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Ethnofood chemistry: Bioactive components in unexploited foods from centres of biodiversity

Edited by
Michael Rychlik, Dejian Huang and Yasmina Sultanbawa

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Ethnofood chemistry: Bioactive components in unexploited foods from centres of biodiversity

Topic editors

Michael Rychlik — Technical University of Munich, Germany
Dejian Huang — National University of Singapore, Singapore
Yasmina Sultanbawa — The University of Queensland, Australia

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Marcello Iriti,
University of Milan, Italy

*CORRESPONDENCE
Yasmina Sultanbawa
✉ y.sultanbawa@uq.edu.au

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Editorial: Ethnofood chemistry: bioactive components in unexploited foods from centres of biodiversity

Yasmina Sultanbawa[1]*, Dejian Huang[2] and Michael Rychlik[1,3]

[1]Centre for Nutrition and Food Sciences, ARC Training Centre for Uniquely Australian Foods, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, QLD, Australia, [2]Department of Food Science and Technology, National University of Singapore, Singapore, Singapore, [3]Analytical Food Chemistry, Department of Life Science Engineering, School of Life Sciences, Technical University of Munich, Freising, Germany

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Editorial on the Research Topic
Ethnofood chemistry: bioactive components in unexploited foods from centres of biodiversity

Billions of people globally lack access to a healthy diet which is a leading cause of many noncommunicable diseases and individual foods and nutrients are important for diet diversity which is critical for maintaining health. Dietary patterns of different populations are based on a combination of different foods and there is an interaction in relation to health between foods and nutrients when consumed together. This is where ethnofoods (traditional foods) as unexploited and underutilized food can make a significant change to health and wellbeing by contributing to the diversity of foods needed in the diet.

Ethnofoods are consumed by Indigenous populations worldwide and form an integral component of the unexploited foods from the Centers of Biodiversity. They are nutrient-dense and are rich sources of micro nutrients. In addition, due to the occurrence of vitamins, trace elements, antioxidants and important dietary phytochemicals, diet diversification with ethno-plant foods is a sustainable and affordable strategy for health promotion of these populations and offers dietary perspectives also for other regions in the world.

The aim of this Research Topic is to feature the importance of incorporating ethno plant foods in the diet not only to provide nutrition and health but also to the role these plants played in different centers of biodiversity. This Research Topic is focussed on looking at ethno plant foods from Centers of Biodiversity (Africa, Asia and Australia, Central America, South America) with bioactive components of nutritional and health value.

There is a total of 10 manuscripts included in this Research Topic. Two studies explored the physicochemical and bioactive characterization and *in vitro* health properties of the Peruvian Andean maize (*Zea mays* L.). Fuentes-Cardenas et al. studied the Peruvian Andean maize race *Cabanita* with respect to its bioactive profiles, physical characteristics, and *in vitro* antioxidant properties. The diversity of the Peruvian Andean maize race *Cabani*ta from two provinces (Caylloma and Castilla) in the Arequipa region showed that it was a promising source of phenolic compounds (p-coumaric and ferulic acid derivatives). The anthocyanins were only present in samples that were partially red and contained purple-pigmented kernels. Ranilla et al. investigated the variability of the metabolites in three maize types (white, red and orange) from the same Peruvian Andean race *Cabanita.* Maize with different pigmentation from the *Cabanita* race showed variations in primary and secondary

metabolite composition (phenolic and carotenoid) and was different at different stages of grain maturity. Most phenolic compounds decreased with kernel maturity in all *Cabanita* maize types. All maize types showed similar fatty acid contents which increased with kernel development. In general, all *Cabanita* maize types had similar *in vitro* health-relevant functional properties which were reduced significantly with grain development. Another two studies presented work on Australian native plant foods that have been consumed by the Indigenous population for thousands of years. A recent study on charred plant food remains from Madjedbebe rockshelter in northern Australia, dated the use of these plants back to between 65,000 and 53,000 years. Fyfe, Hong et al. reported on the folate vitamers of the green plum, a native fruit of Australia that grows on the tree *Buchanania obovata* in the northern parts of the Northern Territory and Western Australia. This study revealed that green plums are a good natural dietary source of folates. The green plum 5-methyltetrahydrofolate form of folate increases and accumulates in the fruit through development, ripening and senescence. Akter et al. studied the *in vitro* bioaccessibility and intestinal absorption of selected bioactive compounds in *Terminalia ferdinandiana* (or Kakadu plum), a native Australian fruit. The intestinal absorption assay revealed that the phenolic compound ellagic acid, had the highest permeability *in vitro*. For ascorbic acid, even though bioaccessibility was high, *in vitro* permeability was low, indicating that different compounds show variation in bioaccessibility and absorption.

Grosshagauer et al. studied the potential of Moringa foods from a food chemistry perspective. *Moringa oleifera* is native to India but is also prevalent in tropical and subtropical regions of Africa, Asia, Central and South America. This mini-review looked at the food chemistry and nutrition-related data on Moringa preparations. The dietary intake of the leaves of *Moringa oleifera* is high and has been considered safe, even though variations in different processing and extraction methods prevent the comparison of data and also affect the formulation of guidelines. High levels of anti-nutrients and contaminants indicate the need for further studies, and this should inform legal regulations for the dietary intake of *Moringa oleifera* products. Another study by Chen et al. shows the incorporation of the extract of *Psychotria viridiflora* stem (an antidiabetic tropical plant found in Sarawak, East Malaysia) in noodles and the effect on α-amylase and α-glucosidase. *P. viridiflora* α-amylase inhibition activity is higher than α-glucosidase inhibition activity. In general, the fortification of *P. viridiflora* to develop functional noodles could provide a healthier alternative to diabetic patients. The next three studies are reporting on the therapeutic properties of ethnoplants used in different applications. Yang et al. reported on Rosa roxburghii Tratt (*R. roxburghii*) tea, a traditional Chinese beverage. Two cultivars of *R. roxburghii* were compared for their phenolics and bioactive properties. Both cultivars showed good phenolic contents and potent antioxidant capacity, therefore the leaves could be a promising source of phenolic compounds for functional beverages. Pathaw et al. did a comparative review of the anti-nutritional factors of herbal tea concoctions. This review focussed on underutilized plants of North-East India, *Cymbopogon citratus* (Lemongrass), *Rhus chinensis* (Heimang), *Nelumbo nucifera* (Lotus), *Mentha spicata* (Spearmint) and others, used as herbal tea and processing methods to understand the reduction in anti-nutrients. Different processing methods such as drying or heating process help remove anti-nutrients, and future research is required on adapting brewing conditions to optimize anti-nutrient reduction. Sultana et al. reported on assessing extracts of *Dillenia pentagyna* Roxb. for pharmacological potential and related to the traditional use of this plant to treat cancer, wound healing, and diabetes by local tribes in Bangladesh. Finally, Fyfe, Smyth et al. presented a food chemists perspective paper on developing a framework for responsible research for Australian native plant foods, most of which are ethnofoods eaten by Australia's Indigenous people. This perspective highlights the importance for food chemists to uphold the rights of Indigenous Australians when working with communities growing the food on their land and complying with both local and international laws and obtaining permits and benefit-sharing agreements where required.

Author contributions

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

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ORIGINAL RESEARCH
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Inhibition Effect of Extract of *Psychotria viridiflora* Stem on α-Amylase and α-Glucosidase and Its Application in Lowering the Digestibility of Noodles

Qimin Chen[1†], *Joanne Yi Hui Toy*[1†], *Cynthia Seta*[2], *Tiong Chia Yeo*[2] *and Dejian Huang*[1,3*]

[1] Department of Food Science and Technology, National University of Singapore, Singapore, Singapore, [2] Sarawak Biodiversity Centre, Kuching, Malaysia, [3] National University of Singapore (Suzhou) Research Institute, Suzhou Industrial Park, Suzhou, China

A collection of tropical medicinal plants from East Malaysia's rainforests are used by indigenous tribes for their curative properties. Despite their purported healing properties, these forest plant species are largely unexplored and hence remain virtually unknown to the outside world. In this study, antidiabetic properties of *Psychotria viridiflora,* a plant used to treat diabetes by a local community in Sarawak, Malaysia were investigated. Ethyl acetate (EA) extract of *P. viridiflora* stem was found to exhibit high starch hydrolase inhibition activity with an IC_{50} value of 15.4 ± 2.1 μg/ml against porcine α-amylase and an IC_{50} value of 32.4 ± 3.7 μg/ml against rat intestinal α-glucosidase. A complex mixture of A-type oligomeric proanthocyanidins containing (epi)fisetinidol, (epi)afzelechin, (epi)guibourtinidol, and (epi)catechin were found. These compounds may be responsible for the starch hydrolase inhibition activity. Ethyl acetate (EA) extract of *P. viridiflora* stem was incorporated into wheat and rice flour to reformulate noodles with slow digestibility and was assessed under *in vitro* simulated gastrointestinal conditions. A dose-dependent effect on digestibility was observed for both noodles upon incorporation of 1-6% (w/w) of EA extract, with noodles containing 6% (w/w) extract exhibiting the greatest reduction in digestibility. As compared to rice noodles containing 6% extract (31.16% inhibition), wheat noodles with the same extract concentration had a smaller decline in digestibility (27.25% inhibition) after 180 min. Overall, our findings highlight the potential of *P. viridiflora* in the prevention of postprandial hyperglycaemia.

Keywords: ***Psychotria viridiflora*, characterisation, proanthocyanidins, noodles, *in vitro* study**

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Shiyi Ou, Jinan University, China
Uma Devi Palanisamy, Monash University Malaysia, Malaysia

***Correspondence:**
Dejian Huang
dejian@nus.edu.sg

[†]*These authors have contributed equally to this work*

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INTRODUCTION

The incidence of type 2 diabetes is increasing at an unprecedented rate worldwide partially due to the increased prevalence of obesity and ageing. The number of type 2 diabetes cases is projected to increase from 415 to 642 million people by 2040 (1). Type 2 diabetes is caused by insulin resistance and an insufficient compensatory insulin secretory response. As a result, starch is rapidly degraded by starch hydrolases after a meal, thereby causing an elevated blood glucose level which is defined as postprandial hyperglycaemia (PPHG) (2). This metabolic disorder is a great concern since a high blood sugar level may lead to

complications such as cardiovascular disease, retinopathy, and amputation (3). Currently, antidiabetic drugs such as acarbose are commonly used to prevent postprandial hyperglycemia by slowing down starch digestion. However, the consumption of acarbose may lead to severe side effects such as flatulence, diarrhoea and bloating. This occurrence is due to the accumulation of undigested oligosaccharides, trisaccharides, and disaccharides in the large intestine, which will undergo fermentation by faecal flora to produce hydrogen and methane gas (4). In view of this, there is a need to explore other starch hydrolase inhibitors, most preferably from natural origins that lack the undesired symptoms.

An immense range of botanicals could be found in Southeast Asia and some of these edible and medical plants are used to treat certain diseases since ancient times (5, 6). Local tribes living in remote rainforests in Sarawak, Malaysia rely heavily on a wide assortment of tropical plants for the restoration of health and disease treatments (such as wounds, snakebites, and diabetes) (7). However, few studies have been conducted scientifically to prove the efficacy and safety of these traditional remedies. On a mission to decode the biodiversity in Sarawak rainforest, we (Sarawak Biodiversity Center) have been collecting samples to establish a plant extract library that contains both local plant species and microbial extracts for collaborators all around the world to uncover the chemical and scientific principles behind their purported medical and health-promoting benefits (8). As such, we have developed high throughput assays to study the starch hydrolase inhibitory potentials of the plant extracts (9). Through this screening process, new bioactive constituents with the potential to combat postprandial hyperglycemia will be discovered and subsequently used for the development of low glycemic index starch-rich food. During the initial stage of our collaboration, we conducted a screening of 25 different plant extracts from Sarawak Biodiversity Center for their starch hydrolase inhibition activity and we found that the ethyl acetate extracts of *P. viridiflora* extracts exhibited the strongest α-amylase and α-glucosidase inhibition effects. As such, we further characterised the active constituents of the EA extracts and evaluated their effects as an ingredient in wheat and rice noodles, based on their *in vitro* digestibility. Reported here are our findings.

MATERIALS AND METHODS

Materials

The *P. viridiflora* samples (Voucher specimen: SABC 10761) were collected from the central region of Borneo, Sarawak, Malaysia, 2017. Porcine pancreatic α-amylase (A3176, type VI-B), rat intestinal α-glucosidase (I1630), pancreas lipase type II (L3126), corn starch (S4126), acarbose (A8980), 3,5-dinitrosalicylic acid (DNSA), bile, pancreatin from porcine pancreas (Cat. No. P754526G), pepsin with an activity of ≥250 U/mg from porcine gastric mucosa (Cat. No. P7000-100) were all purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Amyloglucosidase (3,300 U/ml), α-amylase (thermo-stable, 3,000 U/ml), protease (50 mg/ml, 350 tyrosine U/ml) were purchased from Megazyme. Phosphate buffered Saline and Tris buffer (ultra-pure grade) (PBS) were purchased from Vivantis technologies (Subang Jaya, Selangor, Malaysia). Calcium chloride was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Plain wheat flour produced in Brussel and white rice flour produced in Thailand were purchased from NTUC Fairprice in Singapore. HPLC grade acetone, acetonitrile and methanol were obtained from Tedia Company (Fairfield, OH, USA). The 96-well polystyrene microplates with flat bottom were purchased from Fisher Scientific (Nunc, Rochester, NY, USA). Microplate reader was purchased from Biotek Instruments Inc. (Winooski, VT, USA). Electrospray ionisation mass spectra (ESI-MS) were obtained from a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA).

Extraction of the Starch Hydrolase Inhibitors From *P. viridiflora*

Plant extracts were prepared at SBC. The stem of *P. viridiflora* was dried in an oven at 45°C and then ground into a powder. A total of 6.2 kg powder was obtained and sequentially extracted with hexane, dichloromethane, ethyl acetate, methanol, and water. The five extracts were then filtered through Whatman filter paper No-1 and concentrated *via* rotary evaporation and further dried in a vacuum oven to give a dried powder that was stored in the sample bank and for further research.

Assay of α-Amylase or α-Glucosidase Inhibition Activity

The inhibition activity of the various samples was evaluated using a high throughput method developed in our lab (9). Corn starch (0.5 g) was first dissolved in 25 ml PBS buffer by heating the mixture for 2.5 min at 300 rpm. The corn starch mixture was then cooled to room temperature with continuous stirring at 300 rpm. The α-glucosidase stock solution was first prepared by dissolving 0.5 g of rat intestinal α-glucosidase powder in 20 ml of PBS with constant stirring on an ice-bath for 30 min before undergoing centrifugation at 2,000 g at 4°C for 10 min. On the other hand, a stock solution of α-amylase was prepared by dissolving 4 mg of α-amylase into 1 ml of PBS and subsequently sonicated. During the analysis, 200 μl of α-amylase or α-glucosidase stock solution was diluted in 7.8 ml PBS buffer to give a working solution. In each well, 10 μl of α-amylase or α-glucosidase was mixed with a series concentration of plant extracts (20 μl) or acarbose and subsequently incubated at 37°C for 15 min with medium shaking. After incubation, 60 μl of the corn starch solution was added and the readings were recorded at 660 nm for 1.5 h with one reading per 30 s. The percentage inhibition was determined by Equation 1 where AUC represents the area under the curve. The IC_{50} is the inhibitor concentration required to produce 50% inhibition.

$$\text{Inhibition } (\%) = \frac{\text{AUC inhibitor} - \text{AUC control}}{\text{AUC inhibitor}} \times 100 \quad (1)$$

Additionally, the IC_{50} values of the samples were converted into acarbose equivalent (AE) which is defined in equation 2.

$$\text{AE of a sample} = \frac{IC_{50} \text{ of acarbose}}{IC_{50} \text{ of a sample}} \quad (2)$$

Characterisation of Compounds by High Performance Liquid Chromatography-Mass Spectrometry

EA extract was subjected to LC-MS analysis using the Bruker Amazon ion trap mass spectrometer equipped with a Dionex ultimate 3000 RS Diode array detector (Billerica, MA, USA). The heated capillary and spray voltage were maintained at 250°C and 4.5 kV, respectively. Additionally, nitrogen was operated at 80 psi for sheath gas flow rate and at 20 psi for the auxiliary gas flow rate. The MS^3 collision gas used was helium with a collision energy of 30% of the 5 V end-cap maximum tickling voltage. The mass spectra were scanned from *m/z* 100-2,000 in both the positive and negative ion modes with a scan speed at one scan per second. Each fraction was dissolved in methanol at 1 mg/ml and filtered through a 0.45 μl RC membrane. During the HPLC run, 1 μl of fraction was injected into the Develosil diol column (4.6 x 250 mm, Batch: 250612). The mobile phase consists of solvent A, 2% acetic acid in acetonitrile and solvent B, acidic aqueous methanol (CH_3OH: H_2O: HOAc, 95:3:2 v/v/v). Elution programme started with 7% B for 3 min, ramping up to 37.6% at 60 min, 100% at 63 min and holding for 7 min at a flow rate at 1.0 ml/min at 25°C.

Preparation of Rice and Wheat Noodles Incorporated With EA Extracts of *P. viridiflora*

Rice flour (5 g) was incorporated with powdered EA fraction of *P. viridiflora* at different concentrations (0, 1, 3, and 6%) and mixed together with 2% (w/w) salt and 7.5 ml of deionised water. Rice slurries were then spread evenly on a stainless steel tray and steamed in a steamer for 5 min to form cooked noodle sheets of 2 mm thickness. Wheat flour was processed in the same manner but with the addition of only 3.3 ml of deionised water. Wheat dough sheets were subsequently boiled for 5 min. Noodle sheets were then left to cool at room temperature for 15 min. Thereafter, the noodle sheets were cut into noodle strands (2 mm in thickness and 10 mm in width). The moisture content of these noodles was measured according to the AOAC method 934.01 (10).

In vitro Digestion of Noodles

In vitro digestion process was based on the procedure introduced earlier (11). First, the noodles were blended and weighed based on their carbohydrate content. Simulated salivary fluid (SSF) was prepared freshly before use according to (12) with the following concentration of salts: 15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, 0.75 mM $CaCl_2(H_2O)$. The pH was adjusted to 7.0 with 6M HCl. During the oral digestion, 75 U/ml of α-amylase in 1 ml of SSF was added to 0.75 g of the noodle mixture containing 0% of the extract. To stimulate oral digestion, each mixture was vortex for 20 s and placed in a water bath at 37°C for 1 min and 40 s.

Following oral phase digestion, gastric phase digestion was simulated. First, the pH of the mixture was adjusted to 3.0 using 1.0 M HCl. Simulated gastric fluid (SGF) was prepared freshly before use according to (12) with the following concentration of salts: 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 0.075 mM $CaCl_2$. The pH was adjusted to 3.0 with 6 M HCl. Subsequently, 2,000 U/ml of pepsin in 2 ml of SGF was added to initiate the digestion process. This process was carried out at 37°C for 2 h in a water bath with constant shaking at 150 rpm. After 2 h of digestion, potassium carbonate (1.0 M) was added to adjust the mixture to pH 7.0 to deactivate pepsin. The mixture was then transferred into a Snakeskin dialysis tubing with a molecular weight cut-off at 7 kDa.

Following the gastric phase digestion, small intestinal phase digestion was stimulated. Simulated intestinal fluid (SIF) was prepared freshly before use according to (12) with the following concentration of salts: 6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCL, 0.33 mM $MgCl_2(H_2O)_6$, 0.3 mM $CaCl_2(H_2O)$. The pH was adjusted to 7.0 with 1M NaOH. The small intestinal digestion was initiated by adding 100 U/mL of pancreatin in 4 ml of SIF and 10 mM of bile into the mixture. After which, the dialysis tube was placed in a 100 ml sodium phosphate buffer (0.1 M, pH 7.0) fortified with calcium chloride (40 mg/L). This digestion was carried out in a shaker water bath (37°C) under continuous shaking at 150 rpm for 3 h. During the 3-h duration, aliquots (200 μl) of dialysate were withdrawn at different time intervals (0, 10, 20, 30, 40, 50, 60, 90, 120, 150 and 180 min) and immediately replaced with a fresh sodium phosphate buffer (200 μl). For all sets of experiments, a blank sample was prepared using denatured enzymes.

Determination of Inhibition Activity Using DNSA

DNSA was utilised to measure the amount of reducing sugar that is released from the dialysate (13). DNSA reagent (100 μl) and aliquots of dialysate (100 μl) were mixed in each well of a 96-well microplate and steamed at 100°C for 10 min until a reddish-brown colour was developed. The microplate was subsequently cooled to room temperature in an ice-water bath. Thereafter, 100 μl of mixture solution was transferred to a new 96-well microplate reader and the absorbance was read at 540 nm using a microplate absorbance reader (Biotek Instruments Inc., Winooski, VT, USA). Maltose equivalent (ME) was calculated from a pre-determined maltose calibration curve of $y = 0.6022\ x\text{-}0.1738$ where y is the absorbance at 540 nm and x is the concentration of maltose in mg/ml. The degree of hydrolysis was then expressed as milligram ME per gram of starch. The percentage inhibition of flour digestion over 3 h was calculated using Equation 3. $AUC_{control}$ and AUC_{sample} represent the area under the curve obtained from the maltose released vs. time graph.

$$\text{Percentage Inhibition} = \left(AUC_{control} - \frac{AUC_{sample}}{AUC_{control}}\right) \times 100\% \quad (3)$$

Determination of Starch Content

Starch content of the wheat and rice flour was determined using the AOAC method 991.43 (14). Each sample was prepared by

adding 1 g of flour samples into 25 ml of sodium phosphate buffer (pH 6.0, 0.08 M). After which, 50 µl of α-amylase (thermo-stable, 3,000 U/ml) was added to all samples and subsequently incubated in an 80°C water bath for 25 min with constant shaking at 150 rpm. Thereafter, the pH value of all samples was adjusted to pH 7.5 before protease (0.1 ml) was added. The samples were then incubated at 60°C for 40 min. After 40 min of incubation, the mixture was adjusted to pH 4.5 and 0.2 ml of amyloglucosidase was added. After the second round of incubation at 60°C was carried out for 40 min, ethanol (95%, 140 ml) was added and the mixtures were left overnight. Celite was wet with ethanol (78%) and even out on a crucible. The samples were then filtered and kept for starch analysis using DNSA assay at 540 nm as described in setion Characterisation of compounds by high performance liquid chromatography-mass spectrometry. The absorbance was used to calculate the starch content using a predetermined glucose calibration curve, y = 1.7702x – 0.1731 with a R^2 value of 0.999, where y represents the absorbance at 540 nm and x represents the concentration of glucose solution in g/L.

Colour Analysis of Cooked Noodles

The colour of the cooked rice and wheat noodles were measured using a spectrophotometer (Konica Tokyo, Japan) that was equipped with D65 illuminant based on the CIE 1976 (L*, a*, b*) colour space. The measurement area was set to 8 mm and the spectrophotometer was set to exclude the specular component for all measurements.

Textural Analysis of Cooked Noodles

The textural analysis of the cooked noodles was tested using a texture analyser (TA-XT2i; Stable Micro System Ltd., Godalming, Surrey, UK) (11). The hardness and adhesiveness of the cooked noodle strands with 2 mm thickness and 1 cm width were determined through compressing the noodles using a cylinder probe with a diameter of 35 mm. The TA-XT2i settings were as follows: pre-test speed (1.0 mm/s), test speed (1.0 mm/s), post-test speed (3.0 mm/s), strain (75 %), time (2 s), trigger force (5 g). The chewiness of the noodle was determined from the force-time curve of the texture profile. On the other hand, extensibility and tensile strength of 15 cm noodles with 2 mm thickness and 1 cm width were tested by using an A/SPR probe (Spaghetti/noodle tensile rig). The settings of the analyser were as follows: pre-test speed (1.0 mm/s), test speed (3.0 mm/s), post-test speed (10.0 mm/s), distance (100 mm), trigger force (5 g). The distance between the two arms of the rig was set at 40 mm. The measurements were repeated three times for each sample.

Statistical Analysis

All experiments were carried out in triplicates. The results are given as mean ± standard deviations. One-way analysis of variance (ANOVA) with TUKEY's test ($p < 0.05$) was used to determine the differences within the groups using SPSS software.

FIGURE 1 | An image of *P. viridiflora*.

RESULTS AND DISCUSSION

Inhibition Activities of EA Extract of *P. viridiflora* Stems Against α-Amylase or α-Glucosidase

P. viridiflora is locally known as Engkerabai by the Ibans, an indigenous community from Rumah Bajau, Julau, Sarikei Division. The taxonomy identification (Voucher specimen: SABC 10761) was carried out based on the plant characteristics recorded in the specimens at Sarawak Herbarium (SAR) from the Forest Department Sarawak and the appearance of the *P. viridiflora* is shown in **Figure 1**. Currently, no scientific research has been carried out on this native plant from Sarawak.

A total of six different solvents (hexane, methanol, acetone, water, ethyl acetate, dichloromethane) were used for the extraction process and the inhibition activities of the extracts were evaluated. Since the IC_{50} values are dependent on many factors such as enzyme origin, the concentration of substrates and other factors like pH, temperature and the buffer being used, it is more accurate to compare the AE values instead. In fact, a higher AE indicates higher inhibitory activity. In general, only acetone and ethyl acetate fractions exhibited inhibition activities while no inhibition effects were observed for hexane, methanol, water and dichloromethane extracts against α-amylase and α-glucosidase. The AE values of the acetone extract against α-amylase and α-glucosidase were 0.115 (IC_{50} = 40.0 µg/ml) and 0.020 (IC_{50} = 115.0 µg/ml), respectively. On the other hand, the AE of the EA extract against α-amylase and α-glucosidase were 0.288 ± 0.128 (IC_{50} = 15.4 ± 2.1 µg/ml) and 0.095 ± 0.031 (IC_{50} = 32.4 ± 3.7 µg/ml), respectively (**Figure 2**). These results illustrate that the extract obtained from EA exhibits higher inhibition effects as compared to the acetone extract.

To date, various α-amylase and α-glucosidase inhibitors from natural sources like plants have been identified and characterised (15–17). For instance, grape seed extract, containing procyanidins was reported to possess an AE value of 0.793 (IC_{50} = 8.7 ± 0.8 µg/ml) against α-amylase and an AE value of 75.8 (IC_{50} = 1.2 ± 0.2 µg/ml) against α-glucosidase

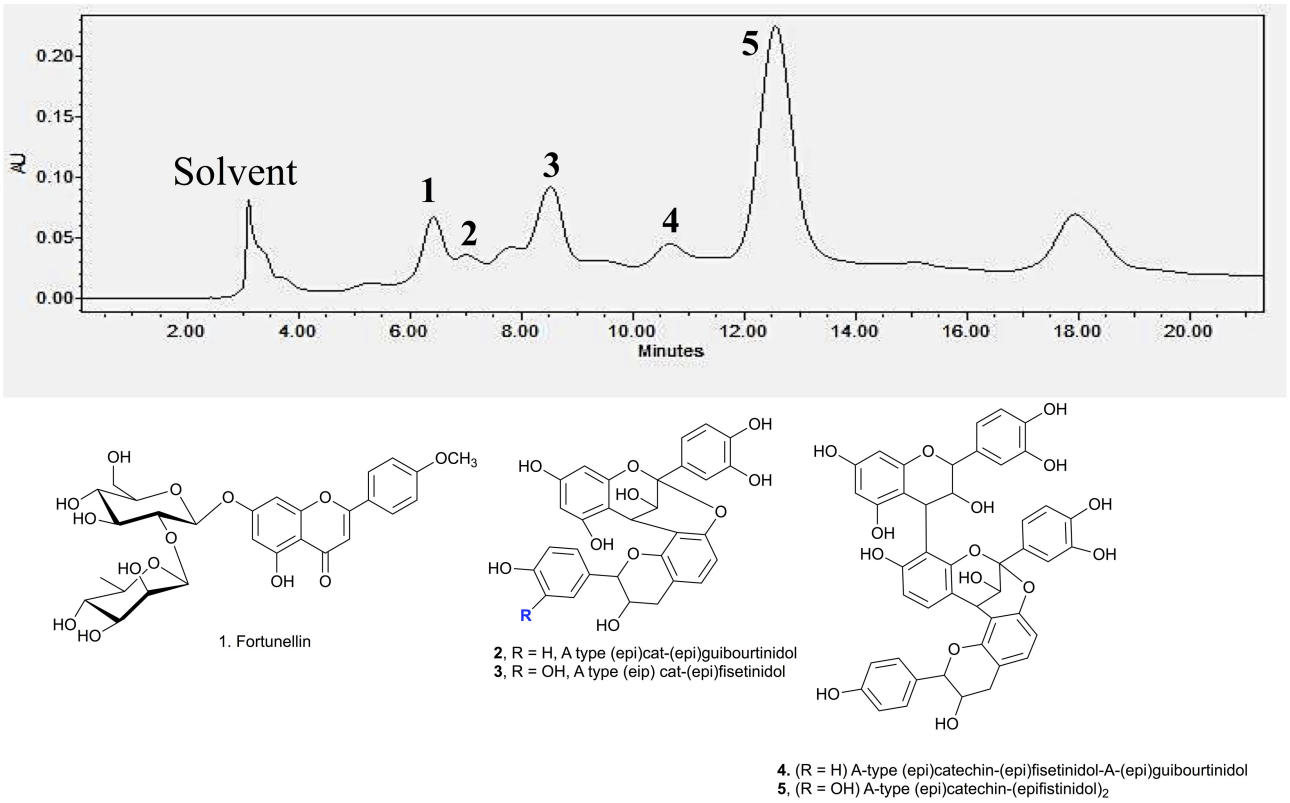

FIGURE 2 | Fragmentation pattern of MS(n) for peak 3 at 8.5 min.

(18) which were much higher than *P. viridiflora* extract. In contrast, the AE values of green tea and white tea extracts (rich in tea catechins) against α-amylase were 0.197 (IC_{50} = 34.9 ± 0.9 μg/ml) and 0.018 (IC_{50} = 378.0 ± 134.0 μg/ml), respectively (18) and were much lower than EA extract of *P. viridiflora*. On the other hand, the inhibition effect of these tea extracts against α-glucosidase were remarkably higher as compared to *P. viridiflora*. In general, the AE value of the green tea extract against α-glucosidase was 182.0 (IC_{50} = 0.5 ± 0.1 μg/ml) and the AE value of the white tea extract against α-glucosidase was 36.4 (IC_{50} = 2.5 ± 0.4 μg/ml) which were notably higher than the EA extract of *P. viridiflora*. Apart from procyanidins, fucoidan that is found in brown marine algae (*Ascophyllum nodosum*) was also reported to have a α-glucosidase inhibition effect with an AE value which ranges from 21.2 to 76.9 (IC_{50} = 13.0 to 47.0 mg/ml) (19). However, no α-amylase inhibition effects were detected from fucoidan obtained from certain algae species like *Fucus vesiculosus* (19). Taken together, these studies reveal that the α-amylase inhibition effect of *P. viridiflora* extract is comparable or even higher than some commonly known plant extracts. On the other hand, the α-glucosidase inhibition effect may be slightly weaker as opposed to certain plant sources. The difference might be due to the concentration of inhibitors in the extracts.

Characterisation of Bioactive Compounds by HPLC-MS

Identification of the active compounds responsible for the starch hydrolase inhibition activity were performed through HPLC-ESI-MS/MS. Additionally, Develosil diol column was utilised for the HPLC analysis due to its good baseline resolution to separate bioactive compounds like proanthocyanidins. **Figure 3** shows the HPLC chromatogram of the EA fraction with the tentatively assigned proanthocyanidins based on the HPLC-ESI-MS/MS results (the MS data for each peak are shown in Supporting Information). Conclusive assignments of their structures would require further separation of each compound and characterisation by single crystal X-ray diffraction.

Peak **1** at 6.7 min was found to be fortunellin with *m/z* value of 593.2 $[M-H]^-$ and MS^2 ions at *m/z* 511, 409, and 289 which is consistent with literature reported fragmentation patterns. To date, limited research has been conducted on Fortunellin. This compound exhibits both anti-inflammation and anti-oxidative activity in a diabetic mice model by regulating the AMPK/Nrf-2 Pathway (20). Fortunellin can also maintain the intestinal barrier function and decrease inflammation in the colitis (21). In view of these properties, fortunellin may be partially responsible for the health promotion activity of *P. viridiflora* stem.

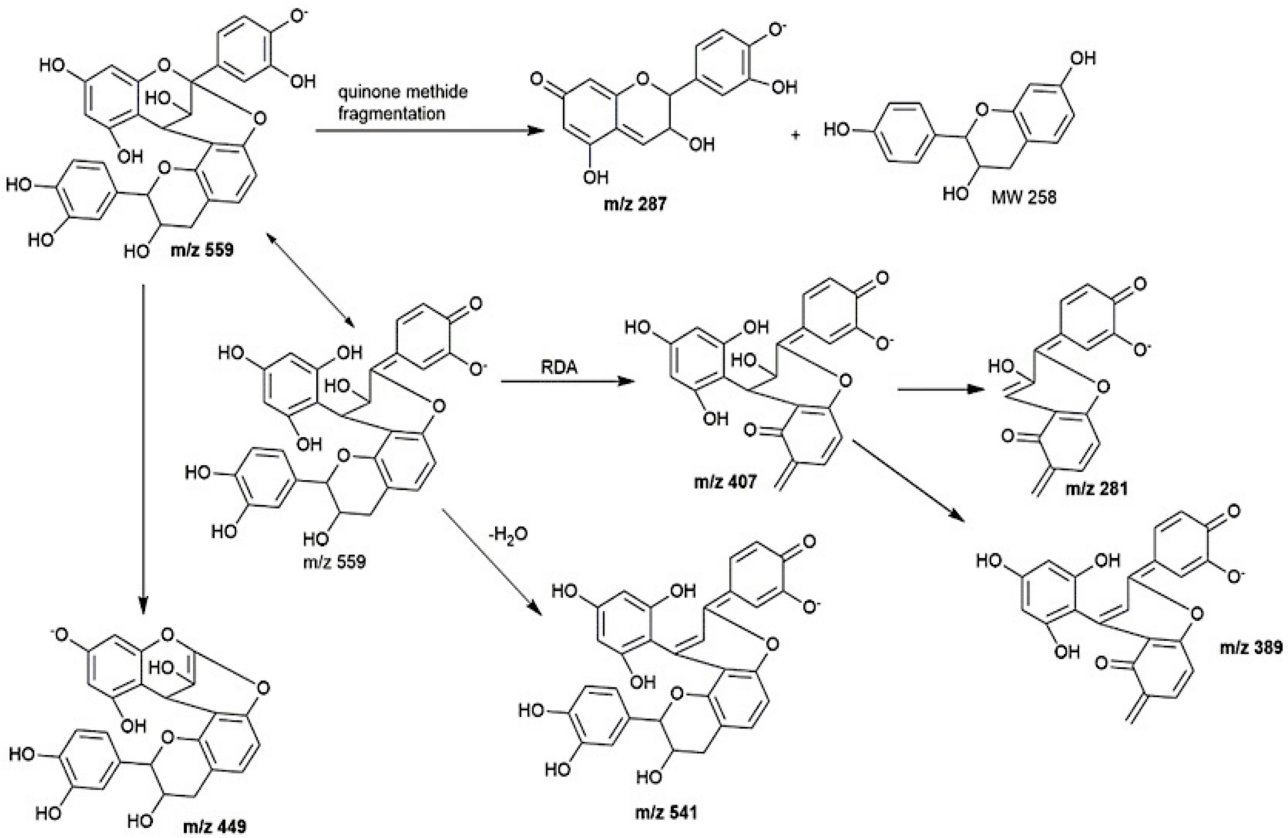

FIGURE 3 | HPLC chromatogram of EA fraction under UV 280 nm and the compounds associated to the major peaks.

Peaks **2** to **6** contain dimeric or trimeric proanthocyanidins, which are composed of different combinations of (epi)catechin, (epi)fisetinidol, (epi)afzelechin, and (epi)guibourtinidol).While procyanidins containing (epi)catechin is most widely found in the plant kingdom, propelargonidins containing (epi)afzelechin is comparatively rare (22). Likewise, only a few plant species containing (epi)guibourtinidol and (epi)fisetinidol monomers that are found in proguibourtinidins and profisetinidin, respectively, have been discovered (22, 23). The peaks were tentatively assigned to contain different combinations of identified monomers based on their MS and MS^n values in conjunction with those reported in the literature (24, 25). From the LC-MS results, dimeric compounds can be identified through the characteristic fragmentation patterns of proanthocyanidins due to quinone methide (QM) reaction, heterolytic ring fission (HRF) and retro-diels-alder reaction (RDA) (26).

Peak 2 at 7.4 min gave *m/z* value of 543 $[M-H]^-$ and MS^2 ions at *m/z* 525, 433, 407. Daughter ion *m/z* 407 $[M-H-136]^-$ was fragmented due to RDA fragmentation. Loss of 136 Da implies that there was one hydroxyl group at ring B of the extension unit. Additionally, daughter ions at *m/z* 433 $[M-H-126]^-$ were obtained due to HRF fragmentation. The loss of 126 Da indicates a 1,3,5-trihydroxybenzene structure at ring A of the extension unit (26). Therefore, HRF pathway indicates the existence of an OH group on ring B which is deduced to be (epi)afzelechin. Taken together, we propose peak 2 to be an A-type (epi)fisetinidol-(epi)afzelechin. Peak **3** at 8.5 min gave *m/z* value of 559 $[M-H]^-$ and MS^n ions at *m/z* 541, 449, 407, 389, 287. Based on the MS^n fragmentation pattern analysis (shown in **Figure 4**), we assigned the identity of the compound to be an A-type dimer of (epi)catechin-fisetinidol dimer (27).

Peak **4** at 11.4 min showed a group of molecular ions at *m/z* 799, 815, 831, and 877. They can be assigned to be trimeric proanthocyanidins with (epi)catechin-(epi)fzalechin-A-(epi)guibourtinidol (molecular weight of 800), and its homologues with one or more OH groups. Taking the MS (2) of the peak at 815 as an example, it fragmented into *m/z* 705, 663, 575, 559, 527, 511, 407, 389, and 287. These fragmentation patterns are observed in typical oligomeric proanthocyanidins MS fragmentations. Fragment 287 is due to quinone methide cleavage of the (epi)catechin terminal unit. For the peak at *m/z* 705 $[M-H-110]^-$, HRF fragmentation ($-C_6H_6O_2$) indicates the presence of an extension unit with a 1,3-dihydrobenzene structure at ring A. The fragment at *m/z* 663.1 $[M-H-152]^-$ is due to RDA fragmentation. Loss of 152 Da through RDA indicates that the ring B of the extension unit has two hydroxyl groups

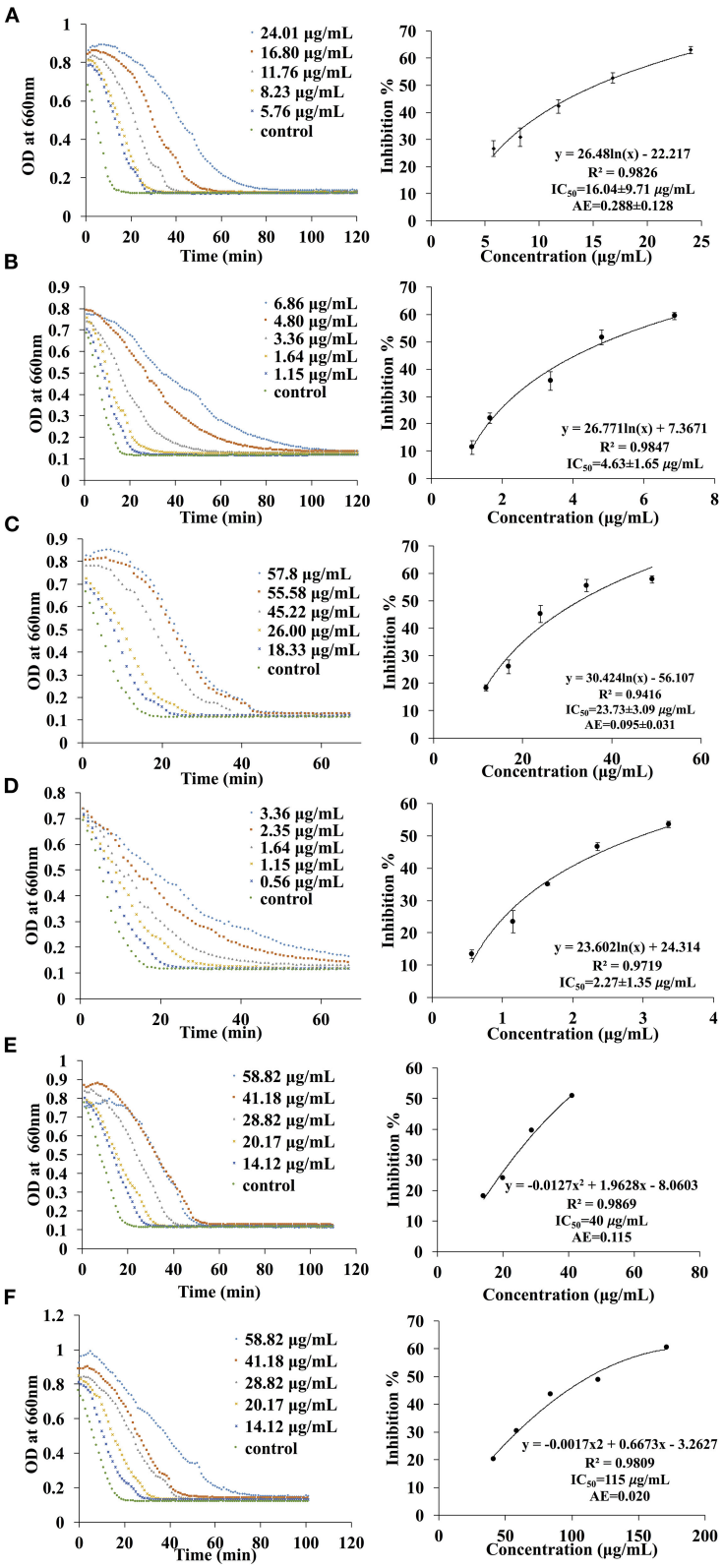

FIGURE 4 | The kinetic curve and dose responsive curve of starch hydrolase in the presence of different concentrations of **(A)** EA extract of *P. viridiflora* against pancreatic α-amylase, **(B)** acarbose against pancreatic α-amylase, **(C)** EA extract of *P. viridiflora* against α-glucosidase, **(D)** acarbose against α-glucosidase, **(E)** acetone extract of *P. viridiflora* against pancreatic α-amylase, **(F)** acetone extract of *P. viridiflora* against alpha-glucosidase.

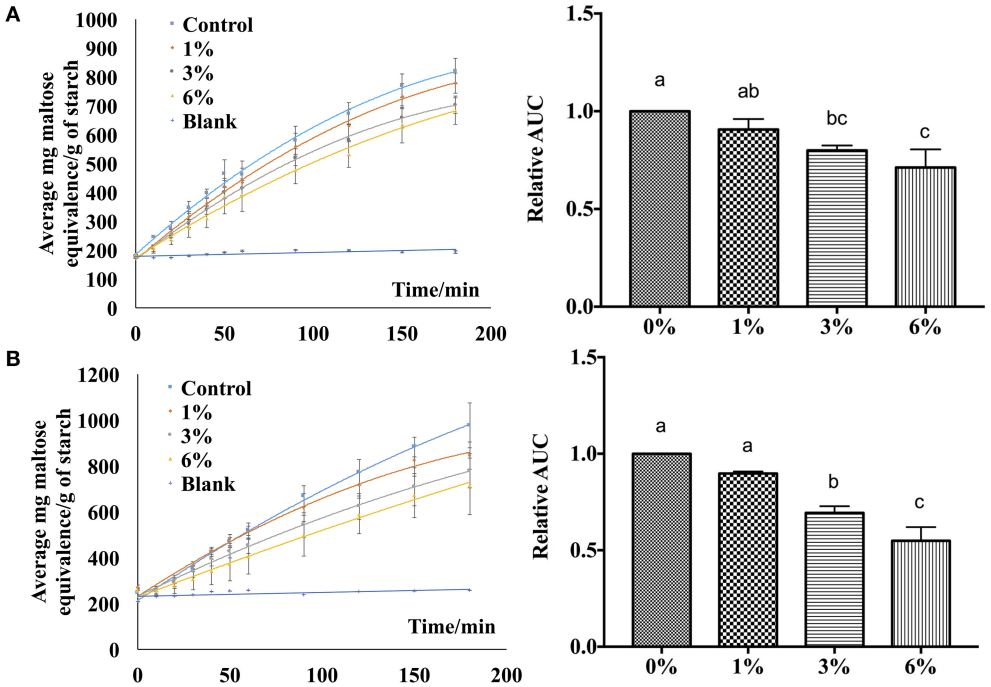

FIGURE 5 | Digestibility profile of **(A)** wheat noodles and **(B)** rice noodles. Figures on the left are expressed as mg maltose equivalence/g of starch in flour during *in vitro* digestibility analysis for 180 min while figures on the right are expressed using the area under the curve. [a,b,c]ANOVA ($n = 3$) at 95% confidence level with same letters indicating no significant difference in area under the curve (AUC) at different concentrations.

which correspond to an (epi)fisetinidol unit. Fragment ion at *m/z* 527 resulted from losing a neutral piece that contains a molecular weight of 288 from the mother ion. Peak **5** at 12.2 min is a trimer with a molecular anion at 831 which indicates the presence of an A-type (epi)catechin-((epi)fisetinidol)$_2$.

Therefore, based on the LC-MS results of the EA extracts, we can conclude that this plant contains mostly A-type dimeric and trimeric proanthocyanidins, which are known starch hydrolase inhibitors.

In vitro Digestion of Rice and Wheat Noodles

In order to verify the effectiveness of the starch hydrolase inhibition activity of *P. viridiflora* plant extract, EA extract was incorporated into rice and wheat noodles and a simulated digestion process was performed using an *in vitro* digestion model. As shown in **Figure 5**, when a higher concentration of EA extract was incorporated into both rice and wheat noodles, a slower release of maltose was observed. Additionally, control samples have a higher starch hydrolysis rate as compared to noodle samples which are incorporated with the EA extracts. This indicates that a higher starch hydrolase inhibition activity was detected when a higher concentration of EA extract was incorporated.

Additionally, the area under the curve was calculated to determine the digestibility of noodles after incorporating EA extract at different concentrations (**Figure 5**). An overall decrease in AUC was observed as the extract concentration increases for both rice and wheat noodles which suggests a dose-dependent relationship on the digestibility of the two noodles since an increase in EA extract from 1 to 6% results in a decline in digestibility.

TABLE 1 | Percentage inhibition of wheat and rice noodles digestion.

EA fraction	% Inhibition	
	Wheat	Rice
1%	8.77[a]	9.14[a]
3%	19.77[ab]	26.89[ab]
6%	27.25[b]	37.16[b]

[a,b]ANOVA at 95% confidence level with same letters indicating no significant difference in the percentage inhibition at different concentration of EA fraction.

Based on the calculated AUC, the inhibition activity of wheat and rice noodles digestion was determined (**Table 1**). The digestibility of wheat noodles containing 1, 3, and 6% of the EA extract were reduced by 8.77 ± 3.64%, 19.77 ± 1.69%, and 27.25 ± 6.77%, respectively. On the other hand, the digestibility of the rice noodles when incorporated with 1, 3, and 6% of EA extract showed a reduction by 9.14 ± 1.64%, 26.89 ± 5.95%, and 37.16 ± 15.29%, respectively. In general, rice noodles had a higher inhibition activity as compared to wheat noodles after the addition of EA extract. This could be caused by the differences in protein content between the two different types of flour which could interact with proanthocyanidins during the

TABLE 2 | Moisture content of noodles incorporated with EA extract.

Noodles	EA extracts			
	0%	1%	3%	6%
Wheat	52.8 ± 0.2[a]	54.1 ± 0.7[b]	54.4 ± 0.3[b]	56.4 ± 0.3[c]
Rice	60.6 ± 0.5[a]	61.3 ± 0.3[a]	62.8 ± 0.6[b]	64.2 ± 0.5[c]

[a,b,c]*Means within rows followed by different letters are significantly different ($p < 0.05$).*

TABLE 3 | Colour properties of cooked rice and wheat noodles incorporated with EA extract.

Noodles	Colour		
	L*(D65)	a*(D65)	b*(D65)
W0%	62.77 ± 0.04[a]	2.44 ± 0.01[a]	19.33 ± 0.02[a]
W1%	44.99 ± 0.03[b]	6.89 ± 0.02[a]	10.31 ± 0.02[b]
W3%	37.88 ± 0.02[c]	8.16 ± 0.01[b]	9.44 ± 0.02[c]
W6%	34.71 ± 0.02[d]	8.46 ± 0.03[c]	10.49 ± 0.02[d]
R0%	69.55 ± 0.02[a]	−0.59 ± 0.01[a]	7.14 ± 0.04[a]
R1%	46.01 ± 0.01[b]	8.90 ± 0.01[b]	9.01 ± 0.01[b]
R3%	35.97 ± 0.01[c]	10.60 ± 0.02[c]	9.53 ± 0.02[c]
R6%	32.61 ± 0.01[d]	10.20 ± 0.02[d]	9.40 ± 0.02[d]

[a,b,c,d]*ANOVA (n = 3) at 95% confidence level with same letters indicating no significant difference.*
W = wheat noodle; R = rice noodle.
L, a*, and b* are hunter L, a, and b values.*

noodle making and cooking process. Since wheat flour contains a higher protein content (10.5%) as compared to rice flour (6.5%), the presence of proteins will interfere with the digestibility of the noodles by binding to proanthocyanidins and thereby reducing its bioavailability. As such, this results in wheat noodles having a lower starch hydrolase inhibition activity as compared to rice noodles despite possessing the same percentage of EA extract.

Moisture Content Analysis

The moisture content of the rice and wheat noodles were measured. As shown in **Table 2**, with an increase in EA extract concentration from 1 to 6% (w/w), the moisture content for both wheat and rice noodles increased slightly. Additionally, no significant differences ($p > 0.05$) in moisture content were observed in the presence of 1 and 3% of the extract in wheat noodles. On the other hand, wheat noodles with 6% EA extract showed the highest moisture content (56.4 ± 0.3%) while the control exhibits the lowest moisture content (52.8 ± 0.2%). Likewise, rice noodles containing the EA extract exhibit a similar trend whereby 6% extract showed the highest moisture content (64.2 ± 0.5%) which is significantly different from the rest ($p < 0.05$). This observation may be due to a decrease in total flour composition in the noodles as the percentage of EA extract increases. Henceforth, less flour is available in the noodle matrix to absorb water in the presence of a higher EA extract concentration. Overall, the moisture content of the control rice noodles (60.6 ± 0.5%) was generally higher than the control wheat noodles (56.4 ± 0.3%). This difference in moisture content is probably due to the starch and protein content differences within the two flours.

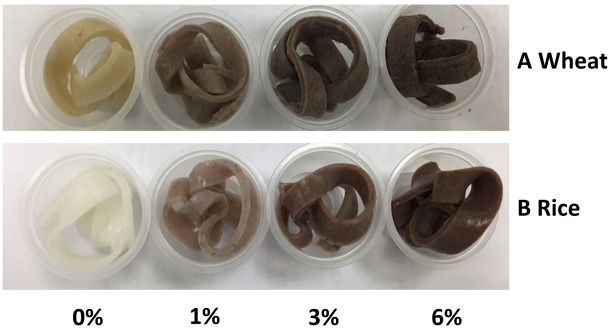

FIGURE 6 | Colour swatches for cooked **(A)** Wheat and **(B)** Rice noodles incorporated with EA extract of different dosages (0%, 1%, 3% and 6%).

Colour Analysis

Colour is one of the major determinants of noodle marketability. In **Table 3**, three parameters were tested whereby L* stands for lightness, a* stands for redness and greenness while b* stands for yellowness and blueness. In general, with an increased in EA extract from 0 to 6% (w/w), the cooked noodles showed significant changes in the L*, a*, and b* values ($p < 0.05$). For instance, the lightness of the noodles decreased with an increased in EA extract. Since the EA extract is dark brown, the addition of these extracts will lead to an increased in darkness and a decreased in brightness of the noodles (**Figure 6**). Due to the colour of the extract, a* values which represent redness and greenness also increased significantly for both noodles. As for b* value (yellowness and blueness), wheat noodles showed a significant decrease while rice noodles showed a slight increase with increased extract concentration. This is because wheat flour is slightly yellow and with the addition of the reddish-brown extract, the darker shade of brown will overpower the light yellow colour in the wheat flour (28).

Generally, functional food products like noodles have become more colourful in recent years. In fact, food that are darker in colour like brown rice are perceived as nutritious to consumers. Research has also shown that red and yellow coloured food could subconsciously help to attract attention from consumers while blue and green coloured food is often associated with food that is healthy (29). Since noodles incorporated with EA extracts of *P. viridiflora* show an increase in a*, these noodles could potentially help in stimulating consumers' appetite (29).

Textural Profile Analysis

Textural attributes of cooked noodles were evaluated by texture profile analysis. Attributes including hardness, chewiness, adhesiveness, tensile strength, and extensibility were analysed. On the whole, the incorporation of EA

TABLE 4 | Textural properties of cooked noodles incorporated with EA extracts.

Noodles	Textural properties				
	Hardness(g)	Chewiness (g)	Adhesiveness (g s)	Tensile strength (g)	Extensibility (mm)
W0%	27222.05 ± 5603.02^{a}	11938.41 ± 1727.37^{a}	−1323.58 ± 261.47^{a}	159.59 ± 8.22^{a}	69.78 ± 15.80^{a}
W1%	21116.9 ± 1965.33^{a}	8060.95 ± 8060.94^{ab}	−1141.22 ± 2.26^{a}	123.98 ± 0.30^{b}	56.30 ± 3.94^{a}
W3%	23289.23 ± 1784.37^{a}	7305.06 ± 1116.65^{b}	−1261.55 ± 14.87^{a}	82.35 ± 12.05^{c}	29.15 ± 9.34^{b}
W6%	25855.26 ± 1095.92^{a}	7248.78 ± 1553.79^{b}	−1066.9 ± 64.37^{a}	77.75 ± 2.49^{c}	18.70 ± 2.20^{b}
R0%	24743.94 ± 2527.74^{a}	17568.87 ± 2844.08^{a}	−160.11 ± 118.48^{a}	177.84 ± 24.81^{a}	26.98 ± 1.18^{a}
R1%	25104.41 ± 2161.74^{a}	17901.22 ± 133.84^{a}	−192.77 ± 99.08^{a}	95.41 ± 1.66^{b}	24.85 ± 1.03^{ab}
R3%	29532.83 ± 5125.49^{a}	19094.58 ± 2918.63^{a}	−249.7 ± 82.99^{a}	86.78 ± 16.87^{b}	18.90 ± 5.66^{bc}
R6%	30800.06 ± 2624.91^{a}	20921.17 ± 2462.71^{a}	−236.51 ± 75.35^{a}	70.91 ± 14.51^{b}	15.04 ± 3.29^{c}

^{a,b,c}ANOVA at 95% confidence level with different letters indicating significant difference ($p < 0.05$). Data expressed as mean± SD of triplicate determination. W = wheat noodle;R = rice noodle.

extracts into the noodles will show several effects on the textural properties. This measurement is imperative to gauge the changes in organoleptic properties which will tend to affect the consumer acceptance of cooked noodles. From **Table 4**, no significant differences were observed for both hardness and adhesiveness of the wheat and rice noodles despite an increased in extract concentration ($p > 0.05$). However, in comparison to wheat noodles, rice noodles were reported to have a lower adhesiveness and a higher extensibility value.

In general, the chewiness, tensile strength, and extensibility values of EA extract incorporated into wheat noodles have all decreased. The decreased in chewiness, tensile strength, and extensibility values with an increased in EA extract concentration indicates that the wheat noodles will become less stretchable and will break easily under force. On the other hand, chewiness value increased, while tensile strength and extensibility values decreased when EA extract concentration in rice noodles increased. From **Table 4**, significant differences in chewiness value were observed between higher concentrations of the EA extract (3 and 6%) and the control wheat noodles. Although a slight increase in chewiness could be observed in the rice noodles with an increasing concentration of extract, this difference is not significantly different ($p < 0.05$). Additionally, the tensile strength of both rice and wheat noodles also decreases significantly with increased concentration of EA extract. However, no significant differences in the tensile strength were observed between wheat noodles containing 3 or 6% of the EA extract. Likewise, no significant differences were observed between the tensile strength of rice noodles with 1, 3%, or 6% of the EA extract. Extensibility values of both the wheat and rice noodles decrease with increasing EA extract concentrations. This is because gluten network formation will be negatively impacted at high EA extract concentrations, thus decreasing the extensibility of noodles. A significant difference in the extensibility value could be observed between the control noodles and noodles with high EA extract concentrations (3 and 6%).

Overall, the incorporation of the EA extract had imposed some significant differences on the textural properties of different types of cooked noodles. For instance, changes in textural properties were observed as the wheat noodles became softer, less chewy and less adhesive with an increased level of EA extract. Rice noodles, on the other hand, were more firm, adhesive and chewier with increasing concentration of EA extracts.

CONCLUSIONS

In conclusion, our data suggest that the EA extracts of *P. viridiflora* could be a viable alternative to inhibit α-amylase and α-glucosidase. In comparison to other plant extracts (green tea, white tea), the α-amylase inhibition activity of *P. viridiflora* is higher while the α-glucosidase inhibition activity is lower (18). Although proanthocyanidins with a low degree of polymerisation were identified to be the major compounds within the extract, future work is required to determine if specific proanthocyanidins are responsible for the inhibitory effects. Overall, the quality attributes of the noodles as well as the digestibility of the noodles were influenced by the addition of *P. viridiflora* extract. In general, the fortification of *P. viridiflora* in the formulation of functional noodles will help to provide a healthier alternative to diabetic patients.

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

The planning of the research was done by QC, DH, CS, and TY. The experiments were performed by JT and QC. All the writers have contributed in the research work and the write-up of this article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.701114/full#supplementary-material

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The Future of Moringa Foods: A Food Chemistry Perspective

Silke Grosshagauer[1†], Philip Pirkwieser[2†], Klaus Kraemer[3,4] and Veronika Somoza[1,2,5]*

[1] Department of Physiological Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria, [2] Leibniz Institute for Food Systems Biology at the Technical University of Munich, Freising, Germany, [3] Sight and Life Foundation, Basel, Switzerland, [4] Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States, [5] Chair of Nutritional Systems Biology, School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany

The tree *Moringa oleifera Lam*. provides its leaves, pods, flowers and seeds for human nutrition. The chemical profile of all these Moringa products varies substantially, not only among the different parts of the plants used. Cultivating, processing as well as storage conditions chiefly determine the contents of nutrients and anti-nutritive constituents. Anti-nutrients, e.g., phytic acid or tannins, are present in notable amounts and may affect micronutrient bioavailability. Although *Moringa oleifera* products have been promoted for several health benefits and are discussed as an alternative treatment in various diseases, risk assessment studies evaluating contamination levels are scarce. Recent investigations have demonstrated alarming contents of heavy metals, polycyclic aromatic hydrocarbons and mycotoxins in *Moringa oleifera* products, indicating the need for a comprehensive risk assessment and contingent legal regulation of these products. In this mini review, we briefly outline pivotal, food chemistry and nutrition related data on Moringa preparations in order to stimulate in-depth research to close the presented knowledge gaps.

Keywords: *Moringa oleifera*, food supplements, food safety, safety assessment, nutrient profile, anti-nutrients, vitamin source

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***Correspondence:**
Veronika Somoza
v.somoza.leibniz-lsb@tum.de

[†] These authors have contributed equally to this work

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INTRODUCTION

The tree *Moringa oleifera Lam.* belongs to the family Moringaceae, genus Moringa, also known as "horse radish tree," "drumstick tree" or simply as "moringa." *Moringa oleifera* is native to India but is also prevalent in tropical and subtropical regions of Africa, Asia, Central and South America. Its multipurpose use as food and feed, dietary supplement or even as functional ingredient of cosmetic products has led to an increased cultivation and global trade (1).

In 2018, the market of Moringa products was evaluated with USD 5.5 billion globally, which is considerably lower than the market size for algae products of USD 32.6 billion in 2017. Nevertheless, the global market of Moringa is expected to rise in upcoming years. Products include leaf powder, which accounted for the largest market share of Moringa products with 30%, but also leaf tea, oil and seeds (1).

Due to its high protein content, its richness in vitamins and minerals and the low demanding cultivation conditions, *Moringa oleifera* is often promoted as a promising plant to combat malnutrition (2). The products of the so-called "miracle tree" are also discussed for medicinal usage, therefore it clearly needs a more critical observation of undesired side-effects, but also a closer look at the bioavailability of nutrients (3). As seen for other complementary foods, increasing

supplementation with, e.g., Moringa leaf powder, negatively correlated with the sensory quality and therefore consumer acceptance (4). Ultimately, the herein discussed differences in nutritional qualities may be associated with consumer acceptance in the future. In this regard, variations found in the different plant parts [e.g., described for polyphenol composition (5)], in wild type vs. domesticated plants, or after different preparation processes may significantly affect both health promoting properties and sensory quality. Chodur et al. (6), for instance, reported that domesticated Moringa plants could be distinguished clearly from a wild type not only through a higher antioxidant potential, but also by a milder, non-bitter taste due to different glucosinolate compositions. Hence, pivotal food quality and safety related data on Moringa preparations are outlined briefly in this mini review in order to stimulate in-depth research for closing the presented knowledge gaps.

Reported Health Effects for Type 2 Diabetes

A supportive effect in type 2 diabetes treatment represents one of the many health benefits for which *Moringa oleifera* products are promoted and will be discussed in this chapter. Other health-promoting properties, such as anti-inflammatory or antioxidant effects and immune regulatory bioactivities, have been thoroughly covered elsewhere [e.g., by Lin et al. (7), Xiao et al. (8), and Afzal et al. (9)] and would go beyond the scope of this perspective.

Moringa oleifera leaves showed glucose lowering effects in various animal studies, which were suggesting this herbal product for the treatment of type 2 diabetes either instead of metformin, a commonly used drug for type 2 diabetes treatment, or in combination with anti-diabetic drugs (10). In one of these studies showing most pronounced effects, six groups of alloxan-induced diabetic Wistar rats plus one group of normoglycaemic Wistar rats (group I) were treated either with distilled water (group I, II), different amounts of an ethanolic *Moringa oleifera* leaf extract without (group III, IV) or with the addition of 150 mg/kg metformin (group V, VI), or with metformin alone (group VII). After 4 weeks, a significantly lower fasting blood sugar level (FBS) of 307 ± 31 mg/kg could be observed in rats administered with 400 mg/kg *Moringa oleifera* leaf extract (group III) compared to the mean FBS of 477 ± 17 mg/kg in the diabetic control group ($p < 0.05$). The glucose levels could even be more reduced to 252 ± 41 mg/kg when receiving 800 mg/kg *Moringa oleifera* leaf extract (group IV) over 4 weeks. The greatest lowering effect of FBS levels could be obtained in group V and VI, when the diabetic rats were either treated with 400 mg/kg or 800 mg/kg *Moringa oleifera* leaf extract, respectively, plus the addition of 150 mg/kg metformin. After 7 days, the FBS level reached 253.0 ± 35.13 mg/kg and 168.5 ± 21.19 mg/kg in group V and VI, respectively, and continued to decrease to 100.6 ± 15.14 mg/kg and 80.8 ± 5.43 mg/kg, respectively, after 28 days of treatment (10). Despite these positive outcomes, data is lacking whether consumption of *Moringa oleifera* products might be associated with unfavorable consequences in treated rats. Omabe et al. (11) not only investigated the positive effect of *Moringa oleifera* leaves on blood glucose level, but also revealed a development of metabolic and anion gap acidosis in Alloxan-induced type 2 diabetic rats: Compared to the control group, in which rats were administered with 1.5 mL PBS twice a day over a period of 5 days, a more than 2-fold increase in anion gap could be observed in rats treated twice daily with 200 mg/kg of an ethanolic extract of *Moringa oleifera* leaves soluted in 1.5 mL PBS, and even a 3-fold increase of anion gap was determined when 10 mg/kg of metformin plus 1.5 mL PBS were administered over the same treatment period. Therefore, this alternative treatment might not be recommended for type II diabetic patients who are susceptible to develop acidosis.

As reviewed by Stohs and Hartman (12), a smaller number of human studies on the effects of *Moringa oleifera* exist compared to a constantly growing number of animal studies, which should be interpreted with caution. As an example for human intervention studies, Kushwaha et al. (13) showed a significant decrease of fasting blood glucose levels in postmenopausal women from 107 ± 7 to 92 ± 3 mg/dL after consuming 7 g of *Moringa oleifera* leaf powder over a period of 3 months. Nonetheless, a comprehensive literature search conducted by Owens III et al. (14) emphasizes that in order to build upon these first promising results on the prevention or treatment of Type 2 diabetes in humans, more studies with standardized approaches, e.g., standardized Moringa preparations as well as treatment durations and doses, are required.

Macronutrient Profiling

In general, the nutrient content of Moringa products chiefly depends on the cultivation conditions, e.g., climate characteristics and soil composition, processing and storage conditions (**Table 1**). For total protein, mean highest amounts of 22.4% were quantitated in dried Moringa leaves, whereas flowers and immature pods contained slightly lower mean contents of 18.9 and 19.3% of total dry mass, as analyzed by means of the commonly used Kjeldahl method (15). Protein digestibility, as one of the main determinants of the nutritional quality of a dietary protein, has not been investigated *in vivo* so far. However, the non-enzymatic hydrolyzability of ground *Moringa oleifera* leaves was studied *in vitro* by Borges Teixeira et al. (16). The authors analyzed a mean total protein content of 277 mg/g in defatted *Moringa oleifera* leaf flour by the Kjeldahl method, and quantitated the amount of protein soluble in sodium dodecyl sulfate (40%) and 2-mercaptoethanole (30%), revealing about 60–70% of the total protein not being hydrolyzed under these conditions.

Another quality indicator of dietary proteins is their content of essential amino acids, for which a wider variation was analyzed among Moringa flowers (44% of total protein) and leaves (31% of total protein), with methionine being the limiting amino acid in all Moringa products (15) (**Table 2**).

