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Main anthocyanins compositions and corresponding H-ORAC assay for wild Lycium ruthenicum Murr. fruits from the Qaidam Basin

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Abstract

Lycium ruthenicum Murr. is a wild plant that is widely been used for juice consumption and medicinal purposes, primarily by individuals residing in the plant's natural growth regions. In this study, high-performance liquid chromatography-diode array detection (HPLC-DAD) and HPLC-electro-spray ionization-mass spectrometry (HPLC-ESI-MS) were used to investigate the composition of major anthocyanins in the fruit of *L. ruthenicum* Murr. (Qaidam Basin). Seven main anthocyanins were effectively identified and quantified, and we detected the rare anthocyanins that naturally present a coumaric acid in both *cis* and *trans* configurations. The content of anthocyanins was measured semi-quantitatively by HPLC. The concentration of Petunidin-3-*O*-rutinose (*trans*-*p*-coumaric acid)-5-*O*-glucose was the major compound (4.477 mg / 100 g), and the total anthocyanins content in the fruit was 5.4 mg / 100 g. The *L. ruthenicum* Murr. Hydrophilic Oxygen Radical Absorbance Capacity (H-ORAC) assay value was 4557 (μmol TE/g) comparable to that of the other fruits that have been reported previously. This study has contributed to the elucidation of the main anthocyanins composition from *L. ruthenicum* Murr. fruits. The results may prove useful in the further development and utilization of the fruit's natural pigment, both as a resource for food additives and for pharmaceuticals.

Keywords: Lycium ruthenicum Murr, HPLC–ESI-MS, anthocyanins, H-ORAC

Introduction

Lycium ruthenicum Murr. is a wild shrub found in northwestern China that is also known as "Mapam" in Tibetan medicine, where it is used in local medicinal practices. Its qualities of salt-resistance and drought-resistance allow it to grow widely in environments that frequently experience water shortage and poor soil conditions. The plant is highly enriched with sugars, flavones, and pigment, and has marked medicinal effects on anti-tumor, hypolipidemic, antioxidation, etc. The ripe fruits have been used to treat heart disease, urethral calculus, abnormal menstruation, etc. Because of its potent medicinal qualities, *L. ruthenicum* Murr. has been recorded as a traditional herb in the Tibetan medical classics, "Jing Zhu Ben Cao" and "Si Bu Yao Dian." The medical effects presented by *L. ruthenicum* Murr. are well known, but despite this, few scholars have systematically studied the chemical composition and antioxidation activities of the plant. Unless addressed, this research paucity will be an obstacle to further research and development of the products within *L. ruthenicum* Murr. However, until now, there have been no comprehensive studies conducted on the anthocyanins composition in the whole plant of *L. ruthenicum* Murr.

Anthocyanins belong to the widespread class of phenolic compounds called flavonids [1], and anthocyanins are considered the most important group of water-soluble pigments in plants. They are responsible for most blue, red, and related colours in flowers and fruits, and are usually connected with sugar moieties. In connection with anthocyanins, glucose, rhamnose, galactose, and arabinose are the most commonly encountered monosaccharides, while disaccharide such as sambubiose, rutinose, and sophorose are also present. Occasionally, the sugar moieties are acylated by organic acids, like acetic acid, oxalic acid, phenolic acids as *p*-coumaric acid, ferulic acid, etc. [2,3], and these acids greatly contribute to the stability of the anthocyanins structure. Until now, 600 kinds of anthocyanins have been found in nature [4,5]. Anthocyanins also possess antioxidants, and the hydrophilic oxygen radical absorbance capacity (H-ORAC) can be used to evaluate the antioxidant efficiency at protecting against radical-induced oxidation. For the measurement of antioxidant capacity, the H-ORAC assay is advantageous

because of its clinical relevance, repeatability, and reliability. Furthermore, it utilizes a more biologically relevant radical source than do DPPH, FRAP, and ABTS [6]. As a result, the H-ORAC assay has been used widely to determine the antioxidant activities of fruit juices, vegetables, and biological fluids [7-9].

In this study, we employed HPLC-DAD-ESI/MS, a well-known and efficient method for anthocyanins identification, to investigate the anthocyanins composition of *L. ruthenicum* Murr. Antioxidant activity of *L. ruthenicum* Murr. was determined using the H-ORAC assay. The objectives of the present study were to establish a reliable method for investigation of anthocyanins composition, and to evaluate the antioxidant activity of *L. ruthenicum* Murr.

