Effect of in vitro gastrointestinal digestion on the composition and bioactivity of anthocyanins in the fruits of cultivated Lycium ruthenicum Murray

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ABSTRACT
The composition of anthocyanins in cultivated Lycium ruthenicum Murray (CLM) before and after in vitro gastrointestinal digestion was determined using an HPLC system coupled with an electro-spray ionization-time of flight mass spectrometry. The antioxidant activity and α-glucosidase inhibition of the anthocyanins before and after digestion were also studied. The results showed that most of the anthocyanins in CLM were found in glycosylated form, containing glycoside ligands. After digestion, the glycosylated anthocyanins were transformed to anthocyanidins (without glycoligands) which exhibited higher antioxidant activity comparatively. Interestingly, although the inhibitory activity of anthocyanins against α-glucosidase was increased by in vitro gastrointestinal digestion, as suggested by IC50 value, the binding affinity of the anthocyanidins to α-glucosidase remained stable, as demonstrated by fluorescence quenching constant. This suggested that the anthocyanidins might have formed the anthocyanidin-α-glucosidase-substrate tertiary complex. Conclusively, in vitro gastrointestinal digestion may enhance the biological activities of CLM anthocyanins, regarding the antioxidant activity and α-glucosidase inhibition.

1. Introduction
Lycium ruthenicum Murray has been bred and developed as a new plant food in Qinghai-Tibetan Plateau to satisfy the increased health demands of human beings. It contains a variety of bioactive functional components including polyphenols, polysaccharides, pectin, essential oils, trace minerals and organic acids, etc. (Zheng et al., 2011). Of these components, anthocyanins are considered as the principal component accounted for high antioxidant, biological and anti-tumor properties (Liobikas, Skemiene, Trumbeikaite, & Borutaite, 2016; Wang et al., 2018; Zheng et al., 2011). Due to limited number of natural sources, cultivated Lycium ruthenicum Murray (CLM) is a promising and cost-effective alternative to the wild one. However, to the best of our knowledge, limited information is available on exploring the overall anthocyanin composition of CLM.

Mass spectrometry (MS) is a powerful tool to characterize the basic constituents of the chemicals and provide authentic information about the structure of the compound (Li, Meng, & Li, 2016). High-performance liquid chromatography (HPLC) coupled with MS (high-resolution) had been widely used for the identification of anthocyanins in various berry fruits including Lycium ruthenicum Murray. For examples, for the first time, it was used to investigate the anthocyanins composition in wild Lycium ruthenicum Murray (WLM) for elucidating anthocyanins composition of it and for their further utilization as healthy food and natural pigment resource (Zheng et al., 2011); Recently, Wang et al.
(2018) have adopted it to study the anthocyanins composition of WLM from different provinces and, based on the identification results, established an effective procedure to trace the geographical origins. Additionally, a high-resolution electrospray ionization-time of flight-MS coupled with an HPLC system (HPLC-ESI-ToF-MS/MS) has been proved as an efficient method to identify the composition of WLM anthocyanins including cis-trans isomers (Wang et al., 2018).

In vivo, anthocyanins may act as free radical scavengers by providing hydrogen atoms and cause disruption of the long-chain oxidation reactions (Rice-Evans, Miller, & Paganga, 1996), which requires absorption of anthocyanins in human gastrointestinal digestion system (Bouayou, Hoffmann, & Bohn, 2011; Tenore, Campiglia, Ritiemi, & Novellino, 2013). On the other hand, gastrointestinal digestion condition may affect the stability and bioactivity of food constituents by causing drastic structural changes (Cilla, Gonzalez-Sarrías, Tomás-Barberán, Espín, & Barberá, 2009). It has been reported that anthocyanins are highly unstable at mild intestinal pH thus the transition from acidic-gastric digestion condition to alkaline-intestinal digestion condition could decrease their total bioaccessibility (Tagliazucchi, Verzeloni, Bertolini, & Conte, 2010). A simulated in vitro gastrointestinal digestion is commonly used to study the changes in dietary components and bioactivity, as it satisfies the factors which may affect the nature, stability and bioactivities of the extractable phytochemicals (Biehler & Bohn, 2010; Cilla et al., 2009). In practice, the simulated in vitro gastrointestinal digestion has been well demonstrated to be correlated with the results of human studies and animal study models (Biehler & Bohn, 2010).

Recently, the practice of using phytochemicals to inhibit carbohydrate-hydrolyzing enzymes (e.g. α-amylase and α-glucosidase) has attracted widespread interests, as the enzyme inhibition can be a potential therapeutic substitute to insulin injection for type II diabetes (Sun, Gidley, & Warren, 2017). α-Glucosidase is a key digestive enzyme hydrolyzing terminal non-reducing (1→4)-linked short chain reducing sugars (e.g. maltose, oligosaccharide, etc.) to release a single α-glucose molecule. Therefore, the enzyme plays an important role in elevating postprandial blood sugar. The inhibition of α-glucosidase by anthocyanins in vitro has been studied previously (You, Chen, Wang, Luo, & Jiang, 2011). α-Glucosidase is located in the brush border of the small intestine, where anthocyanins develop the inhibitory activity against the enzyme. As gastrointestinal environment may change the stability and bioaccessibility of anthocyanins (Tagliazucchi et al., 2010), the inhibitory activity of anthocyanins against α-glucosidase after gastrointestinal digestion process needs further exploration, the aim of which may be achieved by using a simulated in vitro gastrointestinal digestion. Therefore, the objective of this study was to investigate the anthocyanin composition, antioxidant activity and α-glucosidase inhibition activity of CLM anthocyanins before and after simulated in vitro gastrointestinal digestion.