Total lipid contents of 5–7% (15, 16), 3% (15) and 1.3% (15) of dry weight have been reported for leaves, flowers and immature pods, respectively, indicating major variations among the different plant parts. With regard to the fatty acid profile, the highest contents of 6% polyunsaturated fatty acids (PUFAs) of total lipids were analyzed in dried leaves, compared to 50 and

TABLE 1 | Macronutrient profile of *Moringa oleifera* leaves, flowers, immature pods and seeds[a].

Sample	Country	Treatment	Protein (%)	Total fat (%)	Fatty acids (% of total FA)	Dietary fiber (%)	Carbo-hydrates (%)	References
Leaves	Mexico	Oven-dried 60 °C, 8 h	22.4	5.0	SFA 29.9 MUFA 7.2 PUFA 63.2 LA 6.1 ALA 56.9	31.0	27.1	(15)
Flowers	Mexico	Oven-dried 60°C, 8 h	18.9	2.9	SFA 31.8 MUFA 26.3 PUFA 42.2 LA 19.0 ALA 23.0	32.5	36.0	(15)
Immature pods	Mexico	Oven-dried 60°C, 8 h	19.3	1.3	SFA 31.6 MUFA 18.4 PUFA 49.9 LA 23.5 ALA 26.2	46.8	25.0	(15)
Leaves (flour)	Brazil	Oven-dried 35°C, 24 h	28.7	7.1	–	–	44.4	(16)
Seeds	Nigeria	Raw	26.7	30.6	–	1.4	32.0	(17)
Seeds	Nigeria	Boiled			–			(17)
		10 min	21.6	10.4		1.9	49.9	
		20 min	28.9	11.3		2.1	36.5	
		30 min	32.0	12.3		2.3	31.8	
Seeds	Nigeria	Roasted			–			(17)
		10 min	25.9	20.5		3.7	41.0	
		20 min	29.0	10.6		3.7	47.6	
		30 min	26.6	20.5		3.8	42.2	

[a] *Values are presented as means.*

TABLE 2 | Amino acid pattern of *Moringa oleifera* leaves, immature pods and flowers in mg/g dry weight [adapted from Sánchez-Machado et al. (15)].

Amino Acid	Leaves	Immature pods	Flowers
Histidine	7.0 ± 0.4	2.0 ± 0.3	3.1 ± 0.4
Threonine	7.9 ± 0.4	3.3 ± 0.5	5.4 ± 0.2
Tyrosine	4.8 ± 0.9	0.4 ± 0.1	0.4 ± 0.1
Valine	11.3 ± 1.1	4.3 ± 1.0	6.4 ± 0.6
Methionine	1.4 ± 0.3	0.9 ± 0.2	1.0 ± 0.2
Isoleucine	8.9 ± 0.7	3.1 ± 0.4	5.2 ± 0.5
Leucine	17.5 ± 0.2	5.6 ± 0.5	8.7 ± 0.9
Phenylalanine	8.9 ± 0.3	2.3 ± 0.4	3.8 ± 0.5
Lysine	15.3 ± 0.6	2.5 ± 0.6	4.6 ± 0.5
Aspartate	15.8 ± 1.5	7.4 ± 0.3	12.3 ± 0.9
Glutamate	17.1 ± 1.4	14.6 ± 2.3	17.0 ± 2.2
Serine	9.4 ± 0.5	7.5 ± 1.8	7.5 ± 0.4
Glycine	10.3 ± 0.7	4.3 ± 0.5	6.5 ± 0.3
Alanine	12.5 ± 0.6	4.2 ± 0.7	8.1 ± 0.5
Proline	12.4 ± 0.9	4.0 ± 0.6	6.6 ± 0.5
Arginine	12.2 ± 0.8	8.1 ± 2.5	20.1 ± 1.2

42% in immature pods and flowers, respectively. In addition, 7% of the lipid content in Moringa leaves were assigned to monounsaturated fatty acids (MUFAs), whereas saturated fatty acids (SFA) amounted to 30% (15). Although raw *Moringa oleifera* seeds contained more fat than other edible parts of the indigenous tree, with nearly reaching an amount of 31% (17), the lipid content decreased to 20.5% when the seeds were roasted for 30 min and an even higher loss to 12.3% of total fat content was noted when seeds were boiled for 30 min (**Table 1**). Concerning dietary fiber, Sánchez-Machado et al. (15) reported the highest values for oven-dried immature pods (46.8%), which compares to the amounts found in high dietary fiber sources such as edible seaweed. Leaves and flowers yielded lower amounts, with 31.0 and 32.5%, respectively. Seeds ranged from 3.8 (roasted for 30 min) to 1.4% (raw) (17) (**Table 1**).

Micronutrient Profiling

Moringa products are often promoted as nutritional supplements to treat and prevent micronutrient malnutrition such as vitamin A deficiency, which is prevalent in many parts of Africa or South Asia (18). As such tropical and subtropical countries are home to the drumstick tree, several studies investigated the potential of *Moringa oleifera* products as a vitamin A source (16, 17, 19, 20). Borges Teixeira et al. (16) revealed a mean beta-carotene content of 161 μg/g and a mean lutein content of 47 μg/g lyophilized leaf flour. Converting the amount of beta-carotene to retinol equivalents would be equivalent to 26.8 μg RAE per gram leaves. Similar vitamin A levels were

TABLE 3 | Vitamin contents in differently prepared edible parts of *Moringa oleifera* in μg/g (ready to eat)[1].

Sample	Treatment	Vit. A[2]	β-carotene[2]	α-tocopherol	Ascorbic acid	References
Leaves (flour)	Oven-dried 35 °C, 24 h		161			(16)
Seeds (flour)	Raw	20.4				(17)
	Boiled	20.9–28.0				
	Roasted	17.3–18.7				
Leaves (powder)	Fresh		183	369	2,710	(21)
	Lyophilized		821[a]	1602[a]	13,256[a]	
	Cabinet-tray dried		823[a]	1652[b]	5,908[c]	
	Micro-oven dried		659[c]	1355[c]	8,729[b]	
	Oven-dried		778[d]	1596[a]	5,450[d]	
	Sun-dried		544[d]	1123[d]	5,100[d]	

[1] *Values are presented as means or range; different superscript letters (each column) represent significant differences ($p < 0.05$) between dehydration methods.*
[2] *1 μg retinol activity equivalent (RAE) equals 1 μg preformed retinol, or 2 μg β-carotene supplement in oil, or 12 μg β-carotene, or 24 μg other pro-vitamin A carotenoids.*

obtained in *Moringa oleifera* seeds, with 20 μg/g in the raw samples, 21–28 μg/g in boiled seeds and 17–19 μg/g in roasted samples (17). These results have been summarized in **Table 3**. Nevertheless, more profound investigations of *Moringa oleifera* leaves are needed to determine how much of the provitamin carotenoids can be converted to retinol. Furthermore, the study of Sriwichai et al. (22), where bioaccessibility was determined by assessing the amount of compounds transferred into an aqueous micellar phase after an *in vitro* digestion procedure, showed that full bioaccessibility of these bioactive compounds cannot be assumed. The beta-carotene content in raw *Moringa oleifera* leaves was 630 μg/g dry matter, but <5 μg/g dry matter were bioaccessible. Different drying methods as well as grinding and encapsulation were applied in order to allow a destruction of the cell wall and show an efficient way to deliver carotenoids or other bioactive compounds. The best results (<2 mg/100 g dry matter) were obtained when moderate drying temperatures (60°C) were combined with subsequent fine grinding. Encapsulation of the dried and grinded powder lead to similar results of bioaccessible beta-carotene. Although a significant improvement of the bioaccessibility of beta carotene could be obtained with these methods, a great part could not be made bioaccessible (22). Considering these findings, claims and statements of a high carotenoid content should be relativized and further studies about bioavailability and bioconversion to vitamin A are indispensable.

Vitamin A could help to release iron from iron stores and therefore help to reduce anemia. Indeed, in a study by Zimmermann et al. (23) vitamin A supplementation to vitamin A deficient children supported hematopoiesis. Instead of direct vitamin A supplementation, Boateng et al. (19) analyzed the hemoglobin stores and some growth indicators in infants aged between 8 and 12 months in the Eastern region of Ghana after receiving complementary foods fortified with *Moringa oleifera* leaf powder over 4 months. The 237 participating infants were divided into three groups, one group receiving a cereal-legume (35 g/day) blended flour with 5 g Moringa leaf powder, the second group only receiving 5 g of *Moringa oleifera* leaf powder in form of "sprinkles" on top of the usual diet, and the third group receiving 35 g of cereal-legume blended complementary food without *Moringa oleifera* leaves powder. The analysis revealed no significant difference in hemoglobin status or growth parameters between the three groups. This finding might be explained by several factors, including the recruitment of healthy children without treatment of infection prior to intervention, thus leaving the possibility of sub-clinical infections affecting hemoglobin concentrations open, and anemia prevalence at baseline ranging from 53 to 64% in the three groups. The authors hypothesized that the amount of added Moringa (5 g) was too low and the period of intervention (4 months) might have been too short to see any improving effects in hemoglobin status in healthy children. The added amount of 5 g Moringa consists of ∼1.1 mg of iron, which only represents 10% of the daily iron needs recommended by the WHO/FAO for the age group studied (24). Furthermore, no data collection on the use of other vitamin supplementations during the intervention period and a possible contribution of low iron bioavailability due to the presence of phytic acid in *Moringa oleifera* leaves represent additional limitations of the study. The latter was also discussed and observed in the study of Gallaher et al. (25), who evaluated the iron bioavailability of *Moringa oleifera* leaf powder applied to rats. Irrespective to these points, a broader investigation of other iron parameters, such as transferrin receptor or serum ferritin should have been favored instead of observing a single biomarker (hemoglobin). Additionally, a lower adherence in the two groups receiving *Moringa oleifera* leaves powder (61%) was shown compared to the third group who consumed no leaf powder (80%). The adherence was measured by weighing and documenting the amount of leftovers of the supplied study food. This outcome is in accordance with a recent report which compared the acceptability of different products, such as biscuits, cakes, soups or dairy products with a varying amount of bitter-tasting *Moringa oleifera* leaf powder (6, 26). The evaluation studies were conducted with adult study participants (26). In

addition to the high chlorophyll content of the leaves which lead to an intense green color and metallic off taste, the strong herbal smell was discussed to lower the acceptability of Moringa fortified products.

A recent study conducted in iron depleted rats detected a very low bioavailability of iron in air-dried *Moringa oleifera* leaf powder (25). For analyzing iron bioavailability, the hemoglobin regeneration efficiency (HRE) ratio assay was used. The HRE ratios were 2.6 and 1.0 for adding ferrous sulfate and ferric orthophosphate to a purified diet, respectively after 9 days of intervention. An iron repletion for 13 days revealed values of 2.7 when ferrous sulfate was added and 1.2 when iron was added in form of the poorly-absorbed ferric orthophosphate. Consuming a Moringa-rich diet consisting of 38.7 g Moringa/kg diet only showed a HRE ratio of 0.05 and 0.4 after 9 and 13 days, respectively. The HRE ratio decreased from 0.59 after 9 days of repletion to 0.4 after 13 days of iron repletion when Moringa leaf powder was added to a traditional Ugandan recipe, mainly containing rice and ground nuts (5.4 g Moringa/kg diet). The authors assumed that the low bioavailability was caused by the high phytate content of nearly 64 mg/g dry weight existing in the dried leaves (25). In another 3 month intervention study, Senegalese lactating women showed no significant changes of plasma ferritin levels after dietary supplementation of 100 g *Moringa oleifera* leaf powder per week (27). The plasma ferritin levels of 13 women in the Moringa group (n=33) and 14 women in the control group (n=31) were below 12 μg/L at baseline and were classified as iron deficient. After 3 months of intervention, plasma ferritin levels did not change significantly in the Moringa group, whereas only two women showed iron deficiency in the control group who received two tablets weekly, each equivalent to 130 mg of elemental iron plus 0.5 mg folic acid. In this study, the presence of polyphenols in *Moringa oleifera* leaf powder and the subsequent formation of non-bioavailable polyphenol-iron complexes was suggested to explain low iron bioavailability (27).

Influences of Processing on Vitamin Content

Moringa oleifera leaves are promoted for their high vitamin content. Compared to 100 g of oranges, which contain ∼53 mg vitamin C, Saini et al. (21) showed an ascorbic acid content of 271 mg in fresh *Moringa oleifera* leaves (28). Including a realistic consumption quantity of 40 g, which was estimated as an average daily intake from the recommendations on commercially available products (29), would yield 108 mg vitamin C and ≈200 g orange pulp in this equation. The α-tocopherol content of 37 mg per 100 g found in fresh Moringa leaves was comparable to the amount of 41 mg found in 100 g sunflower oil as stated in the USDA Food Databank (28). Furthermore, Saini et al. (21) revealed a trans-lutein content and trans-β-carotene content of 36.9 mg/100 g and 18.3 mg/100 g, respectively, in fresh leaves. Hence, this carotenoid content is higher than the amount stated for raw carrots, which contain ∼8 mg of β-carotene in 100 g (28). However, Saini et al. (21) investigated the retained amounts of vitamins in *Moringa oleifera* leaves when different drying methods are applied, since it is often consumed in a dried form. The highest preservation of these phytoconstituents was obtained by lyophilisation, with a true retention of 89.9% of trans-β-carotene, 51.3% of trans-lutein, 86.7% of α-tocopherol and 97.8% of ascorbic acid (analyzed in triplicates with a standard deviation <5%). With cabinet tray drying, a similar retention rate of trans-β-carotene was obtained, whereas micro-oven-drying, oven-drying and sun-drying decreased the preserved trans-β-carotene content significantly, with sun-drying having the highest impact. A considerable loss in carotenoids should be taken into account when consuming sun-dried Moringa leaves, which is the usual way of drying leaves in African households (21). When sun-dried, only 54.4 mg/100 g trans-β-carotene were retained compared to 82.1 mg/100 g after lyophilisation. The authors stated that α-tocopherol content was the least affected phyto-constituent by all the drying methods, followed by the total phenolic content. Ascorbic acid on the other hand, could only be preserved efficiently by lyophilisation (97.8%) and micro-oven-drying (64.4%). All other drying techniques reported significantly lower total retention of 43.6% by cabinet tray drying, 40.2% by oven-drying and 37.6% by sun-drying (21). To conclude, especially ascorbic acid and carotenoid contents suffer from sun-drying, which would clearly recommend an, albeit more elaborate, lyophilisation procedure. However, the apparent gaps in **Table 3** warrant further, comprehensive investigations on the effects of processing on the vitamin content of all edible Moringa parts and their respective treatments.

Anti-nutrients

Phytic acid was assumed to negatively affect the iron bioavailability in the study of Gallaher et al. (25), who detected 6.4 g/100 g dry weight of phytic acid in dried *Moringa oleifera* leaves. In contrast, Gidamis et al. (30) reported much lower amounts of phytic acid, with 0.23 mg/100 g found in cooked leaves. Additionally, the authors did not observe a significant difference in phytate levels in pods as well as in leaves after cooking. Phytic acid contents of raw and cooked *Moringa oleifera* pods were 0.25 mg/100 g and 0.24 mg/100 g, respectively. After cooking, a decrease of tannins could be shown for both, pods and for leaves (**Table 4**).

A phytochemical evaluation of *Moringa oleifera* seeds was conducted by Mbah et al. (17) in which the maximum contents of phytate, tannins and saponins reached 11.7 mg/100 g, 9.8 mg/100 g and 0.5 mg/g, respectively. The authors additionally evaluated oxalate contents between 2.5 and 3.6 mg/100 g. Even higher oxalate contents were found in dried *Moringa oleifera* leaves, reaching 1,050 mg/100 g (16). Additionally, trypsin inhibitor with a total of 1.45 TUI/g could be detected (16). The trypsin inhibitor content of 0.3 mg/100 g dry weight found in *Moringa oleifera* pods was not affected by cooking (30). As depicted in **Table 4**, the overall lower anti-nutrient contents favor leaves and pods over seeds for consumption. However, more complete data on different products and treatments as well as the comparison to their respective nutrient contents are still required.

TABLE 4 | Anti-nutrient contents in mg/100 g[1].

Sample	Treatment	Phytate	Tannins	Saponins	Oxalate	Trypsin inhibitor	References
Leaves (flour)	Dried	–	2.1		1,050	1.45[2]	(16)
Seeds (flour)	Raw	3.3	9.8	0.1	2.9	na[3]	(17)
	Boiled	3.9–11.7	1.7–7.8	0.4–0.5	2.5–3.1		
	Roasted	3.9–5.6	1.2–7.4	0.2–0.4	2.9–3.6		
Leaves	Raw	0.3[a]	0.22[a]	–	na	nd[3]	(30)
	Cooked	0.2[a]	0.16[bc]			nd[3]	
Immature pods	Raw	0.2[a]	0.2[ab]	–	na	0.3[a]	(30)
	Cooked	0.2[a]	0.1[c]			0.3[a]	

[1] *Values are presented as means or range, different superscript letters per column represent significant differences ($p \leq 0.05$).*
[2] *in TUI/g.*
[3] *na = not analyzed; nd = not detected.*

Contaminants

Heavy Metals

Regarding the contamination of Moringa products with heavy metals, a brief glance into the research fields of biosorption and phytoremediation might raise some concerns. In detail, besides frequently proposed uses of *Moringa oleifera* seeds in the biosorption of heavy metals, a few studies also highlighted the phytoremediation potential for contaminated soil, e.g., as described for cadmium (31) and lead (32). As such, the potential hyper-accumulation of heavy metals from the cultivation sites in edible Moringa parts should be considered, along with a thorough monitoring of the soil and Moringa products in the future.

Aissi et al. (29) evaluated the presence of lead and cadmium in *Moringa oleifera* leaf powders in 24 samples, available from 12 different national and international producers in Benin. The average lead content reached 1.53 mg/kg and a total of 58.3% of the products contained higher levels than the allowed maximum limits set by the European Commission Regulation (EU) No 1881/2006 of 0.3 mg/kg for leafy vegetables (33). Higher lead contents were reported by Limmatvapirat et al. (34) for products purchased in Thailand, with an average value of 2.45 mg/kg for all investigated products and 1.98 mg/kg for leaf powder. Additionally, the authors reported noteworthy levels of arsenic, cadmium and mercury, with 0.362 mg/kg, 0.122 mg/kg and 0.087 mg/kg, respectively. The overall highest contents for the assessed heavy metals were found in tea leaves. While the average cadmium value was below the maximum limit of 0.2 mg/kg defined in the EU regulation No 1881/2006 (33), especially leaf capsule samples exceeded this threshold with contents up to 0.6 mg/kg. Regarding arsenic and mercury, threshold values are available for rice and rice products for arsenic and complementary foods for mercury, with 0.10–0.30 and 0.10 mg/kg, respectively. In light of this, the reported arsenic levels of up to 1.57 mg/kg particularly warrant thorough monitoring.

In comparison, Aissi et al. (29) reported a higher mean cadmium content of 0.25 mg/kg. Although the difference of this value to the maximum allowed limit of 0.2 mg/kg was not statistically significant, the authors stated exceeding levels of cadmium in 75% of the samples, with values up to 0.35 mg/kg. Nevertheless, it was concluded that there is only a low risk of heavy metal intoxication for consumers in general, as the DJE (dose journalière d'exposition apportée par l'alimentation générale) for Moringa was estimated to account for <2% compared to the consumption of other food. The average daily amount of Moringa leaf powder consumed by adults and children was estimated with 40 g according to the recommendations on the packaging.

To summarize, the lead and cadmium contamination through Moringa leaf powders was not categorized as a risk for consumers in general (29). However, the promotion of using *Moringa oleifera* products for treating malnutrition or as a product supporting a healthy lifestyle should still be taken with caution: Individual samples exceeded the existing threshold values of several heavy metals considerably (29, 34) and, when coupled to an increasing daily consumption, a more significant contribution of Moringa products to the total daily intake of these contaminants could be conceivable in the future. Special care also needs to be taken regarding the intended use of Moringa preparations, as food for infants has significantly lower allowed concentrations, e.g., 20 μg/kg for lead and cadmium (33).

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous and persistent organic compounds, some displaying genotoxic and/or carcinogenic activity. Alongside the 16 identified priority PAHs, the amount of Benzo(a)pyrene as well as the sum of PAH4 are often applied as indicators in risk assessment (35, 36). PAHs are formed in various natural and synthetic processes through pyrolysis during the incomplete combustion of organic substances, and may therefore contaminate food through numerous ways—from atmospheric deposition or contaminated soil to industrial food processing and cooking (37). Besides deposition and soil contamination, e.g., a previously described source for tea leaves from drying with combustion gases (38) could also contribute to PAH content of dried Moringa products.

A recent evaluation of PAHs in Moringa herbal tea revealed a $\sum_{16}$PAH content of 5.03 ± 0.84 μg/kg, whereby 5-ring and 6-ring PAHs were the predominant forms (35). Compared to

two or three ring aromatic systems, these molecules are more toxic and less susceptible to degradation processes. This study evaluated the PAH contents in 23 teas, including green, black and herbal teas, in Nigeria. The $\sum_{16}$PAH content of herbal teas ranged between 4.71 and 79.6 μg/kg, whereas the content found in green and black teas were between 1.63–73.5 and 12.5–27.0 μg/kg, respectively (35). For $\sum_{16}$PAH no maximum levels have been established so far in the EU. However, the revealed level of 5.03 ± 0.84 μg/kg in Moringa herbal tea is still lower than the maximum levels of 50 and 10 μ/kg set for $\sum_{4}$PAH and benzo(a)pyrene in dried herbs, respectively (36). Nevertheless, due to the ubiquity of PAHs and various contamination sources, their thorough screening remains necessary.

Mycotoxins

Mycotoxin analysis in *Moringa oleifera* products are very limited, but the outcome of a recent study conducted by Aristil et al. (39), strengthens the need for a more profound mycotoxin evaluation on *Moringa oleifera* products, and in particular on seeds. The main goal of this study was to evaluate the contamination of toxigenic fungi in nine maize, three moringa and six peanut seed samples collected in Haiti. Furthermore, to determine *via* the ELISA technique if the *Aspergillus* section Flavi strains have the potential of producing aflatoxins AFB1 and AFG1. The analysis showed that 71% of the detected fungi in moringa seed samples belonged to *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp., which are potential mycotoxin producers, whereby other fungi made up 28% of the isolated mycobiota. In comparison, these three taxa made up 90% of the fungi in maize samples, and 51% of the mycobiota found in peanut seeds belonged to *Aspergillus* spp. An monoconidial isolation of *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. was carried out in pure culture afterwards. Five isolates of *Aspergillus* section Flavi strains from Moringa seeds were tested for aflatoxin production, whereby three isolates were capable of producing AFB1, and the other two isolates either produced both mycotoxins AFB1 and AFG1 or no aflatoxin, respectively. However, the aflatoxin contamination in the analyzed Moringa samples ranged from non-detectable (LOD: 2.1 μg/kg) to a maximum of 700 μg/kg. Taking the maximum limit of 10 μg/kg for almonds and other kernels for direct human consumption as a reference, the aflatoxin contamination in Moringa samples can be alarmingly high (36).

Safety Assessment

Although an increasing number of investigations addressing the positive health effects of *Moringa oleifera* are conducted, studies about safety and toxicity evaluations are largely lacking.

In the review of Stohs and Hartman (12), *Moringa oleifera* leaves consumed in its different preparations (extract, powder) are considered relatively safe as no adverse effects have been reported in association with human studies until now. Genotoxicity was only observed when rats were administered with an aqueous *Moringa oleifera* leaf extract at a supra-supplementation level of 3,000 mg/kg body weight (40). However, an amount of 1,000 mg/kg body weight, which is still higher than commonly consumed doses, did not exhibit genotoxic effects (40). Administration of a methanolic extract of *Moringa oleifera* leaves at a dose of 200 and 400 mg/kg body weight fed to rats over a period of 8 weeks indicated negative effects on hepatic and renal function (41). Nevertheless, this amount is very unlikely to be consumed on a regular basis, since 400 mg methanolic leaf extract/kg body weight would be equivalent to 12 g leaves/kg body weight (41). Furthermore, the amounts given and results achieved from rodent studies are not transferable one-to-one to humans and need to be extrapolated. An amount of 12 g leaves/kg body weight for a rat would mean ∼156 g Moringa leaves for an 80-kg adult, which is higher than the commonly recommended doses of ∼40 g/day (12, 29).

Several reports about the potential of *Moringa oleifera* leaves to influence fertility, contraception and the reproductive status are existing (42, 43). For example, Attah et al. (42) analyzed the *in vivo* and *in vitro* contraceptive and abortifacient potential of Moringa leaf powder when administered before and after mating of Wistar rats. Uterine contractility was reported for hot as well as for cold extracts of *Moringa oleifera* leaf extract, whereby the highest activity was shown for cold aqueous extracts. This might be caused by the presence of metabolites formed upon digestion and absorption which are degraded at higher temperatures. A dose of 58 mg/kg body weight of cold aqueous extract or 50 mg/kg body weight of a hot aqueous extract which was administered before mating, resulted in 100% infertility and 83.3% infertility, respectively. An amount of 58 mg/kg body weight was stated to be comparable to 250 mg dry plant product. When the Wistar rats received these doses after mating, an 80% abortion activity for cold, and 50% abortion for hot aqueous extracts was observed. No abortion could be shown in animals of the control group, which only received distilled water, whereas a significantly altered morphometry growth was shown by pups delivered by Wistar rats in the hot aqueous extract group.

A study conducted with rabbits investigated differences in reproductive hormone status between female and male rabbits when they received 0–15 g/kg *Moringa oleifera* powder over a period of 12 weeks (43). The follicle stimulating hormone (FSH) was highest in the control group (2.7 ± 0.3 IU/mL) compared to the three Moringa treatment groups which showed serum FSH levels of 1.1 ± 0.1, 0.9 ± 0.1, and 0.6 ± 0.1 IU/mL when the female rabbits were administered with 5, 10 or 15 g/kg *Moringa oleifera* powder, respectively. Contrary, FSH levels of rabbit bucks were higher after receiving 15 g/kg Moringa powder (1.9 ± 0.6 IU/mL), than in the control group (0.9 ± 0.3 IU/mL). Also, semen quality and sperm count improved with an increasing amount of dietary *Moringa oleifera* powder. With an additional investigation of other hormones, such as luteinizing hormone (LH), progesterone and testosterone, the authors could show a supporting effect of *Moringa oleifera* powder on the fertility of bucks, although it led to infertility in female rabbits (43).

When it comes to dietary supplementation with *Moringa oleifera*, the powdered form is rather consumed than the Moringa extracts. However, since no standardized method of aqueous and alcoholic extraction has been established, high variations in the extract constituents have to be expected which do not allow direct comparisons of scientific data from animal studies and their extrapolation to humans.

CONCLUSION

Even though several attempts have already been made to evaluate the composition and safety of *Moringa oleifera* products, variations in processing, preparation and extraction methods impede not only the direct comparison of data, but also the formulation of guidelines for dietary intakes. Despite these challenges, the dietary intake of *Moringa oleifera* leaves has been considered as safe at relatively high doses, and should be re-evaluated in light of individual constituents, e.g., heavy metals and PAHs, as well as mycotoxins. High levels of anti-nutrients and contaminants indicate the need for a more profound analysis accompanied by contingent legal regulations for the dietary intake of *Moringa oleifera* products.

AUTHOR CONTRIBUTIONS

SG reviewed the literature and drafted the manuscript. PP reviewed the literature and c-drafted the manuscript. VS and KK reviewed the literature, conceptualized, and revised the manuscript. All authors approved the finalized version of the manuscript.

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The Framework for Responsible Research With Australian Native Plant Foods: A Food Chemist's Perspective

Selina Fyfe[1]*, *Heather E. Smyth*[1], *Horst Joachim Schirra*[2,3,4], *Michael Rychlik*[1,5] and *Yasmina Sultanbawa*[1]

[1] Queensland Alliance for Agriculture and Food Innovation (QAAFI), Health and Food Sciences Precinct, The University of Queensland, Brisbane, QLD, Australia, [2] Centre for Advanced Imaging, The University of Queensland, Brisbane, QLD, Australia, [3] School of Environment and Science, Griffith University, Nathan, QLD, Australia, [4] Griffith Institute for Drug Discovery, Griffith University, Nathan, QLD, Australia, [5] Chair of Analytical Food Chemistry, Technical University of Munich, Freising, Germany

Australia is a rich source of biodiverse native plants that are mostly unstudied by western food science despite many of them being ethnofoods of Australian Indigenous people. Finding and understanding the relevant policy and legal requirements to scientifically assess these plants in a responsible way is a major challenge for food scientists. This work aims to give an overview of what the legal and policy framework is in relation to food chemistry on Australian native plant foods, to clarify the relationships between the guidelines, laws, policies and ethics and to discuss some of the challenges they present in food chemistry. This work provides the framework of Indigenous rights, international treaties, federal and state laws and ethical guidelines including key legislation and guidelines. It discusses the specific areas that are applicable to food chemistry: the collection of plant foods, the analysis of the samples and working with Indigenous communities. This brief perspective presents a framework that can be utilized by food chemists when developing responsible research involving plant foods native to northern Australia and can help them understand some of the complexity of working in this research area.

Keywords: native Australian plant foods, responsible research, policy, ethical guidelines, framework, Australia

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***Correspondence:**
Selina Fyfe
selina.fyfe@uqconnect.edu.au

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INTRODUCTION

Australia is a rich source of biodiverse native plants that are mostly unstudied scientifically despite many of them being ethnofoods eaten by Australia's Indigenous people. Australia is one of the world's 17 megadiverse countries of flora and fauna with 93% of flowering plants occurring only in it (1). Over the past 10 years native Australian plant foods have become more desired and accepted in mainstream Australian culture and the past five years has seen many becoming common as valued ingredients in food products on supermarket shelves, in gourmet foods and on restaurant menus where they are usually an ingredient featured in the name and marketing of the product. Researching native Australian plant foods offers the possibility of more of them being added to the Australian food supply chain and enabling Indigenous communities to be an important part of this.

Relatively few Australian native plant foods have been studied by western food science to understand their nutritional or functional components or to understand how they can be added to the native food value chain and made commercially available. Some of those that have been studied have been shown to have high values in key nutrients (2, 3) and excellent food preservation properties (4) as well as unique and interesting flavors (5). The Kakadu plum is now used in the Queensland prawn industry as a natural preservative to increase the shelf life of cooked chilled prawns (4).

Food chemists study foods to understand their composition and properties and to identify ways they can be used as food products and as ingredients in other food products. For food chemists to do responsible research on these plant foods they must comply with international, national and state policies, treaties and laws as well as uphold Indigenous rights and obtain the samples and conduct the research in a legal and ethical manner. Finding and understanding all of these requirements is a major challenge.

The European Union's Rome Declaration on Responsible Research and Innovation in Europe (6) defines responsible research and innovation as "the on-going process of aligning research and innovation to the values, needs and expectations of society." It involves engagement of all societal actors, ethics, respecting fundamental rights, governance through policy (7), having research integrity and it should make science more sustainable, ethical and socially beneficial (8). The aim of this paper is to collate the current relevant key legal and policy requirements into a framework for food chemists to study native Australian plants in a responsible way, and to comment on some of the challenges. This brief perspective focuses only on the perspective of food chemists and does not include chemistry related to medicine or pharmacology. It does not include responsible innovation which would require legal analysis of intellectual property laws and legal contracts. It is not a framework for product development or commercialization which would have other requirements including, but not limited to, different types of permits with different conditions and permission requirements, benefit sharing agreements, agreements around branding and market access, the traditional or novel status of the plant food with Food Standards Australia New Zealand and various other regulatory requirements. It is not a legal analysis by lawyers but a perspective of food chemists.

FOOD CHEMISTRY AND AUSTRALIAN NATIVE PLANT FOODS

Food chemistry aims to analyze the composition and properties of food and the compositional changes that occur during handling, processing and storage of food (9). Food chemistry studies of Australian native plant foods include nutrition profiles, flavor chemistry, compounds that give antioxidant and antimicrobial properties, chemical and metabolite composition, compounds that impact safety of these as foods and changes that occur during storage or processing. Recent studies include the nutritional profiling of wattle seeds (10, 11), green plum (3), Kakadu plum kernels (12) and gumby gumby leaves (13); the chemical and metabolite composition of Kakadu plum (2, 14), finger lime, native pepperberry and Davidson's plum (15) and Tasmanian pepper (16); the antioxidant properties of infusions of gulban, anise myrtle and lemon myrtle leaves (17), Kakadu plum extracts (18), wattle seeds (11), the tuckeroo (19), Illawarra plum, Kakadu plum, muntries and native currant (20); and analysis of storage of lemon myrtle, anise myrtle and Tasmanian pepper leaf (21) as well as drying methods of Kakadu plum (22). Food chemistry has the potential to be used in the provenance, traceability and authenticity of native Australian plant foods. Food chemistry studies including metabolomics have been used to assess the authenticity of the geographical locations of other food products (23–25). Metabolomics can be used to give both a fingerprint of the "terroir" metabolome and to identify key compounds to use as biomarkers for specific locations (25, 26) which can be used to guarantee the origins of a product and to protect against food fraud (23). One study of Kakadu plums using attenuated total reflectance mid infrared spectroscopy has shown that adulteration with synthetic ascorbic acid can be detected (27). Further food chemistry analysis in the near future is likely to include more of these types of profiling of other native plant foods and more in-depth chemical and metabolite composition analysis of previously studied ones. This analysis is important for adding these foods as ingredients into other foods, for increasing their use throughout Australia, and for enabling sustainable Indigenous agri-businesses to be created. To conduct these types of food analysis, food chemists need to ensure they understand how to do them responsibly, by obtaining food samples in a legal and responsible way, only doing analysis they are permitted to do, and with responsible and ethical involvement of Indigenous communities.

THE FRAMEWORK FOR RESPONSIBLE RESEARCH

The framework for responsible research presented in **Figure 1** was compiled by finding and reading each individual element of it then connecting and collating these together into a framework. The *Australian Code for the Responsible Conduct of Research* (28) states the principles of responsible research are honesty, rigor, transparency, fairness, respect, recognition, accountability and promotion. Burget et al. (29) describe the conceptual dimensions of responsible research as inclusion, anticipation, responsiveness, reflexivity, sustainability and care. The rights, treaties, laws and policies in the framework provide the governance measures and ethical guidance to enable food chemists to do responsible research on native Australian plant foods.

RIGHTS TO UPHOLD: INDIGENOUS RIGHTS

The rights of Indigenous people add a dimension of inclusion to responsible research. Engagement of all stakeholders is considered essential for innovation to be sustainable, acceptable and desirable (6), and upholding the rights of Indigenous

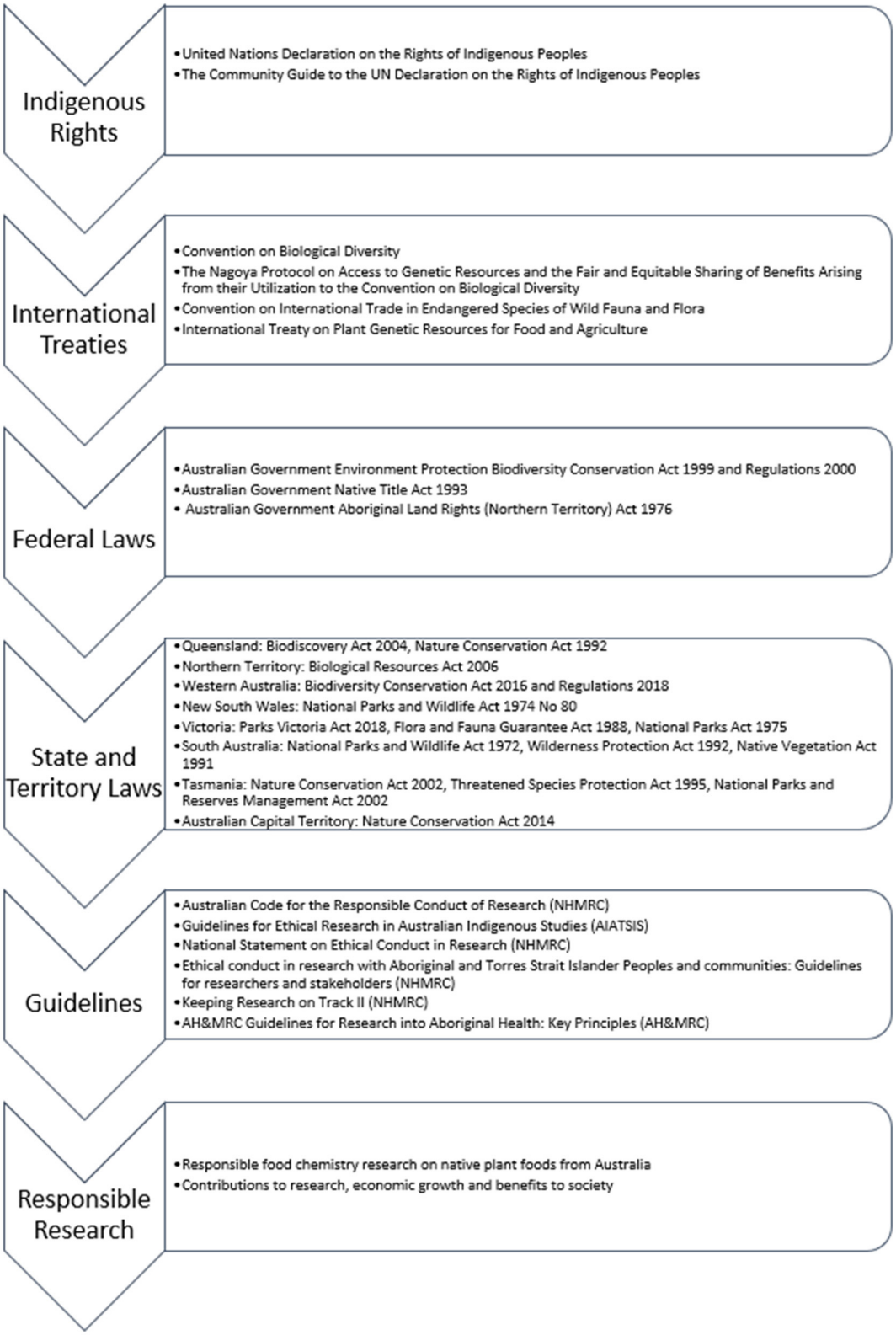

FIGURE 1 | The framework for responsible research for food chemists working with Australian native plant foods. This framework contains key legislation; however, other laws and guidelines may also be relevant.

people means engaging with them and recognizing their right to be engaged in research (28). The *United Nations Declaration on the Rights of Indigenous Peoples* (30) and the Australian *The Community Guide to the UN Declaration on the Rights of Indigenous Peoples* (31) have determined how these rights relate to Indigenous Australians in this context. Article 31 states Indigenous Australians have "the right to maintain, control, protect, and develop their cultural heritage, traditional knowledge and traditional cultural expressions, as well as the manifestations of their sciences, technologies and cultures, including human and genetic resources, seeds, medicines, knowledge of the properties of fauna and flora…" etc. (30). Aboriginal and Torres Strait Islander communities in Australia have knowledge about these plants such as which ones are foods, when they are available, what should not be eaten, and how they should be processed to be made safe or edible (32–34). This extensive plant knowledge has been declared one of the important economic resources of Aboriginal Australians (35). The UN Declaration indicates research on Australian native plant foods used by Indigenous people should involve Indigenous people in the research. This complies with Articles 21 and 32 by giving Indigenous Australians an opportunity to improve their economic conditions and the right to determine and develop strategies for the development or use of their lands and other resources. In addition, such a research partnership allows compliance with Article 29, as Indigenous Australians have the right to the productive capacity of their land. It provides opportunities for communities to produce food from their land, support and create new businesses and develop the economic status of the community which will contribute to societal goals as well as economic growth (36, 37).

TREATIES TO COMPLY WITH: INTERNATIONAL TREATIES

International treaties provide requirements at an international level and protection against the threat of genetic material of native plant species being taken and domesticated in other countries (38). The international *Convention on Biological Diversity* (CBD) was put in place to help with conservation and sustainable use of biological diversity and to ensure there is benefit sharing of genetic resources (39). Article 15 relates to accessing genetic resources and it changed the authority to access natural resources from the "common heritage of mankind" to being that of the national government who now have sovereign rights over natural resources (39, 40) and states access must be on "mutually agreed terms," that "prior informed consent" should be obtained and there should be "sharing in a fair and equitable way the results of research and development and the benefits arising from the commercial and other utilization of genetic resources with the Contracting Party providing such resources." It is from this part of the CBD that the federal, state and territory laws for permits and benefit-sharing agreements for collection and study of native plants has been derived.

The Nagoya Protocol (41) is supplementary to the CBD and is not ratified in Australia (42), but must be complied with if Australian food products or samples are exported to countries that have ratified it. A more detailed explanation of this in relation to Australian native plant foods is given by Adhikari et al. (43). The *Bonn Guidelines* were written to operationalize the relevant provisions in the CBD (44). It gives guidelines about gaining prior informed consent to access genetic resources and basic guidelines of what should be considered in mutually agreed terms.

The *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES) (45) contains a list of endangered wild fauna and flora that CITES regulations relate to. The *International Treaty on Plant Genetic Resources for Food and Agriculture* recognizes that plant genetic resources are depended on for food and agriculture and that most plants that are farmed are being done in places other than where they originated (46). It addresses benefit sharing of plants used for food and agriculture (Article 1), and its purpose is partly to promote the conservation and food production of wild plants by supporting the efforts of local and Indigenous communities (Article 5) and to recognize the contribution they make in conservation and development of plant genetic resources (Article 9).

LAWS TO OBEY: FEDERAL, STATE AND TERRITORY LAWS

Article 15 of the CBD recognizes that states have the sovereign right over their natural resources and national governments have the authority to grant access to them. Governance of biological resources are within the respective jurisdiction of each state, territory, or the Commonwealth government in Australia who all have separate and differing laws and requirements. **Figure 1** contains a list of the key federal and state Acts related to research on native Australian plants (47–63), although other Acts will also be relevant. These Acts enforce the international treaties and Indigenous rights and bring in the appropriate and legal ways to uphold these. They state what land they apply to and how they apply across ownership of land. They use permits and authorizations as a means of ensuring appropriate approval has been given for plant or fruit collection and that the "prior informed consent" required by the CBD has been given from both the government and from owners or title holders of the land. In some circumstances benefit sharing agreements are required to ensure the CBD requirement for "sharing in a fair and equitable way the results of research and development" does occur (39). Some Acts have other relevant requirements for the traceability of samples including for them to be botanically identified by a Herbarium, sampling and storage requirements, the requirement to keep records of the samples and the need for reporting on disposal of plant material.

The federal government has other laws relevant to collecting samples in conjunction with Indigenous communities including the *Native Title Act 1993* (64) and the *Aboriginal Land Rights (Northern Territory) Act 1976* (65) and some states have other relevant Acts too, not included in **Figure 1**. The states add information and requirements in relation to the collection of plant samples on land under Indigenous rights or native title,

FIGURE 2 | A word cloud summary of the principles and core values of the ethical guidelines relevant to the study of native Australian plants. The guidelines used were *AIATSIS Code of Ethics for Aboriginal and Torres Strait Islander Research* (67), the *National Statement on Ethical Conduct in Human Research* section 4.7 (28), *Ethical conduct in research with Aboriginal and Torres Strait Islander Peoples and communities: Guidelines for researchers and stakeholders* (70), *Keeping Research on Track II* (69), the *AH&MRC Guidelines for Research into Aboriginal Health: Key Principles* (71) and *Ten principles relevant to health research among Indigenous Australian populations* (72). The more frequently a key word is used in the respective text, the larger in size it is presented. This word cloud was made using WordClouds.com (Zygomatic).

for example, in NSW the *National Parks and Wildlife Act 1974 No 80* (49) recognizes the cultural significance of certain lands to Aboriginal people (s71D) and details the requirements around Aboriginal land, objects and places.

GUIDELINES TO FOLLOW: ETHICS GUIDELINES

Responsible research involves working in an ethical way with all stakeholders on a project. Ethics approval is mandatory when doing research with human subjects, but the need to obtain approval from an ethics committee for research involving native Australian plant foods may not be as apparent to this research area. The ethics guidelines contribute ways of acting responsibly and upholding Indigenous rights when working and engaging with Indigenous communities. They give contextually appropriate ways of acting and thinking in an ethical manner, to help researchers understand their desire, motivation and the cultural narrative, and help them to act with moral judgement as a way to impede good intentions from having bad effects (66).

When working with Indigenous community members researchers are required to comply with the *AIATSIS Code of Ethics for Aboriginal and Torres Strait Islander Research* (67). One of the functions of the Australian Institute of Aboriginal and Torres Strait Islander Studies (AIATSIS) is to provide ethical guidance for research under the *Australian Institute of Aboriginal and Torres Strait Islander Studies Act 1989* (68). The guidelines for ethical research have the principles of Indigenous self-determination, Indigenous leadership, impact and value and of sustainability and accountability. The National Health and Medical Research Council (NHMRC) *Keeping Research on Track II* (69) contains useful and practical ways and guidance for researchers in having Indigenous communities involved in research. Other relevant ethical guidelines are given in **Figure 1** and also provide useful information about ethical research in this area. The word cloud in **Figure 2** acts as a summary of the ethical guidelines and shows the main themes and terms which appear most frequently in the guidelines and was made using a method slightly modified from Maramba et al. (73).

The two most obvious themes in the ethics guidelines, as shown in **Figure 2**, are respect and cultural. Respect in the NHMRC guidelines relates to respect for Indigenous people and their knowledge (28, 70). Jamieson et al. (72) refers to respect as a mutually respectful partnership framework which comes from an open and transparent relationship. Culture is mentioned in a number of contexts including cultural continuity (69, 70), cultural distinctiveness (28), cultural competency (70), cultural expression (74) and cultural sensitivity (71).

There are general guidelines for ethical and responsible conduct of research which must also be complied with when conducting native plant food research including the *Australian Code for the Responsible Conduct of Research* (28), the *Statement on Consumer and Community Involvement in Health and Medical Research* (75) and, in Queensland, the *Queensland Biotechnology Code of Ethics* (76).

DISCUSSION

The framework above shows the complexity involved in scientifically studying Australian native plant foods. One challenge within the framework is for food chemists

to understand if their planned work will be classed as biodiscovery. The definitions of "biodiscovery" and the related "bioprospecting" are different between the Acts, and definitions are not written with scientific detail. Overall, there is a lack of clarity within these laws about what it means to study chemical composition which could leave them open to multiple interpretations in relation to food chemistry analysis and potential commercial use. Providing guidelines and assistance to scientists in this field of research is essential if a consistent approach is desired. The definitions should clearly state if they apply only to novel or previously undiscovered compounds, or if they apply only to new commercial uses. Current definitions that state they apply to research relating to "biochemical compounds" do not acknowledge that all plant material is made up of chemical compounds including many important but common nutrition and safety compounds. Future policy development to make these definitions clear and interpretable by scientists from various relevant disciplines is highly recommended to facilitate equitable research compliance in this area.

Australian state and federal governments each endorsed a "Nationally consistent approach for access to and the utilization of Australia's native genetic and biochemical resources" on the 11th Oct, 2002 (77), agreeing there should be one approach across all of Australia. However, there are inconsistencies in the land they relate to, the definitions of biodiscovery, the access requirements, and the timing and need for benefit sharing agreements. A nationally consistent approach would help reduce confusion and prevent intentional and unintentional biopiracy from occurring (43).

The need for a benefit-sharing agreement is based on Article 15 of the CBD (39). As part of the Nationally Consistent Approach signed by Australian states it was intended that model contracts and dictionaries of contractual terms be developed for the benefit-sharing agreements so biodiscovery could be facilitated with a certainty of the process (77). The Australian Government has stated that the CBD objective of fair and equitable benefit sharing has been the most difficult to implement (40). Other challenges for food scientists in relation to benefit sharing agreements are understanding and agreeing on what fair and equitable may mean for different parties; working with multiple access providers; what to do when native plants grow over multiple locations; valuing non-monetary and intangible benefits; knowing how to provide reasonable support when handing a remote Indigenous community a legal document; and knowing what reasonable benefit sharing is in regards to Indigenous people's knowledge.

Food chemists collecting samples need to obtain the permits and authorizations required for the specific place where they intend collecting samples and need to understand the requirements for each place they collect from as this varies throughout Australia. This brief perspective shows some of the complexity involved in planning to study native Australian plant foods, before they can even be collected or scientifically studied. Responsible research for food chemists studying native Australian plant foods involves upholding the rights of Indigenous Australians, working with communities when the foods grow on their land, complying with international treaties, following laws and obtaining permits and benefit sharing agreements where required, and working ethically toward and with all stakeholders.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SF, HES, and YS conceptualized the paper. SF researched and wrote the paper. HES, HJS, MR, and YS supervised the project. SF, HES, HJS, MR, and YS edited the paper. All authors contributed to the article and approved the submitted version.

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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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In vitro Bioaccessibility and Intestinal Absorption of Selected Bioactive Compounds in *Terminalia ferdinandiana*

Saleha Akter[1], *Rama Addepalli*[2], *Michael Netzel*[3], *Ujang Tinggi*[4], *Mary Fletcher*[3], *Yasmina Sultanbawa*[1] *and Simone Osborne*[2]*

[1] ARC Industrial Transformation Training Centre for Uniquely Australian Foods, Center for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Indooroopilly, QLD, Australia, [2] Commonwealth Scientific and Industrial Research Organization, Agriculture and Food, St Lucia, QLD, Australia, [3] Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Health and Food Sciences Precinct, Coopers Plains, QLD, Australia, [4] Queensland Health Forensic and Scientific Services, Coopers Plains, QLD, Australia

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***Correspondence:**
Simone Osborne
simone.osborne@csiro.au

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Terminalia ferdinandiana (or Kakadu plum), a native Australian fruit with potential health benefits, contains bioactive compounds such as ellagic acid (EA), ascorbic acid (AA) and calcium, and antinutrients such as oxalic acid (OA). However, few is known about the biological fate of these compounds following ingestion; therefore, the aim of this study was to evaluate *in vitro* bioaccessibility and intestinal absorption of *T. ferdinandiana* compounds using the INFOGEST static digestion model and Caco-2-HT29-MTX-E12 intestinal absorption model. No significant changes ($p > 0.05$) were observed in total AA content throughout *in vitro* digestion, whereas bioaccessibility of EA, OA, and calcium increased significantly from 33, 72, and 67% in the gastric phase to 48, 98, and 90% in the intestinal phase, respectively. The intestinal absorption study revealed variable rates of movement across the cell barrier. Findings reveal novel and important insights for the prediction of *in vivo* bioavailability of selected *T. ferdinandiana* compounds.

Keywords: ***Terminalia ferdinandiana*, Kakadu plum, ascorbic acid, calcium, ellagic acid, oxalate, bioaccessibility**

INTRODUCTION

Fruits are good sources of a range of biologically active (bioactive) compounds including fiber, phenolic compounds, vitamins, and minerals. Fruit phenolic compounds and vitamins possess strong antioxidant capacities (1) and may reduce the risk of some types of cancer, and also cardiovascular, neurodegenerative, and inflammatory diseases (2). Minerals found in fruit, such as calcium, help facilitate muscle contractions and blood clotting and contribute to strong bones and teeth. However, the bioactivity of polyphenols, vitamins, and minerals is limited by bioaccessibility that determines what proportion of these compounds are released from digested food and made available for intestinal absorption and circulation to different tissues (3). Interactions among food matrices and polyphenols, vitamins, and minerals during digestion have been extensively studied in the past decade (4) revealing that food matrix structure and composition impact bioaccessibility, digestibility, and antioxidant capacity of those compounds (3).

From a nutritional perspective, bioaccessibility is defined as the maximum fraction of a food compound that is released from the food matrix in the gastrointestinal tract that becomes available for intestinal absorption (5–7). Bioavailability is defined as the fraction of an ingested compound available for utilization in normal physiological functions (5, 6) and is a critical feature in assessing the role of dietary components in human health (8). Bioactivity refers to systemic circulation of the compound, delivery to a target tissue, and interaction with biomolecules in the tissue followed by a cascade of physiological effects (5). Thus, bioaccessibility is the first step toward achieving bioactivity from a specific compound; therefore, it is important to ascertain the nutritional quality of a nutrient or bioactive compound, not only in terms of quantities required to achieve nutritional requirements, but also to refine the development of functional foods (5).

In vitro assays predict intestinal absorption of food components and involve *in vitro* digestion and measurements of soluble bioactive compounds across a membrane barrier. Some of these *in vitro* assays incorporate mammalian cell lines, such as the Caco-2 human intestinal epithelial cell line, and are commonly employed as alternatives to animal studies (9). The Caco-2 intestinal cell model is the most widely used and validated intestinal epithelial cell model, and although the cell line is colonic in origin, Caco-2 cells undergo spontaneous differentiation in cell culture to form a monolayer of polarized cells that display many of the functional and morphological properties of mature human enterocytes (5). The human HT29-MTX-E12 cell line is also widely used in mimicking human intestinal processes. The HT29-MTX-E12 cell lines are the human HT29 colorectal adenocarcinoma cells differentiated into mature goblet cells using methotrexate. Clones were then selected (E12) from these differentiated cells which possess the characteristics of human goblet cells. The characteristics of the cells include tight junction formation, development of confluent monolayers, and production of mucous layer (10).

Terminalia ferdinandiana, or Kakadu plum, is a native Australian fruit which is well known for its high levels of vitamin C [or ascorbic acid (AA)]. *T. ferdinandiana* also contains ellagic acid (EA) and calcium (Ca^{+2}) and antinutrients such as oxalic acid (OA) (11). Increased uptake of OA has been reported to increase the development of kidney stones in susceptible people. Furthermore, oxalate is produced during mammalian metabolic processes, such as the breakdown of dehydroascorbic acid (i.e., oxidized AA), causing increased accumulation of OA (12). Soluble oxalates can bind with Ca^{2+} and form insoluble salts that limit bioavailability of Ca^{2+} and reduce free intracellular Ca^{2+} potentially leading to Ca^{2+} deficiency (12).

Very few is known about the fate of bioactive compounds from *T. ferdinandiana* during digestion. Subsequently, it is unknown whether EA, AA, and Ca^{2+}, and antinutrients such as OA, are released from the *T. ferdinandiana* fruit matrix under physiological conditions *in vivo,* or whether digestion impacts stability of these compounds. When considering digestive stability of one component, it is important to consider not only the chemical structure of the component, but also the nature of its bond to the food matrix (13). Therefore, static and/or dynamic *in vitro* digestion models that mimic the human gastrointestinal digestion process are a common approach to determine matrix release (bioaccessibility) and stability of polyphenols, vitamins, minerals, nutrients, and nonnutrients in foods as an initial measure to predict potential bioavailability (13).

To the best of our knowledge, there are no previous studies evaluating the *in vitro* bioaccessibility and intestinal absorption of bioactive compounds such as EA, AA, and Ca^{2+}, and antinutrients such as OA, from *T. ferdinandiana*. Therefore, to enhance existing knowledge of the bioaccessibility and absorption of EA, AA, OA, and Ca^{2+} from commonly consumed *T. ferdinandiana* foods, an *in vitro* digestion model (INFOGEST) and Caco-2-HT29-MTX-E12 coculture cell absorption model were applied.

MATERIALS AND METHODS

Chemicals

Pepsin (porcine gastric mucosa) and bovine bile were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia), pancreatin from porcine pancreas was obtained from MP Biochemicals (Irvine, CA, USA), sodium chloride, potassium chloride, sodium bicarbonate, and calcium chloride were obtained from Merck (Bayswater, VIC, Australia), and total bile acids (TBAs) assay kit (colormetric) was purchased from Sapphire Bioscience Pty Ltd. (Redfern, NSW, Australia). Nunc cell culture flasks and 96-well plates were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Dulbecco's modified eagle medium (DMEM), Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS), Hank's balanced salt solution (HBSS), penicillin and streptomycin, heat-inactivated fetal bovine serum (FBS), glutamax, trypsin-EDTA, nonessential amino acids (NEAA), and trypan blue exclusion dye were purchased from Invitrogen (Thermo Fisher Scientific Corporation, Carlsbad, CA, USA). F96 MicroWell™ Black polystyrene plates were purchased from Thermo Scientific (Waltham, MA, USA). CyQUANT® NF assay reagent was purchased from Invitrogen (Molecular Probes, Thermo Fisher Scientific Corporation, Carlsbad, CA, USA). The Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The HT29-MTX-E12 cell line was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Costar Transwell® 24-well polystyrene plates were purchased from Corning Incorporated (Kennebunk, ME, USA).

T. ferdinandiana Samples

Freeze-dried *T. ferdinandiana* powder (0.5 g) was mixed with 20 mL milli-Q water, vortexed, and used as the initial starting material for *in vitro* gastrointestinal digestion based on the INFOGEST model detailed in Section INFOGEST Gastrointestinal *in vitro* Digestion (14). Digestions were performed in duplicate with samples collected at various time points throughout the digestion process. Gastric digesta samples were collected at $t = 0$ (G0) and $t = 60$ (G60) min into the gastric phase, and intestinal digesta were collected at $t = 30$ (I30), $t = 60$ (I60), and $t = 120$ (I120) min into the intestinal digestion phase. The content of AA, EA, OA, and calcium in the freeze-dried *T. ferdinandiana* powder (starting material) was 21.1 g/100 g dry

weight (DW), 2.8 /100 g DW, 1.4 g/100 g DW, and 295 mg/100 g DW, respectively (15, 16).

INFOGEST Gastrointestinal *in vitro* Digestion

A harmonized *in vitro* gastrointestinal digestion method developed in the COST action INFOGEST network was applied (14). Oral phase was not included in this experiment as *T. ferdinandiana* fruit is currently marketed as freeze-dried powder that would not undergo a substantial oral phase or may be incorporated into a nutraceutical product in pill, capsule, or beverage form. The digestive fluids (simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF)) were prepared according to the INFOGEST method (14). The activity of all enzymes was also measured according to the protocols described in the INFOGEST method (14) to ensure standardized application. Pepsin was used at a concentration of 268 units/ mL. Porcine pancreatin was used at 16 units/mL trypsin activity. Bovine bile equivalent to 1.38 mM was also added. **Figure 1** provides an overview of the digestion protocols and conditions employed in the study. At the end of each digestion steps, samples were collected and snap-frozen (−80°C) to stop the digestion. Pefabloc at a concentration of 0.1 M was recommended to stop intestinal digestion; however, considering the cytotoxic nature of Pefabloc at a concentration $\geq$ 0.25 mM, Pefabloc was not applied to avoid future cell toxicity.

Cell Culture

Caco-2 and HT29-MTX-E12 cells were grown in DMEM supplemented with 10% FBS (v/v), 1 X NEAA, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamax in vented culture flasks at 37°C and 5% CO_2. Cells were passaged every 2–3 days upon reaching 90% cell confluency and maintained within passage 10–25 for both cell types.

Cytotoxicity of Digesta Samples

In vitro cell viability assays were employed to determine the dilution of digesta required to produce a noncytotoxic response throughout the *in vitro* absorption assays. Cell viability assays, cell plating, and media and buffer changes were performed on an epMotion® 5075t liquid handling system (Eppendorf, Hamburg, Germany). Caco-2-HT29-MTX-E12 cocultures were prepared by mixing cells at a ratio of 9:1. Caco-2 cells (9,000) and HT29-MTX-E12 (1,000) cells were added in 100 μL media to each well in a Nunc™ F96 MicroWell™ Black polystyrene plate and incubated for 7 days at 37°C and 5% CO_2. On the day of experiment, culture media was removed and replaced with 100 μL HBSS and incubated for 2 h. After 2 h, HBSS was removed and replaced with 50 μL intestinal digesta samples (I30, I60, and I120) diluted 2:1, 5:1, and 10:1 with HBSS, or HBSS for the control wells. Digesta samples were centrifuged with supernatants applied to the cells. The plates were incubated for 2 h at 37°C and 5% CO_2. After 2 h, test compounds were removed and wells were washed with 100 μL HBSS. The HBSS was removed before 74 μL 1X CyQUANT® NF dye binding solution was added to each well using a manual multichannel pipette. The plate was covered and incubated at 37°C for 60 mi. Fluorescence was measured with excitation at 485 nm and emission detection at 530 nm using a Spectramax M3 multimode microplate reader (Molecular Devices, San Jose, CA, USA).

Caco-2-HT29-MTX-E12 Coculture

Caco-2 and HT-29-MTX-E12 cells were seeded on Costar Transwell® plate inserts in a ratio of 9:1 and at a density of 1,09,000 cells/cm^2 and 12,120 cells/cm^2, respectively. Cells were cultured in growth media at 37°C and 5% CO_2 for 21 days to facilitate cell differentiation and formation of an intact monolayer. During the 21-day differentiation period, growth media was removed and replaced with fresh media every 2–3 days. Cell differentiation of an intact monolayer was monitored by measuring the transepithelial electrical resistance (TEER) of the cell monolayer in an apical–basolateral direction. TEER was measured using the Millicell-ERS Voltohmmeter from Millipore (Burlington, MA, USA). TEER is a measurement of resistance and is a sensitive and convenient method to assess and the integrity of the cell monolayer. The values obtained are determined by cellular resistance (between apical and basolateral membrane) and the paracellular resistance (tight junctions). TEER values above 300 Ω.cm^2 are indicative of differentiated Caco-2-HT29-MTX-E12 cells and an intact monolayer (17).

Intestinal Absorption Assay

In vitro absorption was performed by following the procedure described by Osborne and colleagues (18). On the day of the intestinal absorption assay, TEER was measured and growth media was replaced with HBSS in the apical and basolateral chambers for 2 h to enhance uptake of the applied digesta. For the absorption study, intestinal digesta samples (I30, I60, and I120) were diluted 10:1 with HBSS. This dilution was selected based on the results from the cytotoxicity assay that showed no change to cell viability in response to digesta samples diluted 10:1. In the apical chamber, 200 μL diluted intestinal digesta (I30, I60, and I120) were applied for 120 min. HBSS (600 μL) was added in the basolateral chamber. After 2 h, all apical and basolateral samples were collected and stored at−80°C. HBSS was replaced with growth media in all chambers with TEER values measured again immediately after the assay. TEER was also recorded 24 and 48 h after the assay. Intestinal absorption studies were performed in duplicate. The rate of EA, AA, OA, and Ca^{2+} movement across the transwell® membrane was determined by calculating the apparent permeability coefficient (*P*app) in cm/s as follows (19):

$$p_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0} \quad (1)$$

$\Delta Q/\Delta t$ is steady-state appearance rate of compound in receiver (basolateral) compartment (μmol/s).

A is the surface area of the filter (i.e., 0.33 cm^2).

C_0 is the initial concentration in the donor (apical) compartment (μM).

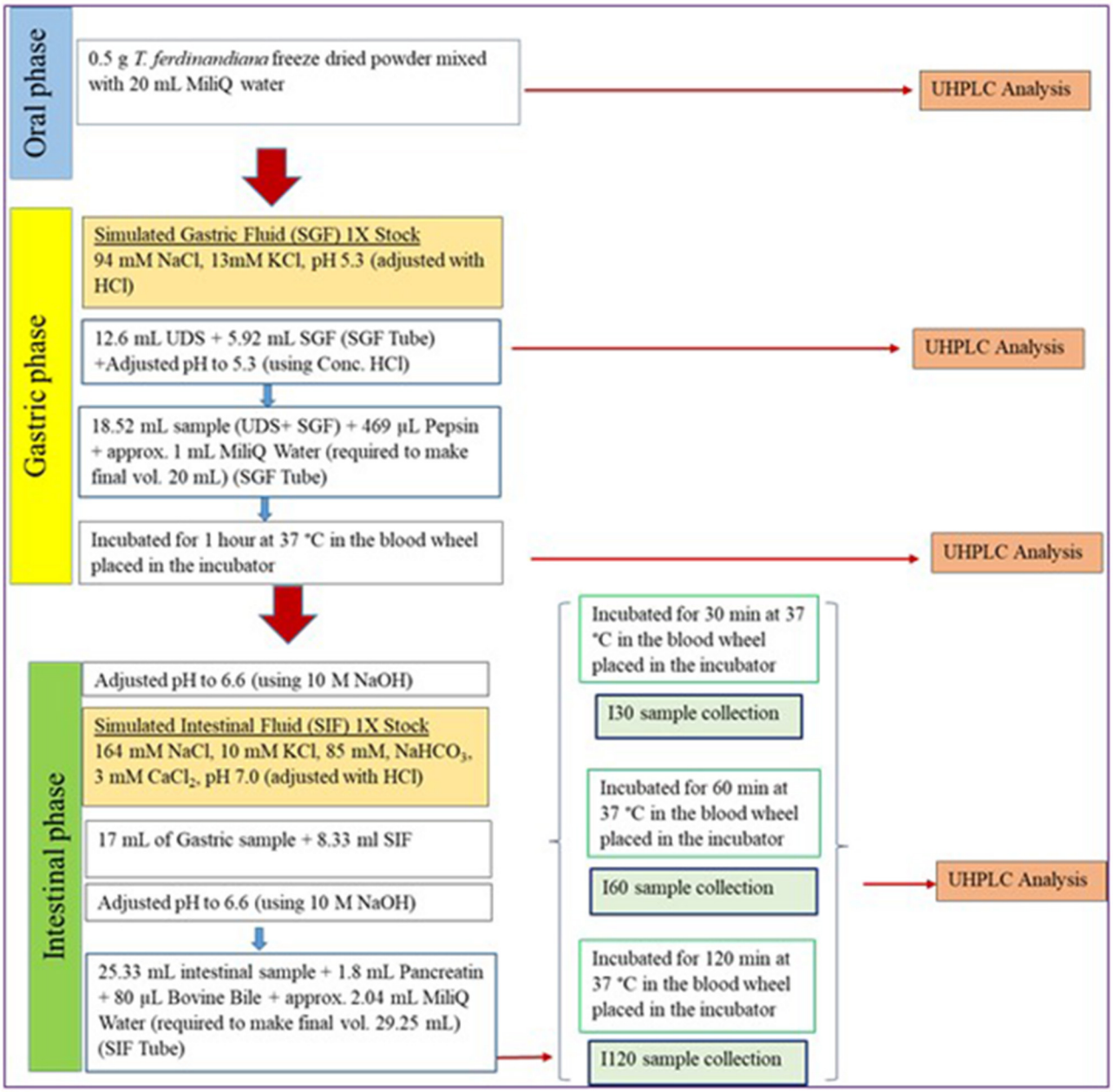

FIGURE 1 | Schematic flow chart of the applied *in vitro* digestion protocol (14). SGF, simulated gastric fluid; UDS, undigested samples; SIF, simulated intestinal fluid.