Experimental

Plant materials

The fresh fruits of *L. ruthenicum* Murr. were randomly collected from Qaidam Basin (Latitude. 36° 2'N, Longitude. 98° 8'E, Altitude. 3000 m), Qinghai-Tibet Plateau, China. They were collected in August 2011, and identified by Mei, L.J., (Engineer, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xi Ning, China).

Chemicals

Trifluoroacetic acid was obtained from Merck (Hohenbrunn, Germany). Malvidin-3, 5-di-O-glucoside chloride (Mv_3G_5G) was acquired from Extrasynthese (Genay, France). Methanol and acetonitrile for HPLC and HPLC-ESI-MS analysis were of chromatographic grade and purchased from Alltech Scientific (Beijing, China). Trolox (6-hydroxy-2, 5, 8-tetramethylchroman-2-carboxylic acid), fluorescein disodium salt, and oxidase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction of anthocyanins

The anthocyanins extraction method was modified from the method used by Zhang J. *et al*., [10]. In brief, we obtained 50g juice from fresh fruits, and then dissolved the juice in beakers with 10 ml methanol containing 2% formic acid and 0.1% Trifluoroacetic. The sample was then twice extracted for 20 min at 40 kHz, 750 W with an ultrasonic extraction device. After centrifugation, suspensions was isolated from fruit residus. The extraction liquids were finally settled to 10 ml with methanol and stored at -20μm for purification.

Purification of anthocyanins

The anthocyanins purification method was modified from the method used by Petko, D. *et al*., [11] In brief, the methanol extraction was first loaded into an SPE C_{18}-column (Phenomenex, Denver, USA). The sugars, formic acid, and other interfering substances were subsequently removed using pure water. Anthocyanins were then eluted with ethanol that contained 2% formic acid and 0.1% Trifluoroacetic acid, and then concentrated via rotary evaporation (EYELA, Tokyo, Japan) at 40μm. Dry extracts were then dissolved in ethanol containing 2% formic acid and 0.1% Trifluoroacetic acid, and passed through a 0.45 μm membrane filter (ANPEL, Shanghai, China) for HPLC analysis.

HPLC analysis

The Agilent 1200 system (Palo Alto, CA, USA) consisted of four G1311A pumps; a G1316A column temperature box; a G1329A auto-sampler; and a G1315A detector. Agilent 1200 Technologies Chemstation software was used for analysis. The analytical column was a C_{18} of ODS 80TS QA (150 mm × 4.6 mm, 5 μm, Tosoh, Tokyo, Japan). Chromatograms were obtained at 525 nm for anthocyanins, and photodiode array spectra were recorded from 200 nm to 800 nm.

The mobile phase consisted of Solvent A: 2% formic acid and 0.1% TFA in water, and Solvent B: 100% acetonitrile. The applied elution conditions were a gradient program for anthocyanins analysis: 0-30 min, 5%-15% B; 30-80 min, 15%-20% B; 80 min-100 min, 20%-5% B. The column temperature was 35μm, flow rate was 0.8 ml/min, and the detection wavelength was 525 nm.

HPLC-DAD-ESI-MS

The mass spectrometry system was an Agilent-1200 HPLC system coupled with a UV detector and ion trap mass detector (Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation conditions were the same as those described above. MS conditions were as follows: positive ion mode; gas (N_2) temperature, 350 °C; flow rate, 8 L/min; nebulizer pressure, 35 psi; HV voltage, 4 KV; octopole RF amplitude, 150 Vpp; skim 1 voltage, 47.7 V; skim 2 voltage, 6.0 V; capillary exit, 127.3 V; cap exit offset, 79.6 V; and scan range, m/z 0-1200.

Hydrophilic Oxygen Radical Absorbance Capacity (H-ORAC) assay

The H-ORAC assay method was modified based on methods described in previously published papers [12]. It was carried out on a microplate fluorescence reader, and data were expressed as micromole Trolox equivalents per gram weight (μmol TE/g).

Results and discussion

HPLC analysis

The HPLC profiles of the sample are presented in Figure 1. The anthocyanins in the sample were well separated under the established HPLC conditions. The chromatogram at 525 nm demonstrated the most abundant component. The results showed that a mobile phase composed of 2% formic acid, 0.1% TFA in water, and acetonitrile, was suitable for the sample separation.