2. Materials and methods

2.1. Plant materials

Fully ripened CLM fruits were collected from three locations of Qinghai-Tibetan Plateau including Hedong farm (located in Golmud: Latitude, 36°25′0″; Longitude, 94°53′0″; Altitude, 2800 m), Delingha farm (located in Delingha: Latitude, 37°13′0″; Longitude, 97°14′0″; Altitude, 2980 m) and Nomuhong farm (located in Dulan: Latitude, 36°2′0″; Longitude, 98°8′0″; Altitude, 2780 m) in July 2017. The fruits were hand-picked and transported to our laboratory immediately. They were freeze-dried using a Sihuan® LG-18C vacuum freeze drier (Beijing, China), crushed to powders by a Zhaoshen® XS-10 pulverizer (Shanghai, China), and sieved through a 40 mesh sieve. The powdered samples were stored at −80°C for further use.

2.2. Chemical reagents

α-Glucosidase from Saccharomyces cerevisiae (EC 3.2.1.20), 6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid (Troloxi), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-Nitrophenol, α-D-glucopyranoside (pNP, >99%), α-amylase, pepsin, pancreatin and bile salts were obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Other chemical reagents including hydrochloric acid (HCl), calcium chloride (CaCl₂), sodium bicarbonate (NaHCO₃) and sodium carbonate (Na₂CO₃) were purchased from Tianli Chemical reagent Co., Ltd. (Tianjin, China).

2.3. Extraction of CLM anthocyanins

The anthocyanins were extracted according to the previous method used by Wang et al. (2018) with some modifications. About 5.0 g of the samples were extracted triply with 50 mL methanol and formic acid (98.2, v/v). After 24 h of extraction at room temperature in the dark, the mixture was filtrated then the filtrates were combined and centrifuged at 3000 g at 4°C for 10 min to remove the fruit residues, protein and polysaccharide sediments. The suspensions were concentrated at 30°C to 10 mL and mixed with same volume ethyl acetate in a separating funnel and stood for 4 h. The extracts were purified by successively rinsing with water (500 mL, containing 0.01% HCl, to remove sugars, formic acid and other interfering substances) and 40% ethanol (500 mL, containing 0.01% HCl) on a Amberlite® XAD-7HP macroporous resin cartridge (2.6 × 50 cm). The purple eluent was collected then evaporated at 30°C and freeze-dried for further use.

2.4. Simulated in vitro gastrointestinal digestion

The digestion was carried out according to the description by Zheng et al. (2018) with some modifications. The freeze-dried CLM powder (2.0 g) was mixed with 20 mL distilled water and 500 μL of 1.30 mg/L α-amylase-CaCl₂ solution. Then, the solution was adjusted to pH 7.0 and incubated at 37°C for 10 min in a Qilinbeier® QL-901 vortex shaker (Haimen, China). Immediately, the mixture was adjusted to pH 2.0 after the addition of 100.0 mg of 14,800 U pepsin and incubated at 37°C for 1 h. After that, the pH was increased to 6.5, followed by the addition of 5 mL of 4.0 mg/L pancreatin and 25.0 mg/L bile salts solution (dissolved in 0.1 mM NaHCO₃). The mixture was then adjusted to pH 7.4 and incubated at 37°C for 2 h. Finally, the solutions were centrifuged after the pH was adjusted to 2.0. The supernatants
were extracted with acetonitrile (with 16% water), concentrated at 30°C and vacuum and freeze-dried. The residue was purified as in Section 2.3 for further analysis.

2.5. Identification of CLM anthocyanins

HPLC analysis was performed as described in our previous study (Wang et al., 2018) on an UltiMate 3000 HPLC system coupled with an ultraviolet-visible detector, a P680 HPLC pump and an AT-330 thermostated column compartment (Thermo Scientific, Waltham, USA). CLM anthocyanins (before and after in vitro gastrointestinal digestion) were dissolved in a methanol-formic acid (98:2, v/v) mixture. Then, an aliquot of 5 μL (filtrated through a Jinteng® 0.22 μm nylon membrane filter (Tianjin, China)) was injected and analyzed at 525 nm. A Merck KGaA reverse phase column C18 column (250 × 4.6 mm, 5 μm, Darmstadt, Germany) was used for separation. The mobile phase was comprised of solvent A (aqueous with 10% formic acid and 0.1% trifluoroacetic acid) and solvent B (acetonitrile with 15% methanol). Gradient elution: 0–3 min (from 3% to 11.5% B); 30–40 min (11.5% B); 40–60 min (from 11.5% to 15.5% B); 60–70 min (from 15.5% to 16% B); 70–80 min (from 16% to 23% B); 80–100 min (from 23% to 3% B). The flow rate and temperature were 0.8 mL/min and 35°C, respectively.

HPLC-ESI-ToF-MS/MS analysis was performed on an Agilent® 6460 QQQ system with an Agilent® 6550 iFunnel Q-TOF (Agilent Technologies, USA) and an Agilent® 1260 HPLC system coupled with an ultraviolet-visible detector, a P680 HPLC pump and an AT-330 thermostatted column compartment (Thermo Scientific, Waltham, USA). CLM anthocyanins were extracted with acetonitrile (with 16% water), concentrated at 30°C and vacuum and freeze-dried. The residue was purified as in Section 2.3 for further analysis.

