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Anthocyanins composition and antioxidant activity of wild *Lycium ruthenicum* Murr. from Qinghai-Tibet Plateau

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ABSTRACT

Lycium ruthenicum Murr. is a nutritional food, and has widely been used for treatment of heart disease, abnormal menstruation and menopause among folks. In present study, the anthocyanins composition and content of *L. ruthenicum* from three different area of Qinghai-Tibet Plateau have been investigated by high-performance liquid chromatography with diode array detector (HPLC–DAD) and HPLC–electrospray ionisation-mass spectrometry (HPLC–ESI-MS). Totally, fourteen anthocyanins have been detected, ten of which were identified and quantified. All of them were first reported in *L. ruthenicum*. Results showed that petunidin derivatives accounted for 95% of the total anthocyanins in fresh fruit. Furthermore, most of the anthocyanins were acylated by coumaric acid, and the rare anthocyanins that naturally presented a coumaric acid in both *cis* and *trans* configurations have been detected in our study. For antioxidant activity, their methanol extracts showed potent antioxidant activity in terms of DPPH·, $ABTS^{\cdot+}$ and ferric reducing antioxidant power (FRAP) assays. The results are valuable for elucidating anthocyanins composition of *L. ruthenicum* fruits and for further utilising them as healthy food and natural pigment resource.

1. Introduction

Lycium ruthenicum Murr. is a unique nutritional food, which widely distributes in salinized desert of Qinghai-Tibet Plateau. Its special physiological characteristics of drought-resistance and salt-resistance make it an ideal plant for preventing soil desertification and alleviating the degree of soil salinity–alkalinity, which are very important for the ecosystem and agriculture in the remote area. In addition to that, *L. ruthenicum* has been recorded in Tibetan medical classic "Jing Zhu Ben Cao" as a traditional herb. Its ripe fruits had been used for treatment of heart disease, abnormal menstruation and menopause. To our best knowledge, although the beneficial effects presented by *L. ruthenicum* were obvious, no scholars have systematically studied its chemical composition. This has largely restricted the further research and development of *L. ruthenicum*. Previous report indicated that *L. ruthenicum* processed abundant anthocyanins (Li, Zhao, Yuan, Zhu, & Shi, 2006), and its pleasing colour (dark purple) has aroused scholars to investigate it. But up to now most of the researches were focusing on the extraction method and total anthocyanins content measurement, no comprehensive study has been conducted to explore the anthocyanins composition of *L. ruthenicum*.

Anthocyanins belong to flavonoids, and widely spread in flowers, fruits and vegetables. They are responsible for the brilliant plant colour (red, blue, and purple). Naturally occurring anthocyanins are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Castaneda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). Anthocyanins molecule usually linked with sugar moieties. Glucose, galactose, rhamnose and arabinose are the monosaccharide most commonly encountered, disaccharides such as rutinose, sophorose, sambubiose also occur (Clifford, 2000). Even trisaccharides have been reported (Cabrita, Frystein & Andersen, 2000). Sometimes, the sugar moieties are acylated by organic acid such as acetic acid, oxalic acid, propionic acid, or phenolic acids as *p*-coumaric acid or ferulic acid etc. (Cuyckens & Claeys, 2004; Wu & Prior, 2005), which greatly help to stabilize the anthocyanins structure. So far, nearly 600 kinds of anthocyanins have been found in nature (Huang, Wang, Williams, & Pace, 2009). The great structure diversities endow anthocyanins with wide range of biological and physiological activities such as antioxidant activity, anticardiovascular disease and antitumor effect (Ichikawa et al., 2001; Lamy et al., 2006;

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Rahman, Ichiyanagi, Komiyama, Sato, & Konishi, 2008; Wang, Cao, & Prior, 1997). The likely mechanism is postulated as that anthocyanins act as potent antioxidants by donating hydrogen atoms to highly reactive free radicals, breaking the free radical chain reaction (Rice-Evans, Miller, & Paganga, 1996). Due to the beneficial effects of anthocyanins, dietitians suggested that add certain amount of fruits and vegetables to daily diet will be beneficial for people's health.

HPLC–ESI-MS, a major efficient method for anthocyanins identification, was employed to investigate the anthocyanins composition of *L. ruthenicum* in present study. Multiple antioxidant assays would be necessary to systematically evaluate the antioxidant activity. For antioxidant activity evaluation, the 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$) and radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) and FRAP were employed. Generally, the objectives of our research are to establish a reliable method to investigate the anthocyanins composition, and evaluate the antioxidant activity of *L. ruthenicum* extracts.

2. Materials and methods

2.1. Plant materials

Fresh fruits of *L. ruthenicum* were randomly sampled from three places, including Delingha (Latitude. 37°13′N, Longitude. 97°14′E, Altitude. 2980 m), Gomud (Latitude. 36°25′N, Longitude. 94°53′E, Altitude. 2800 m) and Dulan (Latitude. 36°2′N, Longitude. 98°8′E, Altitude. 3000 m). The fruits were ripe and hand-picked, then stored in heat preservation box with efficient ice bag right after collected. The samples were preserved in −20 °C for later analysis in laboratory.