Anthocyanins in each sample were measured semi-quantitatively by linear regression of Mv_3G_5G (0.00-0.10 mg/ml), and the content was expressed as milligrams of Mv_3G_5G equivalents per 100 g sample. The result showed

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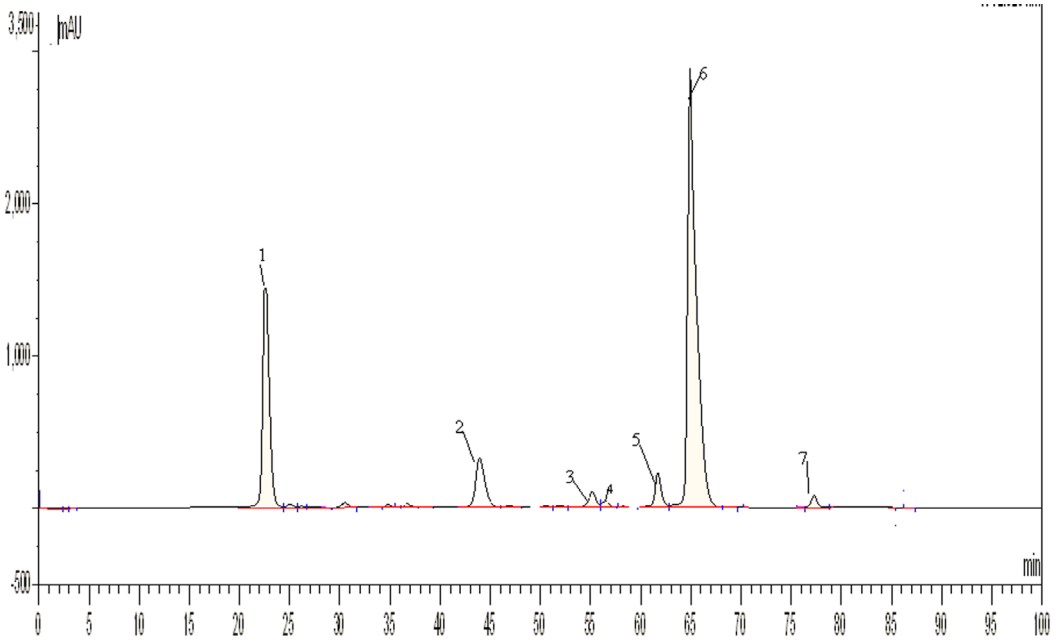

Figure1. The HPLC chromatographic profile of Lycium ruthenicum Murr. The mobile phase consisted of Solvent A: 2% formic acid and 0.1% TFA in water, and Solvent B: 100% acetonitrile. The applied elution conditions were a gradient program for anthocyanins analysis: 0-30 min, 5%-15% B; 30-80 min, 15%-20% B; 80 min-100 min, 20%-5% B. Thecolumn temperature was 35^{0}c, flow rate was 0.8 ml/min, and the detection wavelength was 525 nm.

that the most abundant compound was Petunidin-3-O-rutinose (*trans*-p-coumaric acid)-5-O-glucose (4.477 mg / 100 g), and the total anthocyanins content in the fruit amounted to 5.4 mg / 100 g.

Anthocyanins identification

Sample analyzed by HPLC/ESI-MS/MS are summarized in **Table 1** (retention time in the HPLC system, λ_{max} in the visible region, molecular ion and main fragments observed in MS/MS).

In this study, we applied a gradient program to guarantee the maximum separation of anthocyanins. Individual anthocyanins were identified mainly by retention time, elution order, UV/Vis spectrums, and comparison of MS spectra to previously reported data. Peak 1 was an anthocyanins of high polarity as shown by its short retention time (22.542 min), M^+=m/z 641; MS/MS=m/z 479/317. Peak 2 shared the same fragment, but had a different retention time (45.358 min). Their similar mass spectrums suggested that both were petundin glycosides. As a result of the elution order, the peak 1 compound had a higher polarity than that of peak 2. As reported, the most commonly found sugars in anthocyanins are galactose and glucose, while Abad-García concluded that for glycosylated polyphenols, O-galactoside structures eluted before O-glucoside structures did [13]. Further, if the anthocyanins contained two hexoses, then the two hexoses would likely link to different positions on the aglycone, most likely at the 3- and 5-position [14]. In light of the preceding studies and MS data, peak 1 was tentatively identified as petundin-3-O-galactoside-5-O-glucoside, and peak 2 was tentatively identified as petundin-3-O-glucoside-5-O-glucoside.

Besides glycosylated groups, acylated groups constitute another commonly found form of anthocyanins. Coumaric acid (146 Da) was the major acylated group observed in *L. ruthenicum* Murr. In addition to coumaric acid, three other organic acids were detected in anthocyanins, including caffeic acid (162 Da), malic acid (116 Da), and ferulic acid (176 Da).

