the respiration rate in M1 during post-harvest storage, which reduced environmental pressure.

Similar to the control, the glucose content in all treatments increased in the late storage (**Figure 3C**). A study of broccoli also found that the glucose content increased in the late storage (17). However, the reason for this pattern remains unclear, and additional studies are needed to clarify the underlying mechanism.

Antioxidants and Antioxidant Activity

Ascorbic acid is often used as an indicator of nutrient quality in vegetables because of its lability (18). Previous studies have shown that ascorbic acid can be biosynthesized during storage to regenerate tocopherols to prevent the deterioration of plant tissues (18) and can effectively reduce the content of reactive oxygen species to inhibit tissue browning (12, 19, 20). The ascorbic acid content in baby mustard increased in the early stage of storage. However, in the late storage period, the ascorbic acid content decreased rapidly in the control and M0 but remained stable in M1 and M2 (**Figure 4A**). Similar results also found on *Toona sinensis*, which ascorbic acid content increased in the early stage of storage, however, when the storage prolonged, the

ascorbic acid content declined with advancement in senescence (12). The decrease in ascorbic acid content in the control and M0 treated baby mustard during the late storage period may be due to the accelerated degradation of ascorbic acid, resulting in the degradation and utilization rates of ascorbic acid were likely higher than its biosynthesis rate. While, it is possible that the degradation and utilization rate of ascorbic acid and its biosynthesis rate may remain relatively balanced under M1 and M2 treatments in the late stage of storage. In addition, water loss can also hasten ascorbic acid degradation (21), and MAP significantly slowed weight loss and thus the decrease in ascorbic acid content.

Vegetables produce more secondary metabolites including phenolics to defend against stress during post-harvest storage (22). A similar situation was observed in this study: the total phenolics content of baby mustard increased early during post-harvest storage. However, the total phenolics content in the control decreased after 9 d of storage, which may stem from the acceleration of total phenolics degradation, as the degradation and utilization rates of phenolics were likely higher than the rate of biosynthesis of phenolic compounds late in the storage period (2, 22). However, the total phenolics content in MAP treatments remained relatively stable in the late storage. A previous study of medlar fruit showed that the loss of phenols during the storage can be reduced with MAP (23). An appropriate gas composition also delays the loss of phenols during the storage of guava fruit (24).

The antioxidant activity of fresh vegetables is largely related to the amount of phenolics (7, 25). The observed antioxidant activities in this study are consistent with this previous observation.

Glucosinolates

Glucosinolates not only contribute to taste and flavor but also show anticancer activity by inhibiting the growth of tumor cells (2, 26, 27). However, baby mustard face a series of stresses after harvesting and during storage that may trigger the metabolism of glucosinolates and alter glucosinolate levels (28). The content of aliphatic glucosinolates in baby mustard in all treatments was decreased early during storage. This may stem in part to the contact of myrosinase and aliphatic glucosinolates in the pre-storage pre-treatment and the subsequent hydrolysis of glucosinolates. After storage for 3 d, the content of most aliphatic glucosinolates in the control remained relatively stable. This may stem from the fact that low temperature (4°C) storage retards the decline in the content of glucosinolates in baby mustard during post-harvest storage (2). Although the glucosinolates content decreased as the quality of baby mustard deteriorated during storage, the content of aliphatic glucosinolates in M1 increased after 3 d of storage. This finding is consistent with previous studies of broccoli showing that the content of aliphatic glucosinolates, including glucoiberin and glucoraphanin, in broccoli increased under controlled atmosphere treatments (28–30). MAP treatment significantly delayed the weight loss of baby mustard, indicating that cell integrity was enhanced, and cell damage was lower, under MAP treatment. Consequently, the bursting of vacuoles and the contact between glucosinolates and myrosinase were delayed under MAP treatment during storage (2). Inactivation of myrosinase by elevated CO_2 concentrations (28, 31) might also explain the decreased degradation of glucosinolates observed in this study. In addition, the biosynthesis of aliphatic glucosinolates might be induced during controlled atmosphere storage (28).

Previous studies have shown that stress induces the biosynthesis of indole glucosinolates (32, 33). Similar observations were made in this study: the indole glucosinolates of baby mustard increased early during post-harvest storage. However, similar to aliphatic glucosinolates, the content of indole glucosinolates in the control decreased as the quality of baby mustard decreased during storage. The gas composition surrounding baby mustard gradually became suitable within the package, and this might explain the increase in the indole glucosinolates content in the MAP treatments.

CONCLUSION

The visual quality and health-promoting compounds in the lateral buds of baby mustard were more effectively maintained in M1 and M2 than in the unwrapped control. M1 was the most effective for extending the shelf life and maintaining the content of glucosinolates. However, baby mustard in M0 developed a foul aroma or off-flavors during storage, which led to a decrease in the odor and acceptance score and shortened the shelf life compared with the control. Overall, this study suggests that M1 is a simple, economical, and effective method for maintaining the sensory quality and health-promoting compounds of baby mustard at low temperature (4°C). In addition, microbial analysis has been carried out in several similar studies. Although the problem of microbial contamination in post-harvest baby mustard is not serious, considering the importance of food safety, microbial analysis of baby mustard during the storage should also be done in future research.

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/s.

AUTHOR CONTRIBUTIONS

HL and BS designed the experiments. PL, HD, and GW conducted the experiments. PL, FZ, and ZL analyzed the data. PL and HD wrote the manuscript. FZ and BS revised the manuscript. FZ and HL provided the financial support. All authors have read and agreed to the published version of 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/fnut.2021.730253/full#supplementary-material

Supplementary Figure 1 | Appearance of different baby mustard lateral buds with bags during storage at 4°C under different MAP treatments. M0 indicates packaging with no holes; M1 indicates packaging with 6 mm in diameter holes; M2 indicates packaging with 12 mm in diameter holes.

Supplementary Figure 2 | High performance liquid chromatography chromatograms. **(A)** Soluble sugars; **(B)** Ascorbic acid; **(C)** Glucosinolates. 1, 2, 3, 4, 5, 6, 7, and 8 indicate progoitrin, sinigrin, gluconapin, 4-hydroxy glucobrassicin, ortho-Nitrophenyl β-D-galactopyranoside, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin, respectively.

Supplementary Table 1 | Sensory and nutritional qualities in the lateral buds of baby mustard during storage.

REFERENCES

1. Sun B, Tian YX, Jiang M, Yuan Q, Chen Q, Zhang Y, et al. Variation in the main health-promoting compounds and antioxidant activity of whole and individual edible parts of baby mustard (*Brassica juncea* var. *gemmifera*). *RSC Adv.* (2018) 8:33845–54. doi: 10.1039/C8RA05504A
2. Sun B, Lin PX, Xia PX, Di HM, Zhang JQ, Zhang CL, et al. Low-temperature storage after harvest retards the deterioration in the sensory quality, health-promoting compounds, and antioxidant capacity of baby mustard. *RSC Adv.* (2020) 10:36495–503. doi: 10.1039/D0RA07177C
3. Sun B, Di HM, Zhang JQ, Xia PX, Huang WL, Jian Y, et al. Effect of light on sensory quality, health-promoting phytochemicals and antioxidant capacity in post-harvest baby mustard. *Food Chem.* (2021) 339:128057. doi: 10.1016/j.foodchem.2020.128057
4. Jia CG, Xu CJ, Wei J, Yuan J, Yuan GF, Wang BL, et al. Effect of modified atmosphere packaging on visual quality and glucosinolates of broccoli florets. *Food Chem.* (2009) 114:28–37. doi: 10.1016/j.foodchem.2008.09.009
5. Guo Z, Liu H, Chen X, Huang L, Fan J, Zhou J, et al. Modified-atmosphere packaging maintains the quality of postharvest whole lettuce (*Lactuca sativa* L. Grand Rapid) by mediating the dynamic equilibrium of the electron transport chain and protecting mitochondrial structure and function. *Postharvest Biol Technol.* (2019) 147:206–13. doi: 10.1016/j.postharvbio.2018.09.001
6. Pinela J, Barreira JCM, Barros L, Antonio AL, Carvalho AM, Oliveira MBPP, et al. Postharvest quality changes in fresh-cut watercress stored under conventional and inert gas-enriched modified atmosphere packaging. *Postharvest Biol Technol.* (2016) 112:55–63. doi: 10.1016/j.postharvbio.2015.10.004
7. Ozturk B, Havsut E, Yildiz K. Delaying the postharvest quality modifications of *Cantharellus cibarius* mushroom by applying citric acid and modified atmosphere packaging. *LWT Food Sci Technol.* (2021) 138:110639. doi: 10.1016/j.lwt.2020.110639
8. Burton KS, Frost CE, Nichols R. A combination plastic permeable film system for controlling post-harvest mushroom quality. *Biotechnol Lett.* (1987) 9:529–34. doi: 10.1007/BF01026655
9. Zhang F, Zhang JQ, Di HM, Xia PX, Zhang CL, Wang ZH, et al. Effect of long-term frozen storage on health-promoting compounds and antioxidant capacity in baby mustard. *Front Nutr.* (2021) 8:665482. doi: 10.3389/fnut.2021.665482
10. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem.* (1996) 239:70–6. doi: 10.1006/abio.1996.0292
11. He Y, Fan GJ, Wu CE, Kou X, Li TT, Tian F, et al. Influence of packaging materials on postharvest physiology and texture of garlic cloves during refrigeration storage. *Food Chem.* (2019) 298:125019. doi: 10.1016/j.foodchem.2019.125019
12. Lin S, Chen C, Luo H, Xu W, Zhang H, Tian JJ, et al. The combined effect of ozone treatment and polyethylene packaging on postharvest quality and biodiversity of *Toona sinensis* (A.Juss.) M.Roem. *Postharvest Biol Technol.* (2019) 154:1–10. doi: 10.1016/j.postharvbio.2019.04.010
13. Hansen ME, Sørensen H, Cantwell M. Changes in acetaldehyde, ethanol and amino acid concentrations in broccoli florets during air and controlled atmosphere storage. *Postharvest Biol Technol.* (2001) 22:227–37. doi: 10.1016/S0925-5214(01)00093-X
14. Xu F, Wang H, Tang Y, Dong S, Qiao X, Chen X, et al. Effect of 1-methylcyclopropene on senescence and sugar metabolism in harvested broccoli florets. *Postharvest Biol Technol.* (2016) 116:45–9. doi: 10.1016/j.postharvbio.2016.01.004
15. Elwan MWM, Nasef IN, EI-Seifi SK, Hassan MA. Storability, shelf-life and quality assurance of sugar snap peas (cv. super sugar snap) using modified atmosphere packaging. *Postharvest Biol Technol.* (2015) 100:205–11. doi: 10.1016/j.postharvbio.2014.10.006
16. Itai A, Tanahashi T. Inhibition of sucrose loss during cold storage in Japanese pear (*Pyrus pyrifolia* Nakai) by 1-MCP. *Postharvest Biol Technol.* (2008) 48:355–63. doi: 10.1016/j.postharvbio.2007.10.015
17. Xu F, Tang Y, Dong S, Shao X, Wang H, Zheng Y, et al. Reducing yellowing and enhancing antioxidant capacity of broccoli in storage by sucrose treatment. *Postharvest Biol Technol.* (2016) 112:39–45. doi: 10.1016/j.postharvbio.2015.09.038
18. Barth MM, Zhuang H. Packaging design affects antioxidant vitamin retention and quality of broccoli florets during postharvest storage. *Postharvest Biol Technol.* (1996) 9:141–50. doi: 10.1016/S0925-5214(96)00043-9
19. Nicolas JJ, Richard-Forget FC, Goupy PM, Amiot MJ, Aubert SY. Enzymatic browning reactions in apple and apple products. *Crit Rev Food Sci.* (1994) 34:109–57. doi: 10.1080/10408399409527653
20. Meyer AJ. The integration of glutathione homeostasis and redox signaling. *J Plant Physiol.* (2008) 165:1390–403. doi: 10.1016/j.jplph.2007.10.015
21. Noichinda S, Bodhipadma K, Mahamontri C, Narongruk T, Ketsa S. Light during storage prevents loss of ascorbic acid, and increases glucose and fructose levels in Chinese kale (*Brassica oleracea* var. *alboglabra*). *Postharvest Biol Technol.* (2007) 44:312–5. doi: 10.1016/j.postharvbio.2006.12.006
22. Li XA, Li ML, Han C, Jin P, Zheng YH. Increased temperature elicits higher phenolic accumulation in fresh-cut pitaya fruit. *Postharvest Biol Technol.* (2017) 129:90–6. doi: 10.1016/j.postharvbio.2017.03.014
23. Ozturk A, Yildiz K, Ozturk B, Karakaya O, Gun S, Uzun S, et al. Maintaining postharvest quality of medlar (*Mespilus germanica*) fruit using modified atmosphere packaging and methyl jasmonate. *LWT Food Sci Technol.* (2019) 111:117–24. doi: 10.1016/j.lwt.2019.05.033
24. Singh SP, Pal RK. Controlled atmosphere storage of guava (*Psidium guajava* L.) fruit. *Postharvest Biol Technol.* (2008) 47:296–306. doi: 10.1016/j.postharvbio.2007.08.009
25. Turkoglu A, Duru ME, Mercan N, Kivrak I, Gezer K. Antioxidant and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill. *Food Chem.* (2007) 101:267–73. doi: 10.1016/j.foodchem.2006.01.025
26. Jin P, Yao D, Xu F, Wang HQ, Zheng YH. Effect of light on quality and bioactive compounds in postharvest broccoli florets. *Food Chem.* (2015) 172:705–9. doi: 10.1016/j.foodchem.2014.09.134
27. Wang JS, Yu HF, Zhao ZQ, Sheng XG, Shen YS, Gu HH. Natural variation of glucosinolates and their breakdown products in broccoli (*Brassica oleracea* var. *italica*) seeds. *J Agric Food Chem.* (2019) 67:12528–37. doi: 10.1021/acs.jafc.9b06533
28. Xu CJ, Guo DP, Yuan J, Yuan GF, Wang QM. Changes in glucoraphanin content and quinone reductase activity in broccoli (*Brassica oleracea* var. *italica*) florets during cooling and controlled atmosphere storage. *Postharvest Biol Technol.* (2006) 42:176–84. doi: 10.1016/j.postharvbio.2006.06.009

29. Hansen M, Moller P, Sorensen H, Trejo MCD. Glucosinolates in broccoli stored under controlled atmosphere. *J Am Soc Hort Sci.* (1995) 120:1069–74. doi: 10.21273/JASHS.120.6.1069
30. Rangkadilok N, Tomkins B, Nicolas ME, Premier RR, Bennett RN, Eagling DR, et al. The effect of post-harvest and packaging treatments on glucoraphanin concentration in Broccoli (*Brassica oleracea* var. *italica*). *J Agric Food Chem.* (2002) 50:7386–91. doi: 10.1021/jf0203592
31. Dunford NT, Temelli F. Effect of supercritical CO_2 on myrosinase activity and glucosinolate degradation in canola. *J Agric Food Chem.* (1996) 44:2372–6. doi: 10.1021/jf950753i
32. Bodnaryk PP. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry.* (1992) 31:2671–7. doi: 10.1016/0031-9422(92)83609-3
33. Bartlet E, Kiddle G, Williams I, Wallsgrove R. Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist. *Entomol Exp Appl.* (1999) 91:163–7. doi: 10.1007/978-94-017-1890-5_20

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Optimized Extraction and Characterization of Folates From Date Palm Fruits and Their Tracking During Fruits Wine Fermentation

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Folates belong to the essential B vitamins group and participate in one-carbon metabolism. Date palm fruits (*Phoenix dactilyfera* L. family *Arecaceae*) are consumed by millions of people and are good sources of folates. To date, no detailed study has been carried out on suitable methods for folate extraction from date palm fruits. In the present study, an experimental design using response surface methodology (RSM) was used to maximize the extraction yield of folates from date palm fruits by including enzymatic depectinization. By applying this new strategy and a UHPLC-MS/MS technique for analysis, total folate and different folate vitamers of three cultivars of date palm fruits (Muzafti, Zahdi, and Rubai), brewer's yeast, and fermented date wine were analyzed. The optimized extraction conditions of folates from date palm fruits were found to be a pectinase activity of 47.7 U, an incubation temperature of 40°C, and an incubation time of 38 min, which yielded a total folate content of 191–301 μg/100 g. In brewer's yeast, the extracted total folate content was very high (4,870 μg/100 g), and, in the resulting date wine, it reached a maximum of 700 μg/L on the fifth day. The predominant folate vitamers in date fruit and fruit wine were 5-formyltetrahydrofolate (5-CHO-THF) and 5-methyltetrahydrofolate (5-CH_3-THF). During date palm fruit fermentation for up to 8 days, the 5-CHO-THF content gradually decreased by 20%, while 5-CH_3-THF increased linearly from day 1 to day 5 ($y = 0.058\,x + 0.0284$, $R^2 = 0.9614$). This study shows that date palm fruit and fruit wine are excellent sources of folate, and further study can be focused on different methods to improve folate stability during wine storage.

Keywords: date palm fruits, folate, depectination, wine-making, optimization

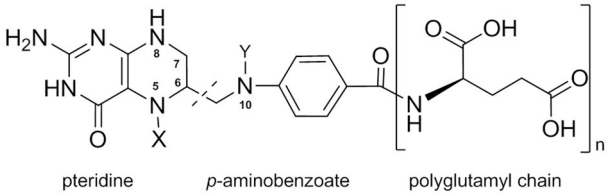
INTRODUCTION

Folates are water-soluble and essential B group vitamins with important functions in normal growth and development. Folates are involved in the nucleic acid synthesis, repair, and methylation as coenzymes in one carbon transfer metabolic reactions (1). Folate deficiency is still prevalent in South Asia and central and western Africa (2). Folate deficiency can induce several syndromes and diseases such as megaloblastic anemia, neural tube defects, spina bifida, anencephaly, cardiovascular disease, and certain cancers (3).

Folate is essential and the human body is unable to synthesize it, and, therefore, it must be obtained from the dietary sources (4). Typical sources of native folate include leafy green vegetables, legumes, dairy products, liver, citrus fruits, and eggs, with quantities ranging from 50 to 200 μg/100 g (5). The FDA-recommended daily intake is high (400 μg per day), and, hence, identifying some non-traditional food sources rich in natural folate would be useful.

Chemically, folate is composed of a pteridine ring, *p*-aminobenzoate and is linked to polyglutamyl chains (a variable number of glutamate moieties) (**Figure 1**) (3). Folate structures differ with the oxidation/reduction state of the pteridine ring, one-carbon substitution at the N5 and N10 positions or numbers of polyglutamyl chain lengths (3). From our previous study, it was found that methyl and formyl substitutions at N5 were the predominant folate forms in common foods which correspond to 5-CH_3-THF and 5-CHO-THF (6). Folate in food is very susceptible to heat, light, and oxygen (7). Therefore, extraction and determining folate levels in food are very challenging. Several methods have been reported for food folate extraction and determination. For folate extraction, the widely applied method is a trienzyme digestion procedure (α-amylase, protease, and conjugase) (7). The enzymes α-amylase and protease digest the complex carbohydrates and protein structures to release the bound folates. Conjugases further hydrolyze folate polyglutamates to monoglutamates for detection. This method was reported to be particularly effective for cereal-based products; however, this method is not compatible with folate extraction from a high-pectin food matrix. In addition, some previous studies also showed that pectin and oligosaccharides or polysaccharides could entrap and slow the release of folate (8, 9). Therefore, a new strategy needs to be developed to release the entrapped folate in the pectin matrix, which is important for accurate folate identification and quantification. For folate quantification, the microbiological assay is the one most commonly used method. Although this method is adequate for determining total folate content, it cannot distinguish diverse folate vitamers (7). Previously, we developed a UHPLC-MS/MS method for quantification of different folate vitamers in different plants with high sensitivity and accuracy (10).

Date palm (*Phoenix dactilyfera* L. family *Arecaceae*) is an important commercial crop grown in Arab countries and has a delicious sugary taste with a soft chewy texture. Date palm fruits are consumed by millions of people, and, although they are high in calories, they are a good source of several nutrients. The sugar content ranges from 65 to 80% on a dry-weight basis and are mostly present in the inverted form (glucose and fructose). Several studies show that date palm fruits are a pivotal source of dietary fibers, minerals, vitamins, and 23 types of amino acids (some of which are not present in the most popular fruits such as oranges, apples, and bananas) (11–13). They are also rich in antioxidants (14). Date palm fruits are usually consumed as fresh (raw) or milk or in partially dried form as soft fruits, which are more stable. In recent years, date palm fruits have been used for fermentation to make wines, which are also becoming popular (15). Unfortunately, there is a serious dearth of scientific studies on the optimization of folate extraction and quantification of folate vitamers in different varieties of date palm fruits or date palm fruit wines. Since date palm fruits contain a significant content of pectin (0.3%), in our preliminary studies, it was observed that depectination of date palm fruits by pectinase during folate extraction helps to increase the folate recovery yield.



X	Y	
H	H	THF
CH_3	H	5-methylTHF
CHO	H	5-formylTHF
CH=NH	H	5-formiminoTHF
H	CHO	10-formylTHF
- CH_2 -		5,10-methyleneTHF
= CH -		5,10-methenylTHF

FIGURE 1 | Chemical structures of folate vitamers and polyglutamyl folate.

Response surface methodology is an effective experimental, mathematical, and statistical method to optimize an extraction process within a set of ranges for key process variables. Response surface methodology (RSM) can be used to obtain statistical details of the influence of independent variables on the process outcomes and to generate a set of mathematical models that could be used to optimize the process. As compared with orthogonal experimental design, RSM reduces the number of tests but still provides a statistically significant model describing the effect of process variables and their interactions. Such designs have been successfully applied to optimize trienzyme (α-amylase, pronase, and conjugase) extraction of folate from cereals in early reports (16, 17).

Therefore, the objectives of this study were to (a) evaluate and optimize the pectinase-assisted extraction of folates from date palm fruits, using the RSM methodology and the UHPLC-MS/MS method for folate quantification; (b) determine the distribution of folate vitamers and the total folate content in different varieties of dates under optimized conditions; and (c) track changes in folates during the fermentation process for making date palm wine. This is the first detailed study on the optimization of pectinase-assisted extraction of folate from high-sugar date palm fruits and folates in date palm wine.

MATERIALS AND METHODS

Chemicals

All the folate standards employed in this study are shown in **Table 1**. Liquid chromatography-mass spectrometry (LC-MS)

TABLE 1 | Folate standards used in this study.

	Standard	Abbreviated name	Formula
1	(6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid, calcium salt	5-CH_3-Glu.Ca	$C_{20}H_{25}N_7O_6$.Ca
2	(6S)-5-methyl-5,6,7,8-tetrahydrofolate-[1^3C_5]Glu, calcium salt 5MTHF [$^{13}C_5$] Glu·Ca	5-CH_3-[$^{13}C_5$]Glu·Ca	$C_{15}^{13}C_5H_{25}N_7O_6$·Ca
3	Folic acid	FA	$C_{19}H_{19}N_7O_6$
4	(6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride	5,10-CH^+THF.Cl	$C_{20}H_{22}N_7O_6$.Cl
5	10-formylfolic acid	10-CHOFA	$C_{20}H_{19}N_7O_7$
6	(6R,S)-5-formyl-5,6,7,8-tetrahydrofolic acid, calcium salt	5-CHOTHF.Ca	$C_{20}H_{23}N_7O_7$
7	(6R,S)-5,6,7,8-tetrahydrofolic acid	THF	$C_{19}H_{23}N_7O_6$

All were purchased from Schircks Laboratories (Jona, Switzerland) except 5-CH_3-[$^{13}C_5$]Glu·Ca, which was from Merck Eprova AG (Schaffhausen, Switzerland).

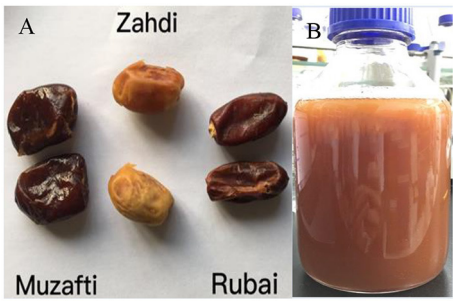


FIGURE 2 | (A) Three different cultivars of date palm fruits investigated in this study. **(B)** Date palm fruits wine with cultivar of Rubai.

grade water, acetonitrile, and formic acid were obtained from Thermo Fisher Scientific (Fair Lawn, NJ). Ammonium acetate, 2-mercaptoethanol and L-ascorbic acid were purchased from Sigma-Aldrich (Shanghai, China). Klerzyme 150 (pectinase: 3.5 $\times$ 10^5 U/ml, cellulose: 1,875 U/ml) was obtained from DSM (Het Overloon, the Netherlands). Recombinant His-tagged human GGH was obtained from Novus Biologicals (Littleton, CO, USA). All other chemicals were obtained from Schircks Laboratories (Jona, Switzerland), except for 5-CH_3-[$^{13}C_5$]Glu·Ca, which was obtained from Merck Eprova AG (Schaffhausen, Switzerland).

Date Palm Fruits

In this study, three different date palm fruit cultivars were investigated. Pictures of the different date palm fruits are shown in **Figure 2A**. The cultivars included were Muzafti, Zahdi, and Rubai. All fruits were obtained from Guangdong Wodelong Biotechnology Co., Ltd (Zhuhai, China). The determination of folate vitamers and total folates in date fruits was based on their dry weight without the seeds. Also shown in the **Figure 2B**, this is the date palm fruit wine with cultivar of Rubai.

TABLE 2 | Factors and levels for Box–Behnken design.

Independent variable	Levels		
	−1	0	1
Pectinase activity (X_1)	25	37.5	50
Incubation temperature (X_2)	30	35	40
Incubation time (X_3)	0.5	1	1.5

Pectinase Treatment for the Extraction of Folates From Dry Date Palm Fruits

Pectinase treatment was employed as a mean of enhancing the extraction and recovery of folate from date samples since preliminary experiments in our lab demonstrated its advantage. Commercial pectinase (Klerzyme 150, DSM, the Netherland) was used, and, in the first phase, individual factors were evaluated for their influence on folate extraction. The three factors were employed: concentration of pectinase enzyme (10-, 25-, 35-, 50-, and 55-U-per-gram dry fruits), incubation time (0.5, 1, 1.5, 2, and 2.5 h) and incubation temperature (30, 35, 40, 50, and 55°C). For all treatments, the pH of the extract was maintained at 6.

For the extraction process, the dry fruits were gently washed with tap water and surface dried with a paper towel. The seeds were then carefully removed. Samples (5 g) were dispensed into 20 ml of an extraction buffer (100-mM ammonium acetate, 1% ascorbic acid, 0.2% 2-mercaptoethanol, pH7.89) and homogenized for 2 min to a fine puree using a homogenizer (IKA T18 Basic; IKA, Staufen, Germany) at 4°C. The homogenates were immediately boiled for 15 min, cooled on ice to room temperature, brought to 25 ml with distilled water, and then adjusted the pH to 6 for further pectinase treatment. The independent factors of pectinase enzymatic activity (X_1), incubation temperature (X_2), and incubation time (X_3) are listed in **Table 2**. A stepwise optimization procedure was employed at this stage. All factors were fixed at one of the three levels (−1, 0, 1), with X_1 (25, 37.5, 50 U/g), X_2 (30, 35, and 40°C) and X_3 (0.5, 1, and 1.5 h). To investigate the initial effects of individual factors, one factor was used at different levels, while the others were maintained at specific levels (preselected). For example, for evaluating the temperature effect, the preselected values were 35-U/g enzyme activity and 1-h treatment time. pH was maintained at 6 for all tests. Once the optimum temperature was selected, this value was fixed for testing the next two variables.

Optimization experiments for pectinase release of folate from date palm fruits were then performed. For this, a three-factor-three-level Box–Behnken design (BBD) was employed to obtain the interactions between factors and to define the optimum extraction conditions. The experiment was designed using Minitab 19.0 (State College, Pennsylvania, USA), and the BBD design is shown in **Table 3**.

Experimental data were fitted for a second-order polynomial model with the following equation:

$$Y = a_0 + b_1X_1 + b_2X_2 + b_3X_3 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3 + d_1X_1^2 + d_2X_2^2 + d_3X_3^2 \quad (1)$$

TABLE 3 | Experimental results for three-level-three-factor Box–Behnken design.

Run	Pectinase activity (X_1)	Incubation temperature (X_2)	Incubation time (X_3)	Total folate (μg/100 g)		
				Experimental	Predicted	Deviation (%)
1	1	0	1	172.73	172.66	0.04
2	0	0	0	190.83	190.88	0.02
3	0	−1	1	184.16	184.79	0.30
4	−1	−1	0	188.92	188.41	0.27
5	0	0	0	191.15	190.88	0.13
6	0	0	0	190.51	190.88	0.19
7	0	1	−1	187.65	187.14	0.27
8	1	−1	0	183.53	183.09	0.24
9	−1	0	−1	176.86	177.04	0.10
10	−1	1	0	180.67	181.22	0.30
11	−1	0	1	182.57	182.59	0.00
12	1	0	−1	186.70	186.80	0.05
13	0	1	1	174.00	173.63	0.21
14	0	−1	−1	179.40	179.88	0.26
15	1	1	0	185.75	186.37	0.33

where Y is the response (total folate content, μg/100 g of the sample), a_0 represents the interception; b_1, b_2, and b_3 are the linearity; c_{12}, c_{13}, and c_{23} represent the interaction coefficient; d_1, d_2, and d_3 represent the squared coefficient; and X_1, X_2, and X_3 represent the independent variables.

Analysis of variance was used to determine the individual linear, quadratic, and interaction regression coefficients (β) using Minitab 19.0 (State College, Pennsylvania, USA). The coefficient of determination (R^2) was used to estimate the fitness of the polynomial equation to the response, and the significance of the dependent variables was statistically analyzed by computing the *F*- value at $p < 0.05$.

After the extraction conditions were optimized for the maximum extraction of folate by employing RSM, the responses were experimentally determined under the optimum enzymatic conditions and compared with the predicted values from the RSM model.

Date Palm Fruit Wine Production

Lab/pilot scale small-volume date palm wine fermentations were carried out in the lab at the Department of Food Science and Technology, Jinan University, China. Well-ripened dry date palm fruits (cultivar: Rubai) were washed and surface dried with a paper towel. The date palm fruit had a high-sugar content of 81% with 3.2% fiber and 0.3% pectin. The pits in the fruits were removed, and the fruits were cut into small pieces. About 100 g of cut pieces were mixed with 300-ml distilled water and boiled for 30 min. The mixture was then cooled to room temperature and homogenized. The homogenate was depectinized under predetermined optimized conditions: pectinase, 48 U/g; incubation temperature, 40°C for 38 min. After depectinization, the mixture was boiled again for 10 min to inactivate the pectinase, cooled to room temperature on ice, and then mixed with 0.036% $NaHSO_3$ and ACTIFLORE F33 yeast (0.03%, Laffort, France) for fermentation, which proceeded anaerobically at 28°C for 8 days. The sugar content was determined and recorded every day, using a refractometer (Mettler Toledo, Leicester, USA). The initial sugar content before fermentation was 27 °Brix, the final sugar content of wine was 7.3°Brix, and the alcohol content was ∼15%. The total folate and distribution of folate vitamers in the wine were determined daily. Subsequently, the wine was packed in separate tubes and stored at −80°C until further folate determination.

Folate Analysis in Fermentation Reagents

To take into consideration folate brought by the fermentation reagents, the endogenous folate content in the fermentation reagents was determined as suggested in early reports (10, 18). Specifically, 5-g samples of fermentation reagents (pectinase and ACTIFLORE F33 yeast) were suspended in 20 ml of an extraction buffer (100-mM ammonium acetate; 1% ascorbic acid; 0.2% 2-mercaptoethanol; pH, 7.89) and homogenized for 2 min using a homogenizer (IKA T18 Basic; IKA, Staufen, Germany) at 4°C. The homogenates were immediately boiled for 15 min, cooled on ice to room temperature, and reconstituted to 25 ml. After this, the extraction was carried out as detailed earlier, and further hydrolysis was carried out as follows.

Folate Deconjugation With Recombinant Human GGH, SPE Cleanup, and UHPLC-MS/MS Determination of Folate Vitamers and Total Folate

For the past two decades, protease and amylase have been applied during folate extraction to improve folate release from the food matrix into the extraction buffer and to determine the concentration of folate vitamers and total folates in a high-protein or starchy food matrix (19). For yeast and pectinase

extract, 1.5 ml of pronase (10 mg/ml) was added to 25 ml of the extraction, followed by incubation at 37°C for 3 h. After treatment with pronase, 1.5 ml of α-amylase was added and incubated again at 37°C for 2 h. The pronase- and amylase-treated yeast extract and the date palm fruit wine were subjected to the following deconjugation. As pointed out earlier, intracellular folates can be found in the form of polyglutamates, and folate conjugase can be used to hydrolyze them into monoglutamyl forms. To determine individual folate vitamers in different samples, 5-ml aliquots of different extracts of date palm fruit extract or fermentation reagents (pectinase and ACTIFLORE F33 yeast) or 20 ml of fermented wine were treated with recombinant GGH (5 or 1 μg/ml) at 37°C for 30 min to deconjugate polyglutamyl folates to its monoglutamyl forms. After incubation, all samples were boiled for 5 min and centrifuged at 20,000 rpm for 10 min. The supernatant was recovered and subjected to solid-phase extraction (SPE). The completeness of the deconjugation reaction was determined by monitoring the profile of polyglutamyl folate and the total folate contents (6).

Solid-phase extraction was carried out before UHPLC-MS/MS analysis to remove the interferences and decrease the matrix effect of water-soluble components, such as sugar. Oasis HLB6 cc Vac cartridges (Waters, Milford, MA) were first activated and conditioned successively with 5 ml of methanol and 5 ml of water. Before application to the SPE cartridge, deconjugated extracts were adjusted to pH 2 with 6 mol/L HCl. After the sample was applied, the cartridge was washed with 5 ml of 5% methanol in water and evaporated to dryness in a vacuum. Folate was eluted with 5 ml of 95% acetonitrile in 5% methanol. Finally, the eluate was evaporated to dryness in a stream of nitrogen, reconstituted with 1 ml of the extraction buffer, and filtered through a 0.22-μm nylon filter before injection into the UHPLC-MS/MS system.

Quantitation of folate vitamers was carried out, following the methods described in Zou et al. (6). UHPLC separation was performed with an Agilent 1290 Infinity UHPLC equipped with a binary pump, an autosampler, a column oven, and degasser. The column was a HILIC column (4.5 × 100 mm, 2.6 μm, Kinetex, Phenomenex), and its temperature was set at 40°C. The mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Its flow rate was 0.8 ml/min. The gradient was as follows: 0–4 min, 0–20% B; 4–5 min, 20–95% B; 5–6.5 min, 95% B; 6.5–9 min, 0% B. The injection volume was 2 μl, and the autosampler was kept at 25°C. The UHPLC eluate was introduced into an Agilent 6460 triple quadrupole mass spectrometer. The MS/MS instrument was operated in the positive-ion electrospray mode at a capillary voltage of +3.5 kV, and a charging voltage of +1 kV. Nitrogen was used as the nebulizing gas at 0.31 MPa along with a carrier gas flowing at 10 L/min at 300°C and a sheath gas flowing at 11 L/min at 350°C. An Agilent Mass Hunter workstation was used to control the equipment for data acquisition and analysis. Acquisitions were performed by selected reaction monitoring.

Statistical Analysis

Microsoft Excel was used to compute the mean ± standard deviation (SD) concentrations of each folate form. Significant differences were determined by *ANOVA*, using Tukey's *post-hoc* test and a paired *t*-test using Minitab 19.0 (State College, PA). A significant difference between the means was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Impact of Individual Factors on Pectinase-Aided Folate Extraction Efficiency

Three factors, namely, pectinase concentration, incubation time, and incubation temperature were tested as independent factors in this study to identify their importance in the extraction of folate from date palm fruit extract. The effect of incubation temperature (first variable tried for optimization) on the folate extraction yield is shown in **Figure 3A** with the yield positively correlating with increasing incubation temperature from 30 to 35°C; however, it then decreased when the temperature was above 35°C. Although the supporting documents from the manufacturer suggested that pectinase activity was optimum at 45°C, it was observed that total folate actually decreased when temperature was above 35°C. This phenomenon could be attributed to the folate degradation induced by higher incubation temperatures beyond 40°C (20, 21). According to an early report, folates are very sensitive molecules and can degrade ∼10–20% after incubation at 37°C for 2 h, which leads to the cleavage of folate into a pteridine and *p*-aminobenzoic acid linked to a polyglutamate tail (22). Neither pteridine nor *p*-aminobenzoic acid has biological functions. This optimum level of 35°C was used when testing the other two factors.

The extraction yields at different incubation times (second variable) are shown in **Figure 3B**, which increased with incubation time between 0.5 and 1.5 h and decreased at longer treatment times. The initial increase in folate markedly increased with the extension of incubation time, which was attributed to the gradual decrease in resistance to intracellular folate diffusion during pectin hydrolysis. Still, longer incubation time caused the degradation of folate due to its thermal susceptibility. Also, there was no significant difference when the extract was incubated with enzyme for 1 and 1.5 h. Therefore, the incubation time was set at 1 h to test the effect of pectinase concentration on the extraction yield.

The influence of pectinase concentration was finally tested on the extraction yield and is detailed in **Figure 3C**, which, like before, showed that the total folate yield increased rapidly with increasing concentration of pectinase up to a middle level of 35 U/g; after which, the further increases were not significant. Hence, a 35 U/g enzyme concentration was considered appropriate. This effect could be explained by the fact that a high pectinase concentration promoted the decomposition of pectin in the cell walls of date palm fruits, which contributed to enhance the permeability of cell membranes and the solubility of folate. It is well-known that pectinase helps to increase the juice yield by degrading cell wall polysaccharides and improving the compressibility of pulp for extraction (23).

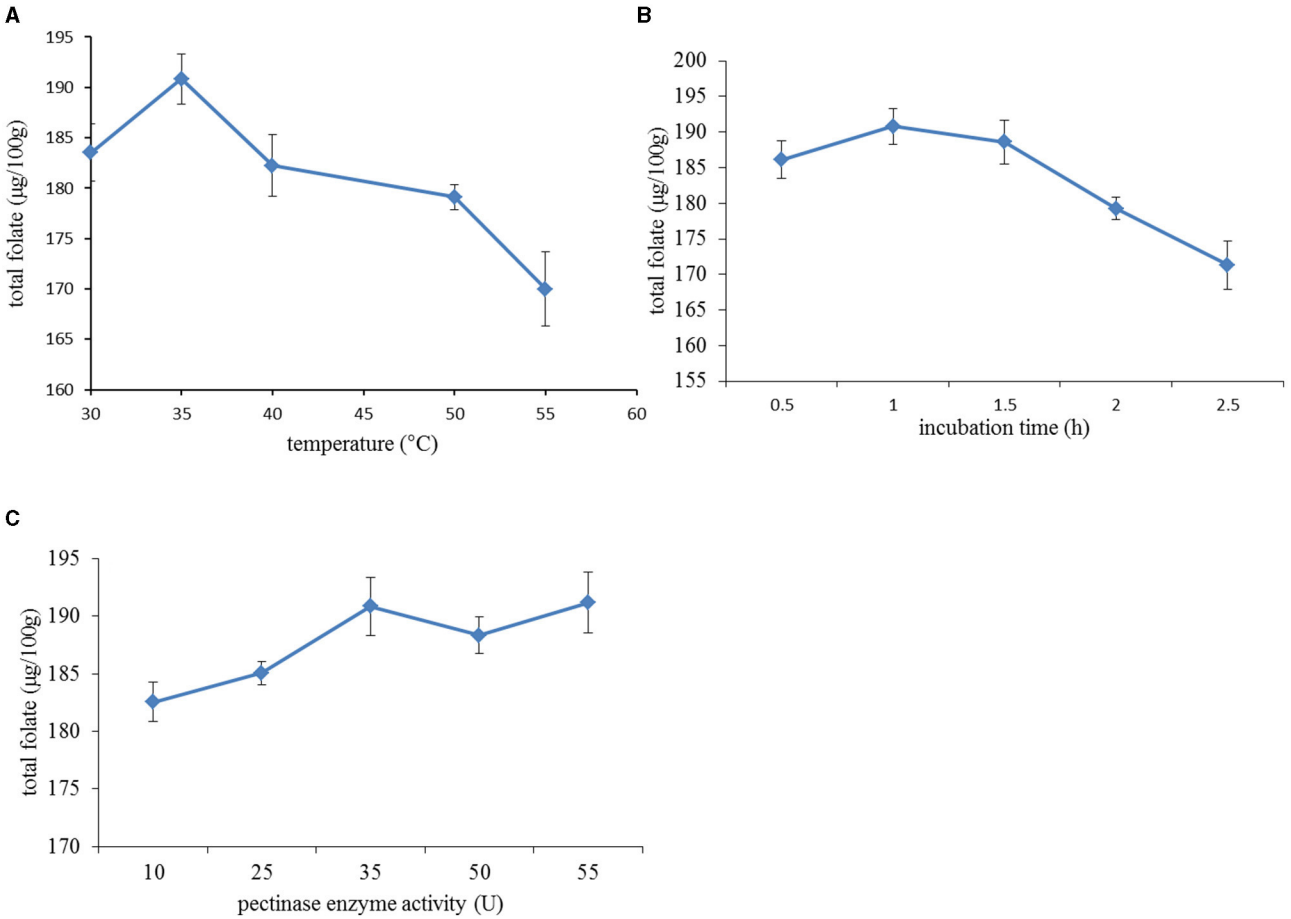


FIGURE 3 | Effect of different extraction variables on total folate content: **(A)** extraction temperature: the incubation time and pectinase concentration were fixed at 1 h and 35 U/g; **(B)** incubation time: incubation temperature and pectinase concentration were set at 35°C and 35 U/g; **(C)** pectinase enzyme activity: the incubation temperature and time were maintained at 35°C and 1 h.

RSM Optimization of Pectinase-Assisted Extraction of Folate

In the previous section, three test factors in folate extraction were evaluated over a broader span but without considering their interactions. In the past, that was one of the optimization pathways, achieving the goal in a stepwise fashion. Such an approach is simple and good for identifying suitable conditions where the factors could influence the independent output. However, to be meaningful, the interactive effects of the different factors need to be considered because they can have synergistic (positive) or antagonistic (negative) effects. An RSM approach is generally used for this purpose. A three-factor, three-level RSM experimental design was used with the central level at the near-optimum conditions as determined from the previous experiments. The levels of the three factors used [pectinase concentration (X_1), incubation temperature (X_2), and incubation time (X_3)] are detailed in **Table 3**.

The effects and interactions for each variable were optimized by BBD. A second-degree polynomial equation was developed as indicated by the following equation as the relationship between the total folate content and coded factors (**Table 4**):

$$\begin{aligned}\text{Total folate content } (\mu g/100\,g) = & -11.4 + 1.165X_1 + 6.41X_2 \\ & + 147.80X_3 - 0.02464X_1^2 \\ & - 0.0905X_2^2 - 29.05X_3^2 \\ & + 0.04191X_1X_2 - 0.7874X_1X_3 \\ & - 1.842X_2X_3 \end{aligned} \quad (2)$$

The analysis of variance (ANOVA) results applied to validate the regression equation and confirm the influence of each factor on total folate content. The results are shown in **Table 4**. The *P* and *F*-values are used to evaluate the importance of each parameter. Low *P* and high *F*-values indicated that the experimental factors were highly significant (24). The relationship between the above regression equation and the response surface was highly significant ($P = 0.000$). Additionally, all of the factors were extremely significant variables ($p \leq 0.01$) (**Table 4**) except X_1. This was probably because its individual effects were confounded by the interactions with the other two factors, and they were highly significant; therefore, the X_1 factor is still a significant

TABLE 4 | Results of *ANOVA* of the regression equation.

Source	DF	Adj SS	Adj MS	*F*-value	*P*-value
Model	9	496.829	55.203	115.27	0.000
X_1	1	0.013	0.013	0.03	0.877
X_2	1	7.876	7.876	16.45	0.010
X_3	1	36.748	36.748	76.74	0.000
X_1^2	1	54.727	54.727	114.28	0.000
X_2^2	1	18.898	18.898	39.46	0.002
X_3^2	1	194.786	194.786	406.75	0.000
X_1 X_2	1	27.448	27.448	57.32	0.001
X_1 X_3	1	96.886	96.886	202.32	0.000
X_2 X_3	1	84.788	84.788	177.05	0.000
Error	5	2.394	0.479		
Lack of fit	3	2.193	0.731	7.25	0.124
Pure error	2	0.202	0.101		
Total	14	499.223			

player in the overall model. In addition, three factors had extremely significant quadratic effects on the extraction of folate ($p < 0.01$). The *F*-value of the model was 115.27, indicating that model was statistically significant. The relativity between lack of fit and pure error is significant because the "lack-of-fit *F*-value" was not significant. The high R^2-value ($R^2 = 0.93$) indicated that the predicted values compared well with the experimental values. R^2 will be slightly increased if all variables and their interaction terms were included in the model regardless whether it was statistically significant or not. An adjusted R^2 (R^2_{Adj}) was used instead of R^2 because R^2_{Adj} would not change a given variable when the interaction term was added or deleted. In this model, an R^2_{Adj} of 0.99 was obtained, revealing that 99% of the total variations were explained by the model. Consequently, the regression equation could well-reflect the real relationship between experimental factors and the total folate and could be employed to obtain the optimal extraction parameters for the extraction of folate from date palm fruits.

Three-dimensional surface plots and two-dimensional contour plots are presented to visualize general variation trends between operational parameters, and the shapes of the response surface curves indicated significant interactions between the extraction variables and the yield parameters (**Figure 4**), as done in other studies (25). In these 3-D plots, the influence of two variables is shown by keeping the third variable at its central level (because it is not possible to show the fourth dimension). 3D response surfaces (**Figure 4**) show a convex shape, which means that the ranges used for extraction variables are appropriate, and an optimal shape can be obtained within the range.

An early study found that steep 3D response surface plots show that the experimental factors have a great influence on the extraction of total folate, and the interaction of the two experimental factors is highly related (23). Additionally, early studies have implicated that, in the 2D contour, the oval and circular contours reveal the interaction between the two factors is significant and not significant, respectively (23). Here, in this study, the 2D contour plots were all oval, especially the interactions between X_2 and X_3, X_1 and X_2 were more significant than X_1 and X_3, which is consistent with the *ANOVA* results in **Table 4**.

Through the above data analysis, the optimized conditions were obtained as follows: pectinase activity: 47.5 U, incubation temperature of 40°C, and incubation time of 38 min. Experimental rechecking was performed to compare the predicted total folate with the experimental value evaluated under the optimal processing conditions. For this experiment, the best extraction processing conditions were rounded as follows: pectinase activity: 48 U, incubation temperature of 40°C, and incubation time of 38 min. The results showed that, under these conditions, total folate reached 193 ± 3 μg/100 g, which is close to the predicted total folate of 191 μg/100 g (error within 1%).

Response surface methodology has also been used in other studies involving folate extraction. For example, the trienzyme extraction of folate from different starchy or high-protein foods was optimized by RSM, demonstrating a shorter extraction time and minimization of folate loss (16, 17). However, this is the first study to optimize RSM for folate extraction from fruit with a high-pectin content by the application of pectinase, while some previous studies have hinted that pectin and oligosaccharides or polysaccharides could entrap and slow the release of folate (8, 9). Furthermore, the optimized method could possibly be applied for folate determination in some high-pectin vegetables and fruits, such as carrots, strawberries, gourds, and many others.

Folate Vitamer Distribution and Total Folate in Different Date Palm Cultivars by Optimized RSM Extraction

The RSM-optimized method was applied for folate extraction from three cultivars of date palm fruit cultivars: Zahdi, Rubai, and Muzafti. In all of them, 5-CHO-THF was the predominant form of folate vitamer, accounting for 91–97%, and 5-CH_3-THF was a minor vitamer, accounting for 3–9% of the total folates. The UHPLC-MS/MS chromatogram is shown in **Figure 5**. The total folate contents were found to be 191 (±2.52), 229 (±3.80), and 301 (±2.29) μg/100 g for Zahdi, Rubai, and Muzafti, respectively. According to Institute of Medicine, the recommended dietary allowance (RDA) of folate is 400 μg per day (26). Thus, serving 100 g of date fruits (without seeds) would provide 48, 57, and 75% of the total folate, respectively, with the cultivar Muszafti contributing the most. In general, all date palm fruits investigated have high-folate content and can use as supplements for folate nutrition. Different folate vitamers and total folate contents in date palm fruits have never been characterized before; therefore, this is valuable information (27).

Total Folate Tracking During Date Palm Fruit Fermentation

Date palm fruits are a staple food, and they are not only eaten raw but are also fermented into wine. The course of the date palm fruit fermentation process is shown in **Figure 6**. The sugar content of the syrup decreased during fermentation until from

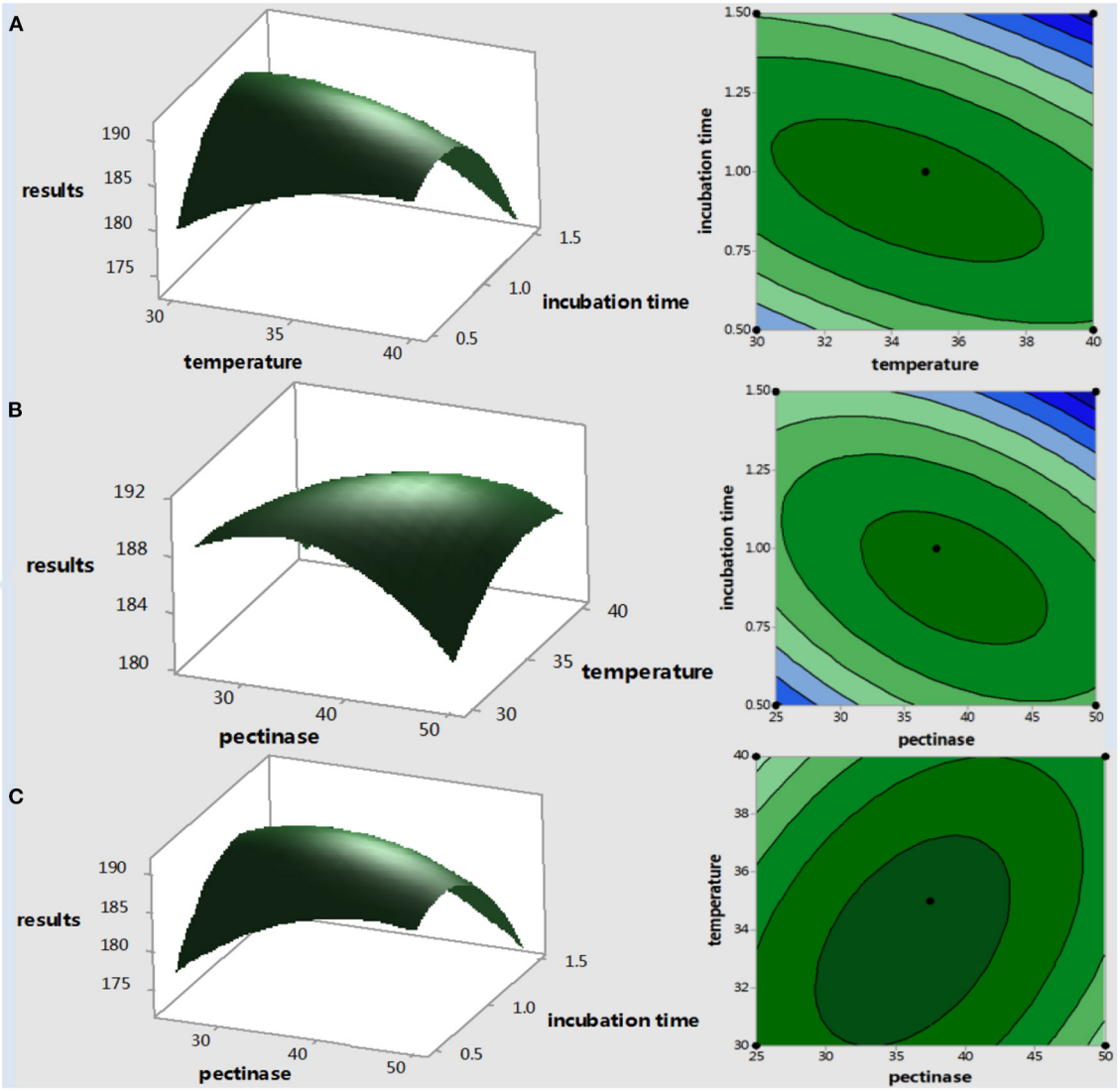


FIGURE 4 | Response surfaces and contour plots for the effect of **(A)** incubation temperature (X_2) and incubation time (X_3), **(B)** pectinase enzyme activity (X_1), and extraction temperature (X_2) **(C)** pectinase enzyme activity (X_1) and incubation time (X_3).

27°Brix to 7.3°Brix in 8 days, resulting in ∼15% alcohol. The relationship between sugar content and the fermentation day was almost linear.

The folate vitamer and total folate in pectinase and brewer's yeast were first evaluated (**Figure 7**). The results showed that there were four types of folate vitamers in brewer's yeast: 5-CH_3-THF (4,460 ± 191 μg/100 g), 5-CHO-THF (330 ± 12 μg/100 g), 10-CHOFA (45 ± 2 μg/100 g), and THF (31 ± 1 μg/100 g). The total folate content in the yeast was 4,870 μg/100 g. The yeast used in the study is the common strain used in grape wine fermentation. This is the first report on folate vitamers and total folate in brewer's yeast; most other studies reported it on bakers' yeasts. Brewer's yeast contains higher total folate than the baker's yeast, which is usually reported to be ∼4,000 μg/100 g (18, 28). However, the distribution of folate vitamers in brewer's yeast was similar to that in baker's yeast, which also had 5-CH_3-THF as the predominant form (92%), followed by 5-CHO-THF (6.8%) and minor constituents of THF and 10-CHOFA. Therefore, in general, yeast is a good source of folate. Additionally, endogenous folate in pectinase was too low to be detected.

Further changes in folate vitamers and total folate during fermentation were also evaluated daily (**Figure 8**). The results clearly showed that there were two types of folate vitamers in date palm wine: 5-CHO-THF and 5-CH_3-THF. During fermentation, the content of 5-CHO-THF gradually decreased from day one, resulting in a total decrease of ∼20% after fermentation for 8 days, although 5-CHO-THF is the most stable form of natural folate. The significant loss of 5-CHO-THF during wine making could be because, during yeast proliferation, some enzymes or compounds might be produced that could lead to 5-CHO-THF degradation. However, this has never been reported earlier. In future studies, the mechanism of 5-CHO-THF degradation could

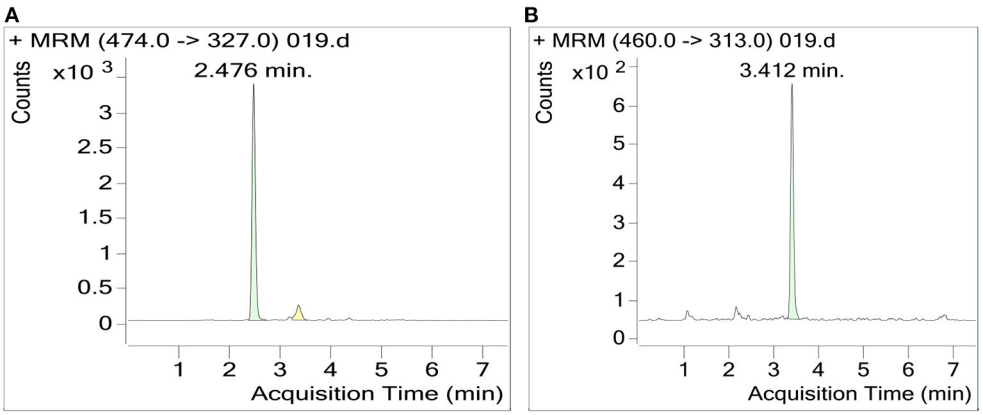


FIGURE 5 | UHPLC-MS/MS chromatogram of 5-CHO-THF **(A)** and 5-CH_3-THF **(B)**. The MS/MS transition of 5-CHO-THF and 5-CH_3-THF: 474 > 327; 460 > 313, respectively.

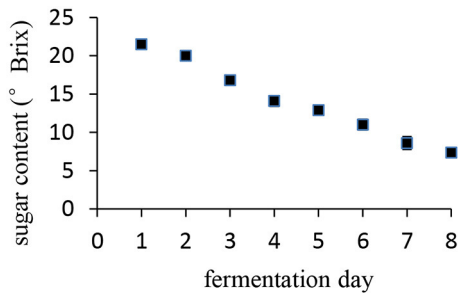


FIGURE 6 | Change of sugar content during date palm wine making.

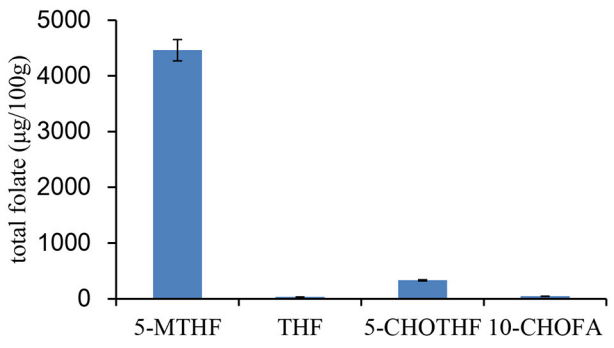


FIGURE 7 | The distribution of folate vitamer and total folate content in brewer's yeast.

be explored. Additionally, there was a significant increase in 5-CH_3-THF during fermentation from day 1 to day 5. This increase followed linear rate kinetics ($y = 0.058x + 0.0284$, $R^2 = 0.9614$). The increased 5-CH_3-THF supposedly came from the growth of yeast. 5-CH_3_THF is an important folate derivative that plays a key role as a methyl donor in the regeneration of methionine from homocysteine, produced in the methylation cycle of one carbon (7). Several studies have reported the biofortification of folate in different food systems with baker's yeast (29). Hjortmo et al. (30) detected 3–5-fold higher total folate in white bread leavened with *Saccharomyces cerevisiae* strain CBS7764 compared to commercial yeast. Walkey et al. (31) reported the enhancement of folate levels in wine by bioengineering the yeast and found that the folate level in wine was mainly contributed by the yeast used, with strain dependence (31). *Saccharomyces cerevisiae* has the ability to synthesize folates because of its genetic apparatus (32). During folate biosynthesis by yeast, three major genes FOL2, FOL3, and DFR1, which encode three important enzymes, were identified (32). GTP-cyclohydrolase I, encoded by FOL2, is the first enzyme in the folate biosynthesis pathway. Dihydrofolate synthase, encoded by FOL3, produces dihydrofolate, while dihydrofolate reductase, encoded by DFR1, converts dihydrofolate into tetrahydrofolate. Tetrahydrofolate will then interconvert to 5-CH_3-THF in one-carbon transfer reactions (32). In addition to yeast, other fermented foods can also biosynthesize folate. D'Aimmo et al. (33) screened 19 strains of bifidobacteria for different folate forms and total folate and found that most strains had a total folate content above 4,000 μg/100 g dry matter, demonstrating that bifidobacteria may contribute to folate intake by applying suitable growth conditions (33). Additionally, some lactic acid bacteria (LAB) species are able to produce folates in fermented milk (34). Moreover, cocultures of different LAB species have been used to increase the content of folates by ∼30% compared to single cultures (35). All of these studies have proved that food fermentation could be a useful tool to increase total folate intake. Furthermore, the stability of folate during date palm fruit wine fermentation must also be considered to properly evaluate the folate content since the degradation and interconversion of folate vitamers can have a deep impact on the final concentration of folates (35). After fermentation, the total folate in the wine reached a maximum level of 700 μg/L. Thus, it was demonstrated that when date palm fruit was processed into wine, total folate was conserved and the sugar content and calories drastically decreased.

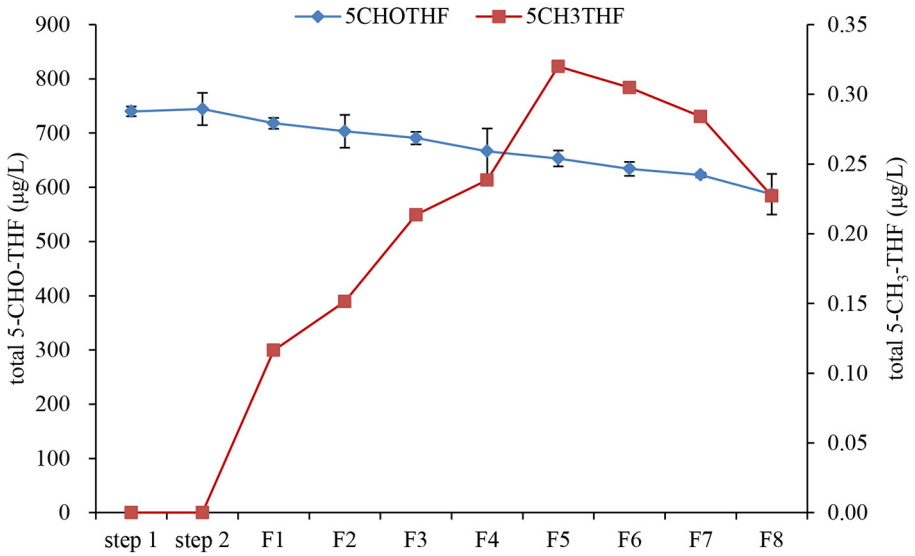


FIGURE 8 | Further changes of folate vitamers and total folate during fermentation step 1: pectinase hydrolysis; step 2: inactivation of pectinase after hydrolysis; F1 to F8: fermentation day 1 to day 8.

CONCLUSIONS

The pectinase-mediated facilitation of the extraction of folate from date palm fruits was characterized using UHPLC-MS/MS analysis. This is the first such study on folate extraction from date palm fruits, demonstrating the influence of the principal variables, pectinase activity, incubation temperature, and incubation time for efficient release of folate from the pectin matrix. It is suggested that, for other fruits and vegetables containing high levels of pectin, pectinase should be considered for improving the extraction efficiency of folate as a cost-effective means of folate extraction.

By applying an optimized RSM methodology, folate vitamer distribution, and total folate content in three cultivars of date palm fruits (Muzafti, Zahdi, and Rubai) were determined. This is the first study to demonstrate that date palm fruits are good sources of dietary folate due to their high-folate content (191–301 μg/100 g) and will add a new ingredient to the USDA Nutrient Database.

Changes in folate vitamers and total folate during date palm fruit wine fermentation were tracked. Two types of folate vitamers were identified as predominant 5-CHO-THF and minor 5-CH_3-THF. Interestingly, during fermentation, 5-CHO-THF decreased by ∼20%, while 5-CH_3-THF first increased from 0 to 0.32 μg/L and then significantly decreased. Further studies are needed to understand the mechanism of the loss of 5-CHO-THF and the generation of 5-CH_3-THF. Additionally, a new strategy should be designed to improve folate stability during fermentation and storage.

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/s.

AUTHOR CONTRIBUTIONS

ZM and LY: methodology, formal analysis, investigation, and writing–original draft preparation. QH and ZL: validation. HR: conceptualization, writing—review, editing, and supervision. CW: conceptualization, writing—review and editing, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Stover PJ. Polymorphisms in 1-carbon metabolism, epigenetics and folate-related pathologies. *J Nutrigenet Nutrige.* (2011) 4:293–305. doi: 10.1159/000334586

2. Busso D, Echeverría G, Passi-Solar A, Morales F, Farías M, Margozzini P. Folate status in women of childbearing age in the Urban Metropolitan Region of Chile: results from the National Health Survey 2016-2017. *Public Health Nutr.* (2021) 24:385–92. doi: 10.1017/S1368980020002608

3. Strobbe S, Van Der Straeten D. Folate biofortification in food crops. *Curr Opin Biotechnol.* (2017) 44:202–11. doi: 10.1016/j.copbio.2016.12.003
4. Scott J, Rébeillé F, Fletcher J. Folic acid and folates: the feasibility for nutritional enhancement in plant foods. *J Sci Food Agric.* (2000) 80:795–824. doi: 10.1002/(SICI)1097-0010(20000515)80:7<795::AID-JSFA599>3.0.CO;2-K
5. Hou SY, Man XX, Lian BY, Ma GF, Sun ZX, Han LD, et al. Folate metabolic profiling and expression of folate metabolism-related genes during panicle development in foxtail millet (Setaria italica (L.) P. *Beauv). J Sci Food Agric.* (2021). doi: 10.1002/jsfa.11355. [Epub ahead of print].
6. Zou YC, Duan HY, Li L, Chen XJ, Wang C. Quantification of polyglutamyl 5-methyltetrahydrofolate, monoglutamyl folate vitamers, and total folates in different berries and berry juice by UHPLC-MS/MS. *Food Chem.* (2019) 276:1–8. doi: 10.1016/j.foodchem.2018.09.151
7. Saini RK, Nile SH, Keum Y-S. Folates: chemistry, analysis, occurrence, biofortification and bioavailability. *Food Res Int.* (2016) 89:1–13. doi: 10.1016/j.foodres.2016.07.013
8. Cavalcanti Albuquerque MA, Yamacita DS, Bedani R, LeBlanc JG, Saad SMI. Influence of passion fruit by-product and fructooligosaccharides on the viability of streptococcus thermophilus TH-4 and lactobacillus rhamnosus LGG in folate bio-enriched fermented soy products and their effect on probiotic survival and folate bio-accessibility under *in vitro* simulated gastrointestinal conditions. *Int J Food Microbiol.* (2019) 292:126–36. doi: 10.1016/j.ijfoodmicro.2018.12.012
9. Estevinho BN, Lazar R, Blaga A, Rocha F. Preliminary evaluation and studies on the preparation, characterization and *in vitro* release studies of different biopolymer microparticles for controlled release of folic acid. *Powder Technol.* (2020) 369:279–88. doi: 10.1016/j.powtec.2020.05.048
10. Luo SY, Duan HY, Zu YC, Qiu RX, Wang C. Quantification of total folate, folate species and polyglutamyl folate distribution in winged beans (Psophocarus tetragonolobus (L) DC) from different cultivars and growth stages by ultra-high performance liquid chromatography tandem mass spectrometry. *J Nutr Sci Vitaminol.* (2017) 63:69–80. doi: 10.3177/jnsv.63.69
11. Aslam J, Khan SH, Khan SA. Quantification of water soluble vitamins in six date palm (Phoenix dactylifera L.) cultivar's fruits growing in Dubai, United Arab Emirates, through high performance liquid chromatography. *J Saudi Chem Soc.* (2013) 17:9–16. doi: 10.1016/j.jscs.2011.02.015
12. Al-Harrasi A, Rehman NU, Hussain J, Khan AL, Al-Rawahi A, Gilani SA, et al. Nutritional assessment and antioxidant analysis of 22 date palm (Phoenix dactylifera) varieties growing in Sultanate of Oman. *Asian Pac J Trop Med.* (2014) 7:S591–8. doi: 10.1016/S1995-7645(14)60294-7
13. Parvin S, Easmin D, Sheikh A, Biswas M, Sharma SCD, Jahan MGS, et al. Nutritional analysis of date fruits (Phoenix dactylifera L.) in perspective of Bangladesh. *Am J Life Sci.* (2015) 3:274–8. doi: 10.11648/j.ajls.20150304.14
14. Al-Alawi RA, Al-Mashiqri JH, Al-Nadabi JSM, Al-Shihi BI, Baqi Y. Date palm tree (Phoenix dactylifera L.): natural products and therapeutic options. *Front Plant Sci.* (2017) 8:845. doi: 10.3389/fpls.2017.00845
15. Matloob MH. Zahdi date vinegar: production and characterization. *Am J Food Tech.* (2014) 9:231–45. doi: 10.3923/ajft.2014.231.245
16. Cho S, Choi Y, Lee J, Eitenmiller RR. Optimization of enzyme extractions for total folate in cereals using response surface methodology. *J Agric Food Chem.* (2010) 58:10781–6. doi: 10.1021/jf102751w
17. Choi Y-M, Eitenmiller RR, Kim S-H, Lee J-S. Optimization of tri-enzyme extraction procedures for the microbiological assay of folate in red kidney bean and roasted peanut using response surface methodology. *Food Sci Biotechnol.* (2009) 18:31–5. doi: 10.1016/j.foodpol.2008.07.001
18. Gmelch L, Wirtz D, Witting M, Weber N, Striegel L, Schmitt-Kopplin P, et al. Comprehensive vitamer profiling of folate mono- and polyglutamates in baker's yeast (Saccharomyces cerevisiae) as a function of different sample preparation procedures. *Metabolites.* (2020) 10:301. doi: 10.3390/metabo10080301
19. Wang C, Riedl KM, Schwartz SJ. A liquid chromatography-tandem mass spectrometric method for quantitative determination of native 5-methyltetrahydrofolate and its polyglutamyl derivatives in raw vegetables. *J Chromatogr B.* (2010) 878:2949–58. doi: 10.1016/j.jchromb.2010.08.043
20. Wang C, Riedl KM, Schwartz SJ. Fate of folates during vegetable juice processing - deglutamylation and interconversion. *Food Res Int.* (2013) 53:440–8. doi: 10.1016/j.foodres.2013.05.011
21. Aprodu I, Dumitraşcu L, Râpeanu G, Bahrim GE, Stănciuc N. Spectroscopic and molecular modeling investigation on the interaction between folic acid and bovine lactoferrin from encapsulation perspectives. *Foods.* (2020) 9:744. doi: 10.3390/foods9060744
22. Brouwer VD, Zhang G-F, Storozhenko S, Van Der Straeten D, Lambert WE. pH stability of individual folates during critical sample preparation steps in prevision of the analysis of plant folates. *Phytochem Anal.* (2007) 18:496–508. doi: 10.1002/pca.1006
23. Xue HK, Tan JQ, Li Q, Tang JT, Cai X. Ultrasound-assisted enzymatic extraction of anthocyanins from raspberry wine residues: process optimization, isolation, purification, and bioactivity determination. *Food Anal Method.* (2021) 14:1369–86. doi: 10.1007/s12161-021-01976-8
24. Liu Y, Wei SL, Liao MC. Optimization of ultrasonic extraction of phenolic compounds from Euryale ferox seed shells using response surface methodology. *IND Crop Prod.* (2013) 49:837–43. doi: 10.1016/j.indcrop.2013.07.023
25. Vajic U-J, Grujic J, Živković J, Savikin K, Godevac D, Miloradović Z, Bugarski B, et al. Optimization of extraction of stinging nettle leaf phenolic compounds using response surface methodology. *Ind Crop Prod.* (2015) 74:912–7. doi: 10.1016/j.indcrop.2015.06.032
26. Finglas P. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. *Trends Food Sci Tech.* (2000) 11:296–7. doi: 10.1016/S0924-2244(01)00010-3
27. Al-Orf SM, Ahmed MHM, Al-Atwai N, Al-Zaidi H, Dehwah A, Dehwah S. Review: nutritional properties and benefits of the date fruits (Phoenix dactylifera L.). *Bull Natl Nutr Instit Arab Republic Egypt.* (2012) 39:98–129.
28. Patring JDM, Jastrebova JA, Hjortmo SB, Andlid TA, Jägerstad IM. Development of a simplified method for the determination of folates in baker's yeast by HPLC with ultraviolet and fluorescence detection. *J Agric Food Chem.* (2005) 53:2406–11. doi: 10.1021/jf048083g
29. Rai AK, Pandey A, Sahoo D. Biotechnological potential of yeasts in functional food industry. *Trends Food Sci Technol.* (2019) 83:129–37. doi: 10.1016/j.tifs.2018.11.016
30. Hjortmo S, Patring J, Jastrebova J, Andlid TA. Biofortification of folates in white wheat bread by selection of yeast strain and process. *Int J Food Microbiol.* (2008) 127:32–6. doi: 10.1016/j.ijfoodmicro.2008.06.001
31. Walkey CJ, Kitts DD, Liu YZ, van Vuuren HJJ. Bioengineering yeast to enhance folate levels in wine. *Process Biochem.* (2015) 50:205–10. doi: 10.1016/j.procbio.2014.12.017
32. Goncerzewicz A, Misiewicz A. The sequence diversity and expression among genes of the folic acid biosynthesis pathway in industrial Saccharomyces strains. *Acta Biochim Pol.* (2015) 62:841–50. doi: 10.18388/abp.2015_1144
33. D'Aimmo MR, Modesto M, Mattarelli P, Biavati B, Andlid T. Biosynthesis and cellular content of folate in bifidobacteria across host species with different diets. *Anaerobe.* (2014) 30:169–77. doi: 10.1016/j.anaerobe.2014.09.018
34. Revuelta JL, Serrano-Amatriain C, Ledesma-Amaro R, Jiménez A. Formation of folates by microorganisms: towards the biotechnological production of this vitamin. *Appl Microbiol Biotechnol.* (2018) 102:8613–20. doi: 10.1007/s00253-018-9266-0
35. Saubade F, Hemery YM, Guyot J-P, Humblot C. Lactic acid fermentation as a tool for increasing the folate content of foods. *Crit Rev Food Sci Nutr.* (2017) 57:3894–910. doi: 10.1080/10408398.2016.1192986

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Changes in Metabolite Patterns During Refrigerated Storage of Lamb's lettuce (*Valerianella locusta* L. Betcke)

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Lamb's lettuce is a popular winter salad, often grown in private vegetable plots, small local farms or in intensive vegetable production. It is usually marketed as a ready-to-eat produce in supermarkets. The aim of the study was to evaluate the changes in biochemical composition and degradation of bioactive compounds during consumer-relevant time of home-grown and store-bought *Valerianella locusta* "Vit" salad. Primary metabolites, assimilatory pigments as well as secondary metabolites were monitored during 1 week of refrigerated storage. Home-grown lamb's lettuce exhibited highest levels of total sugars, total organic acids, vitamin C, and total phenolic content as well as enhanced levels of most individual phenolic compounds and chloroplast pigments. Locally produced samples of lamb's lettuce also contained high levels of analyzed bioactive components. All samples retained most bioactive components during the entire period of refrigerated storage. The results underline the instability of vitamin C during refrigerated storage of lamb's lettuce and pinpoint this parameter as being the most affected by storage.

Keywords: ***Valerianella locusta*, primary metabolites, phenolics, carotenoids, chlorophyll, storage**

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INTRODUCTION

Valerianella locusta L. Betcke or lamb's lettuce is a common minimally processed ready-to-eat vegetable. It can be used either as a leafy salad, as an ingredient in salad mixes or in more complex culinary dishes (1, 2). Traditionally, this appealingly termed vegetable was sown in every kitchen garden or in small local greenhouses and consumed during the colder period of the year. It is extremely tolerant to low temperatures and can even survive under snow cover. Now, increased market demand throughout the year has encouraged producers to grow lamb's lettuce on a larger scale, either in greenhouse cultivation or on open fields, and sell their produce to supermarkets and restaurant supply chains.

Valerianella locusta is known for its balanced mineral and chemical composition resulting in high nutritional value and favorable taste qualities (3). The rosettes contain many bioactive compounds, such as vitamin C, carotenoids, phenols, folic acid, sterols and omega-3 fatty acids similar to other salads (4–7). Długosz-Grochowska et al. (8) performed a detailed compositional study on lamb's lettuce and reported the presence of three folate forms and seven phenolic

compounds. Phenolic acids are the prevalent group of secondary metabolites in *V. locusta*, followed by flavonoid glycosides and free flavonoids (9). Above all, its leaves are one of the richest sources of chlorogenic acid among leafy vegetables and contain very high tetrahydrofolate (also known as vitamin B9) (8) and carotenoid levels (4). During storage bioactive compounds undergo a different rate of degradation and therefore it is preferable that lamb's lettuce, as most leafy salads, is consumed within a week after harvest (9, 10).

Interest for phytonutrient-rich vegetables has increased during the last decades and studies point out that consumers are more and more aware of the benefits of a balanced diet (11). But are pre-packed and ready-to-eat alternatives to home-grown salads really offering the consumer the same portion of health-beneficial compounds? Are the products advertised in supermarkets as locally grown superior to products transported from greenhouses or fields further away? And how do the internal parameters of these products change during storage? Lamb's lettuce (*V. locusta*) "Vit" was chosen as a model plant as it is rich in phenolic compounds, highly perishable and readily available year-round. Moreover, a steady increase of consumer demand for this pre-packed produce can be registered in Europe (12) and on the other hand, its pest free and easy-to-grow characteristics make it one of the most popular salads to grow in private vegetable plots. Home food gardening has received a boost in the last years with more and more people cultivating fresh food (13).

To elucidate the turnover of biochemical compounds in supermarket-bought and home-grown lamb's lettuce this study focused on (1) determining internal quality parameters of different sources of "Vit" lamb's lettuce, and (2) detecting the stability of metabolites during domestic refrigerated storage of lamb's lettuce. A detailed HPLC and HPLC-MS supported analysis of primary and secondary profiles was performed for the period of 1 week to simulate the common consumer's practice of storing lamb's lettuce in sealed plastic bags in household refrigerators. We hypothesized that home-grown samples and locally produced lamb's lettuce are characterized by superior biochemical composition to that of supermarket-bought samples of non-local origin.

MATERIALS AND METHODS

Plant Material

Several bags (4 per treatment, each containing 0.125 kg) of ready-to-eat lamb's lettuce (*V. locusta*) were purchased at three different supermarkets in Ljubljana, Slovenia, at the first day of their commercial life (T0), 24 h after packaging on March 26th 2018 (treatments SM1, SM2, and SM3). All supermarket-bought samples had the same expiry date, were field-grown in an organic production and one of them (SM1) was advertised as a locally grown product. On the same day (March 26th 2018), organic home-grown (HG) lamb's lettuce was collected at a private garden in Lendava, Slovenia (46.56°N, 16.45°E) and transferred immediately to the laboratory facility at Biotechnical faculty in Ljubljana. The cultivation practice for home-grown lamb's lettuce followed the same technological practices applied in large-scale organic vegetable production. All samples were a dark green short leaf "Vit" commercial variety preferred by European consumers and often cultivated on a garden scale. Each treatment was combined into a single batch containing 0.500 kg lamb's lettuce. Prior to analyses, the rosettes were washed in distilled water and gently tapped dry with tissue paper. A portion of each treatment/batch was subjected to immediate analyses on day 1 (T0). To mimic common domestic use, the rest of the batch was stored in plastic bags (4 L) with a zipper, deposited in a refrigerator at 4°C and reopened and analyzed after 2 days (T2), 4 days (T4), and 7 days (T7). On each sampling date, five individual portions (each containing seven rosettes) were ground with liquid nitrogen and all chemical analyses were performed on this source material ($n = 5$).

Extraction and Determination of Sugars and Organic Acids

Rosettes (1.5 g) were homogenized in 2 ml of double distilled water with an Ultra-Turrax T-25 (Ika-Labortechnik). Extraction was performed at room temperature with constant stirring for 30 min (14). Samples were centrifuged (Eppendorf Centrifuge 5810 R) at 10,621 × g for 10 min at 10°C and the supernatant was filtered through 0.20 μm cellulose ester filters (Macherey-Nagel, Germany) into vials. Five repetitions were carried out ($n = 5$) per treatment and sampling; each repetition consisted of several rosettes. Primary metabolites were analyzed on a high-performance liquid chromatography (HPLC) system (Thermo Separation Products, San Jose, CA, USA) with injection volume of 20 µl and flow rate maintained at 0.6 ml min^{-1}. Sugar separation was carried out on a Rezex RCM-monosaccharide column from Phenomenex (Ca+ 2%) at 65°C (300 × 7.8 mm). Double distilled water was used as a mobile phase, total run time was 30 min, and a refractive index (RI) detector was used for carbohydrate detection as described by Weber et al. (15). Analyses of organic acids were performed with a UV detector at 210 nm and 65°C with total run time of 30 min and flow rate of 0.6 ml min^{-1}. A Rezex ROA-organic acid [H+ (8%)] column from Phenomenex (300 × 7.8 mm) was used as described by Mikulic-Petkovsek et al. (14) and Weber et al. (15). The elution solvent was 4 mM sulfuric acid in double distilled water. A PDA detector was used to monitor the eluted organic acids.

Corresponding external standard curves were used for quantification of primary metabolites and all compounds were expressed in mg g^{-1} fresh weight (FW). Total sugar content, total organic acids content and sugar/organic acid ratio were calculated as sums (or ratios) of all detected sugars or organic acids.

Extraction and Determination of Vitamin C

Vitamin C was determined by separately detecting the amount of L-ascorbic acid (AA) and L-dehydroascorbic acid (DHA) according to the method described by Helland et al. (16), with some modifications. Fresh leaves of lamb's lettuce were ground to a powder using liquid nitrogen. For the analysis of AA 1.5 g of leaf powder was extracted with 2 ml of ice-cold 6% *m*-phosphoric acid and for the analysis of DHA with 2 ml of ice-cold 6% *m*-phosphoric acid containing 4 mM EDTA. Extraction was performed with homogenisation on a shaker for 30 s.

A similar HPLC method as for other organic acids was utilized for the quantification of ascorbic. However, it was operated at room temperature and at a different wavelength (245 nm). The identification and quantification were achieved with comparison of the retention time and corresponding external standard curves.

DHA was calculated by subtracting the ascorbic acid content in reduced extract from that of the non-reduced extract. Vitamin C content was defined as the sum of AA and DHA contents.