2.2. Chemicals

Malvidin-3,5-di-*O*-glucoside chloride (Mv3G5G) was purchased from Extrasynthese (Genay, France). Gallic acid (GA) was purchased from Must Bio-Technological Co., Ltd. (Chengdu, Sichuan, China). Trifluoroacetic acid was purchased from Merck (Hohenbrunn, Germany). $DPPH^{\cdot}$, 2,2′-azinobis-(3-etlhylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripyridyl-S-triazine (TPTZ) and Folin–Ciocalteu's phenol reagent were purchased from Sigma–Aldrich (St. Louis, MO). Methanol and acetonitrile for HPLC–DAD and HPLC–ESI-MS analysis were of chromatographic grade and purchased from Alltech Scientific (Beijing, China). Methanol and formic acid were of analytical grade and purchased from Beijing Chemical Works (Beijing, China). HPLC-grade water was prepared by Milli-Q System (Millipore, Billerica, MA, USA).

2.3. Extraction of anthocyanins

The anthocyanins extraction method was modified from the method used by Zhang et al. (2007) with slight modification. Eight grams fresh fruit of each sample (Delingha, Gomud and Dulan) was extracted triply with 15 mL methanol (2% formic acid). The procedure was conducted in 50 mL conical beaker with its orifice sealed by parafilm at room temperature in the dark for 24 h. Then the suspensions were combined and filtered through sheet of qualitative filter paper (Hangzhou Special Paper Industry, Zhejiang, China) to remove the fruit residues, protein and polysaccharide sediment. The filtrate was further passed through 0.22 μm reinforced nylon membrane filter (Shanghai ANPEL, Shanghai, China) for HPLC analysis. For Folin–Ciocalteu and antioxidant activity assay, 2 mL of filtrate was evaporated at 30 °C, and the residue was dissolved in 2 mL of water and then purified by solid-phase extraction cartridge (SPE), C_{18} Supelclean ENVI-18 cartridge (Supelco park, Bellefonte, PA, 500 mg, 3 mL), which had been previously activated by methanol and water. The cartridge was successively rinsed with water (to remove sugars, formic acid, and other interfering substances) and methanol (to elute the polyphenolic fraction). The methanolic eluate was concentrated at 30 °C, and the residue was redissolved in 2 mL of methanol for Folin–Ciocalteu and antioxidant activity assay.

2.4. HPLC–DAD analysis

The samples were analysed by Dionex HPLC system (Sunnyvale, CA, USA), equipped with a P680 HPLC pump, an UltiMate 3000 autosampler, a TCC-100 thermostated column compartment and a Dionex PDA100 photodiode array detector. The analytical column was C_{18} column of ODS 80Ts QA (150 × 4.6 mm, 5 μm i.d.,Tosoh, Tokyo, Japan) protected with a C_{18} guard cartridge (4.6 × 10 mm, 5 μm Kromasil C_{18}). An aliquot of 10 μl solution was injected. Chromatograms were obtained at 525 nm for anthocyanins, and photodiode array spectra was recorded from 200 to 800 nm.

Gradient program was applied for anthocyanins analysis. The eluents were: A, 10% aqueous formic acid with 0.1% TFA; B, 15% methanol in acetonitrile. The applied elution conditions were: 0–30 min, linear gradient from 3% to 11.5% B; 30–40 min, 11.5% B isocratic; 40–60 min, linear gradient from 11.5% to 15.5% B; 60–70 min, linear gradient from 15.5% to 16% B; 70–80 min, linear gradient from 16% to 23% B; 80–100 min, linear gradient from 23% to 3% B. The flow rate was 0.8 mL/min, and temperature 35 °C.

2.5. HPLC–DAD–ESI-MS

Mass spectrometry system was Agilent-1100 HPLC system coupled with a UV detector and ion trap mass detector (Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation condition was the same with the HPLC/DAD analysis mentioned above. The MS conditions were listed as follow: 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.

2.6. Quantitative analysis of individual anthocyanin

Mv3G5G (with the range from 0.01 to 0.80 mg mL^{-1}) was applied as the standard compound to semi-quantify the anthocyanins, and the calibration curve was Y (peak area) = 504.06 × X (Mv3G5G equivalents content) + 0.2275 (r = 0.9999). The individual anthocyanin was expressed as milligrams of Mv3G5G equivalents per 100 g of fresh weight (FW). All of the samples were analysed in triplication.

2.7. Estimation of the total polyphenol content

Total polyphenol content (TPC) in the methanol extract was determined according to the Folin–Ciocalteu method (Mansouri, Embarek, Kokkalou, & Kefalas, 2005). GA was used as standard compound. The TPC was calculated from the calibration curve Y (absorbance) = 4.3781 × X (GA equivalents content) + 0.0523 (r = 0.9994), and the result was expressed as GA mg equivalents per 100 g FW.

2.8. DPPH assay

The DPPH assay was performed according to method of Li et al. (2009) with slight modification. GA was used as the reference compound. The scavenging percentage of $DPPH^{\cdot}$ in each sample was