Preparation and Analysis of Digesta Samples in UHPLC

Ascorbic Acid

T. ferdinandiana digesta and intestinal absorption samples were analyzed for AA. The samples were centrifuged and filtered through 0.22 µm syringe filters prior to analysis. An Acquity UHPLC system (Waters Corp., Milford, MA, USA), equipped with a Waters Acquity UHPLC photodiode array (PDA) detection system, was used to carry out the analysis. Empower™ software (Waters Corp., USA) was used to process and quantify peaks after recording the signals. A Waters Acquity HSS T3 analytical column (100 mm × 2.1 mm, 1.8 µm particle size) (Waters Corp., USA), with an isocratic mobile phase of 0.1% aqueous (v/v) formic acid at a flow rate of 250 µL/min, was used with an injection volume of 2 µL. The absorbance was measured at room temperature at 245 nm.

Ellagic Acid

T. ferdinandiana digesta and intestinal absorption samples were analyzed for EA. All samples were centrifuged and filtered through 0.22 μm PTFE membrane filters (Millipore, CA, USA) before injection into the chromatographic system. Samples were analyzed on a Waters Acquity UHPLC system (Waters Corp., Milford, MA, USA) equipped with PDA detector. A Waters BEH Shield RP18 Column 130 °A, 1.7 μm (100 mm X 2.1 mm) from Waters Corporation (Milford, Massachusetts, USA) was used. Column temperature was maintained at 40°C, the injection volume was 2 μL, PDA spectrum was scanned from 200 to 400 nm with a rate of 2.5 Hz, and detection was carried out at 254 nm. A combination of mobile phase A and phase B was used during 23 min of elution. Formic acid 0.1% was used for mobile phase A (Milli-Q water) and phase B (acetonitrile) at a flow rate of 0.4 mL/min. The gradient program started at 90% mobile phase A for 1 min, followed by a linear gradient to 85% for 4 min, to 65% A at 7 min, then 50% in the next 7 min, then 10% for 4 min, hold at this composition for 1 min, and then 90% at 20 min before reequilibration for 3 min.

Oxalic Acid

T. ferdinandiana digesta and intestinal absorption samples were cleaned up with a SPE step (20) for OA analysis. Mixed reversed phase-weak anion exchange sorbent Strata® SAX (55 μm, 70 Å, 500 mg/3 mL) was purchased from Phenomenex (Torrance, CA, USA) and used in this study. The SPE process was maintained through an Analytichem VAC ELUT SPS 24 Port Vacuum Manifold from Agilent Varian (Santa Clara, CA, USA). The SPE columns were sequentially conditioned with 3 mL of methanol and 3 mL of milli-Q water. Digesta and intestinal absorption samples at a volume of 1 mL dripped through the cartridges by gravity (with a flow rate of about 1 mL/min). The cartridges were washed with 3 mL of water and 3 mL of methanol. The columns were dried under maximum vacuum for 5 min prior to elution. Water with 1% HCl/H_2O with 5% NH_4OH (2 mL) was used as eluent (20). All extractions were performed in triplicate. Afterward, samples were filtered through a 0.2 μm syringe filter and injected in the chromatograph. A Phenomenex Synergi 2.5 μm Hydro-RP 100 Å (100 mm X 3.00 mm) column and isocratic conditions were used to elute OA. The mobile phase consisted of 200 mM phosphate buffer (pH 1.5) with a flow rate 0.1 mL/ min. Run time was 10 min. PDA spectrum was scanned from 190 to 400 nm with a rate of 2.5 Hz, and detection was carried out at 210 nm. Column temperature was maintained at 25°C, and injection volume was 2 μL.

Calcium

Levels of Ca^{2+} were analyzed by ICP-OES (Agilent 700, Australia) after hot block digestion (A.I. Scientific, Australia). Briefly, 0.5–2.0 g samples were accurately weighed into digestion tubes before 4 mL of high-purity nitric acid (69% v/v, Seastar Chemicals, Canada) was added. The digested samples were made up to 20 mL with high-purity water (Aqua Cure, England). Appropriate reference materials were used for quality control.

Statistical Analysis

Data are presented as the mean percentage of six replicates ± standard error of the mean (SEM) for each treatment. Cytotoxicity is expressed as the percentage of viable cells remaining after extract treatment compared to the HBSS control. The *in vitro* gastrointestinal digestion of *T. ferdinandiana* was carried out two times for each compound in triplicate with results expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was performed to determine significant differences ($p \leq 0.05$) between the concentration of bioactive compounds in nondigested and digested *T. ferdinandiana* samples. Significant differences between two groups were determined using nonparametric unpaired *t*-test. One-way ANOVA with Tukey's multiple comparison was used when comparing groups. A significance level of $p \leq 0.05$ was used for all tests. All statistical tests were conducted using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

In vitro Digestion and Bioaccessibility

Bioaccessibility was calculated by expressing compounds released during digestion as a percentage of the initial and total concentration of the compound in the plant material before digestion. Statistical significance was determined throughout gastrointestinal digestion by comparing bioaccessibility of the compounds during the gastric and intestinal digestion phases with bioaccessibility measured from the initial gastric $t = 0$ (G0) digesta sample. No significant differences were observed in bioaccessibility of AA throughout the *in vitro* digestion; however, OA and Ca^{+2} showed significant ($p \leq 0.05$) increases in bioaccessibility throughout the intestinal phase, with respect to the G0 digestion phase, whereas EA significantly increased throughout all digestion phases (**Figure 2**).

Ascorbic Acid

Previous studies by our research group report the amount of AA in *T. ferdinandiana* fruits to be 21.1 /100 g DW (15). Results from this study indicate no significant changes ($p > 0.05$) in total AA content throughout *in vitro* digestion, suggesting that AA was not degraded during this process. AA is a water-soluble antioxidant and this property may account for the almost complete release of AA observed in this study from the solid to liquid phase. This is evident from the calculated bioaccessibility of 93% at the beginning of the gastric phase (G0) (**Table 1**). Approximately 90% of AA was then released from the solid phase into the liquid phase following digestion with pepsin for 60 min (G60). Digestion with pancreatin and bile in the small intestinal phase resulted in 93%, 100%, and 79% bioaccessibility after 30, 60, and 120 min, respectively (**Figure 2A**). Bioaccessibility of AA decreased toward the end of the intestinal phase; however, an unpaired *t*-test between AA bioaccessibility at I60 and I120 did not reveal any significant differences. Our present findings are in agreement with previous findings that reported minimal impact on AA stability during *in vitro* gastric conditions (at pH 2 or 3) (21). Rodríguez-Roque et al. (2) have also demonstrated that gastric digestion had few effect on AA stability by recovering 83%

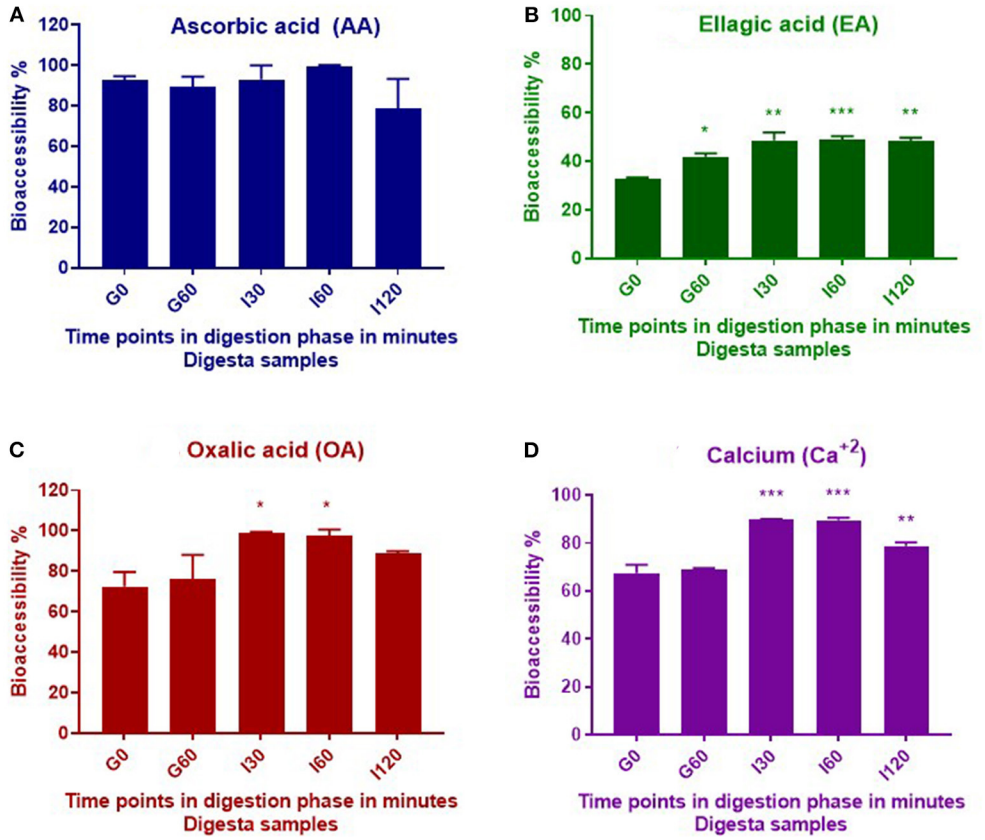

FIGURE 2 | Bioaccessibility of AA **(A)**, EA **(B)**, OA **(C)**, and calcium **(D)** during *in vitro* digestion of *T. ferdinandiana* freeze-dried powder. Asterisks (*) indicate differences that are statistically significant between gastric $t = 0$ (G0) digesta and digesta from other stages of digestion (*$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$). G0, gastric 0 min; G60, gastric 60 min; I30, intestinal 30 min; I60, intestinal 60 min; and I120, intestinal 120 min.

TABLE 1 | Bioaccessibility of AA, EA, OA, and calcium (Ca^{2+}) released from the *T. ferdinandiana* fruit matrix.

	AA (%)	EA (%)	OA (%)	Ca^{2+} (%)
Gastric $t = 0$	93 ± 2^a	33 ± 1^b	72 ± 7^c	67 ± 4^c
Gastric $t = 60$	90 ± 5^a	42 ± 1^b	85 ± 2^a	69 ± 1^a
Intestinal $t = 30$	93 ± 7^a	49 ± 3^b	$99^a \pm 0.0$	$90^a \pm 0.0$
Intestinal $t = 60$	100 ± 1^a	49 ± 1^b	98 ± 3^a	90 ± 1^c
Intestinal $t = 120$	79 ± 5^a	48 ± 1^b	89 ± 1^a	79 ± 2^a

Data are expressed as the mean ± SD (n = 2). Bioaccessibility (%) = bioaccessible content/total content x 100. Statistical analysis consisted of comparing the bioaccessibility of AA, EA, OA, and calcium (Ca^{2+}) of T. ferdinandiana digesta within each digestion phase using one-way ANOVA. Different letters within rows indicate significant differences ($p \leq 0.05$) between the analyzed compounds within each digestion phase.

of this bioactive compound in a blended fruit juice containing orange, pineapple, and kiwi.

Ellagic Acid

Polyphenols occur in foods mainly as esters, glycosides, and polymers that cannot be absorbed intact but require hydrolysis by digestive enzymes, or intestinal microbiota, to degrade or metabolize these compounds into a form that can be absorbed across the intestinal barrier. It is estimated that 48% of polyphenols are "digested" in the small intestine, 42% in the large intestine, and 10% remain undigested and intact within the food matrix (22).

In this study, significant increases ($p \leq 0.05$) were observed in the bioaccessibility of EA across the different digestive phases compared to early gastric phase (**Figure 2B**). The content of total EA in *T. ferdinandiana* fruit (starting material) was 2.8 /100 g DW (16). Bioaccessibility of EA significantly increased from 33% at the beginning of the gastric phase to 48% at the end of the intestinal phase (I120). The increase in EA bioaccessibility may be due to the release of EA from ellagitannins and other EA derivatives produced during digestion. Studies involving other fruits, such as pomegranate and raspberries, suggest that during *in vitro* gastrointestinal digestion of blended fruit juices, EA is liberated from ellagitannins (2). It is also possible that bile salts and pancreatin present in the intestinal phase helped release EA from the food matrix by contributing to the breakdown of complex ellagitannins and other EA derivatives to produce free EA. Similarly, the potential role of bile salts and pancreatin has been highlighted previously in the release and bioaccessibility of carotenes from processed carrots (23).

Oxalic Acid

Figure 2 shows OA bioaccessibility from *T. ferdinandiana* fruit powder. Total OA content in *T. ferdinandiana* fruits was previously reported to be 1.4/100 g DW (15). Bioaccessibility of OA was estimated to be 72% at the beginning of the gastric digestion phase (G0) (**Table 1**) before significantly increasing to 98% in the intestinal phase (I30). At the end of the intestinal phase (I120), OA bioaccessibility decreased to 89% but was not significantly different to OA released in the early gastric phase (G60) (**Figure 2C**). An unpaired *t*-test was also performed between OA bioaccessibility at I60 and I120; however, no significant differences were observed.

Calcium

The Ca^{2+} content in *T. ferdinandiana* fruit was previously reported to be 295 mg/100 g DW (15). The release of Ca^{2+} at the beginning of the gastric phase was 67% and remained steady throughout the gastric phase as with no significant difference observed between G0 and G60 (**Figure 2D**). Bioaccessibility of Ca^{2+} significantly increased to 90% after 30 (I30) and 60 (I60) min in the intestinal phase before significantly decreasing to 79% at the end of the intestinal phase (I120) (**Table 1**) ($p \leq 0.05$). The decrease in the bioaccessibility of Ca^{2+} at the end of the intestinal phase can be correlated with the fact that calcium has a tendency to bind with fatty acids in the lumen forming insoluble soaps (24, 25). In the *in vitro* settings of this study, Ca^{2+} might bind with the bile components or bioactive compounds released from the matrix of *T. ferdinandiana* fruits and formed insoluble complexes and reduced the availability of free Ca^{2+}.

The recommended dietary intake (RDI) of Ca^{2+} for adults is 840 mg/day in Australia (26). The Ca^{2+} content is high in *T. ferdinandiana* fruits (295 mg/100 g DW) (15) compared to some other Australian native fruits, such as lemon aspen (133.3 mg/100 g DW), Davidson's plum (217.3 mg/100 g DW), and Quandong (133.3 mg/100 g DW) (27), qualifying *T. ferdinandiana* as a good source of Ca^{2+}. Bioavailability of Ca^{2+} from matrix-bound Ca^{2+} salts is reportedly influenced by gastric acid secretion and simultaneous ingestion of other foods (28). Cilla et al. (5) have suggested that mineral bioaccessibility can be enhanced by applying thermal treatments to foods to soften the food matrix and increase the release of protein bound minerals. Part of the *T. ferdinandiana* fruits pureeing process involves steaming and may have loosened the food matrix in this study helping to release Ca^{2+} throughout the gastric and intestinal phases of *in vitro* digestion.

In vitro Cytotoxicity of Digesta Samples

Cytotoxicity in response to digesta samples was assessed *in vitro* with the CyQUANT® NF cell viability assay using Caco-2-HT29-MTX-E12 cocultures. **Figure 3** represents the impact of different dilutions (2:1, 5:1, and 10:1) of intestinal digesta samples (I30, I60, and I120) on the viability of Caco-2-HT29-MTX-E12 cocultures. Average cell viability in response to the digesta ranged from 67 to 79% for the 2:1, 84 to 89% for 5:1, and 94 to 96% for 10:1 dilution with no significant impact on cell viability observed in response to any of the diluted digesta. However, cell viability decreased when the digesta samples were more concentrated

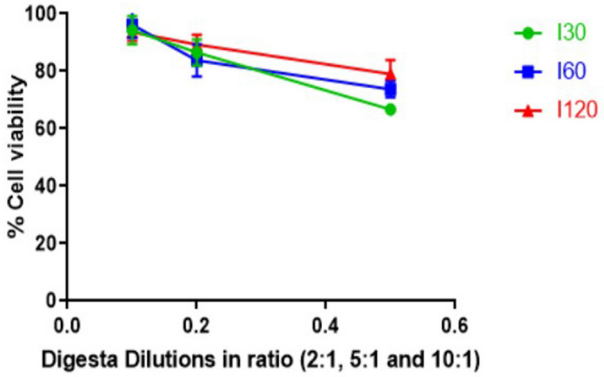

FIGURE 3 | Effect of digesta prepared from *T. ferdinandiana* on Caco-2-HT29-MTX-E12 coculture cell viability *in vitro* using CyQUANT® NF cell proliferation assay. Results are expressed as mean ± SEM of two independent experiments with 4 replicates in each ($n = 8$). I30, intestinal 30 min; I60, intestinal 60 min; and I120, intestinal 120 min.

(**Figure 3**) with the 2:1 dilution having a greater impact on cell viability compared to 5:1 and 10:1 dilution. These cell viability results informed the selection of the dilution for the intestinal absorption study. Overall, the 10:1 dilution was selected for all digesta samples as cell viability was close to 100% in response to all 10:1 dilution.

Transepithelial Electrical Resistance Measurement

Transepithelial electrical resistance is reflective of cell barrier integrity; therefore, TEER values were measured before and after the intestinal absorption assay, and again posttreatment at $t =$ 24 and 48 h. All TEER values remained close to or above 300 $\Omega.cm^2$, indicating Caco-2-HT29-MTX-E12 cell viability and an intact monolayer. Maintaining a TEER value of $\geq$300 $\Omega.cm^2$ is an indication of cell monolayer integrity and ensures formation of a confluent cell monolayer with well-established tight junctions that allow the passage of the compounds by the paracellular route (17). Lower values indicate that tight junctions between cells have not developed enough or are damaged such that small molecules could pass *via* a paracellular route to the basolateral chamber (29).

For all intestinal absorption studies, TEER values ranged from 309 to 413 $\Omega.cm^2$ before application of digesta, and between 273 and 370 $\Omega.cm^2$ after treatment. Overall, the average change in TEER before and after the intestinal absorption assays ranged from 1 to 5%. Constant TEER values suggest that application of the diluted digesta did not disrupt the monolayer that is associated with intestinal absorption *via* paracellular routes (30). A small number of wells showed reduced TEER values immediately after the treatment and may have been due to lack of serum in the coculture model. It has been reported that cells grown on transwell membranes in a medium without serum display a loosening, or even loss of tight junction (31). Based on the results from the cytotoxicity assay, it is possible that the decline in initial TEER values

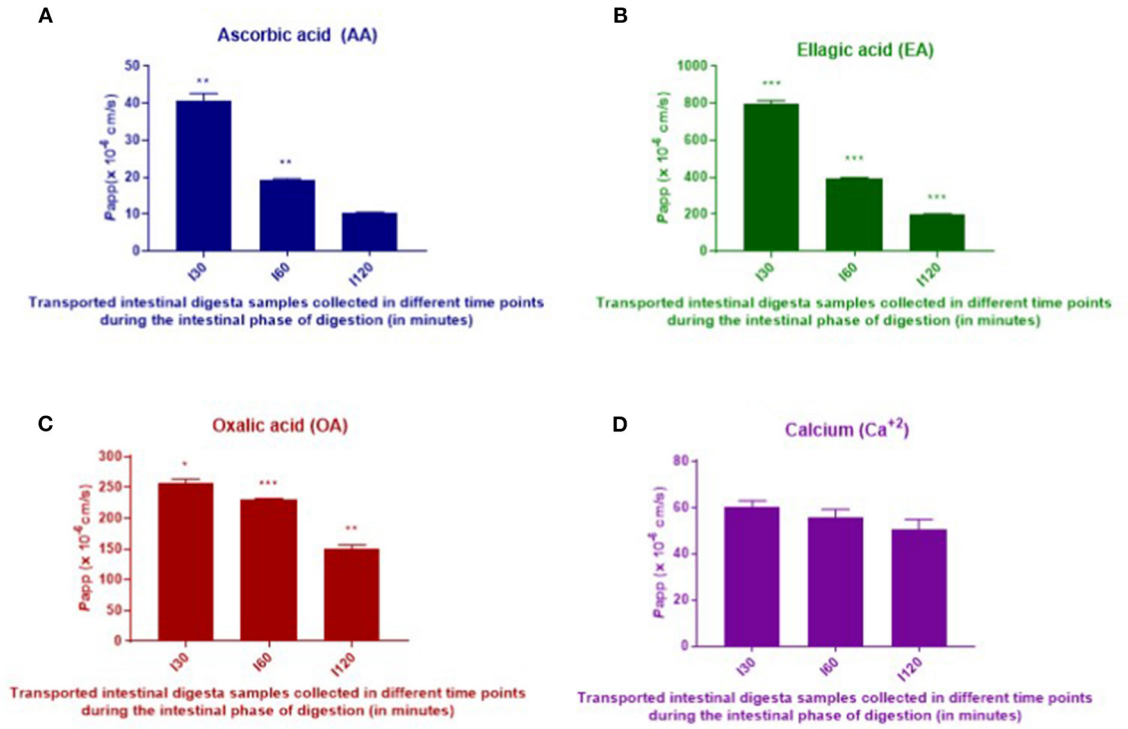

FIGURE 4 | *In vitro* intestinal absorption of AA **(A)**, EA **(B)**, OA **(C)**, and calcium **(D)** from digested *T. ferdinandiana* powder across Caco-2-HT29-MTX-E12 cell barriers. Caco-2-HT29-MTX-E12 cocultures grown on transwells were incubated with I30, I60, and I120 intestinal digesta at 10:1 dilution for 2 h. Results are expressed as the mean ± SEM of 2 determinations ($n = 2$). One-way ANOVA followed by Tukey's multiple comparison tests was performed to compare apparent permeability (*P*app) for each compound from I30, I60, and I120. Asterisks (*) indicate differences that are statistically significant among the applied digesta (I30, I60, and I120) samples within each compound (*$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$). I30, intestinal 30 min; I60, intestinal 60 min; and I120, intestinal 120 min.

TABLE 2 | Apparent permeability (*P*app) of AA, EA, OA, and calcium (Ca^{2+}) from *T. ferdinandiana* digesta.

Samples	AA	EA	OA	Ca^{2+}
	***P*app (x 10^{-6} cm/s)**	***P*app (x 10^{-6} cm/s)**	***P*app (x 10^{-6} cm/s)**	***P*app (x 10^{-6} cm/s)**
I30	41 ± 5^a	796 ± 7^a	258 ± 5^a	60 ± 6^a
I60	19 ± 0.4^b	394 ± 4^b	231 ± 1^b	56 ± 7^a
I120	10 ± 0.1^b	201 ± 2^c	150 ± 7^c	50 ± 9^a

Data are expressed as the mean ± SD ($n = 2$). Statistical analysis consisted of comparison of I30, I60, and I120 digesta samples within each compound using one-way ANOVA with Tukey's multiple comparison. Different letters within columns indicate significant differences ($p \leq 0.05$).

in this study was not due to cell death but due to a change in Caco-2-HT29-MTX-E12 permeability. TEER measurements 24 h postdigesta application showed increased values where decreases in TEERs were observed immediately after application. Consecutive measurements 48 h postdigesta application revealed steady TEER values that were similar to initial measurements further indicating that the diluted digesta samples were not toxic to the cells (31).

Caco-2-HT29-MTX-E12 Intestinal Absorption Model

Intestinal absorption of AA, EA, OA, and Ca^{2+} from digested *T. ferdinandiana* powder was measured using Caco-2-HT29-MTX-E12 coculture cell monolayers. Intestinal digesta samples that were collected at different time points (I30, I60, and I120) throughout the *in vitro* digestion were applied to the coculture transwell model for 120 min before apical and basolateral samples were collected. The amount of AA, EA, OA, and Ca^{2+} was measured in the digesta initially applied to the apical chamber of the transwell model, and in samples collected from the basolateral chamber. Apparent permeability (*P*app) was calculated with statistical significance observed ($p \leq 0.05$) in *P*app values obtained from the applied I30, I60, and I120 digesta samples (**Figure 4** and **Table 2**). Overall, *P*app of absorbed AA, EA, and OA was significantly higher from I30 digesta compared to I60 and I120 digesta. However, absorption of Ca^{2+} did not significantly change when comparing any digesta.

In vitro digestion and Caco-2-HT29-MTX-E12 cell monolayer models were utilized in this study to determine *in vitro* absorption as an indicator for bioavailability, of selected bioactive

compounds in *T. ferdinandiana* fruit powder. In general, bioavailability of bioactive compounds depends on digestive stability and efficiency of the transepithelial passage. Generally, *P*app values $< 1 \times 10^{-7}$ and $>1 \times 10^{-6}$ cm/s in an apical (AP) to basolateral (BL) direction (AP to BL) are considered low and high permeation, respectively (32). According to the traditional Caco-2 cell assay used to investigate intestinal absorption of drugs, *P*app values $> 3 \times 10^{-6}$ cm/s suggest highly permeable compounds and represent an intestinal absorbed fraction in humans of more than 60% whereas *P*app values $< 3 \times 10^{-6}$ cm/s are characteristic of low permeability compounds representing an intestinal absorbed fraction in humans of <60% (17). Another study reports that the intestinal absorption of xenobiotics is considered negligible if the *P*app is $<0.1 \times 10^{-6}$ cm/s and essentially complete if the transepithelial *P*app is $>5.0 \times 10^{-6}$ cm/s (33). The *P*app values obtained for EA were higher than those calculated for AA, OA, and Ca^{2+}. The *P*app values of EA measured in this study are higher than the *P*app values of EA ($0.347 \pm 0.018 / 10^{-6}$ cm/s) reported previously (34). An interesting finding from the study presented here is that *P*app values were higher in response to I30 samples compared to I60 and I120 samples.

From the *P*app observed in this study, it is apparent that EA, AA, OA, and Ca^{2+} demonstrated significant intestinal absorption. However, *in vitro* results cannot replace *in vivo* studies, and there are several factors that can affect intestinal absorption of bioactive compounds *in vivo*. Compound loss during gastric digestion, pH changes, solubility, interactions with other compounds and enzymes, food matrix, removal by blood stream, metabolism, and microbial breakdown of complex compounds can all affect bioavailability of the compound (32).

With respect to intestinal absorption of EA *in vitro*, *P*app of 1 mg/mL EA across a Caco-2 cell barrier (at a density of 1.2×10^5 cells/insert in a 12-well transwell plate) for 4 h in an absorptive direction (apical–basolateral) was previously reported to be 0.347×10^{-6} cm/s (34). The *P*app values obtained in this study are higher than the previous report. In the previous report, Caco-2 cell lines were used, and in this study, a coculture of Caco-2-HT29-MTX-E12 was used. The use of a coculture of Caco-2-HT29-MTX-E12 could accurately imitate the small intestine because the tight junctions of HT-29 cells are not as tight as the Caco-2 cells (35). Moreover, the generation of mucus and the TEER value in a coculture of Caco-2-HT29-MTX-E12 are more similar to that of human small intestine (35). Additionally, for compounds undergoing passive intestinal absorption, permeabilities were generally higher in cocultures than in Caco-2 monolayers (36). Lan et al. (37) reported that absorption of EA by HepG2 cells was detected just 15 min after application with peak absorption measured after 4 h. Bioactive compounds encounter several challenges throughout ingestion, digestion, and absorption across the intestinal barrier. There are numerous reports on the *in vitro* absorption behavior of different compounds with some reports based on Caco-2 monolayers, and some on a coculture system involving Caco-2 and HT29-MTX-E12 cells.

Based on the previous findings, the freeze-dried *T. ferdinandiana* powder (1 g) used in this study was predicted to contain 210 mg of AA. Based on bioaccessibility being 100% for AA at I60, it can be inferred that a recommended serve of *T. ferdinandiana* freeze-dried powder (i.e., 1 g) could provide an adequate amount of bioaccessible AA compared to the minimum RDI of 45 mg per day, but would still be within the upper intake level of 2,000 mg/day (26). However, the low apparent permeability of AA measured in this study could suggest reduced levels of AA for distribution to tissues. In this study, bioaccessibility of EA only reaches a maximum of 49% in the I30 and I60 phases; however, apparent permeability was higher compared to the other phytochemicals tested.

CONCLUSION

T. ferdinandiana is a nutritious fruit with potential health benefits that could alleviate chronic human diseases. However, no previous studies have investigated bioaccessibility and intestinal absorption of important bioactive compounds, such as EA, AA, OA, and Ca^{2+}, present in *T. ferdinandiana*. Findings from this study showed that each compound exhibited different bioaccessibilities and intestinal absorption *in vitro*. The intestinal absorption assay revealed that EA had the highest permeability *in vitro*. This study also demonstrated that even though bioaccessibility of AA was high, *in vitro* permeability was low. *In vitro* experiments do not always reflect the complex *in vivo* environment; however, *in vitro* models can be valuable screening tools for informing *in vivo* trials. The challenges that impact bioaccessibility and intestinal absorption of different *T. ferdinandiana* compounds, such as solubility, permeability, and cell accumulation, need to be addressed when formulating food products and additives using *T. ferdinandiana* fruit. The findings from this study have provided novel and important data that could be used to predict *in vivo* bioavailability of EA, AA, OA, and Ca^{2+} from *T. ferdinandiana*. This will also assist in the development of healthy and functional food products based on *T. ferdinandiana* fruit.

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

SA, MN, UT, MF, YS, and SO conceived and designed the study. SA performed the experiments, analyzed data, and wrote the manuscript. RA helped with the experiments. UT helped with the calcium analysis. MN, MF, YS, and SO critically revised and edited the manuscript. All authors approved the final manuscript.

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Free and Bound Phenolic Profiles of *Rosa roxburghii* Tratt Leaves and Their Antioxidant and Inhibitory Effects on α-Glucosidase

Yuzhe Yang[1†], Wu Li[2†], Wenyan Xian[1], Wei Huang[1,3] and Ruili Yang[1,3]*

[1] Guangdong Provincial Key Laboratory of Food Quality and Safety, College of Food Science, South China Agricultural University, Guangzhou, China, [2] School of Biotechnology and Health Sciences, Wuyi University, Jiangmen, China, [3] Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, China

Rosa roxburghii Tratt (*R. roxburghii*) tea is a traditional Chinese beverage. This study aims to investigate and compare the phenolics in free and bound forms of two cultivars of *R. roxburghii* leaves, and their bioactivities. The total phenolic content of free and bound fractions was 72.71 and 17.75 mg GAE/g DW in Gui Nong No. 5 (GNN5) and 94.28 and 11.19 mg GAE/g DW in Seedless Cili (SC). A total of 37 phenolic compounds were characterized and quantified by UPLC-Q-Exactive Orbitrap/MS with ellagic acid, quercitrin, isoquercitrin, and quininic acid in free fraction, while gallic acid, ellagic acid, and hyperoside were main compounds in bound fraction. The free fraction with higher phenolic contents also showed excellent performances on antioxidant activities and α-glucosidase inhibitory potency than bound phenolics. Therefore, the results highlight that *R. roxburghii* leaves are a promising source enriched in phenolic constituents for functional beverages and nutritional foods.

Keywords: phenolic profiles, antioxidant activity, alpha-glucosidase, UPLC-Q-Exactive Orbitrap/MS, *Rosa roxburghii* Tratt leaves

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***Correspondence:**
Ruili Yang
rlyang77@scau.edu.cn

† These authors have contributed equally to this work

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INTRODUCTION

Rosa roxburghii Tratt (*R. roxburghii*), a member of the *Rosaceae* family, is widely planted in the mountainous and central hilly areas of the southwest and central south regions of China. Currently, Gui Nong No. 5 (GNN5) and Seedless Cili (SC) are the largest two cultivars (1). According to the records of "Encyclopedia of Guizhou" and "A Supplement to the Compendium of Materia Medica," the fruits, leaves, and roots of *R. roxburghii* are widely used to treat many diseases, such as invigorating spleen and dissolving heat stroke. As a medicinal food homoeologous plant, *R. roxburghii* has recently attracted increasing attention, as it is rich in nutritional and functional constituents. The tea made from *R. roxburghii* leaves not only has a unique tea aroma, but also possesses various health benefits, including antioxidant (2), anti-inflammatory, and lowering blood sugar activities (3). It has been historically used as an edible and medicinal resource for the treatment of diabetes in southwestern China. Based on these advantages, *R. roxburghii* tea has been selected into "the national famous, special and excellent new agricultural products catalog." These healthcare benefits have been mainly ascribed to high contents of phenolics. Studies on *R. roxburghii* tea have focused on process optimization and functional activities, but there are a few reports on the polyphenol species and contents in them.

As the most abundant secondary metabolites of plants, polyphenols are often widely present in plant and plant-derived products in the daily diet, which have various biological functions, such as antioxidant, anti-inflammatory, and antidiabetic activities (4). In particular, polyphenols strongly inhibit α-glucosidase, which is located on the brush border of the small intestine villi and is a key carbohydrate-hydrolyzing enzyme, mostly the terminal α-bonds of oligosaccharides, form glucose, which is the final stage of carbohydrate digestion (5). Therefore, inhibition of α-glucosidase is an effective method for alleviating postprandial hyperglycemia without diarrhea and other intestinal disturbances caused by current drugs, such as acarbose and miglitol (6). Exploration of effective natural polyphenolic α-glucosidase inhibitors with minimal side effects is required and is considered important for the management of diabetes mellitus.

Polyphenols exist in plants in two main forms, namely, those in the free form are often found in the vacuole of plant cells, and those in the bound form are linked to other natural chemical products through ester linkages, ether linkages, and glycosidic linkages (7, 8). Currently, most studies mainly concentrate on the composition and functional activities of free phenolics than that of bound phenolics. In fact, bound phenolics is the more prevalent form of phenolics in foods. For instance, about 50–95% of phenolic contents were contributed in bound form in vegetables (9), fruits, and legume/seeds (10). The significant amounts of bound phenolics released slowly and continuously by the digestion and colonic fermentation may allow improving the bioaccessibility, bioavailability, and bioactivity of phenolics for a comparatively long time. As a result, both free and bound phenolics should be considered to fully estimate the bioactive values of foods, which is conducive to the rational development and utilization of natural chemical resources. However, there are few studies on thorough polyphenols identification based on mass spectrometry and evaluation of antioxidant and α-glucosidase enzyme inhibition activity of *R. roxburghii* leaves. Hence, the characterization of phenolic metabolites of *R. roxburghii* leaves and assessment of their potential biological activities are of great interest in the food and pharmaceutical industries.

Thus, the aim of this study was to characterize the phenolic profile (free and bound) of the largest two cultivars of *R. roxburghii* leaves using ultra-performance liquid chromatography along with Q-Exactive Orbitrap tandem mass spectrometry (UPLC-Q-Exactive Orbitrap/MS). Furthermore, the antioxidant capacities of each phenolic extract were determined by different antioxidant methods and hypoglycemic function was evaluated by the inhibition of α -glucosidase.

EXPERIMENT

Chemicals

The standard reference materials were purchased from Sigma Aldrich (Shanghai, China). The 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid ammonium salt (ABTS)], 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3',6'-dihydroxyspiro [isobenzofuran-1 (3 H),9'-(9 H) xanthene]-3-one (FL)2,2'-azobis (2-amidinopropane)dihydrochloride (AAPH), chromato-graphic-grade methanol and Folin-Ciocalteu phenol reagent (2 M), *p*-nitrophenyl-β-D-galactopyranoside (*p*-NPG), α-glucosidase were obtained from Sigma Aldrich (Shanghai, China). Other analytical grade materials were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China).

Leaf Sample

Leaves of *R. roxburghii* (GNN5 and SC) were harvested from mountainous areas in Guizhou Province (103° 36′ to 109° 35′ E and 24° 37′ to 29° 13′ N) in late December 2020 and precooled and stored at −80°C for 12 h. Using a vacuum lyophilizer (Christ, Osterode am Harz, Germany) and grinder, a fine powder was made and stored at −20°C.

Extraction of Polyphenols

Free and bound phenolics were extracted according to the method reported by Li et al. (11). For free phenolics, the frozen leaves sample (0.5 g) was mixed with 15 ml of chilled 80% methanol (1% HCl). The mixture was ultrasonicated for 20 min and then centrifuged (4,000 rpm, 20 min, 4°C) to collect the supernatant (three times). The combined supernatants were evaporated using a rotary vacuum evaporator (BUCHI, China) at 35°C, and then dissolved in methanol (final volume, 10 ml) to obtain a free phenolic fraction. After the free phenolic extraction, obtained residues were used to extract bound phenolic compounds by a two-step sequential solvent extraction with base hydrolysis and acid hydrolysis. First, the residue was added to 15 ml NaOH (3 M), after hydrolyzing for 4 h at 30°C under a stream of N_2, acidified with HCl to pH 2 (6 M), and then centrifuged at 10,000 rpm for 20 min. The supernatant was extracted by equal volumes of ethyl acetate three times. The upper organic phase was collected and evaporated using a rotary evaporator (BUCHI, China). After base hydrolysis, the remaining residue was hydrolyzed with 15 ml of HCl (3 M) for 1 h at 85°C. Once the hydrolysis was completed, this mixture was acidified to pH 2 with NaOH (6 M) and then immediately centrifuged and extracted by the similar procedures described above. The two parts of extracts obtained by alkaline and acid hydrolysis were combined, evaporated, and resolubilized with 10 ml of methanol, which was considered bound phenolics.

Determination of Total Phenolics Content and Total Flavonoid Content

The total phenolics content was determined by the Folin-Ciocalteu colorimetric method (12). Briefly, 25 μl of sample solution or standard solution and 125 μl of Folin-Ciocalteu reagent were reacted for 10 min at room temperature, and then 125 μl Na_2CO_3 was added. The reaction was continued for 30 min after shaking by an oscillator. The absorbance was measured at 765 nm using a spectrophotometer (Molecular Devices, United States). The gallic acid was used as a standard

(y = 0.0052x + 0.3651, R^2 = 0.9982) and expressed as mg GAE (gallic acid equivalents)/g DW (dry weight) of leaves.

The total flavonoid content of the extracts was determined based on the aluminum chloride colorimetric method as previously described (13). Briefly, 25 μl of sample solution or standard was mixed with 110 μl of $NaNO_2$ solution (0.066 M) for 5 min. The mixture was allowed to stand at room temperature for 5 min, and then 15 μl of $AlCl_3$ solution (0.75 M) was added and incubated for 6 min. Then, 100 μl of 0.5 M NaOH solution was added. The absorbance was measured immediately at 510 nm by a spectrophotometer (Molecular Devices, United States). The catechin was used as a standard (y = 0.002x + 0.0341, R^2 = 0.9973) and expressed as mg CAE (catechin equivalents)/g DW (dry weight) of leaves.

UPLC–Q–Exactive Orbitrap/MS Analyses

The phenolic compounds of *R. roxburghii* leaf extracts were characterized and quantified using a UPLC system connected to a Q-Exactive Orbitrap MS (Thermofisher Scientific, China) equipped with an electrospray ionization (ESI) source according to the method from our previous study (14). Separation of phenolics was carried out with an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, particle size 1.7 μm, Waters, Milford, MA, United States), eluting with solvent A (acetonitrile) and solvent B (0.1% formic acid in Milli-Q grade water) gradient elution as the mobile phase. The gradient was formed as follows: 0–3 min 95–85% B, 3–11 min 85–70% B, 11–15 min 70–50% B, 15–21 min 50–10% B, 21–22 min 10–95% B. The flow rate was 150 μl/min with a sample injection volume of 2 μl, and the column temperature was equilibrated to 20°C. MS experiments work with the following conditions: auxiliary gas (N_2), 10 arb; sheath gas (N_2), 35 arb; capillary voltage, 3,200 V; capillary temperature, 320°C; scan range, 100–1,500 m/z.

The total ion current chromatogram was presented in negative ionization mode because of the presence of hydroxyl, glycoside, and/or carboxylic acid groups. Phenolics compounds were identified by comparison of their spectra and retention times with those of externally injected standards such as quininic acid, gallic acid, (-)-gallocatechin, protocatechuic acid, neochlorogenic acid, chlorogenic acid, catechin, cryptochlorogenic acid, *p*-hydroxybenzoic acid, 6,7-dihydroxycoumarin, caffeic acid, benzoic acid, vanillin, rutin, *p*-hydroxy-cinnamic acid, *p*-coumaric acid, ellagic acid, hyperoside, isoquercitrin, ferulic acid, kaempferol -3-*o*-rutinoside, proanthocyanidins, isoferulic acid, astragaline, quercitrin, phlorizin, quercetin, naringenin, kaempferol, and isorhamnetin. For compounds with no available standards, identification was made using the precise mass of the parent ion [(M–H)] and typical MS fragmentation pattern compared with references and OTCML database. The quantification of the characterized phenolic compounds was carried out by the external calibration curve of each standard. When the standard was not available, the compound quantification was expressed as equivalent to the structurally closest phenolic compound.

Evaluation of Antioxidant Activity

Assay of DPPH Radical Scavenging Activity

The DPPH assay was performed by the reported method (15). In dark conditions, a 100 μl sample or blank solution was mixed with 100 μl DPPH (0.35 M) and incubated for 30 min. Its absorbance was measured at 517 nm using a spectrophotometer (Molecular Devices, United States). The result was calculated as mg Trolox equivalents per gram of dry weight (mg TE/g DW) (Trolox standard curve, y = 0.0204x − 0.0393, R^2 = 0.9951).

Assay of ABTS Radical Scavenging Activity

The ABTS assay was performed as previously described (16). A total of 5 ml 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) (7.0 mM) and 88 μl potassium persulphate (140 mM) were allowed to react for 12 h in dark in order to prepare the fresh stock solution. The ABTS working solution was freshly prepared by diluting 80% methanol to get an absorbance of 0.70 ± 0.02 units at 734 nm. A total of 20 μl sample or blank solution was mixed with 200 μl working solution for 6 min. Its absorbance was measured at 734 nm. The results were calculated as mg Trolox equivalents per gram of dry weight (mg TE/g DW) by comparing with the Trolox standard curve (y = 0.0015 x − 0.0492, R^2 = 0.9989).

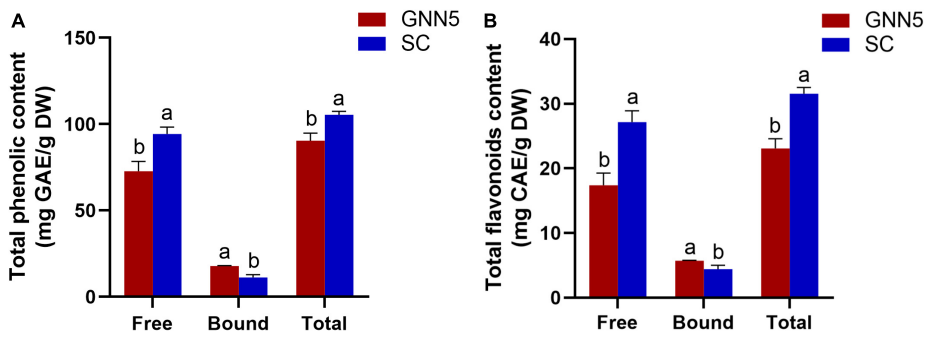

FIGURE 1 | Total phenolic contents **(A)** and total flavonoid contents **(B)** of free and bound phenolic fractions extracted from two *R. roxburghii* leaves. Data represent the mean values ± SD (n = 3), and different letters indicate significant difference (p < 0.05) between two cultivars. Total, the sum of free and bound fractions.

Oxygen Radical Absorbance Capacity Assay

The oxygen radical absorbance capacity (ORAC) assay was performed by the reported method (17). PBS buffer (pH 7.4) was used as the solvent for reagents. A total of 100 μl fluorescein sodium solution (8 mM) and 25 μl sample solution or blank solution were mixed well in a black 96-well plate, and the plate was equilibrated at 37°C for 15 min; then, 75 μl AAPH (119.4 mM) solution was added and recording of fluorescence intensity was started by scheduled recording function of a fluorometer with excitation at 485 nm and emission at 530 nm every 2 min for 2 h. The results were expressed as μmol Trolox equivalents per gram of dry weight (μmol TE/g DW) (Trolox standard curve, y = 303934 x − 647020, R^2 = 0.9966).

α-Glucosidase Activity *in vitro*

The α-glucosidase inhibitory activity *in vitro* was estimated by the method previously described (18). At 37°C, 20 μl different concentrations of sample or control solution were incubated with 45 μl α-glucosidase (1 U/ml) in 0.1 M pH = 6.8 phosphate buffer solution for 10 min, and the reaction was continued for 20 min after 45 μl *p*-NPG (2.5 mM) in 0.1 M. Phosphate buffer solution with pH = 6.8 was added. After the addition of 100 μl sodium carbonate (0.2 M), its absorbance was measured at 405 nm. In the control group, PBS buffer was used instead of the sample. Acarbose (0.625–20 mg/ml) was used as the positive control. The α-glucosidase inhibiting activity of the extracts was repressed as a half-inhibition concentration (IC_{50}) value. The α-glucosidase inhibiting activity was calculated according to the following formula:

$$\alpha - \text{Glucosidase inhibitory activity}(\%) = [1 - (A_{sample}/A_{control})] \times 100\%.$$

Statistical Analysis

All assays were conducted in triplicate and repeated three times, with the data reported as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (one-way ANOVA) with Tukey's HSD test to determine the differences between means. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed and images were generated with Origin 2021b (OriginLab, Northampton, MA, United States). Correlation analyses between phenolics and bioactivities were performed using standard Pearson correlation. All statistical analyses were carried out at a significance level of 5% ($p \leq 0.05$) using IBM SPSS.

RESULTS AND DISCUSSION

Total Phenolic Content and Total Flavonoid Content

Rosa roxburghii leaves rich in phenolics are traditionally processed into healthy beverages, such as tea. However, the qualitative and quantitative analysis of polyphenols in *R. roxburghii* leaves is limited. **Figure 1** shows the phenolic content (free, bound, and total phenolics, and the total phenolic content is the sum of free and bound fractions) of two cultivars of *R. roxburghii* leaves. The free phenolic content of SC was 94.28 ± 4.01 mg GAE/g DW, which was significantly higher than that of GNN5 with 72.71 ± 5.67 mg GAE/g DW, while the bound phenolic content of SC (11.19 ± 1.66 mg GAE/g DW) was lower than that of GNN5 (17.75 ± 0.29 mg GAE/g DW). A similar trend was observed for flavonoids, which was 27.14 ± 1.76 and 17.38 ± 1.90 mg CAE/g DW (free fraction) and 4.42 ± 0.06 and 5.71 ± 0.06 mg CAE/g DW (bound fraction) for SC and GNN5, respectively (**Figure 1**).

The bound phenolics contributed to 10.61% and 19.62% of TPC in SC and GNN5, resepctively, indicating that there also existed a considerable amount of bound phenolics in addition to the free phenolics in *R. roxburghii* leaves. Importantly, the average sum of TPC (free + bound) of these two cultivars was 97.97 mg GAE/g DW, which suggested that *R. roxburghii* leaves are indeed a valuable source of phenolic compounds compared with other foods. For example, these values are greater than that of 14.16–14.54 mg GAE/g DW in mint (19) and comparable to that of 34.78–40.33 and 87.59–88.27 mg GAE/g DW in green tea (20) and raw black tea leaves (21), which were the well-known natural good source of phenolic compounds.

Characterization of Phenolic Compounds

Considering the presence of some possible interfering compounds that react with Folin-Ciocalteu reagent, such as sugars, aromatic amines, and organic acids (22), UPLC-Q-Exactive Orbitrap/MS analysis was conducted to further identify and quantify the compositions of phenolic compounds. A total of 37 individual phenolics were identified in all samples of *R. roxburghii* leaves (**Supplementary Table 1** and **Supplementary Figure 1**). According to the reference standards (retention times, the accurate mass, and the secondary fragment), 30 individual phenolics were directly identified. They included quininic acid, gallic acid, (-)-gallocatechin, protocatechuic acid, neochlorogenic acid, chlorogenic acid, catechin, cryptochlorogenic acid, *p*-hydroxybenzoic acid, 6,7-dihydroxycoumarin, caffeic acid, benzoic acid, vanillin, rutin, *p*-hydroxy-cinnamic acid, *p*-coumaric acid, ellagic acid, hyperoside, isoquercitrin, ferulic acid, kaempferol -3-*o*-rutinoside, proanthocyanidins, isoferulic acid, astragaline, quercitrin, phlorizin, quercetin, naringenin, kaempferol, and isorhamnetin. The other seven peaks were tentatively identified by comparing their MS data with those reported in the literature. Peak 7 was characterized as brevifolin carboxylic acid by an $[M\text{-}H]^-$ ion at m/z 291.0148 and fragment ions at m/z 247.0246 due to the loss of carboxyl moieties (23). Peak 11 (m/z 951.0756) was identified as geranium from its fragment ions at m/z 933.0653 and m/z 300.9991 (24), based on the similar fragmentation pattern reported previously. Peak 12 was preliminarily authenticated as corilagin with parent ion m/z 633.0742 $(M–H)^-$ and the fragment ions at m/z 300.9991 ($C_{14}H_5O_8^-$) and m/z 275.0198 ($C_{13}H_7O_7^-$) associated with

TABLE 1 | Phenolic profiles of two *R. roxburghii* leaves in free and bound fractions (mg/100 g DW).

Phenolic compound	GNN5		SC	
	Free	Bound	Free	Bound
Quininic acid	138.60 ± 2.56^{a}	4.25 ± 0.28^{c}	110.42 ± 2.46^{b}	5.31 ± 1.33^{c}
Gallic acid	3.29 ± 0.22^{c}	281.59 ± 2.95^{a}	7.27 ± 0.37^{c}	132.28 ± 5.91^{b}
Protocatechuic acid	0.46 ± 0.06^{c}	3.39 ± 0.22^{a}	0.20 ± 0.05^{c}	2.50 ± 0.29^{b}
Neochlorogenic acid	16.10 ± 0.68^{b}	1.24 ± 0.22^{c}	64.16 ± 1.67^{a}	0.31 ± 0.09^{c}
Chlorogenic acid	1.08 ± 0.08^{b}	ND	25.25 ± 0.40^{a}	ND
Brevifolin carboxylic acid	71.85 ± 8.32^{b}	11.76 ± 0.80^{c}	100.68 ± 3.43^{a}	9.59 ± 0.36^{c}
Cryptochlorogenic acid	1.39 ± 0.08^{a}	ND	ND	0.06 ± 0.00^{b}
p-Hydroxybenzoic acid	ND	63.25 ± 2.35^{a}	ND	ND
Caffeic acid	ND	11.91 ± 0.99^{b}	ND	15.09 ± 0.62^{a}
Benzoic acid	8.65 ± 0.17^{c}	34.27 ± 1.73^{a}	6.41 ± 0.15^{c}	29.22 ± 3.21^{b}
p-Hydroxy-cinnamic acid	ND	6.72 ± 0.18^{a}	ND	7.54 ± 0.64^{a}
p-Coumaric acid	ND	7.17 ± 0.21^{b}	ND	7.71 ± 0.37^{a}
Ellagic acid	237.62 ± 3.95^{b}	182.60 ± 5.10^{c}	264.21 ± 6.13^{a}	179.72 ± 5.33^{c}
Ferulic acid	0.88 ± 0.05^{c}	11.00 ± 0.11^{a}	0.07 ± 0.02^{d}	3.58 ± 0.26^{b}
Isoferulic acid	1.97 ± 0.09^{c}	26.99 ± 1.49^{a}	0.44 ± 0.18^{d}	13.43 ± 0.56^{b}
ΣPhenolic acids	482.02 ± 13.40^{c}	644.54 ± 11.92^{a}	579.33 ± 9.84^{b}	406.2 ± 6.01^{d}
(-)-Gallocatechin	2.95 ± 0.08^{b}	ND	3.95 ± 0.17^{a}	ND
Catechin	73.16 ± 1.63^{a}	1.60 ± 0.05^{c}	66.66 ± 1.19^{b}	1.91 ± 0.26^{c}
Rutin	0.33 ± 0.04^{b}	ND	11.50 ± 0.29^{a}	ND
Hyperoside	97.90 ± 2.57^{b}	85.64 ± 0.39^{c}	176.71 ± 2.10^{a}	66.05 ± 5.86^{d}
Isoquercitrin	125.17 ± 3.30^{b}	2.18 ± 0.44^{c}	218.34 ± 2.99^{a}	ND
Kaempferol -3-*o*-rutinoside	0.17 ± 0.01^{c}	ND	6.32 ± 0.07^{a}	0.51 ± 0.17^{b}
Quercetin 3-*O*-6″-acetylglucoside	2.04 ± 0.14^{b}	ND	12.13 ± 0.81^{a}	ND
Proanthocyanidins	ND	ND	0.07 ± 0.01^{a}	ND
Kaemperol-3-*O*-glucuronide	1.33 ± 0.13^{a}	0.22 ± 0.03^{b}	ND	ND
Astragaline	10.59 ± 0.41^{a}	8.50 ± 0.24^{b}	9.57 ± 0.30^{b}	4.36 ± 0.16^{c}
Quercitrin	153.25 ± 4.08^{a}	0.38 ± 0.02^{d}	138.92 ± 1.98^{b}	6.94 ± 0.75^{c}
Kaempferol pentoside	0.65 ± 0.06^{b}	ND	10.06 ± 0.54^{a}	0.42 ± 0.07^{b}
Phlorizin	0.33 ± 0.04^{b}	ND	0.60 ± 0.06^{a}	ND
Quercetin	2.15 ± 0.19^{c}	13.61 ± 0.21^{a}	2.42 ± 0.15^{c}	10.56 ± 0.99^{b}
Naringenin	34.04 ± 0.84^{a}	13.18 ± 0.54^{c}	24.81 ± 0.95^{b}	26.11 ± 1.17^{b}
Kaempferol	4.47 ± 0.18^{c}	28.58 ± 0.73^{a}	1.46 ± 0.08^{d}	15.98 ± 1.05^{b}
Isorhamnetin	0.02 ± 0.00^{bc}	0.21 ± 0.02^{a}	ND	0.03 ± 0.00^{b}
ΣFlavonoids	506.59 ± 7.68^{b}	154.43 ± 0.37^{c}	682.00 ± 4.25^{a}	134.06 ± 6.48^{d}
Geranium	ND	ND	0.15 ± 0.04^{a}	ND
Corilagin	33.34 ± 1.21^{a}	ND	21.18 ± 0.44^{b}	ND
6,7-Dihydroxycoumarin	0.06 ± 0.01^{b}	ND	0.08 ± 0.02^{a}	ND
Castalagin	9.62 ± 0.64^{a}	ND	9.52 ± 0.41^{a}	ND
Vanillin	0.09 ± 0.01^{c}	0.86 ± 0.06^{a}	ND	0.51 ± 0.02^{b}
ΣOthers	43.11 ± 1.78^{a}	0.86 ± 0.06^{c}	30.94 ± 0.85^{b}	0.51 ± 0.02^{c}
ΣPhenolic compounds	1031.72 ± 22.01^{b}	799.83 ± 11.61^{c}	1292.27 ± 8.75^{a}	540.77 ± 8.40^{d}

Data represent the mean values ± SD (n = 3). ND, not detected/determined. Brevifolin carboxylic acid, geranium, corilagin, and castalagin were quantified in ellagic acid equivalents; quercetin 3-O-6″-acetylglucoside was quantified in quercetin equivalents; kaemperol-3-O-glucuronide and kaempferol pentoside were quantified in kaempferol equivalents. Different letters in same line indicate significant difference ($p < 0.05$).

hexahydroxydiphenoyl group of corilagin (25). Peak 16 (m/z 933.065) may be associated with castalagin, with the ions at m/z 915.0599 indicating fragmentation of these core molecules after the release of one water molecule. Peak 26 (m/z 505.0993) was tentatively characterized as quercetin 3-*O*-6″-acetylglucoside because of its fragment ions at m/z 301.0348, corresponding to the quercetin moiety (26). Peak 29 and peak 32 were identified as kaemperol-3-*O*-glucuronide and kaempferol-pentoside, respectively, at their $[M-H]^-$ ion and the fragment ions at m/z 285.0389 (27). Among the abundant individual phenolics, protocatechuic acid, neochlorogenic acid, brevifolin carboxylic acid, catechin, benzoic acid, ellagic acid, hyperoside, ferulic acid, isoferulic acid, astragaline, quercitrin, quercetin, naringenin, and kaempferol derivative were detected in the free and bound

TABLE 2 | Antioxidant capacity of free and bound phenolic compounds extracted from two *R. roxburghii* leaves.

	DPPH (mg TE/g DW)	ABTS (mg TE/g DW)	ORAC (μ mol TE/g DW)
Free phenolics (GNN5)	310.15 ± 4.55^{a}	1355.48 ± 182.13^{a}	1730.52 ± 8.57^{b}
Free phenolics (SC)	311.80 ± 3.53^{a}	1336.87 ± 70.49^{a}	2017.38 ± 20.95^{a}
Bound phenolics (GNN5)	50.53 ± 1.43^{b}	250.42 ± 12.74^{b}	839.58 ± 34.95^{c}
Bound phenolics (SC)	28.35 ± 0.52^{c}	147.99 ± 12.16^{b}	610.25 ± 23.05^{d}

Data represent the mean values ± SD (n = 3) and different letters in the same column indicate significant difference ($p < 0.05$).

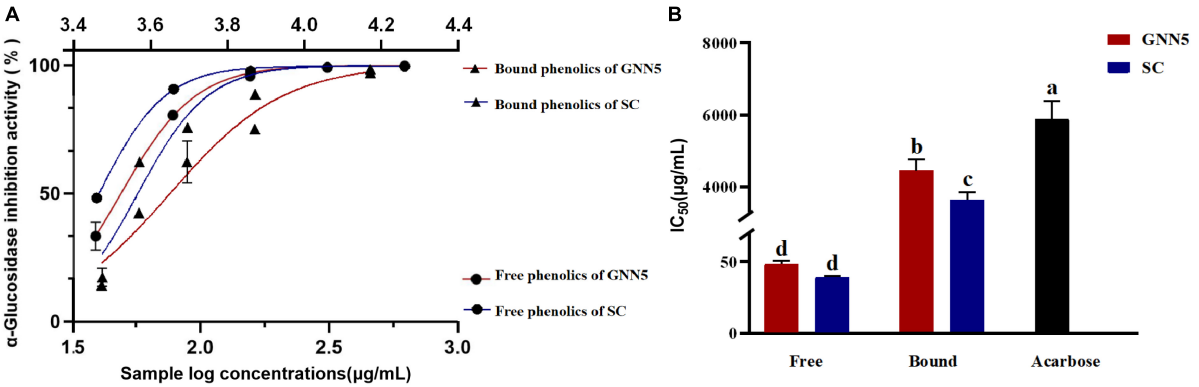

FIGURE 2 | The α-glucosidase inhibitory potency of free and bound phenolic compounds extracted from two *R. roxburghii* leaves **(A)**. Panel **(B)** represents the corresponding IC_{50} values of the samples. Different letters denote statistically significant differences at $p < 0.05$.

phenolic extracts. (-)-Gallocatechin, chlorogenic acid, corilagin, 6,7-dihydroxycoumarin, castalagin, rutin, quercetin 3-*O*-6″-acetylglucoside, and phlorizin derivative were found only in the extracts of free phenolics. In addition, caffeic acid, *p*-hydroxycinnamic acid, and *p*-coumaric acid derivatives were found only in the extracts of bound phenolics (**Table 1**). Interestingly, many individual phenolics detected in *R. roxburghii* leaves such as gallic acid, catechin, rutin, *p*-coumaric acid, hyperoside, isoquercitrin, and kaempferol also abundantly exist in green tea (20).

Quantitative Analysis of Individual Phenolic Compounds in Various Extracts

The quantitative analysis of individual phenolic compounds from *R. roxburghii* leaves was performed with the corresponding relative standards (**Supplementary Table 2**). As shown in **Table 1**, abundant individual phenolics of SC and GNN5 in free form were ellagic acid, quercitrin, quininic acid, isoquercitrin, hyperoside, brevifolin carboxylic acid, and catechin. Compared with those abundant in free phenolics, those abundant phenolics in bound fractions were gallic acid, ellagic acid, and hyperoside for SC and GNN5, respectively. The bound phenolics contributed to 29.50% and 43.67% of total phenolics content in SC and GNN5, respectively. Acosta-Estrada et al. (9) reported that 16.7–76.3% of phenolics compounds exist fundamentally in the bound form in fruits. Considering the effect of bound phenolics on gut health after liberation by microbiota in the colon, exploring bound compounds of *R. roxburghii* leaves is significant to estimating the scientific support for healthcare. In addition, the total concentration of phenolics detected by UPLC-ESI-MS/MS in *R. roxburghii* leaves was up to 1,831.55 mg/100 g DW (GNN5) and 1,833.04 mg/100 g DW (SC), which suggested *R. roxburghii* leaves as a good source of phenolic compounds. The high phenolics (free + bound forms) clearly indicated that *R. roxburghii* leaves have a great potential application in the development of functional and value-added foods. In addition, the abundant individual phenolics of green tea, including catechins, gallic acid, and ellagic acid (28), were also found in *R. roxburghii* leaves.

Antioxidant Capacity

The healthy function of polyphenols has been mainly ascribed to their antioxidant activity, which can be measured by scavenging or reducing radical species (e.g., DPPH, ABTS, and ORAC assays) (29). Therefore, DPPH, ABTS, and ORAC assays were employed to assess the antioxidant potential of the different phenolic extracts from *R. roxburghii* leaves. As summarized in **Table 2**, the DPPH values of free phenolics in SC (311.80 ± 3.53 mg TE/g DW) and GNN5 (310.15 ± 4.55 mg TE/g DW) were significantly higher than that of their bound phenolics (28.35 ± 0.52 mg TE/g DW and 50.53 ± 1.43 mg TE/g DW) ($p < 0.05$). However, no significant difference was observed between the two cultivars for free phenolic extracts ($p > 0.05$). With similar results found in ABTS, ABTS values of free phenolics from SC (1,336.87 ± 70.49 mg TE/g DW) and GNN5 (1,355.48 ± 182.13 mg TE/g DW) exhibited 9.03 and 5.41 times that of their bound phenolics, respectively. Moreover, free phenolics of SC exhibited the highest ORAC value (2,017.38 ± 20.95 μmol TE/g DW), followed by free phenolics of GNN5 (1,730.52 ± 8.57 μmol TE/g DW), bound phenolics of GNN5 (839.58 ± 34.95 μmol TE/g DW), and SC

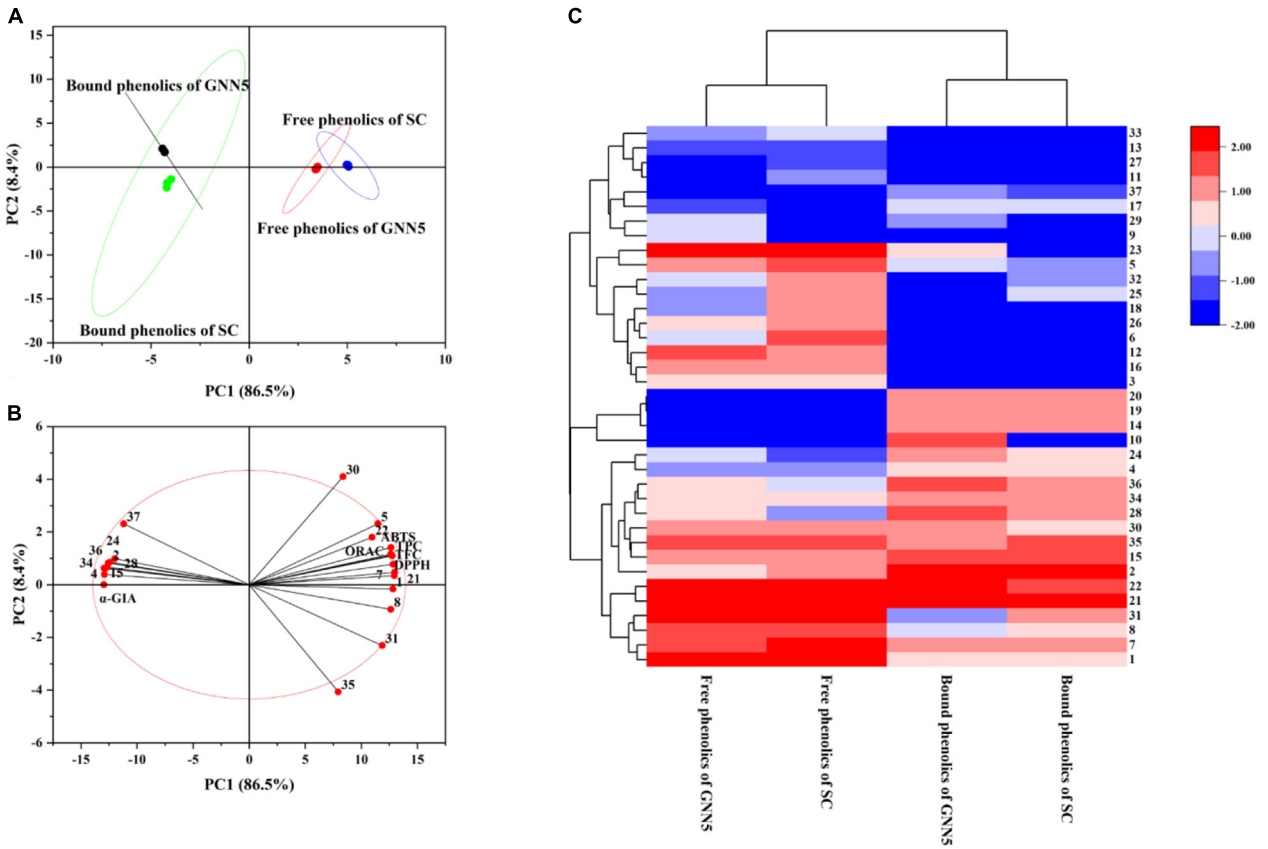

FIGURE 3 | Multivariate analysis on the datasets of two *R. roxburghii* leaf extracts. **(A)** The PCA loading plot; **(B)** the PCA score plot; **(C)** Hierarchically clustered heat (HCA) map. The component numbers are in one-to-one correspondence with **Supplementary Table 1**.