Extraction of Phenolic Compounds

Five repetitions were carried out ($n = 5$) per treatment and sampling date; each repetition included several rosettes. Rosettes (1.5 g) were chopped and extracted with 2 ml methanol containing 3% (v/v) formic acid in an iced ultrasonic bath for 1 h as described by Mikulic-Petkovsek et al. (14) and Weber et al. (15). Then, the extracts were centrifuged at 10,621 × g at 4°C for 10 min, filtered through 0.2 μm Chromafil AO-20/25 polyamide filters (Macherey-Nagel, Germany) and transferred to vials. Identical samples were used for determination of total phenolic content.

Determination of Individual Phenolic Compounds Using HPLC-DAD-MS^n Analysis

Thermo Finnigan Accela HPLC system (Thermo Scientific, San Jose, USA) coupled with mass spectrometer with a diode array detector at 280 nm (derivatives of hydroxycinnamic acids) or 350 nm (flavones, flavonols, and flavanone) were used for identification of individual phenolics as described by Weber et al. (15). Spectra were recorded between 200 and 600 nm. A 150 × 4.6 mm i.d., 3 μm, Gemini C_{18} (Phenomenex, Torrance, CA, USA) column operated at 25°C was used. The elution solvents were (A) 3% acetonitrile with 0.1% formic acid in double distilled water (v/v/v) and (B) 3% double distilled water with 0.1% formic acid in acetonitrile (v/v/v). Samples were eluted according to the linear gradient described by Weber et al. (15). The injection amount was 20 μl and the flow rate maintained at 0.6 ml min^{-1}.

Phenolics were identified on a mass spectrometer with electrospray ionization (ESI) operating in negative ion mode using full scan data-dependent MS^n scanning from *m/z* 115 to 1,500. Operating conditions were previously reported by Weber et al. (15). Spectrometric data were elaborated using the Excalibur software (Thermo Scientific) and retention times, UV spectra and MS^n fragmentation were used for compound identification (SM, **Table 1**). Quantification was achieved by comparing peak areas of the sample and corresponding standard curves. Compounds were expressed in mg kg^{-1} fresh weight (FW). For compounds lacking standards, quantitation was carried out using comparable compounds. Therefore, luteolin-pentosylhexoside, kaempferol-3-*O*-rutinoside, genistin, hesperidin and diosmetin were quantitated in equivalents of luteolin-4'-*O*-glucoside.

Total Phenolic Content

Total phenolic content (TPC) was determined using Folin-Ciocalteau (FC) reagent (five replicates per sampling date) as described by Mikulic-Petkovsek et al. (17). Gallic acid was used as standard and TPC was expressed in mg of gallic acid equivalents (GAE) per kg of fresh weight (FW).

Extraction and Determination of Chloroplast Pigments

The extraction of chloroplast pigments from lamb's lettuce (1.5 g) was performed with 2 ml ice-cold acetone under dimmed light as described by Sircelj and Batic (18). The extracts were filtered through 0.2 μm polyamide filters and immediately analyzed on the HPLC system.

Analysis of individual carotenoids was carried out on a HPLC system with a DAD detector at 450 nm. Separation of samples was achieved on a Gemini C_{18} column at 25°C and flow rate was maintained at 1 ml min^{-1}. The gradient was as follows: from 10 to 70% B in the first 18 min, then linearly to 70% B to 22 min and returning to the initial conditions to the end of the run. Mobile phase A was acetonitrile, double distilled water and methanol (100/10/5; v/v/v) and mobile phase B was acetone with ethyl acetate (2/1; v/v).

Individual carotenoids were further determined on a TSQ Quantum Access Max quadrupole mass spectrometer as previously described by Senica et al. (19). The chromatographic conditions were the same as described above. The MS instrument was operated using an atmospheric pressure chemical ionization (APCI) source in positive ion mode. The APCI parameters were as follows: vaporizer temperature 450°C, capillary temperature 320°C, corona voltage 4.0 kV, sheat gas 55 L/h, auxiliary gas 10 L/h. Mass spectra were scanned in range from *m/z* 70 to 650 and argon was used as collision gas. Data acquisition was performed using Xcalibur 2.2 software. Identification was achieved with mass spectra scans, fragmentation, retention times and spectral properties of target compounds (SM, **Table 2**).

Chemicals and Products

The following standards were used for determination of sugars and organic acids: sucrose, fructose and glucose, as well as citric, malic, oxalic, tartaric and fumaric, ascorbic acid from Fluka Chemie (Buchs, Switzerland); shikimic and quinic acid from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Standards for phenolic compounds were acquired from Sigma-Aldrich Chemicals (3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, luteolin-4'-*O*-glucoside, quercetin-3-*O*-rutinoside).

Zeaxanthin, chlorophyll a and b, β-carotene and lutein were from Sigma-Aldricht Chemie (Steinheim, Germany) and neoxanthin, violaxanthin, antheraxanthin and β-cryptoxanthin from DHI LAB Product (Hørsholm, Denmark). Methanol for the extraction of phenolics was obtained at Sigma-Aldrich Chemicals. The chemicals for the mobile phase were HPLC-MS grade acetonitrile, sulphuric acid and formic acid from Sigma-Aldrich Chemicals. Water for the mobile phase was double distilled and purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Statistical Analyses

Data were statistically analyzed with the program Statgraphics Centurion XVII (Manugistics, Inc., Rockville, MD, USA) using

TABLE 1 | Individual sugars, organic acids, and vitamin C levels (mg kg^{-1} FW ± SE) of different *V. locusta* "Vit" samples prior to refrigerated storage (T0).

	Sample source[a]				
Compound	**HG**	**SM 1**	**SM 2**	**SM 3**	**Significance**
Fructose	2091.0 ± 108.1^{a}	735.4 ± 100.2^{b}	224.9 ± 7.5^{c}	267.7 ± 15.2^{c}	***
Glucose	5227.5 ± 270.2^{a}	1838.4 ± 250.5^{b}	562.2 ± 18.8^{c}	669.4 ± 38.1^{c}	***
Sucrose	59.7 ± 6.3^{b}	214.4 ± 81.9^{a}	82.0 ± 4.4^{b}	280.8 ± 23.6^{a}	***
Citric acid	1746.9 ± 298.0^{a}	1399.9 ± 208.2^{ab}	1289.0 ± 74.8^{ab}	1199.9 ± 76.4^{b}	**
Fumaric acid	66.6 ± 4.0^{a}	50.0 ± 7.5^{a}	37.3 ± 2.1^{b}	33.1 ± 1.2^{b}	***
Malic acid	5721.8 ± 219.0^{a}	5056.3 ± 695.9^{a}	3409.4 ± 145.1^{b}	3161.4 ± 150.4^{b}	***
Oxalic acid	63.0 ± 3.8	64.5 ± 9.1	63.0 ± 3.8	58.0 ± 2.8	NS
Quinic acid	1701.2 ± 109.6^{a}	1226.1 ± 210.1^{b}	776.8 ± 42.8^{c}	795.0 ± 48.9^{c}	***
Shikimic acid	6.5 ± 0.2^{a}	5.2 ± 0.7^{a}	3.7 ± 0.2^{b}	3.7 ± 0.4^{b}	***
Tartaric acid	232.9 ± 49.7^{a}	194.0 ± 43.4^{b}	226.1 ± 11.3^{a}	207.4 ± 9.2^{ab}	***
Vitamin C	406.6 ± 54.1^{a}	239.8 ± 62.8^{ab}	214.2 ± 24.2^{b}	116.9 ± 26.8^{b}	***

[a]*Sample source: HG, home-grown sample; SM1, locally-grown supermarket-bought sample; SM2, supermarket-bought sample; SM3, supermarket-bought sample.*
*Different letters (a-c) denote statistically significant differences in each compound among V. locusta "Vit" sample sources by Duncan's multiple range test at ***$p < 0.001$, **$p < 0.01$, and NS, non-significant. Standard error (SE) following each mean represents the standard deviation of sampling distribution ($n = 5$).*

one-way analysis of variance (ANOVA). Differences between samples, separately for each sampling date, were estimated using the Duncan multiple range test. *P*-values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Sugars, Organic Acids, and Vitamin C

The content of individual sugars, organic acids and vitamin C in *V. locusta* leaves during 1-week refrigerated storage were studied for the first time. In all samples analyzed in our study glucose was present in highest amounts, followed by fructose and significantly lower levels of sucrose (**Table 1**). The content of sucrose in lamb's lettuce was comparable to that of baby leaf lettuce reported by Spinardi and Ferrante (20). Similar sugar composition of lamb's lettuce was also described by Enninghorst and Lippert (1), but their research did not report specific contents of these primary metabolites. Recently, sugar levels in lamb's lettuce were analyzed by anthrone colorimetric method, which detects the total amount of soluble sugars in the matrix but fails to specify the content of individual carbohydrates (3, 6). Their results are comparable to the sum of individual sugars, identified in our samples (**Figure 1A**).

Regardless of the sampling date, HG lamb's lettuce was characterized with up to almost three-fold levels of total sugars (TS) compared to SM1 sample, which was second richest in this group of primary metabolites. Significantly lower levels of TS were recorded in SM2 and SM3 samples and both supermarket-bought samples also demonstrated a comparable sugar turnover during refrigerated storage (**Figure 1A**). Although an ∼36% decrease of TS was detected on the 7th day of HG lamb's lettuce storage, this sample still contained the highest levels of TS compared to other *V. locusta* sources on the last sampling. Comparably, Braidot et al. (4) measured 40% less glucose in lamb's lettuce on the sixth day of refrigerated storage which is consistent with the results on total sugars in our study (**Figure 1A**). Decreased levels of carbohydrates during the postharvest storage period have directly been linked to lamb's lettuce respiration for which the sugars represent the main substrates (1, 20). Similar findings have been reported on shredded cabbage (21) and turnip (16) and the authors clarified the carbohydrate depletion in samples with increased respiration as well as decomposition correlating with longer storage period. The processes have been extensively studied by McKenzie et al. (22) who monitored ethanolic and lactic fermentation pathway in asparagus and broccoli during storage and linked these with reduced levels of glucose, fructose, and sucrose in different cell compartments.

Extensive analysis of organic acids in various fruits and vegetables, including lamb's lettuce, was undertaken by Flores et al. (23), who detected 10 different compounds in this group of metabolites in fresh *V. locusta* leaves albeit several were only present in traces. All samples analyzed in the present study contained highest amounts of citric and malic acids and lower levels of fumaric, oxalic, quinic, shikimic and tartaric acids (**Table 1**), which is consistent with the report of Flores et al. (23). Comparable (and highest) levels of total organic acids (TOA) were measured in HG and SM1 samples during the entire period of refrigerated storage. Only on day 4 did we detect significantly higher levels of TOA in HG sample compared to locally grown supermarket-bought lamb's lettuce. Initial levels of TOA were significantly lower in SM2 and SM3 samples, but the time trend of TOA was similar for both sources with an exception on the second sampling. An increase in TOA was recorded on the second and fourth day of storage in most *V. locusta* samples followed by a decrease on day 7 (**Figure 1B**). TOA pattern (mostly consisting of citric and malic acid in *V. locusta* rosettes) corresponds to the report of Tsuchida et al. (24), who monitored organic acid metabolism during cucumber storage. The authors reported a dramatic increase of malic and citric acid in first 3 days of cold storage, followed by decreased levels of these organic acids at later samplings. The final negative turnover of TOA has been

TABLE 2 | Individual phenolic compounds (mg kg^{-1} FW ± SE) in different *V. locusta* "Vit" samples during refrigerated storage.

		3-CQA[a]	5-CQA	4-CQA	Q-rut	Lut-pentosylhex	K-rut	Genistin	Hesperidin	Diosmetin	Di CQA hex	CQA hex
T0[b]	HG[c]	7.53 ± 0.74^{c}	1043.88 ± 39.80^{a}	56.23 ± 2.02^{a}	11.02 ± 0.37^{a}	36.85 ± 1.59^{a}	196.14 ± 9.97^{a}	10.32 ± 0.33^{a}	34.68 ± 1.18^{b}	18.89 ± 1.39^{b}	102.53 ± 3.29^{b}	0.95 ± 0.16^{d}
	SM 1	8.12 ± 0.99^{bc}	831.22 ± 48.45^{b}	46.81 ± 2.69^{b}	7.96 ± 0.33^{b}	19.88 ± 0.91^{b}	67.42 ± 5.07^{b}	5.65 ± 0.37^{b}	50.99 ± 0.83^{a}	25.77 ± 0.73^{c}	146.69 ± 11.37^{a}	9.11 ± 0.58^{a}
	SM 2	10.75 ± 0.86^{ab}	423.42 ± 33.14^{c}	21.14 ± 1.15^{c}	4.67 ± 0.27^{c}	5.86 ± 0.41^{d}	18.33 ± 1.61^{c}	2.02 ± 0.15^{c}	21.92 ± 1.12^{c}	10.98 ± 0.51^{bc}	54.06 ± 3.73^{c}	3.11 ± 0.28^{c}
	SM 3	13.76 ± 1.51^{c}	730.15 ± 69.84^{b}	42.96 ± 3.88^{b}	5.38 ± 0.29^{c}	9.05 ± 0.90^{c}	27.77 ± 2.66^{c}	1.70 ± 0.22^{c}	31.39 ± 3.12^{b}	15.27 ± 2.46^{b}	97.84 ± 10.71^{b}	6.46 ± 0.73^{b}
		***	***	***	***	***	***	***	***	***	***	***
T2	HG	6.70 ± 0.32^{c}	1014.05 ± 58.18^{a}	56.54 ± 1.79^{a}	11.92 ± 0.49^{a}	36.85 ± 1.59^{a}	214.34 ± 4.55^{a}	10.67 ± 0 42^{a}	44.77 ± 0.84^{a}	19.83 ± 0.49^{a}	116.61 ± 29.48^{ab}	8.18 ± 0.54^{b}
	SM 1	10.59 ± 1.72^{b}	885.02 ± 222.80^{ab}	51.53 ± 1.13^{b}	9.57 ± 0.35^{b}	19.88 ± 0.91^{b}	93.35 ± 11.53^{b}	5.96 ± 0.26^{b}	44.11 ± 1.27^{a}	21.79 ±1.10^{a}	159.58 ± 10.44^{a}	9.38 ± 0.34^{a}
	SM 2	9.12 ± 0.37^{bc}	453.19 ± 33.14^{c}	24.72 ± 2.14^{d}	4.85 ± 0.27^{c}	5.86 ± 0.41^{d}	18.55 ± 1.89^{c}	2.00 ± 0.14^{c}	31.02 ± 2.56^{c}	13.06 ± 1.17^{c}	79.55 ± 9.42^{c}	4.59 ± 0.49^{cd}
	SM 3	14.26 ± 0.99^{a}	639.14 ± 11.77^{bc}	35.11 ± 1.44^{c}	5.37 ± 0.48^{c}	9.05 ± 0.90^{c}	30.06 ± 0.77^{c}	2.53 ± 0.13^{c}	38.58 ± 0.28^{b}	16.26 ± 0.70^{b}	83.41 ± 4.13^{b}	5.69 ± 0.23^{c}
		***	*	***	***	***	**	***	***	***	*	***
T4	HG	7.12 ± 0.65	1048.41 ± 41.87^{a}	52.84 ± 2.28^{a}	12.11 ± 0.85^{a}	36.27 ± 2.30^{a}	200.88 ± 12.02^{a}	10.69 ± 0.68^{a}	32.69 ± 0.94^{a}	19.22 ± 0.70^{a}	149.56 ± 8.82^{a}	8.26 ± 0.45a
	SM 1	6.61 ± 0.93	896.28 ± 27.56^{b}	44.64 ± 2.28^{b}	7.09 ± 0.42^{b}	15.45 ± 1.18^{b}	72.22 ± 5.52^{b}	4.54 ± 0.27^{b}	30.41 ± 1.63^{ab}	18.27 ± 0.90^{b}	153.4 ± 7.14^{a}	7.77 ± 0.61^{ab}
	SM 2	13.13 ± 2.96	490.88 ± 38.36^{d}	26.65 ± 2.18^{c}	5.30 ± 0.32^{c}	7.20 ± 1.20^{c}	23.63 ± 5.94^{c}	2.05 ± 0.34^{c}	22.18 ± 1.37^{c}	12.93 ± 0.65^{b}	82.02 ± 8.15^{c}	4.38 ± 0.48^{c}
	SM 3	9.64 ± 2.27	753.57 ± 27.74^{c}	45.77 ± 1.20^{b}	5.43 ± 0.09^{c}	9.14 ± 0.78^{c}	29.10 ± 1.83^{c}	1.92 ± 0.22^{c}	27.35 ± 1.39^{b}	12.61 ± 3.22^{a}	114.99 ± 3.75^{b}	6.44 ± 0.37^{b}
		NS	***	***	***	***	***	***	***	*	***	***
T7	HG	9.29 ± 0.32	1067.30 ±39.52^{a}	51.51 ± 1.76^{a}	11.00 ± 0.42^{a}	32.32 ± 0.92^{a}	191.47 ± 8.35^{a}	9.05 ± 0.17^{a}	31.04 ± 1.62	16.69 ± 0.69	156.56 ± 6.26^{a}	8.13 ± 0.70^{a}
	SM 1	9.20 ± 0.93	962.78 ± 35.59^{b}	48.79 ± 1.00^{ab}	7.82 ± 0.36^{b}	15.36 ± 1.28^{b}	62.63 ± 6.10^{b}	4.77 ± 0.31^{b}	37.00 ± 3.41	18.17 ± 1.76	185.17 ± 7.47^{a}	8.73 ± 0.28^{a}
	SM 2	12.01± 1.28	456.89 ± 21.58^{d}	23.66 ± 0.95^{c}	5.22 ± 0.48^{c}	6.60 ± 0.55^{c}	20.74 ± 0.97^{c}	1.84 ± 0.09^{c}	23.53 ± 3.44	12.20 ± 1.69	94.25 ± 4.03^{c}	5.30 ± 0.87^{b}
	SM 3	11.72 ± 0.99	776.57 ± 39.23^{c}	43.84 ± 3.04^{b}	5.03 ± 0.19^{c}	9.14 ± 0.31^{c}	31.60 ± 1.21^{c}	2.14± 0.18^{c}	29.75 ± 1.62	13.94 ± 1.90	109.71 ± 6.22^{b}	7.33 ± 0.86^{bc}
		NS	***	***	***	***	***	***	NS	NS	***	*

[a] *Compound identification: 5-CQA, 5-O-caffeoylquinic acid; 3-CQA, 3-O-caffeoylquinic acid; 4-CQA, 4-O-caffeoylquinic acid (cryptochlorogenic acid); Q-rut, quercetin-3-rutinoside; Lut-pentosylhex, luteolin-pentosylhexoside; K-rut, kaempferol-3-O-rutinoside; Di CQA hex, di-caffeoylquinic acid hexoside; CQA hex, caffeoylquinic acid hexoside.*

[b] *Sampling dates: T0, immediately after purchase or harvest, prior to refrigerated storage; T2, two days in refrigerated storage; T4, four days in refrigerated storage; T7, days in refrigerated storage.*

[c] *Sample source: HG, home-grown sample; SM1, locally grown supermarket-bought sample; SM2, supermarket-bought sample; SM3, supermarket-bought sample.*

*Different letters (a-d) denote statistically significant differences in each individual compound among V. locusta "Vit" sample sources by Duncan's multiple range test at ***$p < 0.001$, **$p < 0.01$,*$p < 0.05$ and NS, non-significant separately for each sampling. Standard error (SE) following each mean represents the standard deviation of sampling distribution ($n = 5$).*

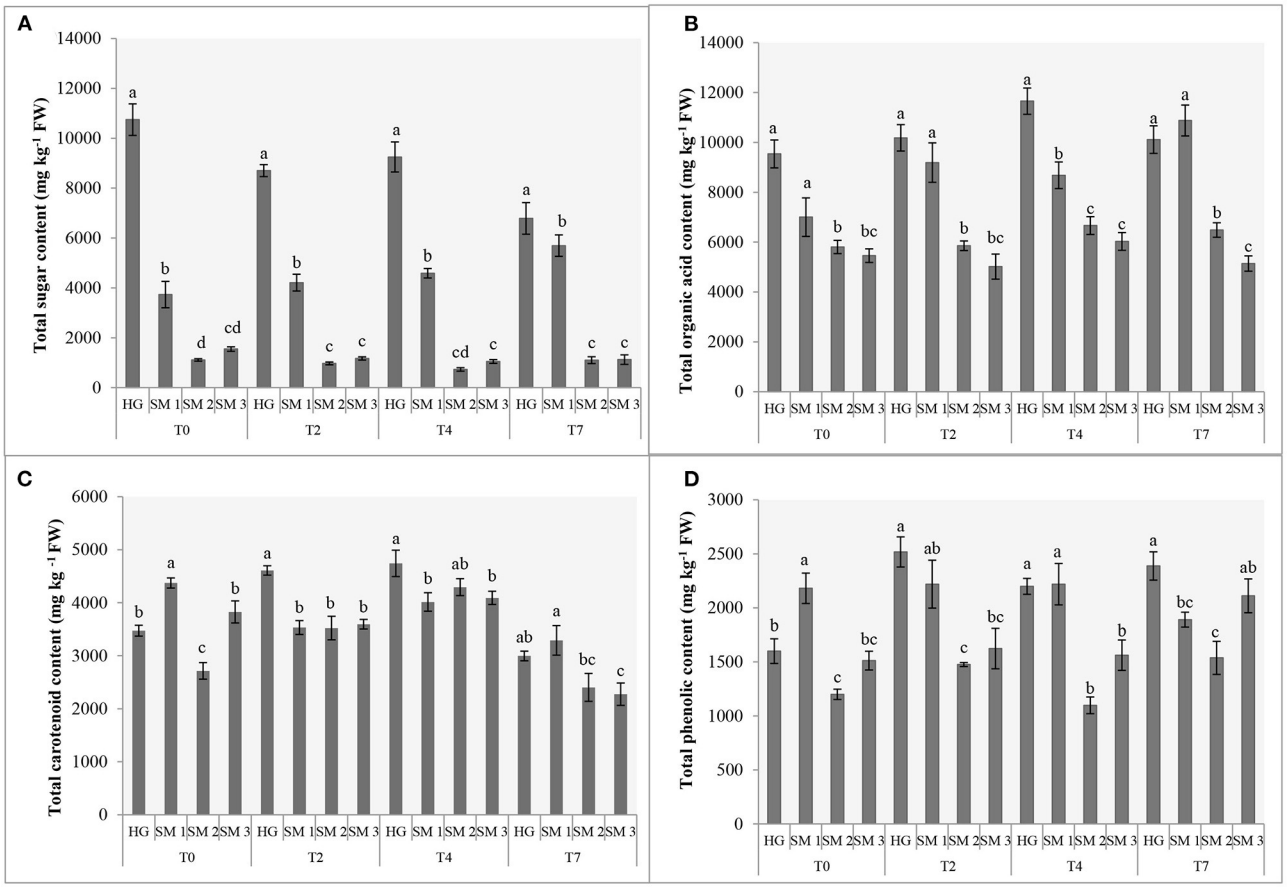

FIGURE 1 | Sums (mg kg^{-1} FW) of sugars **(A)**, organic acids **(B)** and carotenoids **(C)** and total phenolic content **(D)** of different sources of lamb's lettuce subjected to refrigerated storage (T0, prior to storage; T2, 2 days in refrigerated storage; T4, 4 days in refrigerated storage; T7, 7 days in refrigerated storage). Different letters (a-d) above bars denote statistically significant differences in each parameter among *V. locusta* "Vit" sample sources by Duncan's multiple range test at $P < 0.05$ separately for each sampling. Standard errors (SE) are presented with bars within each column.

linked to reduced activity of citrate synthase and the role of the enzyme in the Krebs cycle.

Lamb's lettuce is characterized by high ascorbate content compared to other leafy salads (25). The highest levels of vitamin C were detected in HG samples during the entire period of refrigerated storage, followed by SM1 sample (**Table 1**). Lowest levels of vitamin C were in SM2 and SM3 samples on all sampling dates. The evolution of vitamin C during storage was comparable among all samples and an average of 40% decrease was measured at the end of refrigerated storage. These data are in accordance with the report of Ferrante et al. (25) and Preti and Vinci (26), who detected from 44 to 49% decrease of AA 7 days after *V. locusta* rosettes were stored in a refrigerator. A loss of vitamin C (particularly ascorbic acid) in vegetables during postharvest handling procedures and storage has been reported in other papers, for instance on spinach (27), green beans (28), Japanese radish and green pepper (29) and baby lettuce (20). Vitamin C and specifically, ascorbic acid assimilation in plant tissue is associated with the metabolism of carbohydrates (30) and Wojciechowska et al. (3) confirmed a slight positive correlation between the content of sugars and ascorbic acid in the leaves of lamb's lettuce. A similar correlation was not confirmed for vitamin C in our study. The initial levels of vitamin C in our samples of lamb's lettuce were similar to the results of Wojciechowska et al. (3) as well as Colonna et al. (31). The decrease in ascorbate content is expected during handling and storage of vegetables and has been ascribed to its water solubility, thermic degradation and enzymatic oxidation (32).

Total Phenolic Content

The content of phenolics was determined with the standard Folin-Ciocalteu assay which revealed significant differences among the sources of lamb's lettuce subjected to refrigerated storage (**Figure 1D**). Highest TPC (2,181 mg kg^{-1} GAE FW) of fresh samples was determined in SM1 sample, followed by home-grown *V. locusta* rosettes (1,599 mg kg^{-1} GAE FW). The values were in range with previously reported data on lamb's lettuce (6, 8, 33) but significantly lower compared to the TPC reported by Hawrylak-Nowak et al. (34), who measured from 4,000 to 7,000 mg kg^{-1} GAE FW in lamb's lettuce subjected to high temperature stress. Interestingly, TPC increased on the third day of storage in all samples

and then either remained constant or increased further. The results are in accordance with the report of Preti and Vinci (26) who measured equivalent content of phenolics in lamb's lettuce during a 7-day refrigerated storage. Similarly, Myojin et al. (29) determined increased TPC of shredded red and white cabbage during a 7-day refrigerated storage and Santos et al. (11) reported a comparable TPC turnover of fresh-cut aromatic herbs. Higher TPC of fresh herbs was tentatively explained with stress-related formation of phenolics following postharvest wounding. Galani et al. (32) linked increased synthesis of phenolic compounds in vegetables under low temperature stress during storage with up-regulated activity of phenylalanine ammonialyase, coupled with low level of polyphenoloxidase activity reducing the oxidation level of phenolic substrates.

Individual Phenolic Compounds

Twelve phenolic compounds were identified in all samples of lamb's lettuce: five compounds from the group of hydroxycinnamic acids (all derivatives of caffeoylquinic acid), two flavonols (quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside), two flavones (luteolin-pentosyl hexoside, diosmetin), an isoflavone (genistin) and a flavanone (hesperidin). Hydroxycinnamic acids (particularly 5CQA) represented the most abundant phenolic class in *V. locusta*, regardless of sample origin or duration of refrigerated storage (**Table 2**). Similarly, Grzegorzewski et al. (9), Ramos-Bueno et al. (5) and Długosz-Grochowska et al. (6) measured highest levels of 5CQA in lamb's lettuce. The levels of this compound exceeded 1,000 mg kg^{-1} FW in some of our samples, which is consistent with the report of Długosz-Grochowska et al. (8). Other phenolic acids, specifically 3CQA and 4CQA were present in much smaller amounts (**Table 2**) and have been reported in lamb's lettuce for the first time. The levels of rutin and diosmetin were in range with the reports of Długosz-Grochowska et al. (6) but, contrary, higher levels of hesperidin were detected in our samples.

The highest content of 5CQA was detected in HG samples regardless of sampling date. Levels of hydroxycinnamic acids remained constant or even increased during refrigerated storage of lamb's lettuce. Interestingly, comparable content of most individual phenolic compounds was detected in *V. locusta* samples during refrigerated storage. For instance, the content of kaempferol-3-*O*-rutinoside increased in all samples during the first 4 days in the refrigerator which is in accordance with the study of DuPont et al. (35). The authors evaluated compositional changes of lettuce and endive during cold storage and detected a net gain of the prevalent form of kaempferol and explained it with a release of this component from an unidentified precursor. A similar pattern was also reported by Santos et al. (11), who studied phenolic turnover in fresh-cut aromatic herbs. An increase in phenolic compounds during cold storage of *V. locusta* samples may be explained by the activation of phenylalanine ammonia lyase—a key enzyme in biosynthetic pathway, which is triggered by cold-stress conditions (36). Ferrante et al. (25) also proposed that the increase of phenols may counteract the loss of ascorbic acid and balance the shift in antioxidant status of stored rosettes.

Chloroplast Pigments

Two groups of chloroplast pigments were determined in *V. locusta* samples, namely chlorophylls and carotenoids. Chlorophyll a was the prevalent form of chlorophyll pigments in all samples followed by chlorophyll b, regardless of storage days (**Table 3**). Home-grown sample initially contained highest levels of total chlorophyll but during refrigerated storage this trait was no longer consistent. Total chlorophyll content was in accordance with previously reported data on lamb's lettuce (25, 34). In addition to chlorophyll a and b, pheophytin was also detected in *V. locusta* samples like in the study of Braidot et al. (4). The molecule is structurally analogous to chlorophyll but lacks the central Mg^{2+} ion and is often present as a degradation product of chlorophyll a (37).

The content of chlorophylls in most *V. locusta* samples was significantly reduced only after 7 days of refrigerated storage (**Table 3**). Correspondingly, Braidot et al. (4) detected deleterious effects of storage on pigment contents of *V. locusta* samples after at least 5 days of storage. A different methodological approach in evaluating chlorophyll turnover was undertaken by Manzocco et al. (38), who measured decreased SPAD index on approx. 10th day in refrigerated storage. The reduction of chlorophyll content during cold storage of *V. locusta* seems to be very slow and depends on the duration and storage parameters (25, 39). Similar results were reported for Swiss chard, rocket and baby lettuce (20, 25, 40).

Carotenoids are potent antioxidant molecules and among them lutein is frequently found in leafy vegetables (5). In addition to this carotenoid, six other forms of assimilation pigments were confirmed in *V. locusta* samples: antheraxanthin, β-cryptoxanthin, β-carotene, neoxanthin, violaxanthin and zeaxanthin. Carotenoid identification was in accordance with the study of Ramos-Bueno et al. (5) except for antheraxanthin, which has been determined in lamb's lettuce for the first time. Neoxanthin was the most abundant carotenoid in all samples, followed by lutein (**Table 3**). Similar carotenoid composition and content were reported by Długosz-Grochowska et al. (6) apart from higher levels of β-carotene in their study. However, the transitory nature of this carotenoid may explain higher levels of violaxanthin and zeaxanthin in our samples as β-carotene is an important intermediate in formation of xanthophylls (41). Generally, the highest sum of carotenoids was monitored in home-grown and locally grown rosettes, but the significance was not detected on all samplings (**Figure 1C**). Carotenoids proved similarly stable during the first 5 days of storage compared to chlorophyll pigments, but a decrease after this initial period has been detected in our samples. Consistently, Ferrante et al. (25) and Spinardi and Ferrante (20) reported the reduction of carotenoid content in *V. locusta* rosettes only after 8 days of storage at 4°C or even 10°C but Kołton et al. (33) failed

TABLE 3 | Individual carotenoids (mg kg^{-1} FW ± SE) in different *V. locusta* "Vit" samples during refrigerated storage.

Date		Antheraxanthin	β-cryptoxanthin	β-carotene	Chlorophyll a	Chlorophyll b	Lutein	Neoxanthin	Pheophytin	Violaxanthin	Zeaxanthin
T0[a]	HG[b]	140.05 ± 11.41	8.02 ± 0.81.	7.90 ± 0.89[a]	2030.84 ± 330.16[a]	909.02 ± 46.57[a]	1381.51 ± 174.30[b]	2163.89 ± 89.33	26.67 ± 5.66	130.15 ± 12.30[b]	40.71 ± 3.59[b]
	SM 1	168.89 ± 19.02	13.04 ± 1.65	1.15 ± 0.06[b]	783.90 ± 175.21[b]	624.28 ± 21.13[a]	1926.34 ± 146.99[a]	2212.23 ± 67.29	35.36 ± 3.11	183.30 ± 3.45[b]	67.15 ± 3.00[a]
	SM 2	117.88 ± 17.78	10.59 ± 2.59	1.85 ± 0.07[b]	1003.37 ± 445.19[b]	567.45 ± 67.80[a]	1500.36 ± 87.93[ab]	1870.31 ± 61.36	32.00 ± 1.04	142.82 ± 5.91[b]	70.60 ± 7.01[a]
	SM 3	161.53 ± 2.27	16.26 ± 0.40	0.70 ± 0.03[b]	419.82 ± 13.85[b]	144.41 ± 6.31[b]	1538.50 ± 14.86[ab]	1742.74 ± 71.99	15.23 ± 1.96	288.42 ± 15.49[a]	78.42 ± 1.49[a]
		NS	NS	*	**	**	**	NS	NS	**	**
T2	HG	93.16 ± 5.88	5.28 ± 0.12[ab]	2.00 ± 0.24	1426.02 ± 174.02[b]	771.43 ± 62.05[b]	1432.81 ± 51.50	2600.80 ± 72.68[a]	11.49 ± 2.22	62.22 ± 2.14[b]	52.24 ± 8.27
	SM 1	62.16 ± 2.18	1.91 ± 0.23[c]	4.53 ± 0.15	2288.26 ± 408.07[ab]	1148.67 ± 42.30[ab]	1436.76 ± 89.59	1741.13 ± 82.88[b]	16.63 ± 3.43	37.52 ± 2.37[b]	48.07 ± 2.48
	SM 2	70.22 ± 6.11	7.09 ± 0.69[a]	2.59 ± 0.18	3307.13 ± 722.86[a]	1467.46 ± 98.91[a]	1561.34 ± 91.54	1966.82 ± 52.52[b]	10.90 ± 4.16	54.23 ± 2.62[b]	60.26 ± 5.79
	SM 3	79.19 ± 5.00	4.24 ± 0.29[bc]	1.33 ± 0.45	1456.28 ± 233.91[b]	881.28 ± 62.05[b]	1472.29 ± 32.11	1903.96 ± 60.96[b]	7.33 ± 0.70	81.37 ± 4.94[a]	52.48 ± 2.40
		NS	**	NS	*	*	NS	**	NS	*	NS
T4	HG	80.68 ± 9.40[b]	6.61 ± 0.14b[c]	1.76 ± 0.16[a]	4424.45 ± 317.35[a]	1673.16 ± 16.59[a]	1991.62 ± 171.70[a]	2369.35 ± 164.82[a]	26.79 ± 3.90	37.14 ± 2.45[d]	39.51 ± 1.47[b]
	SM 1	72.48 ± 3.69[b]	5.42 ± 0.15[c]	0.39 ± 0.02[b]	1866.76 ± 151.29[b]	996.66 ± 51.36[c]	1519.61 ± 95.63[b]	1874.11 ± 87.54[b]	16.97 ± 1.21	61.68 ± 4.85[c]	80.40 ± 3.16[a]
	SM 2	100.03 ± 4.00[a]	10.15 ± 1.31[ab]	0.36 ± 0.05[b]	2231.11 ± 290.70[b]	1035.72 ± 15.86[b]	1884.56 ± 43.72[a]	2142.18 ± 118.28[ab]	24.07 ± 0.43	82.44 ± 6.31[b]	74.04 ± 5.30[a]
	SM 3	108.22 ± 2.92[a]	12.17 ± 1.48[a]	0.83 ± 0.20[b]	1167.61 ± 94.10[c]	892.33 ± 59.52[c]	1800.35 ± 81.73[ab]	1950.77 ± 50.44[b]	16.98 ± 2.38	134.78 ± 4.93[a]	83.33 ± 2.78[a]
		**	**	**	***	***	*	*	NS	***	***
T7	HG	36.67 ± 2.10	0.63 ± 0.06[ab]	3.83 ± 0.17	1454.77 ± 248.90	690.36 ± 34.52	1151.34 ± 48.87	1712.05 ± 61.67	7.98 ± 0.12[a]	41.30 ± 3.43[b]	50.07 ± 4.78
	SM 1	32.94 ± 0.99	1.10 ± 0.02[b]	4.91 ± 0.29	1197.10 ± 182.05	521.66 ± 25.72	837.81 ± 37.88	1461.33 ± 118.02	4.12 ± 0.02[b]	91.74 ± 6.31[a]	59.99 ± 2.91
	SM 2	33.11 ± 2.73	0.56 ± 0.11[b]	1.73 ± 0.04	523.58 ± 109.42	299.29 ± 17.72	793.88 ± 28.28	1446.72 ± 107.12	1.91 ± 0.05[b]	57.81 ± 3.36[ab]	69.15 ± 7.18
	SM 3	43.82 ± 3.86	1.41 ± 0.02[a]	4.26 ± 1.23	905.54 ± 179.51	498.70 ± 25.58	999.40 ± 37.52	1443.18 ± 94.61	3.51 ± 0.01[b]	100.94 ± 9.87[a]	82.09 ± 4.14
		NS	**	NS	NS	NS	NS	NS	*	*	NS

[a] *Sampling dates: T0, immediately after purchase or harvest, prior to refrigerated storage; T2, two days in refrigerated storage; T4, four days in refrigerated storage; T7, days in refrigerated storage.*

[b] *Sample source: HG, home-grown sample; SM1, locally-grown supermarket-bought sample; SM2, supermarket-bought sample; SM3, supermarket-bought sample.*

*Different letters (a-d) denote statistically significant differences in each individual compound among V. locusta "Vit" sample sources by Duncan's multiple range test at ***$p < 0.001$, **$p < 0.01$,*$p < 0.05$ and NS, non-significant separately for each sampling. Standard error (SE) following each mean represents the standard deviation of sampling distribution ($n = 5$).*

to report any changes in total carotenoids even after 3 weeks of storage.

CONCLUSIONS

Home-grown lamb's lettuce is generally hand-picked in the garden, cleaned and consumed within the same day. According to UNECE standard for Lamb's lettuce (42), sealed bags of fresh and turgid rosettes should also be dispatched to the supermarket as fast as possible; preferably on the day of harvest. However, the sequence of procedures necessary to produce ready-to-eat product (i.e., washing, removing root tufts or cotyledons, packaging and sealing) and transport to the supermarket shelf promotes the biochemical instability of the product itself (12). A loss of primary and secondary metabolites was expected in all samples after harvest, but particularly in those stored for a longer period of time (i.e., all SM samples). Results underline the instability of vitamin C during refrigerated storage of lamb's lettuce and pinpoint this parameter as being the most affected by storage. Other primary and secondary metabolites were less affected by storage but as a rule, home-grown samples (or in some cases locally produced supermarket-bought lamb's lettuce) demonstrated highest levels of most compounds on all samplings. These samples also retained most bioactive components after a 7-day storage which confirms the superiority of local and home-grown produce compared to other foodstuffs for the entire period of consumer-relevant time.

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/s.

AUTHOR CONTRIBUTIONS

FS provided an initial idea, got in touch with growers and suppliers to ensure Vit samples were all grown organically according to the same measures. AS was in charge of sampling, coordinated laboratory work, and compound identification. JJ was in charge of compound identification and optimization of analytical methods. MS analyzed the samples in the laboratory and did statistical analysis. VS wrote the manuscript. All authors contributed to 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/fnut.2021.731869/full#supplementary-material

REFERENCES

1. Enninghorst A, Lippert F. Postharvest changes in carbohydrate content in lamb's lettuce (*Valerianella locusta*), in: International Conference on Quality in Chains. *Acta Hortic.* (2003) 604:553–8. doi: 10.17660/ActaHortic.2003.604.65
2. Ragaert P, Verbeke W, Devklieghere F, Debevere J. Consumer perception and choice of minimally processed vegetables and packaged fruits. *Food Qual Preferences.* (2004) 15:259–70. doi: 10.1016/S0950-3293(03)00066-1
3. Wojciechowska R, Długosz-Grochowska O, Kołton A, Zupnik M. Effects of LED supplemental lighting on yield and some quality parameters of lamb's lettuce grown in two winter cycles. *Sci Hortic.* (2015) 187:80–6. doi: 10.1016/j.scienta.2015.03.006
4. Braidot E, Petrussa E, Peresson C, Patui S, Bertolini A, Tubaro F, et al. Low-intensity light cycles improve the quality of lamb's lettuce (*Valerianella olitoria* [L.] Pollich) during storage at low temperature. *Postharvest Biol Technol.* (2014) 90:15–23. doi: 10.1016/j.postharvbio.2013.12.003
5. Ramos-Bueno RP, Rincón-Cervera MA, González-Fernández MJ. Phytochemical composition and antitumor activities of new salad greens: Rucola (*Diplotaxis tenuifolia*) and corn salad (*Valerianella locusta*). *Plant Foods Hum Nutr.* (2016) 71:197–203. doi: 10.1007/s11130-016-0544-7
6. Długosz-Grochowska O, Wojciechowska R, Kruczek M, Habela A. Supplemental lighting with LED improves the biochemical composition of two *Valerianella locusta* (L.) Cultivars. *Hortic Environ Biotechnol.* (2017) 58:441–9. doi: 10.1007/s13580-017-0300-4
7. Banjac MZK, Kovacevic SZ, Jevric LR, Podunavac-Kuzmanovic SO, Horecki ANT, Vidovic SS, et al. Artificial neural network modeling of the antioxidant activity of lettuce submitted to different postharvest conditions. *J Food Process Preserv.* (2019) 43:1–9. doi: 10.1111/jfpp.13878
8. Długosz-Grochowska O, Kołton A, Wojciechowska R. Modifying folate and polyphenol concentrations in Lamb's lettuce by the use of LED supplemental lighting during cultivation in greenhouses. *J Function Foods.* (2016) 26:228–37. doi: 10.1016/j.jff.2016.07.020
9. Grzegorzewski F, Ehlbeck J, Schlüter O, Kroh LW, Rohn S. Treating lamb's lettuce with a cold plasma – influence of atmospheric pressure Ar plasma immanent species on the phenolic profile of *Valerianella locusta*. *Food Sci Technol.* (2011) 44:2285–9. doi: 10.1016/j.lwt.2011.05.004
10. Urlic B, Dumicic G, Romic M, Ban SG. The effect of N and NaCl on growth, yield, and nitrate content of salad rocket (Eruca sativa Mill.). *J Plant Nutr.* (2017) 40:2611–8. doi: 10.1080/01904167.2017.1381122
11. Santos J, Herrero M, Mendiola JA, Oliva-Teles MT, Ibáñez E, Delerue-Matos C, et al. Fresh-cut aromatic herbs: nutritional quality stability during shelf-life. *LWT—Food Sci Technol.* (2014) 59:101–7. doi: 10.1016/j.lwt.2014.05.019
12. Beghi R, Giovenzana V, Civelli R, Malegori C, Buratti S, Guidetti R. Setting-up of a simplified handheld optical device for decay detection in fresh-cut *Valerianella locusta* L. *J Food Eng.* (2014) 127:10–5. doi: 10.1016/j.jfoodeng.2013.11.019
13. Mullins L, Charlebois S, Finch E, Music J. Home food gardening in Canada in response to the COVID-19 pandemic. *Sustainability.* (2021) 13:1–12. doi: 10.3390/su13063056
14. Mikulic-Petkovsek M, Schmitzer V, Slatnar A, Stampar F, Veberic R. Composition of sugars, organic acids, and total phenolics in 25 wild or cultivated berry species. *J Food Sci.* (2012) 77:C1064-70. doi: 10.1111/j.1750-3841.2012.02896.x
15. Weber N, Schmitzer V, Jakopic J, Stampar F. First fruit in season: seaweed extract and silicon advance organic strawberry (Fragaria×ananassa Duch.) fruit formation and yield. *Sci Hortic.* (2018) 242:103–9. doi: 10.1016/j.scienta.2018.07.038
16. Helland HS, Leufven A, Bengtsson GB, Skaret J, Lea P, Wold A. Storage of fresh-cut swede and turnip in modified atmosphere: effects on vitamin C. Sugars, glucosinolates and sensory attributes. *Postharvest Biol Technol.* (2016) 111:150–60. doi: 10.1016/j.postharvbio.2015.07.028
17. Mikulic-Petkovsek M, Slatnar A, Stampar F, Veberic R. The influence of organic/integrated production on the content of phenolic compounds in apple

leaves and fruits in four different varieties over a 2-year period. *J Sci Food Agric.* (2010) 90:66–78. doi: 10.1002/jsfa.4093
18. Sircelj H, Batic F. Evaluation of selected nutritional factors in *Aposeris foetida* (L.) Less. During the harvesting period. *J Appl Botany Food Qual Angewandte Botanik.* (2007) 81:121–5. Available online at: https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.1000.3999&rep=rep1&type=pdf
19. Senica M, Veberic R, Grabnar JJ, Stampar F, Jakopic J. Selected chemical compounds in firm and mellow persimmon fruit before and after the drying process. *J Sci Food Agric.* (2016) 96:3140-77. doi: 10.1002/jsfa.7492
20. Spinardi A, Ferrante A. Effect of storage temperature on quality changes of minimally processed baby lettuce. *J Food Agric Environ.* (2012) 10:38–42. doi: 10.1234/4.2012.2526
21. Nei D, Uchino T, Sakai N. Tanaka S. Prediction of sugar consumption in shredded cabbage using a respiratory model. *Postharvest Biol Technol.* (2006) 41:56–61. doi: 10.1016/j.postharvbio.2006.02.008
22. McKenzie MJ, Greer LA, Heyes JA, Hurst PL. Sugar metabolism and compartmentation in asparagus and broccoli during controlled atmosphere storage. *Postharvest Biol Technol.* (2004) 32:45–56. doi: 10.1016/j.postharvbio.2003.09.015
23. Flores P, Hellín P, Fenoll J. Determination of organic acids in fruits and vegetables by liquid chromatography with tandem-mass spectrometry. *Food Chem.* (2012) 132:1049–54. doi: 10.1016/j.foodchem.2011.10.064
24. Tsuchida H, Kozukue N, Han GP,Choi SH, Levin CE, Friedman M. Low-temperature storage of cucumbers induces changes in the organic acid content and in citrate synthase activity. *Postharvest Biol Technol.* (2010) 58:129–34. doi: 10.1016/j.postharvbio.2010.06.006
25. Ferrante A, Martinetti L, Maggiore T. Biochemical changes in cut vs. intact lamb's lettuce (*Valerianella locusta*) leaves during storage. *Int J Food Sci Technol.* (2009) 44:1050–105. doi: 10.1111/j.1365-2621.2008.01891.x
26. Preti R, Vibci G. Nutritional and sensory evaluation of ready-to-eat salads during shelf life. *Agro Food Ind Hi-Tech.* (2016) 27:26–31.
27. Gil MI, Ferreres F, Tomás-Barberán FA. Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. *J Agric Food Chem.* (1999) 47:2213–7. doi: 10.1021/jf981200l
28. Lee SK, Kader AA. Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biol Technol.* (2000) 20:207–20. doi: 10.1016/S0925-5214(00)00133-2
29. Myojin C, Yamaguchi T, Takamura H, Matoba T. Changes in the radical-scavenging activity of shredded vegetables during storage. *Food Sci Technol Res.* (2008) 14:198–204. doi: 10.3136/fstr.14.198
30. Smirnoff N, Conklin PL, Loewus FA. Biosynthesis of ascorbic acid in plants: a Renaissance. *Ann Rev Plant Physiol Plant Mol Biol.* (2001) 52:437–67. doi: 10.1146/annurev.arplant.52.1.437
31. Colonna E, Rouphael Y, Barbieri G, De Pascale. S. Nutritional quality of ten leafy vegetables harvested as two light intensities. *Food Chem.* (2016) 199:702–10. doi: 10.1016/j.foodchem.2015.12.068
32. Galani JHY, Patel JS, Patel NJ, Talati JG. Storage of fruits and vegetables in refrigerator increases their phenolic acids but decreases the total phenolics, anthocyanins and vitamin c with subsequent loss of their antioxidant capacity. *Antioxidants.* (2017) 6. doi: 10.3390/antiox6030059
33. Kołton A, Wojciechowska R, Długosz-Grochowska O, Grzesiak W. The storage ability of Lamb's lettuce cultivated in the greenhouse under LED or HPS lamps. *J Hortic Res.* (2014) 22:159–65. doi: 10.2478/johr-2014-0033
34. Hawrylak-Nowak B, Dresler S, Rubinowska K, Matraszek-Gawron R, Wock W, Hasanuzzaman M. Selenium biofortification enhances the growth and alters the physiological response of lamb's lettuce grown under high temperature stress. *Plant Physiol Biochem.* (2018) 127:446–56. doi: 10.1016/j.plaphy.2018.04.018
35. DuPont MS, Mondin Z, Williamson G, Price KR. Effect of variety, processing, and storage on the flavonoid glycoside content and composition of lettuce and endive. *J Agric Food Chem.* (2000) 48:3957–64. doi: 10.1021/jf0002387
36. Tavarini S, Degl'Innocenti E, Pardossi A, Guidi L. Biochemical aspects in two minimally processed lettuces upon storage. *Int J Food Sci Technol.* (2007) 42:217–9. doi: 10.1111/j.1365-2621.2006.01223.x
37. Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, et al. Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidipsis. *Plant Cell.* (2009) 21:767–85. doi: 10.1105/tpc.108.064089
38. Manzocco L, Foschia M, Tomasi N, Maifreni M, Dalla Costa L, Marino M, et al. Influence of hydroponic and soil cultivation on quality and shelf life of ready-to-eat lamb's lettuce (*Valerianella locusta* L. Laterr). *J Sci Food Agric.* (2011) 91:137345–1380. doi: 10.1002/jsfa.4313
39. Ferrante A, Maggiore T. Chlorophyll a fluorescence measurements to evaluate storage time and temperature of Valeriana leafy vegetables. *Postharvest Biol Technol.* (2007) 45:73–80. doi: 10.1016/j.postharvbio.2007.02.003
40. Ferrante A, Incrocci L, Serra G. Quality changes during storage of fresh-cut or intact Swiss chard leafy vegetables. *J Food Agric Environ.* (2008) 6:132–4. doi: 10.1234/4.2008.1294
41. Couso I, Vila M, Vigara M, Cordero BF, Vargas MA, Rodriguez H, et al. Synthesis of carotenoids and regulation of the carotenoid biosynthesis pathway in response to high light stress in the unicellular microalga *Chlamydomonas reinhardtii*. *Eur J Phycol.* (2012) 47:223–32. doi: 10.1080/09670262.2012.692816
42. *UNECE Standard for Lambs Lettuce (FFV-60).* New York, NY and Geneva: United Nations (2015).

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Widely Targeted Metabolomics Analysis to Reveal Transformation Mechanism of *Cistanche Deserticola* Active Compounds During Steaming and Drying Processes

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Cistanche deserticola is one of the most precious plants, traditionally as Chinese medicine, and has recently been used in pharmaceutical and healthy food industries. Steaming and drying are two important steps in the processing of *Cistanche deserticola*. Unfortunately, a comprehensive understanding of the chemical composition changes of *Cistanche deserticola* during thermal processing is limited. In this study, ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)-based widely targeted metabolomics analysis was used to investigate the transformation mechanism of *Cistanche deserticola* active compounds during steaming and drying processes. A total of 776 metabolites were identified in *Cistanche deserticola* during thermal processing, among which, 77 metabolites were differentially regulated ($p < 0.05$) wherein 39 were upregulated (UR) and 38 were downregulated (DR). Forty-seven (17 UR, 30 DR) and 30 (22 UR, 8 DR) differential metabolites were identified during steaming and drying, respectively. The most variation of the chemicals was observed during the process of steaming. Metabolic pathway analysis indicated that phenylpropanoid, flavonoid biosynthesis, and alanine metabolism were observed during steaming, while glycine, serine, and threonine metabolism, thiamine metabolism, and unsaturated fatty acid biosynthesis were observed during drying. The possible mechanisms of the chemical alterations during thermal processing were also provided by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Furthermore, the blackening of the appearance of *Cistanche deserticola* mainly occurred in the steaming stage rather than the drying stage, which is associated with the metabolism of the amino acids. All results indicated that the formation of active compounds during the processing of *Cistanche deserticola* mainly occurred in the steaming stage.

Keywords: *cistanche deserticola*, widely targeted metabolomics, steaming, drying, active compounds, formation mechanism

INTRODUCTION

Cistanche deserticola, belonging to the orobanchaceae family, is one of the most famous tonic medicines and is mainly distributed in the tropical and subtropical regions of the world, such as China, Iran, India, Mongolia, and so on (1–3). *Cistanche deserticola* serves as one of the most commonly utilized herbal medicines for the treatments of kidney deficiency, impotence, female infertility, morbid leucorrhea, profuse metrorrhagia, and senile (4, 5). Modern pharmacological research showed that *Cistanche deserticola* has the effects of improving immunity, anti-fatigue, anti-aging, and enhancing learning and memorization ability (6). Owing to these health benefits, *Cistanche deserticola* tea made from its stem tubers has been developed as a nourishing supplement and is being increasingly favored by consumers. The active ingredients of *Cistanche deserticola* have been shown to be responsible for its medicinal functions (7). Some active ingredients in *Cistanche deserticola*, such as phenylpropanoids (for example phenylethanoid glycosides), flavonoids, polysaccharides, oligosaccharides, iridoids, and lignans have been reported in the previous studies (6, 8).

Due to the perishable and seasonal features, the all-year-round supply of fresh *Cistanche deserticola* is unavailable, accordingly, the processed *Cistanche deserticola* becomes the main consumption form. The quality of *Cistanche deserticola* is dependent on many factors, such as climate, habitats, hosts, harvest time, processing technology, and position on the plant, among which processing technology is particularly important (4). Steaming and drying are two important steps in the processing of *Cistanche deserticola*. Usually, the harvested *Cistanche deserticola* rhizome was steamed in a steaming boiler at 93°C for 30 min and then dried at 60°C until the moisture content of 10% on a wet basis (w.b.) (9). Previous studies have shown that steaming can promote the accumulation of active ingredients in *Cistanche deserticola*, such as phenylethanoid glycosides, soluble sugars, and polysaccharides, accompanied by blackening of appearance color (9–11). However, most of the previous studies focused on certain specific compounds, very rare research are about the changes in all the chemical compounds and the metabolite conversion mechanism during processing. Therefore, it is necessary to clarify the metabolite changes of *Cistanche deserticola* in different processing stages.

Metabolomics is usually applied to qualitative and quantitative analysis of all small molecules (namely, targeted and non-targeted compounds) detected in the sample (12). Analysis of changes in various chemical components during food processing helps to deepen the understanding of the mechanism of chemical component transformation in food processing (12). In recent years, metabolomics has also been applied to the study of *Cistanche deserticola* for the discrimination of different parts (11) and different *Cistanche deserticola* species (13). The detection methods of metabolites in these studies were mostly based on targeted and non-targeted metabolomics. Among them, targeted metabolomics is based on standard products, with high data accuracy and reliability, however, limited coverage of metabolites. Targeted metabolomics is an important part of metabolomics research, it is the targeted and specific detection and analysis for specific metabolite groups, rather than all the components in the sample. Non-targeted metabolomics technology can qualitatively determine the metabolites based on existing databases, with high coverage of compounds, however, low accuracy. The key metabolites must be confirmed by standard products (14). Widely targeted metabolomics is a new technology that integrates the advantages of non-target and targeted metabolites detection technologies to achieve wide coverage, high throughput, and sensitivity (15). Consequently, this technology has been widely used in the study of ingredient changes in different materials during processing, such as active ingredients in functional foods by different processing methods (16), flavonoids and phenylpropanoids compounds in Chinese water chestnut processed with different methods (17), rice yellowing mechanism during yellowing process (18), and the formation mechanism of characteristic non-volatile chemical constitutes during oolong tea manufacturing process (19). Therefore, it is theoretically feasible to use widely targeted metabolite technology to study the mechanism of the conversion of active ingredients during the processing of *Cistanche deserticola*.

Thus, the objectives of the present study were to (1) provide useful information on the chemical changes in *Cistanche deserticola* during steaming and drying processes by using ultraperformance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) combined with a widely targeted metabolomic approach; (2) identify the differential metabolites and their regulation rules, and reveal the possible conversion pathways in *Cistanche deserticola* during processing. This study is, therefore, expected to provide a theoretical reference for the formation mechanism of high-quality *Cistanche deserticola*.

MATERIALS AND METHODS

Materials and Chemicals

Raw materials: Fresh *Cistanche deserticola* samples were obtained from the Hetian region in Xinjiang Province of China. The samples were carefully selected with the same size (average length, diameter, and weight were 11.7 ± 1.1 cm, 7.0 ± 1.1 cm, and 360 ± 8.9 g, respectively). The samples were stored at room temperature in a dark environment with an initial moisture content of about 78.56% ± 3.47%. Prior to the experiments, *Cistanche deserticola* samples were washed with tap water to remove the dust on the surface. Excess water on its surface was removed by blotting paper.

Chemicals: Methanol, acetonitrile, and formic acid were liquid-chromatography mass spectrometry grade (LC-MS) and purchased from Merck (Sigma Aldrich, MO, USA). The other analytical standards presented a purity higher than 98% (Sigma Aldrich, MO, USA).

Experimental Design

Previous studies have shown that the chemical compounds distribute unevenly in the longitudinal direction of *Cistanche deserticola* (1). Therefore, to obtain the same initial contents of chemical compounds in each sample, in the present research all selected *Cistanche deserticola* was cut into three equal parts for the fresh group (A), steamed without drying group (B),

and dried after steaming group (C), respectively, by longitudinal segmentation with the longitudinal symmetry axis as the center (20).

For group B, the samples were successively steamed for 8 min according to preliminary experiments. A pulsed vacuum steaming equipment (self-developed by China Agricultural University, Beijing, China) was used for steaming treatment of fresh *Cistanche deserticolas*. Steamed samples were dried in a vacuum freeze-dryer (LGJ-25C, Si Huan Scientific Instrument Factory Co., Beijing, China). The heating plate and cold trap temperature were 30 and −60°C, respectively. For group C, the samples were successively steamed using pulsed vacuum equipment for 8 min and dried in the hot air impingement dryer (self-developed by China Agricultural University, Beijing, China) until the final moisture content of 10% (w.b.). The airflow rate and temperature were set at 6 m/s and 60°C, respectively, referring to the research results of Zou et al. (11). All samples were stored at −20°C no more than 7 days before further analysis.

Determination of Appearance Color of Cistanche Deserticola

The appearance color of the *Cistanche deserticola* before and after each thermal processing was measured using a colorimeter (SMY-2000SF, Shengming Yang Co., Beijing, China), and the blackness was characterized by L^* value.

Sample Preparation and Extraction

The metabolite extraction was carried out according to the method reported previously by Chen et al. (21) with some minor modifications. In brief, the dried samples were crushed using a mixer mill (MM 400, Retsch Company, Haan, Germany) with a zirconia bead for 2 min at 60 Hz. Then 50 mg powder (sifted through a 65 mesh sieve) of each sample was precisely weighed, transferred to an Eppendorf tube, and extracted with 1 ml methanol/water mixture (v:v = 3:1). After 30 s vortex, the mixture was homogenized twice at 35 Hz for 4 min, sonicated for 15 min in an ice-water bath, and then shaken overnight at 4°C. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant was collected and filtered through a 0.22-μm membrane, then the obtained extract was transferred to 2-ml glass vials and store at −80°C until the UHPLC-MS/MS analysis.

Metabolites Analysis by UHPLC-MS

UHPLC Conditions

The UHPLC separation was carried out using an EXIONLC system (Sciex Technologies, Framingham, MA, USA). The analytical conditions were as follows: column: Waters ACQUITY UHPLC HSS T3 C18 (1.8 μm, 2.1 × 100 mm); solvent system: mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile containing). The gradient program: 98% A/2% B at 0 min, 50% A/50% B at 10 min, 5% A/95% B at 11 min, 98% A/2% B at 13.1 min, and 98% A/2% B at 15 min. Flow rate: 0.40 ml/min; column temperature: 40°C; injection volume: 2 μl; automatic injection temperature: 4°C.

ESI-QTRAP-MS/MS Conditions

A triple quadrupole (QQQ)-linear ion trap mass spectrometer (QTRAP, API 6500 QTRAP UHPLC-MS/MS) + QQQ spectrometer equipped with an ESI turbo ion-spray interface (Sciex Technologies, Framingham, MA, USA) was applied for MS analysis. The analytical conditions were as follows: ion spray voltage: +5,500 V (positive ion mode)/−4,500 V (negative ion mode), curtain gas: 35 psi, source temperature: 400°C, ion source gas 1: 60 psi, ion source gas 2: 60 psi, declustering potential: ±100 V. QQQ scans were acquired as multiple reaction monitoring (MRM) experiments with collision gas (nitrogen) set to 5 psi.

Qualitative and Quantitative Analysis of Metabolites

Qualitative and quantitative analyses of metabolites were performed according to the methods by Liu et al. (18). Primary and secondary mass spectrometry data were qualitatively analyzed based on the self-built human metabolome database (MWDB) (Metware Biotechnology Co., Ltd. Wuhan, China) and the public database. Meanwhile, to ensure the accuracy of the qualitative analysis of some substances, interferences from repeated signals of Na^+, NH_4^+, K^+ and ions, and repetitive signals of fragment ions derived from other relatively large molecules and isotope signals were removed during identification. Metabolite structural analysis was performed with reference to the public databases (Mass Bank, KNApSAcK, HMDB, MoTo DB, and METLIN).

Metabolite quantification was carried out using the MRM mode of the QQQ mass spectrometry. In the MRM mode, the precursor ions (parent ions) of the target substances and excluded ions corresponding to other substances with different molecular weights were screened first using the quadrupole rod to initially eliminate interference. The precursor ions then break through the collision chamber to form many fragment irons after ionization, which were filtered by QQQ to select single-fragment ions with the desired characteristics while eliminating interference from non-target ions. Finally, after obtaining the metabolite mass spectrometry data of different samples, the mass spectrum peaks of all substances were integrated, and the mass spectra peaks of the same metabolite in different samples were integrated and corrected using Multi Quant version 3.0.2 (ABSCIEX, Concord, Ontario, Canada). The corresponding relative metabolite contents were represented as chromatographic peak area integrals.

Data Processing and Analysis

The metabolic data were processed using orthogonal partial least squares-discriminant analysis (OPLS-DA) and hierarchical cluster analysis (HCA). OPLS-DA was used to discriminate each group; it is more sensitive than other statistical methods to variables with low correlations (17). The OPLS-DA models were validated through a permutation analysis (200 times). The model was considered stable when the model parameters (R^2 and Q^2) were both close to 1. The variable importance projection (VIP) values of metabolites were calculated. Any metabolite with VIP values greater than 1.0 and p-values

less than 0.05 were selected as biomarkers for each paired comparison between different thermal processing stages of *Cistanche deserticola*. The screening of different metabolites was visualized in the form of the volcano plot. Metabolites accumulation among different samples was analyzed by using the R package (www.rproject.org/). The Venn diagram was built according to the program web-based smart diagram® (https://cloud.smartdraw.com/). The commercial databases, such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/kegg/), Pub Chem (https://pubchem.ncbi.nlm.nih.gov/), the Small Molecule Pathway Database (SMPDB) (https://smpdb.ca/), and HMDB (https://hmdb.ca/), were used for enrichment analysis of differential metabolites and finding metabolic pathways.

RESULTS AND DISCUSSION

Appearance Color Changes of Cistanche Deserticola During Thermal Processing

The difference in appearance color of *Cistanche deserticola* between the fresh, steamed and dried samples are representatively displayed in **Figure 1**. From fresh to dried sample with the going of the processing stage, the appearance color of the samples changed from yellow-brown to dark black, and the darkness in color became more and more obvious (the corresponding L^* value was reduced from 50.26 to 24.90). Obviously, the appearance changes of *Cistanche deserticola* mainly occurred in the steaming process. The Maillard reaction, during which sugars react with amino acids under thermal conditions (22), would be greatly responsible for the dark-colored appearance of processed rhizomes of *Cistanche deserticola*. Previous research studies have shown that precursors were converted into colorants and generated substances with dark color in the Maillard reaction (23). Similar findings were also observed in previous studies for steaming of *Polygonum multiflorum* (24) and rhizomes of *Polygonatum cyrtonema* (25). The darkness of the steamed samples was further deepened after drying. This phenomenon was probably due to the occurrence of a decrease in the pigment concentration during the drying process.

Overview of the Metabolites in Raw and Thermal Processed Cistanche Deserticola Samples

The total ion chromatogram (TIC) of quality control (QC) sample (a mixture of all the samples investigated) and a multi-peak detection plot of chemicals in the MRM mode of the same sample are illustrated in **Supplementary Figure 1**. Different colored peaks represented different components in the sample. As shown in **Figure 2**, a total amount of 776 metabolites were identified in the current study (**Supplementary Table 1**) in the fresh *Cistanche deserticola* samples, which were divided into 15 classes, including 40 amino acid and derivatives, 33 phenylpropanoids, 23 flavonoids, 68 flavone, 67 terpenes, 67 phenols, 87 alkaloids, 13 carbohydrates, 28 nucleotide and derivatives, 5 alcohols and polyols, 3 purine nucleosides, 15 carboxylic acids and derivatives, 14 organic acids and derivatives, 12 phytohormone, and 28 other chemicals. Among them, the largest group was amino acid and derivatives, the relative content of which accounted for 30.26% of the total metabolite composition. In addition, 10 kinds of phenylethanoid glycosides, such as echinacoside and verbascoside, were detected and classified in the phenylpropanoids group.

The accumulation pattern of metabolites among different treatment groups was analyzed by HCA. As shown in **Figure 3**, 107 identified metabolites of *Cistanche deserticola* were clustered in heat maps based on Euclidean distance arithmetic. Metabolites identified at different thermal processing stages were gathered into three clusters according to the dendrogram. The brighter color indicates the higher content of a particular metabolite in the respective sample. The heat map of HCA showed larger di?erences in abundance between the fresh and steamed samples than those between steamed and dried samples, indicating that metabolites in *Cistanche deserticola* may have different transformations during the steaming and drying stage, and the types and quantities of metabolites involved in the steaming process are more than those in the drying process.

Differential Metabolite Analysis of Cistanche Deserticola at Different Thermal Processing Stages

For a better understanding of the impact of each processing on the metabolites of *Cistanche deserticolas*, the OPLS-DA scatter scores of pairwise comparison groups are shown in **Figure 4A**, showing that the fresh, steamed, and dried after steaming *Cistanche deserticolas* were significantly different. Moreover, the R^2Y and Q^2 (as shown in **Supplementary Figure 2**) with high-test values indicated that this model was highly reliable without overfitting.

To screen the expression level of metabolites between the fresh, steamed, and dried after steaming *Cistanche deserticola*, the analysis of volcano plot was further applied among all 776 metabolites identified according to the fold-change, combined with VIP values to screen the differentially expressed metabolites. Significant differential metabolites were selected according to the criterion that a fold change score of ≥ 2 or ≤ 0.5 with a VIP ≥ 1. The screening results are illustrated in **Figure 4B**. In the volcanic map, each point represents a metabolite and the color of the scattered dots represents the final screening result. Red represents metabolites that are significantly upregulated (UR), green represents those significantly downregulated (DR), and gray represents those insignificantly different. As shown in **Figure 4B**, 47 metabolites in the fresh vs. steamed group (17 UR and 30 DR), 30 metabolites in steamed vs. dried group (22 UR and 8 DR), and 65 metabolites in the fresh vs. dried group (29 UR and 36 DR) were selected to be significantly differential. The number of significantly different metabolites in the fresh vs. steamed group was higher than those in the steamed vs. dried group, indicating that the influence on metabolites in the steaming process is higher than that of the drying process. The differential metabolites produced during thermal processing of *Cistanche deserticola* were further classified and compared. These

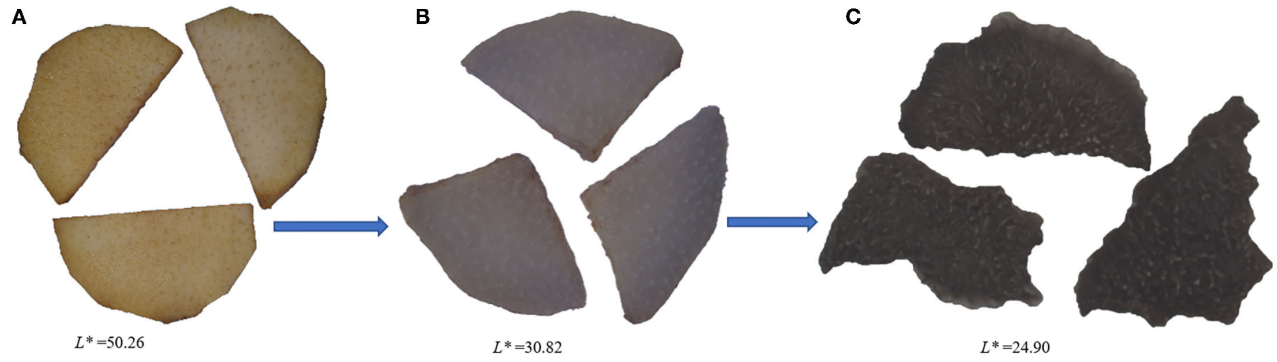

FIGURE 1 | Appearance color characteristics during *Cistanche deserticola* thermal processing. **(A, B, C)** represent samples at the thermal processing stage before steaming, after steaming without drying, and after steaming and drying, respectively.

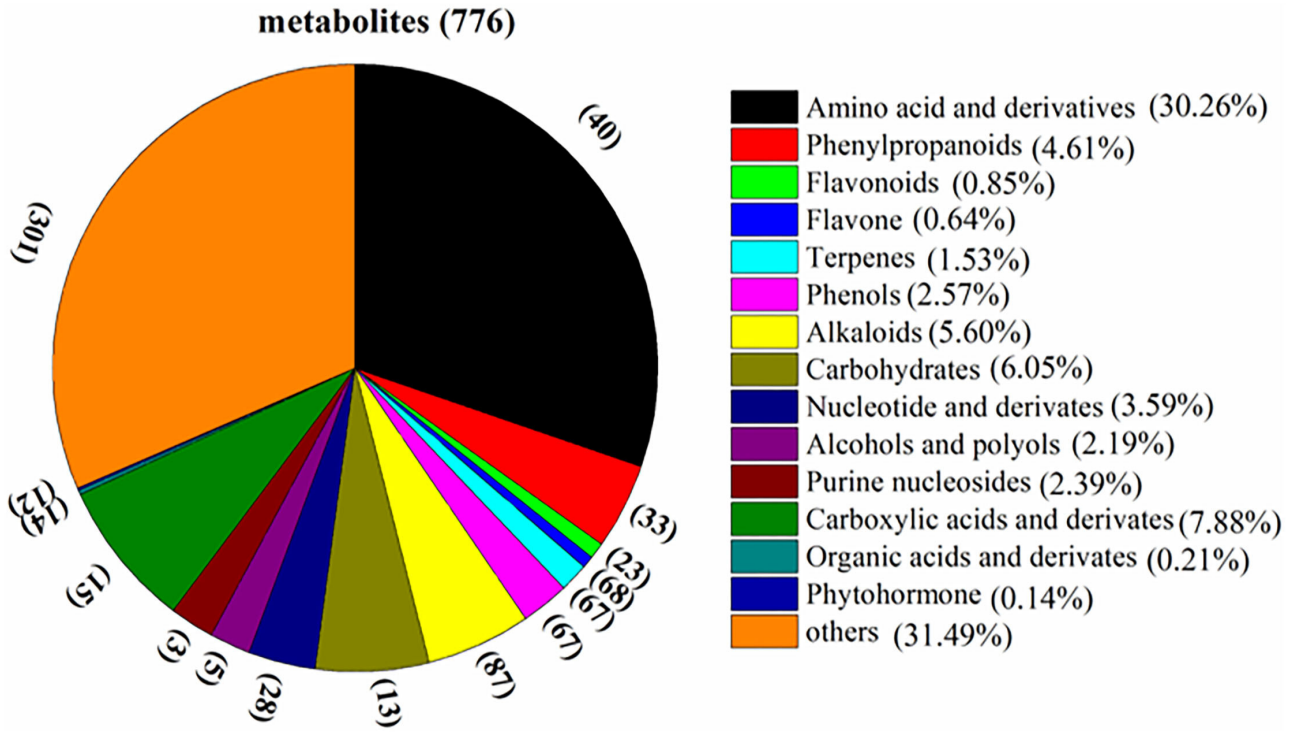

FIGURE 2 | Classification and composition of the 776 metabolites of *Cistanche deserticola*.

differentially expressed metabolites were classified into 21 classes, mainly amino acids and their derivatives, flavonoids and their derivatives, phenylpropanoids, alkaloids, terpenes, phenols, and nucleotide and their derivatives (**Table 1**). In fresh vs. steamed group, it can be found that flavonoids (such as isoquercitrin, troxerutin, cyanidin, and fisetin), phenylpropanoids (such as chlorogenic acid and 3-(3,4-Dihydroxy-5-methoxy)-2-propenoic acid), and nucleotide and their derivatives (uracil and beta-Nicotinamide mononucleotide) were significantly DR, while amino acids and their derivatives (such as N6-Acetyl-L-lysine, 1-Methy-L-histidine, and L-Phenylalanine) were significantly UR. However, in the steamed vs. dried group, the expression trends of these types of differential metabolites were opposite. Some amino and their derivatives (such as N, N-Dimethylglycine), nucleotide and their derivatives (such as 2′-Deoxyuridine; Deoxyuridine) were significantly DR, while most of the phenols (such as methyl gallate and 4′-Prenyloxyresveratrol), flavonoids (such as isoquercitrin and cyanidin), phenylpropanoids (verbascoside),

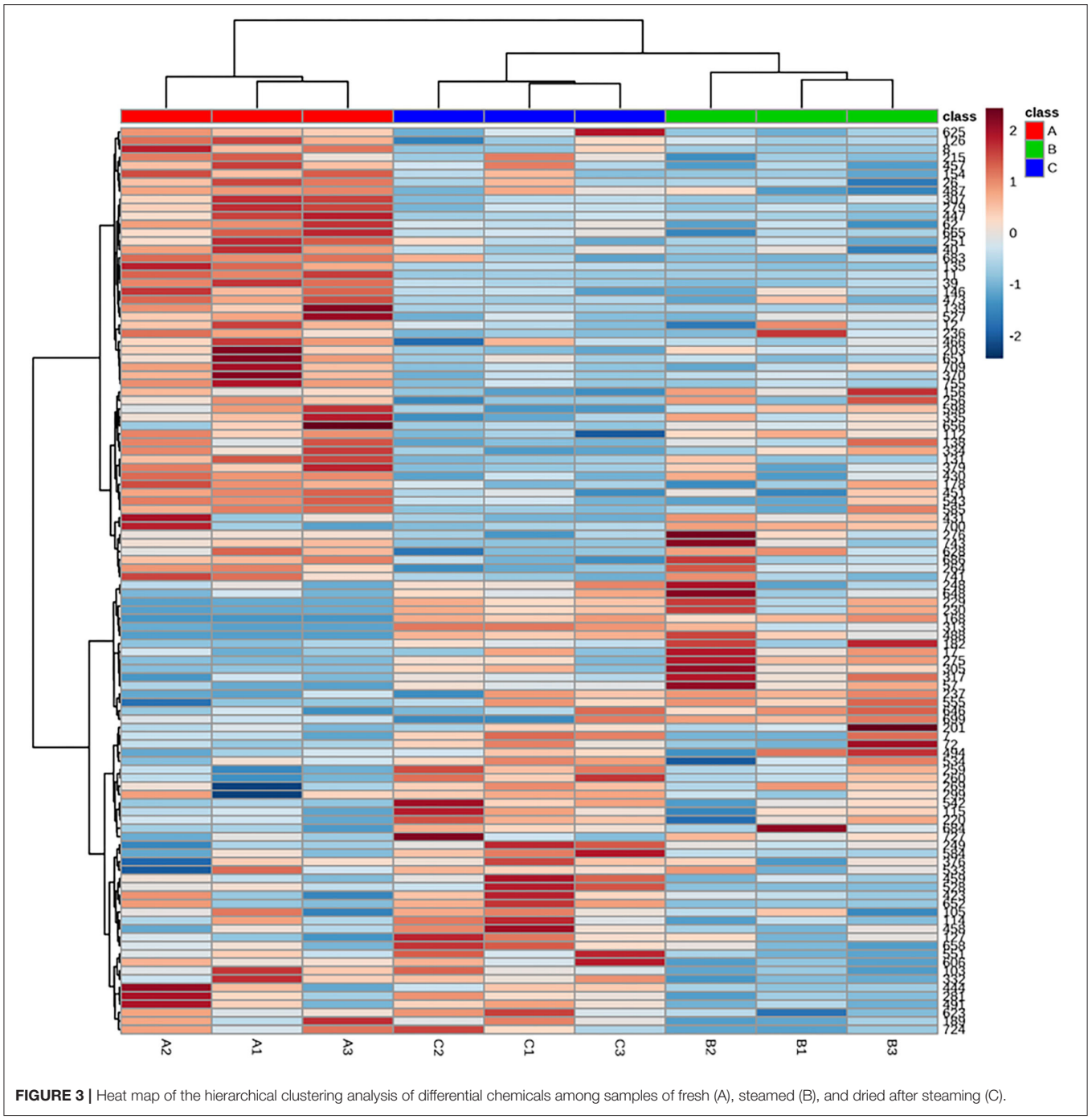

FIGURE 3 | Heat map of the hierarchical clustering analysis of differential chemicals among samples of fresh (A), steamed (B), and dried after steaming (C).

and terpenes (such as terpinolene and furanodiene) were significantly UR.

These results showed that the chemical composition of *Cistanche deserticola* has undergone conversion during thermal processing, which is mainly reflected in the conversion of flavonoids, phenylpropanoids, and amino acids, and the conversion mechanism of these components is different in different processing stages. The use of a high temperature during the steaming and drying processes was previously found to promote hydrolysis, redox, isomerization, substitution, and other thermophysical and chemical reactions of metabolites (26). In this study, it was found that the metabolites, such as flavonoids and phenylpropanoids, were significantly accumulated in the steamed *Cistanche deserticola* compared to their corresponding fresh one, indicating that some key physiological and metabolic activities leading to the synthesis of flavonoids and phenylpropanoids might be activated under high temperature and humidity. This result can also be supported

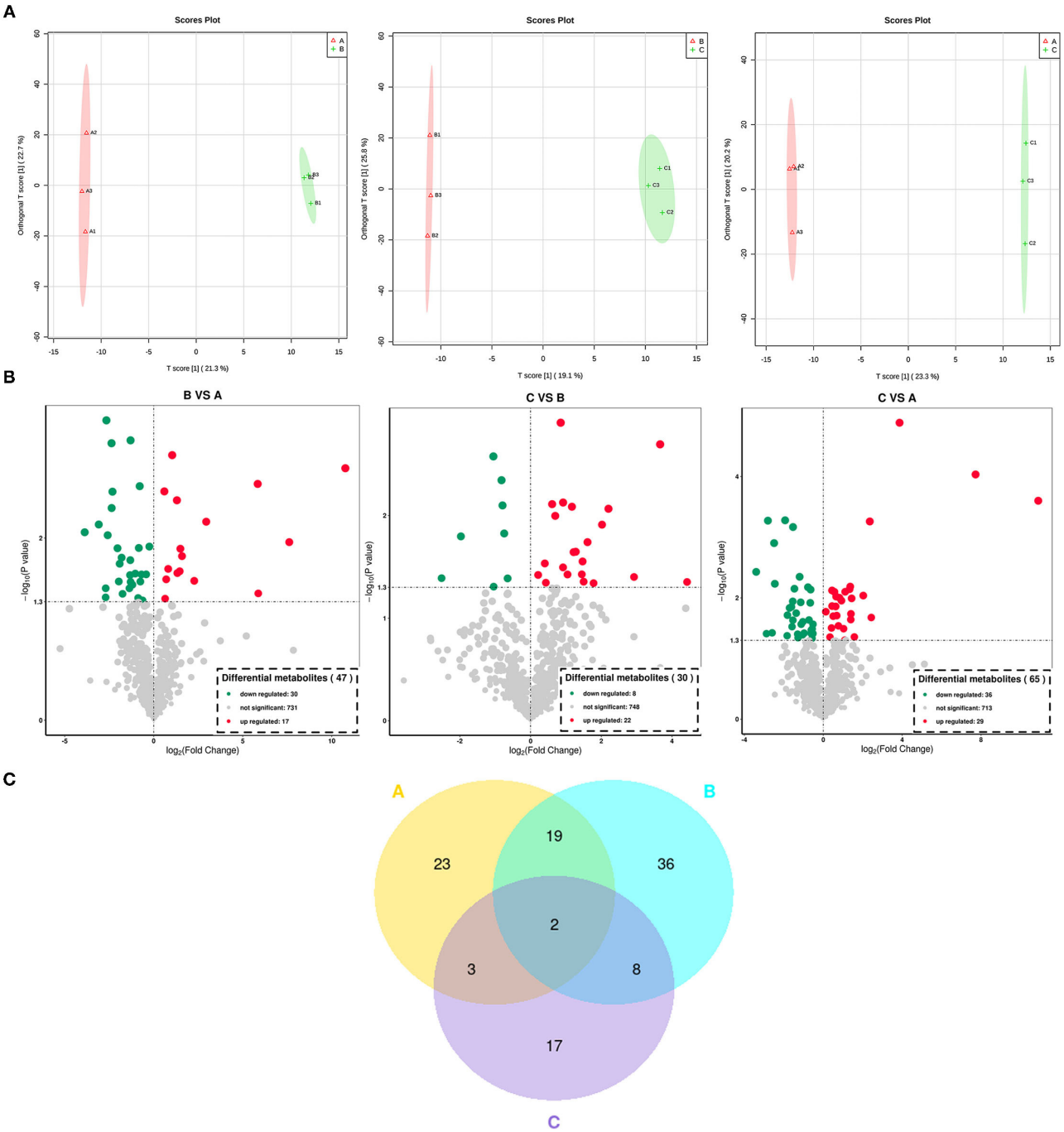

FIGURE 4 | Differential metabolite analysis in all *Cistanche deserticola* samples processed at different stages. **(A)** the score plots of the differential metabolites generated from OPLS-DA; **(B)** the volcano plot of the differential metabolite in different samples; **(C)** the Veen diagram of the differential metabolite in different samples. A, fresh; B, steamed without drying; C, dried after steaming. OPLS-DA, orthogonal partial least squares discriminant analysis.

by the report from Peng et al. (10) who found that the content of PhGs (belonging to phenylpropanoids) increased after steaming. However, the accumulation of these components in the dried sample after steaming showed a significant decrease, which may be attributed to the thermal degradation of these heat-sensitive components during the long-term drying process. Previous studies have shown that flavonoid glycosides can be decomposed into sugar bodies and flavonoid aglycones under thermal conditions, and flavonoids loss during the drying process was synthetically affected by temperature and drying

time (26, 27). The upregulation of amino acids and their derivatives (N6-Acetyl-L-lysine, 1-Methy-L-histidine, and L-Phenylalanine) is attributed to the high-temperature-promoting protein degradation during steaming processing. In addition, it was also observed that some other amino acids and their derivatives of N, N-Dimethylglycine, L- Kynurenine, glycine, serine, and threonine were DR. The decrease in the content of these amino acids might be associated with thermal-induced Maillard reaction during which reducing sugars react with amino acids to generate 5-HMF, contributing to the production of black appearance in *Cistanche deserticola* (22). The results of Section appearance color changes of cistanche deserticola during thermal processing further verified this hypothesis. Therefore, the blackening of *Cistanche deserticola* during steaming was probably related to the metabolism of amino acids.