Peaks 3, 5, 6, and 7 could be identified as coumaric acid acylated anthocyanins by observing their MS spectra. Two pairs of isomers were detected, which differed only in their coumaric acid configuration (*cis* and *trans*): peak 3 and peaks 5 and 6. Peak 3 had molecular ions M^+: 919 m/z, with the aglycon ion (303 m/z) indicating that it was delphinidin derivatives. The acylated group and the fragmentation pattern (MS/MS = m/z 757/627/465/303) indicated that delphinidin was attached with two hexoses and one pentose. Previous research had demonstrated that the *cis*-p-coumaroyl derivatives had a higher polarity than did the *trans* configuration, and *cis*-p-coumaroyl derivatives eluted earlier [15-16]. By comparing the present MS data with

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Table 1. Anthocyanins identified in the extract of *Lycium ruthenicum* Murr.

Peak no.	Category	t_R (min)	UV λ_{max} (nm)	M^+ (m/z)	MS/MS (m/z)
1	Petundin-3-O-galactoside-5-O-glucoside	22.542	525.0	641	479/317
2	Petundin-3-O-glucoside-5-O-glucoside	45.358	275.70 533.64	641	479/317
3	Delphinidin-3-O-rutinose (trans/cis-p-coumaric acid)-5-O-glucose	54.392	280.10 529.40	919	757/627/465/303
4	Petunidin-3-O-rutinose(caffeic acid)-5-O-glucoside	55.583	279.50 529.70	949	787/641/479/317
5	Petunidin-3-O-rutinose (cis-p-coumaric acid) -5-O-glucose	61.275	278.00 533.80	933	771/641/479/317
6	Petunidin-3-O-rutinose (trans-p-coumaric acid)-5-O-glucose	64.625	280.50 531.50	933	771/641/479/317
7	Malvidin-3-O-rutinose (cis-p-coumaric acid)-5-O-glucoside	76.983	281.31 532.24	947	785/493/331

that in previous reports [17], we tentatively identified peak 3 as delphinidin-3-O-rutinoside (*trans/cis*-p-coumaroyl)-5-O-glucoside. For peaks 5 and 6, the molecular ion M^+: 933 mass agreed well with the masses calculated for $C_{43}H_{49}O_{23}$ (933.266). The high molecular weight and long retention time clearly indicated that both anthocyanins were acylated anthocyanins. Accordingly, peaks 5 and 6 were identified as petunidin-3-O-rutinoside (*cis*-p-coumaroyl)-5-O-glucose, and petunidin-3-O-rutinoside (*trans*-p-coumaroyl)-5-O-glucose. For peak 7, we detected 331 m/z, which indicated that it was malvidin derivatives. Further, its fragmentation pattern was similar to those of the four anthocyanins above, and so peak 7 was tentatively identified as malvidin-3-O-rutinoside (*cis*-p-coumaroyl)-5-O-glucoside.

Acylation would decrease the polarity of anthocyanins and extend their HPLC retention time. In light of retention time, elution order, and MS data, peak 4 could be identified as acylated anthocyanins. The molecular ion fragmented to four production ions MS/MS = m/z 787/641/479/317. By comparing the MS data with those reported by Wu & Prior [18], peak 4 was identified as petunidin-3-O-rutinoside (caffeic acid)-5-O-glucoside.

Several anthocyanins compounds could not be analyzed because their contents were prohibitively small. Besides, seven main compounds in *L. ruthenicum* Murr. were identified in the present study.

H-ORAC of sample

The activity of *L. ruthenicum* Murr. was 4557 (μmol TE/g). Most fruits with high antioxidant capacities appear to be intense or dark in colour. Fruit antioxidant content could be affected by the cultivar, and maturity level, as well as growing conditions, i.e. location, soil state, climate, and agricultural practices [19]. *L. ruthenicum* Murr. possesses special physiological characteristics: drought-resistance, salt-resistance, and anti-ultraviolet properties. Therefore, it has many anti-oxidant elements, and more in-depth research must be conducted in this area.

Conclusions

The present study elucidated the main chemical compositions of wild *L. ruthenicum* Murr., as well as their corresponding H-ORAC values. Considering that *L. ruthenicum* Murr. fruit contains an abundance of anthocyanins with extremely high H-ORAC values, the fruit could be regarded as a natural source of pigments and food additives. In this study, only the main anthocyanins compositions and H-ORAC values were investigated. For further understanding of the plant's biological effects, more in-depth research must be conducted.

Competing interests

The authors declare that they have no competing interests

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