(610.25 ± 23.05 μmol TE/g DW). These values are greater than that of 777.00–1,173.00 μmol TE/g in black tea (30) and comparable to that of 934.00–1,302.60 and 1,060.10–1,125.00 μmol TE/g in oolong tea and green tea (31), respectively, which were normally well-known natural antioxidant products. Overproduction of free radicals relates to oxidative stress and plays a significant role in the development of chronic diseases, such as cardiovascular diseases and diabetes, and dietary antioxidants may block the occurrence and progression of these diseases (32). The results indicated that phenolic extracts of *R. roxburghii* leaves may be consumed as natural dietary antioxidants.

α-Glucosidase Inhibition Activity

Located in the small intestine, the role of α-glucosidase is to hydrolyze glycosidic bonds in oligosaccharides and disaccharides and release D-glucose. Glucosidase inhibitors interfere with the role of α-glucosidase, delay the absorption of D-glucose, and reduce postprandial blood glucose levels (18). So far, the widely used drug product is acarbose; for this experiment, it was used as a positive control. As shown in **Figure 2**, the polyphenolic extracts of *R. roxburghii* leaves were superior to the positive control in terms of their inhibitory effects. The IC_{50} values of phenolic extracts of *R. roxburghii* leaves were significantly lower than that of the positive control, among which free phenolic extracts of SC (39.72 ± 0.59 μg/ml) exhibited the lowest ($p < 0.05$) IC_{50} value, which was significantly lower by 99.34% compared with the positive control acarbose (IC_{50} = 5.98 mg/ml). The α-glucosidase inhibition activity of free phenolics of GNN5 (IC_{50} = 49.00 ± 1.71 μg/ml) was also significantly lower than that of acarbose, but higher than that of free phenolics of SC. Meanwhile, bound phenolics of SC and GNN5 also possessed higher α-glucosidase inhibitory activity (IC_{50} = 3.711 ± 0.21 and 4.524 ± 0.30 mg/ml), which were also significantly lower than the positive control acarbose. In line with the previous research (33, 34), phenolics from *Rosa roxburghii* leaves also exhibited a strong inhibition against the α-glucosidase activity. Previous studies reported that gallic acid, quercitrin, isoquercitrin, and catechin inhibited the α-glucosidase activity and significantly decreased the hydrolysis rates of different starches (35). Ellagic acid (180 mg/day for 8 weeks), as a strong α-glucosidase inhibitor (36), significantly reduced the blood glucose level and insulin resistance of patients with type 2 diabetes (37). Therefore, the α-glucosidase inhibiting activity of *R. roxburghii* leaf extracts can be mainly attributed to the presence of phenolics compounds. As the raw material of *R. roxburghii* tea, the good inhibitory effect of *R. roxburghii* leaves on glucosidase provided strong evidence for the validation of its functional activity.

TABLE 3 | Correlation between phenolic compounds and bioactivities.

Parameters	GNN5	SC
Total phenolics (Folin-Ciocalteu) vs. DPPH	0.992**	1.000**
Total phenolics (Folin-Ciocalteu) vs. ABTS	0.958**	0.998**
Total phenolics (Folin-Ciocalteu) vs. ORAC	0.991**	1.000**
Total phenolics (Folin-Ciocalteu) vs. α-glucosidase inhibition	−0.988**	−0.998**
Total phenolics (Folin-Ciocalteu) vs. Total phenolic compounds (HPLC-MS)	0.966**	0.999**
Total phenolic compounds (HPLC-MS) vs. DPPH	0.989**	0.999**
Total phenolic compounds (HPLC-MS) vs. ABTS	0.972**	0.999**
Total phenolic compounds (HPLC-MS) vs. ORAC	0.991**	0.999**
Total phenolic compounds (HPLC-MS) vs. α-glucosidase inhibition	−0.982**	−0.995**
Ellagic acid vs. DPPH	0.996**	0.999**
Isoquercitrin vs. DPPH	0.999**	0.999**
Hyperoside vs. DPPH	0.948**	0.948**
Quercitrin vs. DPPH	1.000**	1.000**
Brevifolin carboxylic acid vs. DPPH	0.978**	0.997**
Ellagic acid vs. ABTS	0.987**	0.995**
Isoquercitrin vs. ABTS	0.970**	0.995**
Hyperoside vs. ABTS	0.988**	0.965**
Quercitrin vs. ABTS	0.978**	0.996**
Brevifolin carboxylic acid vs. ABTS	0.941**	0.993**
Ellagic acid vs. ORAC	0.994**	0.999**
Isoquercitrin vs. ORAC	0.998**	0.999**
Hyperoside vs. ORAC	0.937**	0.943**
Quercitrin vs. ORAC	0.998**	1.000**
Brevifolin carboxylic acid vs. ORAC	0.981**	0.998**
Ellagic acid vs. IC_{50}	−0.989**	−0.997**
Isoquercitrin vs. IC_{50}	−0.995**	−0.997**
Hyperoside vs. IC_{50}	−0.935**	−0.937**
Quercitrin vs. IC_{50}	−0.995**	−0.997**
Brevifolin carboxylic acid vs. IC_{50}	−0.975**	−0.997**

*Positive correlation (+), negative correlation (–), significant differences: **$p < 0.01$.*

Principal Component Analysis and Hierarchical Cluster Analysis

Principal component analysis, which provided direct visualization of the distribution of individual phenolic compounds and bioactivity of different samples, was performed and the maps were generated (**Figures 3A,B**). The loading plot of PCA showed that PC1 86.5% and PC2 8.4% accounted for 94.9% of the total variances, which indicated that these two principal components could load maximum information from the original data. The free phenolics obtained from GNN5 and SC were classified into cluster 1, while the bound phenolics extracted from these two cultivars were classified into cluster 2. Two clusters from the same phenolic fraction of different cultivars with intersections indicated that their compositions were similar. With respect to the score plot of PCA, the cosine values may indicate the relationship between two variables (**Figure 3B**). The results confirmed that the bioactivities of phenolic extracts from *R. roxburghii* leaves were evidently influenced by TPC and TFC. Among them, quininic acid (1), neochlorogenic acid (5), brevifolin carboxylic acid (7), ellagic acid (21), and hyperoside (22) were significantly correlated with ABTS and DPPH scavenging capacity and ORAC (the antioxidant activities). In addition, quininic acid (1), brevifolin carboxylic acid (7), and catechin (8) were significantly correlated with the α-glucosidase inhibiting activity. This study highlighted the significant effect of phenolics on the biological activities of *R. roxburghii* leaf extracts.

In the model of HCA, the data of thirty-seven phenolic compounds in four extractions (**Table 1**) through standardization were used to conduct analysis, and a hierarchically clustered heat map was obtained as the output (**Figure 3C**). On the one hand, four extracts were clearly divided into two groups, and the difference between free phenolics and bound phenolics was more significant than that of cultivars, which is consistent with the result of PCA. On the other hand, thirty-seven phenolic compounds were also classified into three groups. For group 1, they were abundant in all samples. Phenolic compounds, including *p*-hydroxybenzoic acid, caffeic acid, *p*-hydroxy-cinnamic acid, and *p*-coumaric acid, from group 2 were only found in bound phenolics, and phenolics of group 3 were almost present in free form. The result of PCA and HCA demonstrated that the bound fraction was indispensable for the evaluation and utilization of phenolic resources in *R. roxburghii* leaves.

Correlation of Bioactivity With Different Extracts

Table 3 summarizes the correlations between phenolic compounds and bioactivities of leaf extracts from GNN5 and SC. The results showed that the antioxidant activities (DPPH, ABTS, and ORAC) were correlated significantly ($p < 0.01$) with total phenolics (Folin-Ciocalteu) ($r = 0.958$–1.000). Positive high linear correlations ($p < 0.01$) were also observed between the antioxidant activities and total phenolic compounds by UPLC-MS ($r = 0.972$–0.999). IC_{50} was used to express α-glucosidase inhibition, and a smaller IC_{50} value corresponds to stronger α-glucosidase inhibition. Consequently, the negative relationships between IC_{50} value and total phenolics (Folin-Ciocalteu) ($r = -0.988$ to -0.998), and total phenolic compounds (UPLC-MS) ($r = -0.982$ to -0.995) were also observed. The correlations between these bioactivities and phenolic compounds content in *R. roxburghii* leaf extracts were in line with the previous studies (14). The significant and positive correlations between the total phenolic compounds (UPLC-MS) and total phenolics (Folin-Ciocalteu) from SC and GNN5 implied that the method used to extract the phenolic compounds was effective to remove the non-phenolic interfering substances that could react with Folin-Ciocalteu reagent (38). Moreover, the correlation analysis was conducted to evaluate the major phenolic compound contributors to the bioactivities of *R. roxburghii* leaves. The major phenolics of SC and GNN5, quininic acid, brevifolin carboxylic acid, ellagic acid, hyperoside, and isoquercitrin correlated significantly ($p < 0.01$) with the bioactivities (DPPH, ABTS, ORAC, and α-glucosidase

inhibition activity) (r_{DPPH} = 0.948–1.000, r_{ABTS} = 0.941–0.996, r_{ORAC} = 0.937–1.000, r_{IC50} = −0.935 to −0.997). The results revealed that *R. roxburghii* leaves can be sources of natural phenolics for utilization in the development of functional foods and nutraceuticals.

CONCLUSION

For the first time, phenolics fractioned into free and bound forms from *R. roxburghii* leaves of two cultivars were studied and their antioxidant and α-glucosidase inhibition activities were evaluated. The profiles of free and bound phenolics and their bioactivities were quite contrasting. The content of free and bound phenolics in GNN5 was 72.71 and 17.75 mg GAE/g DW and in SC was 94.28 and 11.19 mg GAE/g DW, respectively. Flavonoids comprised the maximum phenolics from the free fraction, whereas phenolic acids made a similar contribution for free and bound phenolics. A total of 37 phenolic compounds were identified and quantified by UPLC-Q-Exactive Orbitrap/MS in which ellagic acid, quercitrin, isoquercitrin, and quininic acid were abundant in the free phenolic fraction, while the bound phenolic fraction contained more gallic acid, ellagic acid, and hyperoside. In addition, multivariate analysis revealed that a strong correlation existed between the TPC/individual phenolic compounds and bioactivities of the *R. roxburghii* extracts. Therefore, the characterization and quantification of phenolic compounds in *R. roxburghii* leaves provide a scientific basis for the traditional practices of the leaves in treating different ailments. The data presented in the phenolic profile of *R. roxburghii* leaves clearly reveal that this leaf provides good sources of natural antioxidants for the development of functional foods, nutraceuticals, cosmetics, medicine, or high value-added products.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YY: formal analysis, investigation, data curation, and writing – original draft. WX: visualization. WL: conceptualization and supervision. WH: resources and funding acquisition. RY: writing – review and editing and project administration. 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.2022.922496/full#supplementary-material

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Biological Functions of *Dillenia pentagyna* Roxb. Against Pain, Inflammation, Fever, Diarrhea, and Thrombosis: Evidenced From *in vitro, in vivo,* and Molecular Docking Study

Nahida Sultana[1‡], Hea-Jong Chung[2‡], Nazim Uddin Emon[1‡], Safaet Alam[3,4†], Md. Tohidul Islam Taki[1], Sajib Rudra[5*†], Afroza Tahamina[6], Rashedul Alam[7], Firoj Ahmed[4] and Abdullah Al Mamun[8]*

[1] Department of Pharmacy, Faculty of Science and Engineering, International Islamic University Chittagong, Chittagong, Bangladesh, [2] Gwangju Center, Korea Basic Science Institute, Gwangju, South Korea, [3] Drugs and Toxins Research Divison, BCSIR Laboratories Rajshahi, Bangladesh Council of Scientific and Industrial Research, Rajshahi, Bangladesh, [4] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka, Bangladesh, [5] Department of Botany, Faculty of Biological Science, University of Chittagong, Chattogram, Bangladesh, [6] Beijing Advanced Innovation Center for Food Nutrition and Human Health, China-Canada Joint Lab of Food Nutrition and Health (Beijing), Beijing Engineering and Technology Research Center of Food Additives, Beijing Technology and Business University, Beijing, China, [7] Department of Pharmacology, Medical School, Jeonbuk National University, Jeonju, South Korea, [8] Molecular Pharmacology Research Center, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, China

Dillenia pentagyna Roxb. is traditionally used to treat cancer, wound healing, diabetes, and diarrhea in local tribes. This study was designed to evaluate the pharmacological potentiality of this plant. *In vivo* analgesic, anti-inflammatory, and antipyretic studies of the methanol extracts of *D. pentagyna* (MEDP) leaves were performed by using acetic acid-induced nociception, formalin-induced paw licking, and yeast-induced pyrexia assay methods, respectively. *In vivo* antidiarrheal activity was carried out in mice by following castor oil-induced diarrhea and gastrointestinal transit manner. *In vitro* thrombolytic experiment was performed employing the clot lysis activity. Besides, a molecular docking study was performed by executing the software (PyRx, Discovery Studio, and UCSF Chimera). In the acetic acid-induced writhing study, MEDP possesses significant writhing inhibition in a dose-dependent manner. It showed 50.86% of maximum inhibition of pain in the case of MEDP at a dose of 400 mg/kg body weight. In the anti-inflammatory study, maximum inhibition rate was observed at a value of 59.98 and 41.29% in early and late phases, respectively, at the dose of 400 mg/kg body weight. In the case of yeast-induced hyperpyrexia, MEDP reduced hyperpyrexia in a dose-dependent manner. In the antidiarrheal assay, MEDP moderately inhibited the occurrence of diarrhea in all the experiments. In the thrombolytic study, a moderate (17.76%) clot lysis potency has been yielded by MEDP. Again, the molecular docking simulation revealed strong binding affinities with almost all the targeted proteins. The present study suggests that the MEDP possesses remarkable pharmacological activity and this finding validated the ethnobotanical significance of *D. pentagyna* as the source of pain, fever, and diarrhea management agent.

Keywords: *Dillenia pentagyna*, pyrexia, antidiarrheal, antipyretic, thrombolytic, anti-inflammatory, molecular docking, ethnomedicinal plant

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***Correspondence:**
Safaet Alam
safaet.du@gmail.com
Sajib Rudra
rudrasajibcu89@gmail.com

†ORCID:
Safaet Alam
orcid.org/0000-0002-1831-2278
Sajib Rudra
orcid.org/0000-0003-1030-1379

‡*These authors have contributed equally to this work*

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INTRODUCTION

Pain is referred to as the response to several noxious stimuli in the immune response and is complicatedly structured through various episodes such as vasodilation, plasma extravasation, cell migration, and release of different mediators (1). In addition, bacteria and viruses trigger pyrexia which has been governed by CNS feedback mechanisms including vasodilation or sweat production and is responsible to reduce body temperature (2). Although NSAIDs are vigorously prescribed for pain, inflammation, and fever management, they can cause serious side effects following mucosal disruptions, ulcers, perforation, and blood suppression along with kidney damage, increased blood pressure, and cardiovascular complications (3). Diarrhea is one of the most prevalent diseases associated with altered bowel movement, wet stool, and abdominal pain is caused by multiple factors, namely, infections, food aversion, intestinal disorders, and also symbolic symptoms of multiple diseases like diabetes mellitus, and inflammatory diseases (4). Acute diarrhea in pediatric patients may be caused by bacteria and viruses *via* self-limiting of fluid and electrolyte replacement. Therapeutic approaches to reducing pain and diarrheal incidence are typically controlled by chemicals or medicines, but the adverse effects of such medicines present a substantial danger (4). Vascular blockage caused by a blood clot (thrombus) developed in the circulatory system due to lack of hemostasis guides to severe cascade sequels in atherothrombotic diseases including myocardial or cerebral infarction followed by death (5). About 1.5 billion (currently about 3.5 billion, i.e., 88%) of the world population are being treated by herbal medicines prepared from medicinal plants which demand new compounds discovery with promising remedial effects and negligible side effects contributing to the treatment of pain, fever, diarrhea, and thrombus (6). In structural molecular biology and computer-assisted drug design, molecular docking is an efficient process. Molecular docking is a structure-based drug design technology that simulates molecular interactions and predicts receptor-ligand binding mechanism and affinity (7). The purpose of ligand-protein docking is to predict the most likely binding modalities of a ligand to a known three-dimensional structure of a protein. Successful docking algorithms may successfully explore high-dimensional regions by using a scoring mechanism that correctly scores dockings. Docking may be used to do virtual screening on vast libraries of compounds, grade the findings, and provide structural insight into how the ligands interact with the target, all of which are immensely advantageous for lead optimization (8). Therefore, molecular docking is becoming more popular as a method for discovering new drug targets because of the growing availability of protein and nucleic acid structures. Docking structure prediction accuracy and screen hit rates have been the subject of a recent study. Docking against homology-modeled targets is now possible for a larger number of proteins as the number of experimentally known structures grows (9). Thus, research should be carried on so that these can lead us to new drug development in the future (10). *Dillenia pentagyna* Roxb. (family: Dillaneaceae) is an ethnomedicinal plant grown in the different district in Bangladesh. The plant is locally known as Banchalta, Hargaza (Bengali), Ajuli (Dhaka-Mymensingh), Argeza (Chittagong), and Ekush (Sylhet). The tribal and folk people utilize this species for the treatment of numerous diseases, for example, bone fracture (leaf), stomach cancer, pain (root), diarrhea and dysentery (leaf), pain (root), etc. (11, 12). In addition, this plant contains a number of flavonoids and triterpenes, e.g.; kaempferol, quercetin, rhamnetin-3-glucoside, isorhamnetin, and naringenin-7 galactosyl (1-4) glucoside, betulin, malic acid, lupeol, betulinaldehyde, β sitosterol, stigmasterol, and betulinic acid (13–15). Many approaches, namely, agitation, maceration, percolation, and soxhlet extraction for concentrating and extracting bioactive components from *D. pentagyna* have been demonstrated previously. Finally, based on previous reports and folkloric usages of this plant, it can be hypothesized that *D. pentagyna* can be a prospective source to develop novel therapeutics in the treatment of pain, fever, inflammation, diarrhea, and thrombosis.

In this study, phytochemical screening has been revealed to predict the elements which could enhance the pharmacological activity of methanol extracts of *D. pentagyna* (MEDP). Furthermore, we will also report the *in vivo* antinociceptive, antipyretic, antidiarrheal, and *in vitro* thrombolytic investigations of this plant extract along with *in silico* study; hence it was not accelerated previously.

MATERIALS AND METHODS

Plant Material

In November 2019, plant leaves of the *D. pentagyna* were obtained from Mirsharai, Chittagong, Bangladesh. The plant was identified by Mr. Sajib Rudra, taxonomist, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh (Accession number: CTGUH SR 7921).

Drying and Grinding

After the collection and identification, the leaves were washed accurately and dried under natural shade at (23 ± 2)°C for about 14 days. The dried leaves were then ground into powder using a high-capacity grinder. Powdered plant material was stored in a well-closed plastic container for further evaluation.

Extraction of the Plant Material

According to the previously established method, from 400 g dried powder of *D. pentagyna*, 18 g crude methanol extract was obtained (16).

Drugs and Chemicals

All drugs and chemicals used in this research were of analytical grade. To appliance the analgesic and anti-inflammatory study, acetic acid and formalin were collected from BDH Chemicals Ltd. (Poole, United Kingdom), diclofenac-Na was obtained from Incepta Pharmaceuticals (Dhaka, Bangladesh), and paracetamol and loperamide were from Square Pharmaceuticals Ltd. (Dhaka, Bangladesh). Streptokinase was obtained from Sanofi-aventis Bangladesh Ltd. (Dhaka, Bangladesh). Castor oil was supplied

by Well Heath (Madrid, Spain), 10% charcoal in 5% gum acacia from Taj Scientific (Chittagong, Bangladesh), and Tween-80 and ethanol were procured from Sigma-Chemical Co. (St. Louis, MO, United States).

Experimental Animals

To propagate experiments, Swiss albino mice (both male and female sex) aged 7–8 weeks and weighing around 25–30 g were collected from the Venom Research Centre (VRC), Chittagong, Bangladesh. Plastic cases were made to keep all animals under the 20 ± 2°C temperature and serve a 12-h light-dark cycle with the standard provision of water and food. P&D committee (Department of Pharmacy, International Islamic University Chittagong, Bangladesh) sanctioned all study protocols directed in an isolated and silent condition. The animals were acclimatized to laboratory conditions for 10 days before experimentation. The handling and taking care of animals were carried out by universal rules for the utilization and maintenance of experimental animals (17). The principles and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) were enforced to maintain the mitigation of pain and stress of the experimental models. Throughout the experiments, "3R" (Replace, Reduce, and Refine) was strictly maintained to prevent extreme pain and suffering. Experienced researchers and laboratory assistants handled the total experiment. Finally, after completion of the experiment, an anesthesia overdose [Ketamine HCl (100 mg/kg) and Xylazine (7.5 mg/kg)] through the intraperitoneal route were administered to the laboratory models followed by euthanasia (18).

Phytochemical Screening

The phytochemical investigations of MEDP to confirm the existence of carbohydrates, alkaloids, flavonoids, tannins, terpenoids, glycosides, steroids, saponin, resin, phenol, polyphenol, protein, anthocyanin, and cholesterol were defined by the established method (19).

Assesment of Pharmacological Actions

In vivo Bioassay

Preparation of Extract Solution

1% Tween Solution Preparation. In total, 1 ml Tween was taken in a beaker and 99 ml distilled water was added to the beaker to prepare 1% Tween-80.

Dose Preparation. For preparing 200 and 400 mg/kg dose, 200 and 400 mg extract were taken, respectively, and dissolved in 10 ml 1% Tween solution.

Acute Toxicity Test. An acute oral toxicity study was performed according to the OECD guidelines for the testing of chemicals (20). To conduct the toxicity test, a total of five animal models were considered. They received a single oral dose of either 500, 1,000, 1,500, or 2,000 (mg/kg BW) of MEDP followed by suspension of food administration for 3–4 h and then observation of the animals was done carefully for the next 72 h. Other changes, including in skin and fur, eyes and allergic reaction, respiratory and circulatory rate, and autonomic and CNS function (excitability and sedation) were observed (21).

In vivo Analgesic Activity

Acetic Acid-Induced Writhing Test. The acetic acid-induced writhing test was performed by an established protocol mentioned by Emon et al. (22). Four groups of male mice (20–25 g) each having six mice were used in this experiment. MEDP (100, 200, and 400 mg/kg; p.o.) had been administered to the individual mice. After 30 min, an intraperitoneal injection of 0.7% v/v acetic acid solution was administered. In transpicuous cages, mice were positioned individually and 5 min had been allowed to endure. For 20 min, the number of acid-induced writhes had been counted. Control and standard group animals were treated with normal saline (10 ml/kg, i.p.) and diclofenac-Na (10 mg/kg, i.p.), respectively.

Formalin-Induced Licking Test. According to Hunskaar et al. method (23), four groups of mice, each having five mice (20–25 g) were injected 20 μl of 1% formalin prepared in 0.9% saline, subcutaneously into the dorsal hind paw and shifted instantly in a transparent box for observation. The length of response time (paw licking or biting) was once determined between 0–5 min (first phase) and 15–30 min (second phase). Animals had been administered MEDP (100, 200, and 400 mg/kg; p.o.). Diclofenac-Na (10 mg/kg, i.p.) had been given to the standard animals. Control animals had been given normal saline (0.1 ml/10 g).

In vivo Antipyretic Activity

According to Brewer's yeast-induced fever method with some modifications (24, 25), at 0 h, the basal rectal temperature of each mouse was once recorded using a medical digital thermometer. Pyrexia was triggered with the aid of a subcutaneous injection of 15% w/v suspension of Brewer's yeast in distilled water at a dose of 10 ml/kg body weight. After 18 h of Brewer's yeast injection, the rise in rectal temperature used to be recorded, and only animals displaying an increase in temperature of at least 0.6°F (or 1°C) had been chosen for the study (26). The animals had been randomly divided into five groups, each group containing six mice. Group I received 1% Tween-80 in normal saline orally. Group II was given the standard drug paracetamol at the dose of 10 mg/kg orally. Groups III, IV, and V received methanol extract at an oral dose of 100, 200, and 400 (mg/kg; p.o). After the treatment, the temperature of all the mice in each group was recorded at 0, 1, 2, 3, and 4 h.

In vivo Antidiarrheal Activity

Castor Oil-Induced Diarrhea. The related method (27) was followed for this study with slight modification. Mice (20–25 g) fasted for 18 h. The selected mice for diarrheal tests were divided into four groups consisting of 6 mice. Group I was given normal saline (10 ml/kg) orally and designated as the control group. Group II obtained loperamide (5 mg/kg) as a standard group. Groups III–V obtained methanolic extract of MEDP (100, 200, and 400 mg/kg; p.o.), respectively. After 1 h, all groups received castor oil 1 ml each orally. Then they were positioned in cages lined with adsorbent papers and observed for 4 h for the presence of characteristic diarrheal droppings. In total, 100% was regarded

as the total number of feces in the control group. The activity was expressed as % inhibition of diarrhea.

$$\%\text{Inhibition of defecation} = \frac{\text{Mo} - \text{M}}{\text{Mo}} \times 100$$

Here, M_o = mean number of feces of the control group and M = mean number of feces of the test group.

Castor Oil-Induced Gastrointestinal Motility. This experiment was done with the strategy of a previously established method (28). All mice were divided into five groups of six mice. In total, 0.5 ml of castor oil was given orally to each mouse to produce diarrhea. After 1 h, the group I was given normal saline (10 ml/kg) orally as the control group. Group II received loperamide (5 mg/kg) as a standard group. Groups III, IV, and V received a MEDP (200 and 400 mg/kg; i.p.), respectively. After 1 h, 1 ml charcoal meal (10% charcoal suspension in 5% gum acacia) was given to all mice. The animals were then sacrificed after an hour and the small intestine was dissected from pylorus to cecum. The distance traveled by the charcoal meal from the pylorus was measured and expressed as a percentage of the total length of the small intestine from the pylorus to the cecum.

$$\text{Peristalsis index} = \frac{\text{Distance travel by the charcoal meal}}{\text{The total length of the small intestine}} \times 100$$

$$\%\text{ of inhibition} = \frac{\text{Mean length of small intestine distance travel by charcoal meal}}{\text{Mean length of the small intestine}} \times 100$$

In vitro Bioassay

In vitro Thrombolytic Activity

This study was conducted by following the method mentioned by Ahmed et al. (29). In total, 5 ml of venous blood had been drawn from wholesome volunteers. A total of 0.5 ml/tube of blood was dispensed in a preweighed sterile Eppendorf tube. Incubation of them at 37°C for 45 min allowed clot formation. The developed serum was eliminated except for disturbing the clot. Each tube was once again weighed to measure the clot weight. A total of 100 μl extract solutions were added separately to the tubes. As a positive control, 100 μl of streptokinase was added separately. A total of 100 μl of water was added separately to the clot of blank tubes. All the tubes were incubated at 37°C for 90 min. After 90-min incubation, the developed fluid from the clot was discarded very carefully and tubes were weighed again.

In silico Studies

Selection of Ligands

The compounds of *D. pentagyna* such as Kaempferol (PubChem CID: 5280863), Quercetin, (PubChem CID: 5280343), Isorhamnetin (PubChem CID: 5281654), Lupeol (PubChem CID: 259846), and Betulin (PubChem CID: 72326) were loaded in 2DSDF format, and the ligands were then minimized and turned to pdbqt format using PyRx tools to calculate the binding affinity in these targets.

Preparation of Targeted Proteins

The three-dimensional structures of the mu-opioid receptor-Gi protein complex (PDB: 6DDF) (30), the structure of celecoxib bound at the COX-2 active site (PDB: 3LN1) (31), human microsomal prostaglandin E synthase 1 (PDB: 3DWW) (32), kappa-opioid receptor (PDB: 6VI4) (26, 33), and tissue-type plasminogen activator (PDB: 1TPM) (34) were downloaded from the RCSB Protein Data Bank in pdb format. Gasteiger charges and hydrogen atoms were employed in a unique way to synthesize proteins. This procedure also avoided the use of any extraneous solvents. Changes in proteins were yielded in different parameters, for example, selenomethionine (MSE) was replaced by methionine (MET), Bromo UMP was replaced by UMP (U), and methylselenyl-dUMP (UMS) was replaced by UMP (U), methylselenyl-dCMP (CSL). The Dunbrack 2010 rotomer library was used to replace several of the side chains that were yet unfinished. In Chimera, the residues were retained in AMBER ff14sB mode and Gasteiger mode. As a result, all proteins have been shrunk to the least level of energy (35).

Docking Analysis

PyRx Autodock Vina was used to accomplish the binding interaction on the generated protein-ligand complexes (36). Docking experiments were conducted using PyRx's semiflexible docking device. The phytochemicals were converted to PDBQT formats using PyRx AutoDock tools. The stiffness of proteins and ligands was maintained throughout this investigation. Ligand molecules had provided ten degrees of freedom (37). During the transformation, a grid box with an active site in the center was built. Finally, utilizing the BIOVIA Discovery Studio Visualizer 2020, docking sites for the best connection strategies were examined (24).

Statistical Analysis

Statistical analysis of data is exhibited as mean ± SEM using Graph pad prism version 5.0. Statistical significance was dictated by one-way ANOVA and was accomplished by Dunnett's multiple comparison test. *P*-values of less than 0.05, 0.01, and 0.001 were considered statistically significant.

RESULTS

Phytochemical Screening

The qualitative phytochemical screening was performed to ensure the presence or absence of secondary plant metabolites. The phytochemical screening result of MEDP showed in **Table 1**.

Acetic Acid-Induced Writhing

Methanol extracts of *D. pentagyna* (MEDP) (100, 200, and 400 mg/kg) produced a dose-dependent analgesic response which moderately reduced acetic acid-induced writhing. Dose 100, 200, and 400 mg/kg produced 19.23 ($^{**}p < 0.01$), 31.27% ($^{***}p < 0.001$), and 50.86% ($^{***}p < 0.001$) inhibition respectively. Diclofenac-Na formed 64.94% ($^{***}p < 0.001$) of inhibition. The result of acetic acid-induced writhing has been shown in **Figure 1**.

TABLE 1 | Qualitative phytochemical screening of the methanol extracts of *Dillenia pentagyna*.

Phytochemicals	Observations
Carbohydrates	+
Alkaloids	+
Flavonoids	+
Tannins	+
Terpenoids	+
Glycosides	+
Steroids	+
Saponin	–
Resin	–
Phenol	+
Polyphenol	+
Protein	+
Anthrocyanin	–
Cholesterol	+

(+) = Present, (–) = Absent.

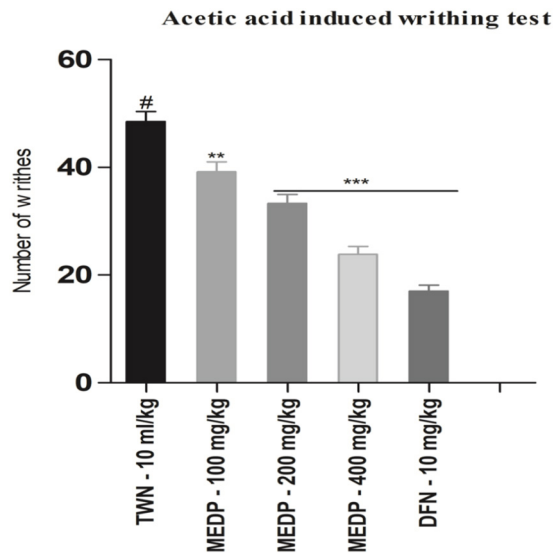

FIGURE 1 | Effects of various test doses of MELM on the acetic acid-induced writhing test in mice (n = 6). Values are presented as mean ± SEM; one-way ANOVA followed by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered as significant compared with the control, where # is designated as control. MEDP = methanol extract of *Dillenia pentagyna*, TWN = 1% Tween-80 and DFN = diclofenac-Na.

Formalin-Induced Licking

Oral administration of MEDP at different concentrations showed a moderate reduction of paw licking in both early and late phases in formalin-induced pain methods. In the early phase, 18.15%, 47.49% ($^{**}p < 0.01$), and 59.98% ($^{***}p < 0.001$) had been reported for MEDP 100, 200, and 400 mg/kg, respectively. On the other hand, 14.63%, 29.92% ($^{**}p < 0.01$), and 41.29% ($^{***}p < 0.001$) licking inhibition was recorded in the late phase for MEDP 100, 200, and 400 mg/kg, respectively. Connecting to the standard drug (diclofenac-Na)

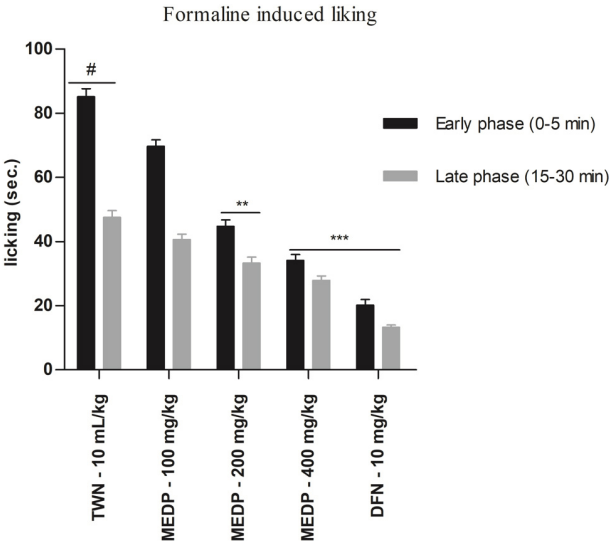

FIGURE 2 | Effects of various test doses of the MELM on the formalin-induced paw licking study in mice (n = 6). Values are presented as mean ± SEM; one-way ANOVA followed by Dunnett's test. $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered as significant compared with the control, where # is designated as control. MEDP = methanol extract of *Dillenia pentagyna* TWN = 1% Tween-80 and DFN = diclofenac-Na.

showed 76.32% ($^{***}p < 0.001$) and 72.18% ($^{***}p < 0.001$) of inhibition in both the early and late phases. The consequence of the formalin-induced paw licking test has been shown in **Figure 2**.

Antipyretic Activity

Methanol extracts of *D. pentagyna* (MEDP) significantly ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) reduces the fever at the dose-dependent manner. The body temperature of mice has been decreased with the administration of MEDP (200 and 400 mg/kg). MEDP 400 mg/kg showed almost similar activity to the standard drug paracetamol. The summary of hyperpyrexia reduction has been shown in **Table 2**.

Castor Oil-Induced Diarrhea

In this experiment, oral administration of MEDP 100, 200, and 400 mg/kg showed significant dose-related inhibition of defecation frequency when compared to loperamide 5 mg/kg. MEDP administered at the dose of 100, 200, and 400 mg/kg showed 29.31%, 51.03% ($^{**}p < 0.01$), and 60.68% ($^{***}p < 0.001$) reduction, respectively, where, standard drug loperamide (5 mg/kg) yielded 78.13% ($^{***}p < 0.001$) of retardation. Result is shown in **Table 3**.

Charcoal-Induced Intestinal Transit in Mice

In this study, the results found that, after the administration of MEDP 100 mg/kg, charcoal was traveled in the small intestine up to (31.93 ± 0.83) cm. Besides, charcoal meal

TABLE 2 | Antipyretic activity of the methanol extracts of *Dillenia pentagyna* in yeast induced pyrexia method.

Group	Initial rectal temperature before yeast injection (°F)	Rectal temperature at yeast injection and after the administration of sample (°F)				
		After 18 h	1st hour	2nd hour	3rd hour	4th hour
Control (Tween – 10 mg/mL)	98.90 ± 0.12	100.40 ± 0.31	100.60 ± 0.29	102.60 ± 0.20	102.30 ± 0.19	102.50 ± 0.19
Paracetamol (100 mg/kg)	98.60 ± 0.10	101.2 ± 0.31	99.20 ± 0.12**	97.90 ± 0.19***	96.90 ± 0.50***	96.30 ± 0.09***
MEDP (100 mg/kg)	98.10 ± 0.08	100.1 ± 0.50	100.0 ± 0.56	99.70 ± 0.33*	99.20 ± 0.65*	98.35 ± 0.33*
MEDP (200 mg/kg)	98.27 ± 0.06	100.4 ± 0.70	98.10 ± 0.40*	98.90 ± 0.30*	98.80 ± 0.65**	97.56 ± 0.09**
MEDP (400 mg/kg)	98.90 ± 0.07	100.8 ± 0.92	97.70 ± 0.84***	97.36 ± 0.66***	96.95 ± 0.58***	96.80 ± 0.77***

*Values are expressed as mean ± SEM or percentage (n = 6). The data were analyzed by one-way ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with control. MEDP = Methanolic extract of Dillenia pentagyna.*

TABLE 3 | Castor oil induced diarrheal test *Dillenia pentagyna.*

Treatment	Dose (mg/kg)	Average no. of feces (4 h)	% of inhibition
TWN#	10 mL/kg	14.5 ± 0.08	0
LPM	5	3.17 ± 0.20***	78.13
MEDP	100	10.25 ± 0.60	29.31
MEDP	200	7.1 ± 1.22**	51.03
MEDP	400	5.7 ± 0.73**	60.68

*Effects of various test doses of the MEDP on the castor oil induced diarrhea test in mice (n = 6). Values are presented as mean ± SEM; One-way analysis of variance (ANOVA) followed by Dunnett's test. **p < 0.01, and ***p < 0.001 is considered as significant compared with the control, where # is designated as control. MELM = methanol extract of Dillenia pentagyna, TWN = 1% Tween-80 and LPM = Loperamide.*

has been traveled almost (24.65 ± 0.73) (*$p < 0.05$) cm and (18.70 ± 1.10) (***$p < 0.001$) cm for MEDP 200 and 400 mg/kg, respectively. Under similar experimental conditions, only (11.23 ± 1.12) (***$p < 0.001$) cm charcoal meal traveled in the small intestine of mice at a dose of loperamide 5 mg/kg. The peristaltic index and percent of inhibition in castor oil-induced gastrointestinal motility have been shown in **Table 4**.

TABLE 4 | Castor oil-induced gastrointestinal motility of the methanol extracts *of Dillenia pentagyna*.

Treatment	Dose (mg/kg)	Distance travel by charcoal meal (cm)	Peristalsis index	Inhibition (%)
TWN#	10 mL/kg	38.60 ± 1.08	10.6	0
LPM	5	11.23 ± 1.12***	38.35	70.90
MEDP	100	31.93 ± 0.83	18.44	17.27
MEDP	200	24.65 ± 0.73*	22.27	36.13
MEDP	400	18.70 ± 1.10***	29.65	51.55

*Effects of various test doses of MEDP on castor oil induced charcoal meal transit study in mice (n = 6). Values are presented as mean ± SEM; One-way analysis of variance (ANOVA) followed by Dunnett's test. *p < 0.05 and ***p < 0.001 is considered as significant compared with the control, where # is designated as control. MEDP = methanol extract of Dillenia pentagyna, TWN = 1 % Tween-80 and LPM = Loperamide.*

Anticoagulant Activity

In vitro thrombolytic test, streptokinase (100 μl) showed 71.42 % (***$p < 0.001$) clot lysis. In addition, 4.70% clots lysis was observed when the mice were treated with sterile distilled water where MEDP showed only 17.76% (*$p < 0.05$) clot lysis in the clot lysis study model. A statistical representation of clot lysis percentage by negative control, positive control, and MEDP has been shown in **Figure 3**.

Molecular Docking Analysis

The molecular interactions of 6DDF and components of *D. pentagyna* ranked as Lupeol > Betulin > Quercetin > Isorhamnetin > Kaempferol. The docking rank for the 3LN1 receptor and selected elements is as follows:

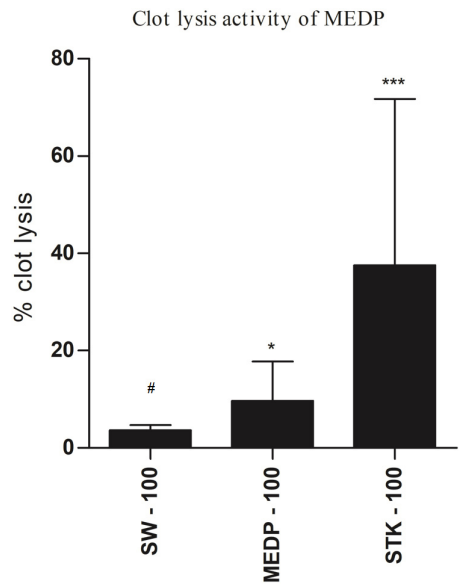

FIGURE 3 | Clot lysis effects by saline water, streptokinase, and MEDP. Anticoagulant values are presented as mean ± SEM (*n* = 5); One-way ANOVA was followed by Dunnett's test. *$p < 0.05$ and ***$p < 0.001$ were considered as significant compared with the control, where # is designated as control. MEDP = methanol extract of *Dillenia pentagyna* leaves, SW = saline water, SPK = streptokinase.

Betulin > Lupeol > Quercetin > Isorhamnetin > Kaempferol. The best hit of 3DWW with the Quercetin was possessed *via* a series of residues (glu77, met76, arg73, his72, and arg73). Besides, betulin and lupeol possessed the best binding affinity to the 6VI4 and 1TPM receptors. Betulin binds to the kappa-opioid receptor through pro238 and phe147 residues where lupeol interacted with the tissue-type plasminogen activator through his18 and ser20 residues and the binding scores of these complexes are –9.2 and 7.0 (kcal/mol), respectively. The overall interactions have been pointed out in **Table 5** and **Figure 4**.

DISCUSSION

In this study, the analgesic, anti-inflammatory, antipyretic, and antidiarrheal activity of MEDP were evaluated to identify the claims which were made in traditional medicine. The results obtained from the study revealed that the MEDP produced a moderate dose-dependent inhibition of pain response. The standard drugs, however, showed a greater effect than the extract. The acetic acid-induced writhing is a manifestation of peripheral pain (38). Induction of several endogenous biochemical pain mediators like PGE2, PGI2, PGF2α (39), etc. are responsible for further stimulation of nociceptive neurons and an additional amplification of pain sensation takes place through capillary permeability (40). The pain inhibitory capability showed by the extract is a potential reflection of the apprehension of prostaglandins release mimicking the same mechanism followed by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). A moderate exhibitory activity showed by MEDP in the formalin-induced licking test can be differentiated between the central and peripheral pain components. Pain induced by formalin is biphasic having an early phase (0–5 min) and the other late phase (15–20 min) (41). Activation of C-fiber neurons potentiates the early phase followed by the release of inflammatory mediators causing the late phase (42). A satisfactory response in both phases is reflected by centrally acting drugs. On the other hand, a peripherally acting drug is only effective in the late phase by inhibiting prostaglandin synthesis. The study result of MEDP reveals a very significant antipyretic effect in Brewer's yeast-induced elevation of body temperature in a dose-dependent way. It is already documented that elevation of body temperature occurs by the proinflammatory cytokines productions like interleukin-1β (IL-1β) and IL-6, interferon-α (IFN-α), and tumor necrosis factor-α (TNF-α), and prostaglandins like PGE2 and PGI2 by acting on the brain (43, 44). Paracetamol and other antipyretics used in fever management work by reducing prostaglandin levels and acting on cyclooxygenase enzymes, enhancing antipyretic messages within the brain and stimulating anti-inflammatory signals at the injury site (45). A significant drop in temperature in yeast-induced pyrexia is noticed in methanol and petroleum ether fractions of *D. pentagyna* which is higher than even paracetamol and suggests that the plant possesses a significant antipyretic property. Prostaglandins produced in pyrexia are also an easy target of flavonoids (46). Several studies have confirmed the use of medicinal plants against diarrhea, i.e., antispasmodic activity slows intestinal movement, suppresses gut motility, promotes water adsorption, or decreases intraluminal fluid aggregation (47). Previously, numerous mechanisms were suggested to understand the diarrheal action of castor oil, including suppression of gastrointestinal Na^+-K^+ ATPase response to minimize normal fluid accumulation, amplification of adenylate cyclase, or effective secretion facilitated by mucosal cAMP (48), enhancement of prostaglandin production, platelet stimulation factor and currently, nitric oxide was believed to lead to the production of prostaglandin (49). Flavonoids and carbohydrates collected from selective conventional medicinal plants in Bangladesh have been claimed to have antidiarrheal capabilities (50). Recently, a variety of medicinal plants had been examined and it was found that the antidiarrheal action of these plants was attributed to flavonoids, alkaloids, tannins, triterpenes, saponins, reducing sugar, and sterols, existing in them (51). Again, the thrombosis or formation of the blood clot is a result of summative cascade action where damaged regions of the endothelial cell surface or blood vessel are barricaded by the platelets deposition, tissue factor, and fibrin (52) where the foundational stage is governed by platelets when the activated platelets form platelets to platelets bonds followed by further binding to the leucocytes and bringing

TABLE 5 | The docking score of screened phytochemical's binding at the active site of the selected proteins.

Compounds	PubChem CID	Analgesic, Anti-inflammatory and Anti-pyretic (Kcal/mol)			Antidiarrheal (Kcal/mol)	Thrombolytic (Kcal/mol)
		6DDF	3LN1	3DWW	6VI4	1TPM
Kaempferol	5280863	–7.0	–6.6	–7.0	–8.7	–5.6
Quercetin	5280343	–7.3	–6.9	–7.5	–8.8	–5.9
Isorhamnetin	5281654	–7.3	–6.7	–7.5	–8.7	–5.7
Lupeol	259846	–8.8	–7.5	–2.6	–9.1	–7.0
Betulin	72326	–8.4	–7.7	–1.4	–9.2	–5.6
Standard drugs (Ibuprofen/Indomethacin/ Loperamide/Streptokinase)	3672/3715/ 3955/9815560	–7.4	–6.4	–8.2	–7.1	–6.4

PDB: 6DDF = Mu Opioid Receptor-Gi Protein Complex, PDB: 3LN1 = Structure of celecoxib bound at the COX-2 active site, PDB: 3DWW = human microsomal prostaglandin E synthase 1, PDB: 6VI4 = Kappa Opioid Receptor, PDB: 1TPM = tissue-type plasminogen activator.

them into a complex process of plaque formation and growth (53). So a thrombolytic agent should possess the aptness to lyse clot by disrupting the fibrinogen and fibrin and plasmin is one of the natural antithrombotic agents (54). Fibrinolysis results from the activation of cell surface-bound plasminogen to plasmin. Streptokinase, which is a bacterial plasminogen activator and a widely used thrombolytic agent, can convert additional plasminogen to plasmin (55). Phytochemicals can be prospective agents in blood clot lysis. Studies have shown that secondary phenolic compounds, such as flavonoids, have a wide range of

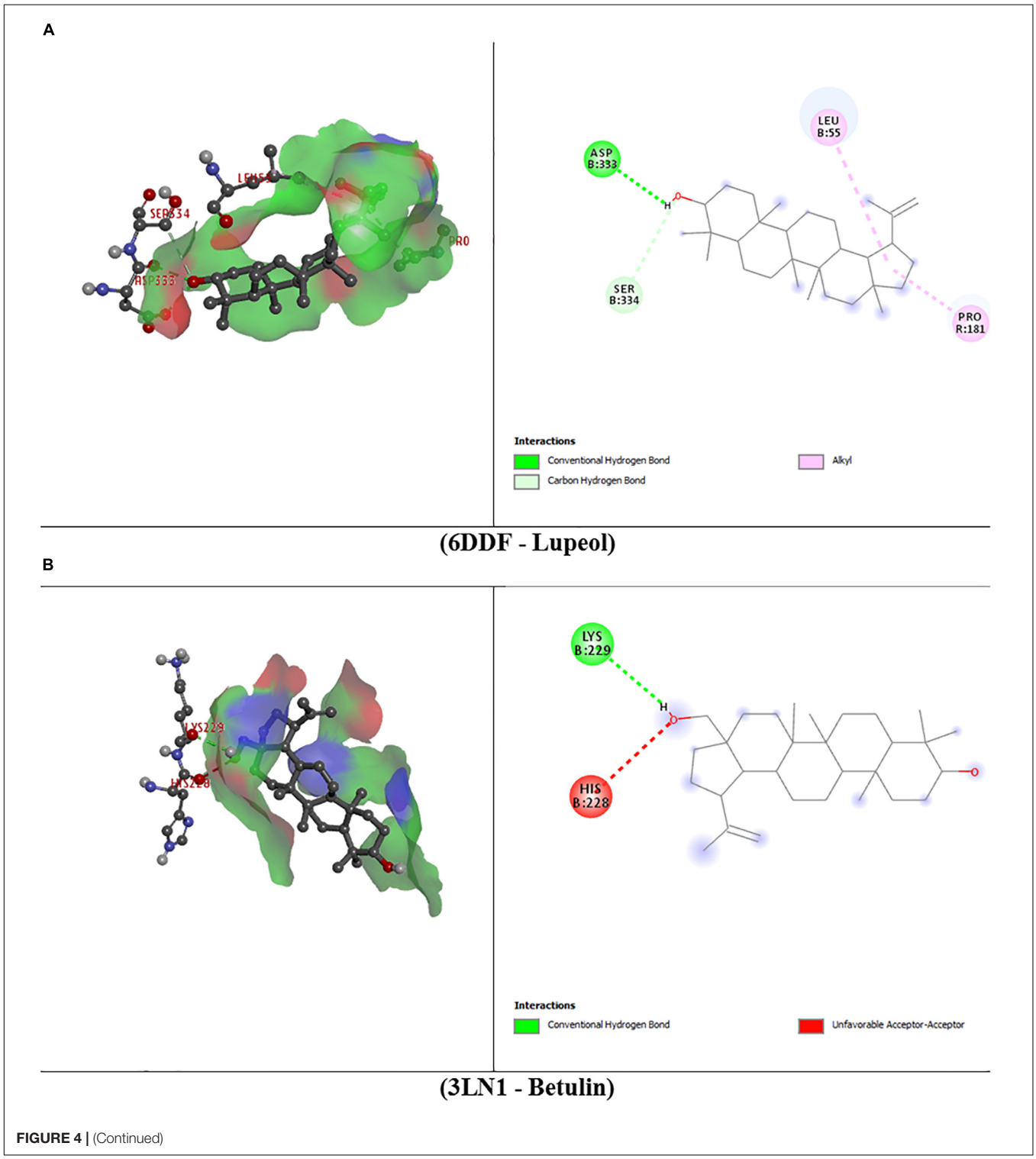

FIGURE 4 | (Continued)

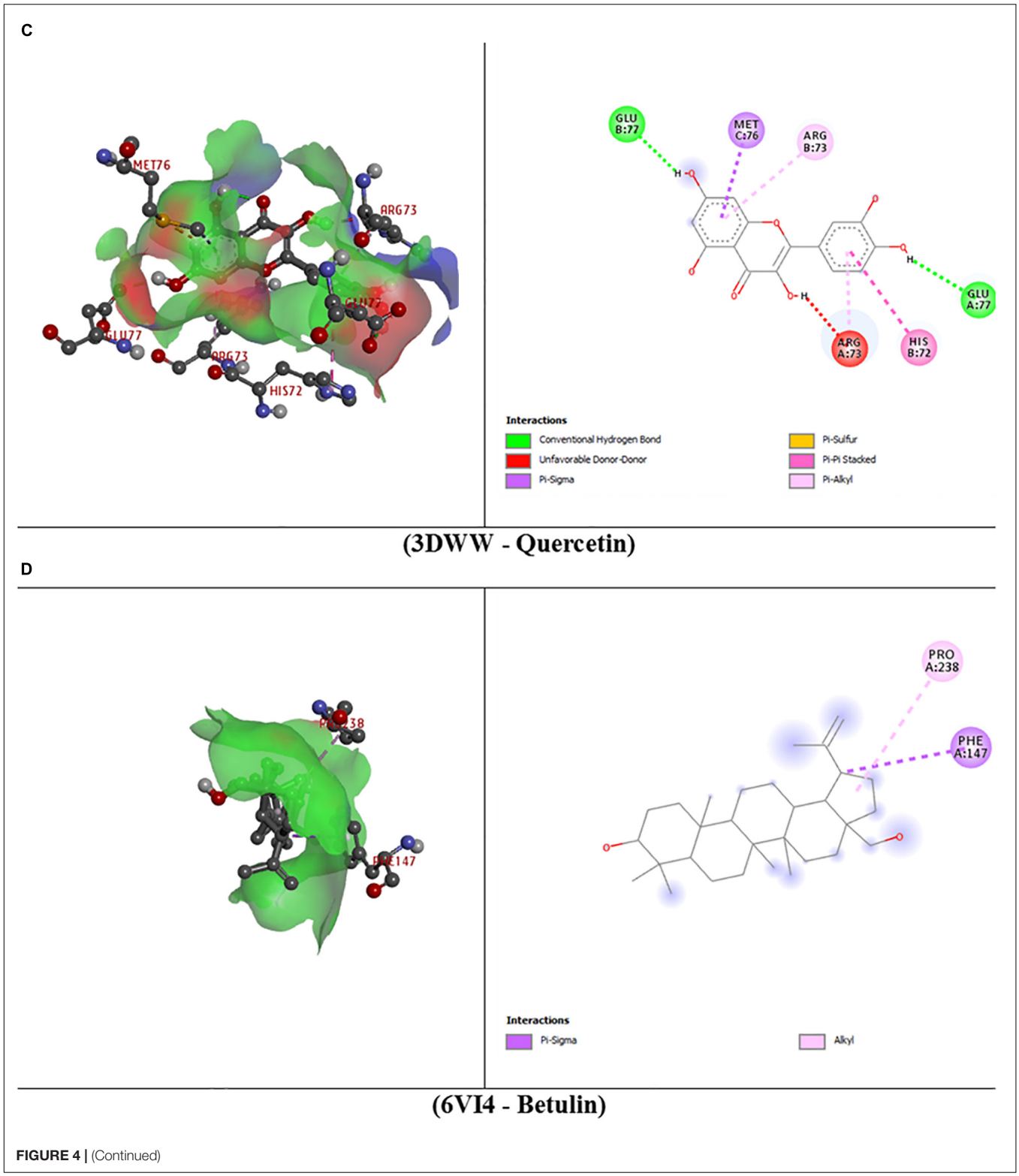

FIGURE 4 | (Continued)

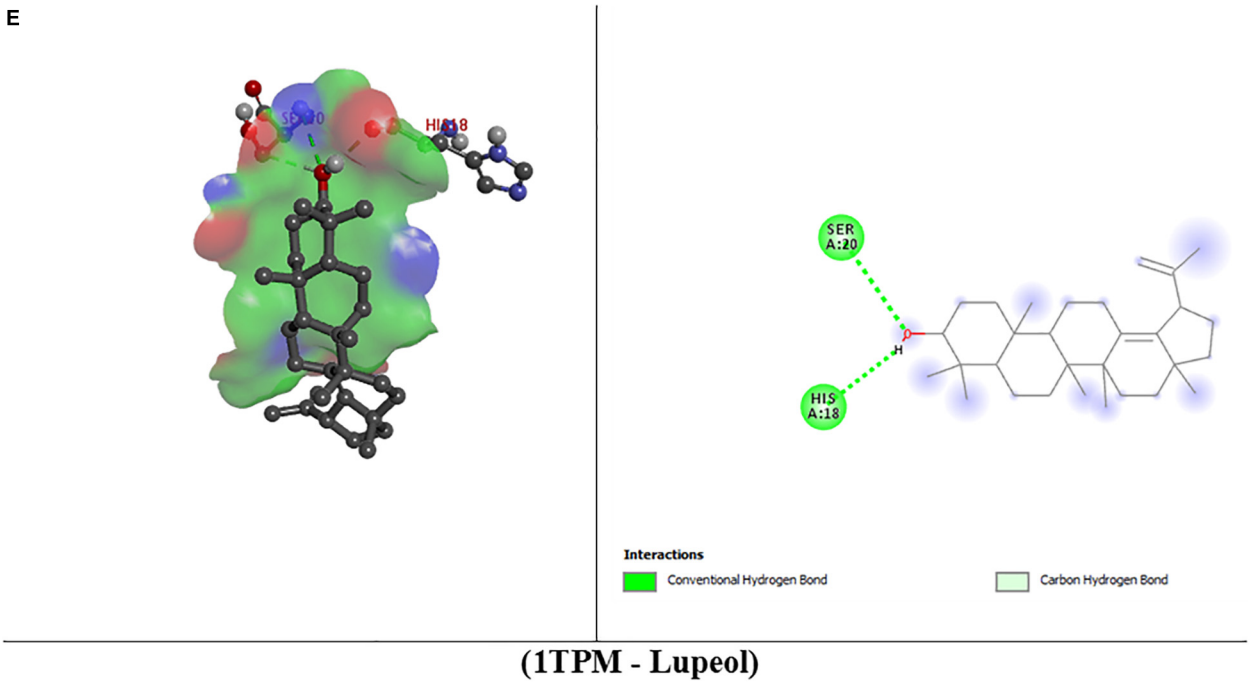

FIGURE 4 | 3D and 2D presentations of the best key interactions in the binding pocket for selected ligands and receptors where panel **(A)** represents the interaction of mu-opioid receptor-Gi protein complex and lupeol, panel **(B)** denotes celecoxib bound at the COX-2 active site and betulin, panel **(C)** denotes microsomal prostaglandin E synthase 1 and quercetin, panel **(D)** denotes kappa-opioid receptor and betulin, panel **(E)** denotes the interaction of tissue plasminogen activator and lupeol, respectively.

pharmacological properties (56). Many studies are being done on flavonoids to see if they help fight free radicals, prevent heart disease and cancer, and even protect the liver from damage and inflammation (57). The immune system and inflammatory cells are significantly impacted by certain flavonoids. Flavonoids such as keampferol, quercetin, apigenin, luteolin, and hesperidin have been found to have anti-inflammatory and analgesic activities (56). Inflammation-inducing enzyme systems, such as serine-threonine protein kinases and tyrosine may be affected directly by the antioxidant capabilities of flavonoids (58). Kaempferol's anti-inflammatory properties were attributed to a slew of mechanisms. This drug inhibits the release of IL-6, IFN-a, and TNF-a (59). The transcriptional activation of Nrf2-regulated genes and the reduction of the DNA-NF-B binding activity of the myeloid differentiation factor 88 (MDF88) are likewise reduced by Kaempferol (60, 61). Quercetin's anti-inflammatory effects also include its capacity to prevent the synthesis of TNF-a in macrophages and the creation of IL-8 in lung cells (A549). It has been shown that quercetin reduces apoptotic neuronal cell death by inhibiting TNF-1 and IL-1 mRNA expression levels in LPS-stimulated glial cells. Quercetin also inhibits inflammation-causing enzymes [cyclo-oxygenase (COX) and lipoxygenase (LOX)] in RAW 264.7 cells and restricts LPS-induced inflammation by inhibiting Src and Syk in RAW 264.7 cells-mediated tyrosine phosphorylation of PI3K-(p85) and subsequent activation of TLR4/MyD88/PI3K (62). Lupeol's anti-inflammatory activities were also demonstrated in studies using A23187-stimulated macrophages and triterpenes such pretreatment with lupeol to reduce PGE2 production (63). In the perspective of these observations, flavonoids and triterpenes are critical for the treatment of a wide range of health conditions, namely, chronic pain, acute inflammation, fever, diarrhea, thrombosis, and more. On the other hand, molecular docking is the process of finding the lead compound and hit compound from molecular databases using a scoring function, which has greatly increased the screening efficiency over the classic screen approach. Virtual screening has a wide range of uses. The integrated approach thrives swiftly, especially considering the exponential rise of high-throughput high-performance computing machine learning and deep learning methods (7). Considering this concept, this study also included a molecular docking study and some of the previously identified compounds of *D. pentagyna* have been docked to the mu-opioid receptor-Gi protein complex, COX-2 receptor, microsomal prostaglandin E synthase 1, and kappa-opioid receptor and the results showed prominent binding affinity of receptors and ligands. Among all compounds, Lupeol, Betulin, and Quercetin yielded the best docking scores which means these compounds have many capabilities of being the best lead compounds and better possibilities of being a good drug for the treatment of prospective diseases. With further research on cell viability tests and *in vivo* studies, this finding may have important implications in the treatment of cardiovascular diseases which is increasing at an alarming rate. Since the drugs used for cardiovascular diseases

are not economical and not accessible to the greater section of society, the application of this study may be a boon for them.

CONCLUSION

According to the experimental results, MEDP possessing an elevated amount of bioactive phytoconstituents can be a remarkable wellspring of analgesic and antipyretic activities with moderate anti-inflammatory, antidiarrheal, and anticoagulant therapies. The study attempts to evaluate its traditional uses in the management of pain, fever, and diarrhea though further studies are still recommended to ascertain its absolute safety and efficacy profile in the long term use and the establishment of a proper mechanism of action for exerted pharmacological activities. Future prospects of MEDP can be evaluated properly if the plant extract/respective phytochemicals can be subjected to extensive preclinical studies followed by clinical trials. Thus, for the drug-development process, future researchers can give the COX inhibitory potentials of MEDP an exclusive focus.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the all biological activity screenings were conducted according to the ethical standards laid down in the Declaration of Helsinki 2013 (64). Animal models were handled and treated according to the principles of the Swiss Academy of Medical Sciences and Swiss Academy of Sciences and were euthanized following the Guidelines for the Euthanasia of Animals: 2013 edition.

AUTHOR CONTRIBUTIONS

NS, NUE, H-JC, and FA conceptualized and designed the study protocol. NS, NUE, and MTIT prepared the plant extract, designed protocols, conducted the investigations, and collected data. NUE, SA, SR, and AAM calculated the data. NUE and SA revised the manuscript. SA, NUE, NS, and SR wrote the manuscript. SA, SR, and FA supervised and monitored the research. All authors read and approved the final manuscript for publication.

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Dejian Huang,
National University of Singapore,
Singapore

REVIEWED BY
Yafang Shao,
China National Rice Research Institute (CAAS), China
Janet Alejandra Gutierrez-Uribe,
Monterrey Institute of Technology and Higher Education (ITESM), Mexico

*CORRESPONDENCE
Lena Gálvez Ranilla
lgalvez@ucsm.edu.pe

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Diversity of the Peruvian Andean maize (*Zea mays* L.) race *Cabanita*: Polyphenols, carotenoids, *in vitro* antioxidant capacity, and physical characteristics

Iraida Sara Fuentes-Cardenas[1], Rody Cuba-Puma[1], Shaneri Marcilla-Truyenque[1], Huber Begazo-Gutiérrez[2], Gastón Zolla[3], Claudia Fuentealba[4], Kalidas Shetty[5] and Lena Gálvez Ranilla[1,6]*

[1]Laboratory of Research in Food Science, Universidad Catolica de Santa Maria, Arequipa, Perú, [2]Estación Experimental Agraria Arequipa, Instituto Nacional de Innovación Agraria (INIA), Arequipa, Perú, [3]Laboratorio de Fisiologia Molecular de Plantas, PIPS de Cereales y Granos Nativos, Facultad de Agronomia, Universidad Nacional Agraria La Molina, Lima, Perú, [4]Escuela de Alimentos, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile, [5]Department of Plant Sciences, North Dakota State University, Fargo, ND, United States, [6]Escuela Profesional de Ingeniería de Industria Alimentaria, Facultad de Ciencias e Ingenierías Biológicas y Químicas, Universidad Catolica de Santa Maria, Arequipa, Perú

The high diversity of the Peruvian Andean maize (*Zea mays* L.) represents a biological and genetic heritage relevant for food security, but few studies are targeted toward its characterization and consequent valorization and preservation. The objective of this study was to evaluate the potential of the Peruvian Andean maize race *Cabanita* with respect to its bioactive profiles (free and bound phenolic and carotenoid composition), physical characteristics, and *in vitro* antioxidant properties. Maize landraces with variable kernel pigmentation were collected from two provinces (Caylloma and Castilla) within the Arequipa region (among ten Andean sites) and the phytochemical profile was evaluated by Ultra High-Performance Liquid Chromatography with diode array detector (UHPLC-DAD). All maize samples were important sources of phenolic compounds mainly soluble *p*-coumaric and ferulic acid derivatives whereas anthocyanins were only detected in maize with partially red pigmented kernels. Major phenolic compounds in the bound phenolic fractions were ferulic acid and its derivatives along with *p*-coumaric acid. Carotenoid compounds including xanthophylls such as lutein, lutein isomers, and zeaxanthin were only detected in orange and white-yellow pigmented maize and are reported for the first time in Peruvian landraces. The multivariate analysis using Principal Components Analysis (PCA) revealed low variability of all data which may indicate a level of similarity among maize samples based on evaluated variables.

However, maize grown in Caylloma province showed more homogeneous physical characteristics and higher yield, whereas higher phenolic contents and antioxidant capacity were observed in maize from Castilla. Samples CAY (yellow-pigmented kernel, Castilla) and COM (orange-pigmented kernel, Caylloma) had the highest total phenolic (246.7 mg/100 g dried weight basis, DW) and carotenoid (1.95 μg/g DW) contents among all samples. The variable Andean environmental conditions along with differences in farming practices may play a role and should be confirmed with further studies. Current results provide the metabolomic basis for future research using integrated omics platforms targeted toward the complete characterization of the ethnic-relevant maize race *Cabanita*.

KEYWORDS

Peruvian maize, phenolic compounds, carotenoids, antioxidant capacity, biodiversity, *Zea mays* L.

Introduction

Peru is in the central western region of South America and has been considered a megadiverse country since 2002 according to the Cancun Statement recognized by the United Nations (1, 2). Around 84 of the 117 life zones in the planet are found in Peru (3). The Andes Mountains located along its territory creating a complex geography with diversity of climates and ecosystems contains unique biodiversity hotspots (3, 4). These natural features together with the targeted selection and adaptation of several plant species by ancient Peruvian inhabitants over thousands of years have given rise to the high crop biodiversity found presently in Peru (3).

Maize (*Zea mays* L. ssp. *mays*) is one of the most important staple foods in Peru and is currently cultivated from regions at sea level to different Andean highland areas (5). The maize genetic diversity is classified into races which are defined as "a group of related individuals with enough characteristics in common to permit their recognition as a group" (6). A race of maize is comprised by landraces that are highly heterogeneous and genetically diverse open-pollinated dynamic populations developed under traditional farming systems (7–9). The landraces belonging to a same race show similar morphological characteristics, geographical distribution, ecological adaptation, and cultural importance (10). Peru and Mexico have been considered the two primary centers of maize domestication and both countries have the largest number of maize races in the world (11, 12). The Andean region is likely the zone with the major diversity of maize races in the world in terms of varied phenotypic characteristics such as kernel and ear morphology and pigmentations (13, 14). Nevertheless, the scientific information about Andean maize landraces is very limited in comparison with the significant research focused on Mexican germplasm (5).