Venn diagram was used to differentiate the common and exclusive metabolites of *Cistanche deserticola* during different thermal processing stages. As shown in **Figure 4C**, both common and unique metabolites exist between the different comparison groups. Twenty-one common metabolites were observed between the fresh and steamed group, while only 5 and 10 metabolites were found common between the fresh and dried group, steamed and dried group, respectively. Thus, a total of 23 and 17 exclusive metabolites ($p < 0.05$) were observed in *Cistanche deserticola* during the thermal processing stage of steaming and drying, respectively. This result further confirmed that steaming was particularly critical for the conversion of metabolites during *Cistanche deserticola* processing.

Enrichment Analysis and KEGG Pathway Impact Analysis of Differential Metabolites

The differential metabolites ($p < 0.05$) in fresh and processed samples were mapped to the KEGG, HMDB, and PubChem online databases, which contain knowledge of the molecular interaction, reaction, and relation networks, and the enrichment results and detailed metabolic pathways are shown in **Supplementary Table 2** and **Figure 5**. As shown in **Figures 5a1,a2**, pathway impact revealed the enrichment of phenylpropanoid biosynthesis, flavonoid biosynthesis, alanine metabolism, riboflavin metabolism, taurine and hypotaurine metabolism, and nicotinate and nicotinamide metabolism during steaming of *Cistanche deserticola*. Whereas, during the drying process after steaming, the metabolic pathways of the differential metabolites mainly contained glycine, serine and threonine metabolism, thiamine metabolism, pyrimidine metabolism, and unsaturated fatty acids biosynthesis. Furthermore, some metabolic pathways between these two pairwise comparisons overlapped, such as nicotinate and nicotinamide metabolism, phenylpropanoid biosynthesis, and flavonoid biosynthesis, but their enrichment levels were very different in two pairwise comparisons. These results suggested that the conversion pathways of metabolites between the steaming and drying processes of *Cistanche deserticola* were different, and the differences in metabolic pathways could explain the differences in the presence of differentially exclusive metabolites during thermal processing. These biochemical alterations might be used to comprehend the impact of thermal processing stages on *Cistanche deserticola* composition.

Based on the KEGG annotation and enrichment analysis, four metabolic pathways (phenylpropanoid biosynthesis, flavonoid biosynthesis, alanine metabolism, and glycine, serine, and threonine metabolism) were chosen as key metabolites to characterize the conversion of the main active components of *Cistanche deserticola* during thermal processing (**Figures 5b1,b2**). The current study indicated that phenylpropanoids and flavonoids were accumulated but amino acids were degraded in steamed *Cistanche deserticola* compared to fresh and dried samples. The phenylpropanoid biosynthetic pathway is upstream of biosynthetic pathway of flavonoid. Similar conclusions were published by Liu et al. (18) who reported that the accumulation level of phenylpropanoids in the process of rice yellowing has increased significantly, compared with normal rice. Phenylpropanoids are derived from cinnamic acid, and their precursor is phenylalanine, which can be synthesized by activating the activity of phenylalanine ammonia-lyase (PAL) when heated (28). Previous studies reported that the phenylpropanoid pathway led to the biosynthesis of coumarins, flavones, isoflavones, and flavanols, which are the important weapons for plant defense (29), and to prevent cell death caused by the strong heat stress in the steaming process, the phenylpropanoid pathway may be enhanced due to the biological stress caused by high temperature (30, 31). Flavonoids are the main secondary metabolites derived from phenylpropanoids (32), and their accumulation could protect plants from oxidative damage by scavenging-free radicals (33). Compared to the fresh and dried *Cistanche deserticola*, the higher biosynthesis of flavonoids in the steamed *Cistanche deserticola* may be associated with enhanced heat stress during the steaming process providing protection against reactive oxygen species (ROS) (34, 35). As shown in **Figures 5b3,b4**, amino acid metabolism played an important role in the thermal processing of *Cistanche deserticola*. Content changes of alanine, glycine, serine, and threonine after steaming found in medicinal herbs have been used to indicate the occurrence of the Maillard reaction (36). Nevertheless, due to the complicated process of the *Cistanche deserticola* steaming, a comprehensive evaluation of the *Cistanche deserticola* steaming, such as blackening in appearance, active compounds, and metabolic biomarkers, should be further investigated.

CONCLUSIONS

In the present study, UHPLC-MS/MS-based widely targeted metabolomics approach was employed to study the formation mechanism of active compounds at different thermal processing stages of *Cistanche deserticola*. The current results revealed that the biosynthesis of some key metabolites, such as phenylpropanoids and flavonoids, was significantly enhanced during the steaming process. The expression level of amino acids in steamed *Cistanche deserticola* was enhanced, indicating the transformation between primary and secondary metabolites. In addition, the blackening of the appearance of *Cistanche deserticola* mainly occurred in the steaming stage rather

TABLE 1 | List of significantly different metabolites up/downregulated in *Cistanche deserticola* under different thermal processing stages.

KEGG-ID	Molecular mass	Metabolite name	Class	VIP	*P*-value	Fold change	Regulation
Thermal processing stage: steaming							
	101.05	1-Aminocyclopropanecarboxylic acid	Phytohormone	2.00	0.01	0.07	Downregulated
C05623	464.09	Isoquercitrin	Flavonoids	2.01	0.01	0.12	Downregulated
C06802	645.25	Acarbose	Alkaloids	1.79	0.04	0.15	Downregulated
C10526	286.12	(–)-Sativan	Flavonoids	1.84	0.04	0.15	Downregulated
	742.23	Troxeruti	Flavonoids	2.13	0.00	0.16	Downregulated
C16959	216.15	Furanodiene	Sesquiterpenoids	1.98	0.01	0.17	Downregulated
C08493	145.05	Indole-3-carboxaldehyde	Phytohormone	2.12	0.00	0.19	Downregulated
C12634	610.15	Kaempferol3-O-beta-sophoroside	Flavonoids	2.04	0.00	0.19	Downregulated
C05905	286.05	Cyanidin	Flavonoids	2.06	0.00	0.20	Downregulated
C09372	367.11	(+)-Bicuculline	Alkaloids	1.95	0.01	0.24	Downregulated
C00106	112.03	Uracil	Nucleotide and derivatives	1.86	0.03	0.26	Downregulated
C00852	354.10	Chlorogenic acid	Phenylpropanoids	1.93	0.02	0.27	Downregulated
C00455	334.06	beta-Nicotinamide mononucleotide	Nucleotide and derivatives	1.94	0.02	0.29	Downregulated
C01965	290.14	Trimethoprim	Phenol ethers	1.82	0.04	0.29	Downregulated
C10414	268.07	Dalbergin	Coumarins	1.82	0.03	0.39	Downregulated
C12312	133.05	Indolin-2-one	Alkaloids	1.89	0.02	0.39	Downregulated
C00345	276.02	6-Phosphogluconic acid	Organooxygen compounds	1.93	0.02	0.40	Downregulated
C12298	198.16	Citronellyl acetate	Monoterpenoids	2.12	0.00	0.41	Downregulated
C01118	219.07	O-Succinyl-L-homoserine		1.86	0.03	0.41	Downregulated
C10851	175.08	Calystegine B2	Alkaloids	1.86	0.03	0.44	Downregulated
C01378	288.06	Fisetin	Flavonoids	1.88	0.02	0.49	Downregulated
C06575	134.11	P-Cymene	Monoterpenoids	1.77	0.05	0.54	Downregulated
C05123	125.99	2-Hydroxyethanesulfonate	Organic acids	1.96	0.01	0.56	Downregulated
C18326	234.14	N-p-Coumaroyl putrescine	Phenolamides	1.77	0.04	0.56	Downregulated
C05610	208.07	Trans-3,5-Dimethoxy-4-hydroxy cinnamaldehydee	Phenylpropanoids	2.07	0.00	0.58	Downregulated
C05619	210.05	3-(3,4-Dihydroxy-5-methoxy)-2 propenoic acid	Cinnamic acids and derivatives	1.83	0.03	0.59	Downregulated
C07650	263.07	Gemcitabine	Pyrimidine nucleosides	1.88	0.03	0.62	Downregulated
C02107	150.02	D-tartaric acid	Organic acids and derivatives	1.76	0.05	0.65	Downregulated
C09922	386.10	Cleomiscosin A	Coumarins	1.88	0.02	0.74	Downregulated
C00568	137.05	P-Aminobenzoate	Benzoic acid derivatives	1.95	0.01	0.84	Downregulated
C02727	188.12	N6-Acetyl-L-lysine	Amino acid and derivatives	2.07	0.00	1.51	Upregulated
C17756	151.06	Leukoaminochrome	Indoles and derivatives	1.76	0.04	1.56	Upregulated
C11045	294.12	Aspartame	Carboxylic acids and derivatives	1.85	0.03	1.62	Upregulated
C05138	332.24	17a-Hydroxypregnenolone	Steroidsand steroid derivatives	1.89	0.02	1.76	Upregulated
C00255	376.14	Riboflavine	Vitamins	2.11	0.00	2.05	Upregulated
C10372	272.10	9-Methoxy-alpha-lapachone	Quinones	2.05	0.00	2.47	Upregulated
C10875	412.12	Podophyllotoxinone	Lignans	1.91	0.02	2.51	Upregulated

(Continued)

TABLE 1 | Continued

KEGG-ID	Molecular mass	Metabolite name	Class	VIP	*P*-value	Fold change	Regulation
C01152	169.09	1-Methy-L-histidine	Amino acids	1.88	0.02	2.75	Upregulated
C09274	310.20	Tabernanthine	Alkaloids	1.95	0.01	2.82	Upregulated
C00079	165.08	L-Phenylalanine	Amino acid and derivatives	1.93	0.02	3.00	Upregulated
C05198	251.10	5'-Deoxyadenosine	Nucleotide and derivatives	1.84	0.03	4.84	Upregulated
C08431	251.10	Cordycepin	Nucleotide and derivatives	1.84	0.03	4.84	Upregulated
C00153	122.05	Nicotinamide	Alkaloids	2.01	0.01	7.72	Upregulated
C02353	329.05	Adenosine 2',3'-cyclic phosphate	Purine nucleotides	2.07	0.00	57.29	Upregulated
C00942	345.05	Guanosine 3',5'-cyclic monophosphate	Nucleotide and derivatives	1.78	0.04	58.52	Upregulated
C10190	372.12	Tangeretin	Flavonoids	1.97	0.01	195.63	Upregulated
	374.28	Ginkgolic acid C17:1	Phenols	2.10	0.00	1737.4	Upregulated
Thermal processing stage: drying							
C05243	299.15	N-Methylcoclaurine	Alkaloids	1.87	0.04	0.17	Downregulated
C01026	103.06	N,N-Dimethylglycine	Amino acid and derivatives	2.03	0.01	0.25	Downregulated
C09202	376.15	Tripdiolide	Diterpenoids	2.20	0.00	0.48	Downregulated
C09868	150.10	(R)-Menthofuran	Prenol lipids	1.89	0.04	0.48	Downregulated
C05380	180.05	Nicotinurate	Carboxylic acids and derivatives	2.15	0.00	0.56	Downregulated
C17496	350.25	10-Gingerol	Phenols	2.09	0.01	0.57	Downregulated
C02666	178.06	Coniferylaldehyde	Phenylpropanoids	2.03	0.01	0.59	Downregulated
C00526	228.07	2'-Deoxyuridine	Nucleotide and derivatives	1.94	0.04	0.64	Downregulated
C10501	624.21	Verbascoside	Phenylpropanoids	1.92	0.04	1.16	Upregulated
C00576	101.08	Betaine aldehyde	Organonitrogen compounds	1.99	0.03	1.32	Upregulated
	184.04	Methyl gallate	Phenols	1.88	0.04	1.35	Upregulated
C08316	338.32	Erucic acid	Fatty Acyls	2.11	0.001	1.53	Upregulated
C10283	312.14	4'-Prenyloxyresveratrol	Phenols	2.07	0.01	1.62	Upregulated
C05905	286.05	Cyanidin	Flavonoids	2.21	0.00	1.81	Upregulated
C17497	194.09	Zingerone	Phenols	1.96	0.03	1.89	Upregulated
C00153	122.05	Nicotinamide	Alkaloids	2.13	0.01	1.89	Upregulated
C00881	227.09	Deoxycytidine	Nucleotide and derivatives	1.95	0.04	2.06	Upregulated
	174.10	6(1H)-Azulenone, 2,3-dihydro-1,4-dimethyl	Miscellaneous	2.09	0.01	2.25	Upregulated
C06075	136.13	Terpinolene	Monoterpenoids	2.01	0.02	2.32	Upregulated
	804.38	Rebaudioside B	Diterpenoids	2.03	0.02	2.43	Upregulated
C07650	263.07	Gemcitabine	Pyrimidine nucleosides	1.90	0.04	2.73	Upregulated
C00328	208.08	L-Kynurenine	Amino acid and derivatives	2.00	0.03	2.77	Upregulated
C10333	367.16	Isatidine	Alkaloids	1.92	0.04	2.83	Upregulated
C09770	334.07	Cedeodarin	Flavonoids	2.06	0.02	3.06	Upregulated
C13202	472.39	DL-alpha-Tocopherylacetate	Phenols	1.86	0.04	3.44	Upregulated
C04294	143.04	4-Methyl-5-thiazoleethanol	Azoles	2.04	0.01	4.06	Upregulated
C10640	372.16	Kadsurin A	Lignans	2.13	0.01	4.61	Upregulated
C16959	216.15	Furanodiene	Sesquiterpenoids	1.87	0.04	7.61	Upregulated
C16968	366.11	Neoglycyrol	Coumarins	2.21	0.00	12.69	Upregulated
C05623	464.10	Isoquercitrin	Flavonoids	1.85	0.04	21.52	Upregulated

VIP, variable importance projection.

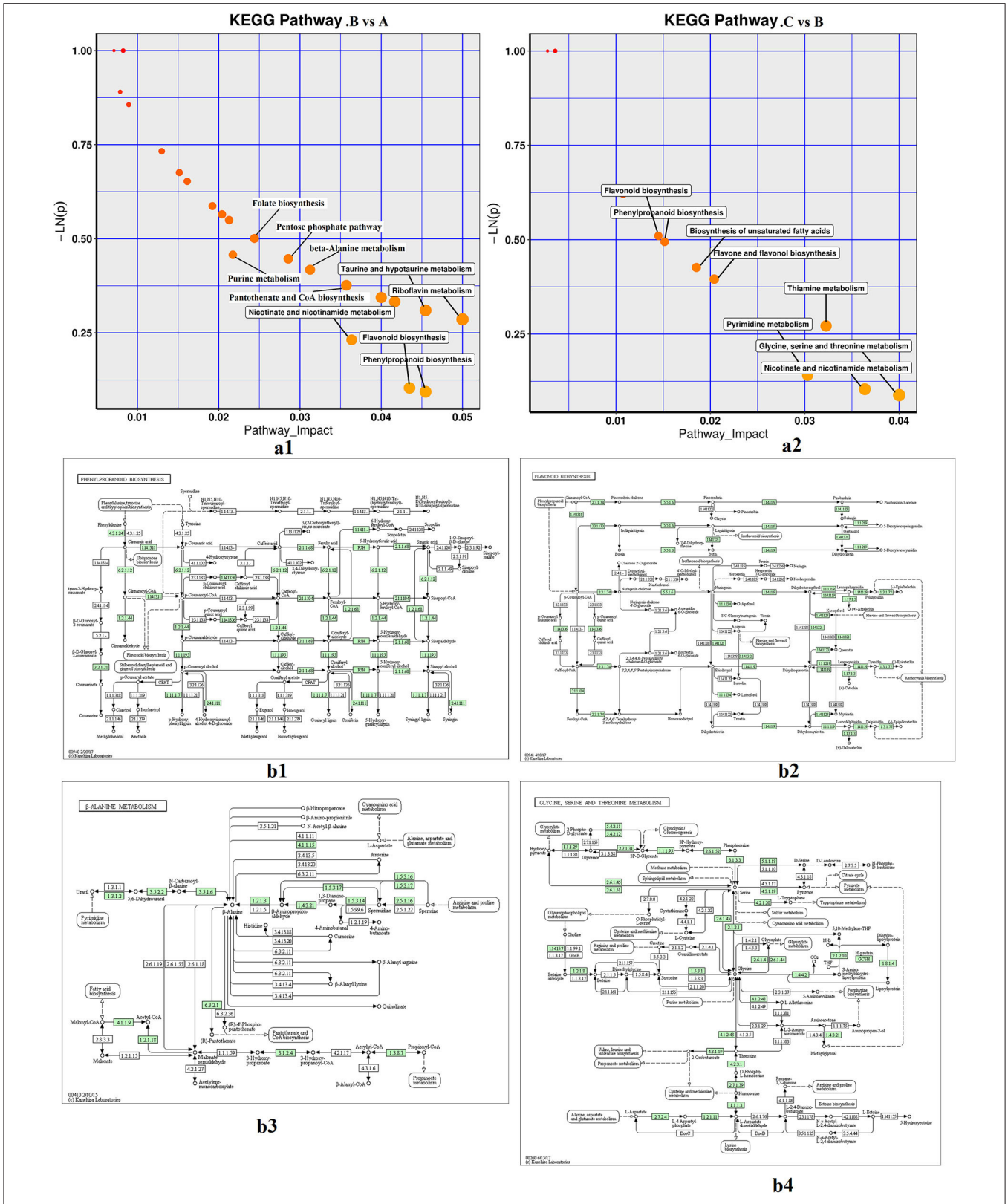

FIGURE 5 | Metabolic enrichment pathway analysis in two comparative groups **(a1,a2)** and important KEGG pathway maps **(b1,b4)**. **(a1,a2)** represent the enrichment analysis of different metabolites in the steaming and drying processes, respectively; **(b1,b4)** respectively represent phenylpropanoid biosynthesis pathway, flavonoid biosynthesis pathway, alanine metabolism pathway, glycine, serine and threonine metabolism pathway. A, fresh; B, steamed without drying; C, dried after steaming. KEGG, Kyoto Encyclopedia of Genes and Genomes.

than the drying stage, this characteristic is associated with the amino acids' metabolism pathway. However, the levels of the above metabolites decreased significantly during the drying process, suggesting the formation of active compounds mainly occurred in the steaming stage during the thermal processing of *Cistanche deserticola*. To the best of our knowledge, this is the first time that the widely targeted metabolomic method was used to reveal that the mechanism of active compounds changes during the thermal processing and their crucial contribution to the *Cistanche deserticola* blackening. However, further investigation is needed for a better understanding of the relationship between the biosynthesis of active compounds and the blackening of the appearance during thermal processing.

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/s.

AUTHOR CONTRIBUTIONS

ZA conducted experimental design, performed the experiments, generated the data, and wrote this manuscript. YZ performed the metabolomics analysis. XL provided the statistical analysis. WS conducted data processing and investigation. Funding acquisition, overall framework, and writing-reviewing were completed by YL. 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/fnut.2021.742511/full#supplementary-material

REFERENCES

1. Wang X, Wang J, Guan H, Xu R, Luo X, Su M, et al. Comparison of the chemical profiles and antioxidant activities of different parts of cultivated Cistanche deserticola using ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry and a 1,1-diphenyl-2-picrylhydrazyl-based assay. *Molecules.* (2017) 22:2011. doi: 10.3390/molecules22112011
2. Fu Z, Fan X, Wang X, Gao X. Cistanches Herba: an overview of its chemistry, pharmacology, and pharmacokinetics property. *J Ethnopharmacol.* (2018) 219:233–47. doi: 10.1016/j.jep.2017.10.015
3. Piwowarczyk R, Carlón L, Kasińska J, Tofil S, Furmańczyk P. Micromorphological intraspecific differentiation of nectar guides and landing platform for pollinators in the Iberian parasitic plant Cistanche phelypæa (Orobanchaceae). *Bot Lett.* (2016) 163:47–55. doi: 10.1080/12538078.2015.1124287
4. Jiang Y, Tu P. Analysis of chemical constituents in Cistanche species. *J Chromatogr a.* (2009) 1216:1970–9. doi: 10.1016/j.chroma.2008.07.031
5. Li Z, Lin H, Gu L, Gao J, Tzeng C. Herba Cistanche (Rou Cong-Rong): One of the best pharmaceutical gifts of traditional Chinese medicine. *Front Pharmacol.* (2016) 7:41. doi: 10.3389/fphar.2016.00041
6. Song Y, Zeng K, Jiang Y, Tu P. Cistanches Herba, from an endangered species to a big brand of Chinese medicine. *Med Res Rev.* (2021) 5:1–39. doi: 10.1002/med.21768
7. Xiong Q, Kadota S, Tani T, Namba T. Antioxidative effects of phenylethanoids from Cistanche deserticola. *Biol Pharmac Bull.* (1996) 19:1580–5. doi: 10.1248/bpb.19.1580
8. Wang L, Ding H, Yu H, Han L, Lai Q, Zhang L, et al. Cistanches herba: chemical constituents and pharmacological effects. *Chin Herbal Med.* (2015) 7:135–42. doi: 10.1016/S1674-6384(15)60017-X
9. Peng F, Xu R, Wang X, Xu C, Liu T, Chen J. Effect of the steaming process on quality of postharvest Cistanche deserticola for medicinal use during sun drying. *Biol Pharm Bull.* (2016) 39:2066–70. doi: 10.1248/bpb.b16-00250
10. Peng F, Chen J, Wang X, Xu C, Liu T, Xu R. Changes in levels of phenylethanoid glycosides, antioxidant activity, and other quality traits in Cistanche deserticola slices by steam processing. *Chem Pharm Bull.* (2016) 64:1024–30. doi: 10.1248/cpb.c16-00033
11. Zou P, Song Y, Lei W, Li J, Tu P, Jiang Y. Application of 1 H NMR-based metabolomics for discrimination of different parts and development of a new processing workflow for Cistanche deserticola. *Acta Pharm Sin B.* (2017) 7:647–56. doi: 10.1016/j.apsb.2017.07.003
12. Zheng J, Wu Z, Yang N, Zhou K, Hu W, Ou S, et al. Widely targeted UHPLC-MS/MS metabolomic analysis on the chemical variation in blueberry-filled pastries during processing. *Frontiers in Nutrition.* (2020) 7:569172. doi: 10.3389/fnut.2020.569172
13. Liu W, Song Q, Cao Y, Xie N, Li Z, Jiang Y, et al. From 1H NMR-based non-targeted to LC–MS-based targeted metabolomics strategy for in-depth chemome comparisons among four Cistanche species. *J Pharmaceut Biomed.* (2019) 162:16–27. doi: 10.1016/j.jpba.2018.09.013
14. Wang H, Hua J, Yu Q, Li J, Wang J, Deng Y, et al. Widely targeted metabolomic analysis reveals dynamic changes in non-volatile and volatile metabolites during green tea processing. *Food Chem.* (2021) 363:130131. doi: 10.1016/j.foodchem.2021.130131
15. Koistinen VM, Da Silva AB, Abrankó L, Low D, Villalba RG, Barberán FT, et al. Interlaboratory coverage test on plant food bioactive compounds and their metabolites by mass spectrometry-based untargeted metabolomics. *Metabolites.* (2018) 8:46. doi: 10.3390/metabo8030046
16. Santin M, Lucini L, Castagna A, Chiodelli G, Hauser M, Ranieri A. Post-harvest UV-B radiation modulates metabolite profile in peach fruit. *Postharvest Biol Tec.* (2018) 139:127–34. doi: 10.1016/j.postharvbio.2018.02.001
17. Nie H, Chen H, Li G, Su K, Song M, Duan Z, et al. Comparison of flavonoids and phenylpropanoids compounds in Chinese water chestnut processed with different methods. *Food Chem.* (2021) 335:127662. doi: 10.1016/j.foodchem.2020.127662
18. Liu Y, Liu J, Wang R, Sun H, Li M, Strappe P, et al. Analysis of secondary metabolites induced by yellowing process for understanding rice yellowing mechanism. *Food Chem.* (2021) 342:128204. doi: 10.1016/j.foodchem.2020.128204
19. Wu L, Huang X, Liu S, Liu J, Guo Y, Sun Y, et al. Understanding the formation mechanism of oolong tea characteristic non-volatile chemical constitutes during manufacturing processes by using integrated widely-targeted metabolome and DIA proteome analysis. *Food Chem.* (2020) 310:125941. doi: 10.1016/j.foodchem.2019.125941
20. Xie Y, Li X, Zhang Y, Zheng Z, Huang L, Liu D, et al. Effects of high-humidity hot air impingement steaming on Gastrodia elata: steaming degree, weight

loss, texture, drying kinetics, microstructure and active components. *Food Bioprod Process.* (2021) 127:255–65. doi: 10.1016/j.fbp.2021.03.005
21. Chen W, Gong L, Guo Z, Wang W, Zhang H, Liu X, et al. A novel integrated method for large-scale detection, identification, and quantification of widely Targeted metabolites: application in the study of rice metabolomics. *Mol Plant.* (2013) 6:1769–80. doi: 10.1093/mp/sst080
22. Arena S, Renzone GD, Ambrosio C, Salzano A M, Scaloni A. Dairy products and the Maillard reaction: a promising future for extensive food characterization by integrated proteomics studies. *Food Chem.* (2017) 219:477–89. doi: 10.1016/j.foodchem.2016.09.165
23. Rizzi G P. Chemical structure of colored maillard reaction products. *Food Rev Int.* (1997) 13:1–28. doi: 10.1080/87559129709541096
24. Liu Z, Chao Z, Liu Y, Song Z, Lu A. Maillard reaction involved in the steaming process of the root of Polygonum multiflorum. *Planta Med.* (2009) 75:84–8. doi: 10.1055/s-0028-1088349
25. Jin J, Lao J, Zhou R, He W, Qin Y, Zhong C, et al. Simultaneous identification and dynamic analysis of saccharides during steam processing of rhizomes of Polygonatum cyrtonema by HPLC–QTOF–MS/MS. *Molecules.* (2018) 23:2855. doi: 10.3390/molecules23112855
26. Wan XC. *Biochemistry of Tea* (Third Edition). Beijing: China Agricultural Publishing House (2003). p. 41–5.
27. Xu Y, Xiao Y, Lagnika C, Li D, Liu C, Jiang N, et al. A comparative evaluation of nutritional properties, antioxidant capacity and physical characteristics of cabbage (Brassica oleracea var. Capitate var L) subjected to different drying methods. *Food Chem.* (2020) 309:124935. doi: 10.1016/j.foodchem.2019.06.002
28. Dixon R A, Paiva N L. Stress-induced phenylpropanoid metabolism. *Plant Cell.* (1995) 7:1085–97. doi: 10.1105/tpc.7.7.1085
29. Gupta R, Min C W, Kim S W, Wang Y, Agrawal G K, Rakwal R, et al. Comparative investigation of seed coats of brown- vs. yellow-colored soybean seeds using an integrated proteomics and metabolomics approach. *Proteomics.* (2015) 15:1706–16. doi: 10.1002/pmic.201400453
30. Commisso M, Toffali K, Strazzer P, Stocchero M, Ceoldo S, Baldan B, et al. Impact of phenylpropanoid compounds on heat stress tolerance in carrot cell cultures. *Front Plant Sci.* (2016) 7:1439. doi: 10.3389/fpls.2016.01439
31. Wahid A, Gelani S, Ashraf M, Foolad M. Heat tolerance in plants: an overview. *Environ Exp Bot.* (2007) 61:199–223. doi: 10.1016/j.envexpbot.2007.05.011
32. Wu X, Yuan J, Luo A, Chen Y, Fan Y. Drought stress and re-watering increase secondary metabolites and enzyme activity in dendrobium moniliforme. *Ind Crop Prod.* (2016) 94:385–93. doi: 10.1016/j.indcrop.2016.08.041
33. Wang Y, Ren W, Li Y, Xu Y, Teng Y, Christie P, et al. Nontargeted metabolomic analysis to unravel the impact of di (2-ethylhexyl) phthalate stress on root exudates of alfalfa (Medicago sativa). *Sci Total Environ.* (2019) 646:212–9. doi: 10.1016/j.scitotenv.2018.07.247
34. Jia X, Sun C, Li G, Li G, Chen G. Effects of progressive drought stress on the physiology, antioxidative enzymes and secondary metabolites of Radix Astragali. *Acta Physiol Plant*. (2015), 37:262. doi: 10.1007/s11738-015-2015-4
35. Paupière M J, Müller F, Li H, Rieu I, Tikunov Y M, Visser R G F, et al. Untargeted metabolomic analysis of tomato pollen development and heat stress response. *Plant Reprod.* (2017) 30:81–94. doi: 10.1007/s00497-017-0301-6
36. Chen J, Ho C. Comparison of volatile generation in serine/threonine/glutamin–ribose/glucose/fructose model systems. *J Agr Food Chem.* (1999) 47:643–7. doi: 10.1021/jf980771a

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An Evaluation of Phenolic Compounds, Carotenoids, and Antioxidant Properties in Leaves of South African Cultivars, Peruvian 199062.1 and USA's Beauregard

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In this study, leaves of sweet potato cultivars from South Africa ("Ndou," "Bophelo," "Monate," and "Blesbok"), "Beauregard," a sweet potato cultivar from the USA, and a Peruvian cultivar "199062. 1" were analyzed using UPLC/QTOF/MS and chemometrics, with the aim of characterizing the locally developed sweet potato cultivars and comparing them with already well-known established varieties on the market. A set of 13 phenolic compounds was identified. A partial least squares discriminant analysis, a hierarchical cluster analysis, and variables importance in projection were used to successfully distinguish sweet potato varieties based on their distinct metabolites. Caffeic acid enabled to distinguish Cluster 1 leaves of varieties ("Beauregard" and "Ndou") from Cluster 2 ("199062.1," "Bophelo," "Monate," and "Blesbok"). The leaves of "Bophelo" contained the highest concentrations of rutin, quercetin 3-*O*-galactoside, 3-caffeoylquinic acid (3-CQA), (5-CQA), 1,3 dicaffeoylquinic acid (1,3-diCQA), 1,4-diCQA, and 3,5-diCQA. Furthermore, Bophelo leaves showed the highest antioxidant activities (FRAP 19.69 mM TEACg^{-1} and IC_{50} values of (3.51 and 3.43 mg ml^{-1}) for DPPH and ABTS, respectively, compared to the other varieties. Leaves of "Blesbok" contained the highest levels of β-carotene (10.27 mg kg^{-1}) and zeaxanthin (5.02 mg kg^{-1}) on a dry weight basis compared to all other varieties. This study demonstrated that the leaves of local cultivars "Bophelo" and "Blesbok" have the potential to become functional ingredients for food processing.

Keywords: phytochemicals, leafy vegetable, caffeoylquinic acid, β-carotene, antioxidant activity

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INTRODUCTION

Sweet potatoes (*Ipomoea batata* L. Lam.) are dicotyledonous plants of the Convolvulaceae family (1). Due to their high yield, drought resistance, and ability to grow in a cultivar of climates and conditions, leafy vegetables, such as sweet potato leaves, are becoming popular as a food security crop in developing countries (2). During the summer season, the leaves can be eaten as green leafy vegetables, possibly alleviating food shortages (2). The elements Na (8.06–832.31 mg 100 g^{-1} dry weight (DW), Mg (220.2–910.5 mg 100 g^{-1} DW), K (479.3–4,280.6 mg

100 g^{-1} DW), Ca (229.7–1,958.1 mg 100 g^{-1} DW, and P (131.1–2,639.8 mg 100 g^{-1} DW) are abundant in sweet potato leaves (3). The leaves, which flourish in poor, wet, and rich soil, can be cropped continuously until the root vegetables are harvested (3). While phytochemical content in sweet potato has been investigated, most of the research has concentrated on β-carotene with little information on variations in total phytochemicals and antioxidant activity among local cultivars.

Sweet potato leaves have been reported to contain phenolics in high levels, which make them superior to other commercial vegetables. Unlike spinach, cabbage, broccoli or kale, sweet potato leaves contain more polyphenols (4). Sweet potatoes high in polyphenol content are increasingly popular with health-conscious consumers (5). Sweet potato leaves, therefore, greatly contribute to the availability of food as well as bioactive compounds for consumers. As a functional food, sweet potato leaves contain a variety of bioactive compounds that have health-promoting properties. Polyphenols and carotenoids are among the compounds in sweet potato leaves reported to have beneficial effects on health (2). The functional compounds found in sweet potato leaves are responsible for a variety of biological functions (antioxidant, anticancer, antimutagenic, immune modulation, and liver protection) (2). As a functional ingredient, powdered sweet potato leaves can be used in food products, such as beverages in the food industry (6). In general, rural populations consume boiled or blanched sweet potato leaves. The sweet potato could become a profitable leafy vegetable crop if appropriate varieties were available or developed.

To date, the Agricultural Research Council of South Africa has released 25 cultivars due to its breeding program (7). "Blesbok," "Bosbok," and "Ribbok" are the main commercialized cultivars currently grown in South Africa, with a cream flesh in most cases (5). On the informal market, the most popular cultivars are "Ndou" (cream fleshed) and "Bophelo" (orange fleshed) (7), while the Peruvian cultivar 199062.1 and the "Beauregard" from the USA are being promoted. Specifically, the breeding effort aims to improve traits of interest to resource-poor farmers, such as high-dry matter content combined with high yield, as well as β-carotene content and resistance to drought and disease (7). However, very limited information is available on the content of phenolic compounds in locally used sweet potato cultivars. Thus far, a study by Nyati et al. (8) promoted the dual-purpose use of cultivar "Bophelo" in South Africa based on the iron, zinc, and β-carotene content. Therefore, for a full understanding of the composition of bioactive compounds and health benefits of the leaves for commercialization of locally grown sweet potato cultivars, it is important to know the predominant bioactive compounds.

In sweet potato leaves, caffeic acid and caffeoylquinic acid derivatives are the main phenolic acids (2). Specific genotypes and stages of leaf development influence these constituents (2). The amount of light exposure affects the concentration of phenolic components of sweet potato leaves (2). Compared with oven drying at 70 or 100°C, freeze-drying produced the most caffeoylquinic acid derivatives in sweet potato leaves (9). Moreover, the amount of lutein in sweet potato varieties varies from 34 to 68 mg 100 g^{-1}, which makes it the main carotenoid component of sweet potato leaves (10). A strong antioxidant capacity is demonstrated by caffeoylquinic acid derivatives (11). However, it is essential to identify the specific caffeoylquinic acid present in the leaves of sweet potato cultivars that contribute to antioxidant activity by analyzing the correlation coefficients between two variables. As sweet potato leaves contain bioactive compounds, an assessment of the leaf compositions of the various cultivars grown locally in comparison to the cultivars already available on the market, such as "Beauragurad" and Peruvian "199062.1," is necessary.

These three objectives serve as the basis for this study. The first objective of the study was to use metabolomic chemometrics to elucidate and characterize the leaves of locally developed sweet potato varieties and compare them with the cultivars "Beauregard" from the United States and the Peruvian cultivar "199062.1." The second objective was to compare the antioxidant properties in the leaves of domestic cultivars, "Beauregard," and Peruvian cultivar "199062.1." The third objective was to compare carotenoid components in the leaves of local sweet potato cultivars with those in the Peruvian cultivar "199062.1" and the "Beauregard" cultivar.

MATERIALS AND METHODS

Chemicals

The following chemicals used in this study: 2.2′-diphenyl-1-picrylhydrazyl (DPPH), methanol, trolox, potassium persulfate, sodium acetate trihydrate, persulfate sodium acetate, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), $FeCl_3 \cdot 6H_2O$, acetone, hexane, isopropyl alcohol, Folin-Ciocalteau reagent, Na_2CO_3, gallic acid, HCl, NaOH, diethyl ether, ethyl acetate, phosphate buffer, butylated hydroxytoluene (BHT), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS), acetic acid, Na_2SO_4, acetonitrile, methanol, *N*-hexane formic acid, NH_4OH, analytical standards (chlorogenic acid, neochlorogenic acid, caffeic acid, quercetin-3-*O*-glycoside, ferulic acid, vanillic acid, p-coumaric acid, β-carotene, lutein, gallic acid, zeaxanthin) were purchased from Sigma Aldrich, Johannesburg, South Africa.

Plant Material

The leaves of four cultivars of sweet potato (*Ipomoea babatas* L.) developed in South Africa (orange-fleshed storage roots "Bophelo," cream-fleshed "Monate," "Ndou," "Blesbok") and the USA's "Beauregard" cultivar (orange fleshed) and Peru's "1999062.1" cultivar (yellow orange flesh) were obtained from Agricultural Research Council (ARC-VIMP), Roodeplaat, Pretoria (**Figure 1**). The planting of the cultivars, according to standard production practices, took place in the middle of October 2020, with an average temperature of 25–31°C. The random harvesting of the leaves, up to the fifth leaf from the tip of the vines, occurred early morning. Leaves free from damage and decay were sorted and the dirt removed by washing under running tap water. Afterwards, the leaves were freeze-dried (United Scientific freeze dryer, Model FM25XL-70 at −55°C), grounded into a fine powder and stored at −20°C for biochemical analysis.

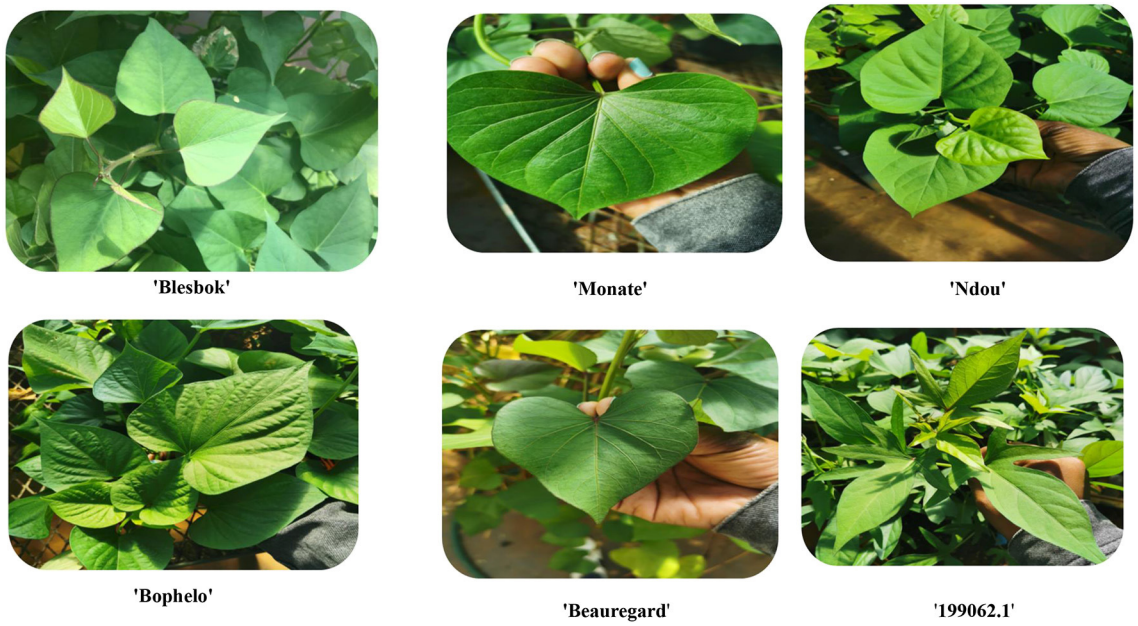

FIGURE 1 | Leaves of different cultivars of sweet potatoes.

Total Phenolic Content (TPC)

Total phenolic content was measured by the Folin-Ciocalteau method (12). A freeze-dried sample (1 g) was extracted with 10 ml of 80% methanol by shaking in a magnetic stirrer for 2 h. Afterward, an aliquot of 100 μl of the extract was mixed with 200 μl of 10% Folin-Ciocalteau, and then 800 μl of 7.5% Na_2CO_3 was added to the mixture, and the absorbance was measured at 736 nm using a spectrophotometer. Quantification was done using gallic acid as a reference standard prepared at concentrations ranging from 0 to 100 μg ml^{-1}. Total phenolic content was expressed as milligrams gallic equivalent per kilogram (mg $GAEkg^{-1}$) on a dry weight basis (DW).

Total Antioxidants Activities

The Ferric Reducing Antioxidant Power (FRAP) assay was performed according to the procedure described by Seke et al. (13) using the FRAP reagent, which was made up of 10 $mmol{\cdot}L^{-1}$ solution of TPTZ in 40 mM of HCl, 20-mM solution of $FeCl_3{\cdot}6H_2O$ and a 20-mM acetate buffer (pH 3.6) mixed in a 1:1:10 ratio, respectively. The sample (0.1 g) was homogenized with 80% methanol and 20 μl of the sample and 150 μl of FRAP reagent and incubated for 10 min, and, thereafter, the absorbance was measured at 593 nm on a microplate reader. Trolox solution ranging from (0 to 30 mM) was prepared for quantification as a reference standard; results were expressed as mM $TEACg^{-1}$.

The radical scavenging activity was assessed using the 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging ability assay with slight modifications, according to the method described by Seke et al. (13), using.1 g of a freeze-dried sample mixed with 1 ml of 80% methanol. The sample mixture was centrifuged at 3,000 × *g* for 5 min at 4°C using a Hermle centrifuge (Model Hermle Z326k, HermleLabortechnik GmbH, Wehingen, Germany). Different sample concentrations (100 μl) were made by serial dilution (0–10 mg ml^{-1}) and 200 μl DPPH solution (13 μl DPPH ml^{-1} methanol) were added to each well, and the absorbance was measured at 517 nm after incubating for 20 min at ± 25°C. The % inhibition was calculated using the equation.

$$\text{DPPH \% inhibition} = (A0 - A1/A0) \times 100$$

Where A0 is the absorbance of the DPPH radical solution, and A1 is the absorbance of a sample. The IC_{50} (mg ml^{-1}) was calculated from the graph of the inhibition percentage vs. the concentration.

2,2′-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic Acid) ($ABTS^+$) radical scavenging was performed based on the method previously described by Seke et al. (13). The production of the ABTS radical cation ($ABTS^+$) was determined by the reaction of the ABTS stock solution (7 mM) with 4.9-mM potassium persulphate at the ratio of 1:1 and allowing the mixture to incubate in the dark at 25°C for 12-16 h before use. An aliquot of 40 μl of a sample (different concentrations from 0-10 mg ml^{-1} made by serial dilution) was pipetted into 200 μl of the $ABTS^+$. The mixture was incubated in the dark in a 96-well microplate reader at 37°C for 10 min; the decrease in absorbance at 734 nm was measured. The % inhibition was calculated using the equation.

$$\text{ABTS \% inhibition} = (A0 - A1/A0) \times 100$$

TABLE 1 | Tentative identification of phenolic compounds in the leaves of different sweet potato cultivars by UPLC–QTOF/MS.

Peak	Retention time (min)	[M-H]-	M-H formula	Error (ppm)	MSE fragments	UV	Tentative identification
1	4.068	353.08893	$C_{16}H_{18}O_9$	−3.17	191, 179, 173, 135	324	Neochlorogenic acid (5-CQA)
2	4.525	369.08224	$C_{16}H_{18}O_{10}$	1.31	207, 192, 167	324	5-Hydroxy-6-methoxycoumarin 7-glucoside
3	5.123	595.16675	$C_{27}H_{32}O_{15}$	0.08	385, 355, 285	290, 330	Eriodictyol 7-O-neohesperidoside (Neoeriocitrin)
4	5.235	179.03531	$C_9H_8O_4$	−1.82	179, 135	290, 323	Caffeic acid
5	5.393	353.08524	$C_{16}H_{18}O_9$	7.28	191, 179, 173, 161, 135	318	Chlorogenic acid (3-CQA)
6	5.967	625.14038	$C_{27}H_{30}O_{17}$	1.04	300, 191, 179, 135	339	Quercetin 3-glucosyl-(1->2)-galactoside
7	6.279	380.99002	$C_{18}H_6O_{10}$	−3.41	301, 179, 151	335	Quercetin derivates
8	6.655	609.14282	$C_{27}H_{30}O_{16}$	5.41	300, 151	255, 353	Quercetin-3-O-rutinoside (Rutin)
9	6.896	463.08786	$C_{21}H_{20}O_{12}$	0.74	300, 271, 285, 179, 151	255, 355	Quercetin 3-galactoside (Q-3-GA)
10	7.116	515.11896	$C_{25}H_{24}O_{12}$	1.06	353, 191, 179, 135	324	3,5-Dicaffeoyquinic acid (3,5-diCQA)
11	7.344	515.11835	$C_{25}H_{24}O_{12}$	2.24	353, 300, 173, 135	324	1,3-Dicaffeoyquinic acid (1,3-diCQA)
12	7.647	515.12085	$C_{25}H_{24}O_{12}$	−2.61	353, 300, 191, 173, 135	324	1,4-Dicaffeoyquinic acid (1,4-diCQA)
13	8.336	515.11902	$C_{25}H_{24}O_{12}$	0.94	353, 300, 203, 191, 173, 179	324	4,5-Dicaffeoyquinic acid (4,5-diCQA)

Where A0 is the absorbance of the ABTS radical solution, and A1 is the absorbance of a sample. The IC_{50} (mg ml^{-1}) was calculated from the graph of the inhibition percentage vs. the concentration.

Untargeted Metabolites

The method of Mashitoa et al. (14) was used to extract and analyze metabolites without any changes. Briefly, freeze-dried samples of leaves (2 g) were homogenized with 15 ml of 80:20 methanol/water (v/v) at 25°C. The samples were vortexed for 1 min and then extracted by sonication (MRC ultrasonic cleaner) for 1 h, centrifuged at 2,000 × *g* using a Hermle centrifuge (Model Hermle Z326k, HermleLabortechnik GmbH, Wehingen, Germany), and the supernatant was taken for analysis. Analysis was done using a Waters Acquity Ultra performance liquid chromatograph (UPLC) hyphenated to a Waters Synapt G2 Quadrupole time of flight (QTOF) mass spectrometer (MS) (Waters, Milford, MA, USA). Concentrations of phenolic compounds were determined using reference standards catechin, epicatechin, caffeic acid, and chlorogenic acid and rutin to quantify compounds based on the areas of their extracted mass chromatograms. **Table 1** presents the chemical formulas, mass fragments, and UV absorbance of the tentatively identified phenolic compounds.

Extraction of Carotenoids

Carotenoids were extracted according to the method of Panfili et al. (15) with minor changes. Freeze-dried leaves (1 g) were extracted using 5 ml of acetone: hexane (1:1) with 0.1% butylated hydroxytoluene (BHT) by keeping them in a dark overnight in tightly closed tubes. The mixture was separated using a centrifuge (HermleLabortechnik, Germany Type 2326K, 2010) at 2,000 × g for 15 min at 25°C using a Hermle centrifuge (Model Hermle Z326k, HermleLabortechnik GmbH, Wehingen, Germany). Afterwards, the residue was rinsed with three additional 5-ml volumes of the extraction solvent, and centrifugation was done as before. The supernatants were pooled, dried with anhydrous sodium sulfate, filtered with a Whatman filter paper (No. 1) and evaporated to dryness under a stream of nitrogen. The extract was redissolved in 1 ml of isopropyl alcohol (10%) in n hexane. Quantification was done by using reference standards of each of β-carotene, zeaxanthin, and lutein at concentrations ranging from (0 to 100 μg ml^{-1}) to quantify compounds based on their areas.

Statistical Analysis

This study was laid out in a completely randomized design with 10 replicates per cultivar, and the experiments were repeated two times by harvesting the leaves in 2020 in December-January. The Genstat (VSN International, Hemel Hempstead, UK) for Windows 13th Edition (2010 version) analyzed the differences between the leaves of different sweet potato cultivars using a one-way ANOVA. To compare the means of the different biochemical components analyzed from the leaves of different sweet potato cultivars, the least significant difference test (LSD) was used, with $p < 0.05$. Each of the six sweet potato cultivars was replicated three times. The results are expressed as mean ± standard deviation. Data sets obtained from the UPLC-Q-TOF/MS analysis for three replicate samples of the leaves of different sweet potato cultivars were imported into MetaAnalyst 5.0 to perform partial least squares discriminant analysis (PLS-DA), variables importance in projection (VIP) scores, and heat maps. Pearson's correlation was conducted, and we used Pearson's correlation coefficients as a distance measure in the graph.

RESULTS AND DISCUSSION

Total Phenols

Figure 2 presents the results of the quantification of total phenolic content. Based on total phenolic content in the leaves of six sweet potato varieties, "Bophelo was the highest, followed by "Beauregard," "Ndou," "Blesbok," "199062.1," and, finally, "Monate." The total phenol content of freeze-dried leaves of six sweet potato cultivars ranged from 2319.10 to 1322.76 mg

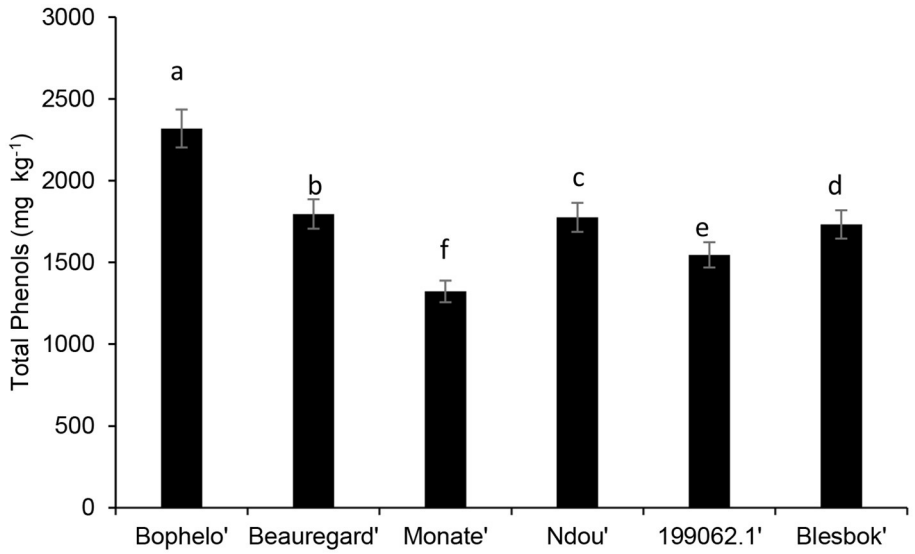

FIGURE 2 | Comparison of the total phenolic content in the leaves of different South African sweet potato cultivar with the USA variety Beauregard and the Peruvian cultivar 199062.1. Bars with similar alphabetic letter are not significantly different at $p < 0.05$ according to Fisher's LSD test.

kg^{-1}. By contrast, Malaysian sweet potato leaves varied between 3,470 and 5,350 mg kg^{-1} in dry weight (11). Furthermore, eight sweet potato cultivars from Japan had leaf total phenolic compounds ranging from 6.3 to 13.5 g GAE100 g^{-1} dry weight, higher than the concretions found in the four South African cultivars and the "Beauregard" and 2000621. Jiang and Koh (16) reported that the leaves of six major North Korean sweet potato cultivars genetically engineered in South Korea contained an average of 650–1,910 g of phenols in 100 g of fresh weight; however, this has no comparison with our data generated on a dry weight basis. Reports of Islam et al. (17) showed that the highest phenolic content in the leaves of "1,389" sweet potato cultivars, lines, and genotypes preserved in the gene bank of NARCKO (National Agricultural Research Center of Kyushu Okinawa Region, Japan) was 6,190 mg kg^{-1} Islam et al. (17). Overall, the total phenolic content of all six sweet potato cultivars was higher than those reported for the common indigenous leafy vegetables *Amaranthus dubius* (516 mg kg^{-1}), *Cleome gynandra* (268 mg kg^{-1}), and *Cucurbita maxima* (394 mg kg^{-1}) (18).

An Untargeted Phenolic Metabolite Profile

Table 1 revealed 13 phenolic compounds tentatively identified by UPLC–QTOF/MS from the leaves of six sweet potato cultivars. The UPLC–QTOF/MS helped to identify caffeoylquinic acid derivatives [(neochlorogenic acid(5-CQA); chlorogenic acid 3-CQA, 3,5-dicaffeoylquinic acid (3,5-diCQA), 1,3-dicaffeoylquinic acid (1,3-diCQA), 1,4-dicaffeoylquinic acid (1,4-diCQA), and 4,5-dicaffeoylquinic acid (4,5-diCQA)], neoeriocitrin (eriodictyol 7-O neohesperidoside), caffeic acid, quercetin 3-glucosyl-(1->2)-galactoside, quercetin derivates, quercetin 3-*O*-galactoside (Q-3-GA), and quercetin-3-*O*-rutinoside (rutin) in the leaves of all six sweet potato varieties. Conversely, the leaves of nine sweet potato cultivars grown in the moderate climate of Poland contained seven polyphenolic compounds, including five caffeoyquinic acid derivatives-—5-CQA, 3-CQA, 4-cryptochlorogenic acid (4-CQA), 3,4-diCQA, 3,5-diCQA—and flavonoids, Q-3-GA and quercetin-3-*O*-glucoside (Q-3-GL) (19). The leaves of six major North Korean sweet potato cultivars genetically engineered in South Korea contained four phenolic compounds, 3CQA, (4,5-diCQA, 3,5-3,5-diCQA, and 3,4-diCQA (16). The observed differences in phenolic components are likely due to the genetic composition of the varieties examined, the climate conditions, and the maturity of the leaves harvested in this study (19). The **Supplementary Figures 1A–N** shows the UV spectrum MS, MS/MS spectrum, and the chemical structures of the tentatively identified compounds.

Metabolomic and Chemometric Profiles

Using the UPLC-Q-TOF/MS data, PCA analysis of unsupervised results helped to identify which sweet potato cultivars contain the most and fewest functional compounds (**Figure 3A**). Two-dimensional scatter plots between PC1 and PC2 explained 90.4% of the total variance (72.4 and 18%, respectively). In **Figure 3A**, three primary groups or clusters of sweet potato cultivars are distinguished based on their leaf phenolic compounds in a systematic and obvious way. As a result, these results confirm that the concentration of different phenolic compounds plays a key role in determining the classification of sweet potato leaves. The loading plot in **Figure 3B** indicates that the greater the distance between a point and its original point, the greater the contribution of the compound to the total variation. The compounds 5CQA, 1,3-diCQA, 1,3-,1,4-diCQA, 3,5-diCQA, Q-3-GA, and 4,5-diCQA were loaded positively on PC1 and separated the leaves of cultivars "199062.1," "Bophelo," and

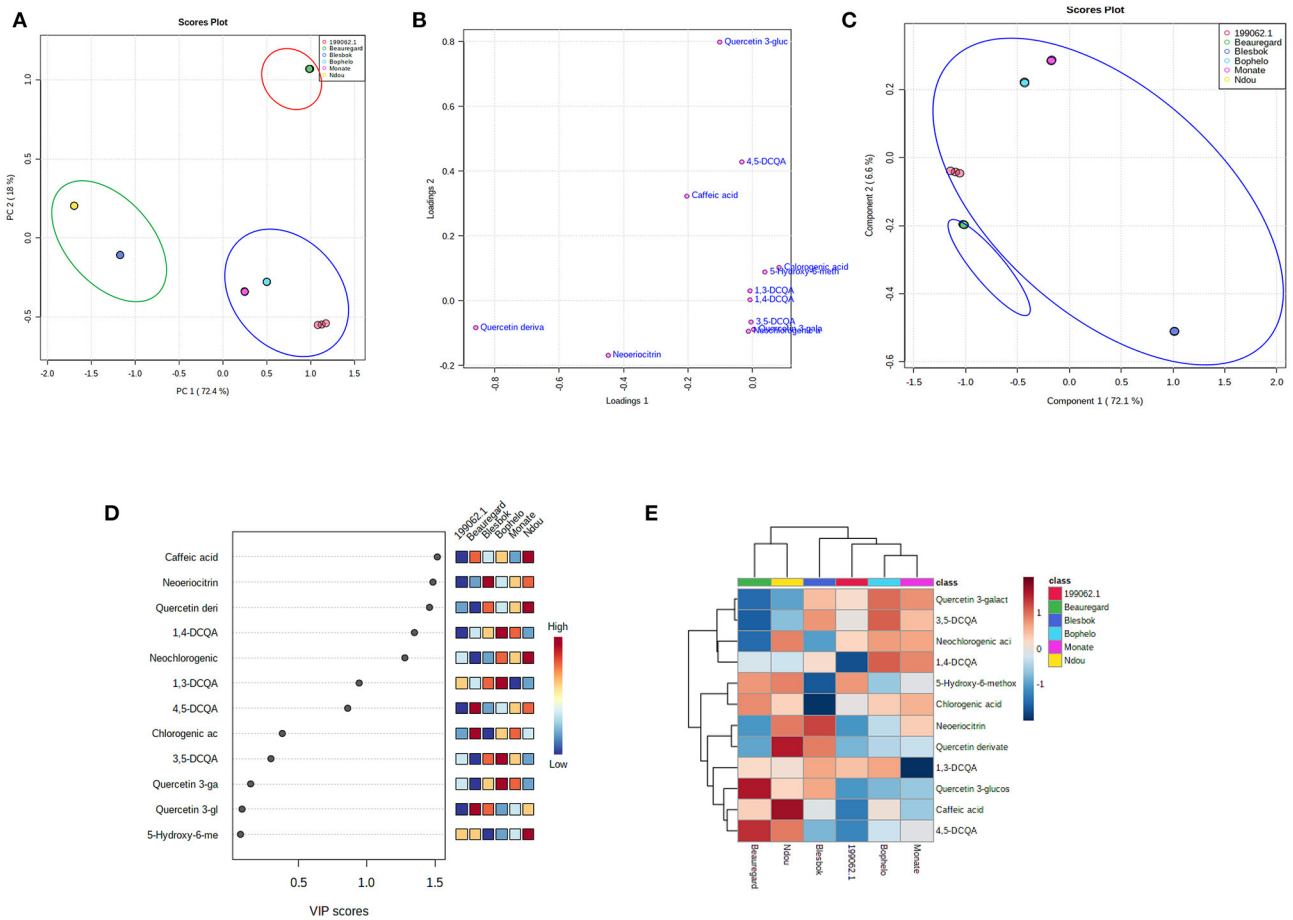

FIGURE 3 | Statistical analyses of bioactive metabolites by Metaboanalyst 5.0 software. **(A)** An unsupervised PCA score plot of phenolic metabolites generated by UPLC-QTOF/MS analysis showing the separation of three clusters. F/MS analysis showing the separation of three clusters. **(B)** A PLS-DA score plot showing six sweet potato cultivars clustered into three groups. **(C)** PLS-DA score plots loaded with different phenolic compounds detected by UPLC-QTOF-MS. **(D)** In PLS-DA, metabolites are assigned VIP scores. The score they receive from low to high determines the importance of variables. The colored boxes on the right show the relative concentration of each of the metabolites. High red levels indicate high levels, and low blue levels indicate low levels. **(E)** Heat map. On the map, the colored areas correspond to the concentrations of different phenolic compounds found in different sweet potato cultivars. Each row represents a phenolic compound, and each column represents the leaf of sweet potato cultivar. Red indicates high levels, and blue indicates low levels.

"Monate" from the rest. Quercetin derivatives and eriodictyol-7-O-neohesperidoside were loaded negatively on PC1, helping to separate the leaves of varieties "Blesbok" and "Beauregard" from the rest. Quercetin 3-glucosyl-(1->2)-galactoside accumulated on PC2 and separated the leaves of "Beauregard" from the others. Therefore, these nine compounds account for most of the variations found between the leaves of six sweet potato cultivars. Despite this, further information must be extracted from the data to provide more specific, meaningful information. Due to this, the complete data set was subjected to partial least squares discriminant analysis (PLS-DA) to determine changes in metabolites according to the type of sweet potato cultivar. PLS-DA has the advantage of not relying on a particular distribution, thus producing more accurate predictions and descriptive models (15). A good fit is observed for the PLS-DA model ($R^2 = 0.90$), and its predictability is high ($Q^2 = 0.85$), allowing us to forecast metabolite changes from the data. Principal component 1 (PC1) accounts for 72.1% of the total variation, while principal component 2 (PC2) accounts for 6.6% of the total variation both together contributing toward 78.1% (**Figure 3C**). PLS-DA was applied to classify the leaves of sweet potato cultivars by their 13 phenolic compounds. PLS-DA plots showed two large groups due to phenolic metabolite distribution. There are four types of sweet potato leaves in Cluster 1: "199062.1," "Bophelo," "Monate," and "Blesbok." "Leaves of "Beauregard" and "Ndou" were placed in Cluster 2. Based on the concept of proximity, it is clear that "199062.1," "Bophelo," "Monate," and "Blesbok" share similar metabolites at higher concentrations. The entire dataset of identified metabolites was analyzed using hierarchical cluster analysis, and clusters of samples with similar chemical composition were shown, providing further evidence about the related metabolites associated with the leaves of different sweet potato cultivars. This analysis was accompanied by a heat map structure based on metabolite concentrations in all samples. The

cladogram at the top of the heat map in **Figure 3D** confirms that there are two major clusters in the PLS-DA plot. Each cluster gram represented a row of data across each column of phenolic compounds as a color block, with dark red boxes representing higher levels of metabolites and dark blue boxes suggesting lower levels. **Figure 3D** shows the 13 metabolites identified in these two groups. The levels of quercetin 3-glucosyl-(1->2)-galactoside and 4,5-diCQA were higher in the leaves of "Beauregard." Leaves of "Ndou" contained higher levels of caffeic acid and quercetin derivates. Leaves of "Blesbok" contained higher concentration of eriodictyol-7-O-neohesperidoside. Furthermore, the heat map represented the tendency of phenolic metabolite compositions in leaves of six sweet potato cultivars.

Additionally, we evaluated the contributions of each metabolite to the separation of groups using Variable Importance in Projection (VIP) scores. A VIP score is determined by summing the squares of the PLS loadings, which indicate how much Y-variance is explained across all dimensions and by adding the weighted sum of the PLS regression coefficients (20). To provide the most meaningful interpretation of the results, only the top metabolites with the highest VIP scores were considered (20) (**Figure 3E**). Among the top six metabolites with VIP scores >1 are caffeic acid, eriodictyol-7-O-neohesperidoside (Neoeriocitrin), quercetin derivates, 1,4-diCQA, quercetin derivates, eriodictyol-7-O-neohesperidoside 3,5-diCQA, and neochlorogenic acid (cis-3-CQA) (**Figure 3E**). Caffeic acid enabled us to distinguish Group 1 (leaves of cultivars "199062.1," "Bophelo," "Monate," and "Blesbok") from Group 2 ("Beauregard" and "Ndou").

Quantified Concentrations of Phenolic Compounds

The concentrations of 13 phenolic compounds determined from six cultivars of sweet potatoes are presented in **Table 2**. Among the leaves of six sweet potato cultivar, "Bophelo" contained the highest concentration of rutin, Q-3-GA, 3-CQA, 5-CQA, 1,3-diCQA, 1,4-diCQA, and 3,5-diCQA. Comparison sweet potato cultivar "Beauregard" showed higher concentrations of quercetin 3-glucosyl-(1->2)-galactoside, 3-CQA, and 4,5-diCQA in the leaves. However, Peruvian cultivar "199062.1" showed the highest concentration of 5-hydroxy-6-methoxycoumarin 7-glucoside and 1,3-diCQA. According to Jung et al. (1), the compound most abundant in sweet potato leaves is 5-neochlorogenic acid (5-CQA), and its amount depends on different cultivars. In contrast, Krochmal-Marczak (19) noted that the chlorogenic acid (3-CQA) was the dominant CQA derivative. Our study found 1,4-diCQA, 3,5-diCQA, and 3-CQA to be the dominant CQA derivatives in the leaves of all six sweet potato cultivars. The phenolic compounds in sweet potatoes have shown the ability to promote human health and can be used as functional foods (1). Therefore, the leaves of the local cultivar "Bophelo" have potential as a functional food. Reportedly, caffeoylquinic acid was antimutagenic in the *Salmonella ames* experiment when caffeoyl groups were bound to quinic acid (21). Additionally, out of the six CQA components, five components were found in higher concentrations in leaves of the local cultivar "Bophelo" while two caffeoylquinic acids (3-CQA, 4,5-diCQA) and one dicaffeoylquinic acid (1,3-diCQA) were detected in the highest concentrations in "Beauregard" and "199062.1" leaves, respectively. The dominant caffeoylquinic acids in the leaves of the local cultivar "Bophelo" were 1,4-DCQA and 3,5-DCQA; additionally, 3,5-DCQA was predominant in the leaves of the USA cultivars "Covington" and "Hernandez" (22). The compound 4-CQA was, however, not detected in the leaves of these sweet potato cultivars. It is likely that 4-CQA is affected by the maturity stage at harvest and is prominent at early maturity stages (19). Furthermore, caffeoylquinic acids and dicaffeoylquinic acids exhibit various biological activities in animals and plants (23).

Antioxidant Capacities

Table 3 compares the antioxidant activities of the leaf extracts of six South African sweet potato cultivars with the USA's Beauregard and Peru's "199062.1" varieties. FRAP, DPPH, and ABTS activities were the highest in leaves of the domestic sweet potato cultivar "Bophelo," compared to the other domestic cultivars and the Peruvian cultivar "199062.1." "Bophelo" leaves showed greater FRAP and DPPH scavenging activities than "Beauregard" leaves despite similar ABTS activity. According to Ghasemzadeh et al. (24), leaf extracts of sweet potato leaves grown in Malaysia with higher total phenolic compounds showed stronger radical scavenging. Compounds exhibiting strong antioxidant activity contain phenolic groups or a large number of conjugated hydroxyl moieties, which are capable of donating electrons to oxidizing radical species (20). It has been established that antioxidant molecules prevent cellular damage and macromolecular degradation by blocking the oxidation of other molecules (20).

A correlation analysis assesses the relationship between two variables by using statistical techniques. A high correlation coefficient indicates a strong relationship between two or more variables, while a low correlation indicates a weak relationship. In order to consider a correlation to be strong, we set a threshold of 0.5. The total phenol content and FRAP activity were strongly and positively correlated ($r = 0.85$, $p < 0.05$) in this study. ABTS radical scavenging activity ($r = 0.75$, $p < 0.05$) and DPPH scavenging activity ($r = 0.88$, $p < 0.05$) are also strongly correlated with total phenol content. Sweet potato leaves contain positive correlations between total polyphenol content and all caffeoylquinic acid derivatives, except for five caffeoylquinic acids and caffeic acids (3). Islam et al. concluded that the correlation between total phenolics and derivatives of the CQA may contribute to the improvement of desirable parameters in the selection of cultivars. Furthermore, a positive correlation ($r = 0.69$ $p < 0.05$) was found between 1,3-diCQA and total phenols in our study. A previous study by Danino et al. (23) demonstrated that 1,3-diCQA exhibits antioxidant properties. In their study, Danino et al. (23) showed that 1,3-diCQA has an IC_{50} of around 2-fold less than trolox, demonstrating greater antioxidant activity than trolox. The same authors found that 1,3-diCQA also significantly inhibits DPPH radicals more effectively than either trolox or caffeic acid. In addition to the abovementioned effects, 1,3-diCQA also

TABLE 2 | Comparison of different phenolic compounds in the leaves of four Southern African sweet potato (*Ipomoea batatas* L.) cultivars with "Beauregard" from the USA and Peruvian "199062.1."

	Orange fleshed storage roots			Cream fleshed roots		
Phenolic components (mg/kg)	"Bophelo" leaves	"Beauregard" leaves	"199062.1" leaves	"Monate" leaves	"Ndou" leaves	"Blesbok" leaves
5-Hydroxy-6-methoxycoumarin 7-glucoside	6.96 ± 0.20c	9.59 ± 0.08a	9.59 ± 0.22a	7.92 ± 0.37b	9.86 ± 0.21a	5.33 ± 0.11d
Eriodictyol-7-O-Neohesperidoside (Neoeriocitrin)	15.94 ± 0.19d	9.50 ± 2.49e	9.35 ± 1.95e	24.43 ± 1.8c	33.86 ± 0.70b	40.26 ± 0.41a
Caffeic acid	41.89 ± 0.17b	44.82 ± 0.56b	26.76 ± 0.34e	34.10 ± 0.64d	65.07 ± 2.00a	39.81 ± 1.55c
Quercetin 3-glucosyl-(1->2)-galactoside	6.97 ± 0.14d	29.52 ± 0.18a	5.46 ± 0.69d	7.87 ± 1.26d	14.38 ± 0.69c	17.89 ± 0.54b
Quercetin derivates	1.56 ± 0.06c	0.26 ± 0.09c	0.60 ± 0.49c	2.24 ± 0.80c	31.13 ± 1.34a	15.98 ± 0.41b
Quercetin-3-O-rutinoside (Rutin)	32.27 ± 0.11a	5.63 ± 1.18d		13.59 ± 4.47c	5.51 ± 0.42d	
Quercetin 3-galactoside	25.04 ± 0.43a	21.33 ± 0.37e	23.73 ± 0.68c	24.68 ± 0.11a	22.09 ± 0.11d	24.19 ± 0.64b
Caffeoylquinic acid components						
Chlorogenic acid (3-CQA)	49.81 ± 0.70a	53.76 ± 0.73a	46.95 ± 0.97b	51.66 ± 0.41a	49.49 ± 1.59a	33.74 ± 0.16c
Trans-5-O-caffeoylquinic acid (Neochlorogenic acid) (cis-3-CQA)	80.49 ± 0.51a	70.11 ± 0.22e	78.04 ± 0.49c	79.89 ± 0.30b	81.02 ± 1.01a	72.23 ± 0.90d
1,3-Dicaffeoylquinic acid (1,3-diCQA)	29.76 ± 0.14a	28.23 ± 0.21b	29.04 ± 0.22a	21.56 ± 7.20c	27.93 ± 0.21b	29.71 ± 0.54a
1,4-Dicaffeoylquinic acid (1,4-diCQA)	49.69 ± 0.70a	47.47 ± 0.16b	44.92 ± 0.74c	49.20 ± 0.58a	47.38 ± 0.22b	48.05 ± 0.47b
3,5-Dicaffeoylquinic acid (3,5-diCQA)	47.90 ± 0.24a	43.06 ± 0.62e	45.86 ± 0.39c	46.71 ± 0.38b	44.60 ± 0.10d	47.10 ± 0.79b
4,5-Dicaffeoylquinic acid (4,5-diCQA)	17.62 ± 0.57c	29.09 ± 0.50a	12.62 ± 0.06d	18.66 ± 0.72c	25.35 ± 0.64b	14.77 ± 0.09d

Means followed by the same letter within the row are not significantly different ($p < 0.05$), each of the samples was replicated three times, and the results are expressed as mean ± standard deviation.

TABLE 3 | Comparison of the antioxidant activities of the leaf extracts of four South African sweet potato varieties with the USA's Beauregard and Peru's 199062.1.

	Orange fleshed storage roots			Cream white fleshed roots		
Antioxidant activity	"Bophelo" leaves	"Beauregard" leaves	"199062.1" leaves	"Monate" leaves	"Ndou" Leaves	"Blesbok" leaves
FRAP (mM TEAC/g)	19.69 ± 0.78a	18.71 ± 0.03b	17.65 ± 0.04c	17.56 ± 0.01c	17.83 ± 0.05c	17.81 ± 0.08c
DPPH (IC_{50} mg/ml)	3.51 ± 0.01d	4.22 ± 0.01c	4.99 ± 0.00ab	5.21 ± 0.00a	4.72 ± 0.03b	4.93 ± 0.07b
ABTS (IC_{50} mg/ml)	3.43 ± 0.00c	3.54 ± 0.02c	4.28 ± 0.04b	4.60 ± 0.01a	3.68 ± 0.05c	4.17 ± 0.03b

Means followed by the same letter within the row are not significantly different at $p < 0.05$. Each of the sweet potato samples was replicated three times, and the results are expressed as mean ± standard deviation.

demonstrated its ability to scavenge the oxidized by reactive oxygen species (ROS) (18). According to Danino et al. (23), 1,3-diCQA's antioxidant activity and ability to scavenge ROS make it a viable candidate for treating conditions, ranging from aging to degenerative disorders.

Carotenoid Components

The different carotenoid components in six varieties of sweet potato leaves are presented in **Table 4**. Leaves of sweet potato cultivar "Blesbok" contained the highest levels of β-carotene (10.27 mg kg^{-1} DW) and zeaxanthin (5.02 mg kg^{-1} DW) compared to other domestic cultivars "Beauregard" and Peruvian cultivar "199062.1." However, the Peruvian "199062.1" and "Beauregard" cultivars contained the lowest levels of β-carotene and zeaxanthin, respectively. In addition, lutein content was highest in leaves of cultivar "Bophelo," followed by "Blesbok." Conversely, the lutein content of Kenyan sweet potatoes varied from 285.7 to 446.6 g kg^{-1} on a dry weight basis was higher than the levels found in four South African cultivars, "Beauregard," and Peruvian "199062.1." Lutein cannot be synthesized in the body; it must be consumed from outside sources. Incorporating lutein into the diet of a consumer at an early age will reduce the severity of age-related macular degeneration (25). Incorporating Lutein into the diet of a consumer at an early age will reduce the severity of age-related macular degeneration. Identification of edible sources of lutein and fortification of foods with lutein could reduce the severity of age-related macular degeneration. Among dark vegetables, Menelaou et al. (26) found sweet potato leaves to be the highest source of lutein. Also, the results of the study showed that β-carotene content is determined by genotype.

TABLE 4 | Comparison of different carotenoid components in the leaves of four South African sweet potato cultivars with the USA's "Beauregard" and Peru's "199062.1" cultivars on a dry weight basis.

	Orange fleshed storage roots			Cream fleshed roots		
Carotenoid components (mg/kg)	"Bophelo" leaves	"Beauregard" leaves	"199062.1" leaves	"Monate" leaves	"Ndou" leaves	"Blesbok" leaves
Lutein	9.50 ± 0.04a	2.62 ± 0.07c	1.29 ± 0.00e	1.29 ± 0.00e	1.88 ± 0.03d	7.19 ± 0.04b
Zeaxanthin	2.27 ± 0.06c	0.14 ± 0.01f	2.13 ± 0.04d	2.96 ± 0.06b	0.50 ± 0.01e	5.02 ± 0.02a
β-carotene	4.36 ± 0.10e	7.33 ± 0.52b	3.67 ± 0.09f	6.36 ± 0.06c	6.21 ± 0.20d	10.27 ± 0.20a
% Vit A RDA male >14/ 100 g	4.04	6.79	3.40	5.89	5.75	9.51
% Vit A RDA female >14 per100 g portion	5.19	8.73	4.37	7.57	7.39	12.23

Means followed by the same letter within the row are not significantly different at $p < 0.05$.

Despite this, indigenous plants, such as *Cleome hitra* (131.705 mg kg^{-1} DW), *Corchorus trilocularis* (54.43 mg kg^{-1} DW), *Moringa oleifera* (100-285 mg kg^{-1} DW), and *Solanum nigrum* (131.705 $mgkg^{-1}$) (27) contain higher levels of β-carotene than sweet potato leaves investigated in this study. For men and women aged 19 to 50, the recommended dietary allowance (RDA) for vitamin A is 900 μg retinol activity equivalents (RAE) and 700 μg RAE, respectively (28). A 100 g of dried "Blesbok" sweet potato leaves contributes 9.51 and 12.23% of the RDA for Vitamin A for men and women, respectively. Alternatively, carotenoid levels in leafy vegetables depend on a number of factors, such as the cultivar, cultivar, the farming method, maturity, as well as environmental factors, such as light, temperature, and soil quality (8). Moreover, "Bophelo" was recommended for dual purpose use (use of both storage roots and leaves) due to the high potential contribution to β-carotene as well as iron and zinc.

CONCLUSION

Using a practical metabolomic chemometrics tool, we discriminated between leaves from four sweet potato cultivars grown in South Africa, USA cultivar "Beauregard," and Peruvian cultivar "199062.1" using their phenolic compounds. It is evident from this study that phenolic compounds are almost identical between the leaves of three diverse groups of sweet potato cultivars, but they differ in composition. The use of sweet potato leaves as leafy vegetables is a new concept in most countries, but, because of its functional properties, it is destined to become a niche market. Additionally, it would be ideal to recommend suitable local sweet potato cultivars that can be used as leafy vegetables in Sub-Saharan Africa. Therefore, breeding and commercialization of sweet potato cultivars "Bophelo" and "Blesbok" for leafy vegetable consumption are encouraged due to their high caffeoylquinic acid and carotenoids content, respectively. It is important to investigate the palatability and antinutritive components of the leaves.

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/s.