2.6. Quantitative analysis of CLM anthocyanins

The contents of individual CLM anthocyanins were determined according to our previous study (Wang et al., 2018) using the HPLC system by ultraviolet-visible detector. Cyanidin 3-glucoside was used as a reference to quantify the anthocyanins with the concentration ranging from 0 to 600 μg/mL. The contents of CLM anthocyanins were expressed as μg cyanidin 3-glucoside equivalents/g dried weight (DW). The total anthocyanins content was the summation of the individual CLM anthocyanins.

2.7. Antioxidant capacities assay

The DPPH, ABTS and FRAP assay were used to evaluate the antioxidant capacities of CLM anthocyanins as described by previous studies (Almeida et al., 2011; Benzie & Strain, 1996; Brand-Williams, Cuvelier, & Berstein, 1995). The antioxidant activities were calculated from the standard curve Y (absorbance) = 0.0008 × X (Troxol equivalents (TE) content) + 0.5152 (R² = 0.9994), Y (absorbance) = 0.0009 × X (TE content) + 0.8941 (R² = 0.9994) and Y (absorbance) = 0.0063 × X (TE content) + 0.0101 (R² = 0.9992), respectively. The results were expressed as μM TE/g DW.

2.8. α-glucosidase inhibition assay

CLM anthocyanins (before and after in vitro gastrointestinal digestion) were dissolved in 0.5% (v/v) ethanol (which has no inhibitory effect on α-glucosidase) and later on diluted with 0.1 M sodium phosphate buffer (PBS) of pH 6.8 as stock solution for the analysis of α-glucosidase inhibition activity.

α-Glucosidase inhibitory activity was determined as reported previously (Yan, Zhang, Pan, & Wang, 2014). A fixed concentration of α-glucosidase (4.0 U/mL) and various amounts of CLM anthocyanins were mixed to a series of assay solutions with the PBS in a 2.0 mL reaction system. The assay was initiated by adding 500 μL of 0.5 mM pNPG after a pre-incubation of 30 min at 37°C. Then, the reaction solution (500 μL) was added to 500 μL of 0.3 M Na₂CO₃ solution. The absorbance was recorded at 400 nm every 2 min on a Unico® 2100 UV-Vis spectrophotometer (Shanghai, China). Relative enzymatic activity I (%) was calculated by the following equation (Sun, Warren, Netzel, & Gidley, 2016):

\[ I = \left(1 - \frac{v}{v_0}\right) \times 100 \]  

where \( v \) and \( v_0 \) are the initial reaction velocity in the presence and absence of anthocyanins, respectively. The α-glucosidase inhibitory activity of CLM anthocyanins was expressed as the median effective concentration for inhibitory activity (IC₅₀), which was calculated by the following equation:

\[ I = I_{\text{max}} \left(1 - \frac{IC_{50}}{[I] + IC_{50}}\right) \]  

where \([I]\) is the anthocyanin concentration, and \(I_{\text{max}}\) is the maximum percentage inhibition.

2.9. Fluorescence quenching assay

The fluorescence quenching of α-glucosidase assay was performed on a Shimadzu® RF-6000 spectrofluorimeter (Tokyo, Japan) in the range of emission wavelength of 295 to 500 nm with the excitation wavelength at 280 nm. Both the slit widths were 2 nm. A 0.2 mL of CLM anthocyanin solution (to give a final concentration of 0.8 mg/mL) or PBS buffer was added to a tube containing 3 mL of 40 U/mL α-glucosidase solution. The fluorescence spectra of these mixed solutions was measured after standing at 4°C for 15 min. To illustrate the quenching mechanism between CLM anthocyanins and α-glucosidase, the fluorescence quenching data were analyzed by Stern-Volmer Equation (3), and the modified one (4) were applied as follows (Ferrer-Gallego, Goncalves, Rivas-Gonzalo, Escrinabo-Ballon, & de Freitas, 2012):

\[ \frac{F_0}{F} = 1 + k_q T_0 [Q] = 1 + K_{Q_0} [Q] \]  

\[ \frac{F_0}{F} = e^{K_{Q_0} [Q]} \]  

where \( F_0 \) and \( F \) are the maximum fluorescence intensity in the absence and presence of CLM anthocyanins, respectively; \( k_q \) is the bimolecular quenching constant, \( T_0 \) is the lifetime of the fluorophore without quencher, and the value of \( T_0 \) of the biopolymer is 10⁻⁸ s (Lakowicz, 2006); [Q] is the concentration of CLM anthocyanins; \( K_{Q_0} \) is the fluorescence quenching constant.
2.10. Statistical analysis

All analyses were conducted in triplicate and results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and principal component analysis (PCA) were performed by SPSS statistics 19.0 (SPSS Inc., Chicago, USA). Differences were considered significant between the samples at \( P < .05 \).