The Peruvian maize genetic diversity was initially classified by Grobman et al. (15) who described 49 races and collected around 3931 accessions that have been preserved *ex situ* by the *Programa de Investigación y Proyección Social en Maíz* (PIPS *Maíz*) located at UNALM (*Universidad Nacional Agraria La Molina*, Lima, Peru). The Peruvian Ministry of Environment has conducted a new collection of maize landraces since 2013 with the aim to update the maize race classification (14). This is an ongoing process which is also considering the relevance of the cultural background and uses associated with each maize race. As a result, 52 races have been identified including new races and removing others (14).

The maize race *Cabanita* has been used as staple food since pre-Hispanic times in the southern Andean region of Arequipa in Peru. Its name likely derives from "Cabanaconde" which is the district name located in the Caylloma province where it is currently grown at around 3000 m above sea level on average. This amylaceous maize has importance at nutritional and economical levels for local indigenous communities since it is used in several traditional food preparations and sometimes represents their main economic income. In fact, the production of amylaceous maize has been partially responsible for the evident overcoming of rural poverty in Peru. However, the introduction of improved hybrids by some farmers may lead to a possible genetic erosion that should be avoided for preserving its genetic heterogeneity, which is the foundation for overall food crop resilience. The race *Cabanita* was not considered in the first classification probably due to the few samples collected from this region at that time; however, the current classification includes this race (14). It has been reported that its geographical distribution encompasses not only Arequipa, but also Moquegua and

Tacna regions which are also located in the southern area of Peru (14).

The Latin American maize diversity has been highlighted as an important biological heritage that may play a fundamental essential role on the worldwide food security, and resilience to climate change (12). Recently, the genetic diversity of maize landraces has been related to variable bioactive profiles mainly phenolic and carotenoid compounds with different health-relevant functional properties (16). In case of Peru, research has been focused mostly on purple maize (*Kculli* race) because of its high content of anthocyanins (16, 17). Different studies have reported the phenolic composition of Peruvian purple maize and highlighted its associated bioactivity such as the antioxidant, antimicrobial, anti-hypertensive, and anti-cancer potential (18–23). However, limited information exists about the bioactive composition of other Peruvian maize races. Ranilla et al. (24) evaluated part of the wide Peruvian maize diversity in relation to its phenolic-antioxidant bioactive compounds and *in vitro* potential for the management of hyperglycemia and obesity. Traditional races from Arequipa including some samples (only 6) from the race *Cabanita* were evaluated in this study. The *Cabanita* group showed the second highest total ORAC (oxygen radical absorbance antioxidant capacity) value and α-amylase inhibitory activity relevant for hyperglycemia prevention after the *Kculli* group (purple-pigmented maize) (24). Hence, strategies integrating critical stakeholders for sustaining conservation coupled with advancing multidisciplinary research of Peruvian maize diversity should be developed.

Based on the previous research advances, the objective of the current study was to evaluate the diversity of the Peruvian Andean maize race *Cabanita* with respect to its bioactive profiles (free and bound phenolic and carotenoid composition), physical characteristics, and *in vitro* antioxidant properties. A collection of new *Cabanita* germplasm from ten Andean locations in Arequipa was performed. In addition, the information about the pre-harvest agronomical practices linked to the cultivation of *Cabanita* race was also documented. Results from this study could be further integrated to molecular approaches aimed at the complete characterization of the race *Cabanita* and the future development of targeted health and climate resilience targeted breeding applications for the benefit of indigenous Andean communities.

Materials and methods

Materials

New germplasm of the maize race *Cabanita* was collected from two Andean provinces (Caylloma and Castilla) within the Arequipa region in Peru according to Ranilla et al. (24). The geographical information (coordinates, altitude, localities of origin) and the codification of maize samples are shown in **Table 1**. Around 9–20 and 9–12 ear units per maize type (red, white, yellow, or mixed pigmentation) and at commercial maturity were collected from Caylloma and Castilla provinces, respectively. At least five locations from each province were randomly sampled (total 10 locations) during the traditional harvest periods (May 2019 and June 2019 in case of maize from Caylloma and Castilla, respectively). All samples from Caylloma were collected from the Cabanaconde district (*Comision de Usuarios La Campiña*) whereas maize from Castilla was collected from three districts (Andahua, Ayo, Chachas) (**Table 1**). Maize ears (with husks) were mostly harvested directly from the plant. However, in some cases, ears were sampled when the maize plants were already cut and piled on the land or recently stored in the farmer's warehouses. Samples were stored in cloth bags, protected from the light, and transported at 18–20°C to the laboratory in a maximum period of 34 h. Since ear samples were partially humid, the husks were eliminated and the ears were dried at environmental conditions with light protection until constant weight trying to mimic farmers' postharvest practices but under controlled parameters (18.5–21.5°C, 29–34% of relative humidity). After drying, samples from each location and type were divided in 3–4 groups according to the similarity of their kernel pigmentation and ear characteristics. Each group corresponded to a biological replicate. **Figures 1**, **2** show pictures of ears and kernel samples from Caylloma and Castilla provinces, respectively. In addition, information about the agronomic pre-harvest practices used by local farmers for the cultivation of sampled maize plants was compiled (**Supplementary Tables 1**, **2** for samples from Caylloma and Castilla, respectively).

Dried ear maize samples and their corresponding kernels were used to evaluate the physical characteristics. Then, kernels were separated, pooled (per replicate) and stored at 5°C. A subsample of 50 g kernel was milled in a A11 Basic analytical mill (IKA, Germany) to a powdered flour (500 μm) and stored at –20°C until analysis.

Reagents

Phenolic standards (ferulic acid, *p*-coumaric acid, cyanidin chloride, gallic acid), carotenoid standards (lutein, zeaxanthin), and the Folin-Ciocalteu reagent were from Sigma-Aldrich (St. Louis, MO, USA). The (±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxilic acid (Trolox), and the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), and 2-2′-azino-bis(3ethylbenothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radicals were purchased from Sigma-Aldrich, USA.

TABLE 1 Geographical information of Cabanita maize samples collected from Caylloma and Castilla provinces.

Province	District	Location	Code	Geographical coordinates	Altitude (masl*)
Caylloma	Cabanaconde	Auqui	CAW	S 15° 37′ 05.6″	3110
			CAR	W 72° 00′ 37.8″	
		Cusqui	CCR	S 15° 37′ 30.9″	2964
			CCY	W 72° 00′ 04.1″	
		Huancce-Tranca	CHW	S 15° 36′ 56.4″	3332
				W 71°58′ 19.5′	
		Occollina-Tuntuiguita	COM	S 15° 37′ 41.6″	3310
				W 71° 58′ 49.5″	
		Liguay	CLY	S 15° 38′ 01.6″	3266
				W 71° 58′ 49.0″	
Castilla	Andahua	Huancarani	CHY	S 15° 29′ 50.7″	3347
				W 72° 20′ 48.6″	
		Ajocha	CAY	S 15° 29′ 57.2″	3399
				W 72° 20′ 45.8″	
	Ayo	Subna	CSW	S 15° 33′ 27.5″	2845
			CSR	W 72° 14′ 16.3″	
	Chachas	Alleachaya	CALR	S 15° 30′ 4.1″	3070
				W 72° 16′ 10.7″	
		Pulluguaya	CPW	S 15° 30′ 5.1″	3043
			CPM	W 72° 16′ 14.4″	

*Meters above sea level.

Moisture analysis and physical measurements

The moisture of kernels was determined by a gravimetric method at 105°C until constant weight (25). The physical characteristics were evaluated in both dried ears and kernels per replicate according to the descriptors defined by the *Programa de Investigación y Proyección Social en Maíz* (PIPS Maíz) (26) and CIMMYT (27). The weight (g), length (cm), tip, center and base diameters (cm), and the pith, rachis and cob diameters (cm) were measured in maize ear samples. Additionally, the number of rows per ear and the number of kernels per row were analyzed. In case of kernels, a row per ear with complete and sound grains was selected and 10 kernels were extracted from the central part. The kernel weight (g), length, width, and thickness (mm) were determined.

Extraction of bioactive compounds from maize samples

Free and bound phenolic fractions

The free and bound phenolic fractions were extracted from the powdered maize samples following the methodology of Ranilla et al. (18) and Ranilla et al. (24). A mix of 0.1% HCl methanol/acetone/water (45:45:10, v/v/v) was used for the extraction of the free phenolic compounds. An alkaline hydrolysis with 3 N NaOH was applied on the free phenolic extraction residue for the release of the bound phenolic fraction (18, 24). Final aqueous extracts were kept at −20°C until further analysis.

Carotenoids

Preliminary trials based on the carotenoid extraction conditions reported by Egesel et al. (28) and Fuentealba et al. (29) were performed. The extraction of carotenoid compounds from powdered maize samples was applied according to Egesel et al. (28) with some modifications (30). An amount of 2–2.5 g of sample was mixed with 6 mL ethanol containing 0.01% (w/v) butylated hydroxytoluene (BHT), and then placed in a water bath at 85°C for 5 min. After this, 120 μL 80% KOH (w/v) was added, vortexed for 15 s, and further heated in the water bath for 10 min (with a 10 s mixing at minute 5). Samples were cooled in an ice bath and 3 mL of methanol:ethyl acetate (6:4, v/v) were added. The mixture was vortexed for 20 s and centrifuged at 2800 rpm for 10 min and the extract was stored under refrigeration (5°C). The resulting pellet was reextracted several times with same solvent volume until obtaining a clear final extract. Processed and stored extracts were pooled, first vacuum-evaporated, then concentrated under nitrogen atmosphere, and set to a known volume (3.5–5 mL) with methanol:ethyl acetate (6:4, v/v). All the procedure was carried out under light

FIGURE 1
Ears and kernels of Cabanita maize samples collected from the Caylloma province located in the Arequipa region in Peru. Letters within each code location show the biological replicates.

and oxygen protection as possible. Carotenoid extracts were analyzed by UHPLC immediately after the extraction process the same day.

Analysis of the total phenolic contents

The Folin-Ciocalteu method was applied to measure the total phenolic contents (TPC) in the free and bound phenolic fractions from maize samples (31). The absorbance was recorded at 755 nm, and results were expressed as mg of gallic acid equivalents (GAE) per 100 g sample (dry weight basis, DW).

Analysis of phenolic compounds by ultra high-performance liquid chromatography

A volume of 5 μL of the free and bound phenolic extracts previously filtered with a polyvinylidene difluoride filter (PVDF, 0.22 μm) was injected in an Ultimate 3000 RS UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) with a quaternary pump, autosampler, and coupled to a Vanquish diode array detector. Phenolic compounds were separated using a Kinetex C18 analytical column (100 × 2.1 mm i.d., 1.7 μm) with a Kinetex C18 guard column (5 × 2.1 mm i.d., 1.7 μm) (Phenomenex Inc., Torrance, CA, USA). The binary gradient elution with 0.1% formic acid and acetonitrile mobile phases, and chromatographic conditions were the same as used previously by Ranilla et al. (18) and Vargas-Yana et al. (32). The chromatograms and peaks were processed using the Chromeleon SR4 software version 7.2 (Thermo Fisher Scientific). Phenolic compounds were identified based on their retention times and spectral characteristics in comparison with external standards and the library spectra. The quantification was performed using calibration curves obtained with aglycone phenolic standards ($r^2 \geq 0.9990$). Peaks corresponding to phenolic acids (*p*-coumaric and ferulic acid) and their derivative compounds (with similar spectral features to those of pure standards, but with different retention times) were detected at 320 nm. The phenolic acid derivatives (*p*-coumaric and ferulic acid derivatives) were quantified using their corresponding

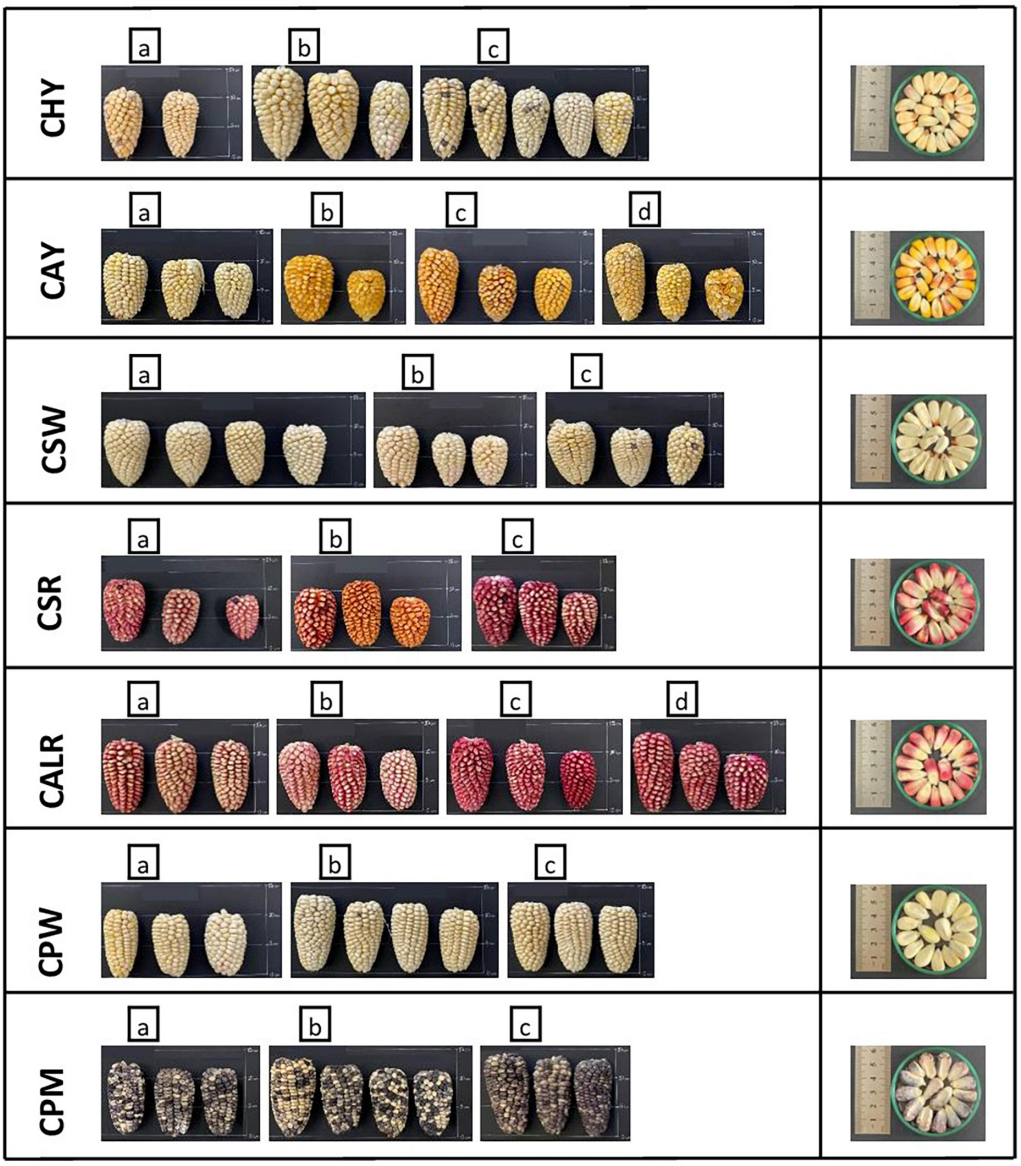

FIGURE 2
Ears and kernels of Cabanita maize samples collected from the Castilla province located in the Arequipa region in Peru. Letters within each code location show the biological replicates.

phenolic acid calibration curves (*p*-coumaric and ferulic acid, respectively). Anthocyanins were detected at 525 nm and quantified as cyanidin chloride. Results were expressed as mg per 100 g sample DW.

Analysis of carotenoid compounds by ultra high-performance liquid chromatography

Carotenoid extracts were filtered with a PVDF filter (0.22 μm) and injected (50 μL) in the same UHPLC system used for the analysis of phenolic compounds. Carotenoids were separated using a YMC carotenoid C30 analytical column (150 × 4.6 mm i.d., 3 μm) protected with a YMC C30 guard column (10 × 4.0 mm, 3 μm) (YMC CO., LTD, Kyoto, Japan). The chromatographic process was carried out at a flow rate of 1.7 mL/min, sample temperature of 10°C, column temperature of 30°C, and eluates were monitored at 450 nm. The reverse phase elution was performed using a ternary gradient elution with methanol (A), dichloromethane (B), and acetonitrile (C). The initial conditions were A/B/C (76/5/19) from 0 to 3.2 min, then solvent B was increased to 34% at 23.3 min (A/B/C: 52.8/34/13.2), and changed to initial

conditions until the 25.1 min. The column was equilibrated for 2.9 more min (total run time of 28 min). The carotenoid peak identification was conducted by comparison of their retention times and spectral characteristics with those of external standards. Lutein and zeaxanthin were quantified using calibration curves ($r^2 \geq 0.9900$) built with pure carotenoid standards. Lutein isomers were quantified as lutein. Results were presented as μg per g sample DW.

2,2-Diphenyl-1-picrylhydrazyl radical inhibition antioxidant capacity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) antioxidant activity was determined in the free and bound phenolic extracts. In addition, the hydrophilic and lipophilic fractions from maize samples were obtained and analyzed. The extraction procedure reported by Fuentealba et al. (29) and Campos et al. (33) was followed with some modifications. For the hydrophilic fraction, a volume of 5 mL 80% methanol was added to 0.2 g of maize sample and mixed in an orbital shaker for 30 min at 230 rpm (environmental conditions). The homogenate was centrifuged at 5000 rpm for 15 min at 4°C and the supernatant (hydrophilic fraction) was separated and kept at – 20°C until use. The residual pellet was used for the extraction of the lipophilic fraction by the addition of 2 mL of dichloromethane, followed by shaking at 230 rpm for 30 min at environmental temperature. The mixture was centrifuged at 5,000 rpm, 4°C for 15 min. The supernatant (lipophilic fraction) was separated and stored at – 20°C until further analysis.

The DPPH method of Duarte-Almeida et al. (34) with modifications was applied. Sample extracts (40 μL) were mixed with 250 μL of diluted 317 μM DPPH solution in methanol. After 25 min of reaction at 25°C, the absorbance decrease was measured at 517 nm in a Biotek Synergy HTX microplate reader (Agilent, Santa Clara, CA, USA). A control with methanol or dichloromethane was included (for the aqueous hydrophilic, or lipophilic extracts, respectively). The antioxidant capacity was expressed as μmol Trolox equivalents per 100 g DW using a standard curve of Trolox (20–160 in methanol and 10–160 μM in dichloromethane, for the aqueous and hydrophilic, and lipophilic extracts, respectively).

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) inhibition antioxidant capacity

The ABTS antioxidant assay was determined in maize phenolic extracts (free and bound fractions), and the hydrophilic and lipophilic extracts according to Fuentealba et al. (29). A volume of 250 μL of 14.3 mM $ABTS^{\cdot+}$ diluted in 96% ethanol was added to 40 μL of sample extract. The reaction was carried out at 25°C for 30 min. Following which, the absorbance was recorded at 734 nm in a Biotek Synergy HTX microplate reader (Agilent). A control with methanol or dichloromethane was included (for the aqueous hydrophilic, or lipophilic extracts, respectively). Results were expressed as μmol Trolox equivalents per 100 g DW using same calibration curves as previously stated for the DPPH assay.

Statistical analysis

All results (from 3 to 4 independent biological replicates) were expressed as means ± standard deviation. The data normality and variance homogeneity were evaluated with the Shapiro–Wilk and Levene tests, respectively ($\alpha = 0.05$). Data were subjected to an analysis of variance (ANOVA) with the HSD Tukey test for multiple comparisons or the Kruskall–Wallis test and the Dunn's test with Bonferoni arrangement for mean comparisons. The R software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria) was used. The unsupervised multivariate principal component analysis (PCA) was also applied to all data using the Unscrambler® X software version 10.4 (Aspen Technology, Inc., Bedford, MA, USA). Further, Pearson correlations among all data were explored using the Statgraphics Centurion XVI software (StatPoint Inc., Rockville, MD, USA).

Results and discussion

Environmental conditions and pre-harvest agricultural management associated with the cultivation of evaluated maize samples

The environmental conditions (maximum and minimum temperature, rainfall, and relative humidity) linked to the period of maize cultivation (August-2018 to May-2019) from each district of sample's origin are shown in **Supplementary Figures 1–4** (Cabanaconde, Andahua, Chachas, and Ayo districts, respectively). The maximum temperature was overall less variable than minimum temperature ranges in all districts. The Andahua district (Castilla province) showed the lowest minimum (2.8–6.0°C) and maximum (16–18.7°C) temperature ranges, whereas the Ayo district (Castilla province) had the highest minimum (6–13°C) and maximum (27.6–29.9°C) temperature values. The relative humidity variability was similar in the Andahua and Chachas districts (Castilla province), but their average values were lower (57.7 and 54.7%, respectively) than values shown in the Cabanaconde and Ayo districts

(81.7 and 70.1%, respectively). The precipitation levels were very low in all districts, showing only some millimeters per day in January and February in all cases. The ultraviolet (UV) index was measured during the collection of maize samples in most of the localities from both provinces (data not shown). Values varied from 7.5 to 10 (at noon time approximately) and are classified as "high to very high" according to the World Health Organization scale (35). Based on these climate characteristics, Cabanita maize plants might have been exposed to variable abiotic stress factors. Garcia et al. (36) have also reported similar environmental harsh conditions in Andean highlands.

Supplementary Tables 1, **2** show information about the agricultural management practices applied by farmers during the cultivation of *Cabanita* maize evaluated in the current research. Maize had a vegetative stage of 8 months and is sown once a year around the same period in both provinces. Farmers from both provinces follow similar farming practices such as the exchange of selected maize kernels among their neighboring communities to be used as seeds, the use of organic fertilizers for soil preparation, and a fallow stage of 3–4 months. The traditional seed exchange systems have shown to generate a flow of genes that is crucial for the *in situ* agrobiodiversity conservation (37). Likewise, fallow systems have been used in traditional agriculture to regenerate and improve soil fertility through the enhancement of soil organic matter following continuous cropping specially in the smallholder farming systems (38, 39). This indicates that farmers from this southern Peruvian Andean region generally preserve ancestral agricultural techniques relevant for biodiversity conservation and overall cultivate maize under organic conditions (control of weeds and pests manually or with flood irrigation, respectively).

However, some differences have been observed in relation to the presence of previous or simultaneous crops, such as the use of organic fertilizers during maize growth, and the origin of the water for irrigation. Caylloma lands seem to be exclusively used for *Cabanita* maize cultivation, the soil fertilization during the maize growth is not frequent, and some *Medicago* species may emerge at this stage (**Supplementary Tables 1**, **2**). In contrast, farmers from Castilla reported previous crops in same lands used for maize cultivation including tuberous species, legumes, and cereals (oat, quinoa, barley), the soil is fertilized with manure during the maize growth, and other plant species such as certain legumes (*Vicia faba* and *Medicago sativa*) are simultaneously grown with maize plants. Legumes including *Vicia fava* have shown high potential for nitrogen fixation, and other legume species have been already used in combination with grasses to improve the soil nutrient regeneration and its physical characteristics in the Peruvian highlands during the fallow stages (40, 41). In addition, some farmers from Caylloma stated out the use of cattle manure or domestic poultry manure, whereas other farmers from Castilla used guinea pig residues besides the cattle manure for soil preparation (information not completely provided). The type of crop rotation and organic fertilization may determine differences in the rhizosphere ecosystems influencing the soil microbiome diversity and composition, and consequently impacting its agronomic performance (42, 43). Additionally, the levels of irrigation have shown to affect the yield and nutritional quality of maize (44). No information about the soil composition and the irrigation frequency from each location were obtained in the current study. The effect of the specific agricultural management differences applied by farmers and the likely interaction with the Andean environmental conditions on soil characteristics along with the influence of these factors on maize quality and composition should be better investigated in future studies.

Physical characteristics of maize samples

Quantitative physical characteristics were measured in dried ears and kernels of *Cabanita* maize as shown in **Table 2**. In general, maize from the Castilla province showed higher variability in their physical descriptors than samples from Caylloma according to the statistical analysis. Maize ears from Caylloma were characterized for showing higher length, center diameter values, along with higher pith, rachis, and cob diameters than ears from Castilla. Samples CHW and CAW had the highest length and center diameters among all samples (12.4 and 6.0 cm, respectively). No statistical differences were observed in the tip and base diameters in maize from both provinces. However, the base diameter ranges were higher than tip values indicating that *Cabanita* maize ears exhibit a conic-cylindrical shape.

The yield-relevant physical parameters such as the ear weight, number of rows per ear and number of kernels per row were higher in maize from Caylloma (136.1–177.8 g, 17.0–18.9, 18.3–21.2; respectively) than ranges observed in samples from Castilla (69.4–170.1 g, 13.8–18.2, 15–20; respectively). Maize CHW, followed by CAW, CCR, and COM all from Caylloma exhibited the highest ear weights (177.8, 162.7, 159.6, and 155.2 g, respectively). In case of Castilla, only CPM sample had a comparable ear weight (170.0 g) as the observed ranges in maize from Caylloma. A strong positive correlation was obtained between the number of rows per ear and the ear weight ($r = 0.7767$, $p < 0.05$). Soil characteristics linked to different fallow and fertilization systems have shown to impact yield in several crops including maize (45, 46). As stated previously, some differences have been observed during the pre-harvest agricultural management of *Cabanita* cultivation that may determine better yields in maize grown in Caylloma than in Castilla. Other factors such as genetic, and environmental conditions might be also involved and need further research.

TABLE 2 Physical characteristics of Cabanita maize samples (ear and kernel) from Caylloma and Castilla provinces.

Characteristic	Caylloma							Castilla						
	CCR	CCY	COM	CAW	CAR	CLY	CHW	CHY	CAY	CSW	CSR	CPW	CPM	CALR
Kernel														
Weight (g)	0.4 ± 0.1bc	0.4 ± 0.0bc	0.5 ± 0.0ab	0.4 ± 0.0bc	0.4 ± 0.0bc	0.4 ± 0.0bc	0.5 ± 0.0ab	0.4 ± 0.1bc	0.3 ± 0.1c	0.5 ± 0.1ab	0.6 ± 0.1ab	0.6 ± 0.0ab	0.6 ± 0.1a	0.6 ± 0.1ab
Length (mm)	16.2 ± 1.1a	15.4 ± 1.1a	17.5 ± 0.5a	17.0 ± 0.6a	16.0 ± 1.0a	16.5 ± 1.0a	16.8 ± 1.2a	15.3 ± 0.6a	14.8 ± 2.4a	17.6 ± 2.2a	16.6 ± 2.5a	16.9 ± 0.7a	16.9 ± 0.1a	15.9 ± 0.6a
Width (mm)	7.7 ± 0.8c	8.4 ± 0.3abc	8.5 ± 0.3abc	8.0 ± 0.7bc	8.3 ± 0.4abc	8.1 ± 0.6bc	8.1 ± 0.2bc	9.1 ± 0.6abc	7.4 ± 0.3c	9.1 ± 0.6abc	8.9 ± 1.3abc	9.6 ± 0.4ab	10.0 ± 0.8a	9.4 ± 0.6ab
Thickness (mm)	5.5 ± 0.1a	5.4 ± 0.3a	5.4 ± 0.3a	5.0 ± 0.3a	5.2 ± 0.2a	5.3 ± 0.3a	5.5 ± 0.1a	6.0 ± 0.5a	5.3 ± 0.3a	5.5 ± 0.2a	5.6 ± 1.2a	6.0 ± 0.4a	5.7 ± 0.2a	6.1 ± 0.2a
Ear														
Weight (g)	159.6 ± 29.3a	136.1 ± 13.4ab	155.2 ± 10.1a	162.7 ± 28.9a	154.4 ± 19.3ab	134.5 ± 21.3ab	177.8 ± 15.7a	97.9 ± 9.0bc	69.4 ± 14.3c	122.1 ± 29.4abc	125.6 ± 23.6ab	139.3 ± 28.7ab	170.1 ± 16.2a	134.9 ± 11.6ab
Length (cm)	10.9 ± 1.4ab	9.9 ± 1.2bc	10.6 ± 0.6ab	11.0 ± 0.3ab	10.9 ± 0.1ab	10.6 ± 1.2ab	12.4 ± 0.9a	10.3 ± 0.2abc	8.1 ± 0.1c	9.2 ± 1.0bc	9.3 ± 0.9bc	10.7 ± 0.9ab	11.4 ± 0.8ab	10.5 ± 0.4ab
Tip diameter (cm)	2.3 ± 0.6a	2.6 ± 0.4a	2.4 ± 0.3a	2.9 ± 0.6a	2.8 ± 0.3a	2.8 ± 0.1a	2.2 ± 0.1a	2.2 ± 0.1a	3.0 ± 0.5a	2.6 ± 0.2a	3.0 ± 0.0a	2.8 ± 0.4a	3.2 ± 0.8a	2.7 ± 0.1a
Center diameter (cm)	6.0 ± 0.3a	5.9 ± 0.2a	5.9 ± 0.3a	6.0 ± 0.4a	5.8 ± 0.3ab	5.8 ± 0.3a	5.9 ± 0.3a	4.9 ± 0.2c	5.1 ± 0.3bc	5.6 ± 0.1abc	5.5 ± 0.3abc	5.5 ± 0.3abc	5.8 ± 0.1ab	5.5 ± 0.1abc
Base diameter (cm)	4.4 ± 0.2a	3.9 ± 0.8a	4.5 ± 0.3a	4.2 ± 0.3a	4.3 ± 0.5a	4.4 ± 0.2a	4.5 ± 0.2a	4.1 ± 0.5a	4.1 ± 0.2a	4.6 ± 0.4a	4.2 ± 0.2a	4.4 ± 0.4a	4.2 ± 0.7a	4.3 ± 0.1a
Pith diameter (cm)	1.1 ± 0.2abc	1.0 ± 0.3abc	1.1 ± 0.2abc	1.4 ± 0.2a	1.2 ± 0.3abc	1.0 ± 0.1abc	1.2 ± 0.1ab	0.9 ± 0.1abc	1.0 ± 0.2abc	0.7 ± 0.1c	0.7 ± 0.1c	0.8 ± 0.2bc	0.9 ± 0.0bc	0.8 ± 0.0bc
Rachis diameter (cm)	2.0 ± 0.3abc	1.9 ± 0.3abc	1.9 ± 0.1abc	2.4 ± 0.4a	2.2 ± 0.3ab	2.0 ± 0.1abc	2.2 ± 0.2ab	1.8 ± 0.1abc	2.0 ± 0.2abc	1.6 ± 0.4bc	1.7 ± 0.1bc	1.6 ± 0.2c	1.9 ± 0.1abc	1.8 ± 0.2bc
Cob diameter (cm)	2.9 ± 0.3abcd	2.8 ± 0.4abcd	2.7 ± 0.1abcd	3.3 ± 0.5a	3.2 ± 0.4ab	2.7 ± 0.2abcd	3.1 ± 0.4abc	2.6 ± 0.1abcd	2.9 ± 0.2abcd	2.2 ± 0.3d	2.4 ± 0.1bcd	2.3 ± 0.2cd	2.6 ± 0.2abcd	2.5 ± 0.2bcd
Number of rows/ear	17.6 ± 1.4abc	18.0 ± 0.6ab	17.0 ± 0.5abcde	18.9 ± 2.2a	17.0 ± 0.9abcde	17.2 ± 0.4abcd	18.6 ± 1.8a	14.0 ± 1.0de	18.2 ± 1.6a	14.5 ± 0.8cde	14.1 ± 1.2de	14.7 ± 1.6bcde	14.6 ± 1.5bcde	13.8 ± 1.0e
Number of kernels/row	19.3 ± 2.5abc	18.9 ± 2.0abc	18.8 ± 1.9abc	20.5 ± 0.5ab	19.6 ± 0.5abc	18.3 ± 1.6abcd	21.2 ± 0.6a	17.1 ± 1.0abcd	15.0 ± 1.0d	17.2 ± 0.8abcd	15.3 ± 1.9cd	17.6 ± 1.4abcd	20.0 ± 1.5ab	16.6 ± 0.7bcd

Different letters within the same row indicate significant statistical differences ($p < 0.05$).

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In relation to the physical characteristics of kernels, no differences were found in the length and thickness of samples from both provinces. Nevertheless, the weight and width values were more variable in samples from Castilla than in maize kernels from Caylloma. The kernel width and weight ranged from 7.7 to 8.5 mm and from 0.4 to 0.5 g, respectively in Caylloma maize. In case of Castilla, the width and weight results varied from 7.4 to 10.0 mm and from 0.3 to 0.6 g, respectively. Sample CPM had the highest width and weight values among all samples.

The variability of kernel pigmentations as shown in **Figures 1**, **2** may be related to differences in the bioactive compound contents and profiles as will be further discussed in next sections. In contrast to a previous investigation where only some *Cabanita* maize samples with white-yellow to mixed yellow-red-pigmented kernels were evaluated (24), a more complete *in situ* collection of *Cabanita* maize samples with variable kernel pigmentations has been performed in the current study. The wide phenotypic diversity of the Peruvian maize races has been reported by the Peruvian Ministry of Environment (14) with limited information about the race *Cabanita*. Results from current study contributes to the physical characterization of this ethnically important maize race for southern Peruvian Andean communities.

Phenolic bioactive composition

The phenolic profiles and contents from *Cabanita* maize samples are shown in **Table 3**. The free phenolic fractions were mostly rich in *p*-coumaric acid derivatives (3.5–8.2 mg/100 g DW), followed by ferulic acid derivatives (1.3–5.9 mg/100 g DW), and free forms of *p*-coumaric and ferulic acids (0.2–0.9 and 0.5–0.8 mg/100 g DW, respectively). Flavonoids such as anthocyanins were only detected in maize with partially red or purple-pigmented kernels (CCR, CAR, CSR, CPM, CALR) at higher concentrations (0.9–5.3 mg/100 g DW) than in white maize with some variegated-purple kernels (0.1 and 0.2 mg/100 g DW for samples CHY and CHW, respectively). The bound phenolic fraction represented on average 95% of the TPC (free + bound) similarly as in previous studies with Peruvian and Chilean maize landraces (24, 47). The major bound phenolic compounds were ferulic acid (98.6–195.1 mg/100 g DW) and ferulic acid derivatives (15.0–28.2 mg/100 g DW). Bound *p*-coumaric acid was found at lower concentrations (10.5–19.3 mg/100 g DW). Ranilla et al. (24) first evaluated the phenolic composition in 6 *Cabanita* maize accessions from the same Peruvian region reporting free *p*-coumaric acid and ferulic acid derivatives ranges below current results (2.6–5.5 and 0.2–0.9 mg/100 g DW, respectively).

Different soluble conjugated phenolic compounds have been found in cereals including maize (48, 49). Hydroxycinnamic acid amides (HCAAs) mainly derived from *p*-coumaric and ferulic acids such as *N,N′*-di-*p*-coumaroylspermine, *N*-*p*-coumaroyl-*N′*-feruloylputrescine and *N,N′*-diferuloylputrescine (DFP) have been detected in the soluble fraction of the yellow maize variety *Amagrano* from Germany (50). These phenolic amides were found at higher concentrations (∼14.8–18.03 mg/100 g DW) than the free forms of *p*-coumaric and ferulic acids (∼3.5–3.9 mg/100 g DW) whereas only traces of mono- and dihydroxycinnamoyl glycerides have been detected (50). Likewise, DFP, feruloylputrescine, cinnamoylputrescine and caffeoylputrescine (0.1–2.4 mg/100 g DW in total in whole grain) have been found in a wide diversity of Mexican maize landraces and have been proposed as taxonomic markers (51). The concentrations of hydroxycinnamic acid derivatives found in this investigation are consistent with ranges reported in above studies. Additional analyses with better analytical detection are needed to confirm the identification of soluble phenolic compounds in *Cabanita* maize. HCAAs are more concentrated in the outer layers (pericarp and aleurone) of maize kernel and have been shown to be part of the natural plant defense against pests and other biotic and abiotic stress factors (51–53). The anthocyanin concentrations found in Cabanita maize agree with the amounts reported by Paulsmayer et al. (54) (0.3–12.8 mg/100 g DW of total anthocyanins with a HPLC method) in diverse pink aleurone maize germplasm from Mexico and the United States with similar kernel pigmentations as those observed in *Cabanita* maize samples (**Figures 1**, **2**). Partially red-pigmented kernels in some evaluated samples indicate lower anthocyanin contents in comparison to purple pericarp-pigmented maize (18).

In case of the bound phenolic fraction, overall higher concentrations of ferulic acid, and ferulic acid derivatives have been detected in current *Cabanita* maize than in a previous study with Peruvian germplasm (107–139 and 17–21 mg/100 DW) (24). In addition, comparable bound *p*-coumaric acid values (19.6–22.5 mg/100 g DW) has been obtained in the same research as those found in this current research. Discrepancy of current results from previously reported phenolic values in same maize race may be related to differences in the pre-harvest practices and sampling conditions and is explained at the end of this section. Ferulic acid and *p*-coumaric acid have also been highlighted as the major bound or insoluble phenolic compounds in other maize landraces and different mature cereal grains such as wheat, rice, and barley (47, 55–57). Other minor bound phenolic compounds in maize comprise of diferulic acids such as 8-*O*-4′-diferulic acid, 5,5′-diferulic acid, and 8,5′-diferulic acid along with triferulic acids (50). It is most likely that ferulic acid derivatives observed in *Cabanita* maize are diferulic or triferulic acids, but this should be further confirmed with mass spectrometry analyses. Total diferulic acid contents found by Zavala-López et al. (58) in several modern and traditional maize hybrids were lower (4.3–13.9 mg/100 g DW) than

TABLE 3 Ultra high-performance liquid chromatography (UHPLC) phenolic profiles and contents (mg/100 g DW) of Cabanita maize samples from Caylloma and Castilla provinces.

Compound	Caylloma							Castilla						
	CCR	CCY	COM	CAW	CAR	CLY	CHW	CHY	CAY	CSW	CSR	CPW	CPM	CALR
Anthocyanins														
Free (Total)	2.8 ± 1.6ab	ND	ND	ND	2.9 ± 1.7ab	ND	0.2 ± 0.2b	0.1 ± 0.1b	ND	ND	0.9 ± 0.9ab	ND	5.3 ± 0.4a	0.9 ± 0.5ab
***P*-coumaric acid**														
Free	0.3 ± 0.0ab	0.3 ± 0.1ab	0.5 ± 0.1ab	0.3 ± 0.0ab	0.2 ± 0.0b	0.4 ± 0.1ab	0.5 ± 0.1ab	0.3 ± 0.1ab	0.9 ± 0.4a	0.8 ± 0.1a	0.6 ± 0.1ab	0.3 ± 0.0ab	0.3 ± 0.0ab	0.3 ± 0.0ab
Bound	11.5 ± 3.0a	11.7 ± 2.4a	10.5 ± 4.4a	11.5 ± 1.2a	11.9 ± 5.2a	10.6 ± 4.0a	13.4 ± 3.6a	14.0 ± 3.6a	19.3 ± 4.5a	10.7 ± 1.6a	15.8 ± 3.6a	11.3 ± 3.3a	11.0 ± 1.7a	13.8 ± 1.8a
Total	11.8 ± 3.0ab	12.0 ± 2.4ab	10.9 ± 4.5b	11.8 ± 1.2ab	12.1 ± 5.2ab	10.9 ± 4.0b	13.9 ± 3.6ab	14.3 ± 3.6ab	20.2 ± 4.8a	11.5 ± 1.5ab	16.3 ± 3.6ab	11.5 ± 3.3ab	11.3 ± 1.7ab	14.2 ± 1.8ab
***P*-coumaric acid derivatives**														
Free*	4.4 ± 0.7ab	4.9 ± 1.4ab	6.3 ± 0.7ab	3.5 ± 0.2b	3.7 ± 0.1ab	4.8 ± 0.4ab	6.0 ± 0.6ab	4.4 ± 0.3ab	8.2 ± 2.4a	7.5 ± 1.0ab	7.4 ± 0.6ab	4.0 ± 0.5ab	4.2 ± 1.0ab	3.7 ± 0.1ab
Ferulic acid														
Free	0.7 ± 0.2a	0.6 ± 0.1a	0.6 ± 0.1a	0.6 ± 0.2a	0.6 ± 0.2a	0.5 ± 0.1a	0.6 ± 0.1a	0.6 ± 0.1a	0.7 ± 0.1a	0.8 ± 0.1a	0.8 ± 0.1a	0.6 ± 0.0a	0.6 ± 0.0a	0.7 ± 0.1a
Bound	152.6 ± 27.4ab	161.8 ± 46.2ab	98.0 ± 36.6b	170.7 ± 25.8ab	139.44 ± 42.4ab	151.4 ± 38.9ab	161.1 ± 28.7ab	177.0 ± 42.5ab	189.0 ± 38.1a	192.0 ± 9.9a	188.6 ± 39.5ab	195.1 ± 25.2a	163.9 ± 5.9ab	192.4 ± 31.5a
Total	153.3 ± 27.4ab	162.3 ± 46.2ab	98.6 ± 36.5b	171.3 ± 25.8ab	140.1 ± 42.4ab	151.9 ± 38.9ab	161.7 ± 28.7ab	177.7 ± 42.6ab	189.7 ± 38.2a	192.8 ± 9.9a	188.4 ± 39.4ab	195.7 ± 25.2a	164.5 ± 5.9ab	193.1 ± 31.6a
Ferulic acid derivatives														
Free	2.0 ± 0.5ab	1.7 ± 0.5ab	3.4 ± 1.0ab	1.3 ± 0.4b	1.4 ± 0.5b	1.6 ± 0.1ab	3.0 ± 0.5ab	3.1 ± 0.4ab	5.9 ± 1.7a	3.3 ± 0.7ab	2.8 ± 0.8ab	2.5 ± 0.7ab	1.3 ± 0.2b	3.4 ± 0.6ab
Bound	16.5 ± 2.9a	17.6 ± 4.7a	15.0 ± 6.0a	18.1 ± 2.4a	16.4 ± 5.1a	16.3 ± 3.6a	19.7 ± 1.8a	24.0 ± 7.6a	22.7 ± 2.9a	22.0 ± 2.0a	18.7 ± 3.1a	28.2 ± 1.7a	26.7 ± 1.8a	16.4 ± 5.1a
Total	18.5 ± 2.7a	19.3 ± 4.3a	18.4 ± 6.0a	19.4 ± 2.1a	17.8 ± 4.9a	17.9 ± 3.7a	22.8 ± 2.2a	27.1 ± 7.2a	28.6 ± 2.5a	25.3 ± 2.5a	21.6 ± 3.1a	30.7 ± 2.3a	28.0 ± 2.0a	27.0 ± 3.9a
Total phenolic acids (Free)	7.4 ± 0.8ab	7.5 ± 1.8ab	10.8 ± 1.2ab	5.7 ± 0.7b	6.0 ± 0.5b	7.2 ± 0.6ab	10.2 ± 0.7ab	8.5 ± 0.9ab	15.6 ± 3.5a	12.4 ± 1.8ab	11.6 ± 1.2ab	7.4 ± 0.5ab	6.5 ± 1.0ab	9.4 ± 1.0ab

Different letters within the same row indicate significant statistical differences ($p < 0.05$). ND, non-detected.

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ferulic acid derivatives quantified in this research (15.0–28.2 mg/100 g DW).

Table 4 shows the TPC determined in the free and bound phenolic fractions using the UHPLC and the Folin-Ciocalteu methods. Bound phenolic contents were overall similar with both methods; however, higher free TPC values were detected with the spectrophotometric method than by UHPLC. This may be explained by the presence of other non-phenolic soluble reducing compounds in evaluated maize extracts. It has been reported that the Folin-Ciocalteu method lacks specificity because of several interfering compounds including proteins, amino acids, aromatic amines, sugars, organic acids, among other organic compounds (59). Overestimated free phenolic contents have been found in yellow corn flour which was related to the presence of soluble interfering compounds such as proteins and reducing sugars (60).

More statistical variability was observed in the UHPLC results than in those obtained by the spectrophotometric method (TPC). In general, phenolic levels in the free fraction were not much affected by the province of origin. UHPLC free phenolic ranges were 8.7–15.6 mg/100 g DW and 5.7–10.8 mg/100 g DW, for Castilla and Caylloma samples, respectively. However, *Cabanita* maize from Castilla had higher bound phenolic contents than Caylloma samples. The total bound phenolic values were 201.6–234.6 and 123.4–200.3 mg/100 g DW for Castilla and Caylloma samples, respectively. A similar trend was observed in the TPCs (free + bound) with both methods. Sample CAY (Castilla) showed the highest UHPLC TPCs followed by CPW sample (Castilla) (246.7 and 242.0 mg/100 g DW, respectively) whereas COM (Caylloma) maize had the lowest concentrations (134.3 mg/100 g DW). CAY maize also exhibited the highest total *p*-coumaric acid and *p*-coumaric acid derivatives along with high ferulic acid contents similarly as in other Castilla samples (CPW, CALR, CSW).

The TPC (free + bound) range of this investigation (201.5–291.1 mg GAE/100 g DW) was almost twofold higher than results obtained previously in kernels from the same Peruvian race (133.5–158.4 mg GAE/100 g DW) (24). This may be explained by differences in the sampling procedure. In current study, the ears collection was generally performed directly from the plant or recently harvested plants. Ranilla et al. (24) evaluated stored kernels from germplasm bank or farmers' warehouses who generally stored dried kernels under indeterminate time and after a sun-drying process for several weeks on the land. The storage time have shown to decrease the phenolic concentrations in sorghum grain and flour (61). Other factors related to the cultivation and harvesting practices also influence the phenolic contents in cereal grains and might also explain observed differences (62). The TPC range obtained in this study was comparable to that reported by De la Parra et al. (63) (243.8–320.1 mg GAE/100 g DW,

TABLE 4 Total phenolic contents of Cabanita maize samples from Caylloma and Castilla provinces.

Compound	Caylloma							Castilla						
	CCR	CCY	COM	CAW	CAR	CLY	CHW	CHY	CAY	CSW	CSR	CPW	CPM	CALR
UHPLC TPC (mg/100 g DW)														
Free	10.3 ± 2.4bcd	7.5 ± 1.8cd	10.8 ± 1.2bc	5.7 ± 0.7d	8.9 ± 1.9bcd	7.2 ± 0.6cd	10.4 ± 0.6bcd	8.7 ± 0.9bcd	15.6 ± 3.5a	12.4 ± 1.8ab	12.6 ± 0.6ab	7.4 ± 0.5cd	11.8 ± 0.8abc	10.3 ± 1.4bcd
Bound	180.6 ± 31.8ab	191.1 ± 52.1ab	123.4 ± 45.6b	200.3 ± 29.3ab	167.7 ± 51.3ab	178.3 ± 44.3ab	194.2 ± 32.7ab	215.0 ± 49.8ab	231.1 ± 44.7a	224.7 ± 10.3ab	223.0 ± 42.4ab	234.6 ± 28.1a	201.6 ± 8.2ab	229.8 ± 36.2a
Total	190.9 ± 31.2ab	198.6 ± 51.3ab	134.3 ± 45.9b	206.0 ± 29.1ab	176.6 ± 49.5ab	185.5 ± 44.7ab	204.6 ± 33.0ab	223.6 ± 49.6ab	246.7 ± 45.2a	237.1 ± 10.7ab	235.6 ± 41.8ab	242.0 ± 28.6ab	213.3 ± 7.9ab	240.1 ± 37.1ab
Folin–Ciocalteu TPC (mg GAE/100 g DW)														
Free	44.4 ± 10.3ab	40.7 ± 4.0ab	47.3 ± 1.2ab	37.2 ± 2.8b	42.8 ± 2.7ab	40.6 ± 1.0ab	42.8 ± 3.8ab	50.8 ± 6.6ab	63.4 ± 5.3a	49.4 ± 4.7ab	48.9 ± 0.7ab	39.4 ± 1.0ab	56.2 ± 4.7ab	47.3 ± 2.8ab
Bound	193.1 ± 31.4a	213.8 ± 27.4a	154.2 ± 51.9a	222.6 ± 14.2a	180.2 ± 59.7a	192.5 ± 53.4a	204.7 ± 27.0a	234.4 ± 33.3a	226.9 ± 50.0a	221.2 ± 7.0a	230.7 ± 48.7a	251.8 ± 32.9a	215.8 ± 11.2a	232.5 ± 26.9a
Total	237.5 ± 35.0a	254.5 ± 30.1a	201.5 ± 51.3a	259.8 ± 16.8a	222.9 ± 57.4a	233.1 ± 54.3a	247.5 ± 23.4a	285.3 ± 33.9a	290.4 ± 48.0a	270.7 ± 6.9a	279.6 ± 49.2a	291.1 ± 32.7a	272.0 ± 13.8a	279.8 ± 28.7a

Different letters within the same row indicate significant statistical differences ($p < 0.05$).

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samples from the United States) and higher than results found in maize landraces with variable pigmentations from Chile (132.2–262.5 mg GAE/100 g DW) (47), Mexico (77.3–123.6 mg GAE/100 g DW) (64) and India (102.3–206.4 mg GAE/100 g DW) (65).

Carotenoid composition

The total carotenoid contents in evaluated samples were lower (0.09–1.95 μg/g DW) than levels of phenolic compounds (**Table 5**). Xanthophylls such as lutein, lutein isomers and zeaxanthin were found in orange-pigmented (COM) and white-yellow samples (CCY, CLY, CHW, CAY, CPW). No carotenoids have been detected in most partially red-pigmented kernels (CCR, CAR, CALR) and some white maize (CHY, CSW). Lutein and lutein isomers were the major carotenoids in all *Cabanita* maize except in CHW sample where higher zeaxanthin (0.39 μg/g DW) than lutein levels (∼0.20 μg/g DW) were found. Sample COM (Caylloma) had the highest total carotenoid values (1.95 μg/g DW), followed by CAY (0.85 μg/g DW, Castilla) and CHW (0.57 μg/g DW, Caylloma). Samples CSR and CPM (with red-orange and purple variegated-pigmented kernels, respectively) which showed anthocyanin compounds (**Table 3**), also had carotenoids but at lower concentrations (0.09–0.31 μg/g DW).

Lutein and zeaxanthin have also been shown as the major carotenoid compounds specially in white, yellow, and red-pigmented maize kernels. Kuhnen et al. (66) reported lower zeaxanthin and lutein ranges (0.08–0.18 μg/g DW and 0.03–0.07 μg/g DW, respectively) in white Brazilian maize landraces compared with yellow landraces (0.07–7.05 μg/g DW and 0.48–3.69 μg/g DW, for zeaxanthin and lutein, respectively). White maize landraces from Malawi have shown ranges of 0.14–0.18 and 0.05–0.11 μg/g DW for lutein and zeaxanthin, respectively (67). In contrast, higher contents of both xanthophylls (4.7–34.97 and 2.36–21.18 for lutein and zeaxanthin, respectively) have been obtained by Uarrota et al. (68) in yellow Brazilian landraces along with moderate ranges of different pro-vitamin A carotenoids. Further, zeaxanthin and lutein have been found at higher concentrations in a red-pigmented Italian landrace (6.6 and 2.2 μg/g DW, respectively) following by lower contents of β-cryptoxanthin and β-carotene (1.2 and 0.2 μg/g DW, respectively) (69).

Carotenoid concentrations observed in the current research are more comparable to those found in white maize, but lower than values reported in yellow and red-pigmented maize. Ryu et al. (70) pointed out that hard maize types such as pop, dent, and flint have significantly higher carotenoid contents than floury types. In addition, the endosperm fraction has been shown to contain the highest carotenoid concentrations in maize and in other cereal kernels, therefore yellow and orange endosperms have higher carotenoid concentrations than white

TABLE 5 Carotenoid profiles and contents (μg/g DW) of Cabanita maize samples from Caylloma and Castilla provinces.

Compounds	Caylloma							Castilla					
	CCR	CCY	COM	CAW	CAR	CLY	CHW	CHY	CAY	CSW	CSR	CPW	CPM	CALR
Lutein	ND	ND	1.37 ± 0.52a	ND	ND	ND	0.10 ± 0.07ab	ND	0.24 ± 0.12ab	ND	0.15 ± 0.13ab	0.07 ± 0.12b	0.04 ± 0.02b	ND
Lutein isomers	ND	0.10 ± 0.08ab	0.23 ± 0.17ab	ND	ND	0.12 ± 0.05ab	0.08 ± 0.06ab	ND	0.36 ± 0.24a	ND	0.11 ± 0.06ab	0.11 ± 0.02ab	0.03 ± 0.03b	ND
Zeaxanthin	ND	ND	0.36 ± 0.09a	ND	ND	ND	0.39 ± 0.61a	ND	0.25 ± 0.23a	ND	0.05 ± 0.06a	ND	0.01 ± 0.01a	ND
Total carotenoids	ND	0.10 ± 0.08b	1.95 ± 0.70a	ND	ND	0.12 ± 0.05ab	0.57 ± 0.60ab	ND	0.85 ± 0.52ab	ND	0.31 ± 0.24ab	0.17 ± 0.13ab	0.09 ± 0.05b	ND

Different letters within the same row indicate significant statistical differences ($p < 0.05$). ND: Non-detected.

endosperm (71, 72). *Cabanita* maize is an amylaceous floury type with white endosperms which likely explains its lower carotenoid levels than other maize landraces. Only sample COM showed a creamy-white endosperm among all samples and had the highest carotenoid values. The orange pigmentation of COM kernel pericarp could be partly linked to the presence of other not detected phenolic compounds in this study such as phlobaphenes. These flavan-4-ols polymers have been identified in Italian maize landraces with brick red pigmentation which is similar as that observed in COM sample (69). Phlobaphenes have been associated with resistance to mycotoxin-producing fungal infection in maize (73). Domínguez-Hernández et al. (16) have recently stated that the carotenoid characterization of maize landrace diversity is very limited and germplasm from few regions of the world including only Mexico and Brazil from the American continent have been investigated. Results from the present study contribute for the first time with information about the carotenoid composition of part of the maize diversity from Peru which is another important primary center of maize domestication in the world.

In vitro antioxidant capacity

Only one sample from each location was selected based on its higher TPCs for the following *in vitro* antioxidant capacity assays. **Table 6** shows the health-relevant antioxidant potential of the free and bound phenolic fractions from *Cabanita* maize samples using the DPPH and ABTS methods. In addition, the hydrophilic and lipophilic fractions were evaluated. The bound phenolic fractions from all *Cabanita* samples showed the highest DPPH and ABTS free radical scavenging capacity and represented 93.5–98.2% of the total antioxidant capacity (free + bound). A significant correlation was found between the UHPLC total bound phenolic contents and the antioxidant capacity (r = 0.6111 and r = 0.5675, $p < 0.05$ with the DPPH and ABTS methods, respectively). Bound hydroxycinnamic acids such as *p*-coumaric and ferulic acids were correlated with this property (r = 0.5168, r = 0.6058 and r = 0.5444, r = 0.5454, $p < 0.05$, with the DPPH and ABTS methods, respectively) suggesting a high contribution of these bound phenolic acids to the antioxidant potential of *Cabanita* maize. The free antioxidant capacity was highly correlated with the anthocyanin contents using both methods (r = 0.8367 and r = 0.8197, $p < 0.05$, DPPH and ABTS assays, respectively) indicating an important contribution of anthocyanins to the free antioxidant property in partially red and purple-pigmented kernels.

The total ABTS antioxidant capacity (free + bound) from this research (2626.0–4103.6 μmol TE/100 g DW) is almost 1.4 times higher than levels reported by Ranilla et al. (24) in some *Cabanita* maize samples (1879.3–2942.1 μmol TE/100 g DW). This may be related to the higher phenolic contents found in this study as was previously discussed. Lower total antioxidant values than those from current investigation were determined in Chilean (1307–1850 μmol TE/100 g DW, ABTS method) and Southern Mexican maize landraces with different kernel pigmentations (377–484 μmol TE/100 g DW, DPPH method) (47, 64). In addition, comparable total antioxidant capacity has been observed in landraces from the Northeast of Mexico (2827–4264 μmol TE/100 g DW, ABTS method) and in diverse waxy maize genotypes grown in Thailand (average of 1096 and 3791 μmol TE/100 g DW with the DPPH and ABTS methods, respectively) (74, 75).

The hydrophilic antioxidant capacity (228.2–397.8 and 566.3–1068 μmol TE/100 g DW, with DPPH and ABTS assays, respectively) was higher than lipophilic antioxidant values (24.1–40.3 and 63.1–87.3 μmol TE/100 g DW, with DPPH and ABTS assays, respectively) which may be due to the higher phenolic concentrations found in *Cabanita* maize than lipophilic phytochemicals such as carotenoids. Significant positive correlations between the total free UHPLC phenolic contents and the hydrophilic antioxidant capacity (r = 0.6492, r = 0.7820, $p < 0.05$, DPPH and ABTS methods, respectively) were obtained. In case of the lipophilic antioxidant capacity, results showed moderate correlation with the total carotenoid levels (r = 0.4443, $p < 0.05$, ABTS method). Other minor lipophilic compounds not evaluated in this study may partially contribute to the lipophilic antioxidant property in *Cabanita* maize. Tocols and phytostherols have been also detected in maize (76). Recently, Lux et al. (50) found tocopherols and tocotrienols in similar ranges as those shown by total carotenoids in yellow maize.

Cabanita maize from Castilla showed higher total antioxidant capacity (free + bound) than Caylloma samples, and differences were high with the ABTS method. Ranges varied from 3410.6 to 4103.6 μmol TE/100 g DW and from 2626.0 to 3331.9 μmol TE/100 g DW for Castilla and Caylloma maize samples, respectively. CAY and CCR samples showed the highest total antioxidant capacity among Castilla and Caylloma maize, respectively. The hydrophilic and lipophilic antioxidant capacity results were in general similar in both provinces, and CAY sample (Castilla) showed the highest values (1068.9 and 87.3 μmol TE/100 g DW for the hydrophilic and lipophilic antioxidant capacity, ABTS assay). Current hydrophilic antioxidant capacity results are much higher than values reported in commercial Chinese maize (28 μmol TE/100 g DW, ABTS method), and other cereal grains such as sorghum (40 μmol TE/100 g DW, ABTS method), wheat (83 μmol TE/100 g DW, ABTS method), and barley (210–250 μmol TE/100 g DW, ABTS method) (77, 78). Comparable values have been shown in quinoa seeds (1280 and 22 μmol TE/100 g DW, hydrophilic and lipophilic fractions, respectively; ABTS method) (79). Differences may be attributed to the specific composition of hydrophilic and lipophilic phytochemicals associated with each grain food.

TABLE 6 *In vitro* antioxidant capacity of Cabanita maize samples from Caylloma and Castilla provinces.

Analyses	Caylloma					Castilla				
	CCR	COM	CAW	CLY	CHW	CHY	CAY	CSR	CPM	CALR
DPPH Antioxidant assay (μ mol TE/100 g DW)										
Free	82.4 ± 19.2a	38.8 ± 3.4ab	61.3 ± 3.9ab	34.6 ± 1.6b	42.2 ± 3.1ab	43.5 ± 8.6ab	58.1 ± 9.5ab	60.3 ± 3.0ab	93.2 ± 12.5a	56.4 ± 6.0ab
Bound	836.2 ± 30.4abc	641.9 ± 50.0d	673.9 ± 79.8cd	776.2 ± 33.8bcd	760.0 ± 51.5bcd	897.7 ± 61.9ab	947.7 ± 93.3a	827.7 ± 138.8abc	831.9 ± 23.6abc	900.6 ± 36.3ab
Total	918.6 ± 36.4ab	680.7 ± 48.7d	735.1 ± 79.8cd	810.8 ± 33.7bcd	801.9 ± 53.8bcd	941.1 ± 68.4ab	1005.8 ± 92.0a	888.0 ± 135.9abc	925.1 ± 35.8ab	957.0 ± 36.8ab
Hydrophilic fraction	348.4 ± 54.1ab	336.2 ± 23.1ab	268.2 ± 50.8bc	294.5 ± 26.9bc	325.3 ± 30.0ab	228.2 ± 29.5c	397.8 ± 34.8a	309.6 ± 15.3abc	290.6 ± 13.6bc	265.9 ± 31.4bc
Lipophilic fraction	35.4 ± 3.4ab	34.0 ± 3.9ab	24.1 ± 6.2b	31.2 ± 6.4ab	34.6 ± 5.3ab	39.9 ± 1.1ab	40.3 ± 10.0a	35.5 ± 4.8ab	33.7 ± 2.7ab	32.9 ± 5.7ab
ABTS Antioxidant assay (μ mol TE/100 g DW)										
Free	103.2 ± 28.4a	46.0 ± 11.0b	40.7 ± 5.6b	36.8 ± 9.1b	53.3 ± 5.1b	43.7 ± 4.3b	62.9 ± 22.1b	56.5 ± 3.1b	106.8 ± 21.0a	49.0 ± 7.7b
Bound	3228.8 ± 135.0bcde	2582.8 ± 327.2f	2585.3 ± 228.2ef	2823.5 ± 95.9def	2951.3 ± 237.8cdef	3801.5 ± 321.0ab	4040.7 ± 283.6a	3519.3 ± 379.8abc	3303.8 ± 206.0bcd	3810.0 ± 176.9ab
Total	3331.9 ± 156.6bcd	2628.8 ± 323.3e	2626.0 ± 224.2e	2860.3 ± 90.1de	3004.6 ± 238.4cde	3845.1 ± 316.9ab	4103.6 ± 280.4a	3575.8 ± 382.9abc	3410.6 ± 220.8bcd	3859.0 ± 182.5ab
Hydrophilic fraction	651.5 ± 41.6b	685.4 ± 23.2b	566.3 ± 11.1b	617.7 ± 48.3b	676.6 ± 32.0b	683.6 ± 50.7b	1068.9 ± 90.1a	689.4 ± 59.3b	652.0 ± 37.8b	661.9 ± 57.8b
Lipophilic fraction	85.0 ± 5.5ab	86.3 ± 3.1ab	73.5 ± 6.3abc	75.9 ± 5.2abc	82.3 ± 5.1ab	76.7 ± 8.0abc	87.3 ± 5.9a	63.1 ± 4.6c	72.0 ± 5.6bc	77.7 ± 7.5abc

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Principal component analysis

Underlying relationships based on all studied variables were explored through the PCA descriptive model (**Figure 3**). The score plot is shown considering the sample code and the district of origin of *Cabanita* samples. This approach retained two principal components (PC1 and PC2) which expressed 48% of the total variance of the sample data set likely indicating a certain grade of homogeneity among all evaluated maize samples from both provinces. This degree of homogeneity could be explained by the endemism of *Cabanita* race. Higher variability with the first two PC (61–71%) was reported by Ranilla et al. (24) when evaluating different maize races from a germplasm bank and collected *in situ* from the Arequipa region in Peru. In the same study, a clear discrimination based on the phenolic composition and some *in vitro* functional properties was found among the *Kculli* (purple-pigmented kernel), *Granada* (red-pigmented kernels), *Arequipeño* (white-yellow kernels) and *Cabanita* maize races (mostly white-pigmented kernels) (24).

Despite the observed low retained variability of the model, PC1 (30% of explained variability) separated samples from the Castilla (Chachas, Ayo, Andahua districts) and Caylloma (Cabanaconde district) provinces (from left to right, **Figure 3A**). The PC2 (18% of explained variability) separated maize grown in Chachas district (samples CALR, CPW, CPM) within the Castilla province. Differences between both provinces were increased by samples CAY (Andahua district, Castilla province) and COM (Cabanaconde district, Caylloma province). A positive correlation among the phenolic compounds including the bound and total UHPLC phenolic contents, bound ferulic acid, bound ferulic acid derivatives, total bound ferulic acid, bound and total *p*-coumaric acid was observed. These variables were correlated with the bound and total antioxidant capacity measured with the ABTS and DPPH free radical inhibition methods. All these variables were higher in samples from the Castilla province, specially from the districts of Chachas (CALR, CAPW, CPM), Ayo (CSW, CSR), and Andahua (CHY). CAY sample (Andahua district, Castilla) showed a different pattern among the other maize samples from Castilla (bottom left side of the score plot). This sample that showed higher TPCs as previously highlighted, also had the highest free UHPLC phenolic compounds including free ferulic acid derivatives, free *p*-coumaric acid derivatives and total free phenolic acids. These variables had a direct relation with higher ABTS hydrophilic antioxidant capacity in CAY sample (**Figure 3B**).

The loading plot also shows that yield-relevant physical characteristics were inversely correlated with phenolic concentrations and the *in vitro* antioxidant capacity indicating that Caylloma samples (right side of the score plot) are linked to lower phenolic contents but had better agronomic yield. Data from sample COM grouped differently compared with the rest of Caylloma maize (bottom right side of the score plot, **Figure 3A**). Variables such as lutein, and total carotenoid concentrations were the highest in this sample and were associated with high ABTS lipophilic antioxidant capacity. Further PCA analysis excluding data from CAY and COM samples was performed and results are shown in **Supplementary Figure 5**. The explained variability with the first two PC decreased to 42% indicating higher homogeneity among evaluated maize samples based on studied variables. However, PC1 (29% of explained variability) consistently separated data according to the province of origin (Castilla and Caylloma samples were in the left and right side of the score plot, respectively, **Supplementary Figure 5A**). Samples from Castilla continued to be linked to higher phenolic contents. Overall, most of the physical kernel and ear parameters (excepting those linked to the yield), and the carotenoid contents were not significant variables (**Supplementary Figure 5B**).