AUTHOR CONTRIBUTIONS

CP performed the experiment, generated the data, and wrote some parts of this manuscript. SL developed the sweet potato cultivars, provided the leaves, advised on the investigations, and contributed to the write-up of the article. TS was responsible for HPLC analysis for carotenoid, visualized and validated the data for phenolic compounds, and interpreted the chromatogram. VM performed the metabolomic chemometric analysis and validated the phenolic compounds. DS conceptualized the research, supervised the CP, and improved the article further. 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/fnut.2021.773550/full#supplementary-material

REFERENCES

1. Jung JK, Lee SU, Kozukue N, Levin CE, Friedman M. Distribution of phenolic compounds and antioxidative activities in parts of sweet potato (*Ipomoea batata* L.) plants and in home processed roots. *J Food Compos Anal.* (2011) 24:29–37. doi: 10.1016/j.jfca.2010.03.025
2. Nguyen H, Chen CC, Lin KH, Chao PY, Lin HH, Huang MY. Bioactive compounds, antioxidants, and health benefits of sweet

potato leaves. *Molecules.* (2021) 26:1820. doi: 10.3390/molecules26071820
3. Islam S. Sweet potato (*Ipomea batatas* L.) leaf: its potential effect on human health and nutrition. *J Food Sci.* (2006) 71:13–18. doi: 10.1111/j.1365-2621.2006.tb08912.x
4. Sun HN, Mu TH, Xi LS. Effect of pH, heat, and light treatmentson the antioxidant activity of sweet potato leaf polyphenols. *Int J Food Prop.* (2017) 20:318–32. doi: 10.1080/10942912.2016.1160410
5. Makori SI, Mu TH, Sun HN. Total polyphenol content, antioxidant activity, and individual phenolic composition of different edible parts of 4 sweet potato cultivars. *Nat Prod Commun.* (2020) 15:1–12. doi: 10.1177/1934578X20936931
6. Luo D, Mu T-H, Sun H, Chen J. Optimization of the formula and processing of a sweet potato leaf powder-based beverage. *Food Sci Nutr.* (2020) 8:2680–91. doi: 10.1002/fsn3.1555
7. Laurie SM, Mtileni MM, Mphela WM, Tjale SS. Performance of informal market sweet potato cultivars in on-farm trials in South Africa. *Open Agric.* (2017) 2:431–41. doi: 10.1515/opag-2017-0047
8. Nyathi MK, Du Plooy CP, Van Halsema TJ, Stomph TJ, Annandale JG, Struik PC. The dual-pupose use of orange-fleshed sweet potato (*Ipomoea batatas* var. Bophelo) for improved nutritional food security. *Agric Water Manag.* (2019) 21:723–37. doi: 10.1016/j.agwat.2019.02.029
9. Jeng TL, Lai CC, Liao TC, Lin SY, Sung JM. Effects of drying on caffeoylquinic acid derivative content and antioxidant capacity of sweet potato leaves. *J Food Drug Anal.* (2015) 23:701–8. doi: 10.1016/j.jfda.2014.07.002
10. Wang S, Nie S, Zhu F. Chemical constituents and health effects of sweet potato. *Food Res Int.* (2016) 89:90–116. doi: 10.1016/j.foodres.2016.08.032
11. Zhang L, Tu ZC, Wang H, Fu ZF, Wen QH, Chang HX, et al. Comparison of different methods for extracting polyphenols from *Ipomoea batatas* leaves, and identification of antioxidant constituents by HPLC-QTOF-MS2. *Food Res Int.* (2015) 70:101–9. doi: 10.1016/j.foodres.2015.01.012
12. Managa MG, Akinola SA, Remize F, Garcia C, Sivakumar D. Physicochemical Parameters and Bioaccessibility of Lactic Acid Bacteria Fermented Chayote Leaf (*Sechium edule*) and Pineapple (*Ananas comosus*) Smoothies. *Front Nutr.* (2021) 8:649189. doi: 10.3389/fnut.2021.649189
13. Seke F, Manhivi VE, Shoko T, Slabbert RM, Sultanbawa Y, Sivakumar D. Effect of freeze drying and simulated gastrointestinal digestion on phenolic metabolites and antioxidant property of the Natal plum (*Carissa macrocarpa*). *Foods.* (2021) 10:1420. doi: 10.3390/foods10061420
14. Mashitoa FM, Manhivi V, Shai JL, Slabbert RM, Sivakumar D. Influence of different types of drying methods on color properties, phenolic metabolites and bioactivities of pumpkin leaves of var. *Butternut squash* (*Cucurbita moschata* Duchesne ex Poir). *Front Nutr.* (2021) 2021:694649. doi: 10.3389/fnut.2021.694649
15. Panfili G, Frantiani A, Irano M. Improved normal-phase high performance liquid chromatography procedure for the determination of carotenoids in cereals. *J Agric Food Chem.* (2004) 52:6373–80. doi: 10.1021/jf0402025
16. Jang Y, Koh E. Antioxidant content and activity in leaves and petioles of six sweet potato (*Ipomoea batatas* L) and antioxidant properties of blanched leaves. *Food Sci Biotechnol.* (2019) 28:337–45. doi: 10.1007/s10068-018-0481-3
17. Islam MS, Yoshimoto M, Yahara S, Okuno S, Ishiguro K, Yamakawa O. Identification and characterization of foliar polyphenolic composition in sweet potato (*Ipomoea batatas* L.) genotypes. *J Agric Food Chem.* (2002) 50:3718–22. doi: 10.1021/jf020120l
18. Moyo S, Amoo SO, Ncube AR, NdhlalaJ F, Finnie J, Van Staden, J. Phytochemical and antioxidant properties of unconventional leafy vegetables consumed in Southern Africa. *S Afr J Bot.* (2013) 84:65–71. doi: 10.1016/j.sajb.2012.09.010
19. Krochmal-Marczak B, Cebulak T, Kapusta I, Oszmiański J, Kaszuba J, Zurek N. The content of phenolic acids and flavonols in the Leaves of nine varieties of sweet potatoes (*Ipomoea batatas* L.) depending on their development, grown in central Europe. *Molecules.* (2020) 25:3473. doi: 10.3390/molecules25153473
20. Carvalho FV, Santana LF, Diogenes V, da Silva A, Costa, L, Zambotti-Villelae L, et al. Combination of a multiplatform metabolite profiling approach and chemometrics as a powerful strategy to identify bioactive metabolites in *Lepidium meyenii* (*Peruvian maca*). *Food Chem.* (2021) 364:130453. doi: 10.1016/j.foodchem.2021.130453
21. Yoshimoto M, Yahara S, Okuno S, Islam MS, Ishiguro K, Yamakawa O. Antimutagenicity of mono-, di, and tricaffeoylquinic acid derivatives isolated from sweet potato (*Ipomoea batatas* L.) leaf. *Biosci Biotechnol Biochem.* (2022) 66:2336–41. doi: 10.1271/bbb.66.2336
22. Truong VD, Mcfeeters RF, Thompson LL, Shofran B. Phenolic acid content and composition in leaves and roots of common commercial sweet potato (*Ipomea batatas* L.) cultivars in the United States. *J Food Sci.* (2007) 72:343–9. doi: 10.1111/j.1750-3841.2007.00415.x
23. Danino O, Gottlieb EH, Grossman S, Bergman M. Antioxidant activity of 1,3-dicaffeoylquinic acid isolated from *Inula viscosa*. *Int Food Res.* (2009) 42:1273. doi: 10.1016/j.foodres.2009.03.023
24. Ghasemzadeh A, Omidvar V, Ze Jaafar H. Polyphenolic content and their antioxidant activity in leaf extract of sweet potato (*Ipomoea batatas*). *J Med Plant Res.* (2012) 6:2971–2776. doi: 10.5897/JMPR11.1353
25. Pratt S. Dietary prevention of age related macular degradation. *J AmerOptom Assoc.* (1999) 70:39–47.
26. Menelaou E, Kachattryan A, Losso J. Lutein content in sweet potato leaves. *HortScience.* (2006) 41:1269–71. doi: 10.21273/HORTSCI.41.5.1269
27. Johnson M, Pace RD. Sweet potato leaves: properties and synergistic interactions that promote health and prevent disease. *Nutr Rev.* (2010) 68:604–15. doi: 10.1111/j.1753-4887.2010.00320.x
28. Van den Berg H, Faulks R, Fernando Granado H, Hirshberg J, Olmedilla B, Sandmann G. The potential for the improvement of carotenoids levels in foods and the likely systemic effects. *J Sci Food Agric.* (2000) 80:880–912. doi: 10.1002/(SICI)1097-0010(20000515)80:7<880::AID-JSFA646>3.0.CO;2-1

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Sprout Caffeoylquinic Acid Profiles as Affected by Variety, Cooking, and Storage

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Various health-promoting properties inherent to plant-based foods have been attributed to their rich bioactive compounds, including caffeoylquinic acids (CQAs). The potential health benefits of CQAs have been well-documented. While sprouts are widely recognized as health-promoting foods owing to their high phytonutrient content, our knowledge regarding the effect of cooking and storage, commonly practiced by consumers, on the CQA content remains limited. First, sunflower sprouts were found to have the highest total CQA content (~ 22 mg/g dry weight) out of 11 commonly available sprouts. Then, the effect of variety, cooking, and low-temperature storage on the CQA profile of sunflower sprouts was investigated. Among the four different varieties of sunflower sprouts, variety 1 harbored the highest total CQA content. Notably, cooking adversely affected the CQA content of sunflower sprouts relative to the uncooked samples in a time-dependent manner, possibly due to the heat sensitivity of CQAs. Under simulated home-refrigeration storage conditions, we observed a significant decline in the content of major CQA compounds (5-monoCQA and 3,5-diCQA) at days 10 and 13 of storage. The results obtained herein provide consumers and food industrialists with increased insight into the effect of cooking and refrigeration on the CQA content of sunflower sprouts.

Keywords: bioactive compounds, caffeoylquinic acids, cooking, low temperature storage, sunflower sprout, variety

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INTRODUCTION

Lifestyle changes and increased awareness among health-conscious consumers have led to an increasing interest in healthy diets of natural origin. There is a large body of evidence indicating a strong association between high plant product intake and reduced risk factors related to major chronic diseases in humans (1), including cardiovascular disease (CVD) (2), cancer (3), and age-related degeneration (4). The health-promoting properties of plants are attributed to the rich content of bioactive compounds, such as vitamins C and E, carotenoids, and phenolic compounds, that possess antioxidant activity (5).

Phenolic compounds exist in plants in various forms, including aglycones (free phenolic acids), esters, glycosides, and bound complexes (6). Among these, caffeoylquinic acids (CQAs) are characterized as esters of hydroxycinnamic acids and quinic acid, which are divided

into three main groups: monocaffeoylquinic acids (monoCQAs with at least three isoforms: 3-CQA, 4-CQA, and 5-CQA, known as chlorogenic acid), dicaffeoylquinic acids (diCQAs with at least three isoforms: 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) (7–9), and tricaffeoylquinic acid (triCQA with one isoform: 3,4,5-triCQA) (10). Owing to their structural diversity, CQAs exert a wide range of biological activities. CQAs have numerous health-promoting characteristics, such as antimicrobial (antibacterial and antiviral), antioxidant, and anti-inflammatory properties, which can substantially reduce the rates of chronic and CVDs, as described by several *in vivo* and *in vitro* studies (11–14). In addition, CQAs interfere with the absorption and biosynthesis of cholesterol and contribute to low-density lipoprotein (LDL) reduction (15). Notably, 5-CQA [chlorogenic acid (CGA)] modifies glucose metabolism, which benefits the treatment of injuries caused by diabetes (16) and protects endothelial cells from oxidative stress (17). They can also inhibit HIV replication and integration (18). In plants, CQAs can enhance resistance to biotic (19, 20) and abiotic (21) stressors by acting as antioxidants and scavenging free radicals.

Seed germination is a complex stage of plant ontogenesis that activates the seed embryo and allows for seedling growth (22, 23). Seven- or 10-day-old sprouts are ideal for harvesting, allowing for post-harvest handling and commercialization (23). Notably, sprouts have a higher content of phytochemicals than other vegetables (23). Health promotion guidelines and dietary recommendations widely recommend regular consumption of sprouts due to their high content of nutrients and bioactive compounds, including CQAs (24, 25). In view of the ever-increasing interest in healthy lifestyles and prevention of diseases, sprouts are highly desirable products of plant origin. Apart from their health-promoting properties, sprouts can be easily and quickly produced and are considered innovative culinary ingredients of high popularity due to their delicate texture, unique colors, and high palatability. Various species can be consumed as sprouts, some of which are of great popularity, such as cereals, legumes, oilseeds, and crucifers, which include lentils, soybean, broccoli, alfalfa, radish, mung bean, and sunflower.

Sunflowers (*Helianthus annuus* L.) belong to the Asteraceae family and are grown commercially worldwide. Sunflower seeds are utilized as an important source of vegetable oil worldwide (26). Sunflower seeds and sprouts have health-promoting properties, including antimicrobial, antioxidant, anti-inflammatory, antihypertensive, wound-healing, and cardiovascular benefits, due to its bioactive compounds that include phenolic compounds, polyunsaturated fatty acids, and vitamins (27). The notable nutritional, medicinal, and culinary benefits of sunflower seeds and sprouts have resulted in an ever-growing popularity among health-conscious consumers worldwide. Sun et al. (28) investigated the antiglycative and antioxidant characteristics of four edible sprouts and found that sunflower sprout extract had antiglycative properties similar to those of aminoguanidine, a well-known synthetic antiglycative agent. Moreover, the strong antioxidant and antiglycative capacity of sunflower sprouts was found in its rich diCQA content. Consistently, Cheevarungnapakul et al. (29) profiled the CQA content in sunflower sprouts and found both mono and diCQAs. Notably, seed germination affects the content of CQAs in sunflowers. Pająk et al. (30) investigated the effect of germination on bioactive compound content (total phenolics and flavonoids) and antioxidant properties of sunflower seeds. Total phenolics, flavonoids, and antioxidant capabilities were significantly higher in sunflower sprouts than in seeds. Moreover, the CQA content showed a 3.7-fold increase in sprouts compared with seeds (30).

Storing sprouts in a refrigerator at home and consuming them as desired is a regular practice. Sprouts are used in various ways. They can be used raw as additions to sandwiches, salads, soups, and desserts, and/or they can be cooked. Thus, it is of paramount importance for consumers and food industries to understand the effect of low-temperature storage and cooking on the content of bioactive compounds in sprouts. Previous studies have documented the effect of cold storage on the phytonutrient content and bioactive properties of various fruits and vegetables, suggesting different patterns of variation in the bioactive compound content under simulated home-refrigeration storage conditions, depending on the species and compounds being investigated (31–39). However, our knowledge regarding the effect of low-temperature storage and cooking on the phytonutrient content of sprouts remains poorly understood and limited to only a few studies.

Swieca and Gawlik-Dziki (40) investigated the effect of low-temperature storage on phenolic content, antioxidant activity, and starch content in green pea, lentil, and mung bean sprouts. It was found that the bioactivity and nutritional quality of sprouts were affected by storage at low temperatures. In addition, storage significantly enhanced the starch digestibility and glycemic index value, which was linked to the reduction of resistant starch content. Notably, sprouts are usually consumed after cooking, which can be performed in different ways, according to personal preferences and/or culinary traditions. However, steaming is considered as one of the most common methods of cooking (41, 42). In addition, steaming has been proved to maintain or increase the total phenolic content in some vegetables (43). Hwang (42) investigated the effect of different cooking methods on the total bioactive compound content of Brussels sprouts. Microwaved Brussels sprouts contained the highest amount of total carotenoids and chlorophylls, followed by steamed and uncooked samples. However, microwaving and steaming deteriorated the antioxidant activity and total flavonoid content of Brussels sprouts. In another study by Chiavaro et al. (44), *sous vide* processed Brussels sprouts contained higher carotenoid content but lower phenolic compounds, ascorbic acid, and antioxidant activity when compared to those of steamed samples. Kumari and Chang (45) demonstrated that cooking caused significant losses to the antioxidant capacity and phenolic content of soy sprouts.

Of particular note, different varieties of one sprout species can harbor different health-promoting properties, likely due to the different contents of phytochemicals, which can be attributed to climatic conditions of sprout cultivation and/or different genotypes. Limmongkon et al. (46) reported a significant difference in phenolic content and antioxidant capacity among the five varieties of peanut sprouts.

Herein, considering the significant nutritional and culinary benefits of sprouts, we profiled the CQA content of various sprout cultivars commonly found in the market in Thailand and found that sunflower sprouts had the highest CQA content. We then examined the effect of low-temperature storage, cooking, and variety on the CQA content of sunflower sprouts. To our knowledge, this is the first report on the effect of these factors on the CQA content of sunflower sprouts. The results obtained from this study will increase consumer awareness regarding the effect of refrigerated storage and cooking on the nutritional value of sunflower sprouts.

MATERIALS AND METHODS

Chemicals

The commercial standards of CQAs (monoCQAs: 1-CQA, 3-CQA, 4-CQA, and 5-CQA and diCQAs: 1,3-diCQA, 1,4-diCQA, 1,5-diCQA, 3,4-diCQA, and 4,5-diCQA) were purchased from Biosynth Carbosynth®, UK (purity ≥ 98.0%), and 3,5-diCQA was purchased from TransMIT GmbH PlantMetaChem (Germany). Puerarin (internal standard) was obtained from Sigma Aldrich (St. Louis, MO, USA; purity ≥ 98.0%). Acetonitrile and ethanol (high performance liquid chromatography (HPLC) grade) were obtained from Merck (Darmstadt, Germany). Ultra-pure water used for all experiments was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

Plant Materials and Seed Germination

The sprouts of broccoli (*Brassica oleracea* var. *italica*), Chinese kale (*Brassica oleracea* Alboglabra Group), green pea (*Pisum sativum*), mung bean (*Phaseolus vulgaris*), mustard (*Brassica nigra*), pea (*Pisum sativum*), peanut (*Arachis hypogaea*), purple cabbage (*Brassica oleracea* var. *Capitata* f. *rubra*), soybean (*Glycine max*), sunflower (*Helianthus annuus* L.), and white radish (*Raphanus sativus*) were purchased from a local supermarket in Bangkok, Thailand (pictures of representative sprout species are presented in **Supplementary Figure 1**). Three independent biological replicates were used for each sprout species. The sprout samples were washed with water and freeze-dried. The CQA profiles of each sprout species were analyzed. The species with the highest total CQA content (sunflower sprouts) was selected for further study.

To investigate the effect of variety on CQA content, seeds of four different varieties of sunflower sprouts (1: Lungtop line, 2: Chiatai, 3: 3A, 4: Fourtid) were purchased from a local supermarket in Bangkok, Thailand (pictures of representative seeds and sprouts are shown in **Supplementary Figure 2**). These four varieties are commonly available in Thai local seed shops. The seeds were then washed with tap water, soaked for 8 h, and wrapped with wet cheesecloth overnight. Seeds were then germinated on coconut dust under controlled conditions (temperature 30°C and 60% relative humidity) for 5 days, under dark conditions for the first 48 h, followed by a 12/12 h-light/dark photoperiod for the remaining days (29).

Cooking Treatment and Storage at 4°C

We used steaming to investigate the effect of cooking on the CQA profile of sunflower sprouts relative to the raw (uncooked) samples. We also varied the cooking period (2, 5, 7, and 10 min) on the knowledge of different cooking habits among chefs. For each cooking period, sunflower sprouts were weighed, washed, and dried with tissue paper. After that, the sprouts were cooked (steamed) using an electric food steamer. Following cooking, the samples were immediately frozen in liquid nitrogen and freeze-dried with the application of Gamma 1-16 LSC freeze-dryer at a shelf temperature of 20°C, ice condenser temperature of −55°C and pressure of 63 Pa for 24 h. To investigate the effect of storage conditions (time-period) on the CQA content of sunflower sprouts, sprouts were stored at 4°C (simulated home-refrigerated storage) and sampled at day 1, 2, 3, 5, 7, 10, and 13 of storage.

Extraction of CQAs From Sprout Samples

Methanolic extracts were prepared to quantify the CQA content of the sprout samples. To this end, freeze-dried samples were ground to a fine powder using a mixer mill (MM 400, Retsch, Germany) at 30 Hz for 1 min. After that, 20 mg (dry weight) of each ground sample was extracted with 1 mL aqueous solution of 80% (v/v) methanol containing an internal standard, puerarin (0.05 g L^{-1}) by shaking vigorously at 1,500 rpm for 15 min at 15°C using an Eppendorf Thermomixer® C (Eppendorf, USA). The mixtures were then centrifuged at 12,000 × *g* for 15 min at 4°C using a table-top 5415 R centrifuge machine (Eppendorf, USA). Supernatants were collected, filtered through a 0.2-μm nylon syringe filter, and injected into a high-performance liquid chromatograph to analyze the CQAs (29).

HPLC

CQA profiling of the sprout samples was performed using an UltiMate 3000 HPLC liquid chromatography system coupled with a Dionex UltiMate DAD 3000 detector (Thermo Fisher Scientific, Waltham, MA, USA) with a Kinetex EVO C18 (250 mm × 4.6 mm, 5 μm) (Phenomenex, USA) using UV at 320 nm following the method described by Cheevarungnapakul et al. (29) with minor modifications. The mobile phase was composed of water with 0.1% (v/v) formic acid (TFA) (pH 2.4; eluent A) and acetonitrile with 0.1% (v/v) TFA (eluent B) with the following gradient program: 5% B (20 min), 5–15% B (10 min), 15% B (25 min), a 4-min hold, 100% B (3 min), a 4-min hold, and 5% B (5 min). The flow rate was 1.5 mL min^{-1} with an injection volume of 10 μL. Peaks corresponding to the retention time and UV spectrum of a commercial standard (standards used in this study were mentioned in section Chemicals) were identified as CQAs. Quantification of each CQA was performed according to the calibration curve in the range of 1.30–500 μg/mL. Puerarin was used as an internal standard. **Supplementary Figure 3** presents the chromatograms of the analytical standards and the sunflower sprout sample. More information regarding the standards used in this study, including standard curves, equations, limit of detection (LOD), and limit of quantification (LOQ) are presented in **Supplementary Figure 4** and **Supplementary Table 1**.

Statistical Analysis

Data were subjected to statistical analysis using SPSS software, version 20 (SPSS Inc., IBM). Data are presented as the mean ± standard deviation (SD) of three independent replicates. Statistical comparisons of the means were performed using a one-way ANOVA, followed by Duncan's multiple range test (variety and cooking experiments) or Student's *t*-test (4°C-storage experiment) at a confidence level of 0.05.

RESULTS

CQA Profiling of Different Sprout Species

Using HPLC, we profiled the CQA content of 11 different sprouts commonly found in Thai local markets. A total of nine CQA compounds, including monoCQA (1-, 3-, 5-, and 4-CQA) and diCQA (1,3-, 3,4-, 1,5-, 3,5-, and 4,5-diCQA) were identified and quantified (**Table 1**). Among the sprouts, sunflower sprouts harbored the highest content of both monoCQA and diCQA (22.01 ± 1.07 mg/g), followed by broccoli (15.32 ± 1.22 mg/g), green pea (12.3 ± 0.51 mg/g), and Chinese kale (11.22 ± 0.87) sprouts on dry weight basis (mg/g dry weight) (**Table 1**). However, pea (4.41 ± 0.19 mg/g dry weight) and purple cabbage (3.99 ± 0.55 mg/g dry weight) sprouts contained the lowest total CQA content (**Table 1**). Considering the highest total CQA content among the sprout species, sunflower sprouts were selected as candidate sprouts for our study.

CQA Profile of Different Sunflower Sprout Varieties

We profiled the CQA content of different sunflower sprout varieties from commercialized seeds in Thailand to investigate the effect of variety on CQA content. HPLC profiling identified and quantified a total of five CQA compounds in the sprouts, including monoCQAs (3-CQA and 5-CQA) and diCQAs (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA; **Figure 1**). Among the samples, variety 1 harbored the highest total CQA content (21.02 ± 0.75 mg/g) followed by variety 2 (18.55 ± 0.31 mg/g) on dry weight basis, and the total CQA content did not differ significantly between the other two varieties. The contribution of each of these two classes (mono and diCQA) to the total CQA content did not vary according to the variety. As presented in **Figure 1**, the contribution of diCQA isomers to the total CQA content was predominant. Notably, for monoCQA, 5-CQA was the major isomer in all samples, whereas for diCQA, 3,5-diCQA was the most abundant (**Figure 1**).

Effect of Cooking on the CQA Content of Sunflower Sprouts

Consuming vegetables after cooking is a common practice among consumers. Accordingly, to investigate the possible effect of cooking on the CQA content of sunflower sprouts, we analyzed the CQA profile after controlled cooking using an electric food steamer for different time periods, including 2, 5, 7, and 10 min. Notably, cooking detrimentally affected the total CQA content of sunflower sprouts relative to the raw (uncooked) sprouts in a time-dependent manner (**Figure 2**). The total CQA content reached its lowest value at 7 and 10 min of cooking (**Figure 2**).

TABLE 1 | CQA profiles and contents of 11 sprouts.

Common name	Scientific name	1-CQA	3-CQA	5-CQA	4-CQA	1,3-CQA	3,4-CQA	1,5-CQA	3,5-CQA	4,5-CQA	Total CQA
Sunflower	*Helianthus annuus*	nd	0.95 ± 0.06	6.7 ± 0.78	0.13 ± 0.008	nd	0.55 ± 0.05	nd	12.87 ± 1.08	0.55 ± 0.11	22.01 ± 1.07^{a}
Broccoli	*Brassica oleracea* var. *italica*	0.08 ± 0.005	0.76 ± 0.09	4.98 ± 0.87	0.09 ± 0.007	nd	0.33 ± 0.09	0.09 ± 0.005	6.75 ± 0.98	1.55 ± 0.11	15.32 ± 1.22^{b}
Green pea	*Pisum sativum*	0.09 ± 0.003	1.65 ± 0.13	3.32 ± 0.77	nd	nd	0.21 ± 0.09	0.11 ± 0.08	4.55 ± 0.66	2.87 ± 0.55	12.3 ± 0.51^{c}
Chinese kale	*Brassica oleracea* Alboglabra group	nd	0.43 ± 0.008	3.43 ± 0.009	nd	nd	nd	nd	5.84 ± 0.32	1.08 ± 0.09	11.22 ± 0.87^{c}
White radish	*Raphanus sativus*	nd	0.46 ± 0.07	2.98 ± 0.09	nd	nd	0.14 ± 0.08	nd	4.09 ± 0.41	2.88 ± 0.21	10.88 ± 0.09^{d}
Mung bean	*Phaseolus vulgaris*	0.07 ± 0.002	0.21 ± 0.08	4.88 ± 0.42	nd	nd	0.08 ± 0.01	nd	3.44 ± 0.32	0.22 ± 0.07	9.54 ± 0.44^{e}
Soybean	*Glycine max*	nd	nd	1.08 ± 0.08	nd	nd	0.11 ± 0.09	nd	4.98 ± 0.09	1.98 ± 0.11	8.08 ± 0.33^{f}
Mustard	*Brassica nigra*	nd	nd	3.11 ± 0.86	0.09 ± 0.008	0.08 ± 0.006	0.23 ± 0.08	nd	4.21 ± 0.54	0.21 ± 0.09	7.99 ± 0.07^{f}
Peanut	*Arachis hypogaea*	nd	0.33 ± 0.03	3.44 ± 0.33	nd	nd	nd	nd	2.77 ± 0.11	1.11 ± 0.33	7.98 ± 0.09^{f}
Pea	*Pisum sativum*	nd	nd	2.31 ± 0.08	nd	nd	0.11 ± 0.06	nd	2.07 ± 0.08	0.08 ± 0.008	4.41 ± 0.19^{g}
Purple cabbage	*Brassica oleracea* var. *Capitata* f. *rubra*	nd	nd	2.09 ± 0.08	nd	nd	0.11 ± 0.08	nd	1.09 ± 0.07	0.87 ± 0.11	3.99 ± 0.55^{g}

Data are presented as the mean ± standard deviation (SD) of three independent replicates, expressed in mg/g of dry weight. For total CQA content, values followed by different superscript letters are significantly different according to Duncan's multiple-range test ($p < 0.05$). nd, not detected. 1,4-diCQA was not detected in any of the studied samples. Therefore, it is not included.

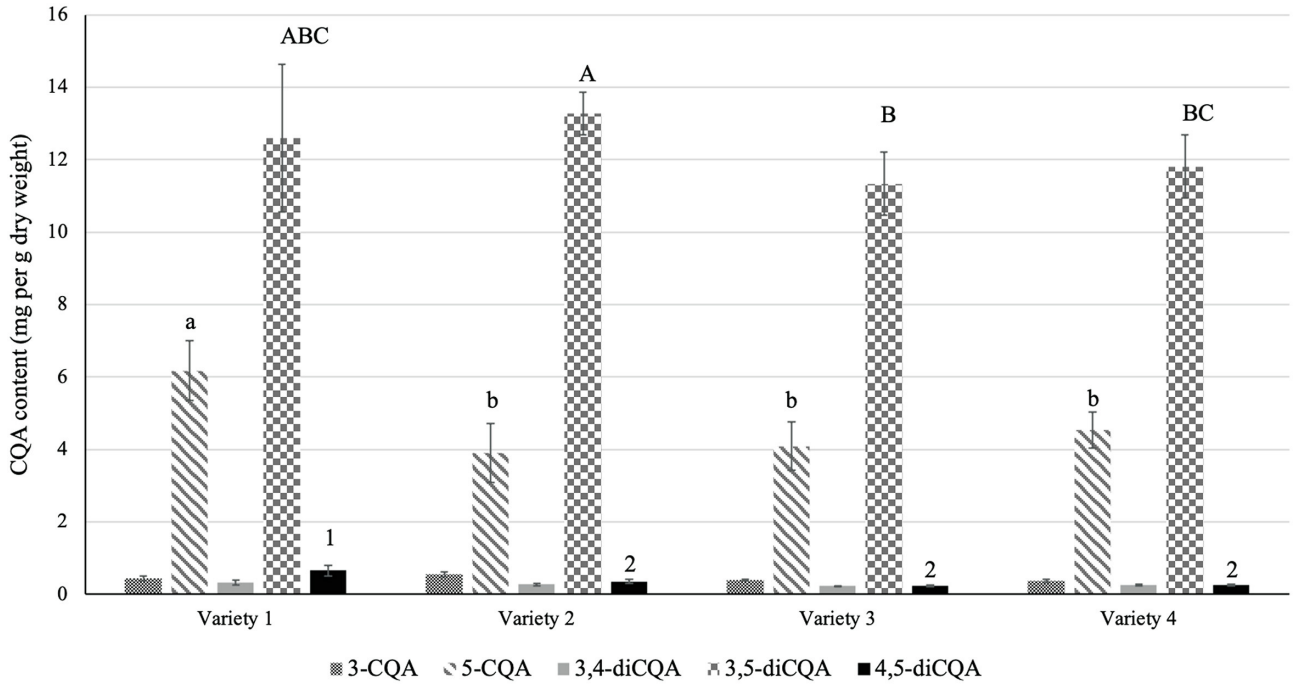

FIGURE 1 | CQA content of different sunflower sprout varieties. Seeds of four different varieties of sunflower sprout (1: Lungtop line, 2: Chiatai, 3: 3A, 4: Fourtid) were purchased and germinated. Sprouts were then extracted for CQA profiling. Error bars represent means ± standard deviations (SD), and three independent biological replicates were performed. For each CQA derivative, comparisons are made among varieties; bars with different letters or numbers differed significantly according to Duncan's multiple range test ($P < 0.05$). The content of 3-CQA and 3,4-diCQA did not vary significantly among the varieties according to Duncan's multiple range test ($P > 0.05$).

For each class of CQA, the content of the major isomer was negatively affected by cooking. For monoCQAs, the content of 5-CQA was significantly reduced at 7 and 10 min compared to the raw sprouts (**Figure 2**). Among the diCQAs, we observed a significant decrease in the predominant isomer (3,5-diCQA) at each time point during cooking compared to the raw sprouts, with the lowest values at 7 and 10 min (**Figure 2**).

Effect of Low-Temperature Storage on the CQA Content of Sunflower Sprouts

Storing sunflower sprouts in a refrigerator at home and consuming as desired is a regular practice among consumers. Therefore, to evaluate the effect of storage period on the CQA profile of sunflower sprouts, we analyzed the CQA content of sprouts after storage under simulated home-refrigeration storage conditions for 13 days. As shown in **Figure 3**, the total CQA content of sunflower sprouts remained unchanged until day 7 post-storage. However, on days 10 and 13, the total CQA content declined as a result of the significant decrease in the content of major isoforms (5-CQA and 3,5-diCQA), which reached their lowest values at days 10 and 13 post-storage (**Figure 3**).

DISCUSSION

Plants have been commonly used as food for centuries. A large body of scientific evidence has suggested a strong association between a high intake of plant-based food products and reduced risk factors for major chronic diseases in humans, such as cancer, CVD, and age-related degeneration. These health-promoting properties are attributed to their rich content of bioactive compounds that possess antioxidant activity, including CQAs. Previous studies have documented the health-promoting characteristics of CQAs, including the antioxidant, anti-inflammatory, anticancer, antidiabetic, antihypertensive, and anti-neurodegenerative properties.

In view of the ever-increasing interest in healthy lifestyles among health-conscious consumers, sprouts are recognized as wellness- and health-promoting foods, widely recommended by dietitians, owing to their high content of bioactive compounds, including CQAs (24, 25). Apart from their health-promoting properties, sprouts are considered innovative and popular culinary ingredients because of their delicate texture, high palatability, and unique color.

Herein, we profiled the CQA content of some sprout species commonly found on the market, including pea, white radish, peanut, sunflower, mung bean, broccoli, Chinese kale, purple cabbage, mustard, soybean, and green pea sprouts, out of which sunflower sprouts harbored the highest total CQA content at around 22 mg/g dry weight. Despite the numerous health benefits of CQAs in humans, data on their content and distribution in plants and foods are limited. In addition, most of the existing data include the content of 5-monoCQA (chlorogenic acid),

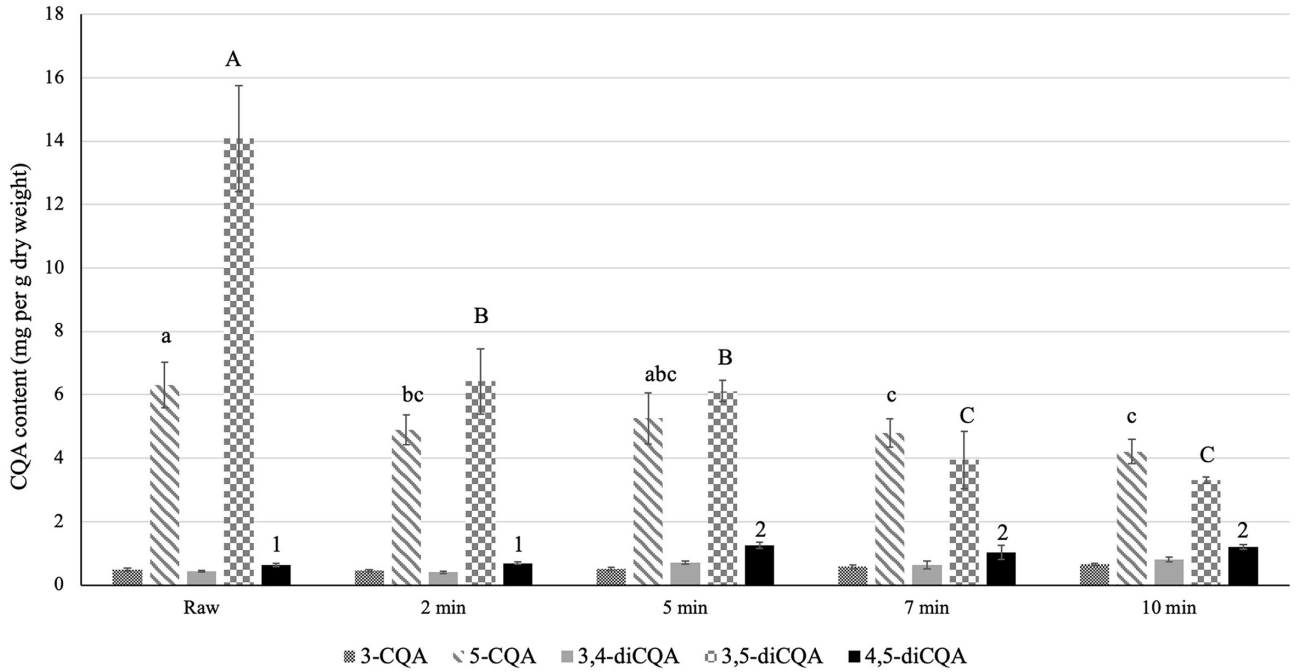

FIGURE 2 | Effect of cooking on the CQA content of sunflower sprouts. The CQA profile of sunflower sprouts were analyzed after cooking under controlled conditions (steaming for different time periods, including 2, 5, 7, and 10 min). Error bars represent means ± standard deviations (SD), and three independent biological replicates were performed. For each CQA derivative, comparisons were made among uncooked (raw) and cooked samples at different timepoints; bars with different letters or numbers differed significantly according to Duncan's multiple range test ($P < 0.05$). The content of 3-CQA and 3,4-diCQA did not vary significantly among raw and cooked samples according to Duncan's multiple range test ($P > 0.05$).

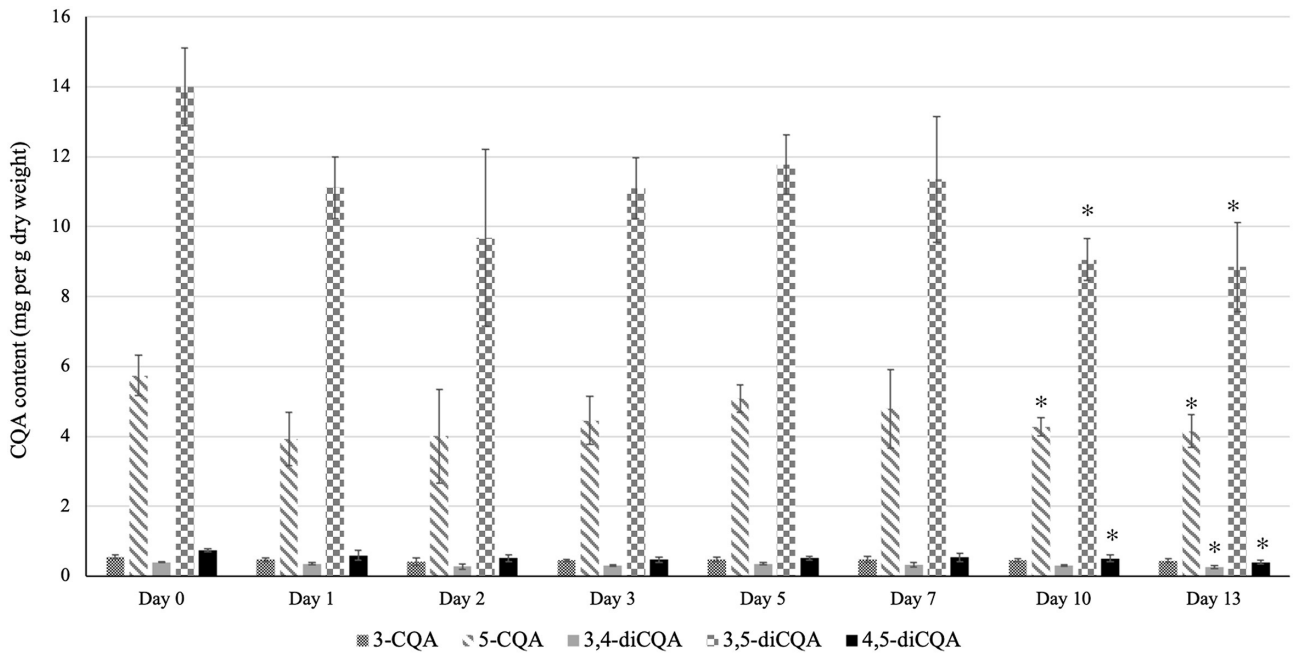

FIGURE 3 | Effect of low-temperature storage on the CQA content of sunflower sprouts. The CQA profile of sunflower sprouts were analyzed after storage under simulated home-refrigeration storage conditions for 13 days. Error bars represent means ± standard deviations (SD), and three independent biological replicates were performed. For each CQA derivative, an asterisk (*) above the bars indicates a significant difference compared to the sample at day 0 (control) (Student's *t*-test, $P < 0.05$). The content of 3-CQA did not vary significantly during storage compared to the control sample (Student's *t*-test, $P > 0.05$).

which is the most abundant CQA in nature. Of particular note, the chlorogenic acid content of sunflower sprouts (∼6.7 mg/g dry weight) is greatly higher when compared to those of other plants reported in the literature, including common buckwheat sprouts (∼0.27 mg/g) (47), red radish sprouts (∼0.1 mg/g), mizuna sprouts (∼0.5 mg/g), Chinese cabbage sprouts (∼0.4 mg/g), turnip sprouts (∼0.2 mg/g) (48), and red cabbage sprouts (∼0.4 mg/g) (49) on a dry weight basis. Although the total CQA content of sunflower sprouts (∼22 mg/g) was still lower than that of the green coffee bean (41 mg/g), it is still comparable to that of roasted coffee beans [light: 19 mg/g, medium: 10 mg/g, and dark roasted one: 5 mg/g; (50)]. Considering the easy and quick production (∼5–7 days after germination) of sunflower sprouts along with their significant nutritional and culinary benefits, this sprout species deserves attention regarding the pharmacological properties attributed to its rich CQA content.

It should be noted that although the CQA content of sunflower sprouts is significantly higher than that of other sprout species reported in the current and previous studies, it should not be concluded that the lower contents observed in some of the investigated plants are not important to humans, owing to the fact that the metabolism and requirements of these compounds seem to vary among individuals, with no dietary recommendations for them.

Among the CQA isomers found in sunflower sprouts, 5-monoCQA and 3,5-diCQA were the most abundant (**Table 1**). Our findings are partially in line with those of previous studies by Sun et al. (28) and Cheevarungnapakul et al. (29), who reported a rich abundance of 5-CQA in sunflower sprouts. However, these studies reported 1,5-diCQA as the predominant diCQA isomer. The inconsistency between our results and those reported previously is likely due to the mis-annotation of 1,5- and 3,5-diCQA in previous reports. Of particular note, the precise identification of individual CQAs by means of chromatographic analysis is challenging due to the difficulty in distinguishing between the positional isomers, especially when they are present at low concentrations. Hence, for more accurate peak identification, the retention times and UV spectra were compared with those of the corresponding green coffee bean extract [known sample (51)]. Accordingly, we found that the peak identity was 3,5-diCQA, not 1,5-diCQA.

Sprouts are consumed in many ways. They can be added as raw ingredients to sandwiches, salads, soups, and desserts, and/or they can be cooked. Cooking may enhance the palatability of sprouts by softening the tissues, inactivating toxic and anti-nutritional compounds and microorganisms, and forming color and flavor compounds (52). In addition, cooking may soften vegetable tissues, facilitating the extraction of phenolic compounds from the cellular matrix (34). However, cooking might have adverse effects on the antioxidant capacity and nutritional quality of sprouts. Hence, it is of great interest to consumers and food industries to gain a better understanding of the effect of cooking on the content of bioactive compounds in sprouts. We assessed the effect of cooking on the CQA profile of sunflower sprouts by comparing the CQA content of cooked sprouts (steamed for 1, 5, 7, and 10 min) to that of raw (uncooked) samples. Notably, cooking had adverse effects on the total CQA content of sunflower sprouts relative to the raw (uncooked) sprouts in a time-dependent manner. The total CQA content reached its lowest value at 7 and 10 min of cooking (**Figure 2**). The decrease in CQA content, especially after a longer cooking period, could likely occur as CQAs are heat sensitive. The decline in CQA content as a result of heating has previously been reported for roasted coffee beans (50) and yerba mate (53) when compared to the CQA profile of green beans. Comparing mono and diCQAs, we observed that diCQAs are more heat liable than monoCQAs (**Figure 2**) The higher stability of monoCQAs under heat might be due to the fact that the ester bond linkage in the quinic acid moiety is less stable in an axial bond configuration than in an equatorial bond configuration (54, 55). Notably, a significant decline in the content of the predominant isomer (3,5-diCQA) was observed in cooked sprouts relative to the raw samples, with the lowest values at 7 and 10 min (**Figure 2**). 3,5-diCQA synthesis was found to be optimal at pH 6, which would likely take place in an acidic compartment of the cell, such as the vacuole (56). The changes in pH led to the instability of the 3,5-diCQA structure (56). The significant decrease in 3,5-diCQA content as a result of cooking might be due to the detrimental effect of heat shock on the cells, which could negatively affect not only the cell membrane but also the vacuolar membrane as a possible cellular site for injury, leading to changes in pH and instability of 3,5-diCQA, which could then be degraded and/or converted to other CQA isomers.

Of particular note, considering the effect of cooking methods and periods on the antioxidant capacity and content of bioactive compounds, there is no consensus in the literature regarding the best way to consume vegetables because of the inconsistent results obtained from different studies. Some studies have documented increases, while others have reported decreases in bioactive compound levels and antioxidant activity after cooking (42, 45, 57–59).

Low-temperature storage of sprouts at home and consumption as desired is a common practice among consumers. In our study, we investigated the effect of low-temperature storage on the CQA profile of sunflower sprouts by profiling the CQA content of sprouts after storage under simulated home-refrigeration storage conditions for 13 days. Notably, we did not observe any significant changes in the CQA content of sprouts until day 7 post-storage (**Figure 3**). However, on days 10 and 13, a significant decrease in the content of major isoforms (5-CQA and 3,5-diCQA) was observed, leading to a decline in the total CQA content (**Figure 3**) which might likely occur as a result of cell injury under cold shock. Similar to the effect of cooking, previous studies have reported different patterns of variations in the phytonutrient content of various fruits and vegetables under simulated home-refrigeration storage conditions, depending on the species and the compounds under investigation (31–39). To the best of our knowledge, our study is the first to report on the effect of low-temperature storage on the CQA content of sunflower sprouts. It should be noted that in the present study, we used targeted metabolomics to quantify the CQA content of sunflower sprouts under different conditions since CQAs are the predominant phenolic compounds in sunflower sprout with numerous health-beneficial properties. This

point justifies the application of targeted metabolite profiling in our study. However, we do not mean to overlook other potential metabolites which might contribute to the nutritional quality of sunflower sprouts. Using non-targeted metabolomics to profile the bioactive compound content of sunflower sprouts under different conditions could be the subject of further investigation.

In summary, we investigated the effect of variety, cooking, and low-temperature storage on the CQA content of sunflower sprouts. All measured parameters significantly affected the CQA profiles of sprouts. Considering the numerous health-promoting benefits of CQAs, our findings provide consumers and food scientists with an improved understanding of the effect of cooking and home refrigeration on the CQA content of sunflower sprouts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SS conceived the study. KC prepared the samples and performed the CQA analysis. GK analyzed the data. SS and GK drafted the manuscript. PB helped in optimizing the CQA analysis conditions. All authors read and approved the final 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/fnut.2021.748001/full#supplementary-material

REFERENCES

1. Slavin JL, Lloyd B. Health benefits of fruits and vegetables. *Adv Nutr.* (2012) 3:506–16. doi: 10.3945/an.112.002154
2. Bazzano LA, Serdula MK, Liu S. Dietary intakes of FandV and risk of cardiovascular disease. *Curr Atheroscler Rep.* (2002) 5:492–9. doi: 10.1007/s11883-003-0040-z
3. Van't Veer P, Jansen M, Klerk M, Kok FJ. Fruits and vegetables in the prevention of cancer and cardiovascular disease. *Public Health Nutr.* (2000) 3:103–7. doi: 10.1017/S1368980000000136
4. De Mello-Andrade JM, Fasolo D. Polyphenol antioxidants from natural sources and contribution to health promotion. *Polyphenols Hum Health Dis.* (2014) 1:253–65. doi: 10.1016/B978-0-12-398456-2.00020-7
5. Podsedek A. Natural antioxidants and antioxidant capacity of Brassica vegetables: a review. *LWT Food Sci Technol.* (2007) 40:1–11. doi: 10.1016/j.lwt.2005.07.023
6. Ross KA, Beta T, Arntfield SD. A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. *Food Chem.* (2009) 113:336–44. doi: 10.1016/j.foodchem.2008.07.064
7. Clifford MN, Wight J. The measurement of feruloylquinic acids and caffeoylquinic acids in coffee beans. Development of the technique and its preliminary application to green coffee beans. *J Sci Food Agric.* (1976) 27:73–84. doi: 10.1002/jsfa.2740270112
8. Kremr D, Cocovi-Solberg DJ, Bajerová P, Ventura K, Miró M. On-line monitoring of *in-vitro* oral bioaccessibility tests as front-end to liquid chromatography for determination of chlorogenic acid isomers in dietary supplements. *Talanta.* (2015) 166:391–8. doi: 10.1016/j.talanta.2015.12.082
9. Shin HS, Satsu H, Bae MJ, Zhao Z, Ogiwara H, Totsuka M, et al. Anti-inflammatory effect of chlorogenic acid on the IL-8 production in Caco-2 cells and the dextran sulphate sodium-induced colitis symptoms in C57BL/6 mice. *Food Chem.* (2015) 168:167–75. doi: 10.1016/j.foodchem.2014.06.100
10. Jeng TL, Lai CC, Liao TC, Lin SY, Sung JM. Effects of drying on caffeoylquinic acid derivative content and antioxidant capacity of sweet potato leaves. *J Food Drug Anal.* (2015) 23:701–8. doi: 10.1016/j.jfda.2014.07.002
11. Bajko E, Kalinowska M, Borowski P, Siergiejczyk L, Lewandowski W. 5-O- Caffeoylquinic acid: a spectroscopic study and biological screening for antimicrobial activity. *LWT Food Sci Technol.* (2010) 54:158–68. doi: 10.1016/j.lwt.2015.08.024
12. Butiuk AP, Martos MA, Adachi O, Hours RA. Study of the chlorogenic acid content in yerba mate (*Ilex paraguariensis* St. Hil): effect of plant fraction, processing step and harvesting season. *J Appl Res Med Aromatic Plants.* (2016) 3:27–33. doi: 10.1016/j.jarmap.2015.12.003
13. Peng BJ, Zhu Q, Zhong YL, Xu SH, Wang Z. Chlorogenic acid maintains glucose homeostasis through modulating the expression of SGLT-1, GLUT-2, and PLG in different intestinal segments of sprague-dawley rats fed a high-fat diet. *Biomed Environ Sci.* (2015) 28:894–903. doi: 10.3967/bes2015.123
14. Liu W, Li J, Zhang X, Zu Y, Yang Y, Liu W, et al. Current advances in naturally occurring caffeoylquinic acids: structure, bioactivity, and synthesis. *J Agric Food Chem.* (2020) 68:10489–516. doi: 10.1021/acs.jafc.0c03804
15. Arantes AA, Falé PL, Costa LCB, Pacheco R, Ascensão L, Serralheiro ML. Inhibition of HMG-CoA reductase activity and cholesterol permeation through Caco-2 cells by caffeoylquinic acids from Vernonia condensata leaves. *Rev Brasil Farmacogn.* (2016) 6:11. doi: 10.1016/j.bjp.2016.05.008
16. Bagdas D, Etoz BC, Gul Z, Ziyanok S, Inan S, Turacozen O, et al. *In vivo* systemic chlorogenic acid therapy under diabetic conditions: wound healing effects and cytotoxicity/ genotoxicity profile. *Food Chem Toxicol.* (2015) 81:54–61. doi: 10.1016/j.fct.2015.04.001
17. Jiang R, Hodgson JM, Mas E, Croft KD, Ward NC. Chlorogenic acid improves *ex vivo* vessel function and protects endothelial cells against HOCl-induced oxidative damage, via increased production of nitric oxide and induction of Hmox-1. *J Nutr Biochem.* (2016) 27:53–60. doi: 10.1016/j.jnutbio.2015.08.017
18. Gu R, Dou G, Wang J, Dong J, Meng Z. Simultaneous determination of 1,5-dicaffeoylquinic acid and its active metabolites in human plasma by liquid chromatography-tandem mass spectrometry for pharmacokinetic studies. *J Chromatogr B Anal Technol Biomed Life Sci.* (2007) 852:85–91. doi: 10.1016/j.jchromb.2006.12.055
19. Niggeweg R, Michael A, Martin C. Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nat Biotechnol.* (2004) 22:746–54. doi: 10.1038/nbt966
20. Leiss K, Maltese F, Choi Y, Verpoorte R, Klinkhamer P. Identification of chlorogenic acid as a resistance factor for thrips in *Chrysanthemum*. *Plant Physiol.* (2009) 150:1567–75. doi: 10.1104/pp.109.138131
21. Cle C, Hill L, Niggeweg R, Martin C, Guisez Y, Prinsen E, et al. Modulation of chlorogenic acid biosynthesis in *Solanum lycopersicum*; consequences for phenolic accumulation and UV-tolerance. *Phytochemistry.* (2008) 69:2149–56. doi: 10.1016/j.phytochem.2008.04.024

22. Silva LR, Pereira MJ, Azevedo J, Gonçalves RF, Valentão P, de Pinho PG, et al. sprouts: a natural source of bioactive compounds. *Food Res Int.* (2013) 50:167–75. doi: 10.1016/j.foodres.2012.10.025
23. Baenas N, Gómez-Jodar I, Moreno DA, García-Viguera C, Periago PM. Broccoli and radish sprouts are safe and rich in bioactive phytochemicals. *Postharvest Biol Technol.* (2017) 127:60–7. doi: 10.1016/j.postharvbio.2017.01.010
24. Xiao Z, Codling EE, Luo Y, Nou X, Lester GE, Wang Q. Microgreens of Brassicaceae: mineral composition and content of 30 varieties. *J Food Compos Anal.* (2016) 49:87–93. doi: 10.1016/j.jfca.2016.04.006
25. Kyriacou MC, El-Nakhel C, Graziani G, Pannico A, Soteriou GA, Giordano M, et al. Functional quality in novel food sources: genotypic variation in the nutritive and phytochemical composition of thirteen microgreens species. *Food Chem.* (2019) 277:107–18. doi: 10.1016/j.foodchem.2018.10.098
26. Guo S, Ge Y, Na Jom K. A review of phytochemistry. Metabolite changes, and medicinal uses of the common sunflower seed and sprouts (*Helianthus annuus* L). *Chem Cent J.* (2017) 11:95. doi: 10.1186/s13065-017-0328-7
27. Fowler MW. Plants, medicines and man. *J Sci Food Agric.* (2006) 86:1797–804. doi: 10.1002/jsfa.2598
28. Sun Z, Chen J, Ma J, Jiang Y, Wang M, Ren G, et al. Cynarin-rich sunflower (Helianthus annuus) sprouts possess both antiglycative and antioxidant activities. *J Agric Food Chem.* (2012) 60:3260–5. doi: 10.1021/jf300737y
29. Cheevarungnapakul K, Khaksar G, Panpetch P, Boonjing P, Sirikantaramas S. Identification and functional characterization of genes involved in the biosynthesis of caffeoylquinic acids in sunflower (*Helianthus annuus* L.). *Front Plant Sci.* (2019) 10:968. doi: 10.3389/fpls.2019.00968
30. Pajak P, Socha R, Gałkowska D, Roznowski J, Fortuna T. Phenolic profile and antioxidant activity in selected seeds and sprouts. *Food Chem.* (2014) 143:300–6. doi: 10.1016/j.foodchem.2013.07.064
31. Howard LA, Wong AD, Perry AK, Klein BP. Beta-carotene and ascorbic acid retention in fresh and processed vegetables. *J Food Sci.* (1999) 64:929–36. doi: 10.1111/j.1365-2621.1999.tb15943.x
32. Serrano M, Martinez-Romero D, Guillén F, Castillo S, Valero D. Maintenance of broccoli quality and functional properties during cold storage as affected by modified atmosphere packaging. *Postharvest Biol Technol.* (2006) 39:61–8. doi: 10.1016/j.postharvbio.2005.08.004
33. Rapisarda P, Lo Bianco M, Pannuzzo P, Timpanaro N. Effect of cold storage on vitamin C, phenolics and antioxidant activity. *Postharvest Biol Technol.* (2008) 49:348–54. doi: 10.1016/j.postharvbio.2008.02.002
34. Blessington T, Nzaramba MN, Scheuring DC, Hale AL, Reddivari L, Miller JC. Cooking methods and storage treatments of potato: effects on carotenoids, antioxidant activity, and phenolics. *Am J Potato Res.* (2010) 87:479–91. doi: 10.1007/s12230-010-9150-7
35. Phillips KM, Tarragó-Trani MT, Gebhardt SE, Exler J, Patterson KY, Haytowitz DB, et al. Stability of vitamin C in frozen raw fruit and vegetable homogenates. *J Food Compos Anal.* (2010) 23:253–9. doi: 10.1016/j.jfca.2009.08.018
36. Madiwale GP, Reddivari L, Holm DG, Vanamala J. Storage elevates phenolic content and antioxidant activity but suppresses antiproliferative and pro-apoptotic properties of colored-flesh potatoes against human colon cancer cell lines. *J Agric Food Chem.* (2011) 59:8155–66. doi: 10.1021/jf201073g
37. Piljac-Žegarac J, Šamec D. Antioxidant stability of small fruits in postharvest storage at room and refrigerator temperatures. *Food Res Int.* (2011) 44:345–50. doi: 10.1016/j.foodres.2010.09.039
38. Galani YJH, Mankad MP, Shah AK, Patel NJ, Acharya RR, Talati JG. Effect of storage temperature on vitamin C. Total phenolics, UPLC phenolic acids profile and antioxidant capacity of eleven potato (*Solanum tuberosum* L). *Varieties Hortic Plant J.* (2017) 3:73–89. doi: 10.1016/j.hpj.2017.07.004
39. Khaksar G, Assatarakul K, Sirikantaramas S. Effect of cold-pressed and normal centrifugal juicing on quality attributes of fresh juices: do cold-pressed juices harbor a superior nutritional quality and antioxidant capacity? *Heliyon.* (2019) 5:e01917. doi: 10.1016/j.heliyon.2019.e01917
40. Swieca M, Gawlik-Dziki U. Effects of sprouting and postharvest storage under cool temperature conditions on starch content and antioxidant capacity of green pea, lentil and young mung bean sprouts. *Food Chem.* (2015) 185:99–105. doi: 10.1016/j.foodchem.2015.03.108
41. Preti R, Rapa M, Vinci G. Effect of steaming and boiling on the antioxidant properties and biogenic amines content in green bean (Phaseolus vulgaris) varieties of different colors. *J Food Qual.* (2017) 2017:5329070. doi: 10.1155/2017/5329070
42. Hwang E-S. Influence of cooking methods on bioactive compound content and antioxidant activity of brussels sprouts. *Prev Nutr Food Sci.* (2017) 22:353–8. doi: 10.3746/pnf.2017.22.4.353
43. Minatel IO, Borges CV, Ferreira MI, Gomez HAG, Chen CYO, Lima GPP. *Phenolic Compounds: Functional Properties, Impact of Processing and Bioavailability, Phenolic Compounds - Biological Activity, Marcos Soto-Hernandez, Mariana Palma-Tenango and Maria del Rosario Garcia-Mateos*. London: IntechOpen (2017).
44. Chiavaro E, Mazzeo T, Visconti A, Manzi C, Fogliano V, Pellegrini N. Nutritional quality of sous vide cooked carrots and Brussels sprouts. *J Agric Food Chem.* (2012) 60:6019–25. doi: 10.1021/jf300692a
45. Kumari S, Chang SK. Effect of cooking on isoflavones, phenolic acids, and antioxidant activity in sprouts of prosoy soybean (Glycine Max). *J Food Sci.* (2016) 81:1679–91. doi: 10.1111/1750-3841.13351
46. Limmongkon A, Janhom P, Amthong A, Kawpanuk M, Nopprang P, Poohadsuan J. et al. Antioxidant activity, total phenolic, and resveratrol content in five cultivars of peanut sprouts. *Asian Pac J Trop Biomed.* (2017) 7:332–8. doi: 10.1016/j.apjtb.2017.01.002
47. Sharma P, Ghimeray AK, Gurung A, Jin CW, Rho HS, Cho DH. Phenolic contents, antioxidant and α-glucosidase inhibition properties of Nepalese strain buckwheat vegetables. *Afr J Biotechnol.* (2012) 11:184–90. doi: 10.5897/AJB11.2185
48. Park H, Shin Y, Kim YJ. Antioxidant contents and activities of twelve varieties of vegetable sprouts. *Korean J Food Sci Technol.* (2019) 51:207–13. doi: 10.9721/KJFST.2019.51.3.207
49. Drozdowska M, Leszczyn ska T, Koronowicz A, Piasna-Słupecka E, Dziadek K. Comparative study of young shoots and the mature red headed cabbage as antioxidant food resources with antiproliferative effect on prostate cancer cells. *RSC Adv.* (2020) 10:43021. doi: 10.1039/D0RA07861A
50. Ludwig LA, Mena P, Calani L, Cid C, Del Rio D, Lean ME, et al. Variations in caffeine and chlorogenic acid contents of coffees: what are we drinking? *Food Funct.* (2014) 5:1718. doi: 10.1039/C4FO00290C
51. Wianowska D, Gil M. Recent advances in extraction and analysis procedures of natural chlorogenic acids. *Phytochem Rev.* (2019) 18:273–302. doi: 10.1007/s11101-018-9592-y
52. Martínez-Hernández GB, Artés-Hernández F, Gómez PA, Artés F. Induced changes in bioactive compounds of kailan-hybrid broccoli after innovative processing and storage. *J Funct Foods.* (2013) 5:133–43. doi: 10.1016/j.jff.2012.09.004
53. Marques V, Farah A. Chlorogenic acids and related compounds in medicinal plants and infusions. *Food Chem.* (2009) 113:1370–6. doi: 10.1016/j.foodchem.2008.08.086
54. Xue M, Shi H, Zhang J, Liu Q-Q, Guan J, Zhang J-Y, et al. Stability and degradation of caffeoylquinic acids under different storage conditions studied by high-performance liquid chromatography with photo diode array detection and high-performance liquid chromatography with electrospray ionization collision-induced dissociation tandem mass spectrometry. *Molecules.* (2016) 21:948. doi: 10.3390/molecules21070948
55. Alcázar Magaña A, Kamimura N, Soumyanath A, Stevens JF, Maier CS. Caffeoylquinic acids: Chemistry, biosynthesis, occurrence, analytical challenges, and bioactivity. *Plant J.* (2021) 107:1299–319. doi: 10.1111/tpj.15390
56. Miguel S, Legrand G, Duriot L, Delporte M, Menin B, Michel C, et al. A GDSL lipase-like from Ipomoea batatas catalyzes efficient production of 3,5-diCQA when expressed in Pichia pastoris. *Commun Biol.* (2020) 3:673. doi: 10.1038/s42003-020-01387-1
57. Turkmen N, Sari F, Velioglu YS. The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chem.* (2005) 93:713–8. doi: 10.1016/j.foodchem.2004.12.038
58. Pellegrini N, Chiavaro E, Gardana C, Mazzeo T, Contino D, Gallo M, et al. Effect of dif- ferent cooking methods on color, phytochemical concentra- tion, and antioxidant capacity of raw and frozen brassica vegetables. *J Agric Food Chem.* (2010) 58:4310–21. doi: 10.1021/jf904306r
59. Murador, D. C., Mercadante, A. Z., and deRosso, V. V. (2016). Cooking techniques improve the levels of bioactive compounds and

antioxidant activity in kale and red cabbage. *Food Chem*. 196, 1101–1107. doi: 10.1016/j.foodchem.2015.10.037

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Effect of Methyl Jasmonate Treatment on Primary and Secondary Metabolites and Antioxidant Capacity of the Substrate and Hydroponically Grown Chinese Chives

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Hydroponic culture has become a commercial planting model for leafy vegetables, herbs, and other plants with medicinal value. Methyl jasmonate (MeJA) is involved in primary and secondary plant metabolism; moreover, it regulates plant bioactive compounds and enhances the nutritional and medicinal value of plants. We performed targeted metabolomic analysis of the primary and secondary metabolites in substrate-grown and hydroponic Chinese chive leaves sprayed with MeJA (0, 300, 500, and 800 μM). Using ultra-performance liquid chromatography (UPLC), UPLC tandem mass spectrometry, and chemometric tools, and analyzed the antioxidant activity of these plants. We identified the biomarkers of amino acids (serine, proline, lysine, and arginine) and phenolic compounds (4-coumaric acid and protocatechuic acid) using chemometric tools to distinguish between substrate-grown and hydroponic Chinese chives treated with MeJA. MeJA (500 μM) treatment significantly increased the total sugar and amino acid (essential and non-essential amino acids and sulfur-containing amino acids) contents of hydroponically grown Chinese chives. However, the changes in total sugar and amino acid contents in Chinese chive grown in substrates showed the opposite trend. The organic acid content of hydroponically grown Chinese chives treated with MeJA decreased significantly, whereas that of substrate-grown plants treated with 300 μM MeJA increased significantly. Further, MeJA treatment significantly increased the phenolic content of substrate-grown Chinese chives. Treatment with 800 μM MeJA significantly increased the carotenoid content of substrate-grown Chinese chives and the phenolic content of hydroponic Chinese chives. In addition, the 500 μM MeJA treatment significantly increased the antioxidant activity of Chinese chives in both substrate-grown and hydroponic cultures, and promoted the accumulation of nutrients and bioactive substances. This treatment also improved the flavor quality of these plants and their nutritional and medicinal value. Thus, the results suggested that MeJA-treated plants could be used as value-added horticultural products.

Keywords: hydroponic agriculture, methyl jasmonate, antioxidant capacity, plant metabolites, Chinese chive, *Allium tuberosum*, metabolic profile

INTRODUCTION

Allium species are known for their beneficial effects on human health and biological functions. These health benefits are attributed to the high content of bioactive compounds in *Allium* plants (1, 2). The Chinese chives (*A. tuberosum* Rottl. ex. Spreng.) have a long history of cultivation in China (3). As a perennial herbaceous root vegetable, it is one of the most widely used and studied species in the genus *Allium* (4). Chinese chives have a unique pungent garlic-like flavor and are rich in organic sulfur compounds, polysaccharides, phenols, flavonoids, and other bioactive substances. Moreover, the Chinese chives is rich in nutrients and has high medicinal value (5, 6). Chinese chives extract also plays an important role in the treatment of cardiovascular disease, diabetes, and cancer, and has antibacterial, anti-inflammatory, and antioxidant activities (7, 8). In addition, the characteristic flavor of the Chinese chives has received attention from researchers in recent years (9). As it is used for both food and medicine, improving the nutritional quality, bioactive substances, and antioxidant activity of the Chinese chives can help in increasing its potential nutritional and medicinal value. However, compared with the conventionally cultivated Chinese chives, research on the nutritional quality and bioactive substances of hydroponic Chinese chives has not been conducted extensively.

Plants are natural sources of ascorbic acid (10, 11), minerals (12, 13), and metabolites, such as sugar, amino acid, organic acid, phenolic compounds (14), flavonoids (15, 16), non-flavonoids, beta-carotene (17), betalains (18), and carotenoids (19, 20). Methyl jasmonate (MeJA) is an important cellular regulator that regulates plant development and defense responses to biotic and abiotic stresses (21, 22). In addition, MeJA is certified as a safe compound for all food items when used before harvesting (23). Phytohormone induction induces a response to adversity in plants, and is the main method of increasing the content of secondary metabolites in vegetables (24). Jasmonic acid (JA) and MeJA are the most commonly studied and widely used excitons that used to promote the accumulation of secondary metabolites in horticultural products to improve their nutritional quality (25). Exogenous MeJA can alter the levels of various primary metabolites, including plant sugars, organic acids, and amino acids (26). Treatment with MeJA promotes the production of bioactive compounds in broccoli (*Brassica oleracea* L. var. *italica* Plenck) (27). In addition, MeJA treatment promotes the biosynthesis of volatile compounds and the accumulation of secondary metabolites and non-volatile secondary metabolites (28, 29). Exogenous JA promotes the accumulation of β-carotene and the production of aromatic compounds (30). Liu et al. (31) found that MeJA promoted lycopene accumulation in tomato (*Lycopersicon esculentum*) by upregulating the expression of genes related to carotenoid biosynthesis. Shafiq et al. (32) reported that the application of MeJA promoted the accumulation of phenolic compounds, such as anthocyanin 3-galactoside, chlorogenic acid, and flavonols in the pericarp of apples (*Malus pumila* Mill.). Studies in the field of medical science have shown that a human diet rich in natural plant-synthesized polyphenols can reduce the risk of chronic and degenerative diseases, such as cancer. This is mainly because phenolic compounds rich in anthocyanins, flavonoids, and phenolic acids often exhibit strong free radical scavenging activity (33). MeJA treatment in pomegranate (*Punica granatum* L.) (34) and blackberry (*Rubus fruticosus* Pollich) (35) promotes bioactive compounds and enhances their antioxidant activities and properties beneficial for human health.

Despite their growing popularity, the nutritional quality and bioactive substances of Chinese chives have been relatively less studied, especially compared to those of other *Allium* vegetables, such as garlic and onions. To the best of our knowledge, previous studies have not reported the effects of MeJA treatment on primary and secondary metabolites in Chinese chives. In addition, there is a lack of information on the effects of MeJA treatment on primary and secondary metabolites in other *Allium* crops. Compared to soil cultivation, hydroponics can provide more efficient nutrient requirements for the plants (36). In addition, hydroponic cultivation mainly improves the yield and accumulation of functional components (37). We hypothesized that the two cultivation methods, substrate culture and hydroponic culture, could induce differences in the content of primary and secondary metabolites in Chinese chives, while the application of MeJA could promote primary and secondary metabolites in substrate-grown and hydroponic Chinese chives, consequently, increasing their antioxidant activity and medicinal value. The objective of this study was to determine how foliar spraying with MeJA affected the metabolites of substrate-grown and hydroponic Chinese chives. To better understand the metabolic fluxes between primary and secondary metabolites, we analyzed primary metabolites (including amino acids, organic acids, and sugars) and secondary metabolites (including phenolic compounds and carotenoids) in substrate-grown and hydroponic Chinese chives treated with MeJA. Our findings are important for the development of cultivation systems that can enhance the nutritional characteristics of vegetables.

MATERIALS AND METHODS

Plant Material and Experimental Design

Seeds of *A. tuberosum* cv. "Chive God F1" were used as the experimental material. On May 8, 2020, the seedlings were cultivated in Wushan (34°25′–34°57′ N, 104°34′–105°08′ E), China, in the core demonstration area for Chinese chives. On July 20, 2021, we harvested the aboveground leaves and old roots of 1-year-old Chinese chive seedlings, removed the leaves and extra old roots while retaining 2–3 cm of old roots to promote the development of new roots. The plants were then transplanted into hydroponic and substrate cultivation systems in a glass solar greenhouse at Gansu Agricultural University in Lanzhou (36°03′ N, 103°40′ E) (**Figure 1**). The temperature and relative humidity conditions in the greenhouse were 20 ± 3°C/15 ± 3°C (day/night) and 60–70%, respectively. The nutrient solution for the hydroponic system was prepared according to Wu et al. (38) (**Table 1**), and the nutrient solution electrical conductivity and pH were 2.13 ms cm^{-1} and 6.15, respectively. Seedlings were fixed with cotton in 16 rectangular hydroponic boxes

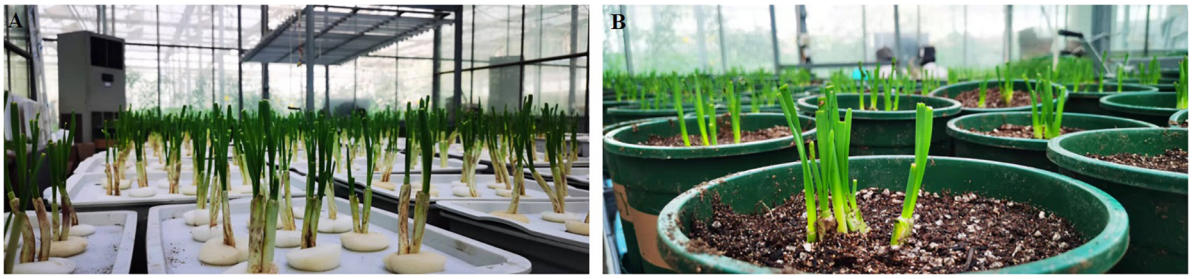

FIGURE 1 | Hydroponic **(A)** and substrate-grown **(B)** Chinese chives grown in a glass solar greenhouse.

(0.37 m × 0.25 m × 0.2 m), each having 11 holes. Two seedlings were fixed in each hole; correspondingly, 88 plants were fixed in four boxes per treatment. The nutrient solution was continuously aerated with an air compressor and replaced every 3 d. In the substrate systems, the cultivation substrate, perlite, and vermiculite were provided by Gansu Green Energy Ruiqi Biotechnology Co., Ltd. (Tianzhu, China). The cultivation substrate, which was mainly composed of peat, coconut bran, cow dung, and perlite, was mixed with perlite, and vermiculite in a ratio of 3:1:1 in a 4-L plastic pot, and four seedlings were transplanted in each pot, that is, out of the total 88 pots for four treatments, 22 pots containing 88 seedlings were used per treatment. Each pot of substrate was supplied with 150 mL of the nutrient solution, the composition of which is listed in **Table 1**, on the first day after transplanting Chinese chive seedlings. In addition, substrate cultivation was watered once or twice per week.

The experiment was conducted in a completely randomized design with three replicates. The hydroponic Chinese chives were treated with the following exogenous hormones: an aqueous solution of MeJA (containing 0.1% ethanol and 0.1% Tween-20) at three concentrations (300 μM, HM300; 500 μM, HM500; and 800 μM, HM800) and a control solution (0 μM, HCK, containing 0.1% ethanol and 0.1% Tween-20). The substrate-grown Chinese chives were treated with the following exogenous hormones: an aqueous solution of MeJA (containing 0.1% ethanol and 0.1% Tween-20) at three concentrations (300 μM, SM300; 500 μM, SM500; and 800 μM, SM800) and a control solution (0 μM, SCK, containing 0.1% ethanol and 0.1% Tween-20). When the seedlings had grown to approximately 15 cm, 150 mL of the aforementioned aqueous solutions were sprayed on the plant leaves in each treatment group (i.e., total 88 plants) at 7:00–8:00 every morning from August 11, 2021, to August 17, 2021. After 35 d (August 24, 2021), Chinese chives were harvested from all treatment groups. In total, 60 Chinese chive plants were randomly selected for each treatment, with 20 plants in each of three biological replicates. All samples were ground to a powder in liquid nitrogen and stored at −80°C prior to analysis.

TABLE 1 | Composition of the nutrient solution for hydroponic.

Compounds	Concentration (mg L^{-1})
$Ca(NO_3)_2{\cdot}4H_2O$	240
KNO_3	619
$(NH_4)_2SO_4$	270
$MgSO_4{\cdot}7H_2O$	248
NaFe-EDTA	30
H_3PO_4	0.14
H_3BO_3	2.86
$MnSO_4{\cdot}4H_2O$	2.13
$ZnSO_4{\cdot}7H_2O$	0.22
$CuSO_4{\cdot}7H_20$	0.08
$(NH_4)_6Mo_7O_{24}{\cdot}7H_2O$	0.02

Analysis of Soluble Sugars and Organic Acids by High-Performance Liquid Chromatography

The sugar contents of samples were determined using High-Performance Liquid Chromatography (HPLC) (Agilent series 1100, Agilent Technologies, Santa Clara, CA, United States). The extraction and determination methods were based on Wei et al. (39), with minor modifications. Briefly, frozen Chinese chive powder (fresh weight, 0.5 g) was homogenized with ultrapure water (2.5 mL) and extracted by ultrasonication at 30°C for 60 min, followed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant (2 mL) was filtered through a 0.22-μm aqueous microporous membrane into a liquid chromatography vial for measurement. The conditions for separating the soluble sugars were as follows: detector, differential refractive index detector (Agilent series 1100, Agilent Technologies); column, LC-NH2 (4.6 × 250 mm); column temperature, 30°C; phase, acetonitrile: water (3:1, v: v); flow rate, 1.0 mL min^{-1}; and injection volume, 25 μL. The concentrations of sugar compounds were determined based on the areas of their extracted chromatograms using the reference standards of fructose, glucose, and sucrose. **Supplementary Figure 1** shows the chromatogram of sugars.

The levels of organic acids were determined using ultra-performance liquid chromatography (UPLC, Waters Corp., Milford, MA, United States). The extraction and determination methods were based on Coelho et al. (40), with minor modifications. Briefly, frozen Chinese chive powder (fresh weight, 1.5 g) was mixed with ultrapure water (7.5 mL) to extract the organic acids. The extract was centrifuged at 4°C and 10,000 × g for 10 min, and the supernatant was aspirated

and filtered through a 0.22-μm aqueous phase microporous membrane into a liquid chromatography vial for measurement. The conditions for separating the organic acids were as follows: detector, UV detector; column, Thermo Hypersil COLD AQ (4.6 × 150 mm, 3 μm); column temperature, 30°C; phase, 20 mmol L^{-1} NaH_2PO_4 (pH = 2.7); flow rate, 0.5 mL min^{-1}; and injection volume, 20 μL. The concentrations of organic acids were determined based on the areas of their extracted chromatograms using the reference standards of oxalic acid, citric acid, and malic acid. **Supplementary Figure 2** shows the chromatogram of organic acids.

Analysis of Amino Acids by Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

The underivatized amino acids in Chinese chives were quantitatively analyzed by hydrophilic interaction chromatography (HILIC) using liquid chromatography/mass spectrometry (LC/MS) detection (Agilent 1290–6460, LC/MS, Agilent Technologies) (41). In brief, frozen Chinese chive powder (fresh weight, 0.1 g) was extracted with 0.5 M aqueous hydrochloric acid (1 mL). The solution was mixed by vortexing at 8,000 × g for 20 min, sonicating for 20 min in a 25°C water bath, and then centrifuging at 20,000 × g for 20 min. The supernatant (250 μL) was transferred to a liquid chromatographic vial with ^{15}N-enriched deuterated internal standard (ISTD), and then diluted to 1 mL with 80% acetonitrile aqueous solution. The HPLC conditions were as follows: column, Agilent Infinity Lab Poroshell 120 HILIC-Z (2.1 × 100 mm, 2.7 μm); phase A, 20 mM ammonium formate (pH = 3) with water at a ratio of 9:1; phase B, 20 mM ammonium formate (pH = 3) with 90% aqueous acetonitrile at a ratio of 9:1; flow rate, 0.5 mL min^{-1}; column temperature, 25°C; injection volume, 1 μL; total running time, 15 min; gradient time (min), 0, 11.5, and 12; and gradient concentration (%B), 100, 70, and 100. The mass spectrometry conditions were as follows: ionization mode, electrospray ionization in positive ion mode; dryer temperature, 330°C; gas flow rate, 13.0 L min^{-1}; atomizer, 35 psi; sheath gas temperature, 390°C; sheath gas velocity, 12 L min^{-1}; capillary voltage, 1,500 V; and nozzle voltage, 0 V. **Supplementary Figure 3** shows the chromatogram of amino acids. The limit of detection (LOD) and limit of quantification (LOQ) values are listed in **Supplementary Table 1**.

Analysis of Phenolic Compounds by High-Performance Liquid Chromatography and Determination of Total Phenol Content

We analyzed polyphenols using an HPLC system (Waters Corp.) equipped with a 1525 pump and a 2998 photodiode array detector. The phenolic compounds were determined according to Liu et al. (42), with minor modifications. Frozen Chinese chive powder (fresh weight, 0.1 g) was added to methanol (2 mL) and placed in a refrigerator at 4°C for 60 min after shaking several times. The solution was centrifuged at 8,000 × g for 10 min at 4°C, and the supernatant was filtered through a 0.22-μm filter membrane into a liquid chromatography vial for detection. The conditions for separating the phenolic compounds were as follows: column, Waters Symmetry C18 column (4.6 × 250 mm, 5 μm); mobile phase, methanol and 1% acetic acid for gradient elution; flow rate, 1.1 mL min^{-1}; column temperature, 30°C; and injection volume, 10 μL. The phenolic compounds were detected at 240, 280, and 322 nm (**Table 2**). P-hydroxybenzoic acid, protocatechuic acid, quercetin, chlorogenic acid, rutin, cinnamic acid, 4-coumaric acid, gallic acid, benzoic acid, ferulic acid, erucic acid, caffeic acid, artichoke element, kaempferol, and gentilic acid (Sigma-Aldrich, Burlington, MA, United States) were used as external standards. **Supplementary Table 2** provides the retention time, wavelengths of maximum absorption in the visible region (λ_{max}) and temporary identification of the phenolic components. **Supplementary Figure 4** shows the phenolic acid chromatogram. The LOD and LOQ values are listed in **Supplementary Table 3**. Total phenol content was determined according to the method of Hand et al. (43) with minor modifications. Briefly, a crude extract of phenol was extracted with 50% methanol, mixed with distilled water, and then extracted with Folin-Ciocalteu phenol reagent and 20% sodium carbonate solution. After 30 min in a water at 50°C in the dark, the absorbance was measured at 760 nm using a UV-1780 spectrophotometer (Shimadzu Instruments, Suzhou, China). Total phenol content was calculated using a calibration curve for gallic acid.

Analysis of Carotenoids by High-Performance Liquid Chromatography

Carotenoids were determined according to Li et al. (44), with minor modifications. Frozen Chinese chive powder (fresh weight, 2 g) and butylated hydroxytoluene (0.1 g) were homogenized by grinding with a small volume of liquid nitrogen and transferred to a 50-mL centrifuge tube. This was added to 20% KOH-methanol solution (8 mL), and the mixture was sealed and placed in a constant water bath at 55°C for 30 min. The extract was added to a 1:2 solution of acetone: ethyl acetate, ultrasonicated for 40 min, and centrifuged at 8,000 r min^{-1} at 4°C. The supernatant was concentrated by rotary evaporation, and the volume was made up to 10 mL with acetone. For HPLC detection, the solution was filtered into the injection bottle with a 0.22-μm organic membrane. Samples were analyzed using an HPLC

TABLE 2 | Determination of phenolic compounds by HPLC at three wavelengths.

Wavelength	240 nm	280 nm	322 nm
Phenolic compounds	P-hydroxybenzoic acid	Cinnamic acid	Erucic acid
	Protocatechuic acid	4-coumaric acid	Caffeic acid
	Quercetin	Gallic acid	Artichoke element
	Chlorogenic acid	Benzoic acid	Kaempferol
	Rutin	Ferulic acid	Gentilic acid

system (Analytical HPLC, 1260 Infinity II LC System; Agilent Technologies) coupled to a diode array detector. The conditions for separating the carotenoids were as follows: column, a special Welch Ultimate C30 column (4.6 × 250 mm, 5 μm) for carotenoids; column temperature, 30°C; flow rate, 1.0 mL min^{-1}; injection volume, 20 μL; phase A, acetonitrile; phase B, water; phase C, methyl tertbutyl ether: methanol (V: V = 1: 1); and phase D, ethyl acetate. The carotenoids were detected at 470, 443, and 286 nm (**Table 3**) and quantified using the reference standards of α-carotene, monoepoxy zeaxanthin, zeaxanthin, lycopene, violaxanthin, lutein, and β-carotene. **Supplementary Figure 5** shows the chromatogram of carotenoids. The LOD and LOQ values are listed (**Supplementary Table 4**).