3. Results and discussion

3.1. Effects of the digestion on composition of CLM anthocyanins

3.1.1. Composition of CLM anthocyanins before gastrointestinal digestion

The chromatographic fingerprints of the anthocyanins in the samples before in vitro gastrointestinal digestion were analyzed by using HPLC and are presented in Figure 1(a). Total 13 peaks were detected in both samples, and the anthocyanin profiles of CLM form three different cultivation locations were found similar (Figure 1(a)), confirming that the anthocyanin profiles are genetically controlled (Li et al., 2016). Individual anthocyanins were then identified using HPLC-ESI-ToF-MS/MS, by comparing the detailed MS data (Table 1) with published values (Wang et al., 2018). Table 1 summarizes the characterization results of CLM anthocyanins, and Figure 2 displays the chemical structures of CLM anthocyanins before in vitro gastrointestinal digestion. It was noted that HPLC-ESI-ToF-MS/MS analysis revealed 14 anthocyanins because peak 2 corresponded to two different-coeluted anthocyanins (Table 1). The co-elution is a commonly observed phenomenon in the study of anthocyanins by HPLC (Zheng et al., 2011). The anthocyanins in CLM are mostly in the glycosylation form and are connected with sugar moieties including galactoside, glucoside and rutinoside at third position in the C ring and at fifth in the A ring (aglycones) of petunidin, delphinidin and malvidin (Table 1 and Figure 2). Furthermore, most of the anthocyanins in CLM are in the acylation form. The acylated groups involve coumaric acid, caffeic acid, malic acid and ferulic acid. This could be induced by the unique geography and tough weather (high altitude, cold, dry and robust sunlight) of Qinghai-Tibetan Plateau which could associate anthocyanins with different sugar moieties (Cabrita, Frystein, & Andersen, 2000) and turn anthocyanins into stable acylated forms (Abad-García, Berrueta, Garmón-Lobato, Gallo, & Vicente, 2009). Notably, the anthocyanins in CLM and WLM were same (Jin et al., 2015; Wang et al., 2018; Zheng et al., 2011). This may come from the fact that CLM samples were transplanted from the wild ones, so both the wild and cultivated types have similar genetic background.

The contents of individual anthocyanins and total anthocyanins of CLM samples are presented in Table 1.
Table 1. Identification and the contents of individual anthocyanins and total anthocyanins before and after *in vitro* gastrointestinal digestion.

| Peak no. | TR (min) | Molecular (M⁺) | Fragment (m/z) | Identification                        | Concent of anthocyanins (µg/g) | Proportion of anthocyanins (%) |
|----------|----------|----------------|----------------|---------------------------------------|-------------------------------|--------------------------------|
|          |          |                |                | Hedong                                 | Delingha                      | Nomuhong                        |
|          |          |                |                | 1560.96 ± 56.82a | 1120.72 ± 42.05a | 1283.48 ± 74.11b | 9.77 ± 0.17a | 7.67 ± 0.16b | 8.45 ± 0.41b |
|          |          |                |                | 513.82 ± 30.89b | 670.81 ± 27.27a | 617.89 ± 13.21ab | 3.22 ± 0.18b | 4.59 ± 0.17b | 4.07 ± 0.11b |
|          |          |                |                | 0.63 ± 0.01 | 0.94 ± 0.04 | 0.94 ± 0.10 | 0.19 ± 0.00 | 0.61 ± 0.04 | 0.91 ± 0.07 |
|          |          |                |                | 4.88 ± 0.03 | 157.63 ± 2.15 | 143.05 ± 15.71 | 0.95 ± 0.16 | 0.94 ± 0.10 | 0.94 ± 0.10 |
|          |          |                |                | 169.86 ± 4.43 | 131.85 ± 8.94 | 138.15 ± 8.94 | 0.68 ± 0.01 | 0.84 ± 0.04 | 0.91 ± 0.07 |
|          |          |                |                | 1.78 ± 0.06 | 4.05 ± 0.14 | 3.24 ± 0.15 | 2.80 ± 0.14 | 0.52 ± 0.03 | 0.60 ± 0.05 | 0.58 ± 0.09 |
|          |          |                |                | 138.60 ± 8.90 | 187.87 ± 7.25 | 1.00 ± 0.06 | 0.61 ± 0.04 | 0.95 ± 0.16 | 2.80 ± 0.14 |
|          |          |                |                | 556.55 ± 30.04 | 1.53 ± 0.05 | 0.94 ± 0.10 | 0.94 ± 0.10 | 0.19 ± 0.00 | 0.61 ± 0.04 | 0.91 ± 0.07 |
|          |          |                |                | 1560.96 ± 56.82a | 1120.72 ± 42.05a | 1283.48 ± 74.11b | 9.77 ± 0.17a | 7.67 ± 0.16b | 8.45 ± 0.41b |
|          |          |                |                | 513.82 ± 30.89b | 670.81 ± 27.27a | 617.89 ± 13.21ab | 3.22 ± 0.18b | 4.59 ± 0.17b | 4.07 ± 0.11b |
|          |          |                |                | 0.63 ± 0.01 | 0.94 ± 0.04 | 0.94 ± 0.10 | 0.19 ± 0.00 | 0.61 ± 0.04 | 0.91 ± 0.07 |
|          |          |                |                | 4.88 ± 0.03 | 157.63 ± 2.15 | 143.05 ± 15.71 | 0.95 ± 0.16 | 0.94 ± 0.10 | 0.94 ± 0.10 |
|          |          |                |                | 169.86 ± 4.43 | 131.85 ± 8.94 | 138.15 ± 8.94 | 0.68 ± 0.01 | 0.84 ± 0.04 | 0.91 ± 0.07 |
|          |          |                |                | 1.78 ± 0.06 | 4.05 ± 0.14 | 3.24 ± 0.15 | 2.80 ± 0.14 | 0.52 ± 0.03 | 0.60 ± 0.05 | 0.58 ± 0.09 |
|          |          |                |                | 138.60 ± 8.90 | 187.87 ± 7.25 | 1.00 ± 0.06 | 0.61 ± 0.04 | 0.95 ± 0.16 | 2.80 ± 0.14 |
|          |          |                |                | 556.55 ± 30.04 | 1.53 ± 0.05 | 0.94 ± 0.10 | 0.94 ± 0.10 | 0.19 ± 0.00 | 0.61 ± 0.04 | 0.91 ± 0.07 |

a Data with the same superscript in the same row are not significantly different (P > 0.05).