Several studies have emphasized that the genotype has more influence on the bioactive composition of maize than differences in the agroecological factors. Giordano et al. (80) found no differences in the main phenolic acids, anthocyanins, carotenoids (β-cryptoxanthin and β-carotene) and the antioxidant capacity from several pigmented Italian landraces under different nitrogen rates. However, the genotype showed a significant effect in same study (80). Some free and bound phenolic compounds along with lipophilic compounds such as carotenoids and tocochromanols from yellow maize were increased by sowing time, but not affected by phosphate fertilization (50). Furthermore, Paulsmeyer et al. (54) pointed out that the anthocyanin profiles and concentrations were strongly influenced by genetic factors with minimal influence of environmental conditions. Based on the PCA analysis in current study (low retained variability of the models), evaluated maize from Caylloma and Castilla provinces would belong to the same landrace population or race (*Cabanita*); however, the heterogenous Andean climatic factors seem to affect the bioactive composition of *Cabanita* maize to a higher extent than the sample type. Differences between maize from both provinces could be attributed to different agroecological factors. Districts from Castilla provinces showed more extreme climatic conditions than Caylloma locations (**Supplementary Figures 1–4**). The phenylpropanoid metabolism in plants has shown an extreme plasticity under changes in the environmental conditions redirecting the metabolic flux to produce phenolic-derived metabolites for plant protection (81). Increased levels of TPCs, bound ferulic acid, and DPPH antioxidant capacity have been reported in kernels from Peruvian purple maize grown at highland Andean locations (with lower temperature ranges and high UV radiation) compared with maize from lowland sites (18). The biosynthesis of ferulic acid and its derivatives was induced in maize seedlings and roots, stems, and leaves of maize plants under salt stress (82). The increase of phenolic metabolites under

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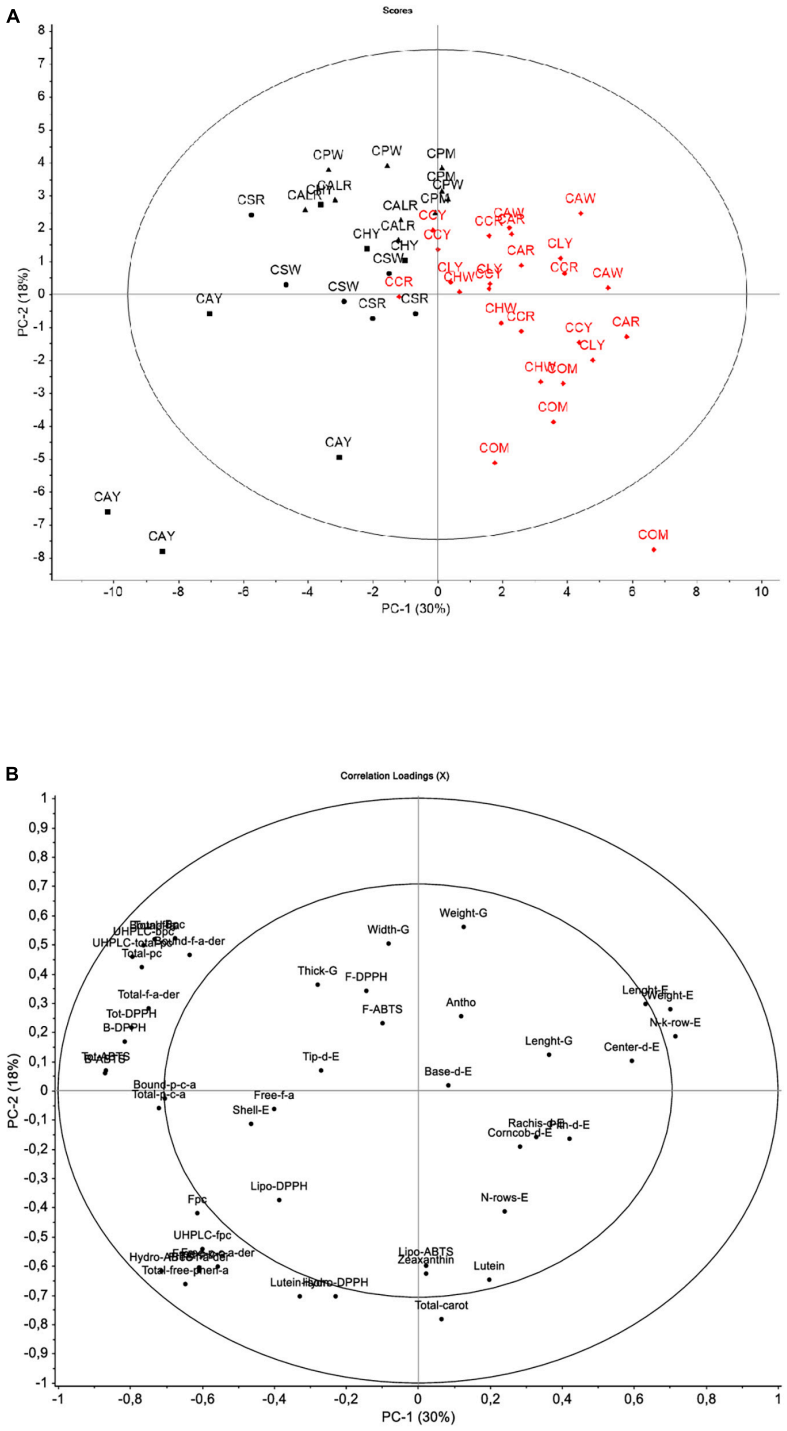

FIGURE 3
Score plot **(A)** and loading plot **(B)** for the principal component analysis (PCA) model for the first two factors considering of all data. In **(A)** Districts: box (Andahua), dot (Ayo), triangle (Chachas), diamond (Cabanaconde). Provinces: black (Castilla), red (Caylloma).

several abiotic stress factors has been previously reported in other cereal crops (83, 84). Cold stress has also shown to impair the photosynthesis leading to a decrease in grain yield (85). This may explain the lower yield physical parameters in maize from Castilla. Nevertheless, specific agricultural management differences observed in each province as previously stated could also play a role and should be investigated in future studies.

Conclusion

The diversity of the Peruvian Andean maize race *Cabanita* from two provinces (Caylloma and Castilla) in the Arequipa region is a promising source of phenolic compounds with *in vitro* antioxidant capacity. Major free phenolic compounds in all maize samples were *p*-coumaric and ferulic acid derivatives whereas anthocyanins were only detected in samples with partially red and purple-pigmented kernels (CCR, CAR, CHW, CHY, CSR, CPM, CALR). The bound phenolic fractions were rich in ferulic acid and its derivatives, followed by *p*-coumaric acid. The hydrophilic antioxidant capacity was correlated with the free phenolic fraction, whereas bound phenolic acids highly contributed to the bound antioxidant capacity. Orange (COM) and white-yellow pigmented maize (CCY, CLY, CHW, CAY, CPW) showed carotenoid compounds mostly xanthophylls such as lutein and zeaxanthin. The multivariate analysis (PCA) revealed a low variability of integrated data indicating a grade of similarity among evaluated maize samples based on their physical, phytochemical, and antioxidant properties. However, Caylloma samples were characterized by their more uniform physical characteristics and higher yield than Castilla maize which exhibited higher phenolic contents and antioxidant capacity. Samples CAY (Castilla) and COM (Caylloma) were remarkable due to their highest phenolic and carotenoid concentrations among all samples (246.7 mg/100 g DW and 1.95 μg/g DW, respectively). The heterogeneous environmental conditions in the Andean region along with differences in the pre-harvest agricultural practices may play a role, but genetic factors may also be involved and should be further investigated. This research provides the foundations of metabolomic base for future molecular studies to better characterize the ethnic-relevant maize race *Cabanita*. Results from this research contribute to current efforts to ensure a good characterization of the high Peruvian maize diversity to give extra value to Peruvian biodiversity for the development of Andean indigenous health-targeted food systems.

Data availability statement

The original contributions presented in this study are included in the article and **Supplementary material**. Further inquiries can be directed to the corresponding authors.

Author contributions

LR and GZ conceived and designed the study. LR directed the research and wrote the manuscript. IF-C and RC-P performed the experiments and analyzed the data. SM-T helped with the experiments. HB-G coordinated and helped with the sample collection. CF performed the multivariate statistical analysis. GZ, CF, and KS critically reviewed the manuscript. All authors have read and approved the final manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.983208/full#supplementary-material

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EDITED BY
Yasmina Sultanbawa,
The University of Queensland, Australia

REVIEWED BY
Thuan Chew Tan,
Universiti Sains Malaysia (USM), Malaysia
Saleha Akter,
The University of Queensland, Australia

*CORRESPONDENCE
Konjengbam Sarda Devi
sardakonj@gmail.com

†These authors have contributed equally to this work

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A comparative review on the anti-nutritional factors of herbal tea concoctions and their reduction strategies

Neeta Pathaw[1], Konjengbam Sarda Devi[1]*, Redina Sapam[1†], Jyotsana Sanasam[1†], Sapam Monteshori[1†], Sumitra Phurailatpam[2†], Huirem Chandrajini Devi[3†], Wangkhem Tampakleima Chanu[4†], Baby Wangkhem[3†] and Naorem Loya Mangang[1†]

[1]Indian Council of Agricultural Research, Research Complex for North Eastern Hill Region, Imphal, Manipur, India, [2]Multi Technology Testing Centre and Vocational Training Centre, College of Agriculture, Central Agricultural University, Imphal, Manipur, India, [3]College of Agriculture, Central Agricultural University, Imphal, Manipur, India, [4]Department of Agriculture, Himalayan University, Itanagar, Arunachal Pradesh, India

Tea is an important beverage consumed worldwide. Of the different types of tea available, herbal tea is an important beverage consumed owing to its popularity as a drink and stress relieving factors, several different herbal concoctions made from seeds, leaves, or roots are currently consumed and sold as herbal teas. The herbal teas are not the usual tea but "tisanes." They are caffeine free and popular for their medicinal property or immune boosters. Herbal tea formulations are popularly sold and consumed by millions owing to their health benefits as they are rich in antioxidants and minerals. However, plants are also known to contain toxic and anti-nutritional factors. Anti-nutritional factors are known to interfere with the metabolic process and hamper the absorption of important nutrients in the body. These anti-nutritional factors include saponins, tannins, alkaloids, oxalates, lectins, goitrogens, cyanogens, and lethogens. These chemicals are known to have deleterious effects on human health. Therefore, it is important to understand and assess the merits and demerits before consumption. Also, several techniques are currently used to process and reduce the anti-nutrients in foods. This review is focused on comparing the contents of various anti-nutritional factors in some underutilized plants of North-East India used as herbal tea along with processing methods that can be used to reduce the level of these anti-nutrients.

KEYWORDS

anti-nutritional, herbal tea, underutilized plants, saponins, phytic acid, alkaloids, anti-nutrients

Introduction

Plants and plant products have been a major source of dietary consumption for humans. Apart from being consumed for nutritional sources, they have also been used for medicinal purposes. The writings of many ancient civilizations suggest the extensive use of plants or plant products as herbal concoctions to treat various ailments. These herbal drinks or beverages later gained popularity owing to their health benefits. Tea is one of the most popular beverages consumed by millions worldwide. It is known to have many health benefits viz., anti-oxidative, anti-hypertensive, and hypolipidemic activities (1). Tea made from leaves of the plant Camellia sinensis dates centuries back and consists of black and green drinks with health benefits contributed by polyphenols such as catechins and theaflavin (2). Along with all the health benefits tea also contains caffeine, hence as a healthy alternative low calorie and decaffeinated traditional herbal drinks are gaining popularity (3). Herbal teas are made with boiling or water infusions of leaves, flowers, roots, barks, etc. (4). Herbal teas are actually mixtures of several ingredients, and are more accurately known as "tisanes" which is derived from the Greek word "ptisane" that means crushed barley. Tisanes are made from combinations of dried leaves, seeds, grasses, nuts, barks, fruits, flowers, or other botanical elements that give them their taste and provide the benefits of herbal teas (5). Herbal Tea comes under the list of beverages with aroma, taste, and healing properties. It has a variety of health benefits, including relaxation, decreasing body temperature, alleviating a poor stomach, and reducing fluid retention in the body (6). It is made by steeping various herbs in water and serving it hot or cold, depending on the user's desire. Herbal teas are popular for their therapeutic and invigorating characteristics that aid in body cleansing and strengthening the immune system. Some of the most popular herbal teas are chamomile, ginger, cardamom, and peppermint with each one having therapeutic potential (4). The past few decades have seen an increased use of plants with medicinal properties being consumed as herbal drinks worldwide. Plants or plant products form an essential part of people's lives as a source of fulfilling their nutritional needs. In spite of plants providing nutrition, it is also a source of many anti-nutrients including tannins, steroids, alkaloids, saponins, phytic acid, flavonoids, and cyanogens. These anti-nutrients interfere with the assimilation of nutrients or inhibit the utilization of nutrients such as proteins and minerals such as iron and zinc (7). The anti- nutritional factors that are known to lower the nutritional value of food can also be reduced by processing or following preparations such as heating, steaming, fermenting, cooking, and boiling. These processing techniques followed traditionally not only aid in storing the plant products but also help in reducing the anti-nutrients. The current paper is an attempt to highlight the use of some of the most popular herbal drinks prepared from some underutilized as well as commonly consumed plants from the north-eastern part of India with the emphasis on the anti-nutrients contents and strategies to reduce the effect of these anti-nutrients.

Underutilized plants as beverages and their processing

Herbal teas are the primary sources of dietary antioxidants in many cultures, of which polyphenolic compounds, in addition to vitamins and carotenoids, have been the focus of the scientific community for the past few decades (8). The World Health Organization in its 2014–2023 strategy has prioritized the screening of plants with medicinal potential with the aim to capitalize on the use of traditional medicine as a source for providing an effective and low cost alternative healthcare coherent to their cultural practices. As these strategies become policies that will affect healthcare practices in the future, therefore, it has become even more pertinent to address issues related to the safe and effective use of these medicinal plants as herbal drinks. As one of the biodiversity hotspots, the North Eastern region of India houses a plethora of medicinal plant species, many of which are commonly consumed. The region, having vast forest cover, is also home to many underutilized plants as a nutritional source or consumed as beverages. Figure 1 shows some of the commonly consumed beverages in North east India. viz., *Cymbopogon citratus* (Lemongrass), *Phlogacanthus thyrsiformis* (Nongmangkha), *Centella asiatica* (Peruk), *Oscimum sanctum* (Basil), *Garcinia pedunculata* (Heibung), *Clitoria ternatea* (Blue pea), *Hibiscus sabdariffa* (Roselle), *Rhus chinensis* (Heimang), *Nelumbo nucifera* (Lotus), *Mentha spicata* (Spearmint), *Rosa damascena* (Rose), and *Zingiber officinale* (Ginger).

Cymbopogon citratus is a tropical herb of the Poaceae family. The identified compounds in *C. citratus* are mainly terpenes, alcohols, ketones, aldehydes, and esters. Some of the reported phytoconstituents are essential oils that contain citral α, citral β, nerol geraniol, citronellal, terpinolene, geranyl acetate, myrcene, and terpinyl methyl heptenone. The plant also contains phytoconstituents such as flavonoids and phenolic compounds, which consist of luteolin, isoorientin 2′-O-rhamnoside, quercetin, kaempferol, and apigenin. Studies indicate that *C. citratus* possesses various pharmacological activities such as anti-amoebic, antibacterial, anti-diarrheal, anti- filarial, anti-fungal, and anti-inflammatory properties. Various other effects such as antimalarial, anti- mutagenicity, anti-mycobacterial, antioxidants, hypoglycemic, and neurobehavioral have also been studied (14).

Phlogacanthus thyrsiformis (Nongmangkha) is a shrub belonging to the Acanthaceae family. It is an evergreen shrub that grows to a height of 2.4 m, leaves are normally 13–35 cm long, oblanceolate, elliptic-oblong, acute, or acuminate. The

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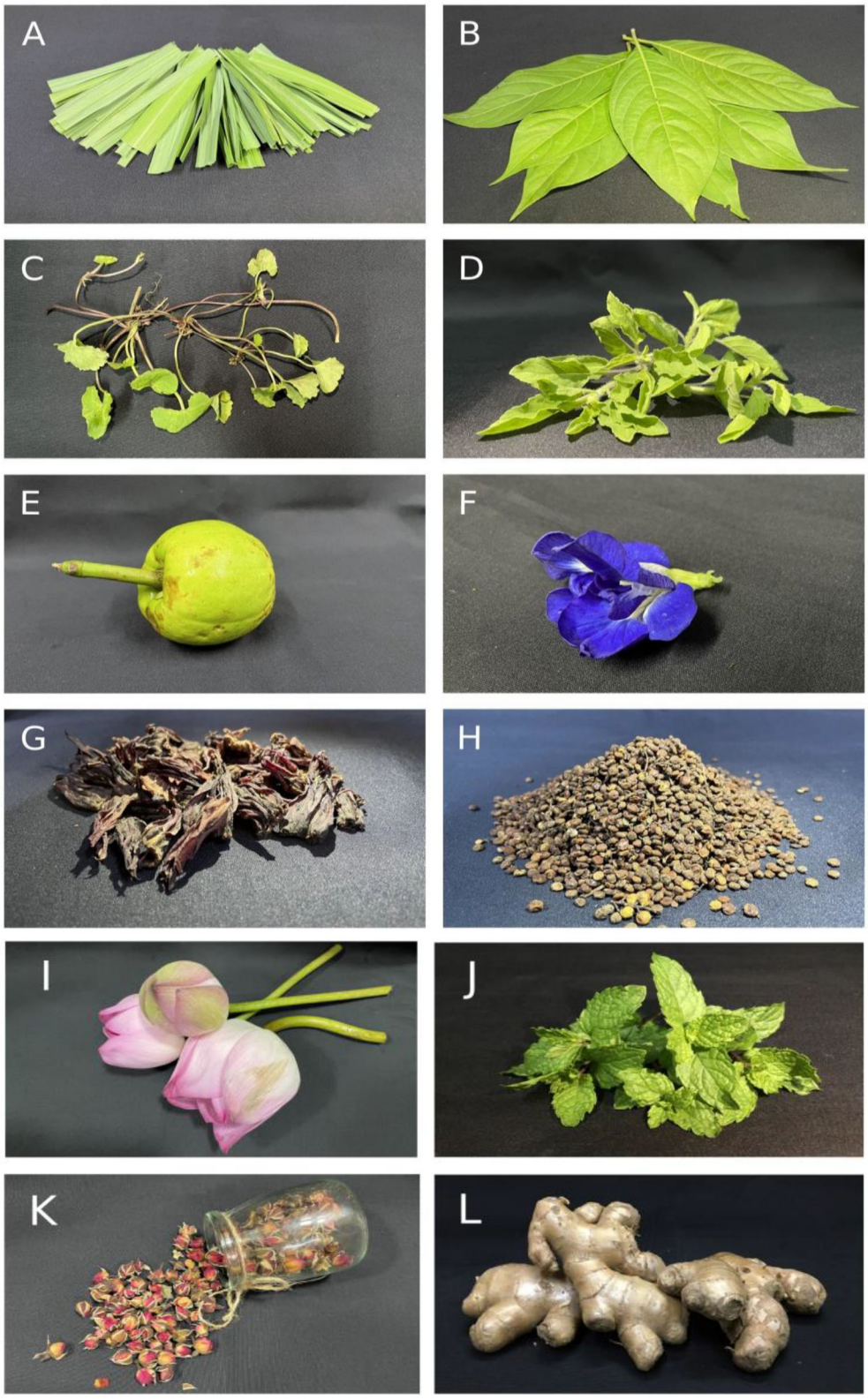

FIGURE 1
Pictures of different plants that are consumed as herbal tea studied in the current review. **(A)** Lemongrass (*Cymbopogon citratus*); **(B)** Nongmangkha (*Phlogacanthus thyrsiformis*); **(C)** Peruk (*Centella asiatica*); **(D)** Holy Basil/Tulsi (*Ocimum sanctum*); **(E)** Heibung (*Garcinia pedunculata*); **(F)** Blue pea (*Clitoria ternatea*); **(G)** Roselle (*Hibiscus sabdariffa*); **(H)** Heimang (*Rhus chinensis*). **(I)** *Nelumbo nucifera* (Lotus), **(J)** *Mentha spicata* (Spearmint), **(K)** *Rosa damascena* (Rose), and **(L)** *Zingiber officinale* (Ginger).

flowers are terminal elongated, thysoid panicles, up to 30 cm long, orange or brick red in color (32). The whole plant is extensively used for its great medicinal value like antipyretic, anti-diabetic, cough, colds, and anti-asthma. The inflorescence is consumed as vegetables and leaves are medicinal (8). The herbal tea is prepared from the dried leaf of *P. thyrsiformis*.

Centella asiatica is the herbaceous plant of the Apiaceae family. A major component of its bioactive constituents is asiaticoside, asiatic acid, madecassoside, and madecassic acid. The whole plant of *Centella* consumed as herbal tea has the properties of antioxidant activities, and treats gastrointestinal disease, gastric ulcer, asthma, wound healing, and eczema (41). Leaves of *C. asiatica* (L) are a rich source of valuable primary and secondary metabolites such as carbohydrates, tannins, steroids, terpenoids, alkaloids, flavonoids, cardiac glycosides, and saponins. *C. asiatica* accumulates large quantities of pentacyclic triterpenoid saponins, collectively known as centelloids. *Centella* is also rich in Vitamin C, Vitamin B1, Vitamin B2, niacin, carotene, and Vitamin A and is reported to possess various pharmacological activities such as antimicrobial activity, anticancer activity, wound healing activity, neuroprotective activity, immunomodulatory activity, anti-inflammatory activity, hepatoprotective activity, insecticidal activity, and antioxidant activity. *C. asiatica* plant possesses potential thrombolytic and antioxidant effects (9).

Ocimum sanctum (Basil) is an aromatic shrub in the basil family Lamiaceae also known as tulsi or holy Basil. It has been found to protect organs and tissues against chemical stress from industrial pollutants and heavy metals, and physical stress from prolonged physical exertion, ischemia, physical restraint, and exposure to cold and excessive noise (30). Tulsi has also been shown to counter metabolic stress through normalization of blood glucose, blood pressure, and lipid levels, and psychological stress through positive effects on memory and cognitive function and its anxiolytic and antidepressant properties (30).

Garcinia pedunculata (Heibung) belongs to the Clusiaceae family. Traditionally, it has been used for the treatment of asthma, bronchitis, coughs, dysentery, fever, and maldigestion (35). A variety of beneficial effects have been reported from the fruit extract, including antimicrobial, antioxidant, anti-inflammatory, hepatoprotective, nephroprotective, and cardioprotective properties (35). According to a recent study, the fruit of *G. pedunculata* contains phenolic compounds, flavonoids that produce total anti-oxidant activity, as well as anthocyanins and ascorbic acid. There are also several benzophenones, including pedunculo, garcinol, and cambogin, as well as organic acids, such as citric acid, hydroxycitric acid, hydroxycitric acid lactone, and oxalic acid (35).

Clitoria ternatea (Blue pea) is a plant from the Fabaceae family. The flowers are ornamental and are traditionally used as a food colorant. *C. ternatea* is also referred to as the butterfly pea, blue pea, aprajita, Cordofan pea, or Asian pigeonwings, and is commonly found throughout South East Asia. The bright blue petals from the flowers of the butterfly-pea plant have been used as an ingredient in herbal tea, caffeine-free, or tisane. *C. ternatea* flower extracts were found to possess antimicrobial, antioxidant, anti-inflammatory, cytotoxic, and antidiabetic activities, and also has been used as a memory enhancer, nootropic, anti-stress, anxiolytic, antidepressant, anticonvulsant, tranquilizer, and sedative agent (12). Various secondary metabolites including triterpenoids, flavonol glycosides, anthocyanins, and steroids have been isolated from the plant. Its extracts possess a wide range of pharmacological activities including antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, local anesthetic, anti-diabetic, insecticidal, blood platelet aggregation-inhibiting, and for use as a vascular smooth muscle relaxing properties (42) (Table 1).

Hibiscus sabdariffa (Roselle) is a sub herb of the Malvaceae family, infusions of the leaves or calyces are regarded as diuretic, choleretic, febrifugal, and hypotensive, decreasing the viscosity of the blood, and stimulating intestinal peristalsis (18). The dried calyces are consumed as an herbal tea. It has a high content of bioactive compounds such as phenolic acids, flavonoids, polysaccharides, and anthocyanins. The calyx extract of *H. sabdariffa* was mainly composed of anthocyanins, which contributed to its antioxidant capacity. It has been reported that *H. sabdariffa* extract is able to exhibit anticancer activity through its ability to protect against oxidative damage in rat primary hepatocytes (43).

Rhus chinensis (Heimang) belongs to the Anacardiacae family. Commonly known as the Nutgall tree or Chinese sumac, it is a deciduous tree abundantly grown in China, Japan, and north eastern India. In Manipur, North-East India, it is popularly known as "Heimang." The whole fruit and seed are traditionally used for their digestive properties and possess strong antiviral, antibacterial, anticancer, hepatoprotective, anti-diarrheal, and antioxidant activities (12, 32). It was found that the fruit of *R. chinensis* contained a high level of crude fiber, crude fat, and total titratable acidity. Ascorbic acid, malic acid, and citric acid were detected as the major organic acids (36).

Nelumbo nucifera (Sacred lotus) is an aquatic species belonging to Nelumbonaceae having herbal remedial benefits in every part of the plant (44). Widely used in Ayurveda and traditional medicines (22, 23, 45), the lotus is native to tropical and subtropical zones of Asia and almost all parts of it, such as flower, seed, leaf, stem, and root are edible (44) whose extract contains various phytochemicals, including alkaloids, flavonoids, phenolic acids, and steroids (22, 23, 45–49), which promote antioxidant (23, 45, 50) anti-inflammatory (51, 52), anti-diabetic (24), anti-obesity (25), and anti-cancer (26) activities. These biological activities are beneficial for individuals with declined immune functions, especially to the aged and advanced age people (53, 54), and immunosuppression in diabetic patients (27).

Mentha spicata (Spearmint) is an aromatic herb belonging to the family Lamiaceae. The leaves or powder of this aromatic herb can be used as a seasoning and flavoring herb or traditionally as an herbal tea (20). Various pharmacological properties have been found in the leaves, such as sedative, carminative, antispasmodic, hypoglycemic action, diuretic action, antimicrobial activity, antioxidant activity, breath problems, and anti-inflammatory action. Typically, *M. spicata* leaves are prepared as tea infusions to treat high blood pressure, menstrual irregularities, digestive issues, and respiratory problems (21, 54, 55). Spearmint essential oil has been used to treat digestive problems, obesity, asthma, coughing, and colds in traditional Arabic Palestinian medicine. Volatile oil, phenols, flavonoids, and lignans are abundant in *M. spicata* leaves (55).

Rosa damascena (Rose) belongs to the Rosaceae family and is one of the most important rose species widely used in the pharmaceutical and food industries and producing high-value essential oil. *R. damascena* is called Damask Rose in English and Gole Mohammadi in Persian (37, 56). Traditionally, *R. damascena* has been used to treat chest pain, constipation, depression, gastrointestinal disorders, inflammation, respiratory problems, and menstrual bleeding. Furthermore, *R. damascena* is one of the most potent sources of antioxidants, including phenolics, flavonoids, carotenoids, and anthocyanins (37).

Zingiber officinale (Ginger) belongs to the family Zingiberaceae and is also a major crop, grown primarily in India, China, and Nigeria. It is used worldwide as a spice, condiment, and herbal intact. The plant is upright ranging from 60 to 90 cm in height with a pseudo-stem and possesses perennial tuberous or rhizomatous roots (38). The characteristic odor and flavor of ginger are caused by a mixture of zingerone, shogaols, and gingerols, volatile oils that are composed of 1–3% of the fresh ginger weight and the constituents of the ginger plant have physiologic effects and also contain various phytochemicals having biological activities such as antimicrobial, antioxidant, and other pharmacological effects (38). The plant is used in traditional medicine for the treatment of several ailments such as Rheumatism, diabetes, snake bite, wounds, baldness, stomach disorder, toothache, bleeding, arthritis, respiratory disorders, and rash, in different parts of the world (39).

Various parts of the plant are used for the preparation of decoction and consumption as herbal tea. Leaves of lemongrass, nongmangkha, peruk, mentha, and basil, fruits of heibung, and heimang, flower, calyx, and flower buds of a blue pea, roselle, lotus, rose, respectively, the rhizome of ginger are used for herbal tea preparation; most of them belongs to under-utilized plants which are preferably consumed for its medicinal and soothing properties. The procedure of herbal tea processing includes drying, an essential thermal process. Oven (cabinet) drying still remains an attractive option for bulk drying of fragrant leaves such as coriander, olive mint, basil, thyme, bay leaves, and olive leaves (57–60). The drying process increases the shelf life by slowing microbial growth and, thus, preventing certain biochemical reactions that might alter the organoleptic characteristics. Drying can be carried out simply by air drying or using the aid of machines. Since air drying in humid weather can lead to spoilage and quality reduction, oven drying is preferred over it. It can take up to a week or more to dry herbs depending on the humidity in the air and the moisture content of the plant parts used for herbal tea. Previous studies have demonstrated that drying, an essential thermal process in producing herbal teas, may cause significant losses of bioactive ingredients and thereby decrease the health benefits and quality of the products (61, 62). Drying is a key step in the preservation of lemongrass leaves and processing of the leaves further into value-added products and oil extraction. The processing of lemon grass tea from freshly chopped green lemongrass using the sun, solar dryer, microwave (model P70B17L-T8) at 50 W, and oven (Prolab Instrument—model OTE 80) at 40, 50, 60°C (63, 64) showed the effect of drying on quality and sensory attributes of lemon grass (*Cymbopogon citratus*) tea whose results showed that the sensory properties, moisture content, ash content, pH and color of lemongrass samples differed with respect to the drying methods used. Reportedly, lemongrass tea from samples dried by oven at 40°C was the most preferred in color, aroma, taste, and overall acceptability and most suitable as the retention of appreciable sensory attributes is highest. This herb with its medicinal, therapeutic, and flavoring/culinary uses is very popular in Asian, Thai, and Vietnamese cooking. It can be used in fresh, dried, or powdered forms as seasonings and teas. Spearmint can be air dried (21) under shade for processing as herbal tea. The processing of roselle utilizes fruit and calyxes (dried) to be used as herbal tea. Although there have been some studies on the extraction of roselle using different types of solvent (65–67) and investigations into the drying processes for these materials (68, 69), comprehensive studies to establish a convenient procedure for drying and brewing to achieve high-quality roselle tea infusion are limited. The use of a common hot-air dryer for drying roselle and the classical brewing method was investigated by Nguyen and Chuyen (43), to find out the most suitable drying and brewing conditions in order to produce roselle tea with a high content of bioactive, antioxidant activity and sensory quality. The combination of drying at 80°C and steeping dried roselle for 30 min in 90°C hot water with a 1:10 solid-liquid ratio (g/ml) produces roselle tea with the highest content of beneficial ingredients. The processing of roses utilizes petals and flowerbuds/rosebuds (dried) to be used as herbal tea, and similarly, in Lotus, lotus petals are dried and used as herbal tea. Lotus petals are dried with all their phytochemicals intact at 60°C for 48 h (44). In Manipur, Elizabeth Yambem founded Manipur Dweller tea, which developed Heimang tea, Heibung tea, Peruk tea, and Nongmangkha tea (36). Since heimang is traditional medicine, which offers health benefits, formulating drinks/tea using heimang seeds and other value-added ingredients such

as cinnamon, ginger, tea leaves, and sugar and salt for taste will improve the quality of the product eventually providing additional health benefits. Devi Heirangkhongjam and Singh Ngaseppam (36) reported that dried fruits of heimang are normally used as ingredients in local culinary preparations instead of fresh fruits which are usually dried and stored to avoid spoilage. The whole fruits were dried at 55 ± 5°C in a hot air oven to process them for future use. Heibung being a rich source of secondary metabolites including xanthones, flavonoids, benzophenones, lactones, and phenolic acids with wide range of biological and pharmacological activities (70) are consumed; it has been processed using the fruit, sliced into small pieces and seeds separated. The sliced pulp dried up, which could either be powdered or unpowdered, and then packed for a healthy cup of herbal tea. A herbal tea is made from whole plants of peruk, leaves of nongmangkha, and tulsi, which are dried and powdered before being packaged. Ginger rhizome are sliced and dried which are powdered as its processing method for consumption as herbal tea. The rhizome of *Zingiber officinale* dried in an oven at a steady temperature of 60°C and milled with an electric blender before being ground into powder are used for evaluation of phytochemicals (39, 40). Blue tea is prepared from blue pea flowers, flowers are harvested and oven dried at 45–50°C (13) and can be stored for use as herbal tea. The dried flowers can be ground and packed for decoction of herbal tea.

Anti-nutritional factors

Anti-nutritional factors (ANFs) are substances that reduce digestion, utilization of nutrients, interfere with the absorption of biomolecules and hamper their bioavailability to human beings and monogastric animals, and may produce other adverse effects. The major anti-nutrients found in plant-based foods are phytates, tannins, alkaloids, lectins, oxalates, etc. (7). Phytate, being the most important among all, reduces the bioavailability of micronutrients such as iron and zinc. The anti-nutritional factors may be classified on the basis of their effects on the nutritional value and the biological response. Antinutritional factors may be broadly grouped into, (i) Factors with a depressive effect on protein digestion and on the utilization of protein, such as protease inhibitors, tannins, and saponins, (ii) Factors that affect mineral utilization, which includes phytates, (iii) Factors that stimulate the immune system and may cause a damaging hypersensitivity reaction, such as antigenic proteins, (iv) Factors with a negative effect on the digestion of carbohydrates, such as amylase inhibitors, phenolic compounds, and flatulence factors (7). Also, it can be presented as (a) Non-protein Amino Acids (Mimosine) as in Leucaena, (b) Glycosides (Saponins) as in Acacia, (c) Polyphenolic compounds (Tannins, Lignins) as in all vascular plants, (d) Alkaloids and Oxalate as in Acacia. Table 2 summarizes some anti-nutrients and their effects on us.

Tannins

Tannins are a group of polyphenols of ∼500 Da molecular weight present in plants. Tannins are secondary metabolites found in abundance in leaves, fruits, and bark (78). They are known to form complexes with protein between the hydroxyl and carbonyl group of tannins and proteins, respectively, thereby affecting protein digestibility and leading to inhibition of the utilization of essential amino acids and minerals (79, 80). Green tea and grapes are known to be rich in tannins. These are also found in plenty in berry fruits, beverages, pomegranate, cocoa, and also some cereals and legumes (81, 82). Naturally, there are two types of tannin groups-(a) hydrolysable-composed of gallotannins and ellagitannins and (b) condensed consisting of proanthocyanidin. The legume-bran is rich in tannins and hence affects the digestibility of proteins by forming tannin-protein complexes (83). Previous reports have shown that tea by-products produced in the beverage industry contain high levels of crude protein (CP, 220–350 g/kg dry matter; DM) and tannins (50–80 g/kg DM) (84).

Saponins

Saponins are triterpene compounds or steroids found in many crops such as legumes, cereals, tea, and some spices. It is known to have a bitter taste and is toxic at higher concentrations. Saponins affect nutrient absorption by inhibiting some metabolic or digestive enzymes and binding to essential nutrients such as iron, zinc, and vitamin E. Tea saponins have specific characteristics such as strong foaming, emulsifying, dispersing and wetting properties as well as active biological activities, such as anti-cancer, anti-inflammatory, antibacterial, haemolysis, antioxidant, anti-hypertensive, weight loss, nerve stimulation or neuroprotection and fish toxicity, and insecticide activities (1, 85–88). They are found in nature and known to have different biological functions. Saponins, when present with cholesterol, have been reported to show a hypocholesterolemic effect (89). They can cause hypoglycemia, and restrict protein digestion and absorption of vitamins and minerals leading to a leaky gut (90).

Phytates

Phytates are found in many plants in the form of myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate. They are secondary metabolites that are concentrated naturally in the seeds of legumes, cereals, oil seeds, and all plant-based foods. In the seed, it is stored as phytin or phytate in the husks. Phytates impede the absorption of minerals such as iron, zinc, magnesium, and calcium (90, 91). It can also inhibit the function of digestive enzymes such as pepsin, trypsin, and amylase (92).

TABLE 1 List of herbal teas studied along with their important phytochemical constituents and health benefits as well as its major consuming countries.

Scientific name (local name) & family	Health benefits	Plant part(s) used	Phyto chemicals	Concentration of Phytochemicals (mg/100 g)	Major consuming countries	References
Centella asiatica (Peruk) & Apiaceae	Potential thrombolytic and antioxidant effects, antimicrobial, anticancer, wound healing, neuroprotective, immunomodulatory, anti-inflammatory, hepatoprotective, insecticidal, and antioxidant	Leaves and the whole plant	Tannins Alkaloids Flavonoids Phenolics Phytates Steroids Soluble oxalates Oxalates	1.11 ± 0.02 0.29 ± 0.18 120.73 ± 0.17 109.32 ± 0.23 16.851 188.974 N/A N/A	Southeast Asia, South Africa, and Madagascar	(9–11)
Clitoria ternatea (Blue pea) & Fabaceae	Antimicrobial, antioxidant, anti-inflammatory, cytotoxic and antidiabetic activities, and also has been used as a memory enhancer, nootropic, anti-stress, anxiolytic, antidepressant, anticonvulsant, tranquilizer, and sedative agent	Flower	Flavonoids Phenolics Steroids	N/A N/A N/A	South and Central America, West Indies, Madagascar, India, China, Philippines, and other tropical Asian countries	(12, 13)
Cymbopogon citratus (Lemongrass) & Poaceae	Anti-amoebic, anti-diarrheal, anti-filarial, anti-mycobacterial, antioxidants, hypoglycaemic, anti-inflammatory properties	Leaves	Alkaloids Phenolics Saponins Tannins Flavonoids	1.38 ± 0.02 3.58 ± 0.02 1.25 ± 0.14 15.60 ± 0.03 4.76 ± 0.02	Asia, South America, and Africa	(13–16)
Garcinia pedunculata (Heibung) & Clusiaceae	Antimicrobial, antioxidant, anti-inflammatory, hepatoprotective, nephroprotective, and cardioprotective properties	Fruits	Flavonoids Phenolics Saponins Tannins Alkaloids	23.38 80.14 N/A N/A N/A	Southeast Asia	(17)
Hibiscus sabdariffa (Sougri) & Malvaceae	Diuretic, choleretic, febrifugal and hypotensive, decreasing the viscosity of the blood, and stimulating intestinal peristalsis	Flower (calyces)	Phenolics Alkaloids Flavonoids Saponins Tannins	37.42 N/A N/A N/A N/A	India, Central America, West Indies, and Africa	(18, 19)
Mentha spicata (Spearmint) & Lamiaceae	Sedative, carminative, anti-spasmodic, hypoglycemic action, diuretic action, antimicrobial activity, antioxidant activity, breath problems, and anti-inflammatory action	Leaves	Phenolics Flavonoids Alkaloids Saponins	2.629 ± 0.15 N/A N/A N/A	Europe, western, and central Asia	(20, 21)
Nelumbo nucifera (Lotus) & Nymphaeaceae	Antioxidant, anti-inflammatory, anti-diabetic, anti-obesity and anti-cancer activities, immunosuppression in diabetic patients	Flower	Phenolics Alkaloids Flavonoids Tannins	351.08 ± 4.62 N/A N/A N/A	Southeast Asia and Middle east	(22–29)

(Continued)

TABLE 1 (Continued)

Scientific name (local name) & family	Health benefits	Plant part(s) used	Phyto chemicals	Concentration of Phytochemicals (mg/100 g)	Major consuming countries	References
Ocimum sanctum (Tulsi) & Lamiaceae	Anxiolytic and antidepressant properties	Leaves	Phenolics Flavonoids Terpenoids Steroids	16.5 4.49 N/A N/A	Southeast Asia	(30, 31)
Phlogacanthus thyrsiformis (Nomangkha) & Acanthaceae	Antipyretic, anti-diabetic, cough, colds, and anti-asthma	Leaves	Flavonoids Tannins Saponins Steroids	0.13 0.29 N/A N/A	North East Region of India	(8, 32–34)
Rhus chinensis (Heimang) & Anacardiaceae	Antiviral, antibacterial, anticancer, hepatoprotective, anti-diarrheal, and antioxidant activities	Fruits	Phenolics Flavonoids Tannins Steroids Oxalates	725 ± 2.64 57.33 ± 1.13 N/A N/A N/A	North East Region of India	(35, 36)
Rosa damascena (Rose) & Rosaceae	Antioxidants, anti-constipation, anti-depression, reduces gastrointestinal disorders, inflammation, respiratory problems, and menstrual bleeding	Flower and flower buds	Phenolics Flavonoids	165.16 81.35	Bulgaria, China, India, Iran, Morocco, Ukraine, Libya, south of France, south of Italy, south of Russia, and Turkey	(37)
Zingiber officinale (Ginger) & Zingiberaceae	Anti-inflammatory activity, cardiovascular effects	Rhizome	Saponins Phytates Oxalates Tannins	4.01 ± 0.07 0.28 ± 0.01 0.26 ± 0.002 0.02 ± 0.00	Asia, Africa, and West Indies	(38–40)

The negatively charged structure of phytic acid binds to the positively charged ions such as iron, magnesium, calcium, and zinc to make complexes by lower absorption of these ions and thereby reducing their bioavailability. As a chelating agent phytic acid is considered an important anti-nutrient (93).

Oxalates

Oxalates are oxalic acids present in plants in soluble and insoluble forms. In soluble form, they exist as potassium and sodium salts, and in an insoluble form, it comprises calcium, magnesium, iron salts, or esters. Oxalates are predominantly found in leafy plants and also synthesized in the body. Insoluble forms of oxalates (calcium oxalate) when accumulated in the body lead to harmful effects causing the formation of kidney stones (94). Green leafy vegetables and plants, berries, beans, chocolate, and some nuts contain high amounts of oxalates (95). Although oxalate in normal concentration is easily digestible, people with certain conditions require lower intake as it can lead to complications. In a small population, ingestion of small amounts of oxalate can lead to conditions such as itchy and burning eyes, ears, throat, and mouth, while large amounts may cause stomach upset with nausea and diarrhea (90).

Effect of anti-nutrients on human health

While the term anti-nutrient is synonymous due to its negative effect, these plant secondary metabolites are known to have many benefits. One has to also consider the effects of concentration as most of the metabolites when consumed in low concentrations do not cause any harm. Anti-nutrients are an integral part of plants as most of them have either a role in plant defense or other functional roles. Anti-nutrients are used as active ingredients in food and drinks, compounds such as saponins, phenolic compounds, and phytic acid when consumed at low levels have shown to lower glucose levels and cholesterol (90). Saponins are also reported to help prevent osteoporosis and maintain liver functions (96). Furthermore, phenolic compounds such as phytic acid, saponins, and

TABLE 2 List of anti-nutrients found in herbal tea and its effects.

Anti-nutrient	Its effects	*In vivo*/*in vitro*	References
Oxalate	• It can form insoluble salts which accumulates kidney stones • It can bind to calcium and prevent it from being absorbed	*In vivo*	(71)
Tannins	• It affects protein digestibility and leads to reduction of essential amino acids (by forming reversible and irreversible tannin-protein complexes between the hydroxyl group of tannins and the carbonyl group of proteins)	*In vitro*	(71)
Steroids	• Increase risk of cardiovascular events including stroke or heart attack	*In vivo*	(72)
Phytates	• It impedes the absorption of minerals like iron, zinc, magnesium & calcium and also inhibits enzymes like pepsin, trypsin etc. • It affects the gastrointestinal absorption of minerals which in turn lowers the bioavailability of the minerals	*In vivo*	(73, 74)
Saponins	• It affects the absorption of vitamin A and E as well as lipids • It can lead to hypoglycemia	*In vivo*	(75)
Alkaloids	• Alkaloids are mostly involved in neurotoxicity or cell signaling disruption	*In vitro*	(71)
Phenolics	• Decrease bioavailability of amino acids • Loss of appetite, breathing problems, and cardiac complications	*In vitro*	(76)
Flavonoids	• It chelates metals such as iron & zinc and reduces the absorption of these nutrients • They also inhibit digestive enzymes and may also precipitate proteins	*In vivo* and *in vitro*	(73)
Terpenoids	• It alters the carbohydrate metabolism and also generates toxic effects in liver and kidney	*In vivo*	(74)
Soluble oxalate	• It exerts its effects by binding calcium (Ca), magnesium (Mg), and other trace minerals such as iron (Fe), making them unavailable for assimilation	*In vivo*	(77)

protease inhibitors are known to have anti-cancer properties. Meanwhile, tannins are known to have anti-viral, anti-parasitic, anti-bacterial, antioxidant, anticancer, immuno-regulatory, and cardiovascular-protective effects (90, 97). Therefore, it is wise to have knowledge about the constituents of the food products and make a conscious judgment in consuming them. The various beneficial effects of anti-nutrients also help as a valuable tool for managing different diseases. Although not always nutritious, consuming in correct quantities can rather have a beneficial outlook.

Strategies to mitigate the effects of anti-nutritional factors

Studies have reported the various effects of anti-nutrients on human health by reducing their nutritional significance. Anti-nutritional factors are found widely in plants and plant products, therefore, removing them is essential to enhance their quality. The presence of high amounts of tannins in food products has been shown to affect the bioavailability of iron by inhibiting its absorption and causing deficiency (98, 99). Concurrently, the presence of phytate in foods is known to cause Zinc deficiency (7). Phytate which serves as a phosphate storing molecule in plants has a high affinity to chelate ions such as Zn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, K^{2+}, Mn^{2+}, and Cu^{2+}, thereby reducing the bioavailability and thus affecting growth and development (100). A report on the reduced growth in chicks was reported as a result of the negative impact on the absorption of Vitamin A and E along with lipids in chickens fed with dietary saponins (101).

Anti-nutrients in food are not only known to reduce nutrient utilization causing nutritional deficiency but it is also toxic when consumed at higher concentrations. Therefore, lately, a great interest has been renewed in mitigating this problem. Traditional methods such as soaking, milling, cooking, roasting, debranning, germination, and fermentation have been used since time immemorial for reducing the effects of these anti-nutrient components in foods. Herbal tea is decoctions made out of herbs and is known to contain different types of antinutritional factors in different concentrations. The herbs and medicinal plants used as herbal drinks and studied in the present review contain various phytochemicals including anti-nutrients (Table 2). The antinutritional factors can be reduced during proper processing and correct brewing temperature, boosting the nutritional value by suppressing the antinutritional factors. Here, we describe various processing methods used in reducing the anti-nutrients in these herbal tea products.

Heating, drying, steaming, and cooking

Heating or dry roasting is used as an application for heat treatments in tea processing. Heating and boiling, among all the methods, are found to be the most effective to reduce harmful compounds in food. Heat treatment of vegetables and cereals leads to the activation of the enzyme phytase making it useful and healthy (102). Drying, an essential thermal process, causes a significant reduction in moisture content, crude protein, crude fiber, ash, and mineral contents, and also the antinutritional factors (flavonoids, alkaloids, glycosides, phenolic compounds, saponins) while it may cause an increase in the oil content. Drying of plants is a step that helps in the prevention of microbial growth while inhibiting biochemical changes, it has been identified to improve the quality of the product due to oxidation reactions (103). Proper drying of *Hibiscus sabdariffa* (roselle) calyxes combined with the right brewing duration of the dried roselle reduces the anti-nutrients and boosts the bioactive content, aroma, and sensory quality (43). Reportedly, lemongrass tea dried in an oven at 40°C was most suitable for the retention of appreciable sensory attributes and least anti-nutrients (63, 64). Drying fresh leaves of *Ocimum tenuiflorum* and processing them as dried, fermented, and unfermented herbal tea showed a differential effect on the phenolic, flavonoid, tannin, and anti-oxidant content, of which the unfermented tea showed an increase in the content in all parameters (103). Although the drying or heating process, an important processing technique, is generally understood to improve the quality of herbal tea, it is yet to understand the chemistry behind the reduction of phytochemical constituents. Therefore, in the herbal tea processing unit drying is an essential step that not only reduces the chances of microbial growth but also enhances aroma and taste due to the release of essential oils. Although heating is considered to reduce the effect of most anti-nutrients, it has been reported that the concentration of polyphenols like tannins increased with an increase in the boiling time (104). Soaking and cooking or boiling is known to greatly impact the nutritional value of food products by reducing their antinutritional contents such as tannins and trypsin (105). Boiling at a controlled temperature for at least 15 min is known to reduce the level of anti-nutrients (106). Similarly, studies on the reduction of phytic acid as a result of soaking and cooking have been reported in legumes (107). A 47% reduction in the content of oxalate in taro leaves was observed as a result of boiling in water for 40 mins, although no significant reduction in oxalate was observed by baking for even 40 min at 180°C (108). Rose petal tea which was oven dried initially at 25–27°C followed by brief drying at 80°C in a hot air oven when brewed in boiling water for 15 min showed good sensory attributes along with higher antioxidative properties (109). Therefore, prolonged boiling should be avoided rather a low time frame for boiling should be followed enough to release sensory properties such as essential oils and aroma which will also help in the reduction of the concentration of polyphenols. As anti-nutrients are not always harmful at lower doses, hence concentration-dependent effects must be considered.

Fermentation

Fermentation is a process that helps in reducing bacterial contamination in food products. It aids in digestion as it is probiotic and also helps improve the absorption of essential minerals (110, 111). Fermentation of cereal crops in Africa is a common practice as it helps increase nutritional quality (112). It has helped in the increase of essential amino acids such as methionine, tryptophan, and lysine (113). Coulibaly et al. (114) have reported on the significant reduction of phytic acids, tannins, and polyphenols as a result of fermentation in millets. The reduction in phytic acid content could be due to the enzymatic action of the enzyme phytase. The fermentation process also helps in the degradation of phytate due to optimal pH conditions thereby increasing the bioavailability of ions such as iron, zinc, magnesium, and calcium (115). Fermentation is also an important step in the processing of black tea which is known as a source of phytochemicals such as theaflavins and thearubigins that function as potent antioxidants conferring protection against cellular damage. Reports of a popular indigenous beverage of the South African Western Cape prepared from a shrubby legume, Rooibos, which is processed either by air drying or fermented. The herbal tea air dried contains higher levels of polyphenol and thereby contains a rich source of antioxidants which is reportedly higher than green tea (116). The fermented Rooibos tea although did show anti-mutagenic property, however, due to fermentation contains lesser antioxidants, however, the fermented tea showed better sensory attributes as it has a stronger and sweet taste than the unfermented Rooibos tea.

Genetic engineering

Genetic engineering is a modern technology that has been used to enhance nutritional factors in plants. Using genetic manipulation techniques reduction of phytic acid has been reported in *Zea mays, Oryza sativa, Hordeum vulgare,* and *Glycine max* (117). These genetically modified plants with low phytic acid content could be beneficial for reducing micronutrient malnutrition. Similar reports on the reduction of antinutrients using genetic techniques have been reported in common bean (*Phaseolus vulgaris* L.) seed, which resulted in increased nutrient and iron bioavailability without causing many changes in the agronomic traits (118). Tissue-specific RNAi mediated knockdown of the *ITP5/6K-1* gene in *Oryzae sativa* has reportedly led to a reduction of phytate in seeds

TABLE 3 Some physical processes for reducing antinutrients.

Physical processing	Comments
Heating or drying	• Heating at temperatures 40–80°C • Heating or drying prevents microbial growth also reduces chances of formations of aflatoxins
Boiling or brewing	• Mild boiling at low temperatures for around 15 min or brewing leads to higher sensory attributes with higher antioxidative potential
Fermentation	• Increases nutritional quality and reduced polyphenolic content
Genetic engineering	• Gene manipulation techniques can be a good technique to reduce antinutritional factors, however no work in tea or herbal has been reported

(119). Similarly, reports on mutating genes like the *IPK1* gene responsible for producing high levels of phosphorus and stored as phytic acid have been reported in maize (120). While most work on genetic engineering in the reduction of antinutrients has been concentrated on reducing phytic acid which is a major antinutritional factor in legumes and crops (121–123), genome editing techniques are yet to be utilized in knocking out anti-nutrients from other plants. Also, a major impediment to the use of genome editing tools has been the argument of constructing a genetically modified organism which is still not acceptable to many.

It has been observed physical processes (Table 3) like that of heating the plant parts lead to the activation of some essential oils and enhance the aroma. Boiling of herbal tea leads to the reduction of some harmful factors, however, boiling though beneficial has to be followed only for a short duration as a higher time frame leads to concentrated amounts of anti-nutrients due to excessive boiling. Another important process for reducing the harmful effects is found to be fermentation which is known to enhance nutritional quality. As previously described how anti-nutrients affect the bioavailability of micro nutrients and vitamins by lowering their absorption thereby leading to nutritional deficiency, hence it is of utmost importance to reduce the content of these antinutritional factors. Also, apart from reduction strategies, a concerted effort by the consumer to consume the herbal products in moderation as most of these metabolites do not cause major harm if consumed in lower doses.

Conclusion

The importance of herbal tea consumption with the growing concern of chronic disorders associated with depleted diets increases. The quality, efficacy, and safety of herbal teas is an area not to be overlooked in future research to develop quality assured processes to harness the goodness of herbs and underutilized plants to be sourced and manufactured to a high standard. The present review attempted a comprehensive study of the underutilized plants as an important source of solutions to the emerging health issues owing to the diet and lifestyle led diseases. Although it is understood that the drying or heating process helps remove anti-nutrients, future research is required on optimizing brewing conditions to understand the synergistic effects of phytochemicals in herbal tea. It is also extremely important for the consumer to consume herbal products in moderation, as most of these metabolites are within a permissible range when consumed in moderation and do not necessarily cause major harm if consumed in lower doses. This review presents the need to explore a wider range of herbal teas, their nutrient, and phytochemical constituents.

Author contributions

NP and KD conceptualized the idea, designed the outlines, drafted the manuscript, reviewed, and improved the draft. JS, RS, SM, SP, HD, WC, and BW drafted the manuscript. JS, RS, NM, and KD prepared the illustrations and worked on proofreading the manuscript. All authors read and approved the final version of the manuscript.

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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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EDITED BY
Yanlun Ju,
Northwest A&F University, China

REVIEWED BY
Mohammad Alrosan,
Universiti Sains Malaysia (USM), Malaysia
Miguel Angelo Faria,
LAQV Network of Chemistry and Technology, Portugal

*CORRESPONDENCE
Michael Rychlik
michael.rychlik@tum.de

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Folate vitamers in the Australian green plum: Through growth and ripening and across locations

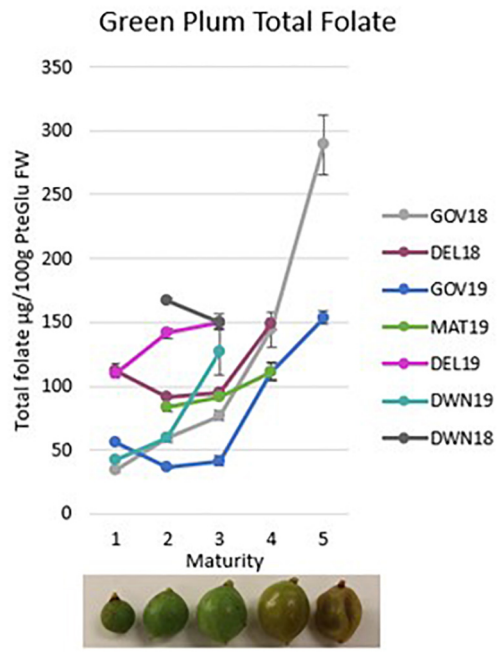
Selina Fyfe[1,2], Hung Hong[1,3], Horst Joachim Schirra[2,4,5], Heather E. Smyth[1], Yasmina Sultanbawa[1] and Michael Rychlik[1,6]*

[1]Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, QLD, Australia, [2]Griffith Institute for Drug Discovery, Griffith University, Brisbane, QLD, Australia, [3]School of Agriculture and Food Science, The University of Queensland, Brisbane, QLD, Australia, [4]School of Environment and Science, Griffith University, Brisbane, QLD, Australia, [5]Centre for Advanced Imaging, The University of Queensland, Brisbane, QLD, Australia, [6]Chair of Analytical Food Chemistry, Technical University of Munich, Freising, Germany

The green plum is a native fruit of Australia that grows on the tree *Buchanania obovata*. This study aimed to confirm the high level of folate in green plums by analyzing a large number of ripe samples from multiple locations and to understand how folate vitamers change as the fruit grows through maturity stages. This study analyzed green plums for five vitamers of folate, H_4folate, 5-CH_3-H_4folate, 5-CHO-H_4folate, 10-CHO-PteGlu, and PteGlu (folic acid) using a stable isotope dilution assay on a liquid chromatograph mass spectrometer (LC-MS). Green plums were tested from four locations, two harvests and five maturity stages. Another 11 ripe samples, each from different tree clumps from one location, were also tested as were ripe red-colored green plums. The results show the 5-CH_3-H_4folate in green plum increases and accumulates in the fruit through development, ripening and senescence. The ripe green plums contain between 82.4 ± 5.5 and 149.4 ± 10.7 μg/100 g Fresh Weight (FW). The red-colored green plums are even higher in folate, with total folate measured as 192.5 ± 7.0 and 293.7 ± 27.4 μg/100 g FW, and further analysis of them is suggested. There is some variation in amounts of folate between fruit from different locations and sets of trees, but all ripe green plums tested are considered good dietary sources of folate.

KEYWORDS

green plum, *Buchanania obovata*, folate, 5-CH_3-H_4folate, 5-methyl-tetrahydrofolate, micronutrients, vitamers, Anacardiaceae



GRAPHICAL ABSTRACT
Green plum total folate results as fresh weight for five maturity stages of green plum growth and by locations and harvest.

Introduction

The green plum is a native fruit of Australia that grows on the tree *Buchanania obovata* in the northern parts of the Northern Territory and Western Australia (1). The flesh and seed of the green plums are harvested and eaten by Aboriginal Australians who live in the remote places where it grows (2). It ripens in the hot humid summer of these tropical areas around the time of the first monsoonal rain (3). Green plums are eaten as a raw fruit from the tree (4), when they have been sundried (5) or when the flesh and seed are pounded together into a pulp (6). Green plums are wild harvested and have not yet been commercialized, however, there is interest from Aboriginal communities in Australia to use the green plum as part of sustainable Indigenous bush food businesses.

An initial study of the nutritional properties of the green plum, that was performed on slightly underripe green plums, discovered it was a good dietary source of folate, containing 752.4 µg/100 g Dry Weight (DW) or 161 µg/100 g Fresh Weight (FW) as pteroylmonoglutamic acid equivalents (7). A further study on ripe green plums found the total folate to be 118 µg/100 g FW (8). This amount of folate indicates the green plum is promising as a dietary source of folate, however, further testing of more green plum samples and from fruit across different locations was necessary to be able to confirm that the folates are consistently high.

Folate vitamers, also known as the B9 vitamins, are essential components of metabolism for all humans, plants and animals. Some plants, bacteria and fungi are able to synthesize folates, whereas humans and animals require it from dietary sources (9). Folate is important in human health as its deficiency can lead to neural tube defects in fetuses, megaloblastic anemia, cardiovascular disease, cancer and neurological disorders (10). Folate is such an important vitamin in health that many foods such as breads and cereals are now fortified with folic acid to counter deficiencies in the diets of whole populations (10, 11). In Australia, wheat flour sold for making bread must contain between 2 and 3 mg/kg of folic acid (12). Finding promising key sources of folate in understudied plant foods could help with decreasing micronutrient deficiencies across the world and contribute to future nutritional resilience (13). Underutilized plant foods, including native Australian plant foods, can add biodiversity to food and agriculture to increase food production and increase options for a balanced diet (14).

The amounts of specific nutritional compounds in fruit can vary across locations and harvest years due to differences in climate conditions, soil types and environmental variables (15). The aim of this study was to analyze green plums for folate vitamers from multiple locations and harvest years, to obtain more results on ripe fruit, and to understand how the vitamers change during fruit growth and ripening. For this study, fruit were obtained from four locations in the Northern Territory, three of these locations were sampled for two harvests, and fruit were harvested at all stages of development and divided into a maturity index to obtain folate results for these maturity stages. Five folate vitamers were measured and total folate was calculated as the sum of them. The folate vitamers measured were tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), 5-formyltetrahydrofolate (5-CHO-H_4folate), and 10-formylfolic acid (10-CHO-PteGlu) which are all generally present in fruit and vegetables, and the fifth vitamer measured was pteroylmonoglutamic acid/folic acid (PteGlu). This information will be useful for Aboriginal communities interested in creating sustainable Indigenous bush food businesses and for the food industry.

Materials and methods

Samples

Samples were collected by staff from Gulkula Mining Company Pty Ltd., Arnhem Land Progress Aboriginal Corporation (ALPA), Wild Orchard Kakadu Plum Pty Ltd., by researchers in this study and with the assistance of botanist Greg Leach (Charles Darwin University) and environmental officer Annemarie van Doorn (Gulkula and ALPA) and were obtained under Northern Territory Parks and Wildlife Permit numbers 64749, 64579, and 66293. Samples were obtained from four

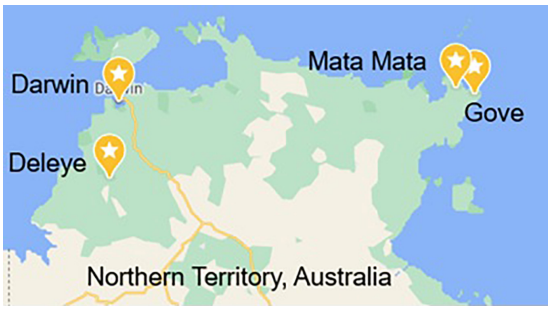


FIGURE 1
Map of the four locations in the Northern Territory, Australia, that the green plums were harvested from Map data© 2022 Google.

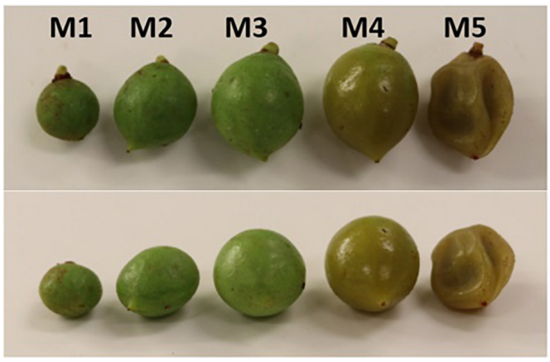


FIGURE 2
Maturity index of Green Plums from left to right: M1, M2, M3, M4, M5 from two different views.

locations, Gove (GOV), Deleye (DEL), Darwin (DWN), and Mata Mata (MAT) (Figure 1), and from two harvests designated 18 and 19, the year in which that green plum harvest began, as the fruit ripen and are harvested between November and February. GOV and MAT were both from the north-east part of the Northern Territory in East Arnhem Land and DEL and DWN were from the north-west part of the Northern Territory (Figure 1). Fruit from MAT were only obtained in the second harvest year. Green plum samples from GOV, DEL and DWN were identified by the Queensland Herbarium as *Buchanania obovata* and were incorporated into the Herbarium collection as AQ952392, AQ952393, and AQ952402. The accepted name for this species is *Buchanania obovata* Engl. from the family Anacardiaceae (16).

The fruit were harvested at all stages of maturity and were divided into five maturity stages to make a maturity index designated M1, M2, M3, M4, and M5 (Figure 2), which are described as:

M1: Smallest size, 0–25% in size, hard green, juvenile
M2: Medium size, 25–50% in size, hard green, juvenile
M3: Largest hard green mature fruit
M4: Yellower and softer, skin more translucent, ripe, edible
M5: Overripe or senescence, yellowish, dryer and wrinkled, edible

M1 and M2 are the juvenile fruit, M3 is the mature green fruit, M4 is the ripe fruit and M5 is ripened fruit that have dried in a similar way to how a sultana is dried. From GOV fruit were harvested from 11 separate locations within a 4-h drive and ripe M4 fruit from each of these locations was analyzed.

Red-colored green plums were harvested in both harvest years from GOV and were also analyzed for folate.

Whole fruit were frozen to −20°C within 1 day of collection and remained frozen during transportation to Brisbane and during storage until further processing. The fruit were cut and had the seeds removed by hand, and the flesh and skin of each composite sample was lyophilized in a Christ Gamma 1–16 LSC Freeze Drying Unit (John Morris Scientific, Osterode, Germany), then ground to a fine powder in a ball mill at 30 rotations per second (Lab Mill, Christy and Norris Ltd., Chelmsford, England) and frozen at −20°C until analysis.

Chemicals

L-ascorbic acid, sodium chloride, sodium hydroxide, disodium hydrogen orthophosphate and potassium dihydrogen orthophosphate were obtained from Chem-Supply (Gillman, SA, Australia). 1,4-dithiothreitol (DTT) was purchased from Roche (Basel, Switzerland). Sodium acetate trihydrate, methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). 2-(N-morpholino)-ethanesulfonic acid (MES) came from Sigma-Aldrich (Steinheim, Germany). Formic acid was from Honeywell (Seelze, Germany). Strata SAX cartridges were from Phenomenex (Aschaffenburg, Germany). Chicken pancreas was from Pel-Freez Biologicals (Rogers, AR, USA) and rat serum was from Bio-Rad (Gladesville, NSW, Australia). The unlabeled folate vitamer reference compounds, pteroylmonoglutamic acid/folic acid (PteGlu), tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), 5-formyltetrahydrofolate (5-CHO-H_4folate), and 10-formylfolic acid (10-CHO-PteGlu) and the ^{13}C-labeled isotopological internal standards, $^{13}C_5$-PteGlu, $^{13}C_5$-H_4folate, $^{13}C_5$-5-CH_3-H_4folate, and $^{13}C_5$-5-CHO-H_4folate were from Schircks Laboratories (Bauma, Switzerland).

Sample and reference preparation

The preparation of the samples, the reference analyses and the isotopological internal standards for the stable isotope dilution assay used to quantify the folate vitamers has previously

been described (17, 18). In short, triplicate replicates of 0.06–0.07 g of freeze-dried powder for each sample were weighed into glass tubes. Extraction buffer (10 mL) containing 200 mM MES, ascorbic acid (20 g/L) and DTT (pH 5.0) (1 g/L) was added and were equilibrated. Internal standards of $^{13}C_5$-PteGlu, $^{13}C_5$-H_4folate, $^{13}C_5$-5-CH_3-H_4folate and $^{13}C_5$-5-CHO-H_4folate were then added in amounts adjusted to the expected content of the analytes to fall in the given calibration line. Tubes were boiled at 100°C for 10 min, cooled on ice and enzymes for deconjugation were added as 2 mL chicken pancreas suspension (1 g/L in phosphate buffer and 1% ascorbic acid) and 0.3 mL of rat serum. Glass tubes were then incubated in the dark in a shaking incubator at 37°C for a minimum of 12 h, then boiled to stop the enzyme activity, cooled on ice, mixed with acetonitrile (10 mL) and centrifuged (4,000 rpm, 20 min, 4°C). The supernatant was purified using strong anion-exchange (SAX, quaternary amine, 55 μm, 70 Å, 500 mg/3 mL) and folates were eluted using an elution buffer of 5% sodium chloride, 1.36% sodium acetate, 1% ascorbic acid and 0.1% DTT. Eluate was membrane filtered at 4°C (0.2 μm PP membrane syringe filter, Pall Corporation, Melbourne, Australia), stored in dark glass vials at −35°C and measured within 10 days of extraction.