Antioxidant Capacity of Chinese Chives

The 2, 2′-diphenyl-1-picrylhydrazyl radical (DPPH) assay for scavenging activity and ferric reducing/antioxidant capacity (FRAP) assay and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay for free radical scavenging activity were performed using a commercially available kit (Suzhou Keming, Suzhou, China) according to the manufacturer's instructions. The sample was homogenized in an ice bath at a 1:10 ratio of sample fresh weight (g): volume of extract (mL), and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was kept in ice for testing, and the supernatant and reagents were added to the enzyme plate in turn according to the instructions. The enzyme plates for the DPPH and FRAP assays were reacted for 20 min at 20–23°C. The absorbance was measured at 515 and 593 nm for DPPH and FRAP assays, respectively, using a multi-function microplate reader (Spectramax i3, Molecular Devices, Sunnyvale, CA, United States). A multifunctional microplate reader was used for the ABTS assay to detect the absorbance at 734 nm immediately after adding the reagents. Trolox was used to establish the standard curve, and the scavenging activity and reducing ability were expressed in Trolox equivalents (μmol Trolox g^{-1} FW).

The oxygen radical absorbance capacity (ORAC) was determined using a kit (Huicheng Biological Technology, Shanghai, China) according to the manufacturer's instructions. In brief, the sample was homogenized in an ice bath at a 1: 10 ratio of sample fresh weight (g): volume of ethanol (mL), and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was placed in ice for further testing. The supernatant (50 μL) and 10 μL mL^{-1} sodium fluorescein (50 μL) was mixed in a black fluorescent enzyme plate and left to react in the dark for 30 min at 37°C. Subsequently, a free radical initiator was added, and the mixture was immediately subjected to fluorescence enzyme kinetic assay using a multi-function microplate reader (Synergy HTX, BioTek Instruments Ltd., Winooski, VT, United States). Readings were recorded every 3 min for 90 min at an excitation wavelength of 485 nm, an emission wavelength of 520 nm, and an incubation temperature of 37°C. The recordings were analyzed using the Trolox calibration curve and the area under the curve (AUC) of the fluorescence decay. The results were expressed as micro-moles of Trolox equivalent per mL. The AUC was calculated as follows:

$$AUC = F_0/F_0 + F_1/F_0 + F_2/F_0 + \ldots\ldots + F_n/F_0$$

where F_0 is the initial fluorescence reading at 0 min, and F_n ($n = 31$) are the fluorescence readings taken every 3 min up to 90 min.

TABLE 3 | Determination of carotenoids by HPLC at three wavelengths.

Wavelength	286 nm	443 nm	470 nm
Carotenoids	Monoepoxy zeaxanthin	Lycopene	α-carotene
			Zeaxanthin
	Violaxanthin		Lutein
			β-carotene

Statistical Analysis

All experimental data were analyzed using IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, United States). The statistical significance of differences in treatment means was evaluated by Duncan's multiple range test ($p < 0.05$). All data are presented as the mean ± standard error of three biological replicates. The figures were generated using Origin Pro 2021. For multivariate analysis, raw data were normalized by the sum, mean-centered, and divided by the square root of the standard deviation of each variable (Pareto scaling). We also conducted principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), and calculated the variables importance in projection (VIP) scores. To evaluate differences between treated and untreated groups, heat maps (with Pearson's correlation coefficients as a distance measure) were illustrated using the MetaboAnalyst 5.0 server (accessed on 20 October 2021).[1]

RESULTS AND DISCUSSION

Effect of Methyl Jasmonate Treatments on Sugars and Organic Acids (Primary Metabolites) in Chinese Chive

The sugars and organic acid contents of substrate-grown and hydroponic Chinese chives treated with MeJA are shown in **Figure 2**. The fructose content of substrate-grown Chinese chives increased with the MeJA treatment (500–800 μM), and was significantly higher than that of the SCK group. In contrast, the MeJA treatment significantly reduced the glucose, sucrose, and total sugar contents of substrate-grown Chinese chives (**Figure 2A**). Tytgat et al. (45) reported that JA treatment reduced the sugar concentration of kale (*Brassica oleracea* L.), which is similar to our results for substrate-grown Chinese chives. However, treatment with 500 μM MeJA significantly increased the fructose, glucose, sucrose, and total sugar contents of hydroponic Chinese chives compared to those in the HCK group. In addition, we found that fructose and glucose constituted the main sugar fraction in hydroponic Chinese chives, accounting for 79–84% of the total sugars. In contrast, fructose constituted the main sugar fraction in substrate-grown Chinese chives,

[1]https://www.metaboanalyst.ca

accounting for 10–65% of the total sugars. Liang et al. (46) reported that the MeJA treatment reduced the levels of most sugars in potted radish (*Raphanus sativus* L.), which was contrary to the results of our hydroponically grown plants. This suggested that the cultivation method, which results in high sugar content in hydroponic plants, is an important factor affecting the sugar contents of hydroponic and substrate-grown Chinese chives.

Citric acid is found in many fruits and vegetables, and its proper supplementation can promote appetite and enhance normal metabolism in the body (47). In this study, we found that citric acid constituted the main organic acid fraction in Chinese chives, accounting for 94–96% (substrate-grown) and 92–96% (hydroponic) of the total acid content. This was followed by malic acid, accounting for 3.6–5.9% (substrate-grown) and 3.7–7.3% (hydroponic) of the total acid content (**Figure 2B**). In addition, treatment with MeJA (300 μM) significantly promoted the citric and total acid contents of the substrate-grown Chinese chives. This is consistent with the findings of Kim et al. (48), who found that MeJA increased the levels of citric and organic acids in potted cabbage (*Brassica oleracea* L.). The MeJA treatment also significantly increased the malic acid content, which accelerates the scavenging of free radicals in human tissues (49). However, organic acid contents in hydroponic Chinese chives decreased significantly with increasing MeJA concentrations (**Figure 2B**). This indicated that the effects of MeJA on the organic acid content of Chinese chives differed between different cultivation methods. Oxalic acid is an anti-nutritional factor that reduces the effectiveness of calcium (a mineral element) in vegetables. It also combines with minerals in other foods to form oxalates, which are difficult for the body to absorb and may cause kidney and urinary tract stones with a poor long-term diet (50, 51). It is generally accepted that vegetables containing oxalic acid should not be consumed at the same time as foods high in calcium (52). In this study, we found that the oxalic acid content of hydroponically grown Chinese chive leaves (0.88 mg g^{-1}) was higher than that of the substrate-grown Chinese chives (0.41 mg g^{-1}). However, the oxalic acid content of the MeJA-treated hydroponically grown Chinese chives decreased significantly with increasing MeJA concentration. This offers a potential way to reduce the oxalic acid content of vegetables with high calcium content, thus, avoiding inadequate calcium absorption by the body due to the high oxalic acid content of vegetables.

Effect of Methyl Jasmonate Treatments on Amino Acids (Primary Metabolites) in Chinese Chive

The levels of 18 amino acids in the substrate-grown and hydroponic Chinese chives treated with MeJA are listed in **Table 4**. Substrate-grown and hydroponic Chinese chives were rich in lysine (73.66 and 62.50 mg g^{-1}), threonine (1.44 and 1.41 mg g^{-1}), phenylalanine (1.51 and 1.45 mg g^{-1}), tryptophan (0.34 and 0.47 mg g^{-1}), leucine (1.76 and 1.65 mg g^{-1}) (1.84 and 1.75 mg g^{-1}), isoleucine (1.84 and 1.75 mg g^{-1}), valine (2.28 and 2.12 mg g^{-1}), and methionine (1.76 and 1.14 mg g^{-1}). Collectively, these accounted for 55 and 54% of the total amino acid content in substrate-grown and hydroponic Chinese chives, respectively, indicating that these plants were good sources of essential amino acids (EAAs). Among non-essential amino acids (NEAAS), glutamate levels were the highest in substrate-grown and hydroponic Chinese chives (26.02 and 18.72 mg g^{-1}, respectively), whereas cysteine levels were the lowest. Amino acids are often involved in various biochemical mechanisms, such as protein synthesis, cell signaling, osmoregulation, and metabolic regulation (53). Tytgat et al. (45) reported that JA treatment reduced the levels of amino acids in kale, and Liang et al. (46) found that MeJA application reduced amino acid content in Brassica. These are similar to our results of substrate-grown Chinese chives, which showed significantly lower levels of total essential amino acids (TEAAs), total non-essential amino acids (TNEAAs), and total amino acids (TAAs) compared to SCK after the MeJA treatment. However, the MeJA (500 and 800 μM)

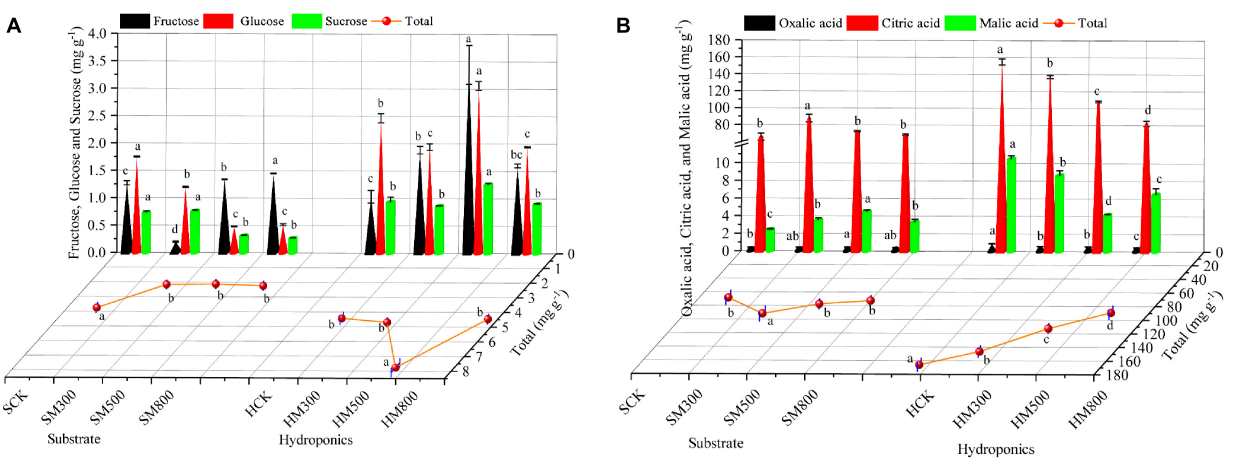

FIGURE 2 | The sugar **(A)** and organic acid **(B)** contents of substrate-grown and hydroponic Chinese chives treated with methyl jasmonate (MeJA). Data represent the mean ± standard error ($n = 3$). Different lowercase letters indicate statistically significant differences according to Duncan's multiple range tests ($p < 0.05$).

treatments significantly increased the levels of TEAAs, TNEAAs, and TAAs in hydroponic Chinese chives compared to those in HCK. This effect was primarily due to a significant increase in the levels of EAAs (such as lysine, isoleucine, valine, and methionine) and NEAAS (such as glycine, aspartic acid, proline, and glutamic acid).

Most amino acids are aliphatic amino acids and function as precursors to aromatic compounds (54). Phenylalanine, tryptophan, and tyrosine are aromatic amino acids that participate in the shikimate pathway, which plays an important role in the aroma development in fruits (55, 56). We found that total aromatic amino acids were inhibited to varying degrees by the MeJA treatment in both substrate-grown and hydroponic Chinese chives. This was probably because MeJA treatment accelerates amino acid deamination and decarboxylation, and degradation of proteins and heat-sensitive amino acids (such as tyrosine) (57). Further studies are required to determine the physiological and molecular mechanisms underlying the effects of MeJA on amino acid synthesis and degradation. Sulfur-containing amino acids are known to play an important role in the production of flavor substances in the Maillard reaction (58). The characteristic flavor and aroma of Chinese chives is produced by the alliinase-catalyzed hydrolysis of metabolites, such as S-alk (alkene) cysteine sulfoxides during cell rupture (59). Volatile components mainly include organosulfur compounds, of which methionine produces methyl sulfide as the main bioactive substance. Methyl sulfide provides the pungent aroma of Chinese chives (60), rather than the fruity aroma provided by aromatic amino acids. We showed that the MeJA treatment significantly increased the cysteine content (sulfur amino acids) in the substrate-grown Chinese chives; moreover, treatment with 500 and 800 μM MeJA significantly increased the methionine content (51–62%) of hydroponic Chinese chives. This suggested that the sulfur-containing amino acids are the primary reason for the flavor of Chinese chives. In addition, sulfur-containing amino acids have an antioxidant capacity comparable to that of tert-butylhydroquinone under deep-frying conditions, and can therefore, be added to foods as antioxidants to extend their shelf life (61).

Effect of Methyl Jasmonate Treatment on Primary Metabolite Profiles in Chinese Chive

An unsupervised principal component analysis (PCA) of primary metabolites (amino acids) using UPLC-MS/MS data confirmed a high degree of reproducibility between the three biological replicates and treatments. These results helped to determine that the MeJA-treated substrate-grown or hydroponic Chinese chives contained the most and least amino acid metabolites, respectively (**Figures 3A**, **4A**). The PC1 and PC2 axes for the substrate-grown Chinese chives explained 79.4% of the total variance (59.3 and 20.1%, respectively) (**Figure 3A**), and those for hydroponically grown Chinese chives explained 88.7% of the total variance (66 and 22.7%, respectively) (**Figure 4A**). The PCA score plot clearly distinguished the MeJA-treated and control groups in a systematic manner according to the amino acids in Chinese chives. This indicated that treatment with different concentrations of MeJA had a considerable effect on the amino acid content of Chinese chives.

To obtain more specific and meaningful information regarding the metabolites, we used PLS-DA to determine the metabolite changes in Chinese chives treated with different concentrations of MeJA. PLS-DA uses a general algorithm based on reduced dimensional discriminant analysis, which provides predictive and descriptive analyses for the selection of discriminant variables (62). This analysis has the advantage of not relying on specific distributions, resulting in more accurate predictive and descriptive models (63). We found that the PLS-DA models for substrate-grown and hydroponic Chinese chives fitted well ($R^2 = 0.97$ and 0.99, respectively) and were highly predictable ($Q^2 = 0.88$ and 0.96, respectively), thus, allowing us to predict the changes in amino acid metabolites. The PC1 and PC2 axes of the substrate-grown Chinese chives explained 77.2% of the total variance (57.1 and 20.1%, respectively) (**Figure 3B**), and those of the hydroponic Chinese chives explained 88.2% of the total variance (65.3 and 22.9%, respectively) (**Figure 4B**). In the PLS-DA score plot, the substrate-grown or hydroponic Chinese chives treated with different concentrations of MeJA were divided into two major groups according to the levels of 18 amino acids. Thus, different concentrations of MeJA and control treatments had significant effects on the amino acid content of the substrate-grown or hydroponic Chinese chives. Based on the proximity of data points, the SCK and SM500 groups of the substrate-grown Chinese chives showed similar amino acid metabolisms. The PLS-DA loading plots indicated that the greater the distance between the metabolite points and the origin, the greater the contribution of the metabolite to the total variation (64) (**Figures 3C**, **4C**). Among the substrate-grown Chinese chives, lysine and glutamine were positively loaded on PC2 and separated the 500 μM MeJA-treated group from other treatment groups. Glycine, valine, phenylalanine, threonine, leucine, tyrosine, and alanine were positively loaded on PC1 and separated the 800 μM MeJA-treated group from other treatment groups. Serine and glutamate showed negative loading on PC1 and PC2 and separated HCK and the 300 μM MeJA-treated group from other treatment groups (**Figures 3B,C**). Therefore, these 11 amino acid metabolites explained most of the variations between substrate-grown Chinese chives treated with different concentrations of MeJA. Among the hydroponic plants, glutamate and glycine were positively loaded on PC1, separating the 800 μM MeJA-treated group from the other treatment groups. Alanine, arginine, and tyrosine were positively loaded on PC2, separating the 300 μM MeJA-treated group from the other treatment groups. Lysine, glutamine, tryptophan, serine, phenylalanine, and threonine were loaded on PC1 and PC2, separating HCK from other treatment groups. Threonine, leucine, proline, asparagine, and methionine were positively loaded on PC1 and PC2, separating the 800 μM MeJA-treated group from the other treatment groups (**Figures 4B,C**). Therefore, these 16 amino acid metabolites explained most of the variations between the hydroponic Chinese chives treated with different concentrations of MeJA.

TABLE 4 | Effect of MeJA treatments on the amino acid contents of substrate-grown and hydroponic Chinese chive.

Amino acid (mg g^{-1})	Substrate				Hydroponics			
	SCK	SM300	SM500	SM800	HCK	HM300	HM500	HM800
Essential amino acids								
Lysine	73.66 ± 1.92a	59.53 ± 1.01b	53.31 ± 1.01c	51.17 ± 1.27c	62.50 ± 0.93b	61.36 ± 1.14b	68.42 ± 0.88a	64.12 ± 2.52ab
Threonine	1.44 ± 0.02a	1.20 ± 0.03b	0.96 ± 0.01d	1.09 ± 0.05c	1.41 ± 0.01ab	1.35 ± 0.02b	1.46 ± 0.01a	1.46 ± 0.05a
Phenylalanine (ArAAs)	1.51 ± 0.02a	1.24 ± 0.02b	1.02 ± 0.02d	1.13 ± 0.02c	1.45 ± 0.01ab	1.4 ± 0.02b	1.51 ± 0.01a	1.50 ± 0.05a
Tryptophan (ArAAs)	0.34 ± 0.01a	0.35 ± 0.00a	0.28 ± 0.00c	0.31 ± 0.00b	0.47 ± 0.01a	0.35 ± 0.01b	0.32 ± 0.00c	0.29 ± 0.01d
Leucine	1.76 ± 0.03a	1.65 ± 0.02a	1.32 ± 0.14b	1.52 ± 0.02ab	1.65 ± 0.02a	1.33 ± 0.09b	1.76 ± 0.01a	1.66 ± 0.04a
Isoleucine	1.84 ± 0.04a	1.76 ± 0.02ab	1.47 ± 0.02c	1.67 ± 0.06d	1.75 ± 0.02b	1.50 ± 0.04c	1.86 ± 0.01a	1.73 ± 0.05b
Valine	2.28 ± 0.04a	1.92 ± 0.03b	1.57 ± 0.08c	1.73 ± 0.08bc	2.12 ± 0.03b	1.70 ± 0.03c	2.40 ± 0.01a	2.24 ± 0.10ab
Methionine (SAAs)	1.76 ± 0.02ab	1.90 ± 0.04a	1.70 ± 0.02b	1.52 ± 0.08c	1.14 ± 0.02c	1.11 ± 0.03c	1.85 ± 0.01a	1.72 ± 0.04b
Non-essential amino acids								
Cysteine (SAAs)	0.01 ± 0.00b	0.04 ± 0.00a	0.03 ± 0.00a	0.04 ± 0.01a	0.01 ± 0.00a	0.01 ± 0.00a	0.02 ± 0.00a	0.01 ± 0.00a
Alanine	8.07 ± 0.19b	6.73 ± 0.16c	4.87 ± 0.05d	9.26 ± 0.06a	9.54 ± 0.08bc	12.43 ± 0.12a	10.17 ± 0.02b	8.93 ± 0.48c
Glycine	1.10 ± 0.05a	0.82 ± 0.03b	0.70 ± 0.04b	0.81 ± 0.06b	0.92 ± 0.02c	1.08 ± 0.02bc	1.13 ± 0.02b	1.46 ± 0.11a
Tyrosine (ArAAs)	0.60 ± 0.00a	0.45 ± 0.02b	0.31 ± 0.01c	0.41 ± 0.02b	0.78 ± 0.00a	0.79 ± 0.03a	0.54 ± 0.01b	0.58 ± 0.03b
Asparagine	1.51 ± 0.05a	1.55 ± 0.07a	1.11 ± 0.09b	1.39 ± 0.04a	1.36 ± 0.02bc	1.24 ± 0.03c	1.50 ± 0.05a	1.48 ± 0.05ab
Proline	6.62 ± 0.09a	6.41 ± 0.09a	5.70 ± 0.10b	5.59 ± 0.22b	5.94 ± 0.08b	5.66 ± 0.06b	6.93 ± 0.10a	6.73 ± 0.19a
Serine	2.96 ± 0.07a	2.29 ± 0.05b	1.70 ± 0.04c	1.33 ± 0.09d	2.37 ± 0.04a	2.23 ± 0.06a	2.39 ± 0.03a	2.47 ± 0.14a
Glutamate	26.02 ± 0.71a	22.58 ± 0.49b	17.90 ± 0.26c	15.95 ± 0.78d	18.72 ± 0.36c	19.57 ± 0.26c	25.05 ± 0.86b	28.06 ± 1.21a
Glutamine	10.62 ± 0.30a	8.40 ± 0.14b	7.58 ± 0.17c	7.10 ± 0.09c	8.92 ± 0.25ab	8.76 ± 0.13b	9.68 ± 0.14a	9.07 ± 0.41ab
Arginine	12.09 ± 0.20a	10.08 ± 0.09b	9.59 ± 0.14b	8.77 ± 0.36c	12.48 ± 0.25a	13.71 ± 0.55a	12.58 ± 0.76a	11.83 ± 0.54a
Total essential amino acids (TEAAs)	84.57 ± 2.08a	69.56 ± 1.15b	61.62 ± 1.12c	60.13 ± 1.46c	72.48 ± 0.95b	70.07 ± 1.17b	79.59 ± 0.88a	74.73 ± 2.85ab
Total non-essential amino acids (TNEAAs)	69.59 ± 1.62a	59.37 ± 1.05b	49.50 ± 0.6c	50.66 ± 1.29c	61.02 ± 0.92b	65.48 ± 0.71ab	69.97 ± 0.63a	70.62 ± 3.13a
Total aromatic amino acids (TArAAs)	2.44 ± 0.04a	2.04 ± 0.04b	1.61 ± 0.03d	1.85 ± 0.03c	2.71 ± 0.01a	2.54 ± 0.02b	2.38 ± 0.01c	2.37 ± 0.08c
Total sulfur amino acids (TSAAs)	1.76 ± 0.03ab	1.93 ± 0.04a	1.74 ± 0.02b	1.56 ± 0.09c	1.15 ± 0.02c	1.12 ± 0.03c	1.87 ± 0.01a	1.73 ± 0.04b
(EAAs: TAAs) * 100 (%)	54.86 ± 0.06b	53.95 ± 0.04c	55.44 ± 0.19a	54.28 ± 0.04c	54.29 ± 0.31a	51.69 ± 0.52b	53.21 ± 0.27a	51.42 ± 0.16b
Total amino acids (TAAs)	154.16 ± 3.69a	128.94 ± 2.2b	111.12 ± 1.7c	110.79 ± 2.74c	133.50 ± 1.68c	135.55 ± 1.31bc	149.56 ± 1.29a	145.34 ± 5.97ab

Data represent the mean ± standard error (n = 3). Different lowercase letters indicate statistical significance by Duncan's multiple range test ($p < 0.05$).

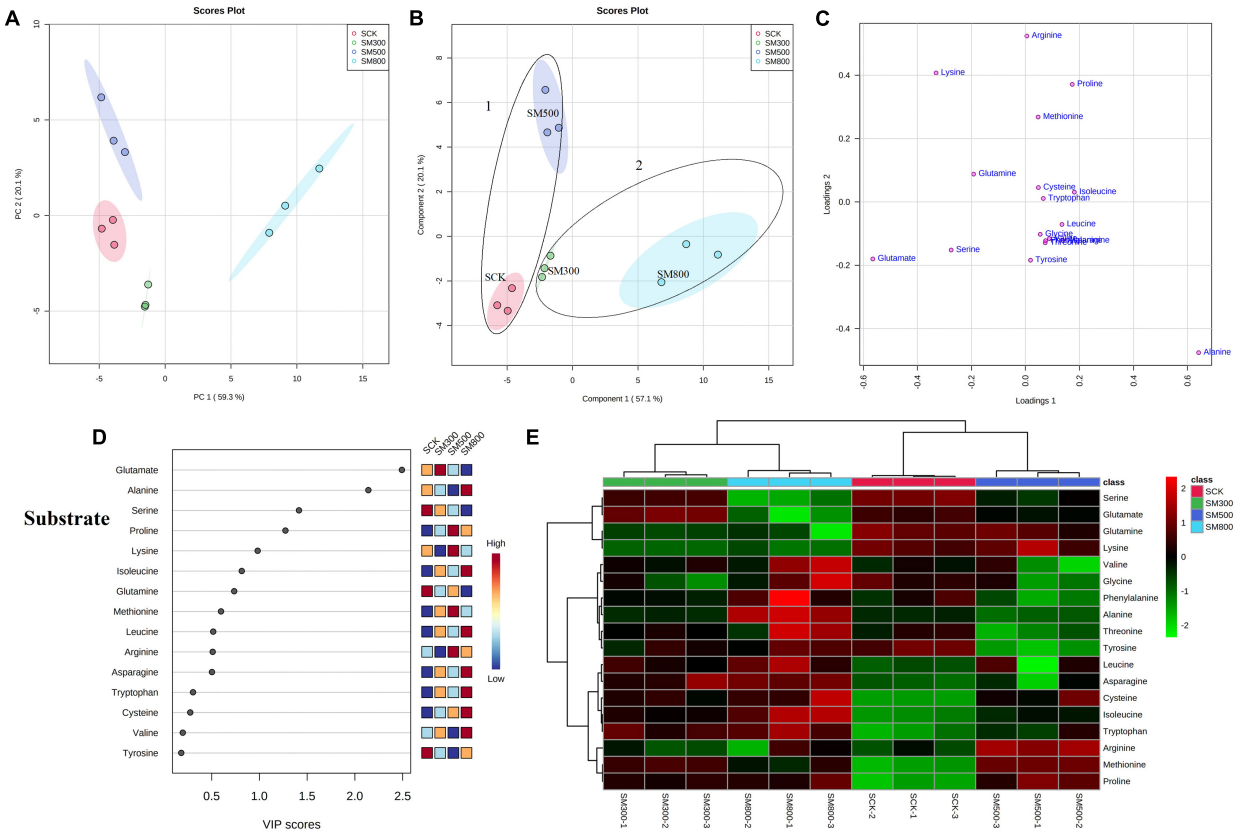

FIGURE 3 | Statistical analyses of bioactive metabolites in substrate-grown Chinese chive treated with methyl jasmonate (MeJA). **(A)** An unsupervised principal component analysis (PCA) score plot of amino acid metabolites. **(B)** A partial least squares discriminant analysis (PLS-DA) score plot of amino acid metabolites. **(C)** PLS-DA score plots loaded with different amino acid metabolites. **(D)** VIP scores of amino acid metabolites in PLS-DA. The scores from low to high determine the importance of variables. The colored boxes on the right show the relative concentration of each metabolite from low (blue) to high (red). **(E)** Heat map of amino acid concentrations. The colored areas correspond to the concentrations of different amino acids from low (green) to high (red) in groups treated with different concentrations of MeJA. Each row represents an amino acid, and each column represents a treatment.

To further elucidate the amino acid metabolites in Chinese chives treated with different concentrations of MeJA, we performed a hierarchical cluster analysis using the entire dataset of identified metabolites. The results yielded different clusters of samples with similar chemical composition, and we prepared a heat map based on metabolite concentrations in all samples. The heat map includes the data of the 18 amino acid metabolites identified in the substrate-grown or hydroponic Chinese chives (**Figures 3E**, **4E**). To some extent, the hierarchical cluster analysis confirmed the two main clusters observed in the PLS-DA score plots (**Figures 3B**, **4B**), which can be inferred from the branches at the top of the heat map (**Figures 3E**, **4E**). In addition, the heat map indicated the trends of amino acid metabolic composition in the substrate-grown or hydroponic Chinese chives treated with different concentrations of MeJA. The substrate-grown Chinese chives in the SCK group contained higher levels of serine and glutamine, the 300 μM MeJA-treated group contained higher levels of glutamate, the 500 μM MeJA-treated group contained higher levels of arginine; and the 800 μM MeJA-treated group contained higher levels of alanine (**Figure 3E**). The HCK treatment group of hydroponic Chinese chives contained high levels of threonine, phenylalanine, and tryptophan, the 300 μM MeJA-treated group contained high levels of alanine, the 500 μM MeJA-treated group contained high levels of cysteine and methionine, and the 800 μM MeJA-treated group contained high levels of glycine and glutamate (**Figure 4E**).

In the PLS-DA analysis, we used the VIP scores to measure the strength of influence and explanatory power of each amino acid metabolite on the categorical discrimination of the sample. Higher VIP scores indicate greater differences between treatments, and are useful for selecting biomarkers that differ between treatments (48). To provide the most meaningful interpretation of the results, we considered only the top metabolites with VIP scores > 1 (65) (**Figures 3D**, **4D**). The top four amino acid metabolites (VIP scores > 1) in the substrate-grown Chinese chives were (from highest to lowest) glutamate, alanine, serine, and proline, which had the highest relative concentrations in the SM300, SM800, SCK, and SM500 treatments, respectively. These patterns allowed us to distinguish between the treatments (**Figure 3D**). The top four amino acid metabolites (VIP scores > 1) in the hydroponic Chinese chives were glutamate, lysine, arginine,

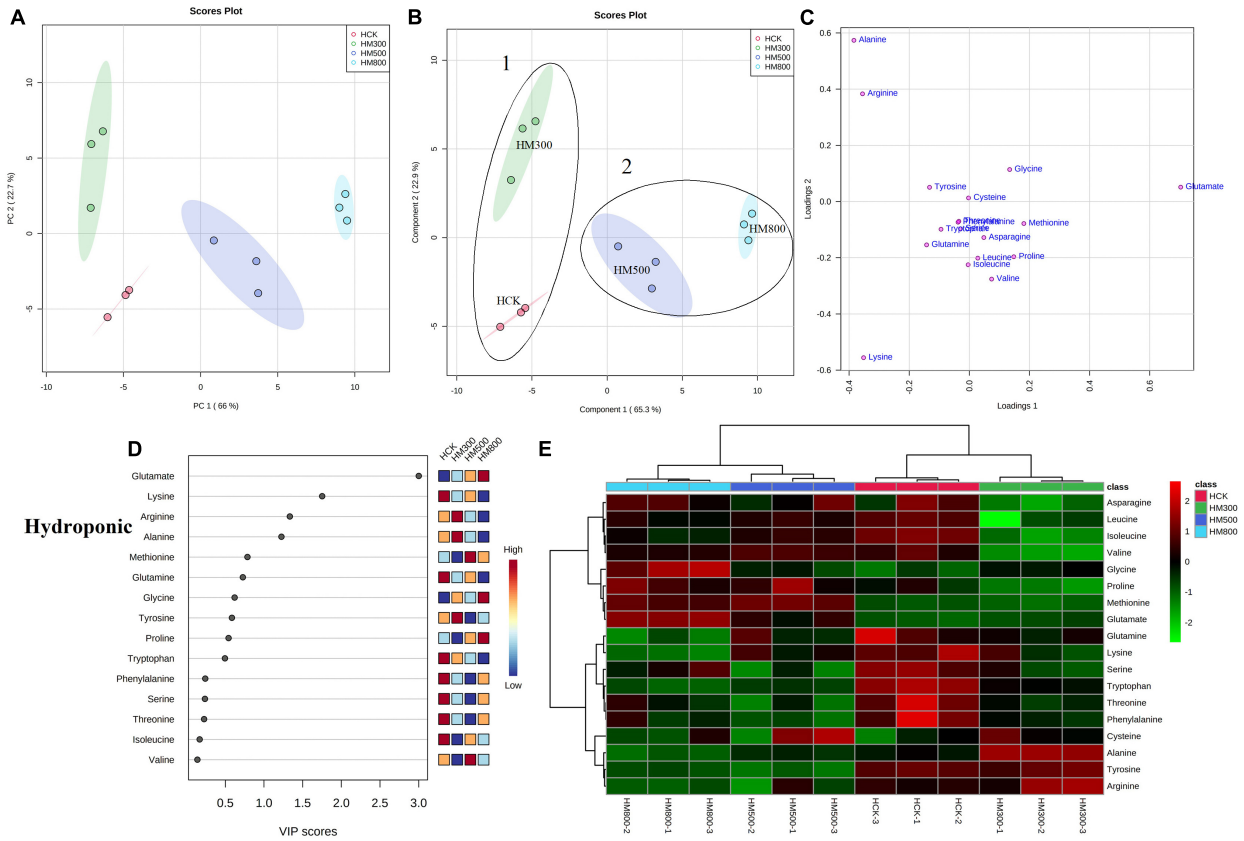

FIGURE 4 | Statistical analyses of bioactive metabolites in hydroponic Chinese chive treated with methyl jasmonate (MeJA). **(A)** An unsupervised principal component analysis (PCA) score plot of amino acid metabolites. **(B)** A partial least squares discriminant analysis (PLS-DA) score plot of amino acid metabolites. **(C)** PLS-DA score plots loaded with different amino acid metabolites. **(D)** VIP scores of amino acid metabolites in PLS-DA. The scores from low to high determine the importance of variables. The colored boxes on the right show the relative concentration of each metabolite from low (blue) to high (red). **(E)** Heat map of amino acid concentrations. The colored areas correspond to the concentrations of different amino acids from low (green) to high (red) in groups treated with different concentrations of MeJA. Each row represents an amino acid, and each column represents a treatment.

and alanine. Glutamate and lysine showed the highest relative concentrations in the HM800 and HCK treatments, respectively, whereas arginine and alanine showed the highest relative concentration in the HM300 treatment (**Figure 4D**). The higher VIP scores for these compounds suggested that they are important biomarkers for describing variations in the primary metabolites in Chinese chives.

Effect of Methyl Jasmonate Treatments on Phenolic Compounds (Secondary Metabolites) and Total Phenol Content in Chinese Chive

The levels of 15 phenolic compounds in the substrate-grown and hydroponic Chinese chives treated with MeJA are listed in **Table 5**. The MeJA treatment significantly increased (2.8–47%) the phenolic content of the substrate-grown Chinese chives compared with that of SCK, and the 500 μM MeJA treatment had the most significant effect. This was consistent with our previous study, where the application of 500 μM MeJA increased the total phenol contents in Chinese chives (66). The phenolic compounds of hydroponic Chinese chives treated with 500 and 800 μM MeJA were significantly higher than those of the HCK group. Phenolic compounds are known to benefit human health, and can be used as functional foods (67). Phenolics includes coumarins, phenolic acids, such as hydroxybenzoic acids (68) and hydroxycinnamic acids (69), flavonoids, such as flavonols (70), flavones (71), flavanols (72), flavanones (73), isoflavones, anthocyanins, chalcones and non-flavonoids, such as tannins, lignans, and stilbenes. Flavonoids, which are widely found in plants, have antioxidant, anticancer, antibacterial, and antimutagenic activities (74), and exhibit high levels of antioxidant activity as plant secondary metabolites (75). Among the substrate-grown Chinese chives, the total phenolic acid content was significantly lower in the MeJA-treated groups; however, the content of total flavonoids in the MeJA-treated groups was significantly higher than that in the control group. In the hydroponic plants, the total flavonoid and total phenolic acid contents of groups treated with 300 and 500 μM MeJA were significantly higher than those of the control group. This suggested that the effect of the MeJA treatment on the flavonoid and phenolic acid contents of Chinese chives depended

TABLE 5 | Effect of MeJA treatments on the phenolic component contents of substrate-grown and hydroponic Chinese chive.

Phenolic components (μ g g^{-1})	Substrate				Hydroponics			
	SCK	SM300	SM500	SM800	HCK	HM300	HM500	HM800
Phenolic acids								
Protocatechuic acid	90.39 ± 0.21d	102.07 ± 0.22b	120.44 ± 1.16a	95.78 ± 0.53c	91.18 ± 0.39c	94.44 ± 1.03c	109.03 ± 1.51b	459.32 ± 7.33a
P-hydroxybenzoic acid	79.83 ± 0.04c	89.45 ± 0.21a	81.25 ± 0.05b	80.93 ± 0.04b	81.20 ± 0.07a	81.02 ± 0.07a	79.76 ± 0.1b	79.85 ± 0.02b
Chlorogenic acid	97.03 ± 0.18c	100.72 ± 0.07b	101.38 ± 0.07a	100.85 ± 0.05b	96.08 ± 0.04c	95.95 ± 0.14c	97.97 ± 0.08b	100.05 ± 0.48a
Gallic acid	288.80 ± 0.17a	216.78 ± 1.33c	216.11 ± 0.86c	247.42 ± 1.13b	270.66 ± 11.08ab	216.30 ± 8.08c	264.88 ± 3.07b	293.54 ± 2.67a
4-Coumaric acid	83.71 ± 0.06a	82.39 ± 0.09b	83.85 ± 0.04a	82.14 ± 0.06c	83.73 ± 0.07c	103.64 ± 0.99a	94.18 ± 0.64b	101.86 ± 0.76a
Ferulic acid	86.71 ± 0.04d	87.43 ± 0.13b	87.07 ± 0.08c	90.30 ± 0.08a	207.99 ± 1.97a	191.33 ± 1.76c	174.73 ± 4.64b	183.08 ± 3.08bc
Benzoic acid	133.04 ± 0.71a	91.05 ± 0.08d	101.90 ± 0.13c	113.11 ± 0.52b	237.68 ± 2.73a	105.34 ± 4.97b	102.07 ± 0.65b	106.26 ± 0.77b
Cinnamic acid	93.61 ± 0.04c	95.00 ± 0.17b	93.79 ± 0.04c	96.33 ± 0.35a	95.09 ± 0.18a	94.23 ± 0.06b	93.54 ± 0.13c	94.71 ± 0.17a
Gentilic acid	103.00 ± 0.12b	99.68 ± 0.03d	108.68 ± 0.30a	101.68 ± 0.07c	107.55 ± 0.61c	111.42 ± 0.32a	109.70 ± 0.76b	101.43 ± 0.14d
Caffeic acid	89.07 ± 0.03b	89.88 ± 0.00a	87.77 ± 0.03d	88.35 ± 0.06c	88.43 ± 0.12b	93.00 ± 0.17a	88.80 ± 0.15b	93.12 ± 0.12a
Cynarin	88.28 ± 0.06b	88.86 ± 0.02a	88.32 ± 0.05b	88.75 ± 0.04a	96.68 ± 0.22c	107.00 ± 0.31a	95.77 ± 0.33c	103.49 ± 0.53b
Erucic acid	100.80 ± 0.75d	108.99 ± 0.65b	113.45 ± 0.43a	105.99 ± 0.35c	134.62 ± 2.10ab	133.10 ± 2.09b	114.08 ± 1.01c	140.00 ± 2.20a
Flavones								
Kaempferol	173.32 ± 0.44b	168.21 ± 0.15d	169.11 ± 0.13c	178.92 ± 0.19a	173.65 ± 0.96b	174.38 ± 1.20b	175.17 ± 0.66b	188.12 ± 3.13a
Rutin	255.78 ± 3.94d	614.30 ± 1.14b	1153.23 ± 3.02a	313.61 ± 1.33c	312.12 ± 2.71b	382.69 ± 10.33a	227.29 ± 3.31c	318.18 ± 2.69b
Quercetin	352.63 ± 0.81c	348.82 ± 0.54d	511.88 ± 0.94a	390.54 ± 0.57b	388.00 ± 2.33b	666.91 ± 37.86a	332.10 ± 2.12b	353.11 ± 7.23b
Total phenolic acids	1334.26 ± 1.96a	1252.29 ± 1.11d	1284.03 ± 0.22c	1291.62 ± 2.41b	1590.89 ± 7.90b	1426.78 ± 16.90c	1424.48 ± 4.5c	1856.71 ± 14.28a
Total flavones	781.73 ± 3.27d	1131.33 ± 0.47b	1834.22 ± 2.83a	883.07 ± 2.08c	873.76 ± 3.89b	1223.98 ± 28.93a	734.56 ± 3.66c	859.41 ± 12.72b
Total	2115.99 ± 1.45d	2383.62 ± 0.98b	3118.25 ± 2.96a	2174.69 ± 3.61c	2464.65 ± 11.46b	2650.76 ± 35.11a	2159.04 ± 2.07c	2716.12 ± 25.37a

Data represent the mean ± standard error (n = 3). Different lowercase letters indicate statistical significance by Duncan's multiple range test ($p < 0.05$).

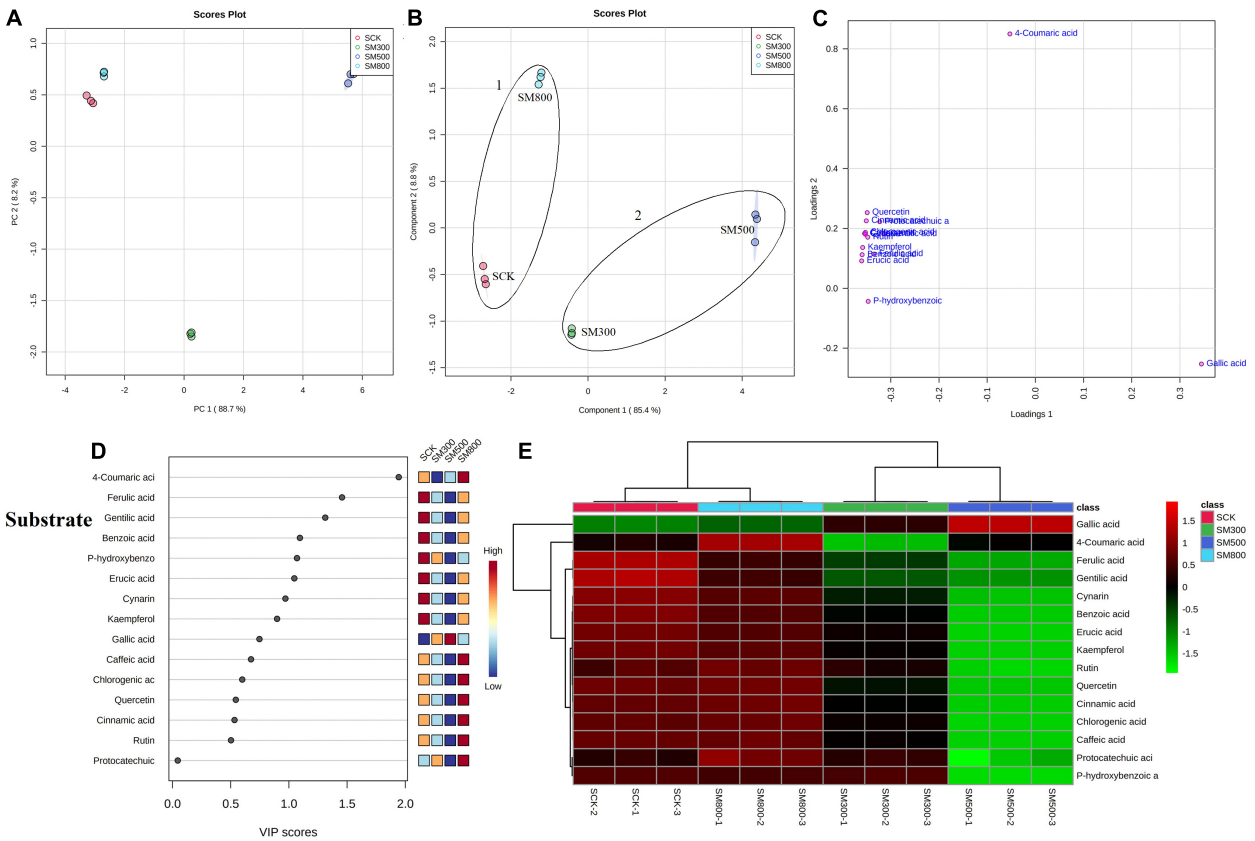

FIGURE 5 | Statistical analyses of bioactive metabolites in substrate-grown Chinese chive treated with methyl jasmonate (MeJA). **(A)** An unsupervised principal component analysis (PCA) score plot of phenolic metabolites. **(B)** A partial least squares discriminant analysis (PLS-DA) score plot of phenolic metabolites. **(C)** PLS-DA score plots loaded with different phenolic metabolites. **(D)** VIP scores of phenolic metabolites in PLS-DA. The scores from low to high determine the importance of variables. The colored boxes on the right show the relative concentration of each metabolite from low (blue) to high (red). **(E)** Heat map of phenolic concentrations. The colored areas correspond to the concentrations of different amino acids from low (green) to high (red) in groups treated with different concentrations of MeJA. Each row represents a phenolic, and each column represents a treatment.

mainly on the cultivation methods. Baek et al. reported similar results after applying MeJA treatment to pak choi (*Brassica rapa* L. ssp. *chinensis*) in soil and hydroponic systems (76). Chinese chives contain high levels of ferulic acid, which has a strong antioxidant capacity and can lower triglyceride levels (77). Protocatechuic acid and rutin have strong free radical scavenging abilities and inhibit lipid peroxidation (78, 79). In the present study, the MeJA treatment in substrate-grown Chinese chives significantly increased the levels of phenolic acids, such protocatechuic acid (6.0–33%), p-hydroxybenzoic acid (1.4–12%), chlorogenic acid (3.8–4.5%), ferulic acid (0.4–4.1%), and erucic acid (5.1–12.5%), and flavonoids, such as rutin (23–351%). In addition, the levels of cinnamic acid, gentilic acid, caffeic acid, cynarin, kaempferol, and quercetin also increased to different degrees. Among the hydroponic Chinese chives, treatment with MeJA (500 and 800 μM) significantly increased the levels of protocatechuic acid (20 and 404%, respectively), chlorogenic acid (2.0 and 4.1%, respectively), and 4-coumaric acid (12 and 22%, respectively) compared with those in the HCK treatment. Treatment with a low concentration of MeJA (300 μM) also significantly increased the levels of 4-coumaric acid, gentilic acid, caffeic acid, cynarin, rutin, and quercetin. These results indicated that the MeJA-treated substrate-grown Chinese chives had higher levels of most phenolic components compared to the hydroponic Chinese chives. In addition, the changes in the total phenol content of MeJA-treated substrate-grown and hydroponic Chinese chives showed similar trends to those of the total phenolic compounds detected by HPLC (**Supplementary Figure 6**).

Effect of the Methyl Jasmonate Treatment on Secondary Metabolite Profiles in Chinese Chive

The PCA analysis showed that the PC1 and PC2 axes for the substrate-grown Chinese chives explained 96.9% of the total variance (88.7 and 8.2%, respectively) (**Figure 5A**), and those for hydroponic plants explained 82.9% of the total variance (48.9 and 34%, respectively) (**Figure 6A**). The PCA score plot distinguished the MeJA-treated groups from controls systematically and clearly based on the phenolic compounds in Chinese chives. The PLS-DA score plot showed that the PC1 and PC2 axes for the

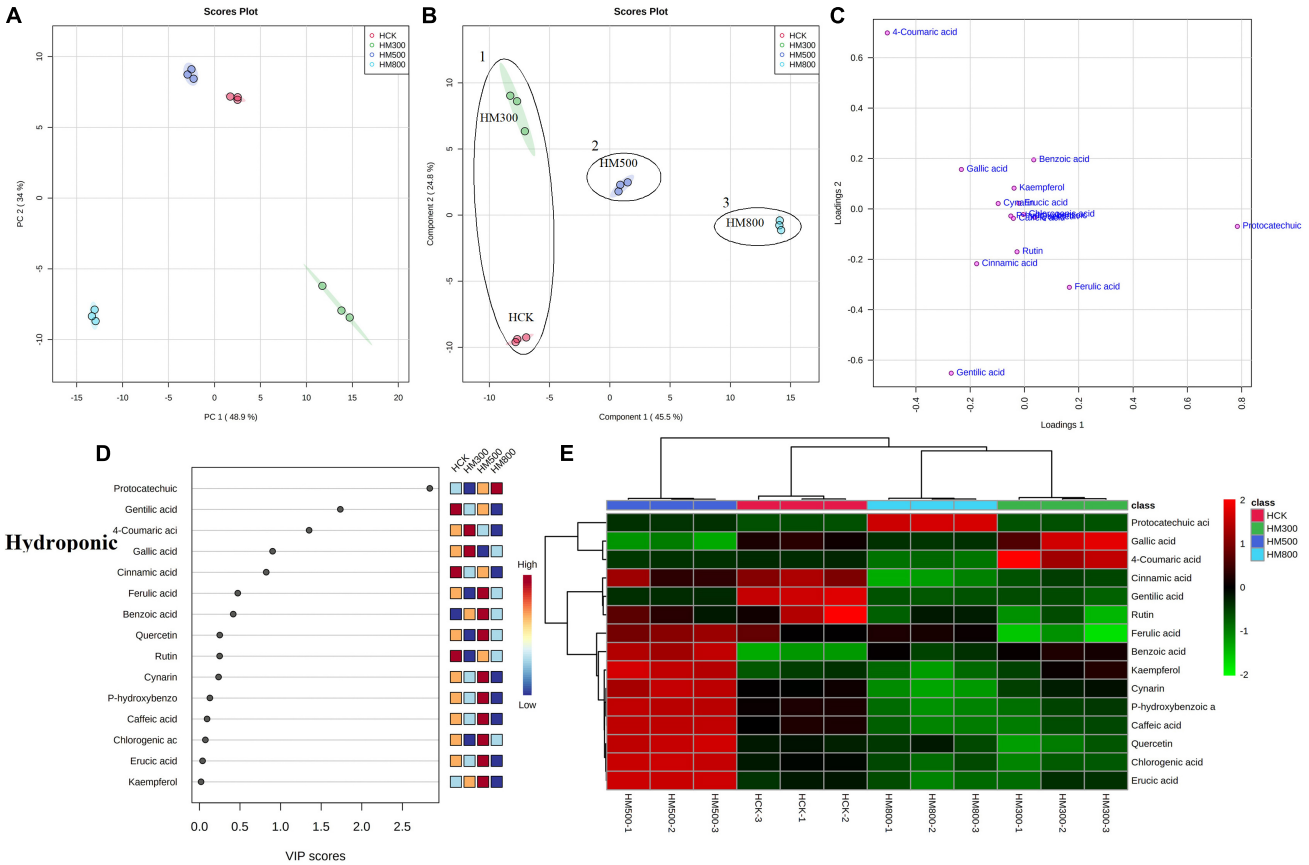

FIGURE 6 | Statistical analyses of bioactive metabolites in hydroponic Chinese chive treated with methyl jasmonate (MeJA). **(A)** An unsupervised principal component analysis (PCA) score plot of phenolic metabolites. **(B)** A partial least squares discriminant analysis (PLS-DA) score plot of phenolic metabolites. **(C)** PLS-DA score plots loaded with different phenolic metabolites. **(D)** VIP scores of phenolic metabolites in PLS-DA. The scores from low to high determine the importance of variables. The colored boxes on the right show the relative concentration of each metabolite from low (blue) to high (red). **(E)** Heat map of phenolic concentrations. The colored areas correspond to the concentrations of different amino acids from low (green) to high (red) in groups treated with different concentrations of MeJA. Each row represents a phenolic, and each column represents a treatment.

substrate-grown Chinese chives explained 94.2% of the total variance (85.4 and 8.8%, respectively) (**Figure 5B**), and those for hydroponic Chinese chives explained 70.3% of the total variance (45.5 and 24.8%, respectively) (**Figure 6B**). Based on the sample distribution in the PLS-DA plot, we hypothesized that there are two large groups in the substrate-grown Chinese chives and three large groups in the hydroponic Chinese chives (**Figures 5B**, **6B**). Based on the proximity of data points, the SCK and SM800 groups of the substrate-grown Chinese chives showed similar phenolic metabolism and were different from the SM300 and SM500 groups. Among the hydroponic Chinese chives, the HCK and HM300 groups had similar phenolic metabolism. Further, among the substrate-grown Chinese chives, the metabolite 4-coumaric acid was loaded positively on PC2, gallic acid was loaded positively on PC1, and p-hydroxybenzoic acid was loaded negatively on PC1 and PC2. Thus, these three phenolic metabolites separated the treatment groups of the substrate-grown Chinese chives (**Figures 5B,C**) and explained most of the variation in plants treated with different MeJA concentrations. For the hydroponic Chinese chives, 4-coumaric acid, gallic acid, kaempferol, cynarin, and erucic acid were negatively loaded on PC1; gentilic acid, cinnamic acid, rutin, caffeic acid, and p-hydroxybenzoic acid were negatively loaded on PC2; and benzoic acid, protocatechuic acid, and ferulic acid were positively loaded on PC1. Thus, these 13 phenolic metabolites separated the various treatment groups of the hydroponic Chinese chives (**Figures 6B,C**) and explained most of the variation between plants treated with different concentrations of MeJA. The hierarchical clustering heat map showed 15 phenolic compound metabolites identified in the substrate-grown or hydroponic cultures of Chinese chives (**Figures 5E**, **6E**). The SCK group contained higher levels of amino acids than other substrate-grown Chinese chives (**Figure 5E**), and the HM500 treatment group of hydroponic plants contained a higher level of amino acids than other hydroponics (**Figure 6E**). The VIP scores plot showed that in the substrate-grown Chinese chives, the top six phenolic metabolites (VIP scores > 1) were 4-coumaric acid, ferulic acid, gentilic acid, benzoic acid, p-hydroxybenzoic acid, and erucic acid (**Figure 5D**). In the hydroponic Chinese chives, the top three phenolic metabolites (VIP scores > 1)

TABLE 6 | Effect of MeJA treatments on the carotenoid contents of substrate-grown and hydroponic Chinese chive.

Carotenoids (μ g g^{-1})	Substrate				Hydroponics			
	SCK	SM300	SM500	SM800	HCK	HM300	HM500	HM800
Violaxanthin	29.71 ± 0.03b	30.61 ± 0.02a	30.67 ± 0.06a	30.65 ± 0.04a	30.24 ± 0.16bc	30.68 ± 0.27ab	31.12 ± 0.06a	29.57 ± 0.33c
Monoepoxy zeaxanthin	77.43 ± 0.48a	68.89 ± 1.52b	65.20 ± 0.18c	78.98 ± 0.6a	63.58 ± 0.55b	66.01 ± 0.66b	57.18 ± 0.25c	70.36 ± 1.65a
Lycopene	223.74 ± 0.29a	90.74 ± 2.94d	204.99 ± 5.03b	166.48 ± 0.94c	121.46 ± 2.19a	117.13 ± 4.84a	59.56 ± 2.98c	94.77 ± 0.93b
β-carotene	2027.16 ± 45.45b	1633.89 ± 37.22c	1648.63 ± 9.59c	2992.70 ± 52.49a	1466.89 ± 8.41a	1369.18 ± 30.71b	1126.55 ± 23.06c	1532.63 ± 32.15a
Zeaxanthin	16.79 ± 0.05c	25.85 ± 0.36b	46.39 ± 4.26a	17.63 ± 0.22c	7.45 ± 0.47c	14.62 ± 0.26a	4.60 ± 0.18d	9.56 ± 0.49b
α-carotene	6.03 ± 0.61d	25.41 ± 1.76c	65.59 ± 3.01a	34.72 ± 0.45b	20.06 ± 0.09b	29.23 ± 1.69a	17.34 ± 0.84b	17.01 ± 0.33b
Lutein	159.21 ± 3.85b	108.88 ± 3.60c	173.89 ± 3.69a	149.50 ± 2.85b	96.54 ± 1.40b	134.22 ± 7.04a	77.29 ± 0.26c	94.74 ± 1.4b
Total	2540.07 ± 48.92b	1984.27 ± 37.26d	2235.35 ± 11.62c	3470.66 ± 55.29a	1806.23 ± 12.07a	1761.07 ± 37.68a	1373.64 ± 24.79b	1848.64 ± 31.87a

Data represent the mean ± standard error (n = 3). Different lowercase letters indicate statistical significance by Duncan's multiple range test ($p < 0.05$).

were protocatechuic acid, gentilic acid, and 4-coumaric acid (**Figure 6D**). The higher VIP scores for these compounds suggested that they were important biomarkers for describing variations in the phenolic compounds of the primary metabolites in Chinese chives.

Effect of the Methyl Jasmonate Treatments on Carotenoids (Secondary Metabolites) in Chinese Chive

Carotenoid is a lipophilic antioxidant and can detoxify various forms of reactive oxygen species (ROS) (80). The levels of different carotenoids in the substrate-grown and hydroponic Chinese chives treated with MeJA are listed in **Table 6**. β-carotene showed the highest levels (2027.16 and 1466.89 μg g^{-1}) in the substrate-grown and hydroponic Chinese chives, followed by lycopene (223.74 and 121.46 μg g^{-1}, respectively), lutein (159.21 and 196.54 μg g^{-1}, respectively) and monocyclic zeaxanthin (77.43 and 63.58 μg g^{-1}, respectively). Carotenoids include, β-carotene, α-carotene (81), and xanthophylls, such as zeaxanthin, neoxanthin, violaxanthin, lutein (82), and lycopene, which have antioxidants capacity (83, 84). The carotenoids in Chinese chives mainly included β-carotene. Lutein enhances the immune function and improves the antioxidant capacity of the body, protects vision, and reduces the severity of age-related macular degeneration (64, 85). The levels of most carotenoids, such as lutein, violaxanthin, zeaxanthin, and α-carotene, were significantly higher in the substrate-grown Chinese chives treated with MeJA (500 μM) than in the SCK group. Treatment with 800 μM MeJA significantly increased the total carotenoid content (37%), mainly because this concentration significantly increased the most abundant β-carotene in Chinese chives (48%). These results were similar to those of Pérez et al. (86). Previous studies have shown that β-carotene content is genotypically determined (64). However, we found that the β-carotene content in the substrate-grown Chinese chives was significantly higher than that in the hydroponic Chinese chives with or without MeJA treatment, suggesting that cultivation method is also a key factor in determining the β-carotene content in Chinese chives. A study on tomato fruits reported that JA treatment had a dose-dependent effect on carotenoid accumulation; that is, when the concentration of JA was > 0.5 μM, carotenoid biosynthesis was inhibited in tomato (31). In our study, hydroponic Chinese chives treated with MeJA (300 μM) showed significantly higher levels of zeaxanthin, α-carotene, and lutein compared to those in the HCK group. Treatment with 500 μM MeJA significantly increased the levels of violet xanthin. However, the carotenoid content in the hydroponic Chinese chives treated with MeJA (500 μM) showed an overall decreasing trend. In addition, the carotenoid content in the substrate-grown Chinese chives was higher than that in the hydroponic Chinese chives with or without MeJA treatment. This may have been because in leafy vegetables, carotenoid levels depend on various factors, such as cultivar species, cultivation method, maturity, and environmental conditions (87). Future studies should investigate the effect of different concentrations of MeJA on carotenoid biosynthesis to improve

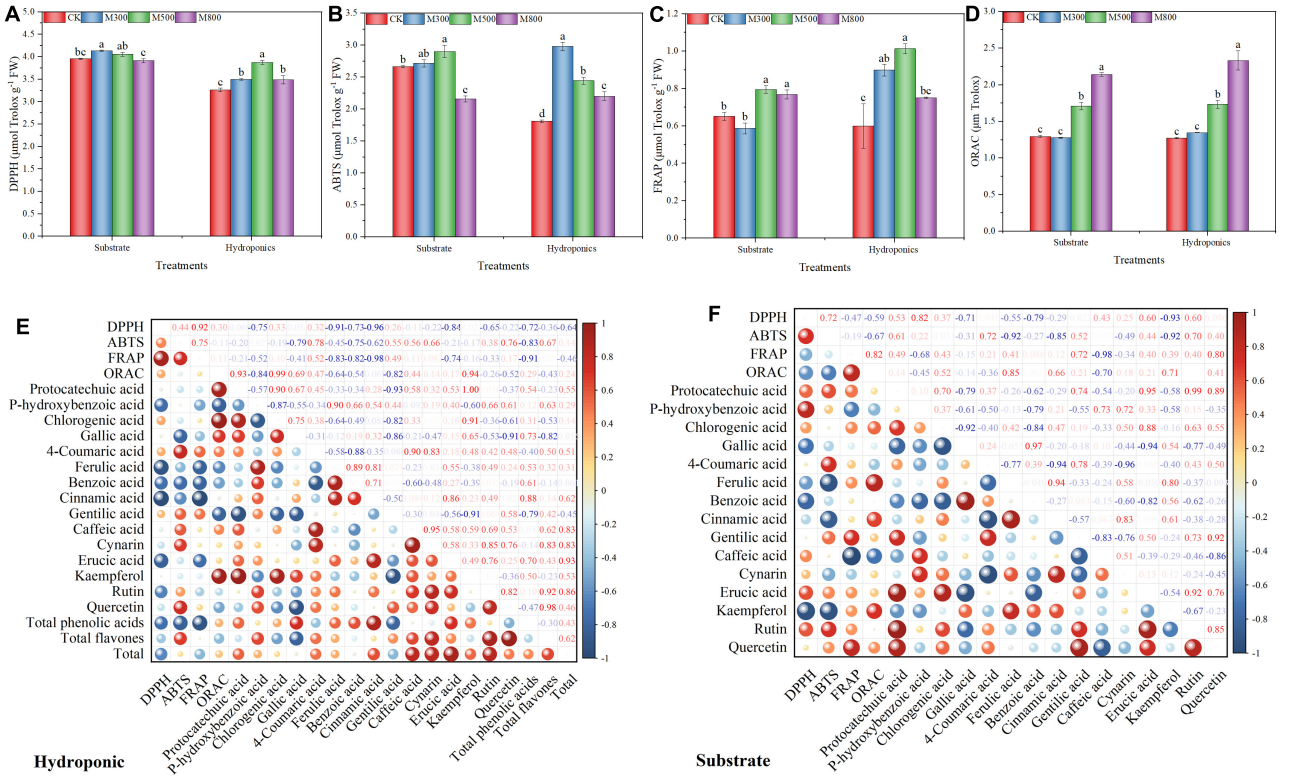

FIGURE 7 | The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity **(A)**, Trolox-equivalent antioxidant capacity (ABTS) **(B)**, ferric-reducing antioxidant power (FRAP) **(C)**, and oxygen radical absorbance capacity (ORAC) **(D)** of Chinese chives grown in substrate or hydroponics as affected by methyl jasmonate (MeJA) treatments. Data represent the mean ± standard error ($n = 3$). Different lowercase letters indicate statistical significance, by Duncan's multiple range tests ($p < 0.05$). The heat map shows the Pearson correlation analysis between the observed parameters of antioxidant capacity and the observed parameters of phenolic compounds in MeJA treated hydroponic **(E)** or substrate **(F)** Chinese chive. The values in the heat map are Pearson's correlation coefficient. The size and color depth of the colored balls correspond to the strength of the correlation, ranging from weak (small balls, blue) to strong (large balls, red).

our understanding on the accumulation of carotenoids in hydroponic Chinese chives.

Effect of the Methyl Jasmonate Treatments on the Antioxidant Capacities of Chinese Chive

The antioxidant activities in the substrate-grown and hydroponic Chinese chives treated with MeJA are listed in **Figures 7A–D**. The ABTS (**Figure 7B**), FRAP (**Figure 7C**), and ORAC (**Figure 7D**) assays showed that the antioxidant activities of the substrate-grown and hydroponic Chinese chives treated with MeJA (500 μM) were significantly higher than those in the SCK and HCK control groups, respectively. Exogenous MeJA can increase the antioxidant activity of plants (88), which is consistent with our findings. The DPPH activities of hydroponic Chinese chives treated with MeJA and of substrate-grown Chinese chives treated with 300 μM MeJA were significantly higher than that of the HCK group (**Figure 7A**). Both phenolics and flavonoids are strongly associated with antioxidant activity (DPPH and ABTS) in *Amaranthus hypochondriacus* (89), *A. tricolor* (84), *A. blitum* (90), weedy species (91), stem amaranth (92), green morph amaranth (93), and red morph amaranth (94), which are corroborative to the present findings. Phenolic compounds are usually positively associated with the antioxidant activity of plant foods (95). In addition, polyphenols have an ideal chemical structure for scavenging free radicals, and higher levels of polyphenols in plants are associated with stronger free radical scavenging ability (96, 97). The correlation heat map further confirmed the relationship between antioxidant activity and phenolic content of the substrate-grown (**Figure 7F**) and hydroponic (**Figure 7E**) Chinese chives. The correlation coefficients indicated the strength of the correlation between the two, and we considered only strong correlations by setting the threshold value to 0.5. In the substrate-grown Chinese chives, we observed strong positive correlations between DPPH activity and the levels of rutin, eruic acid, and p-hydroxybenzoic acid; ABTS and the levels of rutin, protocatechuic acid, and 4-coumaric acid; FRAP and the levels of quercetin and gentilic acid; and ORAC and the levels of kaempferol, cinnamic acid, chlorogenic acid, and ferulic acid (**Figure 7F**). In the hydroponic Chinese chives, we found positive correlations between ABTS activity and the levels of quercetin, cynarin, caffeic acid, gentilic acid, and 4-coumaric acid; FRAP and the level of 4-coumaric acid; and ORAC and the levels of kaempferol, gallic acid, and chloro-genic acid (**Figure 7E**). This further indicated that an

increase in the level of phenolic compounds might be associated with the improved antioxidant capacity of the substrate-grown and hydroponic Chinese chives following the MeJA treatment. Following the MeJA treatment, the ORAC of the substrate-grown and hydroponic Chinese chives increased in a dose-dependent manner. This was similar to the trend of changes observed in the levels of phenolic compounds after the MeJA treatment. In addition to phenolic compounds, sulfur-containing compounds (66), vitamin C (98), chlorophylls (99), carotenoids (100), and polysaccharides (101) also showed strong antioxidant activity.

CONCLUSION

Our results showed that the MeJA-treated Chinese chives showed significant differences in primary and secondary metabolites depending on the cultivation method. After treating the Chinese chives with MeJA, we distinguished between substrate-grown and hydroponic Chinese chives using amino acids and phenolic compounds. The MeJA treatment significantly increased the phenolic content of the substrate-grown Chinese chives and decreased the organic acids content of the hydroponic Chinese chives. Treatment with MeJA (500 μM) significantly increased the total sugar and amino acid (essential and non-essential amino acids and sulfur-containing amino acids) contents of hydroponically grown Chinese chives. However, the total sugar and amino acid contents of the substrate-grown Chinese chives showed trends contrasting to those of hydroponic Chinese chives. In addition, treatment with 500 μM MeJA significantly increased the antioxidant activity of both substrate-grown and hydroponic Chinese chives. The nutritional quality and bioactive substances of Chinese chives were comprehensively analyzed using metabolomics. The results of metabolites under different cultivation methods and MeJA treatments can contribute to increasing the nutritional quality and medicinal value of substrate-grown and hydroponic Chinese chives, and to some extent in promoting the flavor quality of Chinese chives, and promoting Chinese chives as a value-added horticultural product.

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/s.

AUTHOR CONTRIBUTIONS

CW, JZ, JX, and JY conceived and designed the experiments. CW, JZ, and YG analyzed the data. CW wrote the manuscript. CW, JZ, JLi, TN, and JLv involved in the related discussion. JX, JZ, and BP improved the quality of the manuscript. All authors have read and agreed to the published version of 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/fnut.2022.859035/full#supplementary-material

REFERENCES

1. Nicastro HL, Ross SA, Milner JA. Garlic and onions: their cancer prevention properties. *Cancer Prev Res.* (2015) 8:181–9. doi: 10.1158/1940-6207.CAPR-14-0172
2. Zeng Y, Li Y, Yang J, Pu X, Du J, Yang X, et al. Therapeutic role of functional components in alliums for preventive chronic disease in human being. *Evid Based Complement Alternat Med.* (2017) 2017:9402849. doi: 10.1155/2017/9402849
3. Lanciotti L. *The Book of Odes. Chinese Text, Transcription and Translation*. New York, NY: JSTOR (1950).
4. Poojary MM, Putnik P, Kovačević DB, Barba FJ, Lorenzo JM, Dias DA, et al. Stability and extraction of bioactive sulfur compounds from *Allium genus* processed by traditional and innovative technologies. *J Food Compost Anal.* (2017) 61:28–39. doi: 10.1016/j.jfca.2017.04.007
5. Hu G, Lu Y, Wei D. Chemical characterization of Chinese chive seed (*Allium tuberosum* Rottl.). *Food Chem.* (2006) 99:693–7. doi: 10.1016/j.foodchem.2005.08.045
6. Zhang W-N, Zhang H-L, Lu C-Q, Luo J-P, Zha X-Q. A new kinetic model of ultrasound-assisted extraction of polysaccharides from Chinese chive. *Food Chem.* (2016) 212:274–81. doi: 10.1016/j.foodchem.2016.05.144
7. Hong J, Chen TT, Hu P, Yang J, Wang SY. Purification and characterization of an antioxidant peptide (GSQ) from Chinese leek (*Allium tuberosum* Rottler) seeds. *J Funct Foods.* (2014) 10:144–53. doi: 10.1016/j.jff.2014.05.014
8. Hur HJ, Lee AS. Protective effect of *Allium tuberosum* extract on vascular inflammation in tumor necrosis factor-α-induced human vascular endothelial cells. *J Cancer Prev.* (2017) 22:228. doi: 10.15430/JCP.2017.22.4.228
9. Liu N, Tong J, Hu M, Ji Y, Wu Z. Transcriptome landscapes of multiple tissues highlight the genes involved in the flavor metabolic pathway in Chinese chive (*Allium tuberosum*). *Genomics.* (2021) 113:2145–57. doi: 10.1016/j.ygeno.2021.05.005
10. Sarker U, Islam MT, Rabbani MG, Oba S. Genotypic diversity in vegetable amaranth for antioxidant, nutrient and agronomic traits. *Indian J Genet Pl Breed.* (2017) 77:173–6. doi: 10.5958/0975-6906.2017.00025.6
11. Sarker U, Oba S. Response of nutrients, minerals, antioxidant leaf pigments, vitamins, polyphenol, flavonoid and antioxidant activity in selected vegetable amaranth under four soil water content. *Food Chem.* (2018) 252:72–83. doi: 10.1016/j.foodchem.2018.01.097
12. Sarker U, Islam T, Rabbani G, Oba S. Genotype variability in composition of antioxidant vitamins and minerals in vegetable amaranth. *Genetika.* (2015) 47:85–96. doi: 10.2298/GENSR1803995C
13. Chakrabarty T, Sarker U, Hasan M, Rahman M. Variability in mineral compositions, yield and yield contributing traits of stem

amaranth (*Amaranthus lividus*). *Genetika.* (2018) 50:995–1010. doi: 10.2298/GENSR1501085S
14. Sarker U, Islam MT, Rabbani MG, Oba S. Variability in total antioxidant capacity, antioxidant leaf pigments and foliage yield of vegetable amaranth. *J Integr Agric.* (2018) 17:1145–53. doi: 10.1016/S2095-3119(17)61778-7
15. Sarker U, Islam MT, Rabbani MG, Oba S. Phenotypic divergence in vegetable amaranth for total antioxidant capacity, antioxidant profile, dietary fiber, nutritional and agronomic traits. *Acta Agric Scand.* (2018) 68:67–76. doi: 10.1080/09064710.2017.1367029
16. Sarker U, Oba S. Salinity stress enhances color parameters, bioactive leaf pigments, vitamins, polyphenols, flavonoids and antioxidant activity in selected *Amaranthus leafy* vegetables. *J Sci Food Agric.* (2019) 99:2275–84. doi: 10.1002/jsfa.9423
17. Sarker U, Islam MT, Rabbani MG, Oba S. Genotypic variability for nutrient, antioxidant, yield and yield contributing traits in vegetable amaranth. *J Food Agri Environ.* (2014) 12:168–74. doi: 10.1234/4.2014.5378
18. Sarker U, Islam T, Rabbani G, Oba S. Antioxidant leaf pigments and variability in vegetable amaranth. *Genetika.* (2018) 50:209–20. doi: 10.2298/GENSR1801209S
19. Sarker U, Islam MS, Rabbani MG, Oba S. Variability, heritability and genetic association in vegetable amaranth (*Amaranthus tricolor* L.). *Spanish J Agric Res.* (2015) 13:17. doi: 10.5424/sjar/2015132-6843
20. Keleş D, Özgen Ş, Saraçoğlu O, Ata A, Özgen M. Antioxidant potential of Turkish pepper (*Capsicum annuum* L.) genotypes at two different maturity stages. *Turk J Agric For.* (2016) 40:542–51. doi: 10.3906/tar-1601-24
21. Pauwels L, Inzé D, Goossens A. Jasmonate-inducible gene: what does it mean? *Trends Plant Sci.* (2009) 14:87–91. doi: 10.1016/j.tplants.2008.11.005
22. Jang G, Shim JS, Jung C, Song JT, Lee HY, Chung PJ, et al. Volatile methyl jasmonate is a transmissible form of jasmonate and its biosynthesis is involved in systemic jasmonate response in wounding. *Plant Biotechnol Rep.* (2014) 8:409–19. doi: 10.1007/s11816-014-0331-6
23. Bradbury S. Methyl jasmonate; exemption from the requirement of a tolerance. federal register. a rule by the environmental protection agency (EPA). *Fed Regist.* (2013) 78:13–9.
24. Baenas N, García-Viguera C, Moreno DA. Elicitation: a tool for enriching the bioactive composition of foods. *Molecules.* (2014) 19:13541–63. doi: 10.3390/molecules190913541
25. Koo AJ, Gao X, Daniel Jones A, Howe GA. A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. *Plant J.* (2009) 59:974–86. doi: 10.1111/j.1365-313x.2009.03924.x
26. Kim HS. *Functional Studies of Lignin Biosynthesis Genes and Putative Flowering Gene in Miscanthus x giganteus and Studies on Indolyl Glucosinolate Biosynthesis and Translocation in Brassica oleracea*. Champaign, IL: University of Illinois at Urbana-Champaign (2010).
27. Baenas N, García-Viguera C, Moreno DA. Biotic elicitors effectively increase the glucosinolates content in brassicaceae sprouts. *J Agric Food Chem.* (2014) 62:1881–9. doi: 10.1021/jf404876z
28. Cheong J-J, Do Choi Y. Methyl jasmonate as a vital substance in plants. *TRENDS Genetics.* (2003) 19:409–13. doi: 10.1016/S0168-9525(03)00138-0
29. Sun G, Yang Y, Xie F, Wen J-F, Wu J, Wilson IW, et al. Deep sequencing reveals transcriptome re-programming of *Taxus*× *media* cells to the elicitation with methyl jasmonate. *PLoS One.* (2013) 8:e62865. doi: 10.1371/journal.pone.0062865
30. Fan X, Mattheis JP, Fellman JK. A role for jasmonates in climacteric fruit ripening. *Planta.* (1998) 204:444–9. doi: 10.1007/s004250050278
31. Liu L, Wei J, Zhang M, Zhang L, Li C, Wang Q. Ethylene independent induction of lycopene biosynthesis in tomato fruits by jasmonates. *J Exp Bot.* (2012) 63:5751–61. doi: 10.1093/jxb/ers224
32. Shafiq M, Singh Z, Khan AS. Time of methyl jasmonate application influences the development of 'Cripps Pink'apple fruit colour. *J Sci Food Agric.* (2013) 93:611–8. doi: 10.1002/jsfa.5851
33. Weng C-J, Yen G-C. Chemopreventive effects of dietary phytochemicals against cancer invasion and metastasis: phenolic acids, monophenol, polyphenol, and their derivatives. *Cancer Treat Rev.* (2012) 38:76–87. doi: 10.1016/j.ctrv.2011.03.001
34. Sayyari M, Babalar M, Kalantari S, Martinez-Romero D, Guillen F, Serrano M, et al. Vapour treatments with methyl salicylate or methyl jasmonate alleviated chilling injury and enhanced antioxidant potential during postharvest storage of pomegranates. *Food Chem.* (2011) 124:964–70. doi: 10.1016/j.foodchem.2010.07.036
35. Wang SY, Bowman L, Ding M. Methyl jasmonate enhances antioxidant activity and flavonoid content in blackberries (*Rubus* sp.) and promotes antiproliferation of human cancer cells. *Food Chem.* (2008) 107:1261–9. doi: 10.1016/j.foodchem.2007.09.065
36. Palermo M, Paradiso R, De Pascale S, Fogliano V. Hydroponic cultivation improves the nutritional quality of soybean and its products. *J Agric Food Chem.* (2012) 60:250–5. doi: 10.1021/jf203275m
37. Miyagi A, Uchimiya H, Kawai-Yamada M. Synergistic effects of light quality, carbon dioxide and nutrients on metabolite compositions of head lettuce under artificial growth conditions mimicking a plant factory. *Food Chem.* (2017) 218:561–8. doi: 10.1016/j.foodchem.2016.09.102
38. Wu ZH, Maruo T, Shinohara Y. Effect of total nitrogen concentration of nutrient solution in DFT system on the initial growth and nutrient uptake of Chinese chive (*Allium tuberosum* Rottler ex Spreng.). *J Jpn Soc Hortic Sci.* (2008) 77:173–9. doi: 10.2503/jjshs1.77.173
39. Wei X, Wei Y, Liu L, Wang X, Yang Y, Sun B, et al. Determination of sugar and acid components and contents in fruits of 4 blueberry varieties by high performance liquid chromatography. *Chin Fruit Trees.* (2013) 3:64–7. doi: 10.16626/j.cnki.issn1000-8047.2013.03.029
40. Coelho EM, da Silva Padilha CV, Miskinis GA, de Sá AGB, Pereira GE, de Azevêdo LC, et al. Simultaneous analysis of sugars and organic acids in wine and grape juices by HPLC: method validation and characterization of products from northeast Brazil. *J Food Compost Anal.* (2018) 66:160–7. doi: 10.1016/j.jfca.2017.12.017
41. Kennedy A, Bivens A. Method for analyzing underivatized amino acids using liquid mass spectrometry system. *Agilent Technol Appl Note.* (2017) 12:1–8.
42. Liu F. *Optimization of Detection Methods for Polyphenols in Tomato Fruit and Comprehensive Evaluation of Quality*. Lanzhou: Gansu Agricultural University (2021).
43. Hand MJ, Taffouo VD, Nouck AE, Nyemene K, Brice T, Meguekam TL, et al. Effects of salt stress on plant growth, nutrient partitioning, chlorophyll content, leaf relative water content, accumulation of osmolytes and antioxidant compounds in pepper (*Capsicum annuum* L.) cultivars. *Not Bot Horti Agrobot Cluj Napoca.* (2017) 45:481–90. doi: 10.15835/nbha45210928
44. Li J, Xie J, Yu J, Lv J, Zhang J, Wang X, et al. Reversed-phase high-performance liquid chromatography for the quantification and optimization for extracting 10 kinds of carotenoids in pepper (*Capsicum annuum* L.) leaves. *J Agric Food Chem.* (2017) 65:8475–88. doi: 10.1021/acs.jafc.7b02440
45. Tytgat TOG, Verhoeven KJF, Jansen JJ, Raaijmakers CE, Bakx-Schotman T, McIntyre LM, et al. Plants know where it hurts: root and shoot jasmonic acid induction elicit differential responses in *Brassica olracea*. *PLoS One.* (2013) 8:14. doi: 10.1371/journal.pone.0065502
46. Liang Y-S, Choi YH, Kim HK, Linthorst HJM, Verpoorte R. Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by 2-dimensional NMR spectroscopy. *Phytochemistry.* (2006) 67:2503–11. doi: 10.1016/j.phytochem.2006.08.018
47. Wang D. Development and application progress of citric acid (sodium) chemical intermediates: technology. *Industry Ed.* (2004) 1:7.
48. Kim MJ, Chiu YC, Kim NK, Park HM, Lee CH, Juvik JA, et al. Cultivar-specific changes in primary and secondary metabolites in pak choi (*Brassica Rapa*, Chinensis Group) by methyl jasmonate. *Int J Mol Sci.* (2017) 18:17. doi: 10.3390/ijms18051004
49. Wang R. Research progress on production and application of L-malic acid. *Chem Times.* (2002) 5:1–7. doi: CNKI:SUN:HGJS.0.2002-05-000
50. Zhang Y, Lin X, Zhang Y, Zheng SJ, Du S. Effects of nitrogen levels and nitrate/ammonium ratios on oxalate concentrations of different forms in edible parts of spinach. *J Plant Nutr.* (2005) 28:2011–25. doi: 10.1080/01904160500311086
51. Bohn T, Davidsson L, Walczyk T, Hurrell RF. Fractional magnesium absorption is significantly lower in human subjects from a meal served with an oxalate-rich vegetable, spinach, as compared with a meal served with kale, a vegetable with a low oxalate content. *Br J Nutr.* (2004) 91:601–6. doi: 10.1079/BJN20031081
52. Jiang J. Eliminating oxalate from vegetables is beneficial for calcium absorption. *Fam Med.* (2009) 5:1.