Los datos con el mismo superíndice en la misma fila no son significativamente diferentes (P > 0.05).
The total anthocyanin contents (14611.19–15969.89 μg/g DW) were lower than our previously reported samples of WLM (29388.80 μg/g DW) (Wang et al., 2018), indicating that cultivation might have a negative effect on the biosynthesis of anthocyanins. Besides, the total anthocyanin contents of CLM samples from Qinghai Province in this study were higher than the reported CLM from Ningxia Province (8456 μg/g DW) (Tang et al., 2017), suggesting that the environment of Qinghai Province is probably more suitable for the biosynthesis and accumulation of anthocyanins in CLM, which was consistent with the previous study (Lv, Wang, Cheng, Huang, & Wang, 2013). Furthermore, the contents of total anthocyanins in CLM were about 13 times higher than that of cranberry (around 1214 μg/g DW) (White, Howard, & Prior, 2010). The high content of anthocyanins in Qinghai Lycium ruthenicum Murray indicates that its fruit is an excellent source of anthocyanins. Similar results were observed by Wu, Lv, Wang, and Wang (2016) who compared that of Lycium ruthenicum Murray with berries.

The results showed that CLM from Hedong farm had higher total anthocyanin contents as compared to other farms (Table 1), emphasizing that Golmud (where Hedong farm is located) may be the most suitable area of Lycium ruthenicum Murray production. The main reason might be that Lycium ruthenicum Murray has very special physiological characteristics of salt-resistance to grow in drought and salinized deserts, which is the typical feature of Golmud soil (Liu et al., 2013). The difference in total anthocyanins contents among the samples could also be due to other factors (climate, soil constituents, degree of ripeness and year of production). Specifically, the climate factors have great influence on the biosynthesis and accumulation of anthocyanins (Li et al., 2016). For instance, low overnight temperatures and more sunlight hours during the ripening period were found to be helpful for the biosynthesis of anthocyanins (Tomaas-Barberaan & Espin, 2001); similarly, fewer rainfalls and longer sunny days during maturing season could also promote the accumulation of anthocyanins (Stracke, Ruufer, Weibel, Bub, & Watzl, 2009).

Regarding individual anthocyanin in CLM, petunidin-3-O-rutinoside (trans-p-coumaroyl)-5-O-glucoside was the most abundant anthocyanin (10367.60–111239.49 μg/g DW) which accounted for more than 70% of the total anthocyanins (Zheng et al., 2011). The difference could be due to agronomic management practices like irrigation, fertilization, herbicide and pesticide treatments, etc. (Tomaas-Barberaan & Espin, 2001). In addition, it was observed that the distribution of each anthocyanin in CLM samples of different locations were significantly different (Table 1). It could be due to environmental factors such as climate, soil component and year of production which were reported to affect the proportions of anthocyanins (Li et al., 2016). For this reason, PCA was carried out to evaluate the distribution of each anthocyanin. The results showed that the first two principal components (PC) accounted for about 80.53% of the total variance, which is sufficient to explain the total variance of the dataset. Grouping of the CLM samples was
performed visually using the plot, where the samples are represented by the function of the first two PCs (Figure 3). From the loadings of the variables, PC1 explained 64.88% of the variance, and petunidin-3-O-galactoside-5-O-glucoside, malvidin-3-O-rutinoside (cis-p-coumaroyl)-5-O-glucoside and peak 13 (unknown) contributed predominantly in it (Table 2). PC2 yielded 15.65% of explainable results, with petunidin-3-O-glucoside-5-O-glucoside and petunidin-3-O-glucoside (maloyl)-5-O-glucoside loaded heavily (Table 2). The results clearly distinguished the three CLM samples according to the geographical origin, which suggested that anthocyanin composition combined with PCA have the potential to be used for the authentication and traceability of CLM grown in Qinghai Province.