Unlabeled reference compounds of PteGlu, H_4folate, 5-CH_3-H_4folate, 5-CHO-H_4folate, and 10-CHO-PteGlu were measured and pre-dissolved in phosphate buffer. H_4folate, 5-CH_3-H_4folate, 5-CHO-H_4folate and 10-CHO-PteGlu were each mixed with PteGlu and MES extraction buffer and each reference was stored in a dark glass vial until analysis. A response mixture of unlabeled and ^{13}C-labeled reference compounds measured and mixed in MES extraction buffer was made up at the same time as the sample extractions and the unlabeled reference compounds, and stored and measured alongside them.

Instrumental conditions

Instrument conditions were slightly modified from those used in Striegel et al. (17). The analysis was performed on a Shimadzu Liquid Chromatograph Mass Spectrometer (LC-MS) (Shimadzu Corp., Kyoto, Japan) equipped with a Shimadzu 8,060 triple-stage quadrupole mass spectrometer, three Nexera X2 LC-30AC liquid chromatographs, a Nexera X2 SPD-M30A diode array detector, a Nexera X2 C20-20AC prominence column oven, a Nexera X2 SIL-30AC autosampler, and a Raptor ARC-18 column (2.7 μm, 100 × 2.1 mm) (Restek, Bad Homburg, Germany).

The mobile phase used for the binary gradient was a mixture of (A) 0.1% formic acid in distilled water and (B) acetonitrile with 0.1% formic acid and the flow rate was 0.40 mL/min. After 1.5 min the gradient was set to 3% B and raised to 10% B linearly within 3 min. The gradient was held at 10% B for 1.5 min then raised to 15% B over 5 min then to 50% B over 1 min and held for 1 min then reduced to 5% over 1 min and 3% over 4 min. The injection volume used was 5 μL and each injection had a total run time of 18 min. The column effluent was diverted to the mass spectrometer between 2.8 and 11 min and otherwise ran to the waste valve. The MS operated in multiple reaction monitoring mode for MS/MS measurements. The ion source parameters had a heat block temperature of 400°C, dilution line temperature 250°C, interface temperature 300°C, drying gas and heating gas flow were 10 L/min, nebulizing gas flow 2 L/min, column oven temperature was 30°C and collision-induced dissociation gas 270 kPa. Retention time for the folate vitamers were PteGlu 6.4, H_4folate 3.7, 5-CH_3-H_4folate 4.3, 5-CHO-H_4folate 5.9, and 10-CHO-PteGlu 5.8. Each sample replicate was analyzed on the instrument three times.

Calculations and statistics

Results were calculated using the measured amount of PteGlu in comparison to the unlabeled reference compounds in the reference solution. These were used in conjunction with the unlabeled and ^{13}C-labeled folate vitamers in the response measurement following the method of Striegel et al. (17). Results were calculated using Microsoft Excel v 2014 spreadsheets (Microsoft, Redmond, WA, USA). All results are given as μg/100 g as pteroylmonoglutamic acid equivalents in fresh weight (FW) form unless stated as dry weight (DW). The lower limit of quantification was 0.1 PteGlu in μg/100 g FW. Statistical analysis of the results was done using IBM SPSS software (IBM Australia Ltd., Sydney, NSW, Australia) using analysis of variance (ANOVA) and significantly different results were further tested using Tukey Honestly Significant Difference (HSD) tests.

Results and discussion

Folate in green plums through growth and ripening

The folate results for the green plums for the five maturity stages, the four locations and two harvest years are given in Table 1. Results are given for each of the five folate vitamers tested in fresh weight and for the sum of these which is the total folate in fresh weight. Total folate for each sample is also given in dry weight. A strawberry reference sample was analyzed alongside the green plum samples, it was previously determined to be 93.7 ± 6.5 μg/100 g (17), and in the present analysis yielded 87.2 ± 2.6 μg/100 g.

Of the folate vitamers tested, H_4folate, 5-CH_3-H_4folate, and 5-CHO-H_4folate are generally present in fruit and vegetables. The results in Table 1 show that 5-CH_3-H_4folate is the most abundant folate present in green plums at all maturity stages and

TABLE 1 Total folate content and distribution of vitamers in green plums with samples identified by location, harvest year, and maturity stage, results are presented as PteGlu in μg/100 g on fresh weight basis except for far right column which gives total folate on a dry weight basis.

Sample	5-CH3-H4folate	5-CHO-H4folate	10-CHO-PteGlu	H4folate	PteGlu	Total folate	Total folate (dry weight)
Gov 18 M1	24.3 ± 1.7^{a}	8.5 ± 0.9^{a}	0.8 ± 0.1^{b}	$0.3 \pm 0.4^{a,b}$	0.3 ± 0.3^{a}	34.2 ± 1.9^{a}	223.7 ± 12.1^{a}
Gov 18 M2	$38.0 \pm 1.9^{a,b}$	$20.4 \pm 1.1^{d,e,f}$	$<LOQ^{a}$	$1.0 \pm 0.4^{a,b,c,d,e,f}$	$<LOQ^{a}$	$59.5 \pm 3.0^{b,c}$	428.0 ± 21.3^{c}
Gov 18 M3	$55.7 \pm 3.9^{b,c}$	$20.5 \pm 0.6^{d,e,f}$	$<LOQ^{a}$	$0.7 \pm 0.0^{a,b,c,d}$	$<LOQ^{a}$	$77.1 \pm 4.4^{c,d}$	$478.7 \pm 27.6^{c,d}$
Gov 18 M4	$126.1 \pm 12.8^{g,h,i}$	$17.9 \pm 1.2^{c,d,e}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	144.0 ± 13.8	$475.7 \pm 45.4^{c,d}$
Gov 18 M5	260.4 ± 23.3^{j}	26.4 ± 1.8^{g}	$<LOQ^{a}$	$2.2 \pm 1.0^{d,e,f}$	$< LOQ^{a}$	289.0 ± 23.1^{j}	$571.9 \pm 45.6^{e,f}$
Del 18 M1	$73.6 \pm 6.4^{c,d}$	$37.0 \pm 0.7^{h,i,j}$	$<LOQ^{a}$	$1.8 \pm 1.2^{b,c,d,e,f}$	$<LOQ^{a}$	$112.4 \pm 5.4^{e,f}$	$673.2 \pm 32.4^{g,h}$
Del 18 M2	68.0 ± 0.9^{c}	$23.2 \pm 1.3^{e,f,g}$	$<LOQ^{a}$	$0.3 \pm 0.3^{a,b}$	$<LOQ^{a}$	$91.6 \pm 1.0^{d,e}$	$571.9 \pm 6.0^{e,f}$
Del 18 M3	$73.7 \pm 1.8^{c,d}$	$20.5 \pm 1.3^{d,e,f}$	$<LOQ^{a}$	$0.3 \pm 0.4^{a,b}$	$<LOQ^{a}$	$94.5 \pm 2.1^{d,e}$	$589.5 \pm 13.2^{e,f}$
Del 18 M4	$125.8 \pm 5.2^{g,h,i}$	$22.5 \pm 0.6^{d,e,f,g}$	$<LOQ^{a}$	$0.3 \pm 0.3^{a,b}$	$<LOQ^{a}$	$148.6 \pm 4.9^{g,h,i}$	$722.1 \pm 23.8^{h,i}$
Dwn 18 M2	$133.7 \pm 0.6^{h,i}$	33.1 ± 1.9^{h}	$<LOQ^{a}$	$0.6 \pm 0.4^{a,b,c,d}$	$<LOQ^{a}$	167.3 ± 1.7^{i}	1063.5 ± 11.1^{k}
Dwn 18 M3	$114.5 \pm 2.9^{f,g,h}$	$34.4 \pm 0.9^{h,i}$	$<LOQ^{a}$	$0.7 \pm 0.2^{a,b,c,d}$	0.4 ± 0.7^{a}	$150.0 \pm 1.9^{g,h,i}$	882.6 ± 11.1^{j}
Gov 19 M1	$41.4 \pm 1.3^{a,b}$	$14.2 \pm 1.0^{b,c}$	$<LOQ^{a}$	$0.3 \pm 0.1^{a,b}$	$<LOQ^{a}$	$55.9 \pm 1.9^{a,b,c}$	337.4 ± 11.3^{b}
Gov 19 M2	25.8 ± 1.2^{a}	$10.4 \pm 0.9^{a,b}$	0.2 ± 0.3^{a}	$0.5 \pm 0.7^{a,b,c,d}$	$<LOQ^{a}$	$36.9 \pm 1.3^{a,b}$	237.2 ± 8.1^{a}
Gov 19 M3	27.0 ± 2.8^{a}	$14.5 \pm 1.0^{b,c}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$41.5 \pm 3.7^{a,b}$	$272.0 \pm 24.1^{a,b}$
Gov 19 M4*	$97.0 \pm 6.0^{e,f}$	$13.8 \pm 1.0^{b,c}$	$<LOQ^{a}$	$0.2 \pm 0.3^{a,b}$	$<LOQ^{a}$	$111.1 \pm 6.3^{e,f}$	$444.5 \pm 25.2^{c,d}$
Gov 19 M5	139.9 ± 5.8^{i}	$11.0 \pm 0.8^{a,b}$	$<LOQ^{a}$	2.5 ± 0.6^{f}	0.3 ± 0.3^{a}	$153.7 \pm 4.9^{h,i}$	$506.5 \pm 16.2^{c,d,e}$
Del 19 M1	67.6 ± 2.1^{c}	41.7 ± 1.9^{j}	$<LOQ^{a}$	$0.9 \pm 0.2^{a,b,c,d,e,f}$	$<LOQ^{a}$	$110.2 \pm 3.0^{e,f}$	$522.4 \pm 14.2^{d,e}$
Del 19 M2	$100.7 \pm 3.5^{e,f}$	$39.1 \pm 0.8^{i,j}$	$<LOQ^{a}$	$1.6 \pm 0.6^{a,b,c,d,e,f}$	$<LOQ^{a}$	$141.5 \pm 3.7^{g,h}$	$711.9 \pm 18.5^{h,i}$
Del 19 M3	$109.1 \pm 2.9^{e,f,g}$	$39.3 \pm 5.1^{i,j}$	$<LOQ^{a}$	$2.1 \pm 1.5^{c,d,e,f}$	$<LOQ^{a}$	$150.4 \pm 6.6^{h,i}$	791.0 ± 34.6^{i}
Dwn 19 M1	24.2 ± 1.1^{a}	$17.4 \pm 1.5^{c,d}$	$<LOQ^{a}$	$0.5 \pm 0.4^{a,b,c,d}$	$<LOQ^{a}$	$42.1 \pm 2.3^{a,b}$	203.0 ± 11.0^{a}
Dwn 19 M2	$44.0 \pm 2.0^{a,b}$	$14.8 \pm 1.7^{b,c}$	$<LOQ^{a}$	$0.8 \pm 0.3^{a,b,c,d,e,f}$	$<LOQ^{a}$	$59.6 \pm 1.0^{b,c}$	$286.5 \pm 5.0^{a,b}$
Dwn 19 M3	$99.8 \pm 17.2^{e,f}$	$24.9 \pm 1.6^{f,g}$	$<LOQ^{a}$	$2.4 \pm 0.3^{e,f}$	$<LOQ^{a}$	$127.2 \pm 18.5^{f,g}$	$619.1 \pm 90.0^{f,g}$
Mat 19 M2	$58.3 \pm 1.0^{b,c}$	$23.4 \pm 2.8^{f,g}$	$<LOQ^{a}$	$1.6 \pm 0.3^{a,b,c,d,e,f}$	$<LOQ^{a}$	83.4 ± 3.0^{d}	$500.9 \pm 17.8^{c,d,e}$
Mat 19 M3	$68.9 \pm 2.7^{\cdot c,d}$	$22.0 \pm 2.3^{d,e,f,g}$	$<LOQ^{a}$	$0.6 \pm 0.3^{a,b,c,d}$	0.2 ± 0.2^{a}	$91.8 \pm 0.9^{d,e}$	$581.1 \pm 5.4^{e,f}$
Mat 19 M4	$90.7 \pm 7.0^{d,e}$	$20.4 \pm 0.3^{d,e,f}$	$<LOQ^{a}$	$0.4 \pm 0.5^{a,b,c}$	$<LOQ^{a}$	$111.5 \pm 7.5^{e,f}$	$446.2 \pm 30.0^{c,d}$

<LOQ is less than the limit of quantification, *Gov 19 M4 result is the average of the 11 results from Table 2, means followed by the same letter within columns were not significantly different ($p < 0.05$).

TABLE 2 Total folate content and distribution of vitamers in ripe green plums from different locations around Gove in East Arnhem Land, calculated as PteGlu in μg/100 g on fresh weight basis except for the total folate dry weight column.

Sample	5-CH3-H4folate	5-CHO-H4folate	10-CHO-PteGlu	H4folate	PteGlu	Total folate	Total folate (dry weight)
Gov 19 M4 S1	74.5 ± 4.0^{a}	$12.6 \pm 1.2^{c,d}$	$<LOQ^{a}$	0.1 ± 0.2^{a}	$<LOQ^{a}$	87.2 ± 5.0^{a}	348.8 ± 20.0^{c}
Gov 19 M4 S2	129.1 ± 10.3^{e}	19.5 ± 0.7^{e}	$<LOQ^{a}$	0.8 ± 0.5^{a}	$<LOQ^{a}$	149.4 ± 10.7^{c}	597.8 ± 42.7^{a}
Gov 19 M4 S3	$92.6 \pm 3.8^{a,b,c}$	19.3 ± 1.7^{e}	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	111.9 ± 5.5^{b}	$447.8 \pm 22.1^{a,b}$
Gov 19 M4 S4	$79.0 \pm 9.3^{a,b}$	6.4 ± 0.6^{a}	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	85.4 ± 9.5^{a}	$341.8 \pm 37.9^{a,b}$
Gov 19 M4 S5	$96.2 \pm 6.3^{b,c}$	14.9 ± 0.6^{d}	$<LOQ^{a}$	0.2 ± 0.2^{a}	$<LOQ^{a}$	111.3 ± 6.5^{b}	445.3 ± 26.0^{b}
Gov 19 M4 S6	$118.9 \pm 7.5^{d,e}$	18.5 ± 1.0^{e}	$<LOQ^{a}$	0.2 ± 0.3^{a}	$<LOQ^{a}$	137.6 ± 6.5^{c}	550.7 ± 25.9^{c}
Gov 19 M4 S7	$100.1 \pm 1.2^{c,d}$	14.4 ± 1.5^{d}	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	114.5 ± 1.9^{b}	458.1 ± 7.7^{c}
Gov 19 M4 S8	129.6 ± 11.4^{e}	$10.9 \pm 0.6^{b,c}$	$<LOQ^{a}$	0.1 ± 0.1^{a}	$<LOQ^{a}$	140.6 ± 11.1^{c}	562.5 ± 44.2^{a}
Gov 19 M4 S9	$83.7 \pm 2.1^{a,b,c}$	15.0 ± 1.6^{d}	$<LOQ^{a}$	0.7 ± 0.6^{a}	$<LOQ^{a}$	$99.4 \pm 3.0^{a,b}$	397.7 ± 12.0^{a}
Gov 19 M4 S10	73.1 ± 5.9^{a}	$9.1 \pm 0.3^{a,b}$	$<LOQ^{a}$	0.2 ± 0.3^{a}	$<LOQ^{a}$	82.4 ± 5.5^{a}	329.9 ± 22.1^{b}
Gov 19 M4 S11	$90.7 \pm 4.2^{a,b,c}$	$11.2 \pm 0.9^{b,c}$	$<LOQ^{a}$	0.4 ± 0.6^{a}	$<LOQ^{a}$	$102.2 \pm 4.0^{a,b}$	409.2 ± 16.0^{b}
Average:	*97.0 ± 6.0*	*13.8 ± 1.0*	*<LOQ*	*0.2 ± 0.3*	*<LOQ*	*111.1 ± 6.3*	*444.5 ± 25.2*

<LOQ is less than the limit of quantification; means followed by the same letter within columns were not significantly different ($p < 0.05$).

in all locations and both harvests. The green plum results show mostly increases in total folate FW in the green plums through growth, maturation, and ripening and each total folate result is mostly comprised of 5-CH_3-H_4folate. **Table 1** shows that 5-CH_3-H_4folate mostly increases in green plums as it matures between M1 and M3 although DWN 18 and GOV 19 show some decreases. There is a marked increase through ripening between M3 and M4 in all samples sets and again through senescence up to M5. The second highest folate vitamer in green plums is 5-CHO-H_4folate which has a slight decrease in all of the locations and harvest years tested between M3 and M4, through the ripening process, except for DEL 18 which increases slightly. PteGlu, H_4folate, and 10-CHO-PteGlu are only present in very small amounts in green plums, with levels less than 2.5 μg/100 g in all samples for all of these vitamers. PteGlu is only found naturally in trace quantities (11), as is seen in the green plum. The DW total folate results show there is generally an increase between M2 and M3, except for the DWN 18 samples which show a decrease, however, both these results are at higher levels than the other samples. During ripening, between M3 and M4, there is an increase in the total folate in the DEL 18 and GOV 19 samples, a very slight decrease in the GOV 18 and a decrease in the MAT 19. There is an increase in amount of DW folate vitamers from M4 to M5 that occurs during the drying of the ripe fruit.

When comparing the fresh weight results of green plums with those of other fruits, folates in papaya fruit have an increase in 5-CH_3-H_4folate through development and into the ripening stages and then a decrease as it finishes ripening (19). An analysis of six different cultivars of tomato through ripening, from unripe through to full ripe, did not show any clear trend of increasing or decreasing 5-CH_3-H_4folate content through four stages of ripening (20). Tomato fruit that are mature green have been measured as having total folate at 21.93 ± 1.17 μg/100 g FW which was slightly higher than the total folate measured on ripe red tomato's at 18.05 ± 2.01 μg/100 g FW (21). Two other studies also show higher levels of folate in mature green tomatoes then in red ripe ones (22) and one of these showed a decrease from green mature to red ripe tomatoes in 5-CH_3-H_4folate content on three different cultivars (23). Analysis of folates in the kernel of waxy maize through development showed decreasing total folate through four stages of development (24).

Folates are synthesized by plants and the folate vitamers interconvert between themselves as part of the metabolism process (25–27). The biosynthesis pathways of folate in plants have been characterized and well-studied (28). This current analysis adds to plant metabolism knowledge by giving an understanding of the regulation of folate in the green plum. Folates are only synthesized in the mitochondria of plant cells where the pterin and *p*-aminobenzoic acid (*p*ABA) moieties join into a H_4folate molecule, however, they are also found throughout the cell in the plastids, cytosol and vacuoles (26, 29). Folate vitamers, including four of the vitamers in this study, are important in metabolic pathways involving one-carbon transfers. It is the reduced folate forms, including H_4folate and 5-CH_3-H_4folate, which transfer the one-carbon units and are the core of one-carbon metabolism providing single carbon units for anabolic reactions (27). These one-carbon substituents are primarily methyl or formyl groups that exist on the N^5 or N^{10} positions but can also be methylene, methenyl or formimino groups (11, 30). One-carbon transfers are important in amino acid catabolism, 10-CHO-H_4folate is required for purine ring biosynthesis and contributes to formylmethionyl-tRNA synthesis (27) and 5,10-CH_2-H_4folate is required for synthesis of thymidine which is needed for DNA synthesis (30). 5-CH_3-H_4folate acts as a methyl donor during the synthesis of methionine from homocysteine and methionine is converted to S-adenosyl methionine (SAM) (10, 30) and in plants SAM is involved in methyltransferase reactions and the synthesis of biotin and ethylene (27). Folates are essential for the development of plants because of their role in DNA synthesis (26). Folates are present in the green plum from the juvenile stage and at mostly increasing levels as the cells grow and divide enabling DNA synthesis to occur. The 5-CHO-H_4folate has some accumulation in green plums with higher levels in those from DEL and DWN then from GOV and MAT but without major trends in increasing or decreasing seen. The 5-CH_3-H_4folate is the vitamer that is at the highest level and which accumulates the most throughout fruit development. This indicates it is produced at faster rates then the rate it is being used. Further studies on the genetics of green plums, including genomics, transcriptomics, and proteomics, could provide more information about folate biosynthesis and metabolism in this fruit.

FIGURE 3
Red-colored **(top row)** and green-colored **(bottom row)** green plums from GOV location.

The original study on the green plum was from underripe fruit from the same region as the DWN samples and the total folate was 161 μg/100 g FW (7). This is slightly higher than the 18 M3 result from DWN which was 150.0 μg/100 g FW. Ripe, M4, samples were not available for testing for either harvest year from this location, however, these results do confirm the original published green plum folate analysis. Variation in metabolite content in fruit may be caused by many different factors, and the differences seen between location and harvest year in this study could be due to environmental differences such as soil type, rainfall or sunlight, genetic differences, or normal variation between trees.

Folate in ripe green plums

The green plums are eaten when they are ripe (M4) or when they have been semi-dried in the sun (M5). Analysis of the folate vitamers of ripe green plums was done on fruit from 11 different sets of trees in the GOV region during the second harvest to determine the natural variation that occurs in one region. These results are presented in **Table 2** and are a comparison of the natural variation in a location.

The total folate results of the ripe, M4, fruit from GOV were between 82.4 ± 5.5 and 149.4 ± 10.7 μg/100 g FW and all of the M4 samples from other locations also fall in this same range. The total folate on ripe green plums previously published was also from fruit from this location and was found to be 118 μg/100 g FW (8) which is near the middle of the range in this study. **Tables 1**, **2** together show the variation between all of the ripe fruit in this study from all of the locations and harvest years. The two highest vitamers in the ripe fruit are 5-CH_3-H_4folate and 5-CHO-H_4folate. This variation in ripe fruit is similar to the variation seen in strawberry cultivars which have been measured between 93.0 ± 5.3 and 153 ± 5.0 μg/100 g FW using the same method (17). Despite the variation seen in ripe green plums and strawberries, all of the results are still high when compared to other fruits. Green plums and strawberries both have higher folate levels than many other fruits including grape, kiwi, mandarin, watermelon, pineapple (31) and tomatoes (4 to 60 μg/100 g FW) (22). Green plum has higher folate than mango which is also in the family Anacardiaceae (55.8 ± 0.73 to 74.5 ± 2.09 μg/100 g FW) (32). It is higher than the tropical fruits guavas (43.1 ± 5.16–103 ± 4.32 μg/100 g FW), papayas (61.6 ± 3.01–90.7 ± 1.24 μg/100 g FW), jackfruit (52.9 ± 2.61–83.6 ± 5.50 μg/100 g FW), dragon fruit (36.0 ± 0.53 μg/100g FW), prickly pear (23.8 ± 0.44 μg/100 g FW), tamarillo (16.4 ± 0.60 μg/100 g FW) and tamarind (11.4 ± 0.70 μg/100g FW) (32). Despite this, the green plums folate levels are still within the natural range and are below those of the durian, which has been measured as high as 440 ± 7.89 μg/100g FW (18) and passion fruit (136 ± 21.7–271 ± 3.64 μg/100 g FW) (32).

Compared with other Australian native fruit, the green plum has higher levels of folate than most of those that have been analyzed. The green plum range in dry weight for ripe, M4, fruit is between 329.9 ± 22.1 and 597.8 ± 42.7 μg/100 g DW for the GOV and MAT fruit and the DEL 18 M4 fruit was measured at 722.1 ± 23.8 μg/100 g DW. The green plums are higher in folate than the quandong (120 μg/100 g DW), the Kakadu plum, riberry, and lemon aspen (all 110 μg/100 g DW) and Davidson's plum (34–40 μg/100 g DW). The Australian desert lime had levels within the green plum range (420 μg/100 g DW) (33). People in remote Aboriginal communities generally have poor quality diets with many nutrients coming from fortified processed foods (34). The remote locations are difficult to transport fresh fruit and vegetables too, however, bush foods, the fruit and vegetables that are native to these areas, are still eaten by these Aboriginal communities (2). The results show that the green plum is a good dietary source of folate for these remote communities in Australia.

The edible M5 semi-dried fruit had even higher FW and DW results then the ripe M4 fruit. The first harvest year result was 289.0 ± 23.1 and the second 153.7 ± 4.9 μg/100 g FW which is higher than folate levels in dried sultana's, currants, apricots and prunes which have been measured between 13.8 and 83.8 μg/100 g FW (35). These high levels in the M5 fruit also indicate that studies on the degradation of folates in green plums at different storage temperatures should be done to understand their stability, as these vitamers have increased as the fruit has dried.

In humans folate deficiency has been linked to megaloblastic anemia as folate and vitamin B12 are closely linked in metabolite pathways (30). Folate is important for maintaining normal cell growth and division and for the repair, methylation

TABLE 3 Total folate content and distribution of vitamers in ripe (M4) red-colored green plums from East Arnhem Land, calculated as PteGlu in μg/100 g on fresh weight basis except for total folate dry weight column.

	5-CH3-H_4folate	5-CHO-H4folate	10-CHO-PteGlu	H4folate	PteGlu	Total folate	Total folate (dry weight)
Gov 18 red	273.4 ± 25.5	19.7 ± 1.7	<LOQ	0.6 ± 0.4	<LOQ	293.7 ± 27.4	929.6 ± 86.9
Gov 19 red	170.6 ± 6.9	21.3 ± 1.3	<LOQ	0.5 ± 0.4	<LOQ	192.5 ± 7.0	609.7 ± 22.0

<LOQ is less than the limit of quantification.

and synthesis of DNA (10, 36). 5-CH_3-H_4folate is the most abundant form of folate that circulates in the human body and it can cross the intestinal barrier and move into the blood stream (10) or be transported into peripheral tissues (37). Folic acid, PteGlu, is used as a supplement in case of and to prevent folate deficiency (10, 11). Bread is commonly fortified with folate, however, the bioaccessibility of the folate in different bread types ranges from 31 to 120% (38). In strawberry fruit the bioaccessibility of folate vitamers has been found to be between 90 and 95% when subjected to *in vitro* gastrointestinal digestion and colonic fermentation (39). A human pilot study of folate bioavailability of strawberries found a recovery from plasma of 96.2% (40). Biofortified tomatoes analyzed for natural folate bioavailability using a murine model has been found to be comparable to the bioavailability of synthetic 5-CH_3-H_4folate and more than PteGlu (41). 5-CH_3-H_4folate has been found to have advantages over synthetic folic acid in human health as it is absorbed even when gastrointestinal pH has been altered, it is bioavailable even when there are metabolic defects, reduces potential to mask symptoms of B12 deficiency and reduces interactions with other drugs (37, 42). Unmetabolised folic acid from exposure to synthetic folic acid *in utero* at higher concentration in babies at birth has been associated with the development of food allergies and food sensitization (43). Therefore, to find fruit with naturally high levels of folate is important for diet and health. The dry weight results of ripe green plums show that as a freeze-dried powder it is very high in folate and future uses of green plum as a nutraceutical or as a natural source of folate, especially of 5-CH_3-H_4folate, for fortification of foods should be explored. Further studies on the bioaccessibility of folate vitamers in green plums is suggested. The sensory qualities of green plum shows it has well-liked and unique flavor profile of *sweet*, *tart* and *stewed apple* and has potential to be used as a future flavor (8). This analysis confirms that the folate levels in green plums are consistently high across locations, and this, combined with its desirable sensory qualities, suggest it could be used in novel future food products.

Folate in red-colored green plums

Two samples of red-colored green plums from the GOV location were also tested for folate content. **Figure 3** shows representative examples of the ripe red-colored green plums in the top row and the ripe green-colored green plums in the bottom row for comparison.

The folate results of the red-colored green plums, in **Table 3**, show they are even higher in folate then the green-colored green plums in both fresh weight and dry weight. Both harvest years have higher levels of total folate and 5-CH_3-H_4folate than all of the ripe green-colored green plums that were measured. The amount of 5-CHO-H4folate and H4folate are similar to the highest levels in the green-colored ripe fruit. The red-colored fruit also have less than the limit of quantification for 10-CHO-PteGlu and PteGlu, just as all of the ripe green-colored green plums did. Overall, the red-colored green plums are very high in folate and further analysis of red-colored green plums for both its folate vitamers and other nutritional properties is recommended.

Conclusion

This study has shown that green plums are high in folate and are a good natural dietary source of it. The green plum 5-CH_3-H_4folate increases and accumulates in the fruit through development, ripening and senescence. There is some variation between green plums from different locations and sets of trees but all ripe samples are considered good folate sources. The red-colored green plums are even higher in folate than the green-colored green plums and further analysis of them is warranted. Folates are important for plant metabolism and for human metabolism and this study gives insight into folate vitamers in green plums as they grow and ripen, from different locations and as a dietary source.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SF, YS, and MR conceptualized the manuscript. SF and HH performed the analysis. SF wrote the manuscript. HJS, HES, YS, and MR supervised the project. SF, HH, HJS, HES, YS, and MR edited the manuscript. All authors contributed to the article and approved the submitted version.

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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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EDITED BY
Dejian Huang,
National University of Singapore, Singapore

REVIEWED BY
Beatriz Andrea Acosta-Estrada,
Monterrey Institute of Technology and Higher Education (ITESM), Mexico
Ivan Luzardo-Ocampo,
National Autonomous University of Mexico, Mexico

*CORRESPONDENCE
Lena Gálvez Ranilla
✉ lgalvez@ucsm.edu.pe

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Integrated metabolite analysis and health-relevant *in vitro* functionality of white, red, and orange maize (*Zea mays* L.) from the Peruvian Andean race *Cabanita* at different maturity stages

Lena Gálvez Ranilla[1,2]*, Gastón Zolla[3], Ana Afaray-Carazas[1], Miguel Vera-Vega[3], Hugo Huanuqueño[4], Huber Begazo-Gutiérrez[5], Rosana Chirinos[6], Romina Pedreschi[7,8] and Kalidas Shetty[9]

[1]Laboratory of Research in Food Science, Universidad Catolica de Santa Maria, Arequipa, Perú, [2]Escuela Profesional de Ingeniería de Industria Alimentaria, Departamento de Ciencias e Ingenierías Biológicas y Químicas, Facultad de Ciencias e Ingenierías Biológicas y Químicas, Universidad Catolica de Santa Maria, Arequipa, Perú, [3]Laboratorio de Fisiología Molecular de Plantas, PIPS de Cereales y Granos Nativos, Facultad de Agronomía, Universidad Nacional Agraria La Molina, Lima, Perú, [4]Programa de Investigación y Proyección Social en Maíz, Facultad de Agronomía, Universidad Nacional Agraria La Molina, Lima, Perú, [5]Estación Experimental Agraria Arequipa, Instituto Nacional de Innovación Agraria (INIA), Arequipa, Perú, [6]Instituto de Biotecnología, Universidad Nacional Agraria La Molina, Lima, Perú, [7]Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile, [8]Millennium Institute Center for Genome Regulation (CRG), Santiago, Chile, [9]Department of Plant Sciences, North Dakota State University, Fargo, ND, United States

The high maize (*Zea mays L.*) diversity in Peru has been recognized worldwide, but the investigation focused on its integral health-relevant and bioactive characterization is limited. Therefore, this research aimed at studying the variability of the primary and the secondary (free and dietary fiber-bound phenolic, and carotenoid compounds) metabolites of three maize types (white, red, and orange) from the Peruvian Andean race *Cabanita* at different maturity stages (milk-S1, dough-S2, and mature-S3) using targeted and untargeted methods. In addition, their antioxidant potential, and α-amylase and α-glucosidase inhibitory activities relevant for hyperglycemia management were investigated using *in vitro* models. Results revealed a high effect of the maize type and the maturity stage. All maize types had hydroxybenzoic and hydroxycinnamic acids in their free phenolic fractions, whereas major bound phenolic compounds were ferulic acid, ferulic acid derivatives, and *p*-coumaric acid. Flavonoids such as luteolin derivatives and anthocyanins were specific in the orange and red maize, respectively. The orange and red groups showed higher phenolic ranges (free + bound) (223.9–274.4 mg/100g DW, 193.4– 229.8 mg/100 g DW for the orange and red maize, respectively) than the white maize (162.2–225.0 mg/100g DW). Xanthophylls (lutein, zeaxanthin, neoxanthin, and a lutein isomer) were detected in all maize types. However, the orange maize showed the highest total carotenoid contents (3.19–5.87 μg/g DW). Most phenolic and carotenoid compounds decreased with kernel maturity in all cases. In relation to the primary metabolites, all maize types had similar fatty acid contents (linoleic acid > oleic acid > palmitic acid > α-linolenic acid >

stearic acid) which increased with kernel development. Simple sugars, alcohols, amino acids, free fatty acids, organic acids, amines, and phytosterols declined along with grain maturity and were overall more abundant in white maize at S1. The *in vitro* functionality was similar among *Cabanita* maize types, but it decreased with the grain development, and showed a high correlation with the hydrophilic free phenolic fraction. Current results suggest that the nutraceutical characteristics of orange and white *Cabanita* maize are better at S1 and S2 stages while the red maize would be more beneficial at S3.

KEYWORDS

***Zea mays*, Peruvian maize, *Cabanita*, primary metabolites, secondary metabolites, antioxidant capacity, hyperglycemia, biodiversity**

1. Introduction

Maize (*Zea mays* L. ssp. *mays*) originated about 9,000 years ago in Mexico, and Latin America is considered the center of its genetic diversity and primary domestication (1–3). This cereal is staple food in Mesoamerican and Latin American countries since it is the base of many traditional preparations. It has been reported that the conservation and sustainable use of Latin American maize landrace diversity is fundamental for worldwide food security (1). Hence, efforts at multiple levels should be focused on the characterization of the genetically heterogenous landrace material as the base for further breeding improvements relevant for food security and health among indigenous food systems (1, 4).

The diversity of maize landrace populations is represented in races, which are identified according to their common botanical characteristics, geographical distribution, ecological adaptation, and cultural importance (uses and customs) (5–7). Mexico and Peru have concentrated around 30 percent of the Latin American maize diversity including 59 and 52 races, respectively (7, 8). The Peruvian Andean region with its great variety of ecological features has the highest maize phenotypic diversity worldwide (7, 9, 10). However, limited scientific information exist about Andean maize diversity which is compromising its adequate conservation and essential health relevant uses.

Whole cereal grains are valuable sources of carbohydrates, proteins, dietary fiber, minerals, and vitamins along with other critical bioactive metabolites with known health-promoting benefits (11, 12). The regular intake of whole grains has been inversely correlated with lower incidence of several chronic non-communicable diseases including type 2 diabetes (13), cardiovascular disease (14), and some types of cancer (15, 16). Maize contains nutritionally relevant macro and micronutrients mainly carbohydrates, lipids (with mono and polyunsaturated fatty acids), vitamins, minerals, and resistant starch (17). In addition, biologically active functional compounds such as phenolic compounds, carotenoids, tocopherols, and phytosterols have been reported in maize (17). In fact, unique phenolic and carotenoid profiles have been reported in different maize landraces linked to variable nutraceutical properties (18). Accordingly, more studies are needed to fully characterize maize landraces, targeting those that are the base of needs of food security and economy in many geographical areas such as the Andean region.

The maize race *Cabanita* has been cultivated since the Pre-Inca period in the southern Andean region of Arequipa in Peru at around 3,000 meters of altitude (19). Ears of *Cabanita* race have a conic-cylindrical shape and exhibit variable kernel pigmentations with predominance of white and partially red colored-pericarps (19). In a previous study, some Peruvian maize races including *Arequipeño*, *Cabanita*, *Kculli*, *Granada*, and *Coruca* races were evaluated in relation to their phenolic composition, *in vitro* anti-hyperglycemia, and anti-obesity potential (20). *Cabanita* kernels showed the second highest total oxygen radical absorbance antioxidant capacity (ORAC) and hyperglycemia management-relevant α-amylase inhibition following the purple-colored maize group (*Kculli* race) (20). More recently, *Cabanita* maize from two different provinces in Arequipa (Peru) were evaluated in relation to their physical characteristics, bioactive (phenolic and carotenoid) composition and *in vitro* antioxidant capacity (19). Although *Cabanita* samples from both provinces showed a certain grade of similarity according to the multivariate PCA (Principal Component Analysis), in general maize cultivated under Andean environments with naturally higher ecological stress factors such as higher altitudes and lower temperatures showed higher phenolic and antioxidant capacity ranges (19).

The intake of this traditional Andean maize is mostly in the mature dried form. Andean farmers still maintain the postharvest traditional practices along the *Cabanita* maize production chain. Once the maize ears have reached their highest length and a certain moisture level which is subjectively measured based on the farmer's experience, the plants are cut and dried in a piled form in the same land. Thereafter, dried plants are transported to the farmer's warehouses where maize ears are unshelled and exposed directly to the sun until complete drying (19). Dried grains are then consumed roasted, or further milled and used as flour in different culinary preparations.

Several studies focused mostly on sweet and waxy maize improved varieties have shown that bioactive compounds such as phenolic antioxidants and carotenoids vary depending on the kernel maturation stage. Phenolic compounds such as anthocyanins from different Asian colored waxy maize genotypes increased along the kernel maturation from 20 to 35 days after pollination (DAP) (21). Similarly, Zhang et al. (22) observed an increase of the total phenolic contents (TPC) in mature kernels from a yellow maize variety (48 DAP). The increase of carotenoids such as lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin has also been reported during the kernel maturation

of some sweet maize varieties from China (23). On the contrary, the total phenolic and total carotenoids contents decreased at the end of grain maturation in yellow maize bred in United States (116 DAS, days after seeding) (24). These discrepancies reveal that different factors including genetic factors (variety), the time of harvest, and the agroecological conditions of maize cultivation may influence the bioactive composition during the maize kernel maturation. Consequently, the research on this topic should be performed case by case, according to specific ecological environments.

As a second stage follow up studies of previous advances to characterize the Peruvian Andean maize *Cabanita* (19), the objective of current research was to study the primary (polar compounds and fatty acids) and secondary metabolite composition (free and bound phenolics and carotenoid compounds), and the *in vitro* health-relevant functional properties of three selected *Cabanita* types (white, red, and orange pigmented kernels) harvested at different maturity stages, using targeted and untargeted metabolomic platforms. The *in vitro* model based functionality of *Cabanita* maize was evaluated in relation to its antioxidant potential and inhibitory activity against key digestive enzymes (α-amylase and α-glucosidase) relevant for hyperglycemia modulation. Results from this study will contribute with important biochemical and metabolomic information for the characterization, and conservation of the maize race *Cabanita*. In addition, information from this research would be important to diversify the consumption options of this Andean maize beyond the traditional mature form. This would likely lead to potential beneficial effects of food crops at health and economical levels among indigenous communities in the future.

2. Materials and methods

2.1. Cultivation of *Cabanita* maize and sampling

The germplasm of *Cabanita* maize (*Zea mays* L.) collected in a previous study was used (19). Maize with sample codes CAW, CCR, COM representing white, red, and orange kernels were selected for current study considering the pigmentation diversity found in *Cabanita* maize race (19). These maize samples were obtained from the province of Caylloma (Cabanaconde district) located in the southern Andean region of Arequipa in Peru and were stored under refrigeration (2–5°C) (19). The field experiment was performed in the nursery garden *Santa Maria* at the *Universidad Catolica de Santa Maria* located in the Sachaca district (S: 16° 41′ 93.9″; W: 071° 56′ 34.0″; 2,240 meters of altitude), province of Arequipa (Arequipa, Peru).

Cabanita maize was cultivated in 20 L pots under open air and sun light exposure from 25 November 2020 to 28 June 2021. Commercial prepared soil (containing humus, field soil, and manure) was used and its physico-chemical characteristics are shown in Supplementary Table S1. The meteorological conditions during the maize plant development until sample harvest are shown in Supplementary Table S2. Groups of 6 pots were sown in three consecutive weeks (total 18 pots per maize type) and 5 *Cabanita* seeds were sown in each pot (sowing dates: 25 November, 2 December, and 9 December 2020). This procedure was applied to ensure the number of biological replicates (four) at three maturity stages per type of maize (white, red, orange) for the current study. The group of ears harvested from a single pot was considered a biological replicate (from 1 to 4 ears were obtained per pot). Additional supplementation with commercial fertilizers (urea and NPK + micronutrients) was performed during the vegetative period of maize plants (from week 2 to 11 after sowing) and the phytosanitary control was undertaken using conventional practices for the cultivation of maize in combination with the use of ecological insect traps. Well water was used for the irrigation which was carried out under field capacity in a similar way as in field cultivation.

During the plant reproductive stage, the female inflorescences were promptly protected with a plastic bag until the emergence of the styles (silks). The pollination was manually developed using composite pollen collected from mature tassels (male inflorescence) of plants from the same maize type. Once pollinated, each ear was protected with paper bags until physiological maturity. This procedure avoided the cross-pollination among different maize types. Ears were collected at three different grain maturity stages according to the grain physical appearance and moisture contents (25, 26). The milky stage (S1) is characterized by the starch accumulation and the observation of a milky white fluid upon finger pressure (25). In the dough stage (S2), the grain is still soft and humid, with intermediate humidity, whereas the physiological mature stage (S3) corresponds to the completion of kernel development, and a black layer is formed at the base of the kernel (25, 26). In case of the white maize type, S1 corresponded to 28 DAP and 79% moisture, S2 to 39 DAP and 68% moisture, and S3 to 75 DAP and 45% moisture. For the red type maize, S1 was at 33 DAP and 74% moisture, S2 at 36 DAP and 68% moisture, and S3 was at 77 DAP and 45% moisture. In the orange type, S1 corresponded to 32 DAP and 75% moisture, S2 to 43 DAP and 64% moisture, and S3 to 76 DAP and 46% moisture.

After harvest, ear samples were immediately stored under refrigeration (5–8°C) and transported to the laboratory. Husks were eliminated, and samples (ear and kernels) were evaluated in relation to their physical characteristics as will be described in next section. Kernels were separated, pooled per biological replicate, and frozen (−20°C). This process was developed within the 24 h after harvest. Afterwards, samples were freeze-dried in a FreeZone benchtop freeze dryer (Labconco, Kansas, MO, United States) for 60 h, at –40°C, and 0.008 mbar of vacuum pressure. Then, dried kernels were milled in a A11 Basic analytical mill (IKA, Germany) with liquid nitrogen to a powdered flour, packed in 50 ml polypropylene tubes protected from light, and stored at –20°C until analysis.

2.2. Enzymes and reagents

Baker yeast α-glucosidase (EC 3.2.1.20), and porcine pancreas α-amylase (EC 3.2.1.1) were from Sigma-Aldrich (St. Louis, MO, United States). Phenolic standards (gallic acid, vanillic acid, caffeic acid, ferulic acid, *p*-coumaric acid, cyanidin chloride, and quercetin aglycone), carotenoid standards (lutein, zeaxanthin, *β*-cryptoxanthin), and the Folin–Ciocalteu reagent were from Sigma-Aldrich. The (±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxilic acid (Trolox), and the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), and 2–2′-azino-bis(3ethylbenothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radicals were purchased from Sigma-Aldrich. Pyridine, phenyl-β-D-glucopyranoside, methoxyamine hydrochloride, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and methyl undecanoate were from Sigma-Aldrich.

2.3. Physical measurements and moisture determination

Relevant physical descriptors were evaluated in fresh harvested ears and kernels (per maturity stage and maize type) per replicate according to the International Board for Plant Genetic Resources (27). The weight (g), length (cm), and central diameter (cm) were measured in ears whereas the length (mm), width (mm), and thickness (mm) were determined in kernels. The kernel moisture was monitored periodically to characterize the maturity stage for harvest and was determined by a gravimetric method at 105°C (28).

2.4. Extraction of phenolic and carotenoid compounds from maize samples

2.4.1. Free and bound phenolic fractions

The extraction of phenolic compounds from the lyophilized maize samples were performed according to Ranilla et al. (29) with some modifications. An amount of 1 g of maize sample was mixed with 4 ml of 0.1% HCl methanol/acetone/water (45, 45, 10, v/v/v) for the extraction of the free phenolic fraction. The bound phenolic compounds were released from the insoluble free-phenolic residue by alkaline hydrolysis with 3 N NaOH following same procedure as Ranilla et al. (29). Final extracts were reconstituted in milliQ water and stored at-20°C until analysis.

2.4.2. Carotenoids

The procedure of Fuentes-Cardenas et al. (19) was followed. A saponification process was first applied with 80% KOH (w/v) and methanol:ethyl acetate (6, 4, v/v) solvent was used for the carotenoid extraction until a clear final extract was obtained. Carotenoids were extracted under light and oxygen protection and analyzed by ultra high-performance liquid chromatography (UHPLC) after the extraction process the same day.

2.5. Targeted metabolomic analysis

2.5.1. Analysis of phenolic compounds by ultra high-performance liquid chromatography

Free and bound phenolic extracts were filtered using a polyvinylidene difluoride filters (PVDF, 0.22 µm) and the separation was carried out in a Kinetex C18 reverse-phase analytical column (100 × 2.1 mm i.d., 1.7 µm) with a Kinetex C18 guard column (5 × 2.1 mm i.d., 1.7 µm) (Phenomenex Inc., Torrance, CA, United States). The injection volume was 5 µl and samples were injected at 0.2 ml/min flow rate in an Ultimate 3,000 RS UHPLC system (Thermo Fisher Scientific, Waltham, MA, United States) with a diode array detector, a quaternary pump, an autosampler, and column oven. Acetonitrile and 0.1% formic acid in water were used as mobile phases and the same gradient and chromatographic parameters reported by Ranilla et al. (29) and Vargas-Yana et al. (30) were applied. Eluates were monitored from 200 to 600 nm. The Chromeleon SR4 software version 7.2 (Thermo Fisher Scientific) was used for chromatograms and data processing. The identification of phenolic compounds was based on their retention times and ultraviolet–visible spectra characteristics compared with those of the library data and external standards. Calibration curves with external standards were used for the quantification of phenolic compounds ($r^2 \geq 0.9990$). Hydroxybenzoic phenolic acids (HBA) (unidentified 1 and 2) were quantified at 280 nm and expressed as vanillic acid. Vanillic acid derivatives (with similar UV–VIS spectra as that of vanillic acid but with different retention times) were expressed also as vanillic acid. Hydroxycinnamic phenolic acids (HCA) including ferulic, *p*-coumaric, and caffeic acid derivatives were quantified at 320 nm and expressed as ferulic, *p*-coumaric, and caffeic acids, respectively. Flavonoids such as luteolin derivatives (with similar UV–VIS spectra as that of luteolin, but with different retention times), and anthocyanins were detected at 360 and 525 nm, and quantified using quercetin aglycone and cyanidin chloride external standards, respectively. All results were expressed as mg per 100 g DW (dried weight).

2.5.2. Analysis of carotenoid compounds by UHPLC

The analysis of carotenoid compounds was performed with a YMC carotenoid C30 reverse-phase analytical column (150 × 4.6 mm i.d., 3 µm) coupled to a YMC C30 guard column (10 × 4.0 mm, 3 µm) (YMC CO., LTD, Japan) using the same UHPLC system as previously described for the phenolic compound analyses. Filtered carotenoid extracts (0.22 µl, PVDF filter) were injected at 1.7 ml/min flow rate and monitored at 450 nm. A ternary gradient elution was used (methanol, dichloromethane, acetonitrile) and same reverse-phase chromatographic conditions as those reported by Fuentes-Cardenas et al. (19) were applied. The retention time and UV–VIS spectra characteristics of external carotenoid standards and the library data were used for the identification of carotenoid compounds in evaluated samples. In addition, the information of carotenoid analyses in other maize samples from reported literature was also useful for the identification of carotenoid isomers. Calibration curves made with external standards were used for the quantification of carotenoids ($r^2 \geq 0.9900$) and results were presented as µg per g sample DW. Lutein and zeaxanthin compounds and their isomers were quantified as lutein and zeaxanthin, respectively. Unidentified carotenoid compounds, neoxanthin, and violaxanthin isomers were expressed as lutein. *β*-cryptoxanthin isomers were expressed as *β*-cryptoxanthin.

2.5.3. Analysis of the total phenolic contents

The TPC in the free and bound phenolic extracts were evaluated according to Singleton and Rossi (31) using the Folin–Ciocalteu method. Results were presented as mg of gallic acid equivalents (GAE) per 100 g DW.

2.5.4. Fatty acids profiles by gas-chromatography with flame ionization detector

The analysis was adapted from Uarrota et al. (32). The fatty acid methyl ester synthesis (FAME) was obtained by combining ~55 mg of lyophilized maize sample with 70 µl 10 N KOH (prepared in HPLC water) and 530 µl of HPLC grade methanol in a reaction tube. The mix was incubated in a water bath at 55–60°C for 1.5 h with periodic agitation every 30 min. Tubes were then cooled down to room temperature and drops of fuming H_2SO_4 (24 N) were carefully added. An incubation step was repeated as previously described. Samples were cooled, then 500 µl of hexane and 10 µl of internal standard (methyl undecanoate, 26.16 mg/ml) were added. The tubes were vortexed for 2 min and centrifuged at 17,000 *g* for 10 min at 4°C. The

upper layer was transferred to a vial with an insert and 1 µl was injected in an Agilent 7890B gas chromatography system coupled to a flame ionization detector (FID) (Agilent Technologies, Santa Clara, CA, United States). A 2560 capillary gas-chromatography (GC) column (100 m × 250 µm × 0.2 µm) (Supelco, Bellefonte, PA, United States). The injector temperature was set at 220°C, the FID detector at 225°C, air flow (400 ml/min), hydrogen flow (35 ml/min), helium flow (1.6 ml/min), using an injection with a split ratio of 50:1. The chromatographic run was set up at 80°C (initial temperature) and increased to 225°C with a heating ramp at a rate of 25°C per min, and held for 25 min. The retention times of detected peaks from samples were compared with those of external standards for fatty acid identification. Calibration curves ($r^2 \geq 0.9900$) with palmitic, stearic, oleic, linoleic, and α-linolenic acids were used for the quantification of fatty acids in maize samples and results are presented as mg per g sample DW.

2.6. Untargeted metabolomic analysis of polar compounds by gas chromatography mass spectrometry

The extraction of polar metabolites from maize samples, the derivatization process, and the instrumental parameters for the gas chromatography mass spectrometry (GC–MS) analysis were the same as reported by Fuentealba et al. (33). An Agilent 7890B gas chromatography system equipped with a 5977A single quadrupole MS, a PAL3 autosampler, an electron impact ionization source was used (Agilent Technologies). A HP-5 ms Ultra Inert column (30 m × 0.25 mm × 0.25 µm) (Agilent) was used for the separation of polar compounds. The Mass Hunter Quantitative software (Agilent Technologies) was used for the deconvolution and data processing. For peak identification, their retention times and mass spectra were compared with data from NIST14 and a home library (obtained with commercial standards). Results are shown as the relative response of each compound calculated considering their respective sample weight, an internal standard (phenyl-β-D-glucopyranoside), and a quality control (QC) composite sample from all maize samples (33).

2.7. *In vitro* functionality of *Cabanita* maize samples

2.7.1. Extraction of hydrophilic and lipophilic fractions

The soluble hydrophilic and lipophilic fractions from lyophilized maize samples were considered for the *in vitro* assays. The hydrophilic and lipophilic fractions were extracted with 80% methanol and dichloromethane; respectively, following same extraction parameters described by Fuentes-Cardenas et al. (19).

2.7.2. Antioxidant capacity by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

The method of Duarte-Almeida et al. (34) adapted to a microplate reader (Biotek Synergy HTX, Agilent Technologies) with modifications reported by Fuentes-Cardenas et al. (19) was applied. Results are shown as µmol Trolox equivalents per 100 g DW using Trolox calibration curves prepared in methanol (20–160 µM), and dichloromethane (10–120 µM) for the hydrophilic and lipophilic fractions, respectively.

2.7.3. Antioxidant capacity by the 2.2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) scavenging assay

The hydrophilic and lipophilic extracts were evaluated according to Fuentealba et al. (35) using a Biotek Synergy HTX microplate reader (Agilent Technologies). Results were expressed as µmol Trolox equivalents per 100 g DW based on calibration curves built with Trolox standard in methanol and dichloromethane for the evaluation of hydrophilic and lipophilic fractions, respectively. The Trolox concentration ranges used in calibration curves were the same as those shown for the DPPH method.

2.7.4. α-Glucosidase and α-amylase inhibitory activity

The hydrophilic and lipophilic fractions used for the determination of the α-amylase inhibitory activity were obtained similarly as Fuentes-Cardenas et al. (19) but using a different sample and solvent ratio for the extraction (0.5 g sample in 12.5 ml 80% methanol). Final extracts (hydrophilic and lipophilic) were vacuum-evaporated to dryness at 45°C and reconstituted in 2 ml 0.02 M $NaPO_4$ buffer (pH 6.9) (35). In case of the α-glucosidase inhibition analysis, same extraction conditions were assayed as Fuentes-Cardenas et al. (19), and final hydrophilic and lipophilic extracts were also vacuum-evaporated to dryness but resuspended in 1 ml of 0.1 M KPO_4 buffer (pH 6.9) (35). The inhibitory activity against α-amylase and α-glucosidase enzymes were determined with the same methodology reported by Gonzalez-Muñoz et al. (36). The percentage (%) of inhibition at different sample amounts was reported.

2.8. Statistical analysis

Results (from four independent biological replicates) were expressed as means ± standard deviation. A two-way analysis of variance (ANOVA) with the LSD test were carried out to determine significant differences between the means ($p < 0.05$) using the software Infostat[1] (accessed from October to November, 2022). Pearson correlations among all data were explored using the Statgraphics Centurion XVI software (StatPoint Inc., Rockville, MD, United States). All data (from the targeted and untargeted metabolite analyses, the physical characteristics, and the functionality assays) were evaluated through the multivariate principal component analysis (PCA) using the Metaboanalyst software version 5.0[2] (accessed on 2 October, 2022). For the PCA, data were first mean-centered and divided by the standard deviation of each variable. Afterward, an ANOVA with the Tukey's HSD *post hoc* analysis ($p < 0.01$) was carried out to identify significant variables. The heat map or cluster analysis was performed using the Euclidean distance and the Ward algorithm in Metaboanalyst with the top significant metabolites or variables ($p < 0.01$).

1 https://www.infostat.com.ar/

2 https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml

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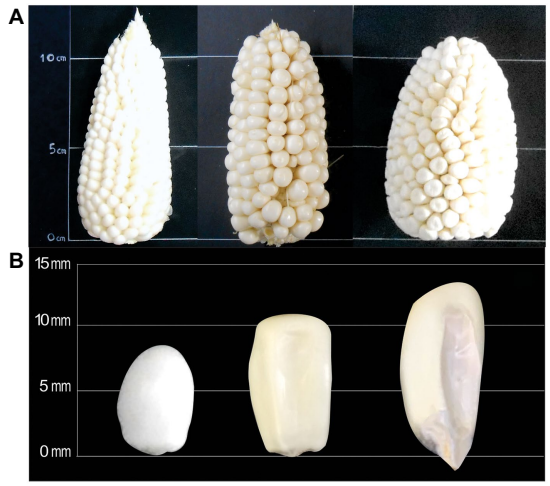

FIGURE 1
Changes of ear **(A)** and kernel **(B)** physical characteristics of white *Cabanita* maize at different maturity stages (S1, S2, S3, from left to right).

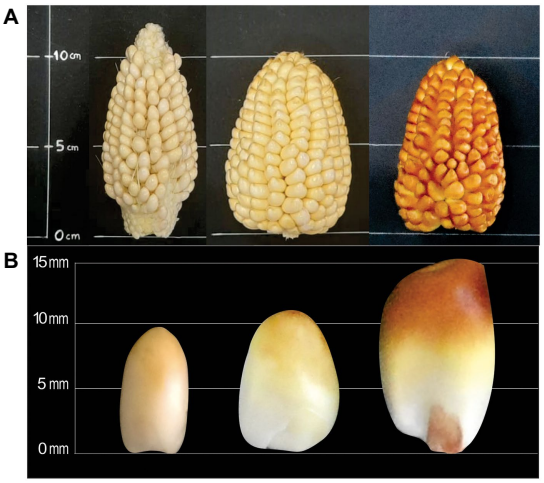

FIGURE 3
Changes of ear **(A)** and kernel **(B)** physical characteristics of orange *Cabanita* maize at different maturity stages (S1, S2, S3, from left to right).

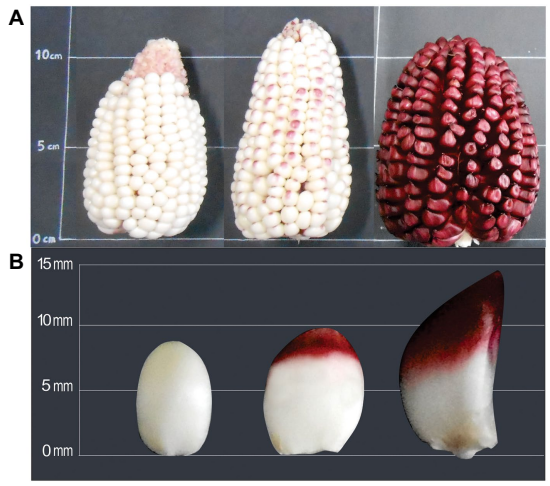

FIGURE 2
Changes of ear **(A)** and kernel **(B)** physical characteristics of red *Cabanita* maize at different maturity stages (S1, S2, S3, from left to right).

3. Results and discussion

3.1. Physical changes of *Cabanita* maize types at different maturity stages

The maturity stages of *Cabanita* maize characterized by the DAP and moisture levels were similar among evaluated maize types. However, their vegetative periods (from the plant emergence stage to the end of the tasseling time) were somewhat different (26). This period occurred at around 9, 10, and 11 weeks after the sowing stage in case of the orange, white, and red maize types, respectively. Consequently, the start of the reproductive period (emergence of the silk or the female inflorescence) was earlier in the orange maize type (13 weeks after sowing), followed by the white (15 weeks after sowing), and the red maize (16 weeks after sowing). This may be important for the adequate planning of maize cultivation periods since Andean farmers traditionally sow maize mixing different *Cabanita* types in the same land.

The physical changes of ears and kernels from the three types of *Cabanita* maize along the maturity stages are shown in Figures 1–3. The development of all maize types was characterized by changes in the pericarp color. The white maize type varied from light-white to white-yellow at S3 which may be related to the accumulation of dry matter with maturity (Figure 1) (37). In case of the red type, the pigmentation appeared in the S2 stage as a small spot at the stigma-end of the kernel that then extended toward almost the half of the grain at S3 (Figure 2). Hong et al. (38) observed a similar trend in a purple-pericarp sweet corn; however, the purple pigment fully spread until the base of the kernel at the highest maturity phase (32 DAP). The orange maize varied from light-yellow at S1 to orange at S3 and this pigmentation only reached the middle of the kernels similarly as in the red case.

Table 1 shows the physical characteristics evaluated in kernels sampled at different developmental stages. No significant interaction between the maize type (M) and the maturity stage (S) factors was found in any of the measured physical parameters. The variation of the ear weight and diameter, and the kernel width along the maturation period was similar in all maize types. Nevertheless, the ear length, kernel length, and thickness were influenced by the maize type. The ear length was higher in the white and red maize than in the orange type, but this latter showed higher kernel length ranges than the former. The maturity stage factor (S) was significant in all the physical characteristics except the ear length which remained almost similar during the maturation. The ear weight, and diameter along with the kernel length, width, and thickness increased with maturation. Overall, the yield-relevant physical parameter (ear weight) was similar among all maize classes; however, some morphological differences have been observed among the kernel types (Figures 1–3). Fuentes-Cardenas et al. (19) also studied the Peruvian maize race *Cabanita* and reported no differences in the quantitative

TABLE 1 Physical characteristics of *Cabanita* maize kernels at different pigmentations and maturity stages.

Maize type	Stage	Ear (cm)			Kernel (mm)		
		Weight	Length	Diameter	Length	Width	Thickness
White	S1	70.4 ± 19.9c	9.1 ± 0.7ab	4.2 ± 0.4f	0.80 ± 0.04e	0.79 ± 0.07d	0.59 ± 0.04e
	S2	150.0 ± 72.9a	9.9 ± 1.2a	5.3 ± 0.2abcd	1.11 ± 0.06 cd	0.80 ± 0.05 cd	0.61 ± 0.02de
	S3	138.4 ± 51.7ab	9.6 ± 0.7ab	5.6 ± 0.7abc	1.44 ± 0.10b	0.97 ± 0.10ab	0.71 ± 0.10abcd
Red	S1	88.8 ± 21.5bc	8.5 ± 0.9abc	4.6 ± 0.5def	0.95 ± 0.15de	0.83 ± 0.17bcd	0.63 ± 0.08cde
	S2	106.9 ± 36.6abc	9.9 ± 1.8a	5.1 ± 0.3 cde	0.93 ± 0.12e	0.89 ± 0.11abcd	0.74 ± 0.08ab
	S3	144.1 ± 64.6ab	9.3 ± 0.4ab	5.9 ± 0.8a	1.43 ± 0.15b	0.95 ± 0.14abc	0.73 ± 0.07ab
Orange	S1	77.0 ± 22.8c	8.1 ± 1.2bc	4.5 ± 0.6ef	1.19 ± 0.06c	0.82 ± 0.06bcd	0.66 ± 0.04bcde
	S2	76.0 ± 16.8c	7.4 ± 0.9c	5.1 ± 0.3bcde	1.21 ± 0.10c	0.82 ± 0.07 cd	0.72 ± 0.01abc
	S3	121.1 ± 18.3abc	8.6 ± 0.7abc	5.8 ± 0.5ab	1.65 ± 0.19a	1.03 ± 0.11a	0.77 ± 0.10a
F value	Maize (M)	1.49^{ns}	7.23**	0.16^{ns}	17.32****	0.46^{ns}	4.40*
	Stage (S)	5.75**	0.99^{ns}	19.68****	66.24****	9.29***	7.75**
	M × S	1.09^{ns}	1.42^{ns}	0.50^{ns}	2.32^{ns}	0.60^{ns}	0.66^{ns}

Different letters in the same column indicate significant statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$); ns, no significant.

physical characteristics between the CAW (white), CCR (red), and COM (orange) maize samples (which are the parental seeds of the white, red, and orange maize types evaluated in the current study). However, mature, and dried ears and kernels were evaluated in such study.

3.2. Phenolic contents and profiles of *Cabanita* maize types at different maturity stages

The phenolic profiles and contents determined in the free and bound phenolic fractions of *Cabanita* maize samples are shown in Table 2. In case of the free phenolic fraction, all maize samples contained phenolic acids such as hydroxybenzoic (HBA) and hydroxycinnamic acids (HCA), but specific flavonoid types such as anthocyanin and luteolin derivatives were detected only in red and orange maize types, respectively.

For the HBA group, the contents were more influenced by the maturity stage (S) than by the maize type (M). The interaction of both factors (M × S) was significant on the vanillic acid derivatives and the total HBA contents. The highest total HBA contents were observed at stage S1, and white and orange maize had higher levels than red maize (44.2, 39.2, and 34.1 mg/100 g DW, for the white, orange, and red maize, respectively). With the kernel maturation, the total HBA concentrations decreased around 80–90% in all cases (from S1 to S3). At least 3 classes of HBA have been detected in all *Cabanita* samples (Supplementary Figures S1–S3). Major HBA at S1 was HBA-1 (λmax = 279 nm), followed by vanillic acid derivatives (λmax = 249, 289 nm). Contents of both HBA then decreased with maturity to reach similar concentrations at S3. A minor HBA compound (HBA-2) was only found at S1 in white and orange maize types. Giordano et al. (39) reported that the free vanillic acid levels found in open-pollinated maize varieties from Italy with variable kernel pigmentations decreased from 1.8–15 to 0–0.08 mg/100 g DW when maturation stages varied from 5 to 76 DAS, respectively. Similarly, the contents of vanillic and protocatechuic acids significantly decreased or disappeared with the grain development of waxy maize from 86–109 to 110–138 DAS (37). Besides vanillic acid, syringic and *p*-hydroxybenzoic acids have been also reported in maize (40, 41). Total free HBA ranges from current study (4.8–44.2 mg/100 g DW) were comparable to levels found in US yellow and Indian specialty maize kernels (~33.7 and 2.7–38 mg/100 g DW, respectively) (40, 41). Other cereals such as barley, wheat, and oat have shown lower free HBA concentrations (~15.5, 12.5, and 4.6 mg/100 g, respectively) (40).

A variable trend was observed in case of the HCA group (Table 2 and Supplementary Figures S4–S6). All *Cabanita* maize types contained *p*-coumaric, and ferulic acid derivatives whereas caffeic acid derivatives were detected at some maturity stages. These HCA derivatives may be soluble conjugated phenolic acids such as hydroxycinnamic acid amides (HCAAs) as was previously reported in different cereals (42, 43). Several HCAAs derived mostly from *p*-coumaric, ferulic and caffeic acids (*N,N*-di-*p*-coumaroylspermine, *N*-*p*-coumaroyl-*N*-feruloylputrescine, caffeoylputrescine) have been previously reported in the free phenolic fraction of maize from different origins (44, 45). Hence, further studies are necessary to better identify the HCA derivatives found in current research. The maturity (S) and maize type (M) showed an important effect on *p*-coumaric and ferulic acid derivatives, but the interaction of both factors was significant only on the ferulic acid derivatives contents. *p*-Coumaric acid and caffeic acid derivatives increased with kernel development. The increase of *p*-coumaric acid derivatives levels from S1 to S3 was on average 2.6-fold, and white and orange maize types exhibited higher ranges than the red maize (0.8–2.2, 0.6–1.6, and 0.4–1.1 mg/100 g DW for white, orange, and red maize, respectively). Conversely, the concentrations of ferulic acid derivatives declined by 32–48% from S1 to S3 in all maize groups. Different studies have shown variable tendencies of the HCA compounds with kernel maturity. The free ferulic and chlorogenic acid contents reduced with kernel maturation in several Italian maize varieties, and the same trend was observed in Chinese waxy pigmented maize samples with ferulic and *p*-coumaric acids (37, 39). Recently, Hu et al. (46) observed an overall increment of ferulic and *p*-coumaric acids during kernel maturation of sweet maize from China, whereas chlorogenic acid

TABLE 2 Phenolic profiles and contents by UHPLC (mg/100g DW) in *Cabanita* maize kernels of different pigmentations and maturity stages.