53. Wu G. Functional amino acids in growth, reproduction, and health. *Adv Nutr.* (2010) 1:31–7. doi: 10.3945/an.110.1008
54. Prasanna V, Prabha T, Tharanathan R. Fruit ripening phenomena–an overview. *Crit Rev Food Sci Nutr.* (2007) 47:1–19. doi: 10.1080/10408390600976841
55. Wang X, Zeng L, Liao Y, Zhou Y, Xu X, Dong F, et al. An alternative pathway for the formation of aromatic aroma compounds derived from L-phenylalanine via phenylpyruvic acid in tea (*Camellia sinensis* (L.) O. Kuntze) leaves. *Food Chem.* (2019) 270:17–24. doi: 10.1016/j.foodchem.2018.07.056
56. Rivero Meza SL, de Castro Tobaruela E, Benedetti Pascoal G, Louro Massaretto I, Purgatto E. Post-harvest treatment with methyl jasmonate impacts lipid metabolism in tomato pericarp (*Solanum lycopersicum* L. cv. Grape) at different ripening stages. *Foods.* (2021) 10:877. doi: 10.3390/foods10040877
57. Deng Y, Luo YL, Wang YG, Zhao YY. Effect of different drying methods on the myosin structure, amino acid composition, protein digestibility and volatile profile of squid fillets. *Food Chem.* (2015) 171:168–76. doi: 10.1016/j.foodchem.2014.09.002
58. Ye Y, Bie Z, Tang L, Tian Y. Study on pretreatment conditions for determination of sulfur-containing amino acids in food. *Food and Ferment Industry.* (2021) 47:236–42. doi: 10.13995/j.cnki.11-1802/ts.025336
59. Wall MM, Corgan JN. Relationship between pyruvate analysis and flavor perception for onion pungency determination. *HortScience.* (1992) 27:1029–30. doi: 10.21273/HORTSCI.27.9.1029
60. Yabuki Y, Mukaida Y, Saito Y, Oshima K, Takahashi T, Muroi E, et al. Characterisation of volatile sulphur-containing compounds generated in crushed leaves of Chinese chive (*Allium tuberosum* Rottler). *Food Chem.* (2010) 120:343–8. doi: 10.1016/j.foodchem.2009.11.028
61. Heng H, Ong XL, Chow P. Antioxidant action and effectiveness of sulfur-containing amino acid during deep frying. *J Food Sci Technol.* (2020) 57:1150–7. doi: 10.1007/s13197-019-04150-5
62. Lee LC, Liong C-Y, Jemain AA. Partial least squares-discriminant analysis (PLS-DA) for classification of high-dimensional (HD) data: a review of contemporary practice strategies and knowledge gaps. *Analyst.* (2018) 143:3526–39. doi: 10.1039/x0xx00000x
63. Carvalho FV, Santana LF, da Silva VDA, Costa SL, Zambotti-Villelae L, Colepicolo P, et al. Combination of a multiplatform metabolite profiling approach and chemometrics as a powerful strategy to identify bioactive metabolites in *Lepidium meyenii* (*Peruvian maca*). *Food Chem.* (2021) 364:130453. doi: 10.1016/j.foodchem.2021.130453
64. Phahlane CJ, Laurie SM, Shoko T, Manhivi VE, Sivakumar D. An evaluation of phenolic compounds, carotenoids, and antioxidant properties in leaves of South African cultivars, Peruvian 199062.1 and USA's beauregard. *Front Nutr.* (2021) 8:773550. doi: 10.3389/fnut.2021.773550
65. Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res.* (2009) 37:W652–60. doi: 10.1093/nar/gkp356
66. Wang C, Lv J, Xie J, Yu J, Li J, Zhang J, et al. Effects of preharvest methyl jasmonate and salicylic acid treatments on the growth, quality, volatile components and antioxidant systems of Chinese chives. *Front Plant Sci.* (2021) 12:767335. doi: 10.3389/fpls.2021.767335
67. Jung J-K, Lee S-U, Kozukue N, Levin CE, Friedman M. Distribution of phenolic compounds and antioxidative activities in parts of sweet potato (*Ipomoea batata* L.) plants and in home processed roots. *J Food Compos Anal.* (2011) 24:29–37. doi: 10.1016/j.jfca.2010.03.025
68. Sarker U, Oba S. Phenolic profiles and antioxidant activities in selected drought-tolerant leafy vegetable amaranth. *Sci Rep.* (2020) 10:18287. doi: 10.1038/s41598-020-71727-y
69. Sarker U, Oba S. Drought stress enhances nutritional and bioactive compounds, phenolic acids and antioxidant capacity of *Amaranthus* leafy vegetable. *BMC Plant Biol.* (2018) 18:258. doi: 10.1186/s12870-018-1484-1
70. Sarker U, Oba S. Antioxidant constituents of three selected red and green color *Amaranthus* leafy vegetable. *Sci Rep.* (2019) 9:1–11. doi: 10.1038/s41598-019-52033-8
71. Sarker U, Oba S. Polyphenol and flavonoid profiles and radical scavenging activity in leafy vegetable *Amaranthus* gangeticus. *BMC Plant Biol.* (2020) 20:499. doi: 10.1186/s12870-020-02700-0
72. Sarker U, Oba S. Nutraceuticals, phytochemicals, and radical quenching ability of selected drought-tolerant advance lines of vegetable amaranth. *BMC Plant Biol.* (2020) 20:564. doi: 10.1186/s12870-020-02780-y
73. Sarker U, Hossain MN, Iqbal MA, Oba S. Bioactive components and radical scavenging activity in selected advance lines of salt-tolerant vegetable amaranth. *Front Nutr.* (2020) 7:587257. doi: 10.3389/fnut.2020.587257
74. Ghasemzadeh A, Jaafar HZ, Rahmat A. Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (*Zingiber officinale* Roscoe). *Molecules.* (2010) 15:4324–33. doi: 10.3390/molecules15064324
75. Zhang R, Zhang B-L, He T, Yi T, Yang J-P, He B. Increase of rutin antioxidant activity by generating maillard reaction products with lysine. *Bioorg Med Chem Lett.* (2016) 26:2680–4. doi: 10.1016/j.bmcl.2016.04.008
76. Baek MW, Choi HR, Solomon T, Jeong CS, Lee O-H, Tilahun S. Preharvest methyl jasmonate treatment increased the antioxidant activity and glucosinolate contents of hydroponically grown pak choi. *Antioxidants.* (2021) 10:131. doi: 10.3390/antiox10010131
77. Srinivasan M, Sudheer AR, Menon VP. Recent advances in Indian herbal drug researchguest editor: thomas paul asir devasagayamferulic acid: therapeutic potential through its antioxidant property. *J Bioorg Med Chem Lett Clin Biochem Nutr.* (2007) 40:92–100. doi: 10.3164/jcbn.40.92
78. Kerry NL, Abbey M. Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation in vitro. *Atherosclerosis.* (1997) 135:93–102. doi: 10.1016/S0021-9150(97)00156-1
79. Sarker U, Oba S. Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected *Amaranthus tricolor* under salinity stress. *Sci Rep.* (2018) 8:12349. doi: 10.1038/s41598-018-30897-6
80. Sarker U, Oba S. Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. *Sci Rep.* (2018) 8:1–12. doi: 10.1038/s41598-018-34944-0
81. Sarker U, Oba S. Color attributes, betacyanin, and carotenoid profiles, bioactive components, and radical quenching capacity in selected *Amaranthus* gangeticus leafy vegetables. *Sci Rep.* (2021) 11:1–14. doi: 10.1038/s41598-021-91157-8
82. Sarker U, Oba S. Leaf pigmentation, its profiles and radical scavenging activity in selected *Amaranthus tricolor* leafy vegetables. *Sci Rep.* (2020) 10:1–10. doi: 10.1038/s41598-020-66376-0
83. Sarker U, Oba S. Drought Stress Effects on Growth, ROS Markers, Compatible Solutes, Phenolics, Flavonoids, and Antioxidant Activity in *Amaranthus* tricolor. *Appl Biochem Biotechnol.* (2018) 186:999–1016. doi: 10.3389/fpls.2020.559876
84. Sarker U, Oba S. The response of salinity stress– induced a. tricolor to growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. *Front Plant Sci.* (2020) 11:559876. doi: 10.1007/s12010-018-2784-5
85. Rohini V, Goodrow-Kotyla EF, Wooten BR, Wilson TA, Nicolosi RJ. Consumption of 2 and 4 egg yolks/d for 5 wk increases macular pigment concentrations in older adults with low macular pigment taking cholesterol-lowering statins. *Am J Clin Nutr.* (2009) 90:1272–9. doi: 10.3945/ajcn.2009.28013
86. Pérez AG, Sanz C, Richardson DG, Olías JM. Methyl jasmonate vapor promotes β-carotene synthesis and chlorophyll degradation in golden delicious apple peel. *J Plant Growth Regulator.* (1993) 12:163–7. doi: 10.1007/BF00189648
87. Nyathi MK, Du Plooy CP, Van Halsema GE, Stomph TJ, Annandale JG, Struik PC. The dual-purpose use of orange-fleshed sweet potato (*Ipomoea batatas* var. Bophelo) for improved nutritional food security. *Agric Water Manag.* (2019) 217:23–37. doi: 10.1016/j.agwat.2019.02.029
88. Szymanowska U, Złotek U, Karaś M, Baraniak B. Anti-inflammatory and antioxidative activity of anthocyanins from purple basil leaves induced by selected abiotic elicitors. *Food Chem.* (2015) 172:71–7. doi: 10.1016/j.foodchem.2014.09.043
89. Sarker U, Oba S. Nutritional and bioactive constituents and scavenging capacity of radicals in *Amaranthus hypochondriacus*. *Sci Rep.* (2020) 10:1–10. doi: 10.1038/s41598-020-71714-3

90. Sarker U, Oba S. Nutrients, minerals, pigments, phytochemical, and radical scavenging activity in *Amaranthus blitum* leafy vegetable. *Sci Rep.* (2020) 10:3868. doi: 10.1038/s41598-020-59848-w
91. Sarker U, Oba S. Nutraceuticals, antioxidant pigments, and phytochemicals in the leaves of *Amaranthus spinosus* and *Amaranthus viridis* weedy species. *Sci Rep.* (2019) 9:20413. doi: 10.1038/s41598-019-50977-5
92. Sarker U, Oba S, Daramy M. Nutrients, minerals, antioxidant pigments and phytochemicals, and antioxidant capacity of the leaves of stem *amaranth*. *Sci Rep.* (2020) 10:3892. doi: 10.1038/s41598-020-60252-7
93. Sarker U, Hossain MM, Oba S. Nutritional and antioxidant components and antioxidant capacity in green morph *Amaranthus* leafy vegetable. *Sci Rep.* (2020) 10:1–10. doi: 10.1038/s41598-020-57687-3
94. Sarker U, Oba S. Protein, dietary fiber, minerals, antioxidant pigments and phytochemicals, and antioxidant activity in selected red morph *Amaranthus* leafy vegetable. *PLoS One.* (2019) 14:e0222517. doi: 10.1371/journal.pone.0222517
95. Duan S-C, Kwon S-J, Eom S-H. Effect of thermal processing on color, phenolic compounds, and antioxidant activity of Faba bean (*Vicia faba* L.) leaves and seeds. *Antioxidants.* (2021) 10:1207. doi: 10.3390/antiox10081207
96. Sokół-Łętowska A, Oszmiański J, Wojdyło A. Antioxidant activity of the phenolic compounds of hawthorn, pine and skullcap. *Food Chem.* (2007) 103:853–9. doi: 10.1016/j.foodchem.2006.09.036
97. Ghasemzadeh A, Omidvar V, Jaafar HZ. Polyphenolic content and their antioxidant activity in leaf extract of sweet potato (*Ipomoea batatas*). *J Med Plants Res.* (2012) 6:2971–6. doi: 10.5897/JMPR11.135396
98. Podsêdek A. Natural antioxidants and antioxidant capacity of *Brassica* vegetables: a review. *LWT Food Sci Technol.* (2007) 40:1–11. doi: 10.1016/j.lwt.2005.07.023
99. Lanfer-Marquez UM, Barros RM, Sinnecker P. Antioxidant activity of chlorophylls and their derivatives. *Food Res Int.* (2005) 38:885–91. doi: 10.1016/j.foodres.2005.02.012
100. Londhe JS, Devasagayam TP, Foo LY, Ghaskadbi SS. Antioxidant activity of some polyphenol constituents of the medicinal plant *Phyllanthus amarus* Linn. *Redox Rep.* (2008) 13:199–207. doi: 10.1179/135100008X308984
101. Ma Q, Kumar SR, Xue Z, Guo Q, Gao X, Chen H. Effect of different drying methods on the physicochemical properties and antioxidant activities of mulberry leaves polysaccharides. *Int J Biol Macromol.* (2018) 119:1137–43. doi: 10.1016/j.ijbiomac.2018.08.023

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Antioxidant Potential of Selected Wild Edible Leafy Vegetables of Sikkim Himalayan Region: Effects of Cooking Methods and Gastrointestinal Digestion on Activity

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Green leafy vegetables or GLVs are one of the main attractions in the local vegetable market and are widely consumed as the main course and side dish in the Sikkim Himalayan region (SHR). This study evaluated the total phenolic (TPC) and flavonoid contents (TFC) and antioxidant potential in different extracts such as methanolic (MeOH), ethyl acetate (EtOAC), and hexane extracts of selected GLVs followed by changes in the antioxidant activity on cooking and stimulated gastrointestinal (GI) digestion. The MeOH extracts of *Urtica dioica* L. (Sisnu), *Nasturtium officinale* W. T. Aiton (Simrayo), *Diplazium esculentum* Retz. Sw. (Ningro), and *Chenopodium album* L. (Bethu) were estimated to have higher TPC [22.73–45.84 μg gallic acid equivalent (GAE)/mg of extract]. In contrast, the plant extracts prepared using EtOAC (except for *N. officinale*, where TFC was found to be higher in hexane extract) were found to contain higher TFC (3.42–14.86 μg quercetin equivalent (QE)/mg of extract). The MeOH extracts also exhibited higher 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (9.55–18.67 μg ascorbic acid equivalent (AAE)/mg of extract), total antioxidant activity (TAA) (0.27–0.32 mg AAE/mg of extract), and reducing power potential (RPP) (1.6–9.9 μg AAE/mg of extract). Among the test MeOH extracts, *U. dioica* demonstrated relatively higher antioxidant activities and was selected for cooking experiments followed by simulated GI digestion. The findings revealed that the loss of antioxidant activity was minimal in steam-cooked leaves (3.5% in 40 min) as compared to the boiled ones (18% in 10 min). The simulated GI (simulated salivary, gastric, and intestinal) digestion performed on raw, steam cooked, and boiled *U. dioica* leaves showed substantial enhancement of antioxidant properties (by 64.63%) through steam cooking in comparison to the raw leaves. Overall the study concludes that higher antioxidant properties can be achieved on the consumption of steam-cooked *U. dioica* leaves.

Keywords: green leafy vegetables, phenolic contents, antioxidant activity, *Urtica dioica*, Sikkim Himalaya

INTRODUCTION

Reactive oxygen species (ROS) which include free radicals such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (OH–), singlet oxygen ($^1O_2$), and alkoxyl radical (RO) are formed continuously in the human body because of different cellular metabolic processes (electron transport chain, phagocytosis, fertilization, energy production in mitochondria, etc.) (1–4). These ROS are reported to cause oxidative damage to living cells on reacting with different molecules of the cell and are responsible for the development of several pathological conditions including DNA damage, lipid peroxidation, protein oxidation, and cellular degeneration. Moreover, uncontrolled generation of ROS has also been associated with various diseases and disorders such as diabetes, cancer, rheumatoid arthritis, osteoporosis, and aging (1, 5). The human body has developed an endogenous defense system consisting of a few antioxidant enzymes (catalase, glutathione reductase, superoxide dismutase, etc.) to protect itself from the harmful effects of ROS; nevertheless, it fails to be effective against the oxidant load in certain conditions. Therefore, the human body needs to be supplied with exogenous antioxidants.

Antioxidants are small molecules that can help to limit or prevent the harmful effects of pervasive free radicals. These molecules are highly essential for the survival of all living beings. Plant-based foods are an excellent source of natural antioxidants such as flavonoids and related phenolic compounds that offer a balance between the oxidants and antioxidants in the body and thereby play role in combating the oxidative stress inside the human body (6). The green leafy vegetables or GLVs which are being commonly used in every household as a part of healthy and promising meals contain a blend of natural antioxidants and phenolic compounds that render them exceptional for several nutritional and important therapeutic properties (7–9). Studies have shown that regular consumption of GLVs rich in antioxidants results in a positive impact by decreasing oxidative damage (10). Conversely, before their consumption, most of the GLVs need to be undergone a cooking process (boiling, steaming, frying, etc.) which is based on taste preference and edibility. Cooking can also induce changes in the physical characteristics, the chemical composition of the food, and influence the concentration, release, and bioavailability of bioactive substances (11, 12). In certain cases, specific culinary methods have resulted in the denaturation of active constituents and thereby reducing the nutritional and therapeutic values. Specific cooking processes have also been shown to enhance the functional properties of selected GLVs (1, 13, 14). The extent of benefits anticipated from foods can also be influenced by the physiological process of digestion where the digestive enzymes act while the food travels through the gastrointestinal (GI) tract (13, 15).

Sikkim, a small Himalayan state of northeastern India, is known for its multiethnic population and notable biodiversity including subtropical and alpine climates. The wild edible leafy vegetables have been valued greatly throughout this region and serve as sources of food for the inhabitants. The mode of consumption and ethnopharmacological significance of some of these vegetables have also been documented (16). Nonetheless, the data on the effects of different cooking processes on the bioactivity of such GLVs are still limited. In addition, the fates of bioactive components during the process of GI digestion have merely been studied. Therefore, this study was aimed at evaluating the antioxidant potential of organic extracts prepared from selected green leafy vegetables commonly used in the Sikkim Himalayan region (SHR), followed by investigating the effects of different cooking methods and *in-vitro* GI digestion on retention of their antioxidant properties. The outcome of the study may contribute to the promotion of consumption of GLVs in SHR and other regions of the country to use a selective cooking approach to assure better antioxidant benefits.

MATERIALS AND METHODS

Materials

Methanol (MeOH), ethyl acetate (EtOAC), hexane, sodium carbonate (Na_2Co_3), Folin–Ciocalteu reagent, aluminum chloride ($AlCl_3$), potassium acetate (CH_3COOK), sulfuric acid (H_2SO_4), sodium phosphate (Na_3PO_4), ammonium molybdate [$(NH_4)_2MoO_4$], 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide $K_4[Fe(CN)_6]$, trichloracetic acid (TCA), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), potassium dihydrogen phosphate (KH_2PO_4), ammonium carbonate [$(NH_4)_2CO_3$], sodium chloride (NaCl), hydrochloric acid (HCl), magnesium chloride ($MgCl_2.6H_2O$), α-amylase, pepsin, pancreatin, calcium chloride ($CaCl_2$), and sodium hydroxide (NaOH) are from Sigma Aldrich.

Collection of Plant Samples and Preparation of Extracts

Green leafy vegetables (GLVs) used in this study including *Urtica dioica*, *Nasturtium officinale*, *Diplazium esculentum*, and *Chenopodium album* were purchased from Lal Bazaar, a local market under Gangtok Municipal Corporation, East Sikkim. Collected leaves were washed in running tap water, oven-dried (40°C), and powdered using a mixer grinder. A definite quantity of leaf powders was extracted with 1:5 (w/v) of three different organic solvents of varying polarity (methanol, ethyl acetate and hexane) using cold maceration. Briefly, the leaf powders (100 g) were dissolved differently in 500 ml of the organic solvent and shaken vigorously for 15 min. Then, the solutions were kept in dark and shaking was performed at every 12 h interval. After 72 h, the solutions were filtered using Whatman No. 1 filter paper and the solvents were made evaporated using Rota evaporator (R 100, Buchi) to obtain the organic extracts which were stored at 4°C for further use.

Estimation of Total Phenolic Content

Total phenolic content (TPC) of the organic extracts was estimated following the Folin–Ciocalteu method previously described by Rai et al. (17). Briefly, 50 μl (2.5 mg/ml) extract was mixed with 2 ml of 2% Na_2CO_3 followed by incubation at room temperature for 2 min. Thereafter, 100 μl of 50% Folin–Ciocalteu reagent was added to the reaction mixture and incubated at room temperature for 30 min. Absorbance was recorded at 720 nm

using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). All the analyses were performed in triplicates and data were expressed in terms of μg gallic acid equivalent (GAE)/mg of the organic extract.

Estimation of Total Flavonoid Contents

Total flavonoid contents (TFCs) of the organic extracts were determined following a standard protocol as described by Aryal et al. (18). To 1 ml (0.625 mg/ml) extract, 0.2 ml of 10% (w/v) $AlCl_3$ (in MeOH) and 0.2 ml (1 M) potassium acetate were added. The volume of the reaction mixture made up to 7 ml by adding distilled water and incubated at room temperature for 30 min. The absorbance was measured at 415 nm using a UV-VIS spectrophotometer. The data were expressed in terms of μg quercetin equivalent (QE)/mg of extract.

Determination of Total Antioxidant Activity

Total antioxidant activity (TAA) of the leaf extracts was determined following the method described by Rai et al. (19). Briefly, 200 μl (0.25 mg/ml) of extract was mixed with 3 ml reagent solution prepared using 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate (1:1:1). The components of the mixture were mixed well and incubated at 95°C in the water bath for 90 min. The reaction mixture was then allowed to cool to room temperature and the absorbance was recorded at 695 nm using a UV-VIS spectrophotometer. The data were expressed in mg ascorbic acid equivalent (AAE)/g of extract.

DPPH Radical Scavenging Activity

The leaf extracts were determined for their DPPH radical scavenging activity using the method described by Rai et al. (20). Briefly, 200 μl (1.25 mg/ml) extracts were mixed with 2 ml 0.16 mM DPPH solution followed by incubation in a dark condition for 30 min. After incubation, the absorbance of content was taken at 517 nm using a UV-VIS spectrophotometer. The DPPH scavenging activity was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \{1 - (S_{abs} - B_{abs}/C_{abs})\} \times 100$$

Where, S_{abs}–absorbance of sample, B_{abs}–absorbance of extract blank, and C_{abs}–absorbance of the control (DPPH).

Determination of Reducing Power Potential

Reducing power potential (RPP) was done according to the method as described by Rai et al. (20). Briefly, 100 μl (2.5 mg/ml) of the extract was mixed with 900 μl phosphate buffer (0.2 M) followed by the addition of 900 μl freshly prepared potassium ferricyanide (1%). The reaction mixture was vortexed and incubated at 50°C for 20 min. After the incubation, 900 μl of 10% TCA was added and centrifuged at 6,000 g for 10 min. The collected supernatant (900 μl) was added to an equal volume of distilled water and $FeCl_3$ (0.1%). Absorbance was taken at 700 nm using a UV-VIS spectrophotometer. The data were expressed in AAE/g of ascorbic acid.

Effects of Cooking on TPC, TFC, and Antioxidant Property of *U. Dioica*

The leaves of the *U. dioica* were cooked with two methods preferred by local people such as (i) boiling and (ii) steaming. Leaves of *U. dioica* (250 g) were divided into 5 equal parts. One part was taken as control and two parts were taken for boiling and the rest two were taken for steaming. Briefly, two sets of 50 g of leaves were taken in 400 ml distilled water and were boiled, respectively, for 5 min (B5) and 10 min (B10). Similarly, two sets of 50 g leaves were subjected to steam cooking for 20 min (S20) and 40 min (S40). The cooked leaves were then oven-dried at 40°C and ground to fine powders, which were then extracted in MeOH for further evaluation. The MeOH extracts were analyzed for TPC, TFC, and antioxidant activities (DPPH scavenging and TAA) as described earlier.

In-vitro Simulated Gastrointestinal Digestion of Cooked and Steamed *U. Dioica*

Simulation of GI digestion was performed *in-vitro* following standard protocol mentioned in Minekus et al. (21) with modifications wherever required. The boiled and steam-cooked *U. dioica* leaves were treated for sequential simulation of mouth, stomach, and small intestine digestion. The compositions of simulated salivary fluid (SSF), gastric fluid (SGF), and intestinal fluid (SIF) are given in **Table 1**. The oral digestion was done independently on 10 g of boiled and steam-cooked leaves which was mixed with 7 ml SSF followed by the addition of 1 ml α-amylase solution (1,500 U/ml in SSF), 50 μl $CaCl_2$ (0.3 M), and 1.95 ml distilled water to attain a paste of food: SSF-1:1 (w/v). The reaction mixture was then incubated for 5 min at room

TABLE 1 | Composition of simulated digestive fluids used in this study.

Simulated digestive fluids	Volume of chemicals used (in mL)					
	KCl (37.3 g/L)	KH_2PO_4 (68 g/L)	$NaHCO_3$ (84 g/L)	NaCl (117 g/L)	$MgCl_2.6H_2O$ (30.5 g/L)	$(NH_4)_2CO_3$ (48 g/L)
SSF, pH 7	30.2	7.4	13.6	-	1.0	0.12
SGF, pH 3	13.8	1.8	25.0	23.6	0.8	1.0
SIF, pH 7	13.6	1.6	85.0	19.2	2.2	-

SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

temperature. To the oral bolus 15 ml SGF, 3.2 ml pepsin solution (25,000 U/ml in SGF), 10 μl $CaCl_2$ (0.3 M), and 1.39 ml distilled water were added for the simulation of the gastric digestion. The pH of the reaction mixture was maintained at 3 and incubated in shaking conditions at 37°C for 120 min. Similarly, simulation of the intestinal phase was performed on the gastric chyme with the addition of 22 ml SIF, 40 ml pancreatin solution (1:4 w/v), 80 μl $CaCl_2$ (0.3 M), 0.3 ml NaOH, and 2.32 ml distilled water and then incubated at 37°C for 120 min at pH 7. After the complete simulation of digestion, the digesta were centrifuged at 4,000 rpm for 45 min and the supernatants were collected. The leftover residues were extracted with MeOH (1:5 w/v) to prepare the extract. TPC, TFC, and antioxidant activities were determined in both cases.

RESULTS AND DISCUSSION

Leafy vegetables are essential sources of minerals, such as microelements, namely, K, Ca, Mg, P, and S, and microelements, namely, Fe, Cu, Mn, Zn, Na, Mo, and B, protein, dietary fiber, carbohydrates, and vitamins for human nutrition (22–26). Most importantly, they are rich in natural antioxidants such as phenolics, betalains, xanthophylls, violaxanthin, ascorbic acids, carotenoids, betacyanins, betaxanthins, chlorophyll A, chlorophyll B, and beta-carotene that have high radical quenching ability (27–31). Phenolics, the nonnutrient secondary metabolites found in fruits, seeds, and vegetables, have long been known for their biochemical and pharmacological importance. Phenolic compounds are considered to be vital in defense responses of the human body including anti-inflammatory, antiaging, antiproliferative, and antioxidative mechanisms (32). They include coumarins, phenolic acids, such as hydroxybenzoic acids and hydroxycinnamic acids, flavonoids, such as flavonols, flavones, flavanols, flavanones, isoflavones, anthocyanins, chalcones, and nonflavonoids, such as tannins, lignans, and stilbenes (33–37). Among others, flavonoids are the most prevalent and ubiquitous group of phenolic compounds that are widely distributed in fruits, plant-derived beverages, and vegetables. Mounting pieces of evidence in terms of epidemiological and clinical findings support their health-promoting and disease-preventing significance (38). Herein, selected GLVs consumed popularly in the SHR such as *U. dioica*, *N. officinale*, *D. esculentum*, and *C. album* (**Figure 1**) were studied for the determination of their TPC, TFC, and *in-vitro* antioxidant potential. The extract yield of MeOH, EtOAC, and hexane extracts of GLVs ranged from 3.97 to 8.05%, 1.77 to 4.50%, and 2.10 to 3.90% (dry weight basis), respectively (**Table 2**). Extract yield was found to be comparatively higher in MeOH extracts than that of EtOAC and hexane extracts. The TPC of GLV MeOH extracts ranged from 22.73 to 52.06 μg GAE/mg, 10.16 to 32.76 μg GAE/mg, and 9.14 to 25.40 μg GAE/mg of extract, respectively. Similarly, TFC of GLV organic extracts ranged from 2.93 to 11.18 μg QE/mg of extract in the MeOH, 3.42 to 14.86 μg QE/mg of extract in EtOAC, and 1.87 to 10.84 μg QE/mg of hexane extracts (**Table 3**). The *N. officinale* MeOH extract among others had the highest TPC (52.06 ± 3.82 μg GAE/mg of extract) followed by that of the *U. dioica*–MeOH extract (45.84 μg GAE/mg of extract). The *C. album* hexane extract was found to have the lowest estimate of TPC (9.14 ± 0.38 μg GAE/mg of extract). The *U. dioica* EtOAC extract was found to have the highest TFC (14.86 μg QE/mg of extract) followed by that of the MeOH (11.18 μg QE/mg of extract) and hexane extracts (10.84 μg QE/mg of extract). TFC was estimated to be the lowest in the hexane extract of *D. esculentum* (1.87 μg QE/mg of extract). The overall results showed variation in the TPC and TFC among different organic extracts of the same GLV.

Biosynthesis and accumulation of phenolic compounds are appeared to be unconventional in each plant and plant organ, and variation in their contents relies on the growth stage and the genotypic composition of the plant species (39). The extractability and recovery of a particular organic component are reported to depend on the polarity of the extraction medium and the ratio of solute to solvent. Likewise, the extractability of phenolic compounds is reliant on the type and polarity index (PI) of the solvents used, and the solubility of the phenolic

FIGURE 1 | Selected green leafy vegetables popularly consumed in the Sikkim Himalayan region.

TABLE 2 | Scientific nomenclature and extract yield of selected leafy greens of Sikkim Himalayan Region.

Scientific names	Common names	Total yield (%)		
		MeOH	EtOAC	Hexane
Urtica dioica	Sisnu	8.05 ± 0.15^{a}	3.87 ± 0.23^{a}	2.92 ± 0.18^{a}
Nasturtium officinale	Simrayo	5.64 ± 0.46^{b}	4.50 ± 0.31^{b}	3.90 ± 0.33^{b}
Diplazium esculentum	Ningro	3.97 ± 0.23^{c}	$4.10 \pm 0.38^{a,b}$	2.87 ± 0.13^{c}
Chenopodium album	Bethu	4.80 ± 0.41^{d}	1.77 ± 0.14^{c}	2.10 ± 0.08^{d}

MeOH, methanolic Extract; EtOAC, ethyl acetate Extract; Hexane, hexane extract. Data expressed as mean ± standard deviation; different alphabets (a, b, c, and d) within the same column indicates significantly different at $p < 0.05$.

TABLE 3 | TPC and TFC in selected wild green leafy vegetables of Sikkim Himalayan region.

Green leafy vegetables	TPC (μg GAE/mg of extract)			TFC (μg QE/mg of extract)		
	MeOH	EtOAC	Hexane	MeOH	EtOAC	Hexane
Urtica dioica	45.84 ± 4.04^{a}	22.86 ± 2.50^{a}	15.87 ± 0.96^{a}	11.18 ± 0.42^{a}	14.86 ± 0.66^{a}	10.84 ± 0.44^{a}
Nasturtium officinale	52.06 ± 3.82^{a}	32.76 ± 0.66^{b}	25.40 ± 3.33^{b}	$3.32 \pm 0.47^{b,c}$	5.02 ± 0.10^{b}	7.20 ± 0.38^{b}
Diplazium esculentum	31.11 ± 2.86^{b}	16.76 ± 2.75^{c}	11.05 ± 1.90^{c}	2.93 ± 0.08^{b}	3.42 ± 0.15^{c}	1.87 ± 0.22^{c}
Chenopodium album	22.73 ± 1.88^{c}	10.16 ± 0.88^{d}	9.14 ± 0.38^{c}	3.61 ± 0.37^{c}	4.15 ± 0.63^{c}	2.50 ± 0.37^{d}

TPC, total phenolic contents; TFC, total flavonoid contents; MeOH, methanolic extract; EtOAC, ethyl acetate extract; Hexane, hexane extract. Data expressed as mean ± standard deviation; GAE, gallic acid equivalent; QE, quercetin equivalent; different alphabets (a, b, c, and d) within the same column indicate significant difference at $p < 0.05$.

compounds in the solvents. In addition, their solubility is contingent on several chemical features including the position of –OH groups, their molecular size, and length of the hydrocarbon chains (40). Polar solvents are often considered suitable to extract most of the phenolic compounds from plant tissues (41, 42). MeOH has been found to be more efficient in extracting high content of low molecular weight phenolics from different plant parts (43). In contrast, flavonoids have an affinity toward both the polar and nonpolar extracting medium because of their diverse chemical structure (O-glycosides and aglycones). Polar solvents have been reported to extract the flavonoid glycosides while nonpolar solvents mostly extract their aglycones (44, 45). Resembling the above-mentioned facts, extracts of GLVs used in the study which were prepared using MeOH and EtOAC (polar extracting medium) appeared to have higher TPC and TFC, respectively. Quite the opposite, TFC in the *N. officinale* hexane extract was found to be higher as compared to other extracts and this can be certainly due to the presence of aglycones in the extract.

The antioxidant activities of the GLV organic extracts were determined using DPPH radical scavenging, TAA, and RPP. Evaluation of antioxidant potential is carried out using different methods as each assay varies in principle and mechanism of action (17). DPPH is a free radical which is a widely applied and acceptable method to study the antioxidant potential of plant extracts (18, 46). The DPPH scavenging activity of the GLV organic extracts ranged from 9.55 to 18.68 μg AAE/mg in MeOH extract, 7.11 to 14.10 μg AAE/mg in EtOAC extract, and 8.09 to 10.85 μg AAE/mg in hexane extracts. Among the test extracts, *U. dioica* MeOH extracts exhibited the highest DPPH radical scavenging activity (18.67 μg AAE/mg of extract), while the *C. album* EtOAC extract exhibited the lowest activity (7.11 μg AAE/mg of extract) (**Figure 2A**). TAA of the test extracts ranged from 0.27 to 0.32 mg AAE/mg of extract in MeOH, 0.23 to 0.26 mg AAE/mg of extract in EtOAC, and 0.25 to 0.28 mg AAE/mg of extract in hexane extracts. Among different plants, TAA of the *U. dioica* MeOH extract was found to be higher (0.32 mg AAE/mg of extract), whereas that of the EtOAC extract exhibited the lowest TAA (0.23 mg AAE/mg of extract) (**Figure 2B**). TAA assay measures the ability of the extract to reduce molybdenum (VI) to molybdenum (V) in acidic condition (47). RPP is an antioxidant method commonly applied for evaluating the presence of reductants, which exhibit antioxidant activity by breaking down of free radical chain on the donation of hydrogen atoms (43). RPP of the test extracts ranged from 1.67 to 9.93 μg AAE/mg of extract in MeOH, 1.2 to 8.60 μg AAE/mg of extract in EtOAC, and 0.23 to 7.80 μg AAE/mg of hexane extracts. Furthermore, the highest RPP was exhibited by *U. dioica* MeOH extract (9.9 μg AAE/mg of extract) and *D. esculentum* hexane extract displayed the lowest RPP (0.23 μg AAE/mg of extract) (**Figure 2C**). The overall results showed that the organic extracts obtained from *U. dioica* demonstrated relatively significant TPC and TFC, and exhibited promising antioxidant activity. Moreover, the *U. dioica* MeOH extract among others was able to exhibit higher antioxidant activity. Higher antioxidant activity in the *U. dioica* MeOH extract could probably be correlated to higher TPC present in the same (48).

The effects of cooking on the antioxidant potential of *U. dioica* leaf extracts were evaluated using steaming and boiling, two major methods used by the local people. Steaming of *U. dioica* leaves was carried out for 20 min (S20) and 40 min (S40), whereas boiling was carried out for 5 min (B5) and 10 min

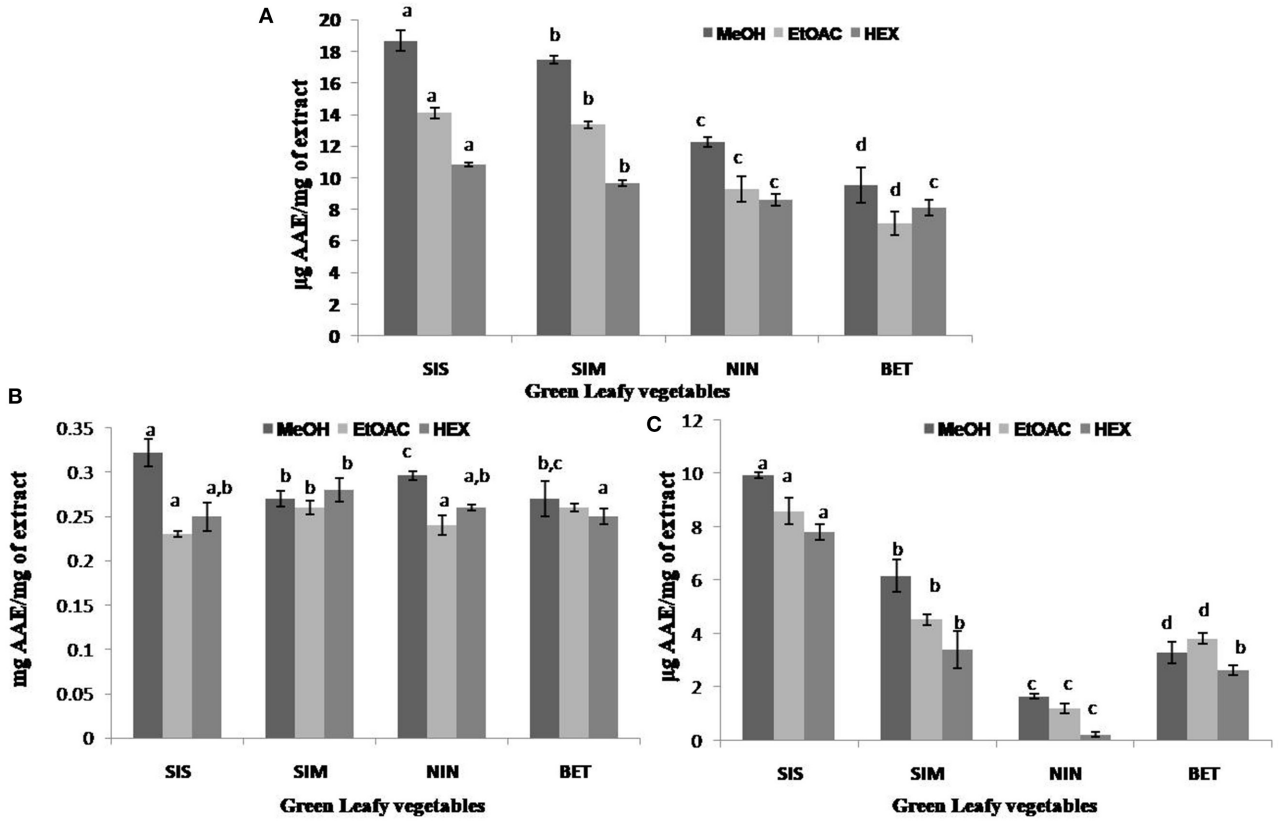

FIGURE 2 | Antioxidant activities of different extracts of selected green leafy vegetables of Sikkim Himalayan region. **(A)** DPPH radical scavenging activity, **(B)** total antioxidant activity, and **(C)** reducing power potential. SIS, Sisnu *(Urtica dioica)*; SIM, Simrayo *(Nasturtium officinale)*; NIN, Ningro *(Diplazium esculentum)*; BET, Bethu *(Chenopodium album)*; MeOH, methanolic extract; EtOAC, ethyl acetate extract; HEX, hexane extract. Values not sharing common alphabets within the same pattern are significantly different ($p < 0.05$).

(B10) to enable proper cooking. The S40 extract of *U. dioica* demonstrated higher TPC (36.19 μg GAE/mg of extract) and TFC (9.84 μg QE/mg of extract) than S20 having 33.52 μg GAE/mg of extract and 9.04 μg Q/mg of extract, respectively. Conversely, B5 extract showed greater TPC (34.28 μg GAE/mg of extract) and TFC (9.23 μg QE/mg of extract) than B10 which is having TPC and TFC of 34.28 μg GAE/mg and 6.86 μg QE/mg of extracts, respectively. The results demonstrated an increase in TPC and TFC on steam cooking of *U. dioica* leaves (**Figure 3A**); however, loss of TPC and TFC was observed with leaf extracts prepared using boiled *U. dioica* leaves. The results resemble the findings made by Salamatullah et al. (49), Rocchetti et al. (50), and Gunathilake et al. (51). Similar observations were also witnessed while assessing the effects of cooking on the antioxidant activities (DPPH radical scavenging and TAA) given in **Figure 3B**. The S40 extract exhibited higher DPPH radical scavenging activity and TAA (18.47 μg AAE/mg of extract and 0.30 mg AAE/mg of extract) than that of the S20 extract (17.28 μg AAE/mg of extract and 0.28 mg AAE/mg of extract). In boiled cooking process, B5 extracts showed higher DPPH (15.54 μg AAE/mg of extract) and TAA (0.3 mg AAE/mg of extract) than B10 (DPPH 13.46 μg AAE/mg of extract and TAA 0.25 mg AAE/mg of extract). Higher boiling time reduced the antioxidant potential of *U. dioica*, as phenolics are released in water used for the boiling method. An increase in antioxidant activity on steam cooking may probably be due to the breakdown of complex structure and release of free from of phenolics on heat treatment (13). Several studies have shown steaming as a useful cooking approach over boiling to retain the antioxidant potential in a few vegetables (14, 49, 51). Therefore, it is essential to standardize the cooking procedure for individual leafy vegetable.

The antioxidant effect of phytochemicals in GLVs will depend not only on their concentration but also on their resistance to the GI environment (13). The sequence of events during cooking followed by GI digestion may lead to an increase or decrease in antioxidant activity (1, 52). Simulated GI digestion of the steam-cooked and boiled leaves was performed using standard referred protocols, and TPC, TFC, and the antioxidant potential (DPPH scavenging and TAA) of the digesta (supernatants and residues) in each case were determined. TPC and TFC were estimated to be higher in steam-cooked and GI digested extracts (0.45 mg GAE/ml extract and 0.13 mg QE/ml extract, respectively) as compared to those estimated in boiled and GI

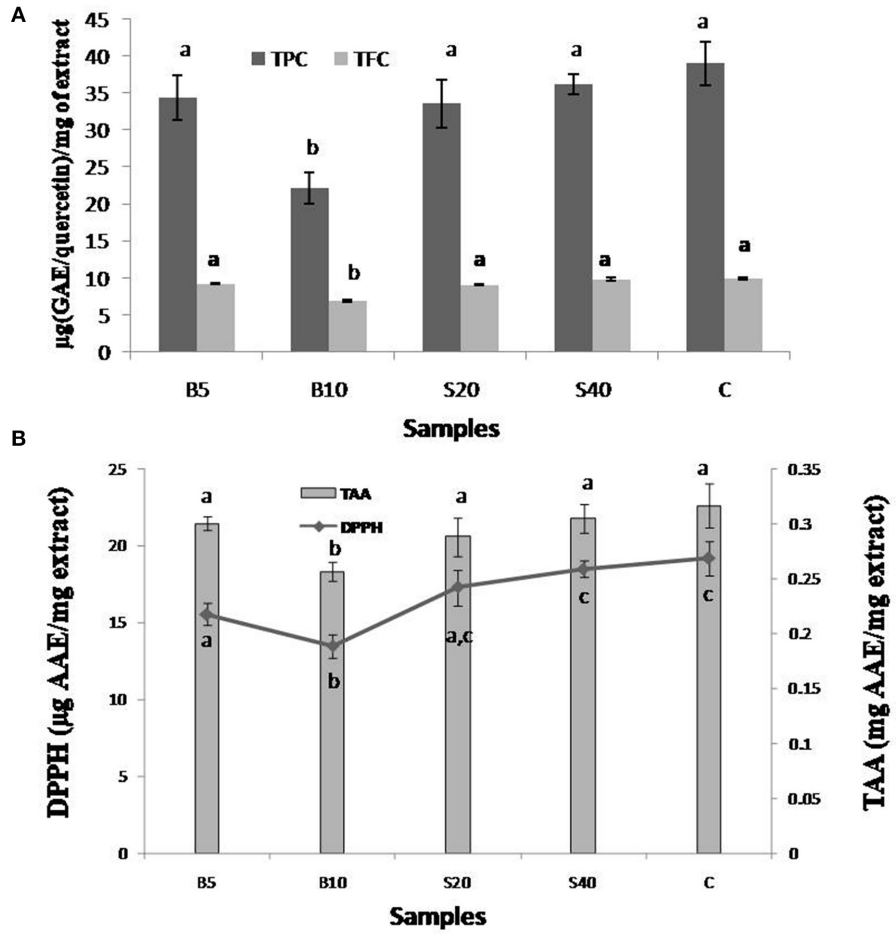

FIGURE 3 | Effects of different cooking methods (steaming and boiling) on **(A)** phenolic (µg GAE/mg of extract) and flavonoid contents (µg QE/mg of extract) and **(B)** DPPH scavenging (µg AAE/mg of extract) and total antioxidant activity (mg AAE/mg of extract). TPC, total phenolic contents; TFC, total flavonoid contents; GAE, gallic acid equivalent; QE, quercetin equivalent; AAE, ascorbic acid equivalent. Values not sharing common alphabets within the same pattern are significantly different ($p < 0.05$).

digested (0.24 mg GAE/ml extract and 0.06 mg QE/ml extract, respectively) (**Figures 4A,B**). Similar findings were also observed in the case of TAA and DPPH scavenging activities where the extracts obtained from the steam-cooked and digested *U. dioica* leaves displayed higher activity (0.27 mg AAE/ml and 5.19 µg AAE/ml of extracts, respectively) as compared to the boiled and GI digested leaves (**Figures 4C,D**). The overall findings of the study suggest that the leafy vegetables consumed in the SHR could be a great source of natural antioxidant metabolites, namely, phenolics and flavonoids. In addition, steam-cooked *U. dioica* leaves could be preferred for consumption to ensure greater bioavailability of its phenolics and higher antioxidant effects. Furthermore, the findings may encourage the inhabitants of the SHR and other parts of the eastern Himalaya to use particular cooking approaches while cooking *U. dioica* leaves to help retain the phenolics and antioxidant properties.

CONCLUSION

In this study, organic extracts of leafy vegetables commonly used by the local people of the Sikkim Himalayan region were evaluated for their phenolic and flavonoid contents, and antioxidant activity. The MeOH extract of *U. dioica* leaves among others was estimated to have higher TPC, TFC, and displayed significant antioxidant activity. The effects of cooking methods and GI digestion on the TPC, TFC, and antioxidant activity demonstrated that steam-cooked and digested leaves retained greater TPC and TFC, and antioxidant activity. The findings of this study could serve as a source of information in promoting the consumption of leafy vegetables in the SHR region and in the use of a definite cooking process to retain their antioxidant properties. Moreover, further research on the chemical efficacies of steam-cooked *U. dioica* leaves can be evaluated *in vivo*.

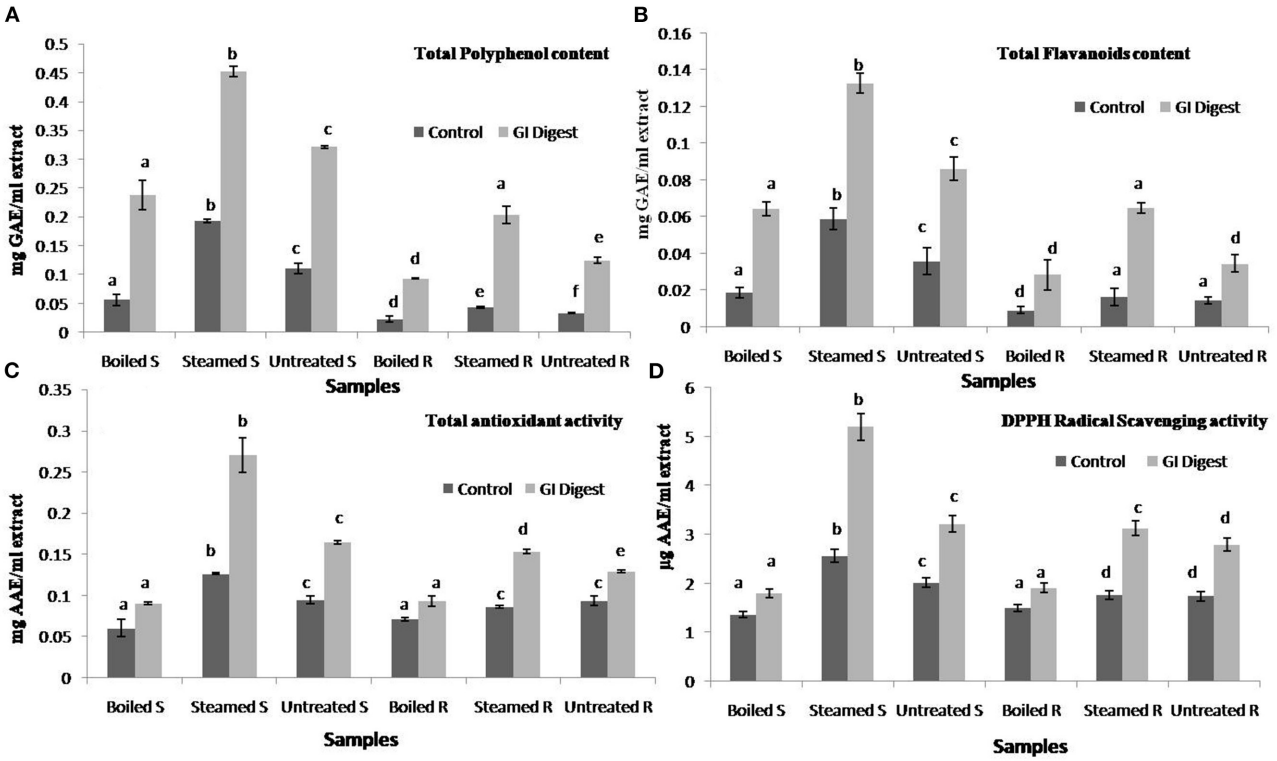

FIGURE 4 | Effects of simulated gastrointestinal (GI) digestion on *Urtica dioica* leaves cooked by different methods (steaming and boiling) on **(A)** phenolics (mg GAE/ml of GI digested extract), **(B)** flavonoids content (mg QE/ml of GI digested extract), **(C)** total antioxidant activity (mg AAE/ml of GI digested extract), and **(D)** DPPH radical scavenging activity (µg AAE/ml of GI digested extract). Boiled S, boil cooked supernatant; Steamed S, steam-cooked supernatant; Untreated S, raw supernatant; Boiled R, boiled cooked residue extract; Steamed R, steam-cooked residue extract; Untreated R, raw residue extract. Values not sharing common alphabets within the same pattern are significantly different ($p < 0.05$).

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/s.

AUTHOR CONTRIBUTIONS

SS: methodology, investigation, validation, formal analysis, visualization, and writing—original draft. SPad: methodology, investigation, visualization, and data curation. MK: methodology, investigation, and data curation. SPat: resources, writing—review and editing, and supervision. DS: conceptualization, resources, writing—review and editing, visualization, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hossain A, Khatun MA, Islam M, Huque R. Enhancement of antioxidant Quality of Green leafy vegetables upon different cooking method. *Prev Nutr Food Sci.* (2017) 22:216–22. doi: 10.3746/pnf.2017.22.3.216
2. Sarker U, Oba S. Drought stress enhances nutritional and bioactive compounds, phenolic acids and antioxidant capacity of Amaranthus leafy vegetable. BMC Plant Biol. (2018) 18:258. doi: 10.1186/s12870-018-1484-1
3. Sarker U, Oba S. Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *A. tricolor*. Sci Rep. (2018) 8: 16496. doi: 10.1038/s41598-018-34944-0
4. Sarker U, Oba S. The response of salinity stress-induced *A. tricolor to growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. Front Plant Sci.* (2020) 11:559876. doi: 10.3389/fpls.2020.559876
5. Gülçin I, Oktay M, Kireçci E. Küfrevioglu ÖI. Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum L*) seed extracts. *Food Chem.* (2003) 83:371–82. doi: 10.1016/S0308-8146(03)00098-0

6. Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr*. (2005) 45:287–306. doi: 10.1080/1040869059096
7. Randhawa MA, Khan AA, Javed MS, Sajid MW. *Handbook of Fertility, Nutrition, Diet, Lifestyle and Reproductive Health*. Cambridge: Academic Press. (2015). p. 205–20. doi: 10.1016/B978-0-12-800872-0.00018-4
8. Tan BL, Norhaizan ME, Liew WPP, Sulaiman RH. Antioxidant and oxidative stress: a mutual interplay in age-related diseases. *Front Pharmacol*. (2018) 9:1162. doi: 10.3389/fphar.2018.01162
9. Arfin S, Jha N, Jha S, Kesari K, Ruokolainen J, Roychoudhury S, et al. Oxidative stress in cancer cell metabolism. *Antioxidants*. (2021) 10: 642. doi: 10.3390/antiox10050642
10. Bacchetti T, Turco I, Urbano A, Morresi C, Ferretti G. Relationship of fruit and vegetable intake to dietary antioxidant capacity and markers of oxidative stress: a sex-related study. *Nutrition*. (2019) 61:164–72. doi: 10.1016/j.nut.2018.10.034
11. Zhang D, Hamauzu Y. Phenolics, ascorbic acid, carotenoids and antioxidant activity of broccoli and their changes during conventional and microwave cooking. *Food Chem*. (2004) 88: 503-509. doi: 10.1016/j.foodchem.2004.01.065
12. Miglio C, Chiavaro E, Visconti A, Fogliano V, Pellegrini N. Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. *J Agric Food Chem*. (2008) 56: 139–47. doi: 10.1021/jf072304b
13. Gunathilake KDPP, Ranaweera KKDS, Rupasinghe HPV. Influence of boiling, steaming and frying of selected leafy vegetables on the in vitro anti-inflammation associated biological activities. *Plants*. (2018) 7: 22. doi: 10.3390/plants7010022
14. Preti R, Rapa M, Vinci G. Effect of steaming and boiling on the antioxidant properties and biogenic amines content in green bean (*Phaseolus vulgaris*) varieties of different colors. *J Food Qual*. (2017) 2017:5329070. doi: 10.1155/2017/5329070
15. Dasgupta S. Effect of cooking on total phenol, total flavonoid, DPPH free radical scavenging assay and total antioxidant capacity of some green leafy vegetables commonly consumed in India. *Indian J Appl Res*. (2021) 11: 1–3. doi: 10.30106/ijar
16. Pradhan S, Tamang JP. Ethnobiology of wild leafy vegetables of Sikkim. *Indian J Tradit Knowl*. (2015) 14:290–7.
17. Rai AK, Prakash M, Anu Appaiah KA. Production of Garcinia wine: changes in biochemical parameters, organic acids and free sugars during fermentation of Garcinia must. *Int J Food Sci Tech*. (2010) 45:1330–6. doi: 10.1111/j.1365-2621.2010.02181.x
18. Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N. Total phenolics content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants*. (2019) 8:1–12. doi: 10.3390/plants8040096
19. Rai AK, Anu Appaiah KA. Application of native yeast from Garcinia (*Garcinia xanthochumus*) for the preparation of fermented beverage: changes in biochemical and antioxidant properties. *Food Biosci*. (2014) 5:101–7. doi: 10.1016/j.fbio.2013.11.008
20. Rai AK, Jini R, Swapna HC, Baskaran V, Sachindra NM, Bhaskar N. Application of native lactic acid bacteria for fermentative recovery of lipids and proteins from fish processing waste: Bioactivities of fermentation products. *J Aquat Food Prod Technol*. (2011) 20:32–44. doi: 10.1080/10498850.2010.528174
21. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. A standardised static in vitro digestion method suitable for food-An international consensus. *Food Funct*. (2014) 5: 1113–24. doi: 10.1039/C3FO60702J
22. Chakrabarty T, Sarker U, Hasan M, Rahman MM. Variability in mineral compositions, yield and yield contributing traits of stem amaranth. *(Amaranthus lividus) Genetika*. (2018) 50:995–1010. doi: 10.2298/GENSR1803995C
23. Sarker U, Islam MT, Rabbani MG, Oba S. Genotypic diversity in vegetable amaranth for antioxidant, nutrient and agronomic traits. *Indian J Genet Pl Br*. (2017) 77:173–6. doi: 10.5958/0975-6906.2017.00025.6
24. Sarker U, Oba S. Protein, dietary fiber, minerals, antioxidant pigments and phytochemicals, and antioxidant activity in selected red morph *Amaranthus* leafy vegetable. *PLoS ONE*. (2019) 14: 222517. doi: 10.1371/journal.pone.0222517
25. Zihad SMNK, Gupt Y, Uddin SJ, Islam MT, Alam MR, Aziz S, Hossain M, Shilpi JA, Nahar L, Sarker SD. Nutritional value, micronutrient and antioxidant capacity of some green leafy vegetables commonly used by southern coastal people of Bangladesh. Heliyon. (2019) 5: *e*02768. doi: 10.1016/j.heliyon.2019.e02768
26. Ejoh SI, Wireko-Manu FD, Page D, Renard CMGC. Traditional green leafy vegetables as underutilised sources of micronutrients in a rural farming community in south-west Nigeria I: estimation of vitamin C, carotenoids and mineral contents. *South Afr J Clin Nutr*. (2021) 34:40–5. doi: 10.1080/16070658.2019.1652963
27. Oboh G. Effect of blanching on the antioxidant properties of some tropical green leafy vegetables. *LWT-Food Sci Technol*. (2005) 38:513–7. doi: 10.1016/j.lwt.2004.07.007
28. Subhasree B, Baskar R, Laxmi Keerthana R, Lijina Susan R, Rajasekaran P. Evaluation of antioxidant potential in selected green leafy vegetables. *Food Chem*. (2009) 115:1213–20. doi: 10.1016/j.foodchem.2009.01.029
29. Sarker U, Oba S. Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected *A. tricolor* under salinity stress. *Sci Rep*. (2018) 8:12349. doi: 10.1038/s41598-018-30897-6
30. Sarker U, Oba S. Response of nutrients, minerals, antioxidant leaf pigments, vitamins, polyphenol, flavonoid and antioxidant activity in selected vegetable amaranth under four soil water content. *Food Chem*. (2018) 252:72–83. doi: 10.1016/j.foodchem.2018.01.097
31. Sarker U, Oba S. Nutritional and bioactive constituents and scavenging capacity of radicals in *Amaranthus hypochondriacus*. *Sci Rep*. (2020) 10:19962. doi: 10.1038/s41598-020-71714-3
32. Hano C, Tungmunnithum D. Plant polyphenols, more than just simple natural antioxidants: oxidative stress, aging and age-related diseases. *Medicines*. (2020) 7:26. doi: 10.3390/medicines7050026
33. Sarkar P, Singh SP, Pandey A, Rai AK. Microbial production and transformation of polyphenols. In: Rai AK, Singh SP. Sacool CR, C Larroche, Pandey A, editors. *Technologies for Production of Nutraceuticals and Functional Food Products*. Elsevier. (2022). p. 189–208. doi: 10.1016/B978-0-12-823506-5.00005-9
34. Sarker U, Oba S. Antioxidant constituents of three selected red and green color *Amaranthus* leafy vegetable. *Sci Rep*. (2019) 9:18233. doi: 10.1038/s41598-019-52033-8
35. de la Rosa LA, Moreno-Escamilla JO, Rodrigo-García J, Alvarez-Parrilla E. Phenolic compounds. In: Yahia EM, editor. *Postharvest Physiology and Biochemistry of Fruits and Vegetables*. UK: Woodhead Publishing. (2019). p. 253–71. doi: 10.1016/B978-0-12-813278-4.00012-9
36. Sarker U, Hossain MN, Iqbal MA, Oba S. Bioactive Components and Radical Scavenging Activity in Selected Advance Lines of Salt-Tolerant Vegetable Amaranth. *Front Nutr*. (2020) 7:587257. doi: 10.3389/fnut.2020.587257
37. do Carmo MAV, Granato D, Azevedo L. Antioxidant/pro-oxidant and antiproliferative activities of phenolic-rich foods and extracts: a cell-based point of view. In: Granato D, editor. *Advances in Food and Nutrition Research*. Cambridge: Academic Press. (2021), p. 253–80. doi: 10.1016/bs.afnr.2021.02.010
38. Anandh Babu PV, Liu D. Flavonoids and cardiovascular health. In: Watson RR, editor. *Complementary and Alternative Therapies and the Aging Population*. Cambridge: Academic Press. (2009), p. 371–92. doi: 10.1016/B978-0-12-374228-5.00018-4
39. Bhandari SR; Kwak JH, Jo JS, Lee JG. Changes in phytochemical content and antioxidant activity during inflorescence development in broccoli. *Chil J Agric Res*. (2019) 79:36–47. doi: 10.4067/S0718-58392019000100036
40. Iloki-Assanga SB, Lewis-Luján LM, Lara-Espinoza CL, Gil-Salido AA, Fernandez-Angulo D, Rubio-Pino, JL and Haines DD. Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of *Bucida buceras* L. and Phoradendron californicum. *BMC Res Notes*. (2015) 8:396. doi: 10.1186/s13104-015-1388-1
41. Johari M, Khong HY. Total phenolic content and antioxidant and antibacterial activities of *Pereskia bleo*. *Adv Pharmacol Sci*. (2019) 2019:7428593. doi: 10.1155/2019/7428593

42. Begić S, Horozić E, Alibašić H, Bjelić E, Seferović S, Cilović Kozarević E, et al. Antioxidant capacity and total phenolic and flavonoid contents of methanolic extracts of *Urtica dioica* L. by different extraction techniques. *Int Res J Pure Appl Chem*. (2020) 21:207–14. doi: 10.9734/irjpac/2020/v21i23 30319
43. Do QD, Angkawijaya AE, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, Ju YH. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *J Food Drug Anal*. (2014) 22:296–302. doi: 10.1016/j.jfda.2013. 11.001
44. De Luna SLR, Ramírez-Garza RE, Serna Saldívar, SO. Environmentally friendly methods for flavonoid extraction from plant material: impact of their operating conditions on yield and antioxidant properties. *Sci World J*. (2020) 2020:6792069. doi: 10.1155/2020/6792069
45. Awouafack MD, Tane P, Morita H. Isolation and Structure Characterization of Flavonoids. In: Justino GC, editor. *Flavonoids*. IntechOpen. (2017). doi: 10.5772/67881
46. Ganesan P, Kumar CS, Bhaskar N. Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. *Bioresour Technol*. (2008) 99:2717–23. doi: 10.1016/j.biortech.2007.07.005
47. Adegbaju OD, Otunola GA, Afolayan AJ. Effects of growth stage and seasons on the phytochemical content and antioxidant activities of crude extracts of *Celosia argentea* L. *Heliyon*. (2020) 6:4086. doi: 10.1016/j.heliyon.2020. e04086
48. Ishiwata K, Yamaguchi T, Takamura H, Matoba T, DPPH. Radical-scavenging activity and polyphenol content in dried fruits. *Food Sci Technol Res*. (2004) 10:152–6. doi: 10.3136/fstr.10.152
49. Salamatullah AM, Özcan MM, Alkaltham MS, Uslu N, Hayat K. Influence of boiling on total phenol, antioxidant activity, and phenolic compounds of celery (*Apium graveolens* L) root. *J Food Process Preserv*. (2021) 45:15171. doi: 10.1111/jfpp.15171
50. Rocchetti G, Lucini L, Chiodelli G, Giuberti G, Montesano D, Masoero F, et al. Impact of boiling on free and bound phenolic profile and antioxidant activity of commercial gluten-free pasta. *Food Res Int*. (2017) 100:69–77. doi: 10.1016/j.foodres.2017. 08.031
51. Gunathilake KDPP, Ranaweera KKDS, Rupasinghe HPV. Changes of phenolics, carotenoids, and antioxidant capacity following simulated gastrointestinal digestion and dialysis of selected edible green leaves. *Food Chem*. (2018) 245:371–9. doi: 10.1016/j.foodchem.2017.10.096
52. Bhatt A, Patel V. Antioxidant potential of banana: study using simulated gastrointestinal model and conventional extraction. *Indian J Exp Biol*. (2015) 53:457–61.

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Optimizing Processing Technology of *Cornus officinalis*: Based on Anti-Fibrotic Activity

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Cornus officinalis, a kind of edible herbal medicine, has been widely used in the protection of liver and kidney due to its medicinal and nutritional effect. Its anti-inflammatory, anti-tumor, and anti-oxidant activities can be enhanced by wine-steamed (WS) processing. Based on the activations of hepatic stellate cells-T6 (HSC-T6) and HK-2, our study used single-factor plus orthogonal design to investigate the anti-fibrosis of *C. officinalis* processed with steamed (S), high-pressure steamed (HPS), WS, high-pressure wine-steamed (HPWS), wine-dipped (WD), and wine-fried (WF). The chemical constituents in processed *C. officinalis* with higher anti-fibrotic activities were detected by ultra-high performance liquid chromatography coupled with hybrid triple quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS/MS). Results showed that *C. officinalis* with HPWS significantly inhibited the activations of HSC-T6 and HK-2. Moreover, compounds in *C. officinalis* with HPWS were obtained *via* UHPLC-Q-TOF-MS/MS, indicating that 27 components were changed compared with raw *C. officinalis*. These results demonstrated that HPWS is the optimal processing technology for anti-fibrosis of *C. officinalis*.

Keywords: ***Cornus officinalis*, fibrosis, wine steamed, processing technology, composition difference**

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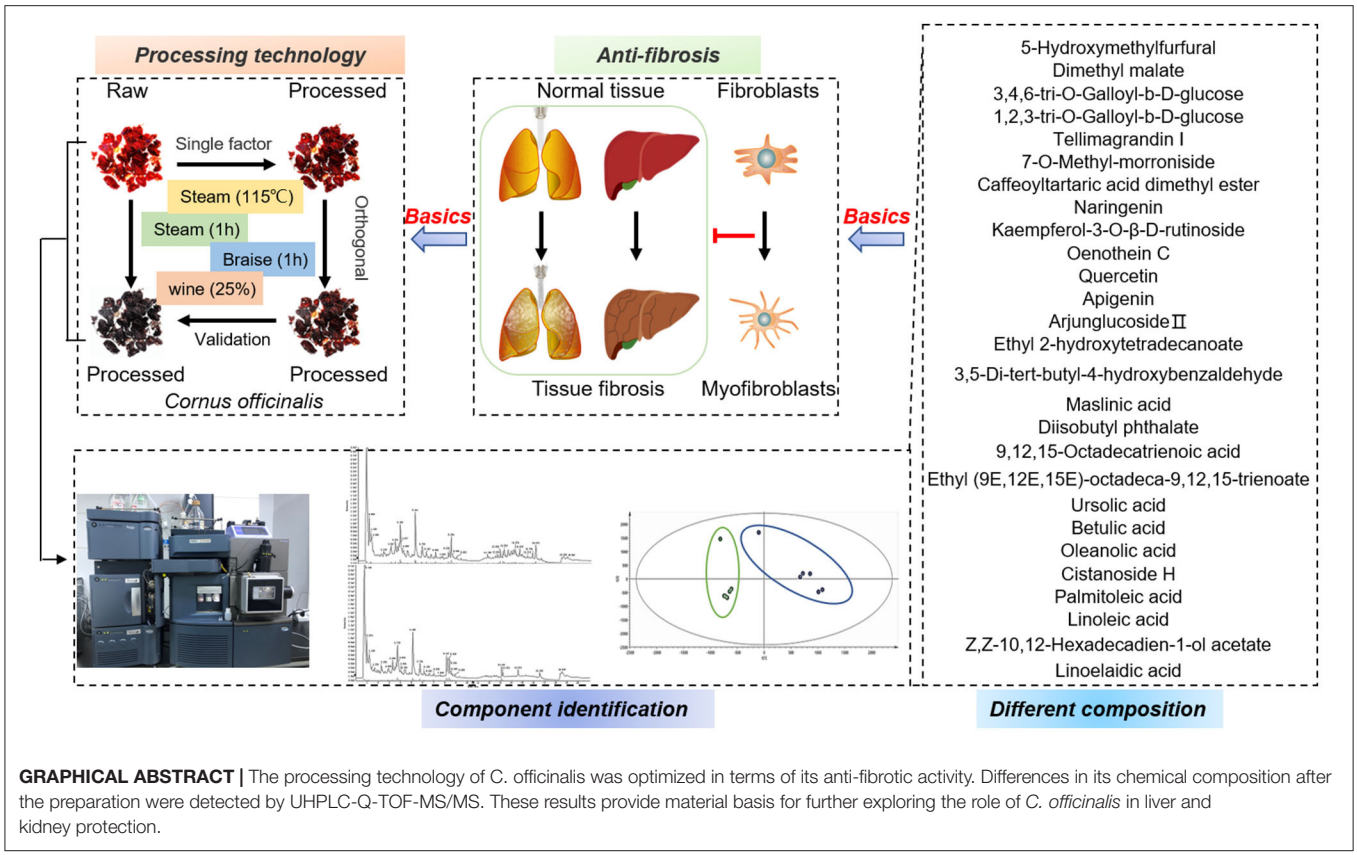
INTRODUCTION

Cornus officinalis (*C. officinalis*), the dry mature fruit of *C. officinalis* Siebold & Zucc, is redefined as a class of herb and edible plant and has been commonly used in traditional Chinese medicine (TCM) (1). *C. officinalis* with mild warm nature, belongs to the meridians of the liver and kidney according to TCM theory; thus, it is commonly used in the prevention and treatments of liver and kidney diseases (2). Moreover, it can be found in foodstuff, such as medicinal dishes, healthcare products, and drinks due to its various pharmacological activities, including anti-inflammatory, antioxidant, and anti-apoptotic (3). To date, about 305 components have been isolated and identified from *C. officinalis*, including iridoids, alkaloids, polysaccharides, flavones, organic acid, essential oils, and terpenoids (1). Among these compounds, loganin and morroniside are active ingredients in *C. officinalis* and could alleviate osteoarthritis in mice by inhibiting pyroptosis and NF-kappaB activity (4, 5). Furthermore, morroniside could ameliorate neuropathic pain through the regulation of glucagon-like peptide-1 (GLP-1) receptors. 5-hydroxymethylfurfural (5-HMF), which are mainly isolated from processed *C. officinalis*, could prevent human umbilical vein endothelial cells (HUVECs) from oxidative stress induced by glucose (6). However, the compounds in *C. officinalis* may change with processing and in turn affect pharmacological activities of



GRAPHICAL ABSTRACT | The processing technology of C. officinalis was optimized in terms of its anti-fibrotic activity. Differences in its chemical composition after the preparation were detected by UHPLC-Q-TOF-MS/MS. These results provide material basis for further exploring the role of *C. officinalis* in liver and kidney protection.

C. officinalis. In traditional crafts of China, *C. officinalis* were often processed with fried, steamed, wined, fated, salted, and others, to meet different clinical effects. For example, wined *C. officinalis* is most commonly used in clinical preparations, such as the Liuwei Dihuang pills in China, which has better effects in nourishing the liver and kidney compared to raw (R) *C. officinalis*. However, there is no unified requirement for steamed, braised times and wine amount, all of which could lead to the difference in the compositions and activities of *C. officinalis*. Therefore, the present study aimed to optimize the processing technology of *C. officinalis* through the single-factor method plus orthogonal experiment design based on the anti-fibrotic activities of *C. officinalis*.

Tissue fibrosis, characterized by excessive deposition of extracellular matrix (ECM), is the outcome of chronic tissue damage, leading to the formation of scar tissue, if without treatments, fibrosis will promote organ dysfunction and even failure (7). The ECM is mainly derived from proliferative and fibrogenic myofibroblasts, which are fibroblast-like cells with contractile properties (8, 9). Activated hepatic stellate cells (HSC-T6) are the main source of myofibroblasts and serve as a key driver of hepatic fibrosis in liver injury (9). In normal liver, HSC-T6 are in quiescent state and involved in the storage of vitamin A (10). During liver injury, quiescent HSC-T6 are activated, which indicated by the high expression of alpha smooth muscle actin (α-SMA) and excessive deposition of ECM (11). Similar to HSC-T6, the human proximal tubular epithelial cell (HK-2) cells play a key role in tubulointerstitial fibrosis (TIF), which is the final result of chronic kidney disease and closely related to the degeneration of renal function (12). Moreover, epithelial mesenchymal transition (EMT) is the main pathogenesis of the renal fibrosis and can transform differentiated epithelial cells into myofibroblasts, which are also characterized by the high expression of α-SMA (13, 14). During fibrogenesis, EMT may be driven by transforming growth factor β (TGF-β), which is the most important pro-fibrotic growth factors (15). Apart from these, TGF-β is regarded as a common vital switch for fibrosis in tissue or organs in response to chronic injuries (16).

In summary, we will evaluate the effects of *C. officinalis* on anti-fibrosis *via* the expressions of α-SMA in TGF-β induced-HSC-T6 and HK-2 cells, with the goal of exploring the protective effect of *C. officinalis* on the liver and kidney, and finally select the processing technology with higher anti-fibrotic activity. Moreover, the changes of chemical constituents in processed

Abbreviations: TCM, traditional Chinese medicine; 5-HMF, 5-hydroxymethylfurfural; HUVECs, human umbilical vein endothelial cells; ECM, extracellular matrix; α-SMA, alpha smooth muscle actin; HK-2, human proximal tubular epithelial cell; TIF, tubulointerstitial fibrosis; HSC-T6, hepatic stellate cells; EMT, epithelial mesenchymal transition; TGF-β, transforming growth factor β; UHPLC-Q-TOF-MS/MS, ultra-high performance liquid chromatography coupled with hybrid triple quadrupole time-of-flight mass spectrometry; ChP, Pharmacopeia of the People's Republic of China; HPS, high-pressure steamed; HPWS, high-pressure wine steamed.

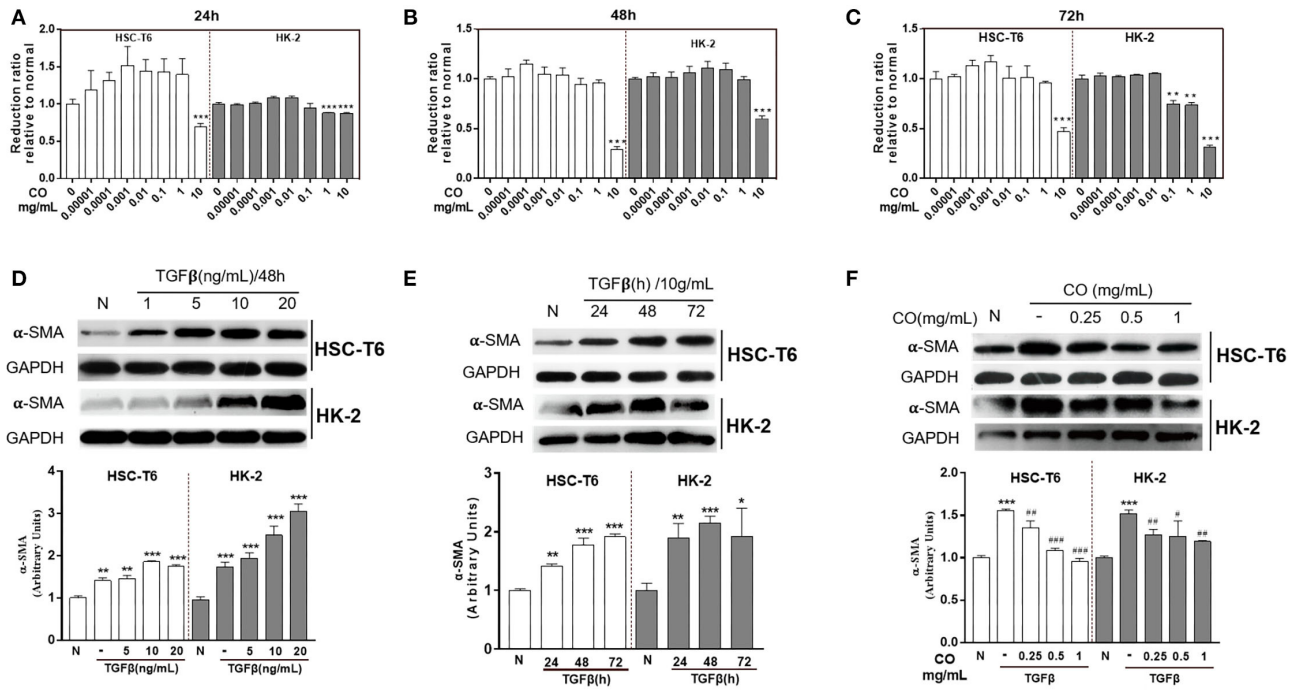


FIGURE 1 | Pharmacodynamic evaluation of *C. officinalis*. The cytotoxicity of *C. officinalis* on HSC-T6 and HK2 cells for 24 **(A)**, 48 **(B)**, and 72 h **(C)**. HSC-T6 and HK2 cells were activated by TGF-β with different concentrations **(D)** and for different times **(E)**. **(F)** The effects of *C. officinalis* on activated HSC-T6and HK2 cells. Data are represented as means ± SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ vs. the control group; $^{\#}p \leq 0.05$, $^{\#}p \leq 0.01$, and $^{\#\#\#}p \leq 0.001$ vs. the TGF-β group.

C. officinalis will be detected by ultra-high performance liquid chromatography coupled with hybrid triple quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS/MS).

MATERIALS AND METHODS

Plant Materials and Processing Technology

The samples of *C. officinalis* were selected from the PanAn City of Zhejiang Province and identified by Professor Jianwei Chen of the Department of Chinese Medicine Identification, School of Pharmacy, Nanjing University of Chinese Medicine. According to single-factor experiments, *C. officinalis* samples were steamed at different times (1, 2, 4, 6, and 8 h), different temperatures (100, 105, 110, 115, 120, and 125°C) and then dried at 60°C, respectively. Next, the samples were braised at different times (0.5, 1, 2, and 4 h) with different dosages of rice wine (w/w) (15, 20, 25, 30, and 40%), respectively. These samples were then steamed again for 1 h at 105°C and then finally dried at 60°C. The orthogonal experimental design was based on the results of single-factor experiments as discussed in the following: The *C. officinalis* samples were processed with three factors (steamed times, steamed temperatures, and braised times) at different dosages of rice wine in line with L^9 (3^4) design orthogonal table shown in S1, S3, and S5, respectively. Moreover, the optimal parameters of *C. officinalis* with high-pressure wine steamed (HPWS) were identified with steamed for 1 h at 115°C, braised time of 1 h, and rice wine dosage (w/w) at 25%. The HPS was obtained with processed for 1 h at 125°C. Additionally, *C. officinalis* could be processed with wine-dipped (WD) and wine fried (WF). *Cornus officinalis* that steamed for 1 h was named as S.

Extraction Preparation

The processed *C. officinalis* (both R and processed) was crushed and sifted through 16-mesh screen. The powder (5 g) was refluxed with 90% ethanol (50 ml) twice and filtered with four layers of gauze. The filtrates were collected and combined. Next, the filtrates were transformed into freeze-dried powder through vacuum concentration and lyophilization.

UHPLC-Q-TOF-MS/MS Analysis

The LC–MS/MS analysis were performed using an UHPLC (Shimadzu LC-30AD, Japan) coupled with a Triple TOF 5600 Plus System (AB Sciex, USA) (17, 18). The parameters that were followed were as follows: Column temperature was 30°C; mobile phase was acetonitrile (B) and 0.1% formic acid in water (A); flow rate was 0.3 ml/min; and the injection volume was 3 μl. The gradient elution procedure was as follows: 0–3.0 min, 5–20% B; 3.0–7.0 min, 20–80% B; 7.0–30 min, 80–90% B; 30–32 min, 90–5% B; 30–32 min, B was maintained at 5%. For UHPLC separation, the samples were analyzed with a 2.1 mm × 100 mm ZORBAX Extend-C18 1.8 μm column (Agilent, USA). The conditions in both electrospray ionization (ESI), positive and negative modes were set as follows: The ion source temperature was 550°C, IonSpray voltage was 5,500–5,500 V, auxiliary spray gas was nitrogen, Ion Source Gas1 (Gas1) was 55 psi, Ion

Source Gas2 (Gas2) was 55 psi, curtain gas (CUR) was 35 psi, declustering potential (DP) was 60 V, and collision energy was 30 V. The scanned ranges of TOF–MS and TOF–MS/MS were 100–2,000 and 50–1,000 Da, respectively.

Cell Culture and Treatment

The HSC-T6 and HK-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Abcam, UK) and Dulbecco's Modified Eagle Media DMEM/F12 (Abcam, UK), respectively, supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (FBS) (Abcam, UK) under 5% CO_2 at 37°C. When establishing liver fibrosis *in vitro*, HSC-T6 and HK-2 cells were cultured in six-well plates and treated with TGF-β (PeproTech, USA), respectively. To detect the effects of *C. officinalis* on anti-fibrosis, cells were treated with extracts derived from *C. officinalis* (both R and processed).

Western Blot Analysis

The cells were lysed using radio-immunoprecipitation assay (RIPA) buffer supplemented with 1% phenylmethanesulfonyl fluoride (PMSF) (Solarbio, Beijing, China) and then quantified by BCA protein assay kit (Beyotime, Shanghai, China). The equivalent protein samples were separated by sodium dodecyl sulfate–polyacrylamid gel electrophoresis (SDS–PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (GE, Freiburg, Germany). The membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated with anti-α-SMA antibody (Abcam, UK) overnight at 4°C. The membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature and visualized with ECL Reagent (Beyotime, Shanghai, China). Finally, the membranes were stripped and probed with GAPDH (loading control). The intensities of bands were quantified by Quantity One software (Bio-Rad, Hercules, CA, USA).