3.1.2. Composition of CLM anthocyanins after gastrointestinal digestion

Chemical compositions of natural extracts have often been used to estimate its available nutrients in daily human diets. Besides, the gut environment can also affect the availability of nutrients during gastrointestinal digestion which may lead to overestimation/underestimation of available nutrients (Bouayed et al., 2011). Additionally, recommendations on nutritional intake should also consider the alterations of nutrients during gastrointestinal digestion which may lead to overestimation/underestimation of available nutrients (Bouayed et al., 2011). Therefore, the anthocyanin profile of CLM after in vitro gastrointestinal digestion was investigated. Five peaks were detected for both samples harvested at different locations (Figure 1b). According to the MS data (Table 1), five anthocyanidins were identified. By comparing with the data published previously (Xu et al., 2012), peak 1 was identified as delphinidin by the characteristic delphinidin aglycone ion (M\(^+\) = m/z 303.0472), and peak 2 to peak 5 were tentatively determined as cyanidin (M\(^+\) = 287.0696), petunidin (M\(^+\) = m/z 317.0646), pelargonidin (M\(^+\) = m/z 321.0219) and malvidin (M\(^-\) = m/z 331.0789), respectively. The results showed that no glycosylated anthocyanin was detected after the gastrointestinal digestion, which may be resulted from the break of the glycosylation of anthocyanins via cutting the O-glycosidic bond during the digestion (Tagliazucchi et al., 2010). In the case, the anthocyanidins (after in vitro gastrointestinal digestion) eluted out earlier than the anthocyanins (before in vitro gastrointestinal digestion) (Figure 1), because most of the anthocyanins are not only in glycosylation status but also in acylation status (Table 1), which would have decreased the polarity of anthocyanins, and extended their retention time in HPLC (Zheng et al., 2011). In addition, two new anthocyanidins (cyanidin and pelargonidin) were detected in the digesta. As discussed above, during in vitro gastrointestinal digestion, the glycoside moieties of anthocyanins were removed and resulted in anthocyanidins which are unstable and differ only in the hydroxyl and methoxyl groups at the positions 3’ and 5’ positions in the B ring (Liobikas et al., 2016). Therefore, the two anthocyanidins might be formed by the linkage of hydroxyl and methoxyl groups at the positions 3’ and 5’ in the B ring (cyanidin, R3’ = -OH, R5’ = -H; pelargonidin, R3’ = -OCH\(_3\), R5’ = -H) (Figure 2) during the gastrointestinal digestion, under the changes from acidic gastric to mild alkaline conditions.

**Figure 3.** PCA two-dimensional plots of the anthocyanin composition in CLM before in vitro gastrointestinal digestion, which shows the grouping of the samples according to the cultivating location.

**Tabla 2.** Cargas de las características en los primeros tres componentes principales de las antocianinas CLM antes de la digestión gastrointestinal in vitro.*

| Variable | PC 1  | PC 2  | PC 3  |
|----------|------|------|------|
| Peak 1   | −0.946 | 0.002 | 0.269 |
| Peak 2   | 0.925  | 0.114 | −0.342 |
| Peak 3   | −0.020 | 0.875 | −0.013 |
| Peak 4   | 0.858  | −0.061 | 0.480 |
| Peak 5   | −0.893 | 0.018 | −0.323 |
| Peak 6   | 0.848  | −0.193 | −0.136 |
| Peak 7   | 0.756  | 0.520 | 0.260 |
| Peak 8   | 0.711  | −0.468 | −0.030 |
| Peak 9   | 0.413  | 0.807 | 0.079 |
| Peak 10  | 0.861  | −0.237 | 0.434 |
| Peak 11  | 0.941  | −0.116 | 0.173 |
| Peak 12  | −0.862 | −0.026 | 0.463 |
| Peak 13  | 0.901  | 0.050 | −0.399 |

*Peak names refer to Table 1.
*Los nombres de los picos se refieren a la Tabla 1.
intestinal environment as well as the impacts of bile acids and pancreatin (Bouayed et al., 2011).

The results suggested that in vitro gastrointestinal digestion might have caused severe structural damage to anthocyanins in CLM samples. The glycoside moieties of anthocyanins were removed during in vitro gastrointestinal digestion and resulted in five anthocyanidins (Table 1). In addition, as shown in Table 1, the total anthocyanins levels after in vitro gastrointestinal digestion were lower than that before digestion. This is consistent with the finding that the transition from acidic-gastric digestion condition to mild alkaline environment could decrease the total amount of bioaccessable anthocyanins (Tagliazucchi et al., 2010).

However, it has not changed the composition of major anthocyanidins in the samples (Table 1). Petunidin was the most abundant anthocyanidin (11083.25–13100.72 μg/g DW) after in vitro gastrointestinal digestion and contributed for almost 95% of the total anthocyanins (Table 1). The contents of the major anthocyanidins were found in the following order: Hedong > Nomuhong > Delingha. For this reason, the anthocyanidins obtained after in vitro gastrointestinal digestion could be used to compare the biological activities with anthocyanins.

### 3.2. Effects of the digestion on antioxidant activity of CLM anthocyanins

CLM anthocyanins before and after in vitro gastrointestinal digestion were exacted with methanol and then purified using a macroporous resin cartridge, yielding a high purity of anthocyanins. A total of 16409.67, 15039.83 and 15651.25 μg CLM anthocyanins were collected from 1 g freeze-dried powder of Hedong, Delingha and Nomuhong samples before in vitro gastrointestinal digestion, respectively (Table 3). According to the quantitative analysis (Table 1), the purity of CLM anthocyanins before in vitro gastrointestinal digestion were over 97% (97.32%, 97.15% and 97.04%, respectively). Similar results were found after in vitro gastrointestinal digestion (Table 3), then, they were used for the analysis of antioxidant activity and α-glucosidase inhibition in this study.