Fraction	Compound	White			Red			Orange			*F*-value		
		S1	S2	S3	S1	S2	S3	S1	S2	S3	Maize (M)	Stage (S)	M×S
Free	HBA-1	30.6 ± 11.2a	16.6 ± 2.6c	2.8 ± 1.0d	23.2 ± 4.7abc	22.2 ± 1.6bc	2.5 ± 1.8d	27.6 ± 9.5ab	17.4 ± 1.3c	5.9 ± 1.8d	0.10^{ns}	58.83****	1.78^{ns}
	HBA-2	0.06 ± 0.05a	ND[1]	ND	ND	ND	ND	0.11 ± 0.08a	ND	ND			
	Vanillic acid derivatives[2]	13.6 ± 1.4a	5.6 ± 1.2c	2.0 ± 1.1d	10.8 ± 3.5ab	9.6 ± 1.2b	2.5 ± 0.5 cd	11.4 ± 4.8ab	5.2 ± 1.2c	2.5 ± 0.5 cd	0.98^{ns}	58.05****	2.88*
	Total HBA	44.2 ± 11.6a	22.2 ± 3.1c	4.8 ± 2.0d	34.1 ± 7.8b	31.8 ± 1.9bc	5.1 ± 2.2d	39.2 ± 14.2ab	22.6 ± 2.1c	8.4 ± 1.8d	0.01^{ns}	69.29****	2.47*
	p-Coumaric acid derivatives[3]	0.8 ± 0.3 cd	0.6 ± 0.2 cd	2.2 ± 0.9a	0.4 ± 0.1 cd	0.3 ± 0.1d	1.1 ± 0.4bc	0.6 ± 0.2 cd	0.53 ± 0.04 cd	1.6 ± 0.8ab	5.39*	22.38****	1.02^{ns}
	Ferulic acid derivatives[4]	3.3 ± 0.5a	1.4 ± 0.2c	1.7 ± 0.5c	2.5 ± 0.5b	2.8 ± 0.5ab	1.7 ± 0.8c	2.5 ± 0.5b	1.2 ± 0.3c	1.7 ± 0.4c	3.96*	18.79****	6.44***
	Caffeic acid derivatives[5]	ND	0.2 ± 0.1bc	1.2 ± 0.6a	ND	ND	1.5 ± 0.6a	0.03 ± 0.00bc	0.02 ± 0.00c	0.6 ± 0.6Bb			
	Total HCA	4.1 ± 0.8abc	2.1 ± 0.2de	5.1 ± 1.3a	3.0 ± 0.6 cde	3.1 ± 0.5bcd	4.3 ± 1.0ab	3.1 ± 0.6bcd	1.7 ± 0.3e	3.8 ± 1.7bc	3.07^{ns}	17.15****	1.78^{ns}
	Luteolin derivatives[6]	ND	ND	ND	ND	ND	ND	22.7 ± 12.5a	11.3 ± 4.9ab	5.1 ± 4.4b			
	Total anthocyanins[7]	ND	ND	ND	0.6 ± 0.2a	1.4 ± 1.3a	14.5 ± 18.7a	ND	ND	ND			
	Total flavonoids	ND	ND	ND	0.6 ± 0.2b	1.4 ± 1.3b	14.5 ± 18.7ab	22.7 ± 12.5a	11.3 ± 4.9ab	5.1 ± 4.4b			
	Total UHPLC free	48.3 ± 11.6b	24.3 ± 3.2 cde	10.0 ± 3.3f	37.6 ± 8.2bc	36.2 ± 3.5bcd	23.8 ± 17.7de	65.0 ± 14.1a	35.6 ± 4.8bcd	17.3 ± 3.9ef	4.80*	38.22****	4.11*
	Free – TPC[8]	57.9 ± 17.3ab	27.0 ± 3.2c	32.3 ± 3.3c	38.0 ± 6.5bc	44.6 ± 6.5abc	53.6 ± 26.1ab	53.0 ± 11.4ab	37.6 ± 6.2bc	58.7 ± 23.5a	1.72^{ns}	3.13^{ns}	2.93*
Bound	*p*-Coumaric acid	4.9 ± 1.2bc	5.2 ± 1.1bc	7.5 ± 1.9ab	6.4 ± 4.3abc	4.1 ± 1.1c	9.4 ± 2.6a	5.9 ± 0.5bc	7.8 ± 2.4ab	7.2 ± 2.2abc	0.77^{ns}	4.28*	1.98^{ns}
	Ferulic acid	163.3 ± 7.4abc	150.1 ± 14.9bc	133.2 ± 21.2c	161.9 ± 42.8abc	142.5 ± 17.7c	177.2 ± 12.0ab	190.3 ± 11.8a	179.3 ± 23.5ab	177.5 ± 29.2ab	6.84**	1.29^{ns}	1.64^{ns}
	Ferulic acid derivatives[4]	8.5 ± 0.7d	10.8 ± 0.5d	11.6 ± 6.0d	12.0 ± 3.0 cd	10.6 ± 4.1d	19.4 ± 2.4ab	13.1 ± 1.2 cd	16.7 ± 6.2bc	22.0 ± 1.9a	11.92***	11.08***	1.75^{ns}
	Total UHPLC bound	176.7 ± 7.9abc	166.1 ± 16.4bc	152.3 ± 28.4c	180.3 ± 49.8abc	157.1 ± 21.9c	206.0 ± 12.4a	209.4 ± 13.2a	203.8 ± 29.4ab	206.6 ± 29.7a	7.68**	0.97^{ns}	1.72^{ns}
	Bound – TPC[8]	155.8 ± 4.5bc	144.3 ± 10.1c	142.2 ± 38.0c	146.0 ± 23.4c	122.6 ± 35.1c	187.0 ± 7.0ab	181.3 ± 14.2ab	188.1 ± 29.9ab	197.1 ± 21.8a	11.23***	3.12^{ns}	2.72^{ns}
Total (free + bound)	UHPLC TPC	225.0 ± 9.2bcd	190.4 ± 14.2de	162.2 ± 29.2e	217.9 ± 44.4bcd	193.4 ± 21.3cde	229.8 ± 22.4bc	274.4 ± 26.8a	239.4 ± 27.4ab	223.9 ± 26.8bcd	12.45***	6.11**	2.70^{ns}
	TPC[8]	213.7 ± 18.8bcd	171.4 ± 8.7e	174.5 ± 35.8de	184.0 ± 19.5cde	167.2 ± 37.6e	240.7 ± 29.1ab	234.3 ± 23.2ab	225.7 ± 32.1abc	255.8 ± 39.4a	10.90***	4.67*	3.14*

Different letters in the same row indicate significant statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$); ns, no significant. *F* values were calculated only in complete dataset per variable. HBA, hydroxybenzoic acids (HBA 1 and 2 indicate different unidentified HBA with different UV–VIS spectra). HCA, hydroxycinnamic acids. S1, S2, S3 indicate maturity stages. [1]Non-detected. [2]Expressed as vanillic acid. [3]Expressed as *p*-coumaric acid. [4]Expressed as ferulic acid. [5]Expressed as caffeic acid. [6]Expressed as quercetin aglycon. [7]Expressed as cyanidin chloride. [8]Folin–Ciocalteu TPC expressed as mg GAE/100 g DW.

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declined (from 15 to 30 DAP) after an initial increase (from 10 to 15 DAP). In the current study, the total HCA contents first decreased from S1 to S2 in white and orange maize types, then increased at S3 in all cases. The origin, maize type (genetic factors), and the harvesting time may explain differences found in this study.

Anthocyanins were present only in red maize, showing an increase from 0.6 mg/100 g DW at S1 to 14.5 mg/100 g DW by the end of kernel maturity. Other flavonoids such as luteolin derivatives were specific for orange maize samples and significantly decreased by 80% from S1 to S3 (from 22.7 to 5.1 mg/100 g DW). No flavonoids were detected in white *Cabanita*. Hong et al. (38) observed a continuous anthocyanin accumulation from 105 mg/100 g DW at 20 DAP to 179 mg/100 g DW at 36 DAP in purple-pericarp "supersweet" sweet maize. The increase of the total monomeric anthocyanin contents with kernel ripening has been also confirmed by different studies (21, 37, 47). The flavone luteolin has been reported in Indian Himalayan pigmented maize accessions and some Chinese maize hybrids (48, 49). In addition, a *C*-glycosylflavone known as maysin (a luteolin derivative) has also been found in mature maize seeds (49). The luteolin derivatives found in the current study (λmax = 256, 270, 349 nm) may be maysin or similar compounds that should be confirmed in future studies. However, higher concentrations of these flavones were found in the orange *Cabanita* maize at all maturity stages compared to levels obtained by Zhang et al. (49) (1.13 ng/g DW of maysin in mature seeds). *C*-glycosylflavones have shown potential neuroprotective properties relevant for Alzheimer's disease prevention (50, 51).

The total free phenolic fraction decreased from S1 to S3, and its composition was variable depending on the kernel stage and maize type. HBA were the most important compounds in white and red maize at S1 and S2. In case of the orange group, HBA and luteolin derivatives highly contributed to the total free phenolic fraction at S1 and S2. This maize showed the highest total free phenolic contents at S1 among all samples (65 mg/100 g DW). The red maize was rich in HBA at S1, and S2 whereas anthocyanins were the major contributors to the free phenolic fraction at S3.

Major compound in the bound phenolic fraction was ferulic acid, followed by ferulic acid derivatives, and *p*-coumaric acid (Supplementary Figures S7–S9). The M × S interaction was not significant for any of the bound phenolic compounds; however, the maize type showed an important effect on the ferulic acid, ferulic acid derivatives, and the total bound UHPLC phenolic contents (Table 2). Orange and red maize types had higher ranges of ferulic acid (177.5–190.3 mg/100 g DW, 142.5–177.0 mg/100 g DW, and 133.2–163.3 mg/100 g DW, for the orange, red, and white maize, respectively), and ferulic acid derivatives than the white group (13.1–22.0 mg/100 g DW, 10.6–19.4 mg/100 g DW, and 8.5–11.6 mg/100 g DW, for the orange, red, and white maize, respectively). Consequently, higher total bound phenolic levels determined by UHPLC were found in orange and red maize, specially at S3 than in the white type. Kernel maturity highly influenced the *p*-coumaric acid and ferulic acid derivatives contents. Both compounds showed an increase of around 1.2–1.7-fold from S1 to S3. Ferulic acid remained almost stable from S1 to S3 in white and orange maize samples, and a similar trend was observed in their total UHPLC bound phenolic contents. In case of the red maize, ferulic acid and the total bound phenolic compounds first decreased from S1 to S2, to further increase at S3. Similar results as those obtained for white and orange *Cabanita* maize have been reported by Zhang et al. (22) in yellow maize. In that study, the total bound phenolic contents were stable with kernel maturation from 15 to 48 DAP (22). Nonetheless, the levels of bound ferulic and *p*-coumaric acids significantly reduced with kernel development (from 5 to 76 DAS) in several Italian maize samples whereas in other research same bound HCA showed a variable tendency depending on the genotype (39, 46).

On the whole, these results suggest differences in the metabolism of phenolic compounds during kernel development among the three types of *Cabanita* maize. A possible metabolic flux of precursors of hydroxybenzoic acids such as some intermediates of the shikimate or the phenylpropanoid pathways toward the biosynthesis of HCA derivatives may occur in case of the white and orange grains (52). HCA may be used as precursors for the biosynthesis of anthocyanins in case of the red maize. Enzymes involved in the biosynthesis of cell wall-relevant phenolic compounds may have been upregulated toward the flavone pathway in the orange maize explaining its overall higher ranges of bound phenolic compounds through the kernel growing process (53, 54).

Ultra high-performance liquid chromatography total phenolic contents (free+bound) declined with kernel maturity in white and orange grains whereas in red maize the contents first decreased from S1 to S2, and then increased at S3. Concentrations at the physiological maturity stage were higher in the case of the orange and red maize type (223.9 and 229.8 mg/100 g DW, for the orange and red maize, respectively) than results obtained by Fuentes-Cardenas et al. (19) in *Cabanita* race (134.3 and 190.9 mg/100 g DW, for the orange and red maize, respectively). However, above authors reported higher total phenolic contents in the white maize type (206 mg/100 g DW) than in the current research (162.2 mg/100 g DW). Differences in the postharvest treatments and the agroecological conditions for the growth of *Cabanita* maize may explain such variations. Generally, phenolic contents measured with the Folin–Ciocalteu method showed the same trend as those analyzed with the UHPLC method. However, the lack of specificity of the Folin–Ciocalteu method may be associated with differences observed specially in results from the free phenolic fraction (55).

3.3. Carotenoid contents and profiles of *Cabanita* maize types at different maturity stages

Cabanita maize types at different maturity stages were also evaluated in terms of their carotenoid composition (Table 3, Supplementary Figures S10–S12). In contrast to the variable effect of studied factors (M and S) on phenolic compounds, carotenoid contents were highly influenced by the maize type. Only xanthophylls were found in all *Cabanita* samples while no carotenes were detected. Moreover, different profiles were observed among studied *Cabanita* maize groups. White and red maize had similar profiles and all-trans-neoxanthin, neoxanthin isomer (~13-*cis*-neoxanthin), all-*trans*-zeaxanthin, and a lutein isomer (~13-*cis*-lutein) were the major carotenoids. All-*trans*-lutein and all-*trans*-zeaxanthin were the main compounds in the orange maize, followed by ~13-*cis*-lutein, and neoxanthin compounds. *β*-cryptoxanthin isomers along with some unidentified carotenoids (2, 3) were only detected in this maize type. The concentrations of all mentioned carotenoids in orange maize were higher than values found in white and red types. A violaxanthin

TABLE 3 Carotenoid profiles and contents (μg/g DW) determined by UHPLC in *Cabanita* maize kernels of different pigmentations and maturity stages.

Compound	White			Red			Orange			*F*-value		
	S1	S2	S3	S1	S2	S3	S1	S2	S3	Maize (M)	Stage (S)	M×S
Neoxanthin isomer[2] (~13-*cis*-neoxantin)	0.18 ± 0.02c	0.14 ± 0.04c	0.11 ± 0.02c	0.11 ± 0.04d	0.15 ± 0.05c	0.08 ± 0.01c	0.39 ± 0.07a	0.29 ± 0.13b	0.31 ± 0.08ab	38.69****	2.42^{ns}	1.24^{ns}
All-*trans*-neoxanthin[2]	0.19 ± 0.03b	0.15 ± 0.11b	0.22 ± 0.01b	0.16 ± 0.16b	0.17 ± 0.07b	0.18 ± 0.02b	0.17 ± 0.11b	0.22 ± 0.11b	0.37 ± 0.12a	2.79^{ns}	2.76^{ns}	1.21^{ns}
Unidentified carotenoid-1[2]	0.05 ± 0.02	ND[1]	ND	ND	ND	ND	ND	ND	ND			
Violaxanthin isomer[2] (~9-*cis*-violaxanthin)	0.06 ± 0.02b	0.04 ± 0.02b	0.05 ± 0.01b	ND	ND	0.04 ± 0.02b	0.13 ± 0.04a	0.14 ± 0.06a	0.13 ± 0.05ab			
Unidentified carotenoid-2[2]	ND	ND	ND	ND	ND	ND	0.14 ± 0.05b	0.26 ± 0.04a	0.17 ± 0.06b			
Lutein isomer[2] (~13-cis-lutein)	0.10 ± 0.03c	0.14 ± 0.08c	0.14 ± 0.05c	0.12 ± 0.06c	0.18 ± 0.11c	0.14 ± 0.03c	0.64 ± 0.27a	0.75 ± 0.24a	0.41 ± 0.04b	48.72****	2.69^{ns}	2.40^{ns}
Unidentified carotenoid-3[2]	ND	ND	ND	ND	ND	ND	0.12 ± 0.03a	0.11 ± 0.03a	ND			
Zeaxanthin isomer[3] (~13-*cis*-zeaxanthin)	ND	ND	ND	ND	0.03 ± 0.01c	ND	0.18 ± 0.05ab	0.21 ± 0.08a	0.12 ± 0.03b			
All-*trans*-lutein	0.14 ± 0.04c	0.07 ± 0.06c	ND	0.06 ± 0.03c	0.03 ± 0.02c	ND	1.48 ± 0.62b	2.00 ± 0.40a	1.02 ± 0.34b			
All-*trans*-zeaxanthin	0.07 ± 0.04c	0.16 ± 0.04c	0.08 ± 0.03c	0.15 ± 0.07c	0.23 ± 0.06bc	0.09 ± 0.03c	1.44 ± 0.35a	1.35 ± 0.20a	0.54 ± 0.22b	155.08****	17.18****	11.56****
Lutein isomer[2] (~9 or 9′-*cis*-lutein)	ND	ND	ND	ND	ND	ND	ND	ND	0.07 ± 0.03			
β-cryptoxanthin isomer[4] (~13 or 13′-*cis*-*β*-cryptoxanthin)	ND	ND	ND	ND	ND	ND	0.17 ± 0.06a	0.20 ± 0.09a	0.06 ± 0.01b			
β-cryptoxanthin isomer[4] (~9 or 9′-*cis*-*β*-cryptoxanthin)	ND	ND	ND	ND	ND	ND	0.10 ± 0.04a	0.19 ± 0.12a	ND			
Total carotenoids	0.77 ± 0.10d	0.69 ± 0.22d	0.62 ± 0.10d	0.66 ± 0.28d	0.84 ± 0.27d	0.56 ± 0.11d	4.97 ± 1.26b	5.87 ± 0.73a	3.19 ± 0.61c	209.22 ****	10.53***	7.21***

Different letters in the same row indicate significant statistical differences (***$p < 0.001$; and ****$p < 0.0001$); ns, no significant. *F*-values were calculated only in complete dataset per variable. S1, S2, S3 indicate maturity stages. [1]Non-detected. [2]Expressed as lutein. [3]Expressed as zeaxanthin. [4]Expressed as *β*-cryptoxanthin.

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isomer (~9-*cis*-violaxanthin) was detected in white and orange maize at all maturity stages, and only at S3 in the red grain. These xanthophylls diversity may be based on the fact that *β*-cryptoxanthin is the metabolic precursor of zeaxanthin which is further metabolized to violaxanthin and then to neoxanthin (56). Several studies have confirmed that predominant carotenoid compounds in maize are generally lutein, zeaxanthin, *β*-cryptoxanthin along with other minor xanthophylls such as zeinoxanthin, antheraxanthin, violaxanthin, neoxanthin, and their isomers (23, 57–59). However, carotene compounds such as α-carotene and β-carotene have been also reported in comparable concentrations in yellow maize varieties (44, 60). Liu et al. (23) reported that two genotypes of Chinese sweet maize showed variable carotenoid profiles and contents during the grain maturation from 10 to 30 DAP. This indicates an important influence of genetic factors and the kernel maturity stage (61).

The maturity stage and the interaction of both factors (M × S) significantly influenced the all-*trans*-zeaxanthin, and the total carotenoid contents. Neoxanthin isomer, all-*trans*-neoxanthin, violaxanthin isomer, and ~13-*cis*-lutein did not show significant changes with kernel development in white and red maize types, but all-*trans*-lutein was not detected at S3. Overall, both maize types showed similar total carotenoid concentrations which were somewhat stable along the kernel growth (ranges of 0.77–0.62 μg/g DW and 0.84–0.56 μg/g DW, for the white and red maize, respectively). In the orange *Cabanita*, all-*trans*-lutein increased by ~35% from S1 to S2 (1.48 and 2.0 μg/g DW at S1 and S2, respectively), but then decreased by 50% at S3 (1.02 μg/g DW). All-trans-zeaxanthin remained almost constant from S1 to S2 (1.44 and 1.35 μg/g DW, respectively). However, it declined by 60% at S3 (0.54 μg/g DW). Similar carotenoid reductions at S3 were observed in case of the 13-cis-lutein, 13-*cis*-zeaxanthin, *β*-cryptoxanthin isomers, and the other unidentified compounds. All-trans-neoxanthin and its isomers showed a certain increase at S3 which indicates the downstream metabolic conversion of *β*-cryptoxanthin, and zeaxanthin (56). The contents of lutein, zeaxanthin, α-cryptoxanthin, and *β*-cryptoxanthin have shown to steadily increase with kernel development from 10 to 30 DAP in sweet corn (23). The increase of zeaxanthin and lutein with kernel maturation from 16 to 24 DAP has been also reported in other sweet maize hybrids (62). Variable zeaxanthin and lutein patterns were observed in some zeaxanthin-biofortified sweet maize depending on the genotype and the kernel position on the cob (61). However, an overall lutein and zeaxanthin accumulation was reported in same study (61). In the current research, higher maturity stages were evaluated (from 28–32 to 75–77 DAP) which likely explains contrasting results compared with previous studies. Xu et al. (24) evaluated a yellow maize variety during maturation from 74 to 116 DAS and found that the contents of zeaxanthin decreased at the end of kernel maturity, whereas lutein increased from 74 to 98 DAS to finally decrease at 116 DAS.

The orange maize exhibited higher total carotenoid contents (3.19–5.87 μg/g DW) than white and red maize (0.77–0.62 μg/g DW and 0.84–0.56 μg/g DW, for the white and red maize, respectively). Nevertheless, carotenoids significantly decreased by ~50% at S3 (from 5.87 to 3.19, at S2 and S3, respectively). Fuentes-Cardenas et al. (19) found lower total carotenoid values in the orange *Cabanita* maize type at physiological maturity (1.95 μg/g DW, COM code) than in the present study. In addition, no carotenoids were detected in the corresponding red and white parental seeds (CCR, CAW) (19). Carotenoids are highly sensitive to light, heat, and oxygen, therefore postharvest practices applied by Andean farmers such as the sun-drying of *Cabanita* ears first in the plant and later on the field for undetermined time may lead to the degradation of carotenoid compounds.

Higher total carotenoid amounts than those from current research have been reported mostly in yellow and sweet maize varieties. Xu et al. (24) found concentrations of 22.78–28.76 μg/g DW at different maturity stages in yellow maize. Ranges of 0.55–43.23 μg/g DW and 11.4–24.0 μg/g DW have been shown in sweet maize harvested at maturity stages from 10 to 32 DAP (23, 63). Floury maize types generally show lower carotenoid contents than hard maize classes such as pop, dent, or flint (64). Therefore, it is expected to find lower carotenoid levels in the amylaceous floury *Cabanita* maize (19).

3.4. Fatty acid composition of *Cabanita* maize types at different maturity stages

The fatty acid composition of *Cabanita* maize is shown in Table 4. No significant effect was found by the maize type indicating similar fatty acid profiles and contents among all samples. Polyunsaturated fatty acids (PUFA) including linoleic and α-linolenic acids represented the major fatty acid fraction in all *Cabanita* maize types (55–59% of the total fatty acid content). The monounsaturated oleic acid contributed with 21–28% of the total fatty acids, followed by saturated acids (palmitic and stearic acids, 18–21%). Among all detected fatty acids, linoleic and oleic acids were the most abundant compounds in maize kernels (50–54%, and 21–28% for linoleic and oleic acids, respectively).

Comparable percentages of linoleic acid have been also reported in Mexican subtropical maize populations (41–51%), sweet maize from the United States (50–63%), and maize varieties from Turkey (50–53%) (65–67). In the case of other Peruvian germplasm, similar fatty acids profiles and concentrations were observed in mature native varieties such as *Chullpi*, *Piscorunto*, Giant *Cuzco*, *Sacsa*, and purple, with ranges of 18.3–25.2 mg/g DW and 9.8–14.6 mg/g DW for linoleic and oleic acids, respectively (68). However, α-linolenic acid concentrations were almost 2 to 3.5-fold higher in *Cabanita* samples (1.3–1.4 mg/g DW, at S3 maturity stage) than in the other Peruvian varieties (0.4–0.7 mg/g DW) (68). Lower α-linolenic acid percentages have been also reported in sweet maize (1.7–2.1%) compared with *Cabanita* maize (3.2–3.7%) (66). Furthermore, this fatty acid was not even detected in several Korean maize hybrids (69). The increase of α-linolenic acid from 0.61 to 4.93% along with the oil contents have been obtained after a long-term breeding process of Mexican maize (65). The contribution of this fatty acid in relation to the total fatty acid content was higher at early maturity stages in *Cabanita* samples (5.1–5.8%).

Linoleic (ω-6) and α-linolenic acids (ω-3) are essential fatty acids that cannot be synthesized by humans (70). After the ingestion, α-linolenic acid is transformed into long-chain ω-3 PUFAs such as docosahexaenoic and eicosapentaenoic acids which play important roles within the organism (71). Dietary α-linolenic acid, and its ω-3 metabolic derivatives have been reported to show antioxidant and anti-inflammatory properties with potential for the prevention of brain malfunction, and cardiovascular disease (72–74). Moreover, diets with ω-6:ω-3 ratios close to 1:1 have been associated with less

TABLE 4 Contents and profiles of fatty acids (mg/g DW) in *Cabanita* maize kernels of different pigmentations and maturity stages.

Maize type	Stage	Saturated fatty acids		Unsaturated fatty acids			Total fatty acids
		Palmitic acid	Estearic acid	Oleic acid	Linoleic acid	α-Linolenic acid	
White	S1	5.1 ± 0.9ab	0.9 ± 0.1bcd	5.9 ± 1.3d	14.7 ± 3.2c	1.6 ± 0.1a	28.0 ± 5.3c
	S2	5.1 ± 0.6ab	0.8 ± 0.1 cd	6.6 ± 1.4 cd	15.6 ± 2.7bc	1.3 ± 0.1de	29.3 ± 4.7bc
	S3	5.9 ± 0.9ab	1.1 ± 0.2a	11.0 ± 2.2a	20.5 ± 4.1a	1.3 ± 0.1e	39.8 ± 7.2a
Red	S1	5.3 ± 0.8ab	0.8 ± 0.1 cd	6.6 ± 1.9 cd	15.3 ± 3.3c	1.5 ± 0.1b	29.5 ± 6.1bc
	S2	5.2 ± 0.6ab	0.8 ± 0.1 cd	7.5 ± 0.9bcd	15.2 ± 2.3c	1.4 ± 0.1 cde	30.1 ± 3.8bc
	S3	6.3 ± 1.3a	1.0 ± 0.2ab	9.8 ± 2.5ab	21.1 ± 4.4a	1.30 ± 0.03e	39.5 ± 8.2a
Orange	S1	4.9 ± 0.6b	0.7 ± 0.1d	5.7 ± 0.9d	14.5 ± 1.5c	1.5 ± 0.1b	27.2 ± 2.8c
	S2	6.0 ± 1.0ab	0.9 ± 0.2bc	9.0 ± 1.9abc	20.4 ± 4.5ab	1.4 ± 0.1bcd	37.6 ± 7.6ab
	S3	5.9 ± 0.6ab	0.9 ± 0.1abc	9.6 ± 1.6ab	21.2 ± 2.9a	1.4 ± 0.1bc	39.0 ± 4.8a
F-value	Maize (M)	0.35^{ns}	0.62^{ns}	0.06^{ns}	0.97^{ns}	2.84^{ns}	0.48^{ns}
	Stage (S)	3.91*	11.72***	17.63****	10.29****	20.45****	11.19***
	M × S	0.81^{ns}	2.01^{ns}	1.55^{ns}	1.08^{ns}	4.24**	1.08^{ns}

Different letters in the same column indicate significant statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$); ns: no significant differences. S1, S2, S3 indicate maturity stages.

incidence of chronic diseases including diabetes and cardiovascular diseases (70). Higher ω-6:ω-3 ratios have been found in unbred maize cultivars from Mexico (59–80:1), sweet maize from US (29–33:1), and Peruvian germplasm from other races (36–50:1) in comparison with ratios found in evaluated *Cabanita* maize at physiological maturity stage (15–16:1) (65, 66, 68). Genetic factors may play a role on observed differences. In addition, differences in the agroecological and post-harvest management conditions may also be involved. Based on the current results, *Cabanita* maize shows potential as a dietary source of health relevant PUFAs.

The maturity stage had a strong influence on fatty acid variability in all *Cabanita* types (Table 4). Saturated acids slightly increased at S3, but their proportions with respect to the total fatty acid contents decreased in all cases from 18 to 15% on average. Contents and percentages of stearic acid almost remained constant with maturation. Oleic acid increased from 21 to 22% at S1 to 25–28% at S3. Linoleic acid concentrations also increased and were high at S3, but their percentages in relation to the total fatty acid contents showed almost no variation with kernel growth (from 52 to 51%, from 52 to 53%, and from 53 to 54% for white, red and orange maize, respectively). The α-linolenic acid concentrations and percentages decreased from S1 to S3 (5.1–5.8% to 3.2–3.7%). Palmitoyl-CoA is a metabolic precursor of palmitic acid, and of stearoyl-CoA which in turn serves as a precursor of oleic, linoleic, and linolenic acids biosynthesis *via* several desaturase enzymes (56). A possible metabolic change toward the biosynthesis of oleic and linoleic acids instead of palmitic acid may explain its percentage decrease with maturity. It is noteworthy that ω-6:ω-3 ratios are lower at S1 stage (9.2–10.4:1) than at S3 (15–16:1) indicating better PUFAs balance when *Cabanita* maize is at milk stage.

3.5. *In vitro* health-relevant functionality of *Cabanita* maize types at different maturity stages

3.5.1. DPPH and ABTS antioxidant capacity

The antioxidant capacity was evaluated with two different *in vitro* methods and only in the bioavailable-relevant soluble hydrophilic and lipophilic fractions of *Cabanita* maize samples (Table 5). The interaction of maize type (M) and the maturity stage (S) was not significant in all cases; however, all variables were influenced by S. The DPPH hydrophilic antioxidant capacity (DPPH-HF) declined with grain growth and there were differences depending on the maize type (M significant). Orange *Cabanita* showed higher values (422.3–821.7 μmol TE/100 g DW) than white and red types (310.7–490.6 and 308.9–520.2 μmol TE/100 g DW, for the white and red maize, respectively). The DPPH-HF decreased from S1 to S3 by 37, 41 and 49% in the white, red, and orange maize, respectively. The opposite trend was observed in case of the DPPH lipophilic antioxidant capacity (DPPH-LF). Values increased from S1 to S3 around 3.4 and 4.8-fold in the orange and red maize, respectively. In the white maize, the DPPH-LF was not detected at S1, but then it increased to 7.8 and 20.8 μmol TE/100 g DW with kernel development at S2 and S3, respectively.

The ABTS hydrophilic antioxidant capacity (ABTS-HF) also decreased with kernel maturity in a range of 36–51%, similarly as in the case of the DPPH-HF. However, the maize type did not show an important effect. Ranges were almost comparable among all *Cabanita* types at all maturity stages (1009.0–2065.2 μmol TE/100 g DW, 1297.5–2012.3 μmol TE/100 g DW, and 1085.2–2194.4 μmol TE/100 g DW, for the white, red, and orange maize, respectively). In addition, the ABTS lipophilic antioxidant capacity (ABTS-LF) increased 1.6–2.0-fold from S1 to S3 as also was noticed in case of the DPPH-LF. The orange maize exhibited the highest value at S3 (94.2 μmol TE/100 g DW) among samples (M significant).

The hydrophilic extracts strongly inhibited both free radicals more than lipophilic fractions indicating higher contents of hydrophilic antioxidants such as soluble polyphenols. In fact, higher concentrations of total free phenolic compounds have been determined in this study in comparison with the total carotenoid contents (ranges of 10–65 mg/100 g DW and 0.56–5.87 μg/g DW, for the total free phenolic and total carotenoid contents, respectively). The UHPLC total free phenolic contents highly correlated with the antioxidant capacity ($r = 0.7709$ and $r = 0.7863$, $p < 0.05$ for the DPPH-HF and ABTS-HF, respectively). Hydroxybenzoic acid

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TABLE 5 *In vitro* antioxidant capacity (μmol TE/100g DW) in *Cabanita* maize kernels of different pigmentations and maturity stages.

Maize type	Stage	Inhibition of DPPH		Inhibition of ABTS	
		HF	LF	HF	LF
White	S1	490.6 ± 57.3bcd	ND	2065.2 ± 98.7ab	36.4 ± 5.0f
	S2	313.5 ± 79.2 cd	7.8 ± 4.6 cd	1959.7 ± 278.1ab	45.1 ± 7.0ef
	S3	310.7 ± 20.7d	20.8 ± 3.5ab	1009.0 ± 86.0d	70.0 ± 3.7bc
Red	S1	520.2 ± 329.6bc	4.1 ± 3.6d	2012.3 ± 121.8ab	40.6 ± 3.0ef
	S2	507.5 ± 106.0bcd	14.3 ± 7.7bc	1819.8 ± 138.6b	48.4 ± 12.9de
	S3	308.9 ± 150.8d	19.9 ± 12.5ab	1297.5 ± 353.1c	81.0 ± 15.0b
Orange	S1	821.7 ± 114.5a	8.0 ± 3.4 cd	2194.4 ± 121.5a	58.9 ± 5.5 cd
	S2	582.2 ± 135.8b	12.7 ± 4.5bcd	1949.6 ± 185.2ab	71.7 ± 1.6b
	S3	422.3 ± 53.1bcd	27.5 ± 4.1a	1085.2 ± 45.9 cd	94.2 ± 6.4a
F-value	Maize (M)	8.51**		0.38^{ns}	30.91****
	Stage (S)	10.05***		92.36****	68.17****
	M × S	1.24^{ns}		2.01^{ns}	0.50^{ns}

Different letters in the same column indicate significant statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$); ns, no significant. *F*-values were calculated only in complete dataset per variable. HF, hydrophilic fraction; LF, lipophilic fraction. S1, S2, S3 indicate maturity stages.

compounds such as vanillic acid derivatives showed a positive correlation with this property ($r = 0.5740$ and $r = 0.7502$, $p < 0.05$ for the DPPH-HF and ABTS-HF, respectively). Likewise, the HBA-1 compound, and the total HBA contents were correlated with the antioxidant capacity measured with both methods ($r = 0.5790$ and 0.7962, $p < 0.05$ for the DPPH-HF and ABTS-HF, respectively in case of the HBA-1; and $r = 0.5900$ and $r = 0.7987$, $p < 0.05$ for the DPPH-HF and ABTS-HF, respectively in case of the total HBA contents). Moreover, free luteolin derivatives also contributed to the antioxidant capacity in the orange maize group ($r = 0.6246$, $p < 0.05$ for the DPPH-HF). Consistent with the current study, soluble phenolic compounds were correlated with high antioxidant capacity evaluated with the ferric reducing antioxidant power (FRAP) and DPPH methods in several Italian maize landraces (75). Flavonoids including anthocyanins have shown to highly contribute to the free radical antioxidant capacity in Mexican red and purple-pigmented maize (76, 77). In the current study, no correlation was found between the total anthocyanin contents and the antioxidant capacity in *Cabanita* red maize which may be due to its lower anthocyanin ranges compared to HBA concentrations specially at S1 and S2 stages.

In case of the lipophilic antioxidant capacity, a moderate correlation was found between this functional quality and the total carotenoid contents ($r = 0.5354$, $p < 0.05$ with the DPPH method). Other lipophilic compounds such as tocopherols and tocotrienols common in the germ of maize grains but not analyzed in the current study may also play a role (17). Some tocopherol compounds from spelt grain (*Triticum spelta*) have shown significant correlation with the antioxidant capacity measured with the DPPH method (78).

A continuous decrease of the DPPH and FRAP antioxidant capacity was observed in the soluble phenolic fractions from yellow maize at different developmental stages (from 74 to 116 DAS) (24). Hu and Xu (37) and Giordano et al. (39) reported that the free radical inhibitory activity along the kernel growth stages was highly variable depending on the maize variety. An increase of the antioxidant response with kernel maturity were reported in sweet and yellow maize in maturity periods shorter (17–25 DAP and 15–48 DAP, respectively) than in the current research (28–77 DAP) (22, 79). Furthermore, Liu et al. (23) observed that the lipophilic oxygen radical absorbance antioxidant capacity (ORAC) increased with grain maturity (10–30 DAP) in sweet maize showing correlation with the total and individual carotenoids such as lutein and zeaxanthin. Differences in the current results from those of above studies indicates an important influence of genetic factors, the maturity stage, and the origin of maize.

In a previous study with Peruvian white, red, and orange *Cabanita* maize, Fuentes-Cardenas et al. (19) pointed out that hydrophilic compounds strongly contributed to the *in vitro* antioxidant capacity (DPPH and ABTS methods) than lipophilic fractions like this study. Nevertheless, lower ABTS-HF was reported by above authors (566.3–685.4 μmol TE/100 g DW) than in this research (1009.0–1297.5 μmol TE/100 g DW) at physiological maturity stage. This may suggest that postharvest management also plays a role in the observed bioactive variability and associated functional quality.

3.5.2. Inhibitory activity against α-amylase and α-glucosidase enzymes

The intake of natural inhibitors of key intestinal carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase may represent an important dietary strategy for hyperglycemia management relevant for the type-2 diabetes prevention (80–82). Table 6 shows the potential *in vitro* inhibitory activity of the soluble hydrophilic and lipophilic fractions from *Cabanita* maize samples against α-amylase and α-glucosidase enzymes.

All hydrophilic (HF) and lipophilic (LF) maize extracts inhibited the α-glucosidase enzyme in a sample dose dependent manner (3–10 mg). However, HF extracts showed higher inhibition than LF fractions. The type of maize (M) was not significant on the α-glucosidase inhibitory activity of both HF and LF fractions at all evaluated sample doses. This indicates similar inhibitory potential among all *Cabanita* maize types. No effect of the M x S interaction was found on results from HF and LF fractions, except at 10 mg (HF). In this case, HF fractions from white and orange maize exhibited greater

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TABLE 6 *In vitro* inhibitory activity against α-amylase and α-glucosidase in *Cabanita* maize kernels of different pigmentations and maturity stages.

Maize type	Stage	α-Glucosidase inhibitory activity (%)						α-Amylase inhibitory activity (%)					
		3mg[1]		6mg		10mg		25mg		62mg		125mg	
		HF	LF	HF	LF	HF	LF	HF	LF	HF	LF	HF	LF
White	S1	17.2 ± 3.5a	6.1 ± 4.3a	31.3 ± 4.0a	7.5 ± 2.6ab	40.0 ± 6.3a	8.9 ± 2.0b	7.0 ± 3.0a	ND[2]	18.6 ± 8.3a	ND	55.0 ± 25.4a	4.3 ± 5.9
	S2	10.7 ± 4.2cd	4.7 ± 4.9a	16.6 ± 4.8de	7.1 ± 1.7ab	20.2 ± 4.9cd	11.9 ± 3.3ab	1.4 ± 1.6b	ND	4.1 ± 3.0c	ND	13.5 ± 4.4b	ND
	S3	6.5 ± 1.4d	5.6 ± 1.6a	10.9 ± 2.9e	7.3 ± 0.3ab	14.8 ± 2.8d	8.6 ± 0.7b	ND	ND	1.8 ± 2.7c	ND	8.1 ± 1.7b	ND
Red	S1	18.4 ± 3.3a	3.4 ± 2.7a	28.0 ± 5.4ab	8.1 ± 4.2ab	32.3 ± 6.9b	12.2 ± 4.0ab	2.4 ± 3.4b	ND	6.6 ± 5.9bc	ND	18.2 ± 8.9b	ND
	S2	16.1 ± 1.7ab	3.8 ± 2.9a	23.0 ± 4.3bc	8.7 ± 2.5ab	31.8 ± 2.8b	12.3 ± 2.8ab	2.0 ± 2.8b	ND	8.0 ± 3.0bc	ND	16.3 ± 3.2b	ND
	S3	7.5 ± 3.8 cd	5.5 ± 4.6a	15.2 ± 6.8de	9.7 ± 3.0a	20.2 ± 7.6 cd	12.2 ± 3.5ab	2.1 ± 2.6b	ND	4.2 ± 4.5c	ND	8.3 ± 2.9b	ND
Orange	S1	20.5 ± 2.3a	4.9 ± 5.8a	30.0 ± 3.4a	5.7 ± 6.2ab	41.0 ± 2.1a	8.6 ± 5.8b	6.8 ± 1.8a	ND	12.8 ± 4.4ab	ND	19.0 ± 3.2b	ND
	S2	10.8 ± 3.0 cd	1.9 ± 2.9a	17.4 ± 3.5 cd	4.6 ± 3.2b	22.7 ± 4.1c	10.2 ± 2.2ab	ND	ND	3.9 ± 1.6c	ND	9.3 ± 4.3b	ND
	S3	12.1 ± 4.2bc	3.4 ± 2.8a	18.3 ± 1.9 cd	8.7 ± 1.3ab	22.6 ± 4.1c	15.1 ± 5.3a	ND	ND	3.0 ± 2.0c	ND	7.9 ± 0.5b	ND
F-value	Maize (M)	3.02[ns]	0.88[ns]	1.22[ns]	1.86[ns]	1.99[ns]	1.39[ns]			0.66[ns]		7.11**	
	Stage (S)	30.13****	0.48[ns]	38.26****	1.08[ns]	43.89****	1.09[ns]			15.76****		19.26****	
	M × S	2.60[ns]	0.29[ns]	2.48[ns]	0.50[ns]	5.12**	1.71[ns]			4.10*		6.78***	

Different letters in the same column indicate significant statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$); ns, no significant. *F*-values were calculated only in complete dataset per variable. HF, hydrophilic fraction; LF, lipophilic fraction.
[1]Sample dose. [2]Non-detected. S1, S2, S3 indicate maturity stages.

inhibition than red maize at S1 (40.0, 41.0, and 32.3%, for the white, orange, and red maize, respectively). Nonetheless, the inhibitory activity of red maize was higher than white and orange at S2 (31.8, 20.2, 22.7%, for the red, white, and orange samples, respectively), whereas results of all HF fractions were almost similar at S3.

The maturity stage (S) had a significant influence on the HF inhibitory activity at all sample doses. At 10 mg, this property decreased with kernel development (from 28–33 to 75–76 DAP) by 63, 37 and 45% in the white, red, and orange group, respectively. This reduction occurred at all sample doses, indicating that hydrophilic inhibitors may be related to the soluble phenolic fraction which also declined with kernel maturity as previously stated. This *in vitro* functional quality had high correlation with the free UHPLC soluble phenolic compounds at all sample doses ($r = 0.7386$, $r = 0.8064$, and $r = 0.8545$, $p < 0.05$ at 3, 6, and 10 mg sample dose, respectively). Specific phenolic compounds such as the vanillic acid derivatives, HBA-1, and the total HBA contents positively correlated with the inhibitory potential of *Cabanita* maize samples ($r = 0.7962$, $r = 0.7728$, and $r = 0.7961$, $p < 0.05$, respectively, 10 mg sample dose). Among free HCA compounds, ferulic acid derivatives also showed a significant correlation ($r = 0.6597$, $p < 0.05$, 10 mg sample dose). In case of LF fractions, results were not influenced by S, showing comparable α-glucosidase inhibition during the grain growth. However, a certain increase (from 5.7 to 8.7% and from 8.6 to 15.1% at 6, and 10 mg of sample dose, respectively) was observed in the orange maize. No significant correlations were found between the LF α-glucosidase inhibitory activity, and any metabolites measured in the current study.

The α-amylase enzyme, relevant for the hydrolysis of α-1,4-glucan polysaccharides into maltose and maltooligosaccharides (83), was inhibited only by HF maize fractions in a dose-dependent manner (Table 6). All values decreased with kernel maturity (S significant) similarly as in the case of the α-glucosidase inhibitory activity. When the maturity stage changed from S1 to S3, the white maize (125 mg dose) showed the highest loss of the inhibitory potential (around 85%), followed by the red, and orange maize (~54 and 58% in red and orange maize, respectively). Both M × S interaction and M factors greatly influenced the α-amylase inhibition. White maize samples had higher α-amylase inhibition at S1 among *Cabanita* maize types. The α-amylase inhibitory potential positively correlated with the free UHPLC total phenolic contents ($r = 0.6358$ and $r = 0.6574$, $p < 0.05$, at 62 and 125 mg of sample dose, respectively). Furthermore, all HBA compounds and free ferulic acid derivatives were correlated with this *in vitro* functional property ($r = 0.5278$–0.6471 at all sample doses, and $r = 0.5340$–0.5599 at 62–125 mg, respectively).

Different studies have highlighted the role of phenolic compounds for hyperglycemia prevention and countering associated oxidative complications through several mechanisms including the modulation of gastric enzymes at intestinal level (84–87). Cereal-derived phenolic acids including several HBA, and HCA compounds have shown inhibitory potential against the intestinal α-glucosidase enzyme which was highly dependent on the number of hydroxyl and methoxy groups in their structure (88). HBA derivatives such as methyl vanillate (a vanillic acid derivative), syringic acid, and vanillic acid from Thai colored rice showed higher inhibition of α-glucosidase than on α-amylase with a mixed-type inhibition mode against α-glucosidase (89). In same study, *in silico* analysis revealed that the inhibition involved the molecular interaction between HBA and the binding sites of digestive enzymes through 3–4 hydrogen bonds depending on the

phenolic compound and the enzyme (89). Conjugated hydroxycinnamic acids amides (HCCA) identified in maize and in other grains from the *Poaceae* family grains such as *N,N*-di-feruloylputrescine, *N*-*p*-coumaroyl-*N*-feruloylputrescine have also been targeted as α-glucosidase inhibitors (90, 91). Recently, another ferulic acid derivative named 6'-*O*-feruloylsucrose isolated from black rice bran showed high α-glucosidase inhibition when *in vitro* and *in silico* studies were performed (92).

Based on above information, the free phenolic fraction including HBA, and some HCA derivatives likely involving ferulic acid derivatives detected in the current research may explain the *in vitro* anti-hyperglycemia potential of *Cabanita* maize HF extracts. However, other polar compounds may also be involved. Some compounds detected with the untargeted GC–MS analysis as will be discussed in next section, have shown direct correlation with results from both enzymatic assays. Alcohol sugars including D-sorbitol, meso-erythritol and phytosterols such as campesterol, and sitosterol positively correlated with the α-amylase ($r = 0.7588$, $r = 0.6556$, $r = 0.6235$, and $r = 0.7850$, $p < 0.05$, at 125 mg dose respectively) and α-glucosidase inhibitory activities ($r = 0.7275$, $r = 0.7062$, $r = 0.6965$, and $r = 0.7218$, $p < 0.05$, at 10 mg, respectively). Some studies have pointed out the role of erythritol and triterpenoid compounds for the management of postprandial blood glucose through the inhibition of α-glucosidase enzyme (93, 94). Stigmasterol among other phytochemicals identified in maize silk have been indicated as potential inhibitors of both α-glucosidase and α-amylase enzymes (95).

The observed LF α-glucosidase inhibitory activity may be ascribed to lipophilic compounds that increase in maize kernel with maturity time specially in case of the orange maize. The contents of α-tocopherol, *β*-tocotrienol, *γ*-tocotrienol, and *δ*-tocotrienol had increased with maturity in grains of *Amaranthus cruentus* (96). Lipophilic extracts from *Vicia fava* L. seeds containing *α*-tocopherol and *γ*-tocopherol compounds exhibited high *α*-glucosidase inhibition (97). It is possible that the inhibitory activity of *Cabanita* maize extracts against digestive enzymes may be due to the synergistic action of HL and LF compounds as also was reported by Parizad et al. (98) in several pigmented cereals. Future studies are necessary to reveal the identity of HF and LF compounds from *Cabanita* maize and their molecular mechanisms of inhibition against hyperglycemia-relevant enzymes.

α-Amylase inhibitory activity results from this study are comparable with those reported by Ranilla et al. (20) in mature *Cabanita* maize kernels from Peru (8.9–10.2%, at 125 mg sample dose). However, higher *α*-glucosidase inhibitory activities were obtained by above authors (34.9–40.8%, at 12.5 mg of sample dose) which may be linked to the higher sample doses evaluated. On the other hand, lower inhibitory activities against *α*-amylase and *α*-glucosidase were found in the free phenolic fraction from Chinese fresh waxy maize harvested at milk stage (~28–72% and ~32–48% for the *α*-amylase and α-glucosidase inhibitory activities, respectively, at 1,000 mg sample dose) than in current study at similar maturity stage (2.4–55.0% at 25–125 mg sample dose, and 17.2–41.0% at 3–10 mg sample dose for the *α*-amylase and *α*-glucosidase inhibitory activities, respectively) (99). Several studies have shown the anti-hyperglycemic potential of maize from different origins (36, 98, 100). Nevertheless, the impact of the maturity stage on this functional property in selected samples from the Peruvian *Cabanita* maize diversity is shown for the first time in this study.

3.6. Primary polar metabolites analysis by GC–MS of *Cabanita* maize types at different maturity stages and principal component analysis

The GC–MS analysis allowed to detect 63 polar metabolites including sugars, amino acids, other nitrogen-containing compounds, free fatty acids, organic acids, sugar alcohols, and phytosterols. A PCA analysis was performed considering all data from the targeted, untargeted metabolomic analyses, and the *in vitro* functional quality to reveal underlying relationships among all variables (Figure 4). The first two principal components from the PCA model explained 58.1% of the total dataset variability. Different groups were observed based on the maize type and maturity stage which were explained by 98 significant variables. The heat map considering the top 60 significant variables is shown in Figure 5. PC 1 (45.2% of explained variance) separated (from top to the bottom) all maize types at S3 (OIII, RIII, and WIII, for the orange, red, and white maize at S3, respectively) from samples at earlier maturity stages (I, II, for S1 and S2, respectively).

Maize samples at S3 were characterized by overall lower concentrations of secondary metabolites (phenolic and carotenoid compounds), along with reduced *in vitro* antioxidant and anti-hyperglycemia potential than maize at S1 and S2. In relation to the primary polar metabolites detected by GC–MS, free monosaccharides (glucose, fructose, galactose), disaccharides (sucrose) among other unidentified sugar molecules decreased with kernel maturity in all maize types (Figure 5). Furthermore, several amino acids (glutamic acid, glycine, alanine, phenylalanine, proline, isoleucine, leucine, valine, and serine) also declined with grain maturity. Simple sugars may have been used as carbon sources for cellular energy metabolism, and the biosynthesis of starch which is known to accumulate in mature cereal grains (101). The decrease of sugar contents with the concurrent increase of starch during grain development was observed by Xu et al. (24) and Saikaew et al. (101) in yellow and purple waxy maize, respectively. Amino acids may have been transformed into proteins or used as metabolic precursors for the synthesis of secondary metabolites (102). The increase of protein with kernel maturation has been reported in maize and rice kernels (101, 103). Among data from all maize types at S3, the orange maize clearly separated and stood out from the red and white groups, whereas some replicates from these last maize types overlapped. This difference was mainly correlated with the highest carotenoid contents in the orange group than in red and white maize.

PC 2 (12.9% of explained data variability) separated orange maize (at all maturity stages) from white and red types (from left to right). The orange maize showed unique flavonoids such as luteolin derivatives and had the highest carotenoid concentrations (especially at S1 and S2) as previously stated. Interestingly, the white maize was different from the other maize types specifically at the S1 stage (Figure 5). Primary metabolites including simple sugars (monosaccharides and sucrose), amino acids, free fatty acids (linoleic and palmitic acids), organic acids (4-aminobutanoic acid or GABA, fumaric acid), amines (putrescine, ethanolamine), myo-inositol and xylitol were more abundant in the white maize at milk stage than in the other maize groups at same maturity period.

Phytosterols such as campesterol and β-sitosterol were detected in all maize groups. The orange and red maize showed comparable

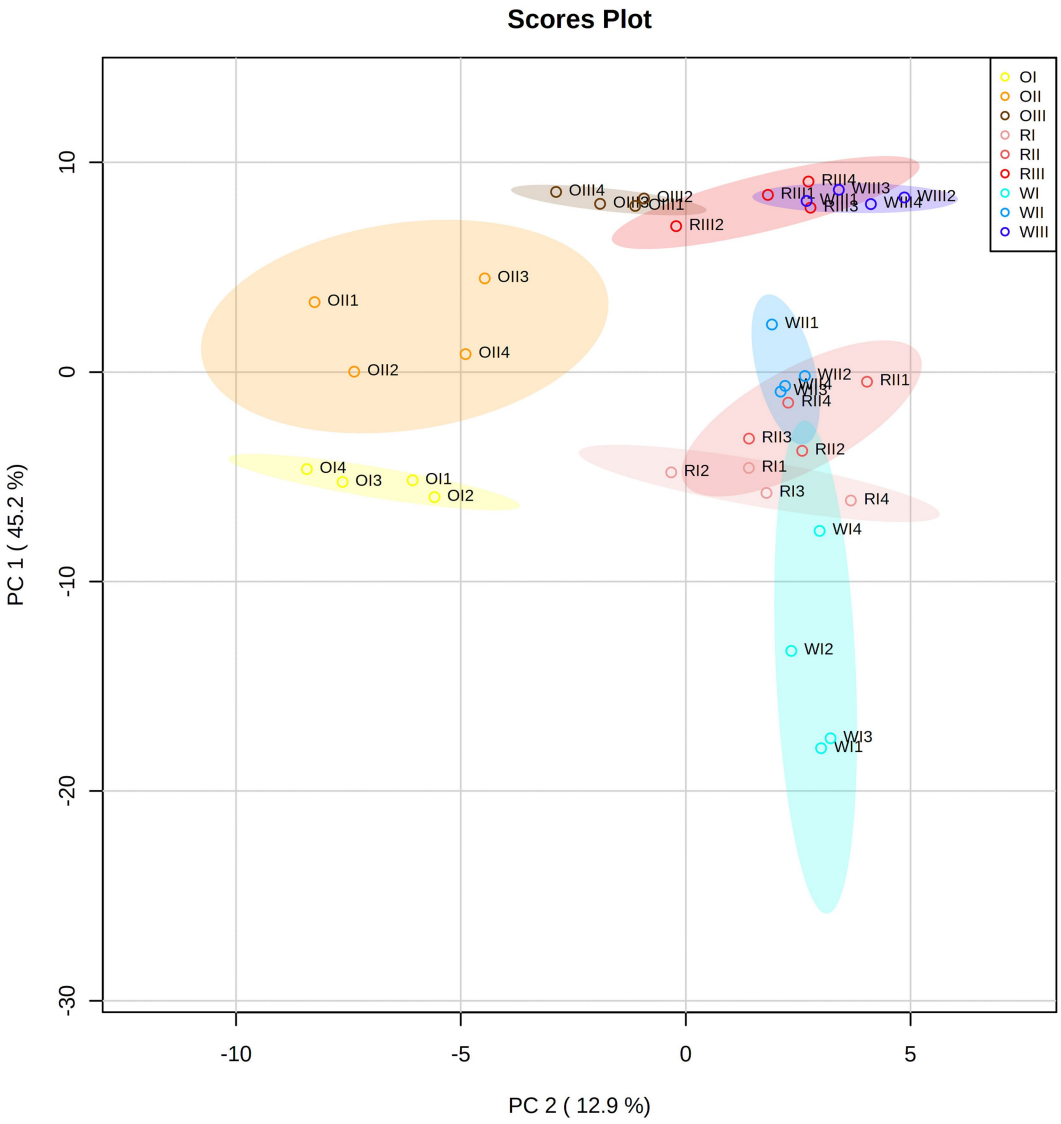

FIGURE 4
Principal component analysis (PCA) score plot of all data from white (W), red (R), and orange (O) *Cabanita* race at different maturity stages (I: S1, II: S2, III: S3).

campesterol contents, but the white maize exhibited the highest sitosterol abundance at S1. Phytosterols are bioactive compounds with potential for the prevention of cardiovascular diseases because of their cholesterol-lowering properties (104). Both detected phytosterols decreased with grain maturity in all *Cabanita* types. The reduction of *β*-sitosterol has been also observed during *Camellia chekiangoleosa Hu*. seeds development (105). *β*-sitosterol is the key precursor of sitosterol-β-glucoside which play a role on the biosynthesis of cellulose (106). The increase of dietary fibre, which is composed of cellulose, hemicellulose, lignin, among other polymers has been reported in *Amaranthus cruentus* with grain maturity (96). Furthermore, the cell wall feruloylation along with the lignin-cross links of the wheat grain outer layers increased during kernel development (107). This likely explains the decrease of free ferulic acid derivatives and the increase of some cell wall phenolics (bound ferulic acid derivatives) specially in the red and orange maize at S3 maturity stage.

Other detected primary metabolites such as free fatty acids (palmitic acid, linoleic acid), organic acids (glyceric acid, fumaric acid, ribonic acid, GABA), amines (ethanolamine, putrescine), myo-inositol, and alcohol sugars (erythritol, xylitol) were also reduced with grain maturity in all maize types. Free fatty acids may have been metabolized for the synthesis of triacylglycerols (TAG) since the total fatty acid contents (derived from the triacylglycerol saponification) increased with grain maturity in all *Cabanita* groups (Table 4). The increase of the total lipid contents during the grain development of yellow maize has been related to the late embryo formation (24). The reduction of ethanolamine and glyceric acid may have been targeted as precursors of glycerophospholids (components of the cell membranes) during grain growth. Some types of phosphatidilethanolamines esterified with variable fatty acids have increased during wheat kernel filling (108). The reduction of intermediate metabolites involved in the tricarboxylic acid cycle (TCA) such as fumaric acid and GABA-derived succinic acid might

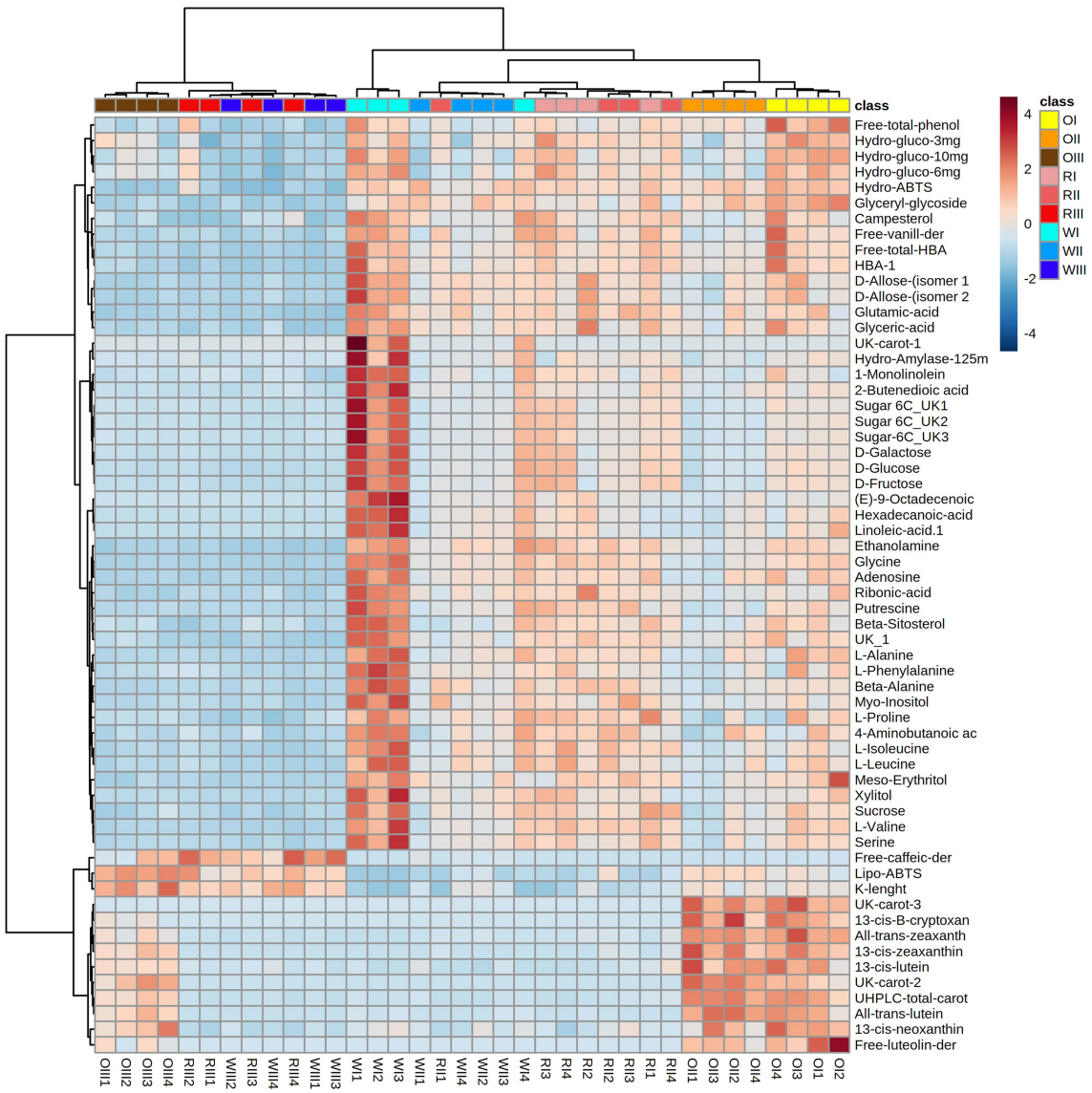

FIGURE 5
Heat map considering 60 significant variables from white (W), red (R), and orange (O) *Cabanita* race at different maturity stages (I: S1, II: S2, III: S3).

reflect a high mitochondrial activity for energy generation during the maize grain development (109). Polyamines including putrescine have essential roles in many biochemical and physiological processes, in particular stress and senescence responses during plant growth and development (110). Putrescine content also decreased with kernel maturity in *Cabanita* maize as observed in other polar metabolites. This polyamine may have been used for the biosynthesis of some HCAAs such as *N*-*p*-coumaroyl-*N*-feruloylputrescine and caffeoylputrescine which have been detected in mature maize (44). In the current study, *p*-coumaric and caffeic acid derivatives (possibly HCAAs) have increased with *Cabanita* kernel maturation.

4. Conclusion

Maize with different kernel pigmentations (white, red, and orange) and representing the diversity of the Peruvian Andean maize race *Cabanita* showed variable primary (polar metabolites), and secondary metabolite composition (phenolic and carotenoid compounds) and were greatly influenced by the grain maturity stage. All maize types showed free HBA and HCA phenolic compounds, but luteolin derivatives and anthocyanins were only detected in orange and red maize, respectively. Major bound phenolic compounds were ferulic acid, followed by ferulic acid derivatives, and *p*-coumaric acid in all *Cabanita* groups. However, orange, and red types had higher bound ferulic acid, and total phenolic contents (free + bound) (223.9–274.4 mg/100 g DW, 193.4–229.8 mg/100 g DW for the orange and red maize, respectively) than the white maize (162.2–225.0 mg/100 g DW). Xanthophylls including lutein, zeaxanthin, neoxanthin, lutein isomer (~13-*cis*-lutein) were detected in all maize types. The orange maize had the highest total carotenoid contents (3.19–5.87 μg/g DW) and contained specific carotenoids such as *β*-cryptoxanthin and zeaxanthin isomers. Most phenolic and carotenoid compounds decreased with kernel maturity in all *Cabanita* maize types. With respect to the primary

metabolites, all maize types showed similar fatty acid contents (linoleic acid > oleic acid > palmitic acid > α-linolenic acid > stearic acid) which increased with kernel development. Other primary metabolites such as simple sugars, alcohols, amino acids, free fatty acids, organic acids, amines, and phytosterols declined with grain maturity and were overall more abundant in white maize at S1. The *in vitro* antioxidant potential and the inhibitory activity against digestive enzymes (α-amylase and α-glucosidase) were high in the hydrophilic fractions and correlated with the free phenolic fraction. In general, all *Cabanita* maize types had similar *in vitro* health-relevant functionality which significantly decreased with grain development. Based on above results, recommended harvesting periods for the consumption of the orange and white *Cabanita* would be at S1 and S2 stages due to their higher phenolic, carotenoid contents (in case of the orange type), *in vitro* functional qualities, phytosterol concentrations, and better ω-6:ω-3 PUFAs balance. The red *Cabanita* maize would be more valuable at S3 because of its higher total anthocyanin, and phenolic contents. Nevertheless, the potential changes on *Cabanita* technological and processing characteristics at lower maturity stages should be further evaluated. Current study provides relevant metabolomic and biochemical information that contribute to the characterization of the Andean *Cabanita* maize diversity. Insights from this research would be important for promoting its consumption beyond its mature form as it is currently applied, and for future improvements at postharvest level. Studies at transcriptomic level would help to reveal the mechanisms involved in the metabolic changes related to the secondary and primary metabolites during *Cabanita* maize maturation. Next level studies should focus on improving the nutraceutical and nutritional properties of *Cabanita* maize with its sustainability and consumer-relevant yield characteristics within the context of Andean food systems.

Data availability statement

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

Author contributions

LR and GZ conceived and designed the study. LR directed the research and wrote the manuscript. AA-C performed the experiments. RC helped with the experimental work. HH contributed with the direction of crop management and pollination control. MV-V helped with the statistical analysis. HB-G coordinated and helped with the original sample collection. RP developed the untargeted metabolomic and free fatty acid analysis. GZ, RC, RP, HH, and KS critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1132228/full#supplementary-material

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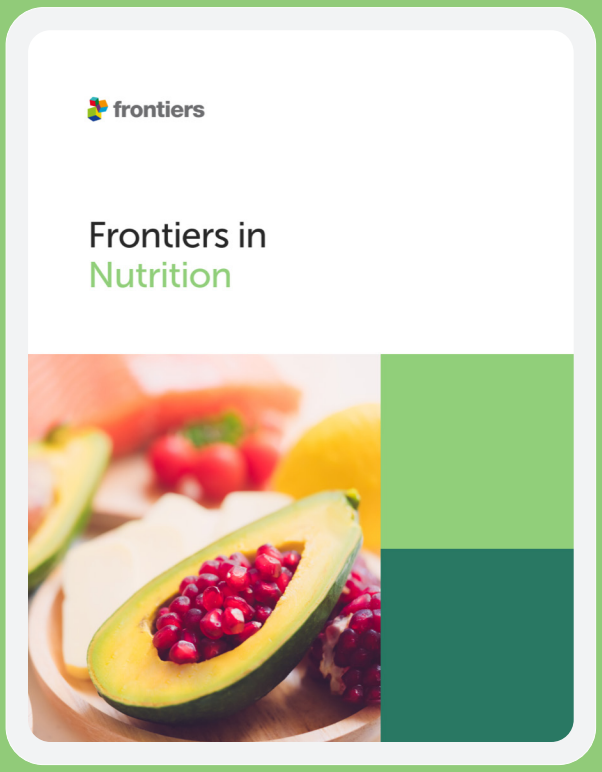

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