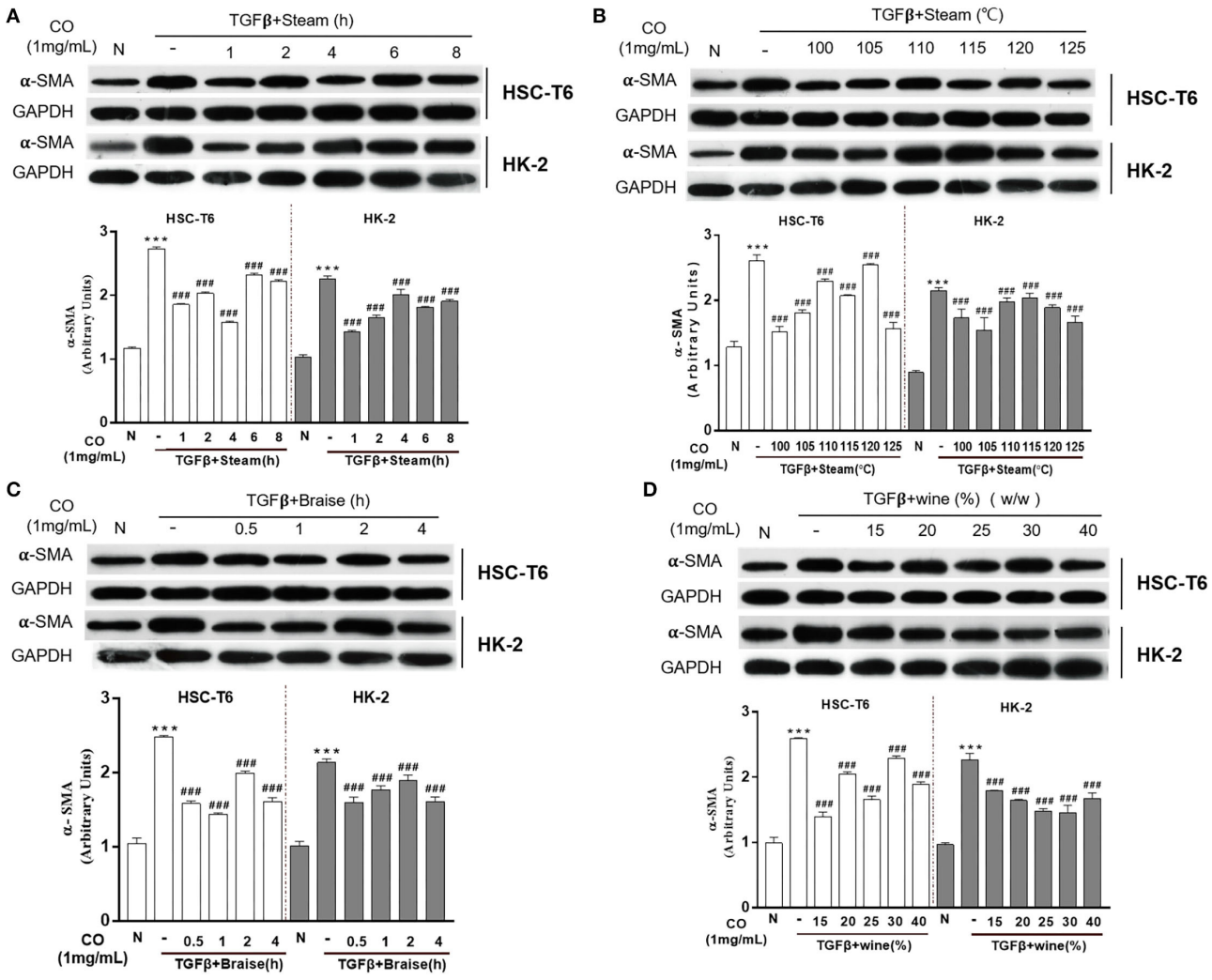


FIGURE 2 | Effects of processed *C. officinalis* on anti-fibrosis changes with univariate elements. Anti-fibrosis activities of the *C. officinalis* samples steamed at different times **(A)**, different temperatures **(B)**, braised at different times **(C)**, and with different rice wine dosages (w/w) **(D)**. Data are represented as means ± SEM. $^{***}p \leq 0.001$ vs. the control group. $^{\#\#\#}p < 0.01$ vs. the TGF-β group.

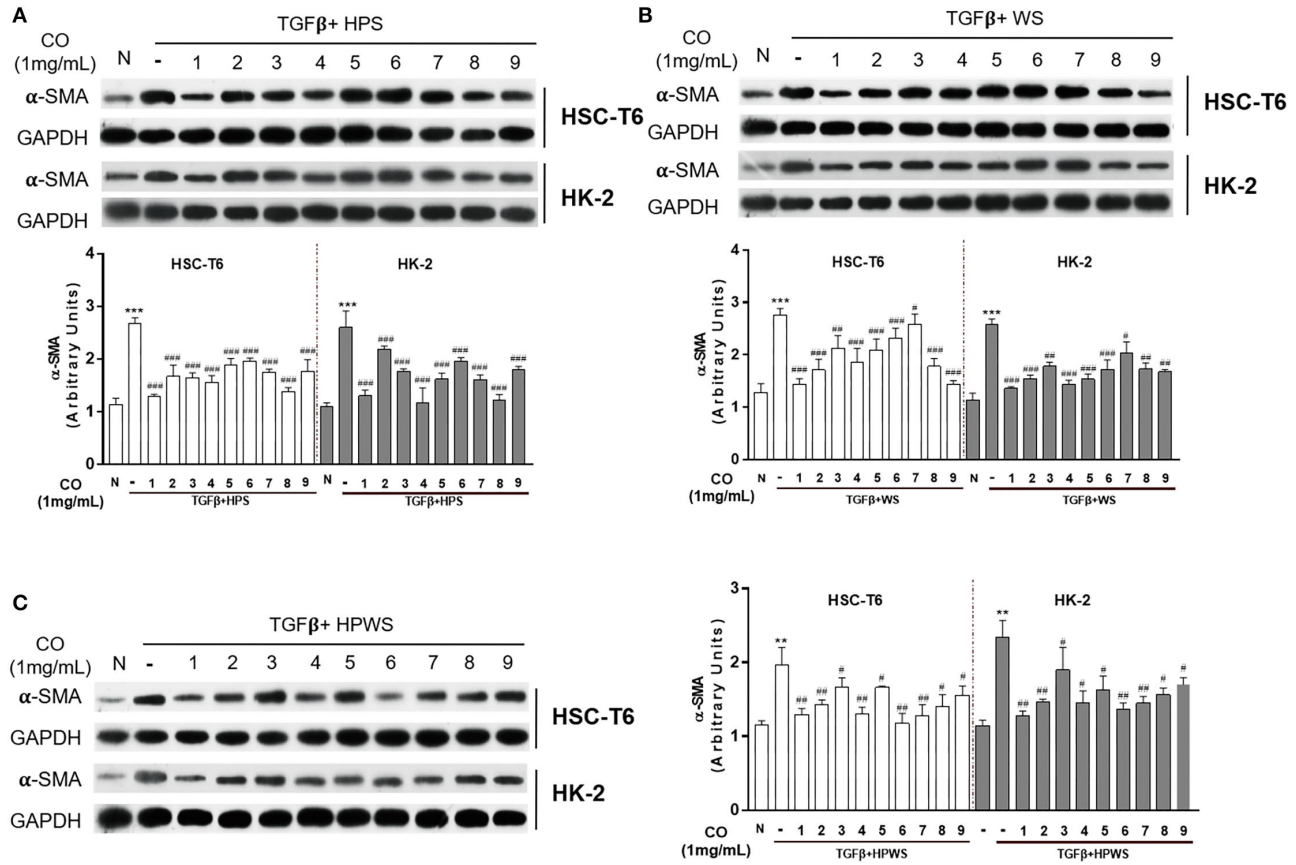


FIGURE 3 | Optimization of the processing technology with orthogonal test. Anti-fibrosis activities of the *C. officinalis* samples processed with HPS **(A)**, WS **(B)**, and HPWS **(C)** technologies. Data are represented as means ± SEM. $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ vs. the control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.01$ vs. the TGF-β group.

Immunocytochemistry

The HSC-T6 cells were cultured in six-well plates and fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then incubated with rabbit polyclonal anti-α-SMA (1:100 dilution) (Abcam, UK) at 4°C overnight. The cells were then incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 488, 1:200 dilution), after which the cell nuclei were stained with DAPI (Beyotime, Shanghai, China). Finally, the images were observed and analyzed with a Nikon TI-E fluorescence microscope.

Statistical Analysis

The data in the experiments were present as mean ± SD. The comparison of the results was evaluated by GraphPad Prism program (Graphpad Software, Inc., San Diego, CA, USA) with one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. Statistical significance between groups was considered with a $p < 0.05$. The difference analyses were detected by MarkerView™ and *t*-test. The original data were imported into MarkerView™ and statistical analysis *via* *t*-test. The *t*-test was employed to identify significant differences among processed products. Here, $p < 0.05$ was considered a significant difference, and a $t > 0$ indicated an increase in ingredient contents.

RESULTS AND DISCUSSION

Evaluation of *C. officinalis* (R) on Anti-fibrosis

The cytotoxicity of *C. officinalis* (R) on HSC-T6 and HK-2 cells were evaluation by CCK8, which is a widely method in the detection of cytotoxicity and drug sensitivity. In this study, HSC-T6 and HK-2 cells were treated with different doses of *C. officinalis* (0–10 mg/ml) for 24, 48, and 72 h. Results showed that *C. officinalis* (R) at a dose range of 0–1 mg/ml had little cytotoxicity on HSC-T6, while the sample at a dose of 10 mg/ml significantly inhibited the survival of cells compared with the normal sample at 24, 48, and 72 h. For HK-2 cells, the minimal cytotoxicity of *C. officinalis* at a dose range of 0–1 mg/ml was observed only at 48 h and was not observed at 24 and 72 h (**Figures 1A–C**, respectively). Therefore, we chose 48 h for the follow-up experiments.

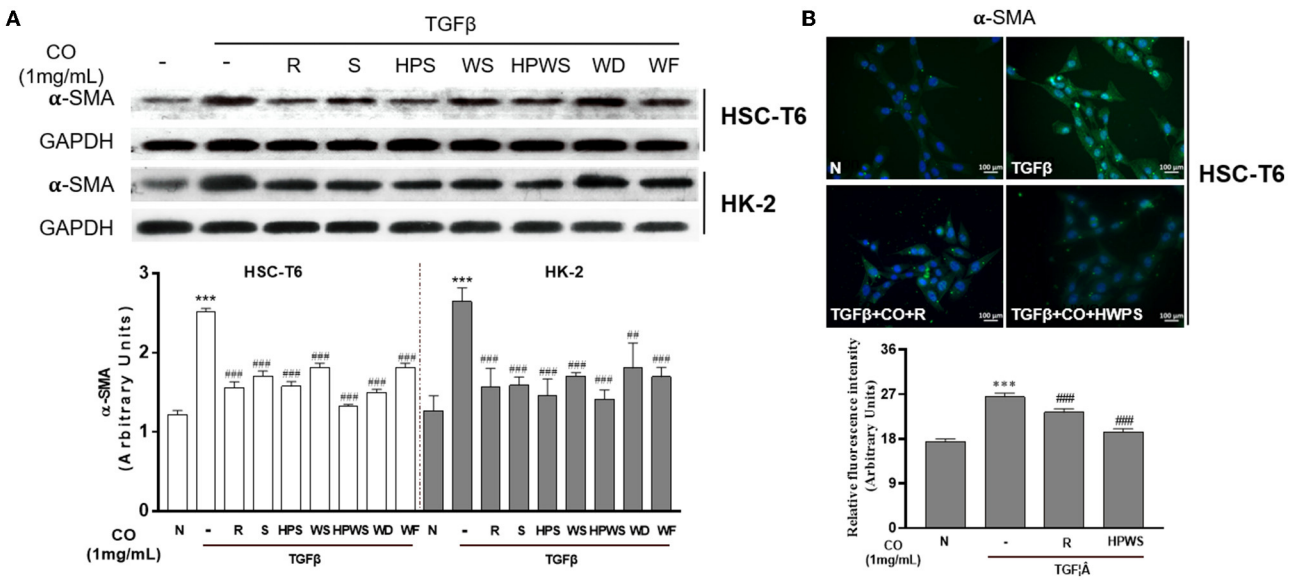


FIGURE 4 | Validation of processed *C. officinalis* in terms of anti-fibrosis activity. The anti-fibrosis activities of all processed *C. officinalis* samples were detected by Western blot analysis **(A)**; the effects of immunofluorescent staining of α-SMA in HSC-T6 treated with *C. officinalis* processed with HPWS present in 400× magnification **(B)**. Data are represented as means ± SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ vs. the control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.01$ vs. the TGF-β group.

To establish a preferable fibrosis model *in vitro*, HSC-T6 and HK-2 cells were treated with different concentrations of TGF-β at different times. The results showed that TGF-β at 1, 5, 10, and 20 ng/ml activated HSC-T6 and HK-2 cells, which manifested by the higher expressions of α-SMA compared to the normal group (**Figure 1D**), moreover, TGF-β at different times 24, 48, and 72 h also up-regulated the expressions of α-SMA in HSC-T6 and HK-2 cells (**Figure 1E**). On comparing the results, TGF-β (10 ng/ml) at 48 h was used to activate the cells. Moreover, **Figure 1D** showed that *C. officinalis* (R) at different doses (0.25, 0.5, and 1 mg/ml) inhibited the expressions of α-SMA increased by TGF-β in HSC-T6 and HK-2 cells, with the best effect identified at a dose of 1 mg/ml (**Figure 1F**).

In TCM, *C. officinalis* was often used in replenishing the liver and kidney due to its tonic effect (1). Furthermore, modern pharmacology indicated that *C. officinalis* showed low toxicity on cells only at high concentrations (19), as confirmed by the results of CCK8. The present study also provided a theoretical basis for further research on liver- and kidney-related diseases. In liver and kidney fibrosis, persistent or dysregulated fibrogenic reactions may hamper regeneration and promote dysfunction (20), which could ultimately raise susceptibility to organ failure and death (21). During these processes, TGF-β contributes to a fibrogenic phenotype by activating fibroblasts cells (20), including HSC-T6 and HK-2 cells. Taking all these elements into account, the present study aimed to develop a therapeutic implementation of anti-TGF-β approaches. The results showed that *C. officinalis* down-regulated the TGF-β-induced expression of α-SMA and provided a direction for us to optimize the processing technology of *C. officinalis* with higher anti-fibrotic activity.

Effects of Processed *C. officinalis* on Anti-fibrosis Changes With Univariate Elements

The processing methods of *C. officinalis* found in the Pharmacopeia of the People's Republic of China (ChP) including wine-steamed (WS) and wine-braised processing. To identify and optimize the processing technology with the best anti-fibrosis activity, single-factor experiment was used in this study. The results showed that *C. officinalis* steamed at different times (1, 2, 4, 6, and 8 h) or temperatures (100, 105, 110, 115, 120, and 125°C) inhibited the expressions of α-SMA induced by TGF-β both in HSC-T6 and HK-2 cells. Among these, for HSCs cells, the anti-liver fibrosis activity of *C. officinalis* steamed for 4 h at 100°C was the best, while steamed at a temperature of 105°C for 1 h had the best anti-fibrosis effects on HK-2 cells (**Figures 2A,B**, respectively). Furthermore, braised *C. officinalis* for 0.5, 1, 2, and 4 h inhibited TGF-β-induced over-expressions of α-SMA in HSC-T6 and HK-2 cells, especially at 1 h in HSC-T6 and 4 h in HK2 cells (**Figure 2C**). *C. officinalis* processed with different dosages of rice wine (w/w) also showed different degrees of anti-fibrosis activities, especially 15% in HSC-T6 and 25% in HK-2 cells (**Figure 2D**).

Wined *C. officinalis* is a traditional and common processing method that applied until now. However, different indexes of evaluation are accompanied by varying wine processing techniques. Thus, this research aimed to explore the suitable wined technology of *C. officinalis* based on the anti-fibrosis synergistic effects produced by each technique. The results showed that the anti-fibrosis effect of *C. officinalis* was negatively correlated with the expression of α-SMA. Thus, the data were

TABLE 1 | Different chemical composition of the raw and HPWS *C. officinalis* products.

		Formula	Mass (Da)	Adduct	Extraction Mass (Da)	Error (ppm)	RT (min)	*t*	*p*	Changedirection
1	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.0317	$[M+H]^+$	127.03897	1.5	1.9	3.548359	0.00528	+
2	Dimethyl malate	$C_6H_{10}O_5$	162.0528	$[M-H]^-$	161.04555	5.2	2.32	−4.381084	0.00138	−
3	3,4,6-tri-O-Galloyl-b-D-glucose	$C_{27}H_{24}O_{18}$	636.0963	$[M-H]^-$	635.08899	4.5	3.87	−2.576338	0.02759	−
4	1,2,3-tri-O-Galloyl-b-D-glucose	$C_{27}H_{24}O_{18}$	636.0963	$[M-H]^-$	635.08899	5.7	3.9	3.263964	0.00852	+
5	Tellimagrandin I	$C_{34}H_{26}O_{22}$	786.0916	$[M-H]^-$	785.0843	5.8	4.54	2.879021	0.01641	+
6	7-O-Methyl-morroniside	$C_{18}H_{28}O_{11}$	420.1632	$[M-H]^-$	419.15589	2.6	4.97	4.730597	0.0008	+
7	Caffeoyltartaric acid dimethyl ester	$C_{15}H_{16}O_9$	340.0794	$[M+H]^+$	341.08671	0.1	5.01	5.844058	0.00016	+
8	Naringenin	$C_{15}H_{12}O_5$	272.0685	$[M+H]^+$	273.07575	0.9	6.73	4.134988	0.00203	+
9	Kaempferol-3-O-β-D-rutinoside	$C_{27}H_{30}O_{15}$	594.1585	$[M+H]^+$	595.16575	0.7	7.29	3.041758	0.01243	+
10	Oenothein C	$C_{34}H_{24}O_{22}$	784.0759	$[M+H]^+$	785.0832	0.1	7.32	5.861379	0.00016	+
11	Quercetin	$C_{15}H_{10}O_7$	302.0427	$[M-H]^-$	301.03538	6.9	10.42	−3.405739	0.00671	−
12	Apigenin	$C_{15}H_{10}O_5$	270.0528	$[M-H]^-$	269.04555	7.1	11.59	4.657361	0.0009	+
13	ArjunglucosideII	$C_{36}H_{58}O_{10}$	650.403	$[M-H]^-$	649.39572	2.5	11.72	3.264763	0.00851	+
14	Ethyl 2-hydroxytetradecanoate	$C_{16}H_{32}O_3$	272.2352	$[M-H]^-$	271.22787	6.5	14	6.580312	6.23E-05	+
15	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	$C_{15}H_{22}O_2$	234.162	$[M-H]^-$	233.1547	6.9	14.12	2.370131	0.03927	+
16	Maslinic acid	$C_{30}H_{48}O_4$	472.3553	$[M-H]^-$	471.34798	5.6	14.16	9.815299	1.89E-06	+
17	Diisobutyl phthalate	$C_{16}H_{22}O_4$	278.1518	$[M+H]^+$	279.15909	0.8	14.62	−8.161049	9.88E-06	−
18	9,12,15-Octadecatrienoic acid	$C_{18}H_{30}O_2$	278.2246	$[M+H]^+$	279.23186	0.8	15.77	3.118799	0.0109	+
19	Ethyl (9E,12E,15E)-octadeca-9,12,15-trienoate	$C_{20}H_{34}O_2$	306.2559	$[M+H]^+$	307.26316	−0.9	15.82	4.285773	0.0016	+
20	Ursolic acid	$C_{30}H_{48}O_3$	456.3604	$[M-H]^-$	455.35307	6.1	16.06	−2.962918	0.01422	−
21	Betulic acid	$C_{30}H_{48}O_3$	456.3604	$[M-H]^-$	455.35307	6.1	16.06	−2.962918	0.01422	−
22	Oleanolic acid	$C_{30}H_{48}O_3$	456.3604	$[M-H]^-$	455.35307	5.6	16.07	−2.962918	0.01422	−
23	Cistanoside H	$C_{16}H_{30}O_2$	254.2246	$[M-H]^-$	253.2173	5.7	16.27	4.714915	0.00082	+
24	Palmitoleic acid	$C_{16}H_{30}O_2$	254.2246	$[M-H]^-$	253.2173	3.9	16.27	4.714915	0.00082	+
25	Linoleic acid	$C_{18}H_{32}O_2$	280.2402	$[M-H]^-$	279.23295	7	16.54	10.40155	1.11E-06	+
26	Z,Z-10,12-Hexadecadien-1-ol acetate	$C_{18}H_{32}O_2$	280.2402	$[M-H]^-$	279.23295	7	16.54	10.40155	1.11E-06	+
27	Linoelaidic acid	$C_{18}H_{32}O_2$	280.2402	$[M-H]^-$	279.23295	4.6	16.55	10.40155	1.11E-06	+

non-negatively analyzed, which are discussed as follows: Xij = max (X1j, X2j, …, Xnj) – Xij/max (X1j, X2j, …, Xnj) – min (X1j, X2j, …, Xnj) + 1; i = 1, 2, …, n, j = 1, 2, …, n). Moreover, the expressions of α-SMA in HSC-T6 and HK-2 cells were used as indicators for comprehensive scoring, each with a weight of 0.5. The composite score was calculated using the following formula: Xij (HK-2) × 0.5 + Xij (HSC-T6) × 0.5. The results showed that *C. officinalis* processed with the following parameters: steamed time of 1 h, steamed temperatures of 105 and 125°C, braised times of 0.5 and 4 h, and rice wine dosage (w/w) at 25%, respectively, had the best inhibitory effects on activated HSC-T6 and HK-2; thus, laying the foundation for the subsequent orthogonal experiments. However, considering the time benefit, the term of orthogonal experiment was finally determined as follows: The steamed times were 1, 2, and 3 h; steamed temperatures were 105, 115, and 125°C; braised times were 0.5, 1, and 1.5 h; and rice wine dosages (w/w) were 25, 30, and 35%.

Optimization of the Processing Technology With Orthogonal Test

In accordance with the terms of the single-factor experiments, the processing parameters were optimized with an orthogonal L^9 (3^4) test design. Our results showed that *C. officinalis* processed with three levels of two factors (steamed times and steamed temperatures), as shown in **Supplementary Table S1**, inhibited the expressions of α-SMA in HSC-T6 and HK-2 induced by TGF-β (**Figure 3A**). The composite score of the anti-fibrosis effects was calculated with non-negative analysis, and the results indicated that the maximum was 1.92 (**Supplementary Table S1**). Furthermore, in selecting the better processing term, the values of K and R are shown in **Supplementary Table S1**, and the variance analysis results are shown in **Supplementary Table S2**. These results showed that *C. officinalis* steamed (HPS) showed optimum anti-fibrosis effect. The craft conditions of *C. officinalis* with WS were also optimized with an orthogonal design (**Supplementary Table S3**), and its anti-fibrosis effects on both HSC-T6 and HK-2 were evaluated *via* the expressions of α-SMA induced by TGF-β (**Figure 3B**). The results indicated that *C. officinalis* with WS inhibited the activation of myoblasts, which was mainly manifested by the decrease in levels of α-SMA (**Figure 3B**). Combined with the results of variance analysis (**Supplementary Table S4**) and the values of *K* and *R* in **Supplementary Table S3**, the optimal processing term with anti-fibrosis effects were as follows: steamed time of 2 h, braised time of 0.5 h, and rice wine dosage (w/w) of 30%. Similarly, steamed HWPS were

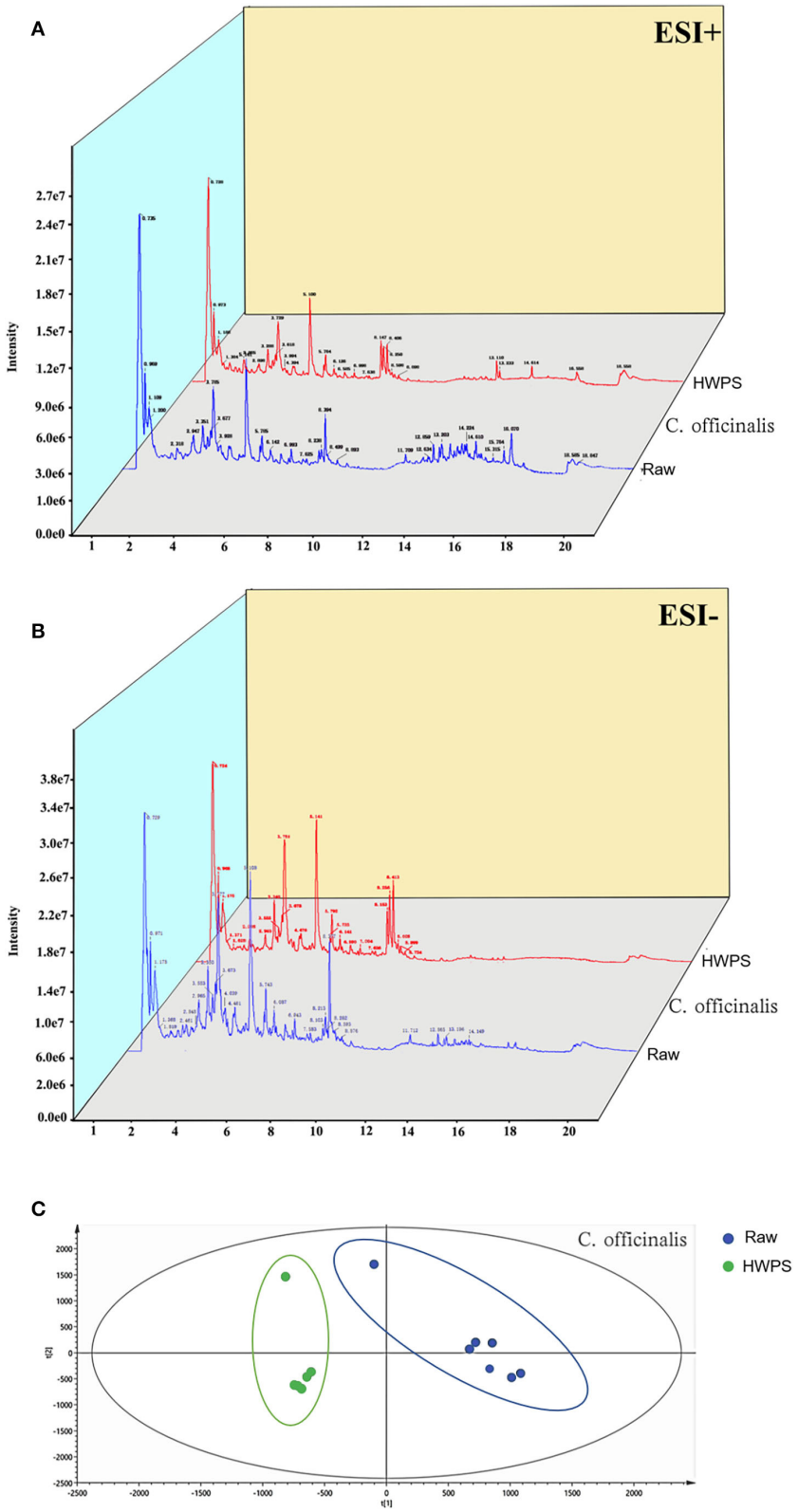


FIGURE 5 | Ingredient identification of *C. officinalis* processed with HPWS. **(A)** Raw sample positive and negative ion pattern diagram. **(B)** The product's positive and negative ion pattern is illustrated. **(C)** PLS-DA score plot.

optimized with orthogonal design (**Supplementary Table S5**), variance analysis (**Supplementary Table S6**), and the expression of α-SMA (**Figure 3C**).

Due to the fact that steamed time, steamed temperature, braised time, and rice wine dosage are vital criteria in the processing of *C. officinalis* (22), processed *C. officinalis* using different technologies have varying antidiabetic effects (23). Besides, the results of our study showed that the *C. officinalis* had different inhibitory effects on activated fibrosis cells. Therefore, optimization of processing criteria is the essential step in screening the technology with the optimal anti-fibrosis effect. Studies have shown that the orthogonal test design is a common method for the optimization of experimental conditions (24, 25). Based on these, the current study used the orthogonal L^9 (3^4) test design to detect the anti-fibrosis of *C. officinalis* processed with different factors for the single-factor experiments and obtained several artifacts of *C. officinalis via* S, WS, and HPWS technologies.

Validation of Processed *C. officinalis* in Terms of Anti-fibrosis Effects

The optimal processing technologies of processed *C. officinalis* were determined by single-factor plus orthogonal experiments. The processed products of *C. officinalis* were prepared according to the terms of HPWS and the stipulation in Chp, including WD and WF technology. The expressions of α-SMA were detected by western blot to verify the anti-fibrosis activity of all processed products of *C. officinalis*. The results showed that *C. officinalis* (both R and processed products) inhibited the expressions of α-SMA induced by TGF-β in both HSC-T6 and HK-2, especially *C. officinalis* processed with HPWS (**Figure 4A**). Then, immunofluorescence assay for α-SMA in HSC-T6 was conducted to confirm the anti-fibrosis effect enhanced by *C. officinalis* processed with HPWS. The results also showed that *C. officinalis* inhibited the positive expression of α-SMA (in green) induced by TGF-β, especially the *C. officinalis* processed with HPWS (**Figure 4B**).

Based on the single-factor, orthogonal tests, variance analysis, and the evaluation of anti-fibrosis activity, *C. officinalis* processed with HPWS showed better anti-fibrosis activity than other processed products of *C. officinalis*. The differences in the anti-fibrosis activities of *C. officinalis* are mainly due to various active components in the *C. officinalis* (26), which are possible to transform with qualitative and quantitative changes during processing. Therefore, exploring changes of composition in *C. officinalis* processed with HPWS can provide material basis for further clarifying the mechanism of *C. officinalis* with HPWS-enhanced anti-hepatic fibrosis.

Ingredient Identification of *C. officinalis* Processed With HPWS

The analysis of the compounds in *C. officinalis* (both R and HPWS) were identified by ESI positive and negative modes (**Figure 5**). The chemical name, molecular mass, molecular formula, and molecular structure of components in *C. officinalis* were retrieved and download from the database, after which the accurate mass-to-charge ratio of plasma morphology were calculated in both ESI positive and negative modes. The raw data were imported into the PeakView™ software. All the chemical components were encoded and a new session was established under the XIC Manager template. Then, the first-level data matching was conducted with reference standards, standard mass spectrometric database, and literature according to m/z. The chromatographic peak with the retention time error within 0.2 min and the m/z error within 10 ppm was identified as a unified compound. Further, the identification validation and chromatographic peak attribution were based on molecular structures and secondary fragments of compounds.

As shown in **Table 1**, 27 components in *C. officinalis* were changed with HPWS, including flavonoids, iridoid glycosides, and organic acids. Moreover, 5-HMF was a typical emerging compound in *C. officinalis* with HPWS. We take quercetin as an example to illustrate the identification procedure: $[M - H]^-$ of peak 11 was 301.0354, formula was calculated as $C_{15}H_{10}O_7$ with mass (Da) 302.0427, the main secondary fragment was 121.0305, and neutral loss was 180.0019. After consulting the literature (27) and comparing the results with standards, we confirmed that the compound is quercetin.

To further excavate the differences in *C. officinalis* between R and HPWS products, the data were standardized with SIMCA 14.1 (version, country) and analyzed *via* PLS-DA under supervised recognition mode. The results in **Figure 5** show the PLS-DA score plot of *C. officinalis* before and after processing. As can be seen, *C. officinalis* processed with R and HPWS s were obviously clustered into two categories, indicating that the processing has changed the chemical composition in *C. officinalis*.

The current results also showed that along with HPWS processing the chemical composition in *C. officinalis* changed qualitatively, including 5-HMF, linoelaidic acid, and quercetin; 5-HMF prevents L02 hepatocytes from injury induced by GalN/TNF-α (28) and attenuates liver fibrosis by inhibiting oxidative stress in mice (29). Furthermore, quercetin can protect the liver and kidney, as reported in another study (30). Thus, this research could reveal a chemical basis for the enhanced anti-fibrosis activity of *C. officinalis* processed with HPWS.

CONCLUSIONS

As a kind of medicine and edible herbal, *C. officinalis* has been shown to suppress liver and kidney fibrosis by inhibiting the activation of HSC-T6 and HK-2 cells. Studies have also shown that its anti-fibrosis activity can change with processing. Based on this information, our experiment used enhanced anti-fibrosis effects as the indicator to optimize the processing technology of *C. officinalis* through single-factor and orthogonal tests. Finally, we identified the processing that can produce optimal anti-fibrosis activity, namely, *C. officinalis* with HPWS, whose chemical composition was identified by UHPLC-Q-TOF-MS/MS analysis. The experimental result is a further step. The results of this study also provided material basis for further exploring the role of *C. officinalis* in liver and kidney protection.

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/s.

AUTHOR CONTRIBUTIONS

GC and PX contributed to the design of the study, acquisition of data, and analysis and interpretation of the data. XH, CD, and YN contributed to the acquisition of data and analysis and interpretation of data. All authors participated in drafting or revising the manuscript and approved the final version of the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.807071/full#supplementary-material

Supplementary Figure 1 | Anti-fibrotic activity of main ingredients in *C. officinalis* processed on HSC-T6.

Supplementary Table S1 | *C. officinalis* with HPS.

Supplementary Table S2 | Variance analysis of *C. officinalis* with HPS.

Supplementary Table S3 | *C. officinalis* with WS.

Supplementary Table S4 | Variance analysis of *C. officinalis* with WS.

Supplementary Table S5 | *C. officinalis* with HPWS.

Supplementary Table S6 | Variance analysis of *C. officinalis* with HPWS.

REFERENCES

1. Huang J, Zhang Y, Dong L, Gao Q, Yin L, Quan H, et al. Ethnopharmacology, phytochemistry, and pharmacology of *Cornus officinalis* Sieb. et Zucc. *J Ethnopharmacol*. (2018) 213:280–301. doi: 10.1016/j.jep.2017.11.010
2. Gao X, Liu Y, An Z, Ni J. Active components and pharmacological effects of *Cornus officinalis*: literature review. *Front Pharmacol*. (2021) 12:633447. doi: 10.3389/fphar.2021.633447
3. Quah Y, Lee SJ, Lee EB, Birhanu BT, Ali MS, Abbas MA, et al. *Cornus officinalis* ethanolic extract with potential anti-allergic, anti-inflammatory, and antioxidant activities. *Nutrients*. (2020) 12:3317. doi: 10.3390/nu12113317
4. Hu J, Zhou J, Wu J, Chen Q, Du W, Fu F, et al. Loganin ameliorates cartilage degeneration and osteoarthritis development in an osteoarthritis mouse model through inhibition of NF-κB activity and pyroptosis in chondrocytes. *J Ethnopharmacol*. (2020) 247:112261. doi: 10.1016/j.jep.2019.112261
5. Yu H, Yao S, Zhou C, Fu F, Luo H, Du W, et al. Morroniside attenuates apoptosis and pyroptosis of chondrocytes and ameliorates osteoarthritic development by inhibiting NF-kappaB signaling. *J Ethnopharmacol*. (2021) 266:113447. doi: 10.1016/j.jep.2020.113447
6. Cao G, Cai H, Cai B, Tu S. Effect of 5-hydroxymethylfurfural derived from processed Cornus officinalis on the prevention of high glucose-induced oxidative stress in human umbilical vein endothelial cells and its mechanism. *Food Chem*. (2013) 140:273–9. doi: 10.1016/j.foodchem.2012.11.143
7. Parola M, Pinzani M. Pathophysiology of organ and tissue fibrosis. *Mol Aspects Med*. (2019) 65:1. doi: 10.1016/j.mam.2019.02.001
8. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev*. (2008) 88:125–72. doi: 10.1152/physrev.00013.2007
9. Higashi T, Friedman SL, Hoshida Y. Hepatic stellate cells as key target in liver fibrosis. *Adv Drug Deliv Rev*. (2017) 121:27–42. doi: 10.1016/j.addr.2017.05.007
10. Puche JE, Saiman Y, Friedman SL, Hepatic stellate cells and liver fibrosis. *Compr Physiol*. (2013) 3:1473–92. doi: 10.1002/cphy.c120035
11. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest*. (2005) 115:209–18. doi: 10.5152/tjg.2018.17330
12. Zhang Y, Li K, Li Y, Zhao W, Wang L, Chen Z, et al. Profibrotic mechanisms of DPP8 and DPP9 highly expressed in the proximal renal tubule epithelial cells. *Pharmacol Res*. (2021) 169:105630. doi: 10.1016/j.phrs.2021.105630
13. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest*. (2002) 110:341–50. doi: 10.1172/JCI1551
14. Loeffler I, Wolf G. Epithelial-to-mesenchymal transition in diabetic nephropathy: fact or fiction? *Cells*. (2015) 4:631–52. doi: 10.3390/cells4040631
15. Meng XM, Tang PM, Li J, Lan HY. TGF-β/Smad signaling in renal fibrosis. *Front Physiol*. (2015) 6:82. doi: 10.3389/fphys.2015.00082
16. Weiskirchen R, Weiskirchen S, Tacke F. Organ and tissue fibrosis: molecular signals, cellular mechanisms and translational implications. *Mol Aspects Med*. (2019) 65:2–15. doi: 10.1016/j.mam.2018.06.003
17. Duan H, Wang GC, Khan GJ, Su XH, Guo SL, Niu YM, et al. Identification and characterization of potential antioxidant components in Isodon amethystoides (Benth.) Hara tea leaves by UPLC-LTQ-Orbitrap-MS. *Food Chem Toxicol*. (2021) 148:111961. doi: 10.1016/j.fct.2020.111961
18. Duan H, Wang W, Li Y, Jilany Khan G, Chen Y, Shen T, et al. Identification of phytochemicals and antioxidant activity of *Premna microphylla* Turcz. stem through UPLC-LTQ-Orbitrap-MS. *Food Chem*. (2022) 373(Pt B):131482. https://doi.org/10.1016/j.foodchem.2021.131482
19. Kao ST, Wang SD, Lin CC, Lin LJ. Jin Gui Shen Qi Wan, a traditional Chinese medicine, alleviated allergic airway hypersensitivity and inflammatory cell infiltration in a chronic asthma mouse model. *J Ethnopharmacol*. (2018) 227:181–90. doi: 10.1016/j.jep.2018.08.028
20. Frangogiannis N. Transforming growth factor-β in tissue fibrosis. *J Exp Med*. (2020) 217:e20190103. doi: 10.1084/jem.20190103
21. Eming SA, Wynn TA, Martin P. Inflammation and metabolism in tissue repair and regeneration. *Science*. (2017) 56:1026–30. doi: 10.1126/science.aam7928
22. Cao G, Cai H, Zhang Y, Cong X, Zhang C, Cai B. Identification of metabolites of crude and processed Fructus Corni in rats by microdialysis sampling coupled with electrospray ionization linear quadrupole ion trap mass spectrometry. *J Pharm Biomed Anal*. (2011) 56:118–25. doi: 10.1016/j.jpba.2011.04.013
23. Ma W, Wang KJ, Cheng CS, Yan GQ, Lu WL, Ge JF, et al. Bioactive compounds from Cornus officinalis fruits and their effects on diabetic nephropathy. *J Ethnopharmacol*. (2014) 153:840–5. doi: 10.1016/j.jep.2014.03.051
24. Cai DJ, Shu Q, Xu BQ, Peng LM, He Y. Orthogonal test design for optimization of the extraction of flavonid from the Fructus Gardeniae. *Biomed Environ Sci*. (2011) 24:688–93. doi: 10.3967/0895–3988.2011.06.015
25. Wei L, Huang X, Huang Z, Zhou Z. Orthogonal test design for optimization of lipid accumulation and lipid property in *Nannochloropsis oculata* for biodiesel production. *Bioresour Technol*. (2013) 147:534–8. doi: 10.1016/j.biortech.2013.08.079
26. Dong Y, Feng ZL, Chen HB, Wang FS, Lu JH. Corni Fructus: a review of chemical constituents and pharmacological activities. *Chin Med*. (2018) 13:34. doi: 10.1186/s13020–018-0191-z

27. Cai H, Cao G, Cai B. Rapid simultaneous identification and determination of the multiple compounds in crude Fructus Corni and its processed products by HPLC-MS/MS with multiple reaction monitoring mode. *Pharm Biol*. (2013) 51:273–8. doi: 10.3109/13880209.2012.720689
28. Jiang ZQ, Ma YX, Li MH, Zhan XQ, Zhang X, Wang MY. 5-Hydroxymethylfurfural protects against ER stress-induced apoptosis in GalN/TNF-α-injured L02 hepatocytes through regulating the PERK-eIF2α signaling pathway. *Chin J Nat Med*. (2015) 13:896–905. doi: 10.1016/S1875–5364(15)30095–9
29. Han XY, Hu JN, Wang Z, Wei SN, Zheng SW, Wang YP, et al. 5-HMF attenuates liver fibrosis in CCl4-plus-alcohol-induced mice by suppression of oxidative stress. *J Nutr Sci Vitaminol*. (2017) 63:35–43. doi: 10.3177/jnsv.63.35
30. Batiha GE, Beshbishy AM, Ikram M, Mulla ZS, El-Hack MEA, Taha AE, et al. The pharmacological activity, biochemical properties, and pharmacokinetics of the major natural polyphenolic flavonoid: quercetin. *Foods*. (2020) 9:374. doi: 10.3390/foods9030374

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De-Oiled Citrus Peels as Feedstock for the Production of Pectin Oligosaccharides and Its Effect on *Lactobacillus fermentum*, Probiotic Source

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Following the extraction of essential oil, citrus (Mousambi, Kinnow, and Orange) peel wastes were used to produce pectin. The yield of essential oil and pectin was maximum in orange. Pectin was characterized by Fourier-transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD) spectroscopy. The degree of esterification (DE) and methoxyl content (MC) was maximum in orange whereas, the equivalent weight was maximum in Mousambi. A significant increase (61.8%) in the *Lactobacillus fermentum* population was observed with pectin as compared with sugar. Three sources followed the Orange > Kinnow > Mousambi trend as a prebiotic source. It was attributed to higher DE as well as higher MC. Enhancement in the bacterial population was in the range of 79.16–87.50%. The present work confirms the potential of pectin as a probiotic source for the enhancement of the bacterial population. Thus, it has a large scope for use in the food industry targeting a circular economy.

Keywords: pectin oligosaccharides, *Citrus* sp, probiotic activity, *lactobacillus fermentum*, XRD

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INTRODUCTION

With an increase in concern about consumer's health, the importance of nutraceuticals and functional foods has increased significantly across the globe. Among a number of functional foods, prebiotics find a special place, and it has attracted consumers' interest in the recent past. With the increase in the research thrust on the human microbiome, the importance of prebiotics has multiplied several times.

In this context, pectin and pectin oligosaccharides have emerged as prominent members of the prebiotic family. Structurally, pectin is a heteropolysaccharide that, remains in the primary cell walls of earthly plants. The principal component is galacturonic acid, a sugar acid derived from galactose. These heterogalacturonans are linear chains of D-galacturonic acid linked *via* α-(1–4)-linkage. Substitution over the galacturonans can also be seen where saccharide residues, such as D-apiose or D-xylose, remain attached to the backbone of residues of D-galacturonic acid in cases of apiogalacturonan or xylogalacturonan, respectively (1). Pectin is widely used in the food industry, cosmetic sector, as well as in pharmaceuticals, mainly as a gelling agent, food stabilizer, and thickener. Pectin can be acquired from a wide range of sources with a variation in yield. About

10–20% of pectin was obtained from sunflower head residues and sugar beets (2). Other sources include cocoa husk (about 9% of dry weight) (3), soya hull with 26–28% of pectin content (4), apple peel waste (1.21% on a dry weight basis) (5), apple pomace having 14–18% pectin on a dry weight basis (6). As mentioned above, citrus peels are also a potential source of pectin obtained from different species and varieties. Approximately 25% of pectin on a dry weight basis was obtained from grapefruit peels (7). A variation in yield was observed, i.e., 15–44% based on different extraction conditions and parameters from the peels of *Citrus limon* (8). Similarly, the endocarp of *Citrus depressa* has been utilized by isolating and detailed structural characterization of pectin (9).

Pectic polysaccharides help to proliferate different gut-friendly microbes as the microbial population gets increased in the course of fermentation and different short-chain fatty acids, such as acetate and propionate, are produced. This property makes these pectic substances prebiotic compounds. The prebiotic potential of pectin was evaluated using *Bifidobacterium* and *Bacteroids* by an *in-vitro* fermentation method that showed an increase in both the population of microbes (10). Pectic oligosaccharides extracted from citrus peel were tested on *Bifidobacterium bifidum* and *Lactobacillus paracasei.* An increase in cell density was observed for both the bacteria in the case of pectin compared with non-pectin substrate (11).

According to a report by the Food and Agriculture Organization out of 124.246 million tons of citrus produced all over the world, only 23.538 million tons have been utilized for processing and trade. That helps to figure out the huge waste generated from the citrus sector, mainly in the form of peels and seeds. Almost 50% of the fruit mass is under-utilized and thrown away as waste (12). But these wastes are a storehouse of various bioactive compounds having numerous health-benefitting effects, making these wastes a huge potential for utilization in various sectors commercially. Among the bioactive compounds, functional carbohydrates especially pectic substances constitute a major fraction (13).

In the field of prebiotic research, the effect of prebiotics on beneficial microorganisms especially probiotics and lactobacilli, and the selective degradation of the prebiotic by the probiotic needs more research thrust to reach a definite conclusion (14).

Considering these facts, the current experiment was designed to evaluate the yield variations of pectin from peels of three different *Citrus* sp (*Citrus limetta*, a hybrid of *Citrus nobilis* and *Citrus deliciosa,* and *Citrus sinensis*). Another objective of the study was to evaluate the effect of these pectins on the *Lactobacillus* population for their prebiotic effect to understand their potential as a potent nutraceutical product.

MATERIALS AND METHODS

Materials

Peels of Citrus

Peels from three different citruses, i.e., peels of Mousambi (*Citrus limetta*), Kinnow [a hybrid between "King" (*Citrus nobilis*) and "Willow leaf" (*Citrus deliciosa*)], and Orange (*Citrus sinensis*) were taken for this study. The reason behind this is that these three types of citruses are mainly consumed in India and eventually generate more waste than others. All the fruits were collected from the local market of New Delhi, India (Azadpur fruit market; N 28.70°, E 77.10°), thereafter fruits were washed and peeled. Afterward, the peels were chopped into pieces and stored at 4°C for further use.

TABLE 1 | Essential oil yield and limonene content in essential oil of three *Citrus* sp.

Source	Oil content (% dry wt. basis)	dl-Limonene content (%area basis)
Mosambi peels	0.74	93.17
Kinnow peels	0.67	91.56
Orange peels	0.79	94.77

Chemicals

For extraction purposes, deionized water was utilized and obtained from a Millipore system with 18.2 MΩ cm resistance. Hydrochloric acid, ethanol, sodium chloride, sodium hydroxide, and phenol red are reagent grade and purchased from Merck® India, New Delhi. Commercially available pectin (HiMedia, Delhi) was used for the comparison of different attributes.

Extraction of Essential Oil

The essential oil was extracted from the citrus peel by hydrodistillation using the Clevenger apparatus. Three different citrus peels (250 g) were taken in a round-bottom flask and connected with the Clevenger apparatus and condenser. Reflux the flask for 6 h to get full extraction of essential oil. After completion of the experiment, the essential oil was separated physically as well as partitioned between water and diethyl ether followed by evaporation of the solvent. The removal of traces of water was eliminated by passing through the minimum quantity of anhydrous ammonium sulfate. The yield of oil was recorded for the three peels.

Characterization of Essential Oil

Essential oils were characterized by gas chromatography–mass spectrometry (GC-MS) (7890A GC, Agilent Technologies equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent Co., CA, USA), connected to a triple-axis HED-EM 5975C mass spectrometer. Carrier gas flow was 1 ml min^{-1} and the injection volume was 1 μl. Helium was used as carrier gas at a head pressure of 10 psi. In GC, the oven temperature was initially held at 40°C for 1 min, and thereafter the temperature was raised with a gradient of 3°C min^{-1} until the temperature reached 220°C. Other settings include 250°C interface temperature and ion source temperature of 200°C.

Extraction of Pectin

Pectin was extracted by following Ranganna's method (15). Normally bleaching is done to inactivate the enzymatic activity. Here, it was not required as it was done during hydrodistillation. Peels were kept in an oven at 60°C overnight for complete drying. After the peels were completely dried, they were ground into fine

powder. About 5 g of powdered peels were put into 50 ml of 0.1 N HCl solution and stirred for 30 min at 60, 70, and 80°C. The hot extract was filtered using Whatman® Grade 1 filter paper and cooled up to 4°C. About 30 ml of ethanol was added to the extract to precipitate pectin. It was separated by filtration and freeze-dried to get pectin powder.

The percentage yield of pectin was determined using the formula,

$$\text{Yield of pectin (\%)} = \frac{\text{Wt. of pectin (g)}}{\text{Wt.of dried peels (g)}} \times 100$$

Physico-Chemical Characterization of Pectin

Moisture Content

About 1 g of pectin substance was kept for drying in an oven for 5 h at 100°C. Then, it was cooled and weighed to obtain its moisture content.

$$\text{\%Moisture content} = \frac{\text{Final wt.} - \text{Initial wt. (g)}}{\text{Initial wt. (g)}} \times 100$$

Equivalent Weight

Pectin (0.5 g) was put in a conical flask of 250 and 5 ml of ethanol was added to it. About 1 g of sodium chloride was also added to sharpen the endpoint and 100 ml of millipore water was taken. Finally, a few drops of phenol red indicator were added followed by titration against 0.1 N NaOH. The endpoint was reached by developing a purple color. This neutralized solution was further used for methoxyl content determination.

$$\text{Equivalent weight} = \frac{\text{Wt.of sample (g)}}{\text{Volume of alkali (mL)} \times \text{Normality of alkali}} \times 1000$$

Methoxyl (MeO) Content

Methoxy content was measured by saponification followed by titration of liberated carboxyl groups (15). The neutralized solution was taken from the equivalent weight determination and 25 ml of 0.25 N sodium hydroxide was added to it. The solution was mixed thoroughly and kept at room temperature for 30 min. After 30 min, 25 ml of 0.25 N HCl was added and titrated against 0.1 N NaOH to the same titration point as in the equivalent weight determination.

$$\text{\%MeO} = \frac{\text{Volume of alkali (mL)} \times \text{Normality of alkali} \times 3.1}{\text{Wt.of sample(g)}}$$

Anhydrouronic Acid (AUA) Content

The determination of anhydrouronic acid content is a prerequisite for estimating the degree of esterification and purity of pectin, using the value of the equivalent weight and methoxyl content. The AUA content of pectin was calculated by using the following formula (16):

$$\text{\%AUA} = \frac{176 \times 0.1 \times y \times 100}{w \times 1000} + \frac{176 \times 0.1 \times z \times 100}{w \times 1000}$$

Where 176 = molecular unit of AUA (g).

y = titer value of NaOH from equivalent weight estimation (ml).

z = titer value of NaOH from methoxyl content estimation (ml).

w = weight of sample (g).

Degree of Esterification

The degree of esterification (DE) of pectin was obtained based on anhydrouronic acid and methoxyl content and measured by the following formula (17),

$$\text{\%DE} = \frac{176 \times \text{\% MeO}}{31 \times \text{\% AUA}} \times 100$$

Where, MeO = methoxyl content.

AUA = anhydrouronic acid content.

Degree of Acetylation

Pectin (0.5 g) and 25 ml of 0.1 N NaOH were mixed and stirred until the pectin was dissolved and kept overnight (15). The whole content was diluted to 250 ml by adding water and 20 ml of aliquot was taken into a distillation apparatus. Then, 20 ml of magnesium sulfate-sulfuric acid solution [magnesium sulfate (100 g) + sulfuric acid (1.5 g), diluted to 180 ml) was also put into the distillation apparatus followed by distillation. The distillate was collected at about 200 ml. This distillate was titrated with 0.05 N NaOH with a phenol red indicator. A blank distillation was also carried out with 20 ml of magnesium sulfate-sulfuric acid solution, and the distillate was titrated.

$$\text{\%DA} = \frac{\text{Volume of alkali (Blank} - \text{Titre) (mL)} \times \text{Normality of alkali} \times 4.3}{\text{Wt.of sample (g)}}$$

Ash Content

Pectin (2 g) was put in a crucible and heated for 6 h at 600°C under a muffle furnace (15). When the crucibles came to room temperature, they were kept in desiccators and weighed precisely. The process was repeated until a constant weight was obtained.

$$\text{\%Ash} = \frac{W2 - W1}{W} \times 100$$

W2 = final weight of crucible and ash (g), W1 = weight of crucible, and W = weight of sample.

Alkalinity of Ash

About 25 ml of 0.1 N HCl was added to the ash obtained upon igniting the pectin. The mixture was then heated to a boil followed by cooling and titrated with 0.1 N NaOH using phenolphthalein as an indicator (15). A blank titration was also carried out with 25 ml of 0.1 N HCl.

$$\text{AlkalinityofAsh} = \frac{\text{Volume of alkali (Blank} - \text{Titre) (mL)} \times \text{Normality of alkali} \times 3.1}{\text{Wt.of sample (g)}}$$

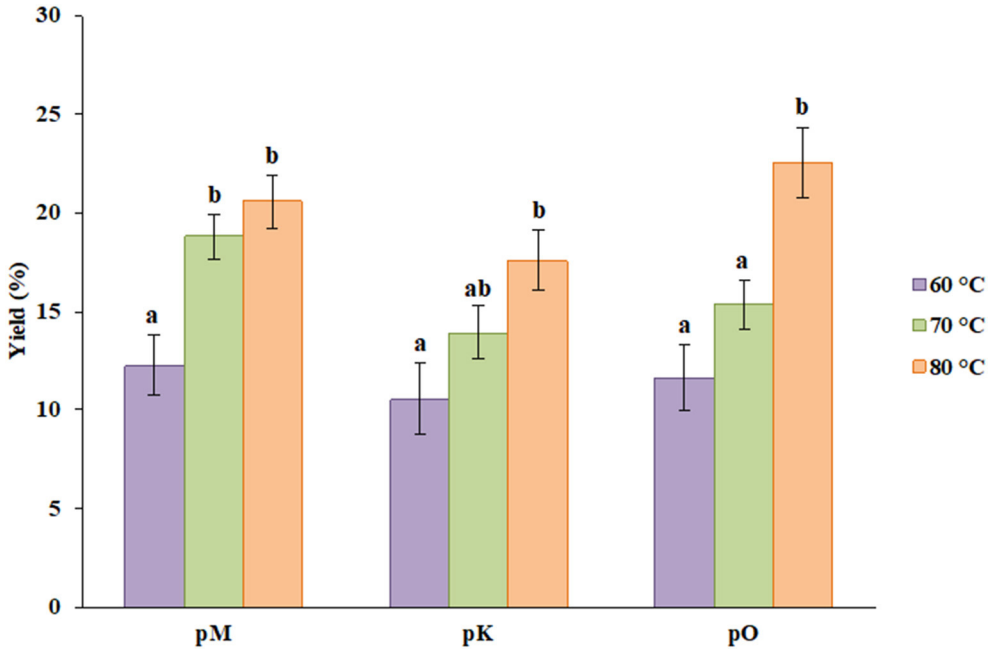

FIGURE 1 | Percent yield of pectin from different sources at different temperatures where pM, pK and pO denotes pectin from Mosambi, Kinnow, and Orange, respectively. Bars sharing the same letter within a treatment are not significantly different ($P < 0.05$). Error bars represent standard deviation.

TABLE 2 | Physicochemical parameters of pectins extracted from three *Citrus* sp.

Parameters	Pectic substances			
	Mosambi peels	Kinnow peels	Orange peels	Commercial
Moisture%	8.19	8.24	9.26	8.08
Equivalent wt.	543.47	510.2	526.32	524.89
Methoxyl content %	6.19	6.54	6.78	7.12
Anhydrouronic acid %	67.82	71.59	71.87	73.93
Degree of Esterification %	51.81	51.86	53.55	54.67
Degree of Acetylation %	0.51	0.47	0.58	0.62
Ash %	1.34	2.12	1.02	1.78
Alkalinity of ash %	10.23	10.74	12.32	11.26

Spectroscopic Analysis of Pectin

Fourier transform infrared spectra of pectin extracted from three different sources along with commercially available pectin were recorded between 4,000 and 600 cm^{-1} on a Nicolet Nexus Avatar 370 FT-IR spectrophotometer (Nicolet, USA) having 4 cm^{-1} of resolution along with 256 scans using KBr disk as a blank (18). To understand the crystallinity of different pectin, X-ray diffraction (XRD) curves were obtained where the scanning speed was set at 1.5^0 2θ per min, and the scanning step of 0.1^0 2θ was selected during the experiment.

Assessment of Prebiotic Effect

Growth Curve of Microbe Taken for Prebiotic Assessment

For this prebiotic effect evaluation of pectin, a pure culture of *Lactobacillus fermentum* was used, which was maintained by the Division of Microbiology, ICAR-IARI, New Delhi. Lactic acid bacteria were isolated from fermented food samples, collected from the native people of Himachal Pradesh, India. Isolation of *L. fermentum* bacteria through sequential methods, such as enrichment technique, acid production based qualitative screening, and followed by quantitative selection based on lactic acid production (19). The microbial strain was grown at 35°C under anaerobic conditions using de man Rogosa Sharp (MRS) broth. The growth of the microbe was monitored at regular time intervals by measuring the optical density (OD) of the medium at 622 nm.

Prebiotic Effect of Pectin

The response of pectin as a prebiotic was assessed by using the same culture media with a similar MRS broth composition but with the replacement of sugar with pectin. The growth of culture was also assessed in MRS broth (composition of MRS broth is given in **Supplementary Table 1**) with no carbohydrate source that was considered as control (20). Carbohydrate concentration was maintained at 2% in all cases. The activated inoculum was incubated with 1% (v/v) and kept at 35°C. Growth of the bacteria was observed at 12 h intervals up to 72 h when the growth of the microbe was in the stationary phase. OD values were measured as an indication of the growth of bacterial cultures (21). For further confirmation, the bacterial count was also done by taking

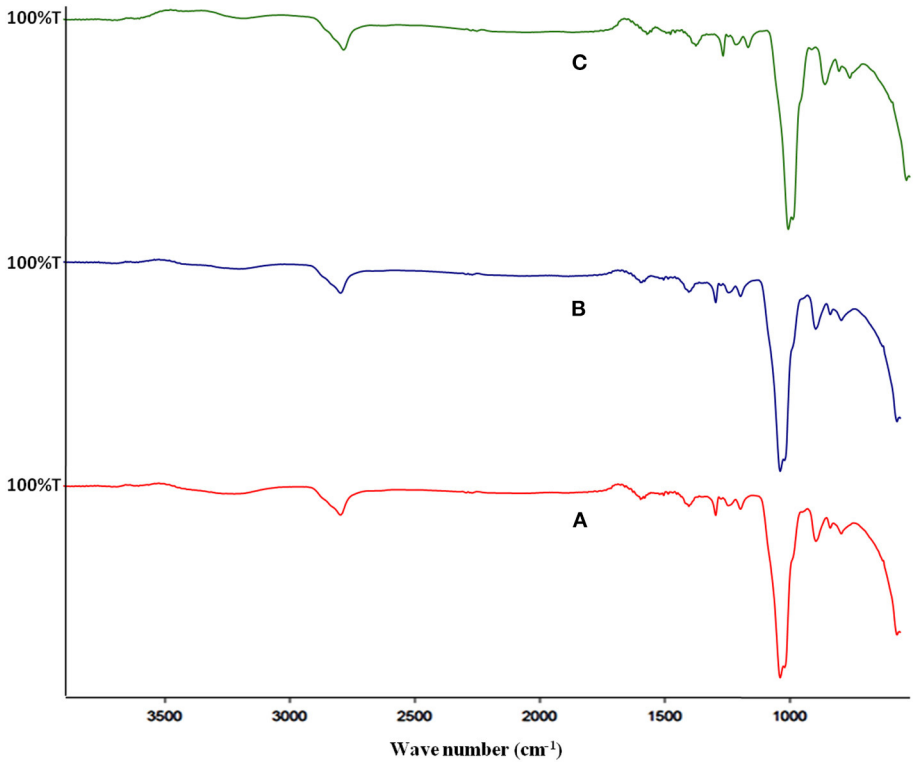

FIGURE 2 | FT-IR spectra of different pectin samples, where **(A–C)** represents pectin from Mosambi, Kinnow, and Orange peels, respectively.

a sample from each respective culture media by the serial dilution method using 0.9% NaCl solution.

Statistical Analysis

Data were statistically analyzed using the open-source statistical program JASP (Version 0.14.1), and represented by using Duncan's multiple range test values. Significant differences were determined at the $p < 0.05$ level. All data were recorded in triplicate.

RESULTS AND DISCUSSION

Essential Oil

With the aim of waste valorization, citrus peel was first utilized for the extraction of essential oil, and then the same de-oiled peel was dried and used for the extraction of pectin. During pectin extraction, blanching was recommended to inactivate the enzyme, which was automatically done during the hydrodistillation process. In this process, we can extract oil followed by pectin for its further utilization.

The essential oil yield was recorded for the three Citrus species and it ranged between 0.67 and 0.79% dry weight basis (**Table 1**). The maximum amount of the oil was reported in the Orange peel. The essential oil extracted by microwave Clevenger apparatus from Orange peel was reported to be 0.42% (22). In total, 18 Citrus species were evaluated and the average oil was reported to be 1.67% on a fresh weight basis (23). A major component of the citrus oil was limonene and it varied between 91.6 and 94.8% (**Table 1**, **Supplementary Figure 1**). Lota et al. (24) reported limonene as a major compound and it ranged between 89.1 and 95.5% across 15 mandarin species.

Yield of Pectin

The percentage yield of pectin from three different sources was obtained at three different temperatures, i.e., 60, 70, and 80°C. As shown in **Figure 1**, the yield from peels of Mosambi was 12.23, 18.77, and 20.53% at particular temperatures. Similarly, 10.56, 13.89, and 17.55% yield resulted from peels of Kinnow, and 11.61, 15.32, and 22.56% yield was obtained from peels of orange. For each source, the yield has been increased by increasing temperature and the highest yield was procured at the highest temperature. This result was found to agree with Rose and Abilasha (8). As pH was fixed during the extraction procedure, the temperature played a major role in the total extraction yield. Better extraction efficiency might be attributed to better cell disruption, which was caused by higher temperature.

Physicochemical Characterization of Pectin

Different physicochemical parameters were evaluated for the characterization of pectin from these three different sources along with commercially available pectin that has been displayed in **Table 2**.

The moisture content of pectin extracted from Mosambi, Kinnow, and Orange peels was 8.19, 8.24, and 9.26%, which is slightly higher than that of commercial pectin (8.08%). Literature data about the moisture content of pectin from various citrus peels lies within 6.4–10% (25).

Equivalent weight was 543.47, 510.2, 526.32, and 524.89 for Mosambi, Kinnow, Orange, and commercial pectin, respectively, which was considerably lower than pectin from apple pomace (833.33–1,666.30) (6). The lower value of equivalent weight could denote higher partial degradation, so an increase or decrease of equivalent weight might have dependence on the presence of free acid (26). The methoxyl content of pectin is an important parameter to understand the gelling property. The values obtained were 6.19, 6.54, 6.78, and 7.12% for different pectin. Kar and Arslan (27) reported methoxyl content as 12.15% for oranges, whereas, Mohamed (7) reported approximately 7.5–8.5% of methoxyl content for grapefruit. Therefore, methoxyl content can vary with the source of material used, the method used for extraction, and other related factors. Low methoxyl pectin gels easily without the addition of sugars in the presence of divalent cations (28).

Anhydrouronic acid content varied between 67.82 and 71.87% as compared with 73.93% for commercial pectin. AUA content indicates the purity of extracted pectin. A lower value (<65%) indicates lower purity of extracted pectin with the possible presence of starch, sugars, and protein in the pectin (29). DE for all pectin was higher than 50% which also have a similarity with that of 57% (sweet orange), 56.1% (orange), and 57.1% (grapefruit) (30). As DE is >50% for the pectins understudy, they can be categorized under high ester pectins with rapid gel setting properties (31). This statement can also be argued by stating the lower values of the degree of acetylation (DA) (0.51–0.58%) for different pectin. Ranganna (15) suggested gelling capacity of pectin can be restored up to a level of 2.4% of acetyl value.

Ash content was obtained as 1.34, 2.12, and 2.12% for three different peels, similar to commercial pectin with 1.78% of ash content. Ismail et al. (29) mentioned approximately 10% of a maximum limit of ash content for good quality pectin. Ash content decreases with an increase in the yield of pectin (32). These data also agree with the statement as pectin having the lowest ash content corresponds to higher yield and vice versa. Results of percentage alkalinity of ash showed the highest value for pectin extracted from orange peels while the lowest for Mosambi peel extracted pectin. No such trend has been observed between percentage content and percentage alkalinity of ash among different pectin.

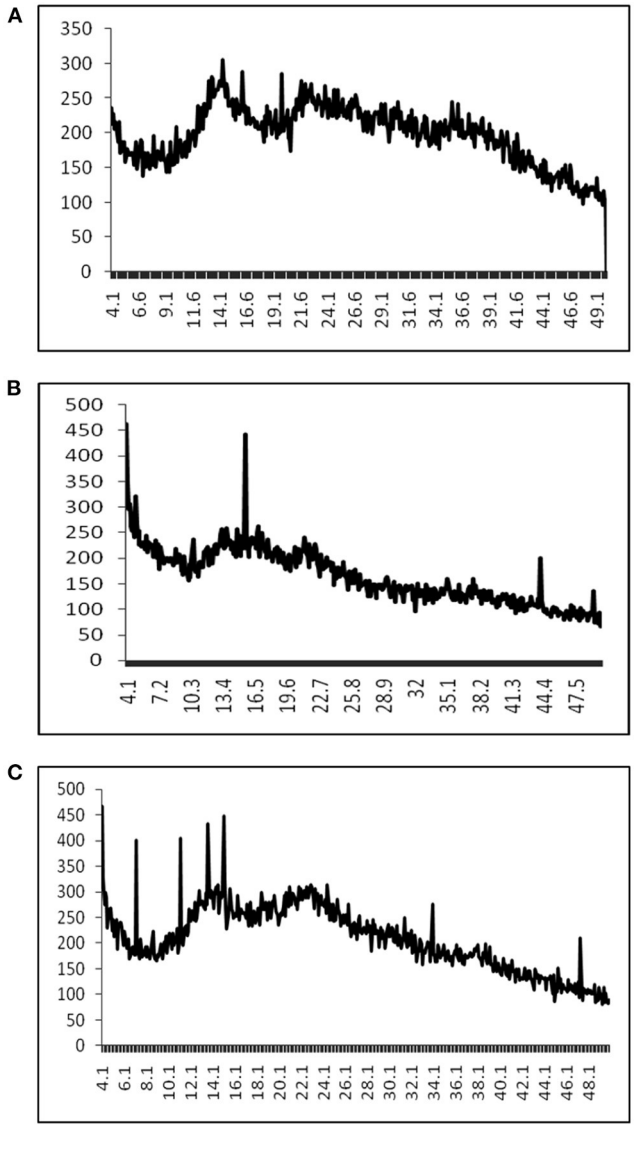

FIGURE 3 | XRD of different pectin samples, where **(A–C)** represents pectin from Mosambi, Kinnow, and Orange peels, respectively.

Spectroscopic Analysis of Pectin

The FT-IR spectra of pectin samples from different sources have shown peaks at approximately 3,300, 2,900, 1,600, and 1,052 cm^{-1} that correspond to –OH stretching, –CH stretching, -C=O stretching (due to ester and acid moiety), and –COC– stretching of galactouronic acid (**Figure 2**). The region in the range of 3,200–3,600 cm^{-1} corresponds to the O–H stretching absorption due to inter- and intramolecular hydrogen bonding of the monomer unit of pectin. Bands (3,000–2,800 cm^{-1}) are assigned to the C–H absorption that includes CH, CH_2, and CH_3 stretching vibrations.

In the fingerprint region (1,200–900 cm^{-1}) of citrus pectin spectra, intense bands at 1,140 and 1,040 cm^{-1} were assigned to stretching vibrations of glycosidic bonds (C–O) and pyranoid rings (C–C). This showed similarity to that of IR spectra of pectin from commercial citrus pectin mixture (33, 34). The intensity of peaks corresponding to –OH stretch and =C O stretch is considerably low, which may be due to a higher concentration of sample as hydrogen bond prevails and decrease the intensity of peaks.

An XRD study of different pectins showed their crystalline nature. Pectin extracted from Kinnow and Orange was more crystalline than compared with pectin from Mosambi as narrow

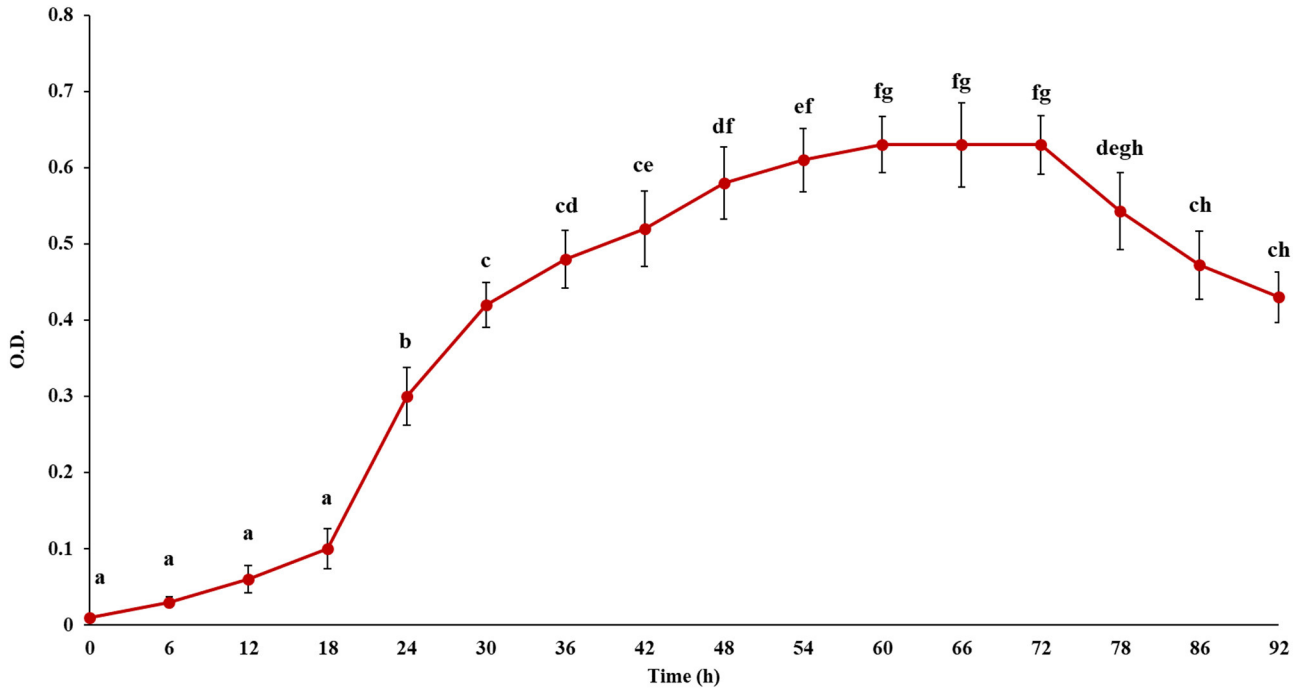

FIGURE 4 | Growth curve of the probiotic, *Lactobacillus fermentum.* Points sharing the same letter are not significantly different ($P < 0.05$). Error bars represent standard deviation.

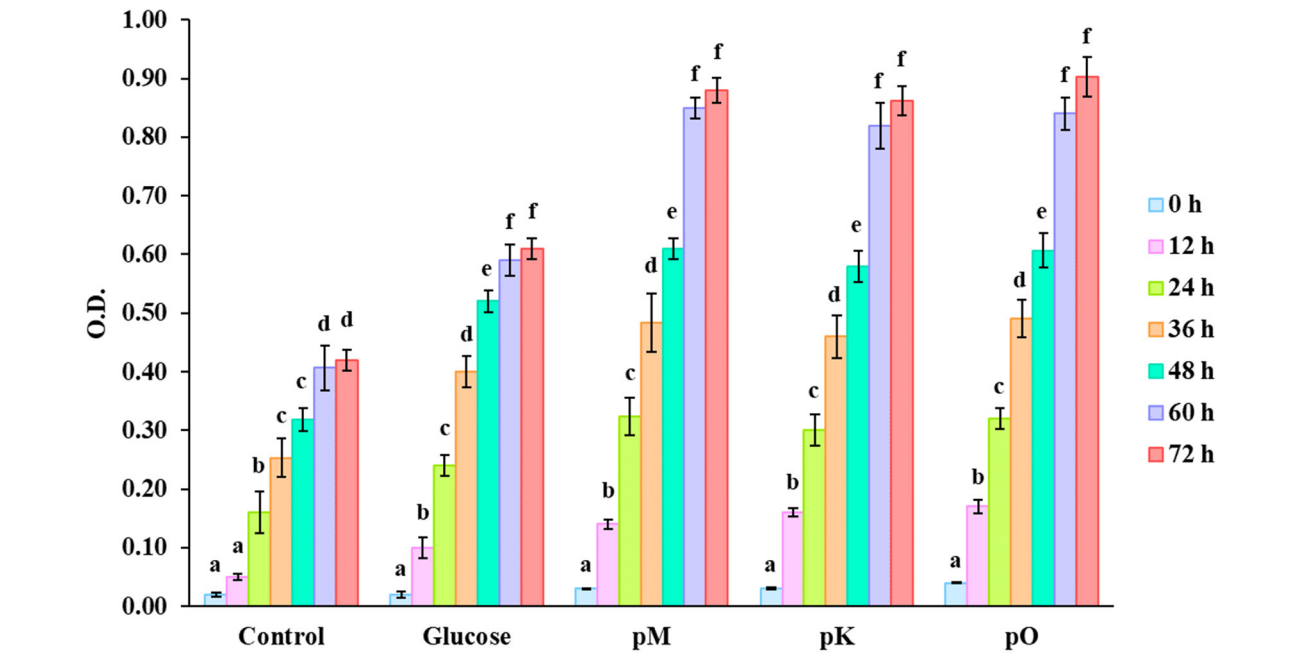

FIGURE 5 | O.D values of culture media with no carbon source; control, carbon source having glucose, pectin from Mosambi peels (pM), Kinnow peels (pK) and Orange peels (pO). Bars sharing the same letter within a treatment are not significantly different ($P < 0.05$). Error bars represent standard deviation.

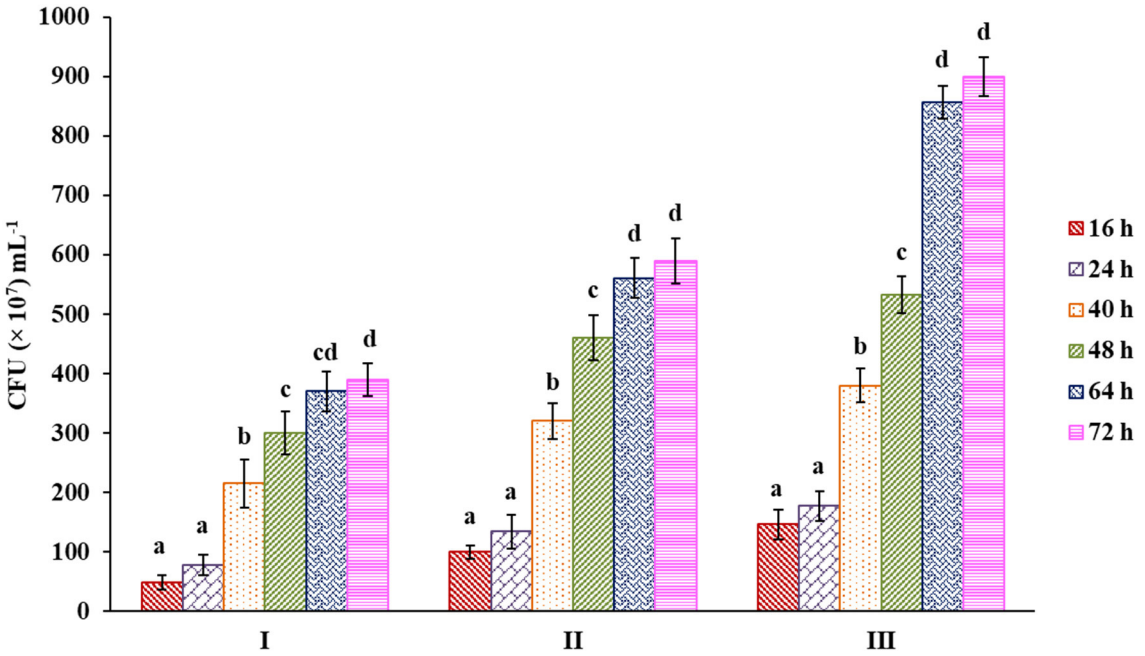

FIGURE 6 | Bacterial count from samples of culture media with control (I), sugar (II) and pectin (III) as carbon source. Bars sharing the same letter within a treatment are not significantly different ($P < 0.05$). Error bars represent standard deviation.

and sharp diffraction peaks have been observed in the spectra for the former two samples but no such distinct peak was observed for latter one (**Figure 3**). In general, amorphous solids generally have a higher solubility and higher dissolution rate because they have higher free energy than corresponding crystals (35). The crystalline nature of pectin was also different for pectin extracted from two different varieties of apples (6) that support the fact of being different crystalline nature of pectin in this study.

Effect of Extracted Pectin on *L. fermentum*

In this study, first the growth of the microbe, i.e., *L. fermentum* was observed to decide the incubation period to perform the prebiotic activity. **Figure 4** depicts the change in OD in course of the growth of the microbe. It has been seen that the lag phase lasted for 18 h. Then the strain grew exponentially, which suggested it was in the logarithmic phase. After that stationary phase began at 60 h and they started to decline after 72 h. Therefore, the prebiotic activity was studied up to 72 h after culture fermentation to understand the effect of pectic substance on the microbial population.

Figure 5 displays an increase in the OD value of culture medium fermented 72 h with pectin extracted from different citrus peels as sources of carbon. A higher OD value denotes better growth of microbe and efficient utilization of pectin. During the logarithmic phase (up to 48 h) as microbes grew rapidly, there was a minute variation between the OD value of medium added with glucose and pectin as carbon source. But in the stationary phase (up to 72 h), the OD value was distinctly higher in the case of a medium with different pectin compared with glucose as a carbon source. This type of trend indicates effective fermentation of pectin by microbe over a longer period. Tingirikari (36) studied *in-vitro* prebiotic analysis of pectic polysaccharides over various gut-friendly microbes and obtained similar kinds of findings. As these pectic substances get fermented, different organic acids are formed that help to increase the microbiome, making these substances an effective prebiotic ingredient. Furthermore, inconsistent data were reported by different authors on the ability of pectin and related oligosaccharides on the growth of specific bacterial populations (37). Decreased or unchanged level of certain bacteria, such as bifidobacteria and *Roseburia*, was reported earlier (38–40).

This result was further confirmed by counting bacterial colonies from each respective culture medium having glucose and pectin as carbon sources along with the control that showed a similar kind of trend as before (**Figure 6**, **Supplementary Figure 2**). A higher bacterial population was observed in the case of pectin than in others up to 72 h, showing a nearly 61.75% increase compared with glucose as a carbon source.

Structure-Function Relationship

Upon comparative characteristic evaluation of three different citrus peel pectin and its related prebiotic activity, the structure-activity relationship was drawn. Methoxyl content was highly correlated with the prebiotic activity. Methoxyl content followed the order pO > pK > pM and the prebiotic activity after 72 h followed a similar trend (enhancement in bacterial population was observed at 79.16, 83.33, and 87.50%, respectively). Pectin,

indigestible by human enzyme, upon fermentation to short-chain fatty acids, contributes significantly to the microbiome. Production of short-chain fatty acids was higher in pectins with high methoxyl content (37). Interestingly, the DE also followed the same trend as methoxyl content. The higher the DE more the production of short-chain fatty acids and it contributed more bioactivity as prebiotics (37). The study recorded important information regarding the prebiotic potential of different pectins varied across molecular weight, structure, DE, etc. The suitability of pectin for its application is governed by the structure of pectin, including its DE, amount of methoxy, and acetyl esters (41, 42). Gomez et al. (43) reported an increase in the population of bifidobacteria and lactobacilli in the range of 19–32%.

CONCLUSION

Citrus peel wastes from *C. limetta*, a hybrid between *C. nobilis* and *C. deliciosa,* and *C. sinensis* were first utilized for the extraction of essential oil, and then they were further processed for the production of pectin. Higher essential oil and pectin yield was observed in the case of orange peels than the other two species. Three different pectins were characterized for their physicochemical and spectroscopic attributes. Three pectins differ in terms of equivalent weight, DE, and methoxyl content. FT-IR spectra showed similar chemical composition but the XRD study displayed differences in crystalline nature among pectin from different sources.

The count of *Lactobacillus* sp. was enhanced significantly in the presence of pectin as compared with sugar source. Among three different pectins, enhancement of the population of the probiotic bacteria followed the order pO > pK > pM. The present study recorded significant enhancement of the probiotic bacteria, which was earlier reported as non-conclusive.

A prebiotic assay over a gut beneficial microbe notified increase in the population, which will help to enrich the gut microbiome. This piece of experiment might be helpful for the utilization of pectin in the food/nutraceutical industry as a promising product by effective use of waste materials generated regularly. Pectin can be used for the production of jams, jellies, frozen foods, and low-calorie foods as a fat and/or sugar replacer.