Antioxidant assay results are presented in Table 4, which shows that CLM anthocyanins before and after in vitro gastrointestinal digestion in both samples exhibited antioxidant activity, and the orders of the three different antioxidant tests (DPPH, ABTS and FRAP) were found same (Hedong > Nomuhong > Delingha). Polyphenols including flavonoids and phenolic acids are considered as promising components accountable for antioxidant activity (Zheng et al., 2011). As shown in Table 1, the total anthocyanins content in the samples followed the given order: Hedong > Nomuhong > Delingha (Table 1). Therefore, the higher antioxidant activity of the samples is likely related to its higher total anthocyanins content. Furthermore, in all the cases, the antioxidant activity of CLM anthocyanins after in vitro gastrointestinal digestion was significantly enhanced as compared to before the digestion in the terms of DPPH, ABTS and FRAP tests (Table 4). It is well known that the chemical structure of

### Table 3. The actual weight and the purity of CLM anthocyanins collected form 1 g samples before and after gastrointestinal digestion.\textsuperscript{a,b}

| Sample   | Weight (μg) Before | Weight (μg) After | Purity (%) Before | Purity (%) After |
|----------|--------------------|-------------------|-------------------|-----------------|
| Hedong   | 16409.67 ± 397.38\textsuperscript{a} | 15039.83 ± 356.27\textsuperscript{b} | 97.32\textsuperscript{a} | 97.63\textsuperscript{a} |
| Delingha | 14071.70 ± 539.49\textsuperscript{a} | 11861.07 ± 356.27\textsuperscript{a} | 97.04\textsuperscript{a} | 97.84\textsuperscript{a} |
| Nomuhong | 15651.25 ± 455.38\textsuperscript{a} | 14071.70 ± 539.49\textsuperscript{a} | 97.04\textsuperscript{a} | 97.84\textsuperscript{a} |

\textsuperscript{a}The purity (%) = (the content of CLM anthocyanins obtained by quantitative analysis (Table 1)/(the actual weight of collected CLM anthocyanins) × 100.

\textsuperscript{b}Data with the same superscript in the same column are not significantly different (P > 0.05).

### Table 4. Antioxidant activities (DPPH, ABTS and FRAP), α-glucosidase inhibition activity (IC\textsubscript{50}) and fluorescence quenching constant (K\textsubscript{Q}) of CLM anthocyanins from the samples before and after in vitro gastrointestinal digestion.\textsuperscript{a,b}

| Sample   | DPPH K\textsubscript{Q} (mg/mL) | ABTS IC\textsubscript{50} (10\textsuperscript{-4} mg/mL) | FRAP | IC\textsubscript{50} (10\textsuperscript{-4} mg/mL) |
|----------|--------------------------------|--------------------------------|-------|--------------------------------|
| Hedong   | 2.3630 ± 0.02\textsuperscript{a} | 95.89 ± 1.32\textsuperscript{b} | 13.2 ± 0.2\textsuperscript{b} | 2.3630 ± 0.02\textsuperscript{a} |
| Delingha | 2.3642 ± 0.01\textsuperscript{a} | 96.51 ± 1.80\textsuperscript{b} | 15.7 ± 0.1\textsuperscript{b} | 2.3642 ± 0.01\textsuperscript{a} |
| Nomuhong | 1.9646 ± 0.01\textsuperscript{a} | 91.77 ± 0.94\textsuperscript{b} | 14.3 ± 0.2\textsuperscript{b} | 1.9646 ± 0.01\textsuperscript{a} |

\textsuperscript{a}Data with the same superscript in the same column are not significantly different (P > 0.05).

\textsuperscript{b}DPPH, ABTS and FRAP content were expressed as μmol TE/g DW.

\textsuperscript{c}Los datos con el mismo superíndice en la misma fila no son significativamente diferentes (P > 0.05).

\textsuperscript{d}Los datos con el mismo superíndice en la misma fila no son significativamente diferentes (P > 0.05).
anthocyanins affects the biological effects. For example, the oxidation-reduction of anthocyanins is mainly depended on the number and position of hydroxyl groups on the aglycones and the extent of glycosylation of aglycones (Liobikas et al., 2016; Rice-Evans et al., 1996). Aglycones have been reported to display higher antioxidant power than their glycosides (Lee, Kim, Kim, Lee, & Lee, 2003), which is consistent with our findings in this study. Besides, petunidin (with O-dihydroxyl substitution), which was the most abundant anthocyanidin after gastrointestinal digestion and contributed for almost 95% of the total anthocyanins (Table 1), is considered as a more active antioxidant because it is more susceptible to oxidation as compared to other one hydroxyl substituent bonded anthocyanidins such as malvidin (Castaneda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Li et al., 2016). Host-related factors such as respiratory bursts of immune cells could produce reactive oxygen species to constantly attack the gastrointestinal tract (Halliwell, Rafter, & Jenner, 2005). It also has been reported that anthocyanins of Lycium ruthenicum Murray possess potent antioxidant activity, thus play a significant role in protecting redox equilibrium from harmful oxidants (Zheng et al., 2011). Thus, CLM, especially from Hedong, can be used as a functional food for preventing gastrointestinal tract diseases related to reactive oxygen species generation during digestion processes.

Figure 4. Fluorescence spectra of α-glucosidase in the absence (black line) and presence (colored lines) of CLM anthocyanins from the samples before (a) and after (b) in vitro gastrointestinal digestion. From top to bottom, the concentrations of CLM anthocyanins are 0.0, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/mL; Stern-Volmer plots for fluorescence quenching of α-glucosidase by CLM anthocyanins before (c) and after (d) in vitro gastrointestinal digestion. The equations for them were fitted according to Eq. (4).

Figura 4. Espectros de fluorescencia de α-glucosidasa en ausencia (línea negra) y presencia (líneas coloreadas) de antocianinas CLM de las muestras antes (a) y después (b) de la digestión gastrointestinal in vitro. De arriba a abajo, las concentraciones de antocianinas CLM son 0.0, 0.05, 0.1, 0.2, 0.4 y 0.8 mg/mL; diagramas de Stern-Volmer para la desactivación de la fluorescencia de la α-glucosidasa por las antocianinas CLM antes (c) y después (d) de la digestión gastrointestinal in vitro. Las ecuaciones para ello se ajustaron de acuerdo con la ecuación (4).
3.3. Effects of the digestion on α-glucosidase inhibition of CLM anthocyanins

Both samples of CLM anthocyanins (before and after in vitro gastrointestinal digestion) inhibited the activity of α-glucosidase in a concentration dependent manner. The respective IC\textsubscript{50} values are showing in Table 4. Notably, the inhibitory effects of CLM anthocyanins before and after in vitro gastrointestinal digestion on α-glucosidase followed the order of Hedong > Nomnhong > Delingha, which was in accordance with the results of antioxidant activity and might be due to higher contents of total anthocyanins (Sun et al., 2016). The inhibitory efficacy of CLM anthocyanins after in vitro gastrointestinal digestion was stronger than before as suggested by lower IC\textsubscript{50} value (Table 4) i.e. the elimination of glycospides from CLM anthocyanins by in vitro gastrointestinal digestion might increase the α-glucosidase inhibitory activity. Enzyme inhibition is reported to be commonly caused by inhibitor–enzyme binding or inhibitor–enzyme-substrate binding (Sun et al., 2017). The binding of glucosidase with the anthocyanins before and after in vitro gastrointestinal digestion was studied through fluorescence quenching in this study. As shown in Figure 4(a,b), both the anthocyanins quenched the fluorescence of glucosidase, indicating the respective binding interactions between them. Both the plots of F/F against anthocyanins concentration concave towards the y axis (Figure 4(c,d)), indicating that both the anthocyanins quenched the enzyme fluorescence through an apparent static or a sphere-of-action mechanism (Castanho & Prieto, 1998). The quenching constant, \(K_Q\)\textsubscript{FQ}, indicates the binding affinity of a quencher to an enzyme, and a higher \(K_Q\)\textsubscript{FQ} value suggests stronger affinity of a quencher to enzyme (Ferrer-Gallejo et al., 2012). The order of \(K_Q\)\textsubscript{FQ} values for the anthocyanins was consistent with that of the inhibition activities (Table 4), suggesting that CLM anthocyanins obtained from Hedong CLM might be more effective in inhibition of α-glucosidase than these from other samples. The \(K_Q\)\textsubscript{FQ} value was calculated as similar for CLM anthocyanins before and after in vitro gastrointestinal digestion in both the samples (Table 4). This suggests that there was no significant (\(P < .05\)) difference in the binding affinity to α-glucosidase between the anthocyanins before and after in vitro gastrointestinal digestion. Therefore, the increased enzyme inhibition by in vitro gastrointestinal digestion may result from the increasing formation of anthocyanins-α-glucosidase-pNPG (Sun et al., 2016). In addition, the anthocyanins after in vitro gastrointestinal digestion existed as deglycosylated form (Table 1); therefore, the similar values of \(K_Q\)\textsubscript{FQ} for the glycosylated and deglycosylated anthocyanins indicated that the glycol-ligands at third position in the A ring and/or fifth position in the C ring in CLM anthocyanins may not influence the binding with glucosidase significantly. It is worth noting that the constant substitutional group in the glycosylated and deglycosylated anthocyanins are the hydroxyl group at fourth and seventh positions. The similar binding affinity of the anthocyanins before and after in vitro gastrointestinal digestion to the enzyme may be attributed to the two hydroxyl ligands that have been reported to play an important role in binding interactions between flavonoids and enzyme protein (Lo Piparo et al., 2008). The enhanced inhibitory activity of CLM anthocyanins against α-glucosidase by in vitro gastrointestinal digestion indicates that the anthocyanins of CLM can be a promising α-glucosidase inhibitor or a food functional ingredient which deserve further in vivo animal studies or clinical trials.

4. Conclusion

This study investigated the anthocyanin profile in CLM before and after in vitro gastrointestinal digestion, as well as its effect on antioxidant activity and α-glucosidase inhibition. The results showed that most of the anthocyanins in CLM were found in glycosylation status and containing glycoside ligands. During in vitro gastrointestinal digestion, the glycosylated anthocyanins were broken by cutting the O-glycosidic bonds, leading to the formation of anthocyanidins. Besides, the antioxidant activity of CLM anthocyanins as assessed by the DPPH, ABTS and FRAP assays, and the α-glucosidase inhibition of CLM anthocyanins were enhanced after in vitro gastrointestinal digestion. These results indicated that CLM anthocyanins can be a potential antioxidant and a vital α-glucosidase inhibitor for further in vivo animal studies or clinical trials (such studies are in process in our laboratory).

Abbreviations

C/WLRM cultivated/wild Lycium ruthenicum Murray (the studied parts of the plant are the fruits)

DW dried weight

PCA principal component analysis

TE Trolox equivalents

TR retention times

Disclosure statement

The authors claim no conflict of interests in this work.

Acknowledgments

Z.-C. Wang thanks China Association for Science and Technology (CAST) and The University of Melbourne for scholarship support.

Funding

This work was financially supported by China Agriculture Research System (CARS-27), Fundamental Research Funds for Sate Central Universities [2017TS043], National Natural Science Foundation of China [21577001], Qinghai Province Basic Research Project [2017-ZJ-774 & 2019-ZJ-913], and Research Fund of Qinghai Normal University.

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