Citrus essential oil is being used in the food as well as cosmetic industry. So, citrus peel, which is a waste material for the juice industry, can be explored for the production of essential oil first and then for the production of pectin. Both these bioactive compounds can be successfully utilized by the food industry.

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/s.

AUTHOR CONTRIBUTIONS

RS: investigation, methodology, and writing—original draft. LN: conceptualization and methodology. AK: conceptualization and formal analysis. AD: validation and resources. DD: methodology and data curation. SSe: data curation and validation. SSa: data curation, formal analysis, and writing—review and editing. 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/fnut.2022.826250/full#supplementary-material

REFERENCES

1. Buchanan BB, Gruissem W, Jones RL. *Biochemistry and Molecular Biology of Plants*. Rockville, MD: American Society of Plant Biologists (2000).
2. Miyamoto A, Chang KC. Extraction and physicochemical characterization of pectin from sunflower head residues. *J Food Sci.* (1992) 57:1439–43. doi: 10.1111/j.1365-2621.1992.tb06878.x
3. Mollea C, Chiampo F, Conti R. Extraction and characterization of pectins from cocoa husks: a preliminary study. *Food Chem.* (2008) 107:1353–6. doi: 10.1016/j.foodchem.2007.09.006
4. Kalapathy U, Proctor A. Effect of acid extraction and alcohol precipitation conditions on the yield and purity of soy hull pectin. *Food Chem.* (2001) 4:393–6. doi: 10.1016/S0308-8146(00)00307-1
5. Virk BS, Sogi DS. Extraction and characterization of pectin from apple (Malus Pumila. Cv Amri) peel waste. *Int J Food Prop.* (2004) 7:693–703. doi: 10.1081/JFP-200033095
6. Kumar A, Chauhan GS. Extraction and characterization of pectin from apple pomace and its evaluation as lipase (steapsin) inhibitor. *Carbohydr Polym.* (2010) 82:454–9. doi: 10.1016/j.carbpol.2010.05.001
7. Mohamed H. Extraction and Characterization of Pectin from Grapefruit peels. *MOJ Food Process Technol.* (2016) 2:31–8. doi: 10.15406/mojfpt.2016.02.00029
8. Rose PAE, Abilasha D. Extraction and characterization of pectin from lemon peel. *Extraction.* (2016) 1:16–20.
9. Tamaki Y, Konishi T, Fukuta M, Tako M. Isolation and structural characterisation of pectin from endocarp of *Citrus depressa*. *Food Chem.* (2008) 107:352–61. doi: 10.1016/j.foodchem.2007.08.027
10. Ferreira-Lazarte A, Kachrimanidou V, Villamiel M, Rastall RA, Moreno FJ. In vitro fermentation properties of pectins and enzymatic-modified pectins obtained from different renewable bioresources. *Carbohydr Polym.* (2018) 199:482–91. doi: 10.1016/j.carbpol.2018.07.041
11. Zhang S, Hu H, Wang L, Liu F, Pan S. Preparation and prebiotic potential of pectin oligosaccharides obtained from citrus peel pectin. *Food Chem.* (2018) 244:232–7. doi: 10.1016/j.foodchem.2017.10.071
12. Cohn R, Cohn AL. Subproductos del procesado de las frutas. In: Arthey D, Ashurst PR, Editors. *Procesado de frutas Acribia.* Zaragoza (1997). p. 288.

13. *McGready RM. 1996 Extraction of pectin from citrus peels and conversion of pectin acid Academic Press.* New York, NY (2005). p. 167–70.
14. Gullón B, Gullón P, Tavaria F, Pintado M, Gomes AM, Alonso JL, et al. Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. *J Funct Foods.* (2014) 6:438–49. doi: 10.1016/j.jff.2013.11.010
15. Ranganna S. *Handbook of Analysis and Quality Control for Fruit and Vegetable Products*. Tata McGraw-Hill Education (1986).
16. Suhaila M, Zahariah H. Extraction and characterisation of pectin from various tropical agrowastes. *ASEAN Food J.* (1995) 10:43–50.
17. Schultz TH. Determination of the degree of esterification of pectin. *Methods Carb Chemist.*(1965) 5:189–94.
18. Gnanasambandam R, Proctor A. Determination of pectin degree of esterification by diffuse reflectance fourier transform infrared spectroscopy. *Food Chem.* (2000) 68:327–32. doi: 10.1016/S0308-8146(99)00191-0
19. Sharma A, Pranaw K, Singh S, Khare SK, Chandel AK, Nain PKS, et al. Efficient two-step lactic acid production from cassava biomass using thermostable enzyme cocktail and lactic acid bacteria: insights from hydrolysis optimization and proteomics analysis. *3 Biotech.* (2020) 10:1–13. doi: 10.1007/s13205-020-02349-4
20. Manderson K, Pinart M, Tuohy KM, Grace WE, Hotchkiss AT, Widmer W, et al. *In vitro* determination of prebiotic properties of oligosaccharides derived from an orange juice manufacturing by-product stream. *Appl Environ Microbiol.* (2005) 71:8383–9. doi: 10.1128/AEM.71.12.8383-8389.2005
21. Li W, Zhang J, Yu C, Li Q, Dong F, Wang G, et al. Extraction, degree of polymerization determination and prebiotic effect evaluation of inulin from Jerusalem artichoke. *Carbohydr Polym.* (2015) 121:315–9. doi: 10.1016/j.carbpol.2014.12.055
22. Ferhat MA, Meklati BY, Smadja J, Chemat F. An improved microwave Clevenger apparatus for distillation of essential oils from orange peel. *J Chrom A.* (2006) 1112:121–6. doi: 10.1016/j.chroma.2005.12.030
23. Di Vaio C, Graziani G, Gaspari A, Scaglione G, Nocerino S, Ritieni A. Essential oils content and antioxidant properties of peel ethanol extract in 18 lemon cultivars. *Sci Hort.* (2010) 126:50–5. doi: 10.1016/j.scienta.2010.06.010
24. Lota ML, de Rocca Serra D, Tomi F, Casanova J. Chemical variability of peel and leaf essential oils of 15 species of mandarins. *Biochem Syst Ecol.* (2001) 29:77–104. doi: 10.1016/S0305-1978(00)00029-6
25. Rehman ZU, Salariya AM, Habib F, Shah WH. Utilization of mango peels as a source of pectin. *J Chem Soc Pak.* (2004) 26:73–6.
26. Nazaruddin R. Effect of ammonium oxalate and acetic acid at several extraction time and pH on some physicochemical properties of pectin from cocoa husks (Theobroma cacao). *Afr J Food Sci.* (2011) 5:790–8. doi: 10.5897/AJFS11.107
27. Kar F, Arslan N. Characterization of orange peel pectin and effect of sugars, L-ascorbic acid, ammonium persulfate, salts on viscosity of orange peel pectin solutions. *Carbohydr Polym.* (1999) 40:285–91. doi: 10.1016/S0144-8617(99)00063-6
28. Gadalla HH, El-Gibaly I, Soliman GM, Mohamed FA, El-Sayed AM. Amidated pectin/sodium carboxymethylcellulose microspheres as a new carrier for colonic drug targeting: development and optimization by factorial design. *Carbohydr Polym.* (2016) 153:526–34. doi: 10.1016/j.carbpol.2016.08.018
29. Ismail NSM, Ramli N, Hani NM, Meon Z. Extraction and characterization of pectin from dragon fruit (Hylocereus polyrhizus) using various extraction conditions. *Sains Malaysiana.* (2012) 41:41–5.
30. Iranzo J, Barandalla PI, Miralles MC. Preparation of dried peel for pectin production from varieties of mandarin, grapefruit bitter orange and lemon produced in Spain. *Revista de Agroquimica Y Tecnologia de Alimentos.* (1980) 20:399–402.
31. Thakur BR, Singh RK, Handa AK, Rao MA. Chemistry and uses of pectin—a review. *Crit Rev Food Sci Nutr.* (1997) 37:47–73. doi: 10.1080/10408399709527767
32. Azad AKM, Ali MA, Akter MS, Rahman MJ, Ahmed M. Isolation and characterization of pectin extracted from lemon pomace during ripening. *J Food Nutr Sci.* (2014) 2:30–5. doi: 10.11648/j.jfns.20140202.12
33. Kyomugasho C, Christiaens S, Shpigelman A, Van Loey AM, Hendrickx ME. FT-IR spectroscopy, a reliable method for routine analysis of the degree of methylesterification of pectin in different fruit-and vegetable-based matrices. *Food Chem.* (2015) 176:82–90. doi: 10.1016/j.foodchem.2014.12.033
34. Monsoor MA, Kalapathy U, Proctor A. Determination of polygalacturonic acid content in pectin extracts by diffuse reflectance fourier transform infrared spectroscopy. *Food Chem.* (2001) 74:233–8. doi: 10.1016/S0308-8146(01)00100-5
35. Chen J, Sarma B, Evans JM, Myerson AS. Pharmaceutical crystallization. *Crystal Growth Design.* (2011) 11:887–95. doi: 10.1021/cg101556s
36. Tingirikari JMR. *In-vitro* prebiotic analysis of microbiota accessible pectic polysaccharides. *Curr Microbiol.* (2019) 76:1452–60. doi: 10.1007/s00284-019-01781-x
37. Larsen N, Bussolo de Souza C, Krych L, Barbosa Cahu T, Wiese M, Kot W, et al. Potential of pectins to beneficially modulate the gut microbiota depends on their structural properties. *Front Microbiol.* (2019) 10:223. doi: 10.3389/fmicb.2019.00223
38. Onumpai C, Kolida S, Bonnin E, Rastall RA. Microbial utilization and selectivity of pectin fractions with various structures. *Appl Environ Microbiol.* (2011) 77:5747–54. doi: 10.1128/AEM.00179-11
39. Aguirre M, Jonkers DMAE, Troost FJ, Roeselers G, Venema K. *In vitro* characterization of the impact of different substrates on metabolite production, energy extraction and composition of gut microbiota from lean and obese subjects. *PLoS ONE.* (2014) 9:e113864. doi: 10.1371/journal.pone.0113864
40. Leijdekkers AGM, Aguirre M, Venema K, Bosch G, Gruppen H, Schols HA. *In vitro* fermentability of sugar beet pulp derived oligosaccharides using human and pig fecal inocula. *J Agric Food Chem.* (2014) 62:1079–87. doi: 10.1021/jf4049676
41. Daas PJ, Arisz PW, Schols HA, De Ruiter GA, Voragen AG. Analysis of partially methyl-esterified galacturonic acid oligomers by high-performance anion-exchange chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Biochem.* (1998) 257:195–202. doi: 10.1006/abio.1997.2554
42. Sila DN, Van Buggenhout S, Duvetter T, Van Loey A, Hendrickx M. Pectins in processed fruits and vegetables: part II-structure function relationships. *Compr Rev Food Sci Food Saf.* (2009) 8:105–17. doi: 10.1111/j.1541-4337.2009.00071.x
43. Gómez B, Gullón B, Yáñez R, Schols H, Alonso JL. Prebiotic potential of pectins and pectic oligosaccharides derived from lemon peel wastes and sugar beet pulp: a comparative evaluation. *J Funct Foods.* (2016) 20:108–21. doi: 10.1016/j.jff.2015.10.029

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Changes of Crocin and Other Crocetin Glycosides in Saffron Through Cooking Models, and Discovery of Rare Crocetin Glycosides in the Yellow Flowers of *Freesia Hybrida*

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Crocetin glycosides such as crocin are noted as functional food materials since the preventive effects of crocin have been reported against chronic disease and cancer. However, it is unclear how these apocarotenoids are structurally changed through cooking for our intake. We examined such changes in crocetin glycosides (crocin, tricrocin, and crocin-3) contained in saffron (stigmas of *Crocus sativus*) through cooking models. These glycosides were almost kept stable in boiling for 20 min (a boiled cooking model), while hydrolysis of the ester linkage between glucose and the crocetin aglycone occurred in a grilled cooking model (180°C, 5 min), along with a 13-*cis* isomerization reaction in a part of crocetin subsequently generated. We further here revealed that the yellow petals of freesia (*Freesia* x *hybrida*) with yellow flowers accumulate two unique crocetin glycosides, which were identified to be crocetin (mono)neapolitanosyl ester and crocetin dineapolitanosyl ester. A similar result as above was obtained on their changes through the cooking models. Utility applications of the freesia flowers as edible flowers are also suggested in this study. Additionally, we evaluated singlet oxygen ($^1O_2$)-quenching activities of the crocetin glycosides contained in saffron and freesia, and crocetin and 13-*cis* crocetin contained in the grilled saffron, indicating that they possessed moderate $^1O_2$-quenching activities (IC_{50} 24–64 μM).

Keywords: saffron, freesia, crocin, crocetin neapolitanosyl ester, cooking

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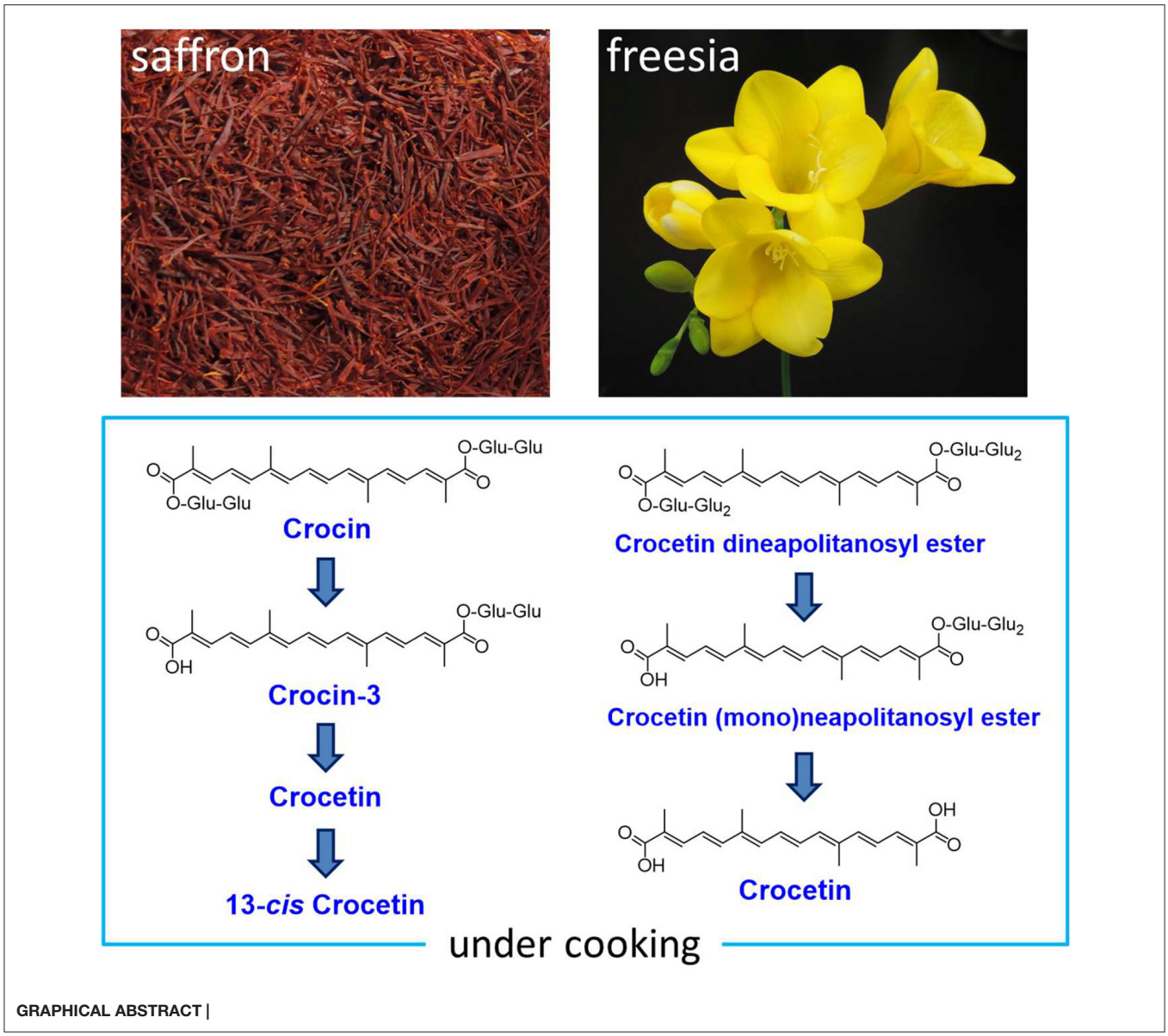
INTRODUCTION

Crocetin is an apocarotenoid composed of 20 carbons, and its digentiobiosyl ester is called crocin, which is the major pigment contained in the dried red stigmas of saffron plants (*Crocus sativus* L.) and gardenia fruits (*Gardenia jasminoides* Ellis), which belong to the Iridaceae and Rubiaceae families, respectively (1). Along with crocin, these plant organs have been reported to accompany



GRAPHICAL ABSTRACT |

several minor components of crocetin glycosides, i.e., crocetin (mono)glucosyl ester, crocin-3 [crocetin (mono)gentiobiosyl ester], tricrocin (crocetin gentiobiosyl glucosyl ester), and crocetin gentiobiosyl neapolitanosyl ester (1, 2). The biosynthetic pathways of these apocarotenoids are shown in **Figure 1A** (3, 4). Saffron is an expensive spice, which has widely been used in cooking as a water-soluble yellow colorant with a distinctive flavor (5). Crocin and relevant glycosides are noted as functional food materials since the preventive effects of crocin have been reported against chronic diseases such as diabetes, fatty liver, and autoimmune diseases, and cancer such as colon cancer (6–9). It is however unclear how crocin and the relevant glycosides are structurally changed through cooking (boiling and grilling) for our intake, although changes of carotenoids that are contained in maize and vegetables through cooking were examined in a few reports (10, 11). In this study, such a change is examined on saffron. In addition, we evaluate singlet oxygen ($^1O_2$)-quenching activities of the generated compounds.

Freesia (*Freesia* x *hybrida*; the Iridaceae family) is a popular flowering plant of South African (the Cape Province area) origin as the cut flowers (12). Many cultivars of freesia plants, whose flowers retain a variety of colors, shapes, and fragrances, have been generated up to the present (13). In Japan, freesia cultivars with yellow flowers maintain an 80% share of the market. Among them, "Aladin" (**Figure 2A**), which retains big yellow flowers, has been the most cultivated freesia cultivar since the 1990s. Cultivar "Ishikawa f2 go" ("f2"; **Figure 2B**) was established by crossing cultivars, "Aladin" and "Rapid Yellow" that retains a characteristic suitable for early forcing, and by selecting one seedling among consequently generated 222 seedlings (14).

This study reveals that yellow pigments in the yellow petals of freesia [cultivars "f2" and "Aladin" in addition to cultivar "Kayak" (**Figure 2C**)] are unique apocarotenoids, crocetin (mono)neapolitanosyl ester and crocetin dineapolitanosyl ester (**Figure 1B**). Plants that produce such apocarotenoids have not been reported so far. Moreover, this is the first report on plants that include the (mono)neapolitanosyl ester. According to Edible Flowers Guide by Thompson & Morgan (https://www.thompson-morgan.com/edible-flowers), freesia is positioned as an unusual edible flower, and described to be "Infused in a tisane

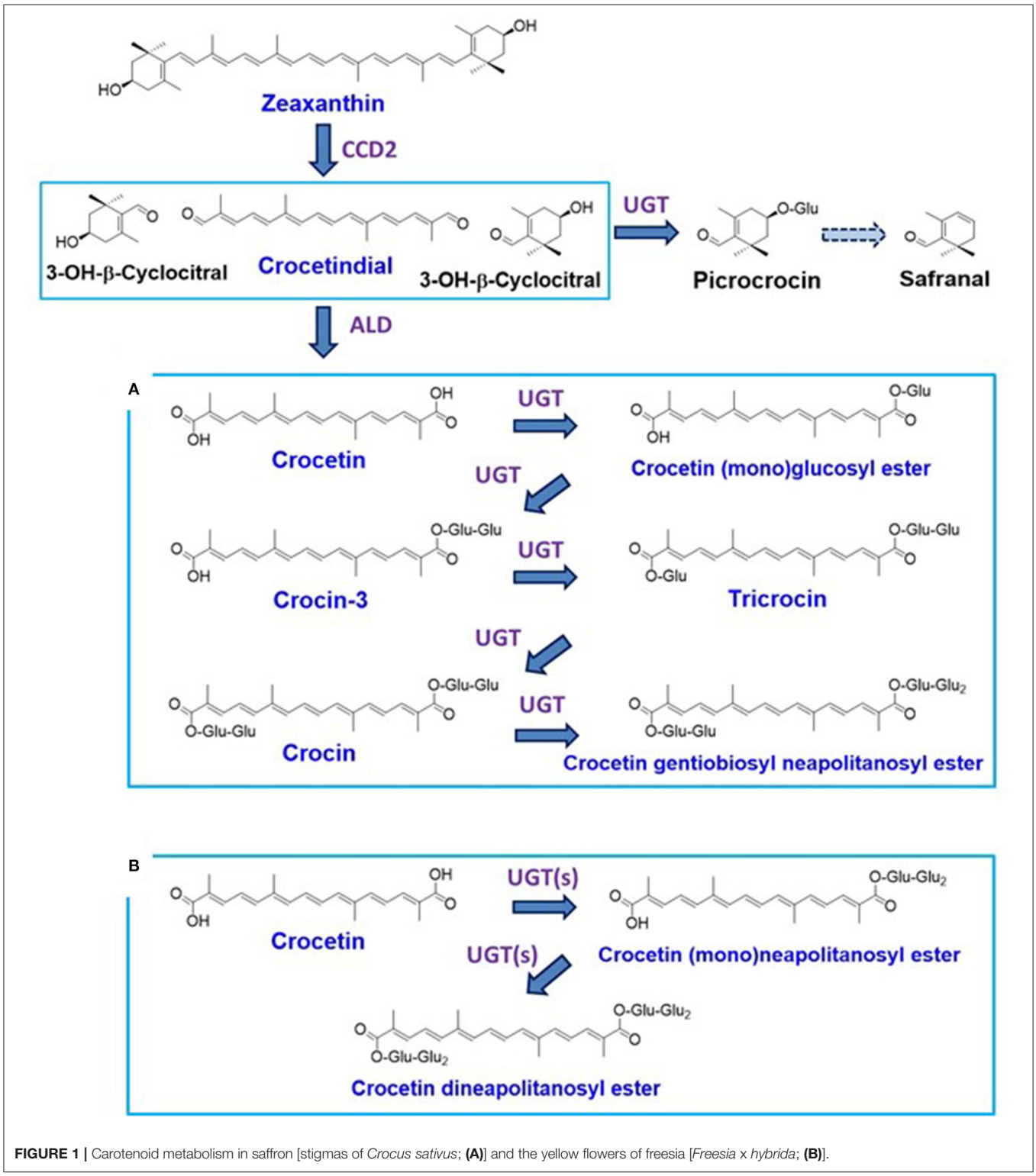


FIGURE 1 | Carotenoid metabolism in saffron [stigmas of *Crocus sativus*; **(A)**] and the yellow flowers of freesia [*Freesia* x *hybrida*; **(B)**].

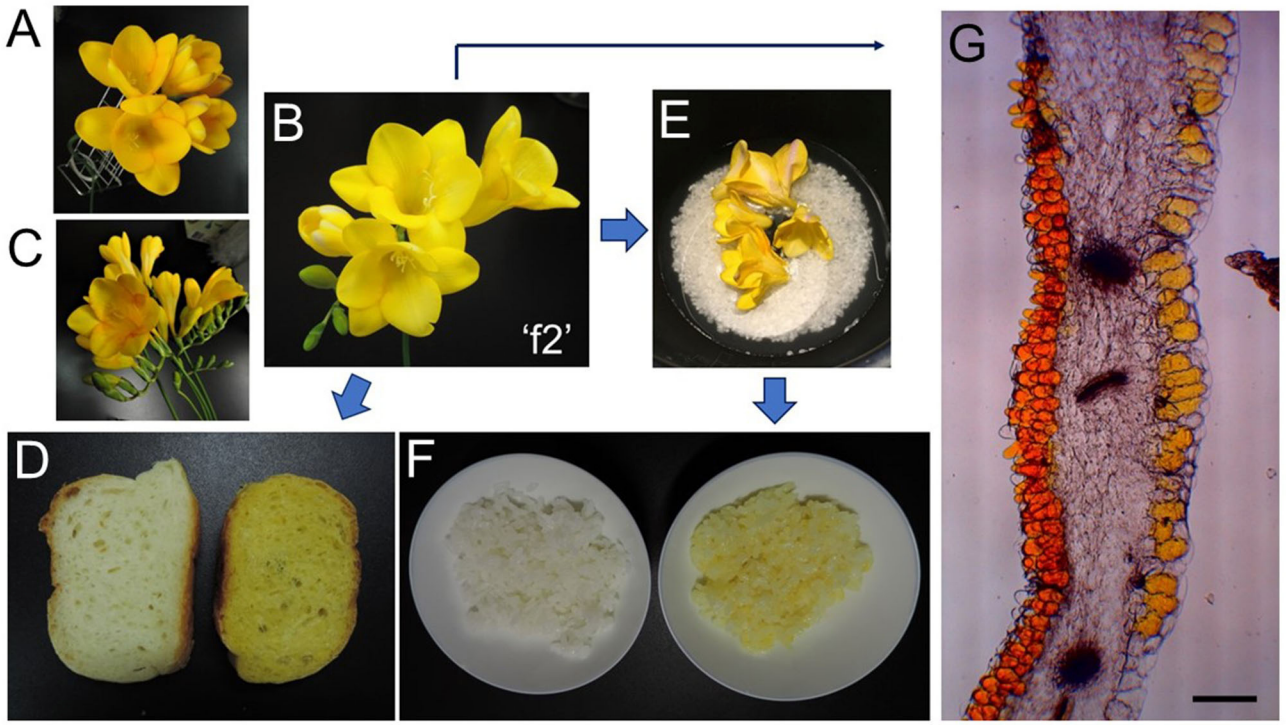


FIGURE 2 | Freesia-relevant pictures: **(A)**, freesia (*Freesia* x *hybrida* L.) cultivar "Aladin;" **(B)**, freesia cultivar "Ishikawa f2 go" ("f2"); **(C)**, freesia cultivar "Kayak;" **(D)**, bread baked without (left) and with (right) freesia "f2" flowers; **(E)**, before cooking with freesia "f2" flowers by a rice cooker; **(F)**, rice cooked without (left) and with (right) freesia "f2" flowers; **(G)**, microscopy image of a freesia "f2" petal (Bar represents 200 μm).

with lemon juice and zest. The peppery scent and bold color are a perfect pick-me-up." Utility applications of the freesia flowers as edible flowers are also suggested in this study.

MATERIALS AND METHODS

Plant Materials

Dried red pistils containing stigmas (called "saffron") from saffron plants (*Crocus sativus* L.) that had been cultivated in Spain were purchased at a spice-special store in Tokyo, Japan. As for freesia (*Freesia* x *hybrida*), we used cultivars "Ishikawa f2 go" ("f2"), "Kayak," and "Aradin." Cultivars "f2" and "Kayak" were grown in a greenhouse, and their flowers were harvested in middle and late March, respectively. Cultivar "Aladin" was purchased at a floral shop in Ibaragi, Japan.

Microscopy pictures of the "f2" petal were recorded by BH2 (Olympus) after cutting it in 3% agar with MicroSlicer ZERO-1 (Dosaka EM, Kyoto, Japan).

Reagents

Dichloromethane (CH_2Cl_2), methanol (MeOH), acetonitrile (CH_3CN), ethyl acetate (EtOAc), and chloroform of analytical grade were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan) or Kanto Chemical Co. Inc. (Tokyo, Japan). The other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, United States).

GC-MS Analysis

In order to detect volatile components of saffron and freesia "f2" flowers, we carried out a gas chromatography-mass spectrometry (GC-MS) analysis. Each 0.1 g of their dried materials was ground with an addition of 0.3 ml of water (H_2O) and 0.3 ml of MeOH, subsequently extracted with 0.6 ml of chloroform (by mixing and centrifugation), and the chloroform extraction was repeated by adding 0.5 ml of H_2O, and finally, each chloroform phase was concentrated to threefold by decompression. The prepared samples were subjected to GC-MS using Shimadzu GCMS-QP5050 (Shimadzu, Kyoto, Japan), as described (15).

NMR and MS Analysis

Nuclear magnetic resonance (NMR) spectra (^1H and ^{13}C NMR, ^1H-^1H DQF COSY, HMQC, HMBC, and NOESY) were measured using an AVANCE 400 MHz NMR spectrometer (Bruker, Rheinstettern, Germany) utilizing standard programs in TopSpin1.3. Chemical shifts were referenced to solvent signals. HR-ESI-MS (+) was measured using a JMS-T100LP mass spectrometer (JEOL, Tokyo, Japan) and accumulated mass calibration was performed using reserpine [$C_{33}H_{41}N_2O_9$, *m/z* 609.2812044 $(M+H)^+$].

Cooking Models and Actual Cooking

As a boiled cooking model, saffron (dried; 0.08 g) or freesia petals [1.8 g (fresh weight)] were added to boiled water (50 ml) and

boiled for 20 min. As a grilled cooking model, saffron (dried; 0.08 g) or freesia petals [1.8 g (fresh weight)] were added to olive oil (7.5 ml) and grilled at 180°C for 5 min.

A paella-type cooking was adopted as an example of the boiled dishes with saffron, as follows: Saffron (0.08 g)-including water (300 ml), polished rice (500 g), and olive oil (30 ml) were put together on a frying pan, and the materials were boiled under medium heat for 20 min. A pilaf-type cooking was adopted as an example of the grilled dishes with saffron, as follows: Olive oil (30 ml), polished rice (500 g), and saffron (0.08 g) were grilled on a frying pan under medium heat for 10 min. Then, water (300 ml) was added to the frying pan and the materials were boiled for 15 min.

Rice was cooked in a rice cooker, using polished rice (Japonica; 75 g), water (90 ml), and "f2" flowers (5 g fresh weight) (**Figure 2E**). After cooking, the flower debris was eliminated (**Figure 2F** right). Bread was baked as follows: Wheat powder (100 g), water (60 ml), NaCl (1.5 g), budding yeast (powder; 1 g), and dried powder from "f2" flowers (10 g fresh weight) by microwave were mixed and left for 2 h 20 min. The material was incubated for 1 h at 37°C, and baked for 21 min at 220°C (**Figure 2D** right).

Extraction of Apocarotenoids From Cooked Materials and Their HPLC-DAD Analysis

Each sample obtained by cooking had an addition of 100 μL of 9,10-dimethylanthracene solution (10 mg/1 ml CH_2Cl_2) as an internal standard to confirm the recovery rate of apocarotenoids. Each of the cooked materials as the cooking models was extracted by CH_2Cl_2-MeOH (1:1) (V:V) (20 ml), MeOH (20 ml), and 50% (V/V) MeOH (20 ml) in a stepwise manner by stirring for 30 min at 20°C. All the extracts (total 60 ml) were combined and concentrated to dryness *in vacuo*.

Each of the actually cooked materials [the paella (boiled) and pilaf (grilled) types] was freeze-dried and powdered by a mill. The powder was extracted by CH_2Cl_2-MeOH (1:1) (1 L), MeOH (1 L), and 50% (V/V) MeOH (1 L) in a stepwise manner by stirring for 30 min at 20°C, and all the extracts (total 3 L) were combined and concentrated to dryness *in vacuo*.

Each extract was dissolved into MeOH (10 ml), and 2.5 μL (saffron) or 20μL (freesia) of the supernatant was analyzed by high-performance liquid chromatography-diode array detector (HPLC-DAD) [column, PEGASIL ODS SP100 (4.6 × 150 mm, Senshu Scientific Co. Ltd., Tokyo, Japan); solvents: A 5% (V/V) CH_3CN containing 20 mM phospholic acid, B 95% (V/V) CH_3CN containing 20 mM phospholic acid. 0→ 5 min 100% A, 5→ 18 min 100% A→ 100% B linear gradient, 18–30 min 100% B, flow rate, 3.0 ml/min; detection, DAD; monitored at 250–600 nm].

Purification of Individual Apocarotenoids (1, 2, 3, and 4) From Saffron

Saffron (0.22 g) was extracted for 30 min at 20°C by stirring with CH_2Cl_2-MeOH (1:1) (V:V) (100 ml), MeOH (100 ml), and 50% (V/V) MeOH (100 ml) in a stepwise manner. CH_2Cl_2-MeOH (1:1) extract was concentrated to dryness to give a yellow oil, and the yellow oil was applied on a diol column chromatography (10 × 150 mm, CHROMATOREX DIOL MB 100-40/75, Fuji Silysia Chemical Ltd., Aichi, Japan) with *n*-hexane-CH_2Cl_2 (5:1) (V:V) and developed with the same solvent. The elution fractions containing yellow pigment were combined and concentrated to dryness (4.7 mg), and further purified by ODS preparative HPLC [column, Develosil C30-UG-5 (10 × 250 mm, Nomura Chemical Co. Ltd., Aichi, Japan); solvent, CH_2Cl_2-CH_3CN (95:5) (V:V); flow rate, 3.0 ml/min; detection, DAD; monitored at 250–600 nm]. The peak eluted at 9.5 min was collected to afford pure **4** (0.4 mg).

MeOH and 50% MeOH extracts were combined and concentrated to dryness (38.2 mg), and **1**–**3** in the extract were purified by ODS preparative HPLC [column, ADME ODS (10 × 250 mm, Osaka Soda Co. Ltd., Osaka, Japan); solvent, 50% (V/V) CH_3CN containing 0.1% (V/V) trifluoro acetic acid (TFA); flow rate, 3.0 ml/min; detection, DAD; monitored at 250–600 nm]. In this HPLC, the mixture of **1** and **2** was eluted at 3.0 min, and **3** was eluted at 5.0 min. The peak of **3** eluted at 5.0 min was collected and concentrated in a small volume to remove CH_3CN and finally freeze-dried to afford pure **3** (7.1 mg). The peak eluted at 3.0 min was also collected and freeze-dried to give yellow oil containing **1** and **2**. This yellow oil containing **1** and **2** was further purified by ODS preparative HPLC [column, PEGASIL ODS SP100 (20 × 250 mm); solvent, 30% (V/V) CH_3CN; flow rate, 8.0 ml/min; detection, DAD; monitored at 250–600 nm]. In this HPLC, **1** and **2** were eluted at 8.0 min (12.8 mg) and 13.0 min (11.4 mg), respectively, as pure compounds.

Purification of Individual Apocarotenoids (5 and 6) From Grilled Saffron

Saffron (0.22 g) in olive oil (10 ml) was grilled for 1 min in a frying pan with medium heat and extracted with CH_2Cl_2-MeOH (1:1) (V:V) (100 ml) twice. The extract (200 ml) was concentrated to dryness, added *n*-hexane (30 ml), and mixed well. The precipitate containing apocarotenoids was purified by ODS preparative ODS HPLC [column, Develosil C30-UG-5 (20 × 250 mm); solvent, 85% (V/V) CH_3CN containing 0.1% (V/V) TFA; flow rate, 8.0 ml/min; detection, DAD; monitored at 250-600 nm]. In this HPLC, **5** and **6** were eluted at 12.0 min (3.2 mg) and 17.0 min (2.1 mg), respectively, as pure compounds.

Purification of Yellow Pigments From Freesia Flowers

Freesia (*Freesia* x *hybrida*) "f2" flowers (179.3 g) were freeze-dried and powdered by mill (18.6 g). This powder was extracted for 30 min by stirring with CH_2Cl_2-MeOH (1:1) (V:V) (1 L), MeOH (1 L), 80% (V/V) MeOH (1 L), and 50% (V/V) MeOH (1 L) in a stepwise manner.

The CH_2Cl_2-MeOH (1:1) and MeOH extracts were combined and concentrated to dryness *in vacuo* (14.50 g). This extract

was dissolved in H_2O (300 ml) and partitioned with EtOAc (300 ml) twice, and a water layer containing yellow pigment was collected. The water layer was concentrated to 200 ml to remove EtOAc, 1 M HCl (10 ml) was added to adjust at pH3, and adsorbed to HP-20 column chromatography (50 × 150 mm, Mitsubishi Chemical Corporation, Tokyo, Japan). The HP-20 column was washed with H_2O (750 ml) and 50% (V/V) MeOH (750 ml), and the yellow compound was eluted with MeOH (750 ml). The MeOH eluate was concentrated to dryness to obtain yellow oil (1.11 g). The yellow oil was mixed with 15 ml of CH_2Cl_2, and the insoluble yellow sediment was collected (repeated twice). The obtained red sediment was dried (0.91 g), dissolved in 3 ml of 50% MeOH, and further purified using preparative ODS HPLC (column, Develosil C30-UG-5 (10 × 250 mm); solvent, 30% (V/V) CH_3CN + 0.1% (V/V) TFA; flow rate, 3.0 ml/min; detection, DAD; monitored at 200–600 nm). The yellow compound was eluted at 17.8 min as pure compound (**7**) (27.1 mg).

The 80% MeOH (including compound **7** also) and 50% MeOH extracts were combined and concentrated in a small volume (300 ml) to remove MeOH and partitioned with EtOAc (300 ml) twice, and the water layer containing yellow pigment was collected. The water layer was concentrated to 200 ml to remove EtOAc, and adsorbed to HP-20 column chromatography (50 × 150 mm). The HP-20 column was washed with H_2O (750 ml) and the yellow compound was eluted with 50% (V/V) MeOH (750 ml). The 50% MeOH eluate was concentrated to dryness to obtain yellow oil (0.75 g). The yellow oil was then purified using preparative ODS HPLC [column, Develosil C30-UG-5 (10 × 250 mm); solvent, 20% (V/V) CH_3CN + 0.1% (V/V) TFA; flow rate, 3.0 ml/min; detection, DAD; monitored at 200–600 nm]. The yellow compound was eluted at 15.3 min as pure compound (**8**) (14.0 mg).

Physico-Chemical Properties of Crocetin Neapolitanosyl Ester (7)

HR-ESI-MS (+) *m/z* 837.31834 ($C_{38}H_{54}O_{19}Na$ calcd. for 837.31570, Δ 3.16 ppm). UV-Vis λmax (ε) in MeOH 254 (9200), 315 (6800), 407 (43000), 428 (62000), 452 (56000). ^{1}H NMR (CD_3OD) δ: 1.96 (3H, s, H-19), 2.00 (9H, s, H-20, H-19', and H-20'), 3.15 (1H, m, H-5'''), 3.17 (1H, m, H-2'''), 3.21 (1H, m, H-2''''), 3.24 (2H, H-4'''' and H-5''''), 3.36 (2H, H-3''' and H-3''''), 3.37 (1H, m, H-4'''), 3.54 (1H, m, H-4''), 3.57 (1H, m, H-5''), 3.65 (2H, H-6'''b and H-6''''b), 3.73 (1H, m, H-2''), 3.78 (1H, m, H-3''), 3.80 (1H, m, H-6''b), 3.83 (2H, H-6'''a and H-6''''a), 4.15 (1H, m, H-6''a), 4.32 (1H, d, J = 7.6 Hz, H-1''''), 4.59 (1H, d, J = 7.8 Hz, H-1'''), 5.65 (1H, d, J = 7.8 Hz, H-1''), 6.47 (3H, H-14, H-9', and H-14'), 6.62 (1H, m, H-11'), 6.64 (1H, m, H-11), 6.70 (1H, m, H-8'), 6.78 (1H, m, H-12), 6.80 (2H, H-15 and H-15'), 7.29 (1H, d, J = 9.1 Hz, H-10'), 7.42 (1H, d, J = 11.9 Hz, H-10). ^{13}C NMR (CD_3OD) δ: 12.7 (C-19), 12.7 (C-19'), 12.9 (C-20), 12.9 (C-20'), 62.2 (C-6'''), 62.6 (C-6''''), 69.4 (C-6''), 70.6 (C-4''), 71.0 (C-4'''), 71.5 (C-4''''), 75.0 (C-2''''), 76.0 (C-2'''), 77.6 (C-3''), 77.6 (C-3''''), 77.7 (C-5''), 77.9 (C-5'''), 77.9 (C-5''''), 78.0 (C-3'''), 82.4 (C-2''), 94.6 (C-1''), 104.5 (C-1''''), 105.6 (C-1'''), 124.6 (C-11), 125.0 (C-11'), 126.3 (C-9'), 127.5 (C-14'), 127.7 (C-9), 132.6 (C-12'), 133.1 (C-12), 136.7 (C-14), 137.9 (C-13), 138.2 (C-13'), 140.4 (C-10'), 142.0 (C-10), 145.0 (C-15'), 146.4 (C-15), 168.1 (C-8). 171.9 (C-8').

$^1O_2$-Quenching Experiment

Methylene Blue (80 μL, 25 μM in ethanol) and linoleic acid (100 μL, 0.24 M in ethanol) were added to 5 ml glass test tubes with and without 40 μl of an apocarotenoid (final concentration, 1–100 μM in ethanol). The tubes were mixed well and then illuminated at 7,000 lux and 22°C for 3 h in a Styrofoam box, after which 50 μl of the reaction mixture was removed and diluted to 1.5 ml with ethanol. OD_{235} was then measured to estimate the levels of conjugated dienes formed in the reaction (16). OD_{235} in the absence of the carotenoid was also measured as a negative control (no $^1O_2$-quenching activity), and the $^1O_2$ quenching activity of each apocarotenoid was then calculated relative to this reference value. The activity was determined as the IC_{50} value, representing the concentration at which 50% inhibition was observed.

RESULTS

GC-MS Analysis of Volatile Components of Saffron and Freesia

Figures 3A,B show the results of GC analysis of the MeOH-chloroform extracts of saffron (**A**) and freesia "f2" flowers (**B**). Peak 1, one of the main peaks in saffron, was found to exist in freesia as peak 3. MS spectra of peak 1 and peak 3 had the highest similarity to safranal in the database of the GC-MS system (**Figure 3C**). Peak 4 appearing in freesia, which corresponded to peak 2 in saffron, was considered 3-hydroxy-β-cyclocitral ($C_{10}H_{16}O_2$; MW 168.23) by their MS spectra (**C**), which should be biosynthesized from zeaxanthin with carotenoid cleavage dioxygenase 2 (CCD2) (3, 17), as shown in **Figure 1**. It is thus likely that these two volatile compounds are present not only in saffron but also in freesia yellow flowers.

Chemical Changes of Apocarotenoids Crocetin Glycosides in Saffron

We extracted apocarotenoids from saffron (dried; 0.08 g) as well as its boiled and grilled samples by the cooking models, and analyzed them by HPLC-DAD (**Figures 4A–C**). Six apocarotenoids corresponding to peaks **1**–**4** and peaks **5**, **6** were isolated from saffron and the grilled saffron, respectively, by the methods described in Material and methods, and subjected to HR-ESI-MS, ^{1}H and ^{13}C NMR analyses. The results, **1**–**6** were identified as crocin (2), tricrocin (18), crocin-3 (18), crocetindial (19), crocetin (20), and 13-*cis* crocetin (21), respectively (their ^{1}H and ^{13}C NMR spectra of **1**–**6** are shown in **Supplementary Figures S1–S12**). The chemical structures of **1**–**6** are exhibited in **Figure 5**.

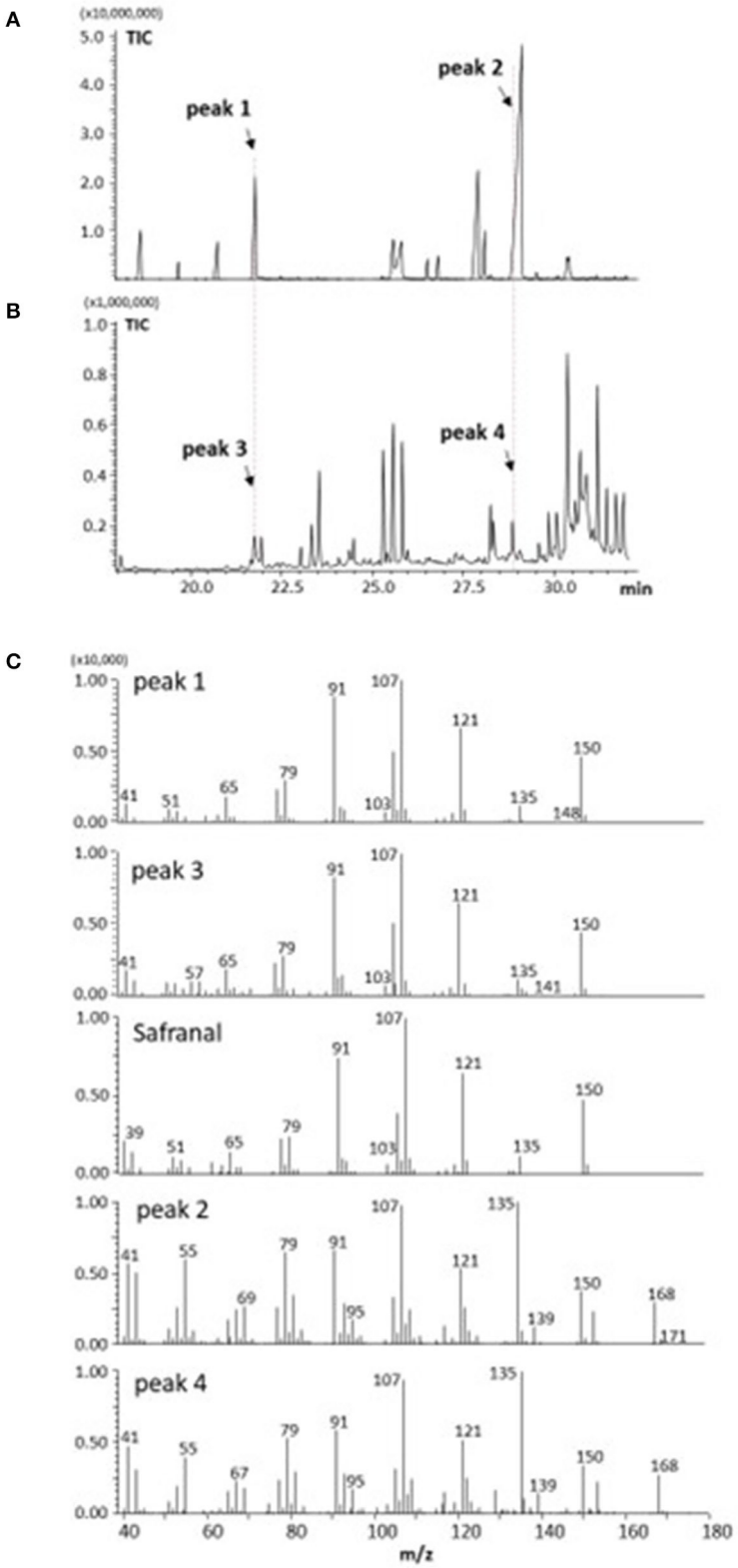


FIGURE 3 | GC-MS analysis of volatile components from saffron **(A,C)** and freesia "f2" yellow-flowers **(B,C)**.

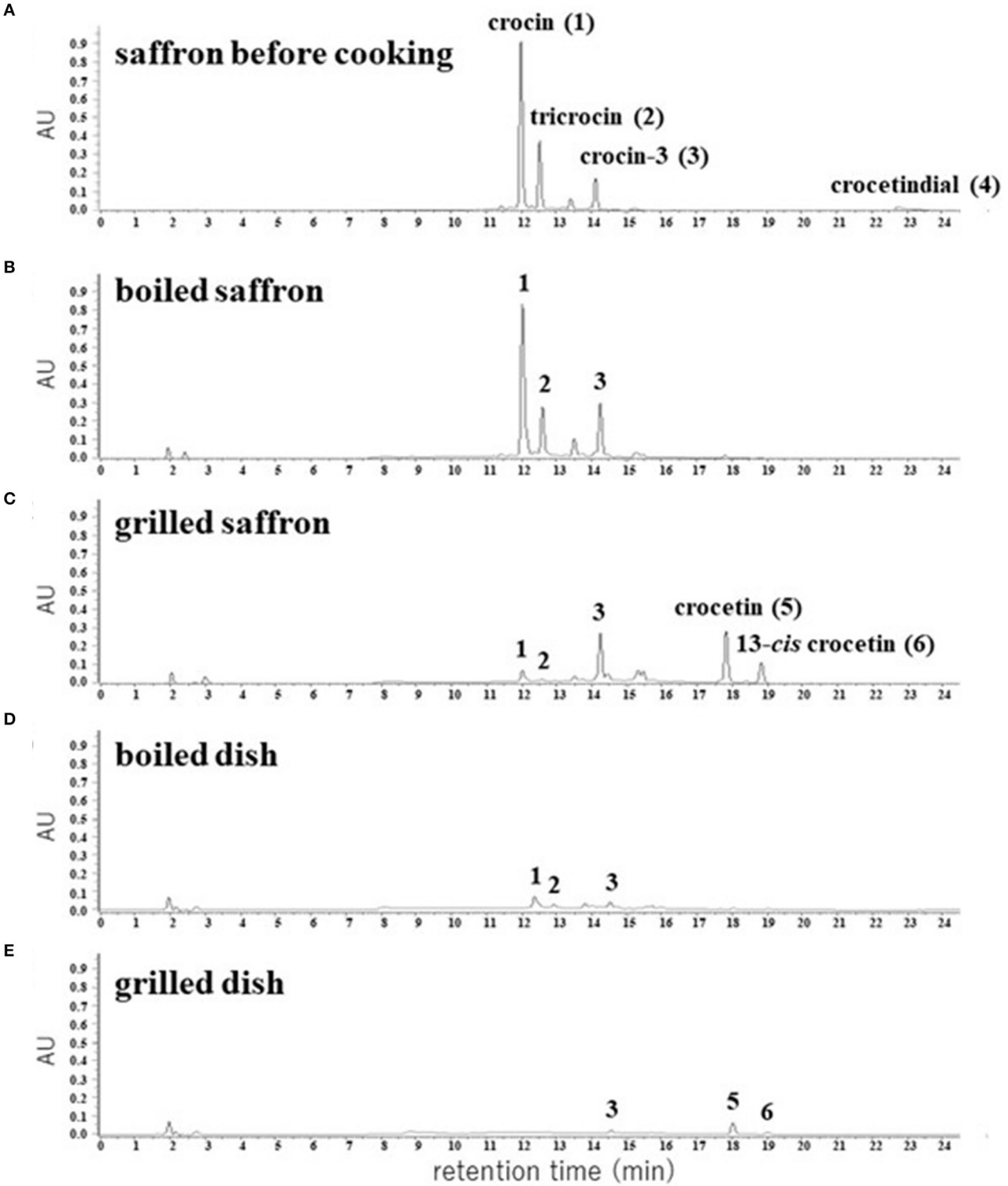


FIGURE 4 | HPLC chromatograms of apocarotenoids extracted from the dried pistils of saffron **(A)** and their cooked materials **(B–E)**. The detection wavelength was 440 nm.

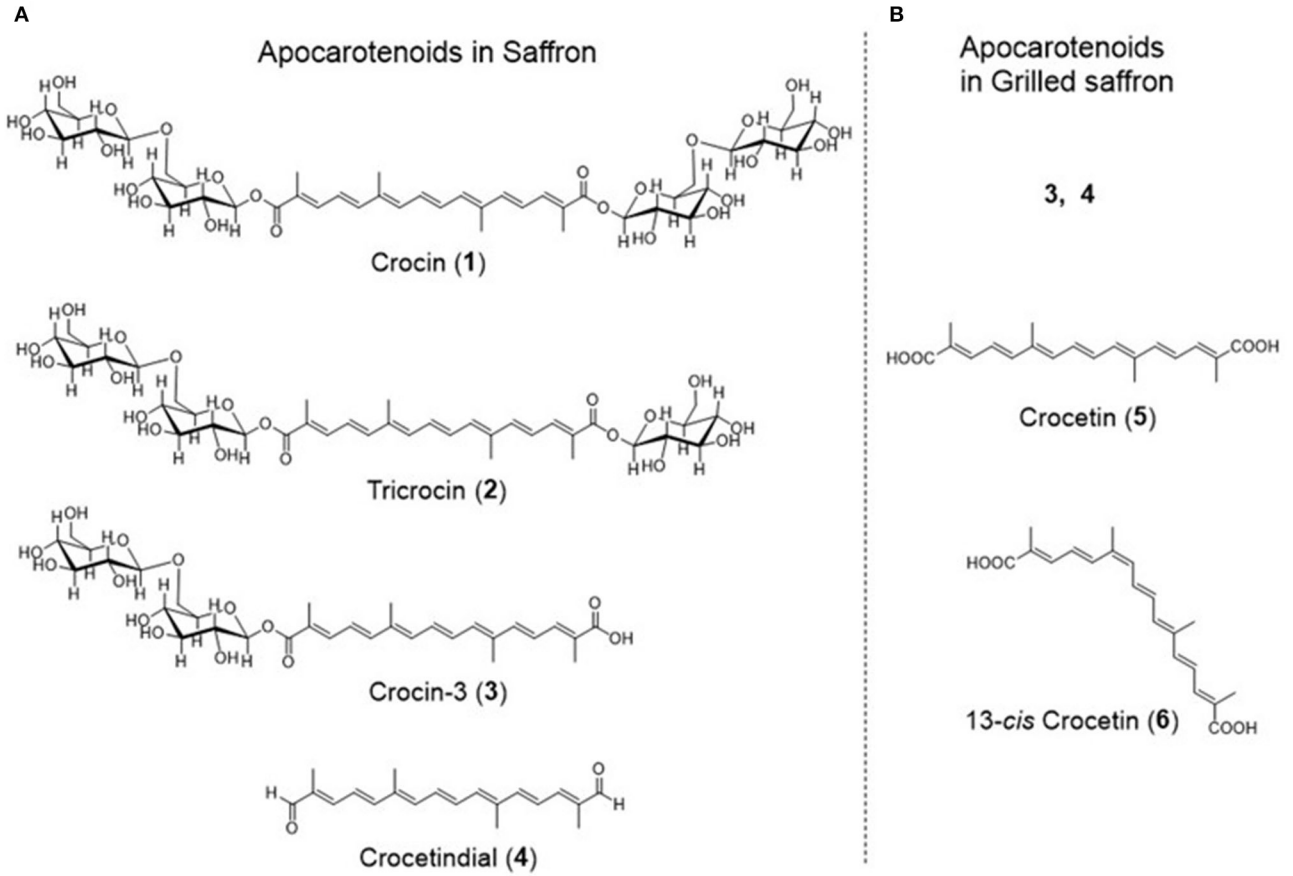


FIGURE 5 | The structures of apocarotenoids in saffron **(A)** and grilled saffron **(B)**.

The predominant apocarotenoid **1** and the other main apocarotenoids **2** and **3** showed the same UV-Vis spectra that possess λmax at 440 nm depending on the same aglycone, and minor apocarotenoids **4**–**6** also exhibited λmax around 440 nm (**Supplementary Figure S13**). Thus, we calculated the peak areas of individual apocarotenoids (**1**–**6**) and total apocarotenoids (**1** + **2** + **3** + **4** + **5** + **6**) at 440 nm in saffron, the boiled saffron, and the grilled saffron in HPLC-DAD analyses, as listed in **Table 1**. In the boiled cooking model of saffron, the amount of crocin (**1**), tricrocin (**2**), and crocin-3 (**3**) almost did not change, while crocetindial (**4**) disappeared, presumably due to its decomposition or a chemical reaction to other compounds. In the grilled cooking model of saffron, the amount of crocin (**1**) and tricrocin (**2**) decreased, the amount of crocin-3 (**3**) slightly increased, and crocetin (**5**) and 13-*cis* crocetin (**6**) appeared (**Figure 4C**). In addition, the total amount of apocarotenoids decreased to about 1/3 (**Table 1**). **Supplementary Figure S18** also shows that the purified crocin (**1**) and crocetin (**2**) were partially changed into crocetin (**5**) and 13-*cis* crocetin (**6**), and into **6**, respectively, at 180°C for 5 min. These observations indicated that hydrolysis of the ester linkage between glucose—the crocetin aglycone occurred, presumably by conversion from crocin and tricrocin to crocin-3 and further to crocetin, along with the subsequent *trans* to 13-*cis* isomerization of a part of crocetin. It is also likely that the degradation of the apocarotenoids occurred through strong heat cooking like grilling.

In order to confirm whether the same chemical changes occur in actual cooking, saffron was mixed with rice and a typical boiling and grilling cooking process to prepare dishes was performed. The apocarotenoids contained in the dishes were extracted and subjected to HPLC-DAD analyses (**Figures 4D,E**; **Table 1**). These results apparently showed that the same chemical changes occurred in the actual cooking. However, the total peak area of detected apocarotenoids in both dishes has decreased to about 1/5-−1/10. It is currently under investigation what happens to such disappearing apocarotenoids.

Identification of Apocarotenoids Crocetin Glycosides in Freesia

The apocarotenoids in freesia "f2" flowers (**7** and **8**) were isolated through chromatographic methods as described in Material and method.

The structures of **7** were analyzed by HR-ESI-MS and 1D (^{1}H and ^{13}C) and 2D (^{1}H-^{1}H DQF COSY, HMQC, and HMBC) NMR spectra. HR-ESI-MS analysis of **7** showed $(M + Na)^+$ at *m/z* 837.31834, and the molecular formula of **7** was determined

TABLE 1 | HPLC peak areas (mV × s) of apocarotenoids in cooked saffron at 440 nm.

Cooking\apocarotenoid	Crocin (1)	Tricrocin (2)	Crocin-3 (3)	Crocetindial (4)	Crocetin (5)	13-*cis* crocetin (6)	Total apocarotenoid peak areas (1 + 2 + 3 + 4 + 5 + 6)
Saffron (before cooking)	3.2×10^6	1.1×10^6	6.0×10^5	3.5×10^4	N.D.	N.D.	5.0×10^6
Boiled saffron	2.6×10^6	8.1×10^5	5.6×10^5	N.D.	N.D.	N.D.	4.4×10^6
Boiled saffron/saffron	81%	74%	93%	0%			88%
Grilled saffron	2.1×10^6	2.9×10^5	7.5×10^5	N.D.	6.3×10^5	3.2×10^5	1.8×10^6
Grilled saffron/saffron	66%	26%	125%	0%			36%
Boiled dish	2.6×10^5	4.5×10^4	6.7×10^4	N.D.	N.D.	N.D.	9.8×10^5
Boiled dish/saffron	8.1%	4.1%	11%	0%			20%
Grilled dish	N.D.	N.D.	3.8×10^4	N.D.	4.0×10^5	3.0×10^4	4.0×10^5
Grilled dish/saffron	0%	0%	6.3%	0%			8.0%

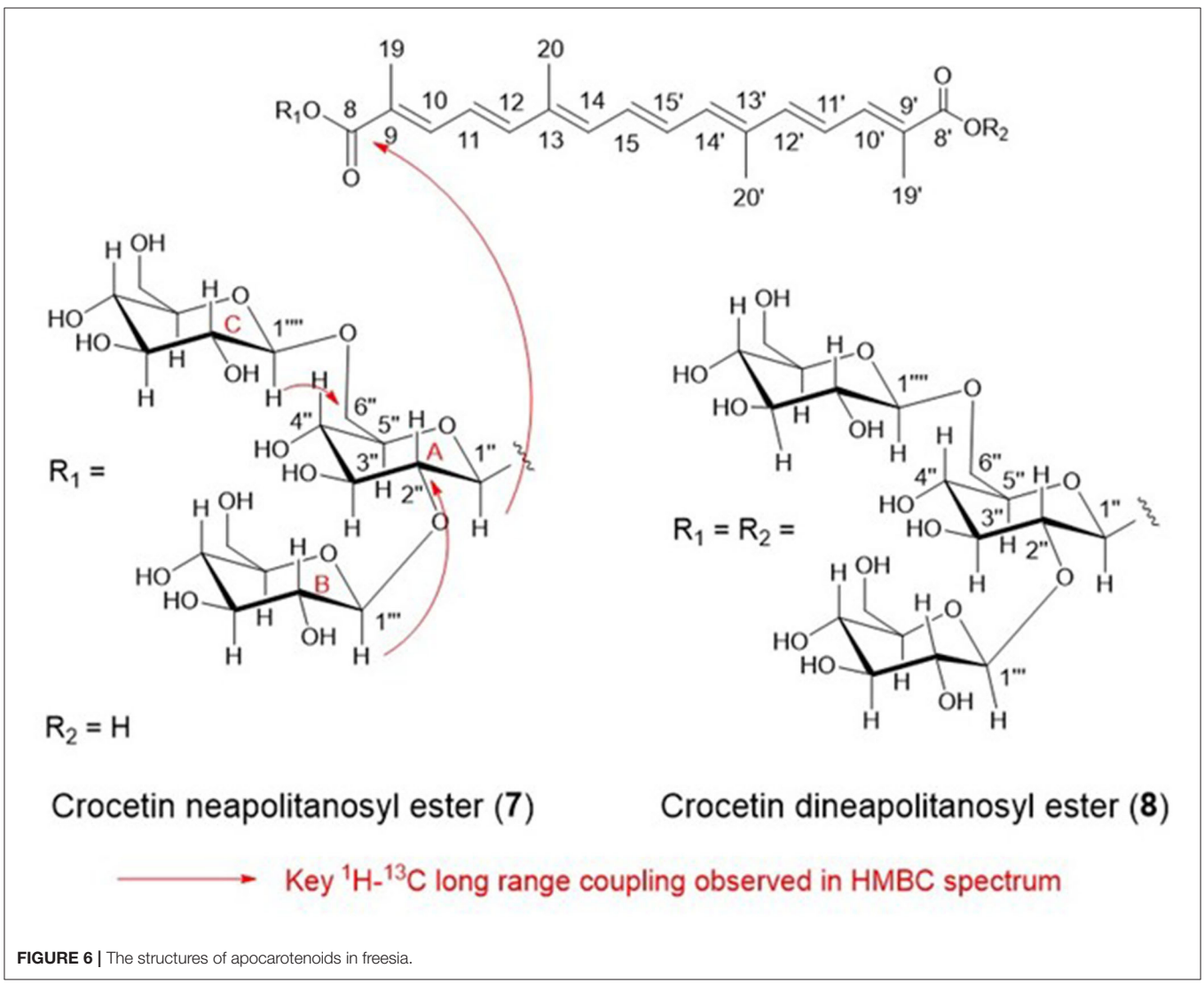


FIGURE 6 | The structures of apocarotenoids in freesia.

as $C_{38}H_{54}O_{19}$ [$(M + Na)^+$ $C_{38}H_{54}O_{19}Na$ calcd. for 837.31570 (Δ3.16 ppm)]. The molecular formula of **7** was identical to that of tricrocin, and the ^1H and ^{13}C NMR spectra of **7** in CD_3OD (**Supplementary Figures S13, S14**) were closely related to tricrocin but not identical. The carotenoid aglycone of **7** proved to be identical to that of tricrocin (=crocetin) by comparison the ^1H and ^{13}C NMR with those of crocetin. Since three anomeric signals [δ_H 4.32 (J = 7.6 Hz), δ_H 4.59

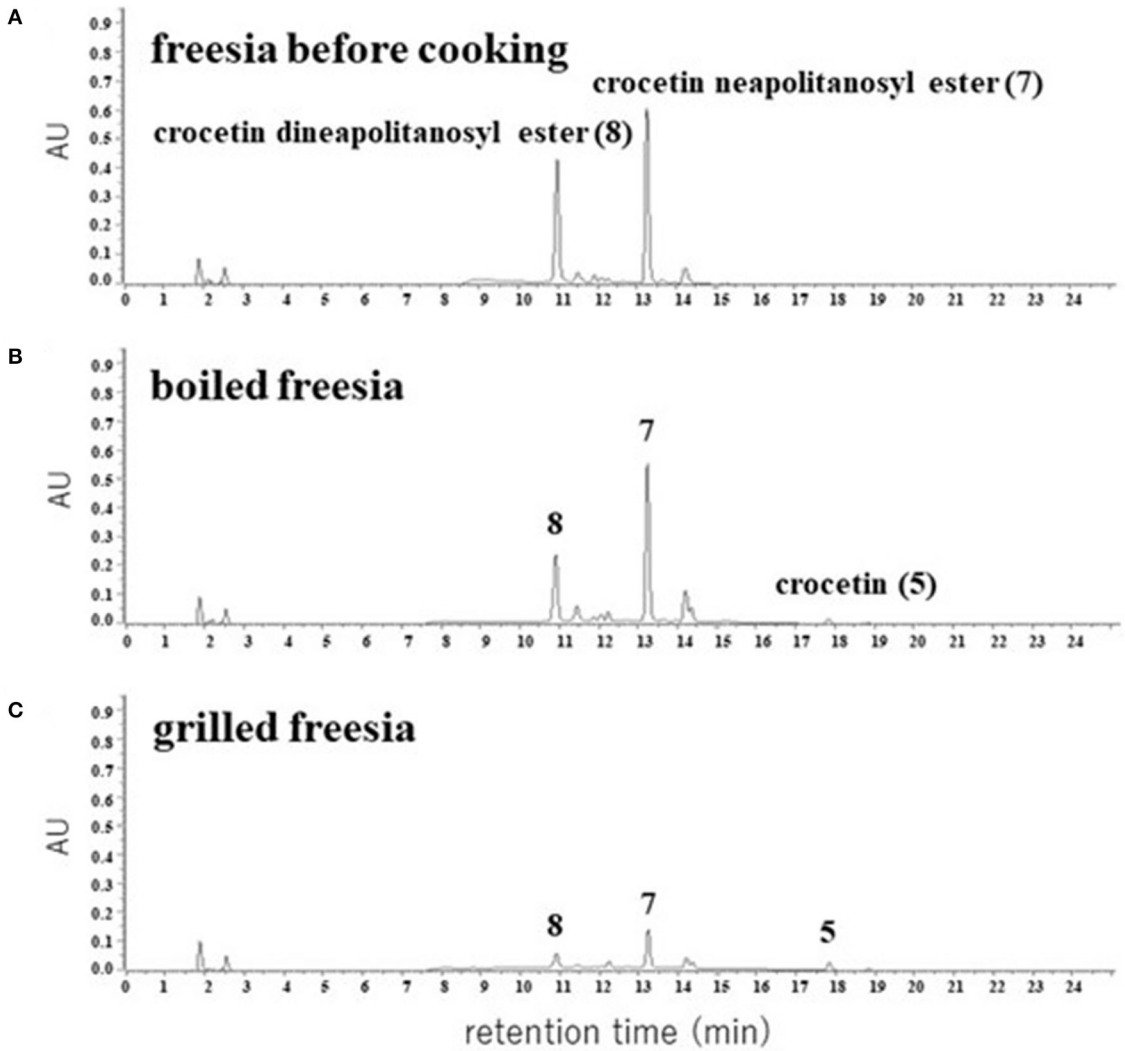


FIGURE 7 | HPLC chromatograms of apocarotenoids extracted from the yellow petals of freesia "Kayak" **(A)** and their cooked materials **(B,C)**. The detection wavelength was 440 nm.

(J = 7.8 Hz), δ_H 5.65 (J = 7.8 Hz)] with β configuration were observed in the ^{1}H NMR spectrum of **7** and since the molecular formula of **7** ($C_{38}H_{54}O_{19}$) was [crocetin ($C_{20}H_{24}O_4$) + 3 hexose ($C_{18}H_{36}O_{18}$)−3H_2O], **7** was proposed to possess a structure in which 3 β-hexoses were bound to crocetin. To determine the type of hexose, **7** (5.0 mg) was hydrolyzed in 2 M HCl (5 ml) under reflux for 2 h, and the hydrolysate was partitioned with EtOAc (5 ml) twice. The H_2O layer containing hexose was concentrated to dryness (2.0 mg), and analyzed by ^{1}H and ^{13}C NMR in D_2O, and $[\alpha]_D$ [+68.3° (c 0.1, H_2O)]. These data clearly proved that hexoses in **7** were all D-glucose. The linkages of 3 β-D-glucose to crocetin in **7** were analyzed by ^{1}H-^{1}H DQF COSY, HSQC, and HMBC, and the linkages among glucoses were proved to be glucoseB(1→ 2)glucoseA and glucoseC(1→ 6)glucoseA and the ester linkage of glucoseA to crocetin was proved by the key ^{1}H-^{13}C long-range couplings observed in HMBC spectrum (from H-1‴ (δ_H 4.59) to C-2" (δ_C 82.4), from H-1"" (δ_H 4.32) to C-6" (δ_C 69.4), and from H-1" (δ_H 5.65) to C-8 (δ_C 168.1) as shown in **Figure 6**. From these observations, the structure of **7** was determined to be crocetin neapolitanosyl ester (**Figure 6**).

The production of **7** by artificial manufacturing processing was reported in two previous studies (22, 23), while this is the first report finding **7** in natural flowers.

HR-ESI-MS analysis of **8** showed $(M-H)^-$ at m/z 1299.47367, and the molecular formula of **8** was determined as $C_{56}H_{84}O_{34}$ [$(M-H)^-$ $C_{56}H_{84}O_{33}$ calcd. for 1299.47657 (Δ 2.23 ppm), and this formula was identical with that of crocetin dineapolitanosyl ester which was reported as a minor yellow pigment produced by *Crocus sativus*. Thus, we compared ^{1}H and ^{13}C NMR spectrum of **8** (**Supplementary Figures S15, S16**) with those of reported crocetin dineapolitanosyl ester (2), and identified **8** as crocetin dineapolitanosyl ester (**Figure 6**).

TABLE 2 | HPLC peak areas (mV × s) of apocarotenoids in cooked freesia yellow flowers at 440 nm.

Cooking\apocarotenoid	Crocetin dineapolitanosyl ester (8)	Crocetin neapolitanosyl ester (7)	Crocetin (5)	Total apocarotenoid peak areas (8 + 7 + 5)
Freesia (before cooking)	5.6×10^6	6.1×10^6	N.D.	1.2×10^7
Boiled freesia	3.3×10^6	5.9×10^6	2.3×10^5	9.2×10^6
Boiled freesia/freesia	59%	97%		84%
Grilled freesia	5.9×10^5	1.4×10^6	1.4×10^5	2.1×10^6
Grilled freeisia/freesia	11%	23%		18%

TABLE 3 | $^1O_2$-quenching activities of apocarotenoids and astaxanthin.

Compound	IC_{50} (μM)
Crocin (**1**)	48 ± 0.4
Tricrocin (**2**)	46 ± 3.5
Crocin-3 (**3**)	64 ± 9.1
Crocetindial (**4**)	24 ± 1.9
Crocetin (**5**)	54 ± 7.4
13-*cis* crocetin (**6**)	54 ± 8.5
Crocetin neapolitanosyl ester (**7**)	56 ± 2.7
Crocetin dineapolitanosyl ester (**8**)	64 ± 6.3
Astaxanthin (control)	1.4 ± 0.1

Chemical Changes of Apocarotenoids Crocetin Glycosides in Freesia

We extracted apocarotenoids from the yellow petals of freesia cultivar "Kayak" [1.8 g (fresh weight)] as well as their boiled and grilled samples by cooking models, and analyzed them by HPLC-DAD (**Figure 7**). **Figure 7A** (**Table 2**) revealed that the petals of freesia "Kayak," like the case of "f2," possessed the dineapolitanosyl ester and (mono)neapolitanosyl ester of crocetin dominantly, which were 48 and 52% of whole apocarotenoids when calculated by peak areas at 440 nm, respectively. In the boiled cooking model in freesia, the amount of crocetin dineapolitanosyl ester was reduced to 59% and a small amount of crocetin appeared, while the level of crocetin (mono)neapolitanosyl ester was not changed (**Table 2**; **Figure 7B**). In the grilled cooking model in freesia, the amounts of crocetin dineapolitanosyl ester and crocetin neapolitanosyl ester were reduced to 11 and 23%, respectively, and a small amount of crocetin also appeared (**Table 2**; **Figure 7C**). These results indicated that hydrolysis of the ester linkage between glucose—the crocetin aglycone occurred in both cooking models, presumably by conversion from crocetin dineapolitanosyl ester to crocetin neapolitanosyl ester and further to crocetin. The degradation of the apocarotenoids is likely to occur specifically through strong heat cooking like grilling.

Significance of This Study on Freesia

We also extracted and analyzed apocarotenoids from the flowers of freesia cultivar "Aladin" in the same way, and found that freesia "Aladin" possessed the dineapolitanosyl ester and (mono)neapolitanosyl ester of crocetin dominantly, like "f2" and "Kayak."

This study revealed that the main pigments in the yellow flowers of freesia (*Freesia* x *hybrida*) were unique apocarotenoids, crocetin (mono)neapolitanosyl ester, and crocetin dineapolitanosyl ester. Plants that include the (mono)neapolitanosyl ester of crocetin have not been reported so far. The stigmas of *Crocus sativus* (saffron) and the fruits of *Gardenia jasminoides*, retain crocin predominantly (1, 2). Recently, fully open mature flowers of *G. jasminoides* were shown to accumulate crocin-3 and crocetin as main apocarotenoids (24). These findings suggest that, among plants producing crocetin glycosides, only freesia retains UDP-glucose transferase(s) [UGT(s)] that has activity sufficient for constructing the neapolitanosyl group composed of three molecules of glucose, as shown in **Figure 1B**. The microscopy image of the petal of cultivar "f2" showed that such yellow apocarotenoids accumulate not in plastids (chromoplasts) but vacuoles (**Figure 2G**).

In addition, we demonstrated that the freesia apocarotenoids can keep yellow color after bread and rice cooking, as shown in **Figures 2D,F**. This study opens the feasibility of freesia yellow flowers as new edible flowers with beneficial functions for human health.

$^1O_2$-Quenching Activities of Apocarotenoids

Many carotenoids were reported to possess $^1O_2$-quenching activity depending on their olefin structures (14). Although such activity of crocin (**1**) was reported (25), $^1O_2$-quenching activity of **2**–**8** has not been reported so far. Thus, we examined these $^1O_2$-quenching activities and proved that **1**–**8** possessed almost equivalent moderate $^1O_2$-quenching activity (IC_{50} 24–64 μM), presumably due to their same conjugation number (=9) (**Table 3**). On the other hand, apocarotenoids **1**–**8** did not show DPPH radical scavenging activities and lipid peroxidation-inhibiting activities even at 100 μM, according to our experiment.

CONCLUSION

In this study, we first reported chemical changes of apocarotenoids in saffron and freesia through cooking models as well as identification of unique apocarotenoids in freesia flowers. We also evaluated the $^1O_2$-quenching activities of apocarotenoids in saffron and freesia.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

This research was conceived and supervised by KS and NM. Laboratory experiments were done by KS, YS, SS, CT, MA, SU, YU, J-iH, and II. Field experiments were carried out by AK, MK, and MM. Data were analyzed by KS, J-iH, MT, and NM. Pictures were recorded by NM. The manuscript was written by KS and NM. 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/fnut.2022.885412/full#supplementary-material

REFERENCES

1. Britton G, Liaaen-Jensen S, Pfander H, *Carotenoids Handbook*. Basel; Boston; Berlin: Birkhäuser (2004).
2. Pfister S, Meyer P, Steck A, Pfander H. Isolation and structure elucidation of carotenoid–glycosyl esters in gardenia fruits (Gardenia jasminoides Ellis) and saffron (*Crocus sativus Linne*). *J Agr Food Chem.* (1996) 44:2612–5. doi: 10.1021/jf950713e
3. Frusciante S, Diretto G, Bruno M, Ferrante P, Pietrella M, Prado-Cabrero A, et al. Novel carotenoid cleavage dioxygenase catalyzes the first dedicated step in saffron crocin biosynthesis. *P Natl Acad Sci.* (2014) 111:12246–51. doi: 10.1073/pnas.1404629111
4. López-jimenez AJ, Frusciante S, Niza E, Ahrazem O, Rubio-Moraga Á, Diretto G, et al. A new glycosyltransferase enzyme from family 91, UGT91P3, is responsible for the final glucosylation step of crocins in saffron (*Crocus sativus L.*). *Int J Mol Sci.* (2021) 22:8815. doi: 10.3390/ijms22168815
5. Carmona M, Zalacain A, Salinas MR, Alonso GL. A new approach to saffron aroma. *Crit Rev Food Sci.* (2007) 47:145–59. doi: 10.1080/10408390600626511
6. Garc-Olmo DC, Riese HH, Escribano J, Ontan J, Fernandez JA, Atiénzar MM, Garcí-Olmo D. Effects of long-term treatment of colon adenocarcinoma with crocin, a carotenoid from saffron (*Crocus sativus L.*): an experimental study in the rat. *Nutr Cancer.* (1999) 35:2; 120–6. doi: 10.1207/S15327914NC352_4
7. Colapietro A, Mancini A, D'Alessandro AM, Festuccia C. Crocetin and crocin from saffron in cancer chemotheraphy and chemoprevention. *Anti-cancer Agent Me.* (2019) 19:38–47. doi: 10.2174/1871520619666181231112453
8. Korani S, Korani M, Sathyapalan T, Sahebkar A. Therapeutic effects of crocin in autoimmune diseases: a review. *Bio Factors.* (2019) 45:835–43. doi: 10.1002/biof.1557
9. Pashirzad M, Shafiee M, Avan A, Ryzhikov M, Fiuji H, Bahreyni A, et al. Therapeutic potency of crocin in the treatment of inflammatory diseases: Current status and perspective. *J Cell Physiol.* (2019) 234:14601–11. doi: 10.1002/jcp.28177
10. Zhang S, Ji J, Zhang S, Guan C, Wang G. Effects of three cooking methods on content changes and absorption efficiencies of carotenoids in maize. *Food Funct.* (2020) 11:944–54. doi: 10.1039/C9FO02622C
11. Miglio C, Chiavaro E, Visconti A, Fogliano V, Pellegrini N. Effect of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. *J Agri Food Chem.* (2008) 56:139–47. doi: 10.1021/jf072304b
12. Gilbertson-Ferriss, TL. Freesia × Hybrida En., Fr., Ge., Sp., Freesia. Halevy AH, editor. *CRC Handbook of Flowering Vol III*. Boca Raton: CRC Press (2019). p. 4.
13. Wang L. Freesia. Flower Breeding and Genetics (2006). ISBN: 978-1-4020-4427-4
14. Murahama M, Matsuda E, Hirano H, Isu H. Breeding of a new freesia cultivar 'Ishikawa f2 go' (Freesia spp). *Hort Res.* (2020) 19:309–11. doi: 10.2503/hrj.19.309
15. Hattan J, Shindo K, Sasaki T, Ohno F, Tokuda H, Ishikawa K, et al. Identification of novel sesquiterpene synthase genes that mediate the biosynthesis of valerianol, which was an unknown ingredient of tea. *Sci Rep.* (2018) 8:12474. doi: 10.1038/s41598-018-30653-w
16. Hirayama O, Nakamura K, Hamada S, Kobayashi Y. Singlet oxygen quenching ability of naturally occurring carotenoids. *Lipids.* (1994) 29:149–50. doi: 10.1007/BF02537155
17. Fang Q, Li Y, Liu B, Meng X, Yang Z, Yang S et al. Cloning and functional characterization of a carotenoid cleavage dioxygenase 2 gene in safranal and crocin biosynthesis from Freesia hybrida. *Plant Physiol Biochem.* (2020) 154:439–50. doi: 10.1016/j.plaphy.2020.06.035
18. Zhang H, Zeng Y, Yan F, Chen F, Zhang X, Liu M. Semi-preparative isolation of crocins from saffron (*Crocus sativus L*). *Chromatographia.* (2004) 59:691–6. doi: 10.1365/s10337-004-0311-z
19. Frederico D, Donate PM, Constantino MG, Bronze ES, Sairre MI. A short and efficient synthesis of crocetin-dimethyl ester and crocetindial. *J Org Chem.* (2003) 68:9126–8. doi: 10.1021/jo034545y
20. Pfander H, Schurtenberger H. Biosynthesis of C20-carotenoids in Crocus sativus. *Phytochemistry.* (1982) 21:1039–42. doi: 10.1016/S0031-9422(00)82412-7
21. Samaha H, Chahine N, Sobolev AP, Menghini L, Makhlouf H. 1H-NMR metabolic profiling and antioxidant activity of saffron (Crocus sativus) cultured in Lebanon. *Molecules.* (2021) 26:4906. doi: 10.3390/molecules26164906
22. Dufresne D, Cormier F, Dorion S, Niggli UA, Pfister S, Pfander H. Glycosylation of encapsulated crocetin by a *Crocus sativus L.* cell culture Enzyme. *Microb Tech.* (1999) 24:453–62. doi: 10.1016/S0141-0229(98)00143-4
23. Zareena AV, Variyar PS, Gholap AS, Rongirwar DR. Chemical investigation of gamma-irradiated saffron (*Crocus sativus L.*). *J Agr Food Chem.* (2001) 49:687–91. doi: 10.1021/jf000922l
24. Sommano SR, Suppakittpaisam P, Sringarm K, Junmahasathien T, Ruksiriwanich W. Recovery of crocins from floral tissue of Gardenia jasminoides Ellis. *Front Nutrition.* (2020) 7:106. doi: 10.3389/fnut.2020.00106

25. Pham TQ, Cormier F, Farnworth E, Tong VH, Calsteren MV. Antioxidant properties of crocin from Gardenia jasminoides Ellis and study of the reactions of crocin with linoleic acid and crocin with oxygen. *J Agr Food Chem.* (2000) 48:1455–61. doi: 10.1021/jf991263j

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