



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^{•+} 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.

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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, Ichianagi, 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[•]) and radical monocation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS^{•+}) 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[•], 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripiryridyl-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), C₁₈ 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 C₁₈ column of ODS 80Ts QA (150 × 4.6 mm, 5 µm i.d., Tosoh, Tokyo, Japan) protected with a C₁₈ guard cartridge (4.6 × 10 mm, 5 µm Kromasil C₁₈). 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 (N₂) 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[•] in each sample was

calculated according to the formula: $\text{absorbance}_{(\lambda=515\text{ nm})t} - \text{absorbance}_{(\lambda=515\text{ nm})0} / \text{absorbance}_{(\lambda=515\text{ nm})0}$. Where t was the time the reaction reaching the plateau stage, and 0 represents the beginning of the reaction. The scavenging ratio was plotted against GA concentration, and the amount of GA equivalents in each sample was calculated by the calibration curve Y (scavenging ratio) = $0.3786 \times X$ (GA equivalents content) + 0.1632 ($r = 0.9991$). All of the samples were analysed in triplication.

2.9. ABTS assay

Previous literature was taken as reference (Re et al., 1999). ABTS⁺ reagent was produced by reacting 10 mL of 7 mM ABTS solution with 178 μ l of 140 mM potassium persulfate aqueous in the dark at room temperature for 13 h before use. The ABTS⁺ solution was diluted to appropriate absorbance. One μ l sample was added to 799 μ l diluted ABTS⁺ solution to react in the dark at room temperature for 10 min, then the absorbance at 732 nm was recorded. GA was used as standard compound with the final concentration ranging from 0.25 to 2 mM. And the calibration curve was Y (scavenging ratio) = $0.4635 \times X$ (GA content) + 0.0075 ($r = 0.9973$). Results were expressed as GA equivalents antioxidant activity (GA equivalents mg per 100 g of FW). All of the samples were analysed in triplication.

2.10. FRAP assay

The FRAP assay was based on the method of Benzie and Strain (1996). Reagents included 300 mM acetate buffer (pH 3.6); 10 mM TPTZ in 40 mM hydrochloric acid; 20 mM FeCl₃·6H₂O. The FRAP reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL FeCl₃·6H₂O solution. 10 μ l sample was added into 790 μ l FRAP solution, the reaction was maintained for 20 min when the absorbance reading was constant at 593 nm. GA was introduced as standard with the final concentration ranging from 0.25 to 2 mM, and the calibration curve was Y (absorbance) = $0.0352 \times X$ (GA content) + 0.1376 ($r = 0.9989$). The results were expressed as GA equivalents reducing activity (GA mg equivalents per 100 g FW). All of the samples were analysed in triplication.

2.11. Statistical analysis

One-way analysis of variance test (ANOVA) and bivariate correlation analysis were performed by SPSS 16.0. Post hoc multiple comparisons were executed with LSD method in ANOVA analysis. The differences were considered to be significant when $p < 0.05$.

3. Results and discussion

3.1. HPLC analysis

The RP-HPLC method provided repeatable and good separation for all three samples (A stands for sample from Delingha; B stands for sample from Gomud and C stands for sample from Dulan) (Fig. 1). In present study, thirteen peaks have been detected in each sample at 525 nm. Based on the chromatograms, the anthocyanins fingerprints of three samples were similar. Moreover the anthocyanins composition patterns were same between *L. ruthenicum* from different place. According to the previous literatures, anthocyanins have been successfully used as biochemical marker in taxonomical study (OrtegaRegules, RomeroCascales, LopezRoca, RosGarcia, & GomezPlaza, 2006; Ryan & Revilla, 2003). And with the unique anthocyanins fingerprint we could possibly distinguish *L. ruthenicum* from other species.

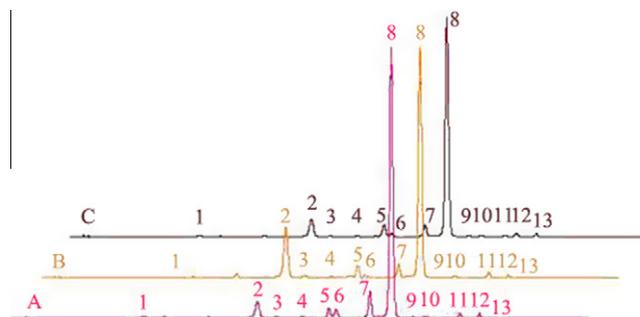


Fig. 1. Comparison of HPLC chromatograms at 525 nm of three different samples. A stands for sample from Delingha, B stands for sample from Gomud and C stands for sample from Dulan.

3.2. Anthocyanins identification

The detailed anthocyanin chromatographic profile at 525 nm obtained from reversed-phase HPLC was displayed in Fig. 2. Five major anthocyanins (peak 2, 5, 6, 7 and 8) accounted for 95% of the total peak area. Although thirteen peaks are visible, MS revealed that there were fourteen anthocyanins. Peak 2 corresponded to two different coeluted anthocyanins. The coelution phenomenon was common in anthocyanins study. Coeluted anthocyanins were structurally different but with similar polarity, which lead them elute at the same time and form only one peak. It is very difficult to check the peak purity only with DAD detector. Fortunately, however, we can examine the peak purity simultaneously with online MS. If no HPLC-MS is available, different eluents or gradient programs would be an ideal method to distinguish the coelution phenomenon. Coelution phenomenon should be avoided because it could complicate the mass spectrum, and make accurate quantification impossible. In this study gradient program was applied to guarantee the maximally separation of anthocyanins. The individual anthocyanin was identified mainly by retention time, elution order, UV/Vis spectrum, and comparison of MS spectra to previously reported data.

The determination of the aglycone ion by ESI-MS showed that only three aglycones, namely, delphinidin (m/z 303), petunidin (m/z 317), malvidin (m/z 331), have been found in *L. ruthenicum*. Among them petunidin was the most abundant aglycone, its derivatives accounted almost for 95% of the total anthocyanins. The detailed MS data of all anthocyanins are summarised in Table 1.

According to previous reports, most of the anthocyanins were linked with sugars. Wu, Prior, & USDA (2005) suggested that the hydroxyl and sugar moiety would increase the anthocyanin polarity and make it elutes early in reverse C₁₈ column. Deducing from the principle, peak 1 was an anthocyanin of highly polarity in terms of its short retention time (22.542 min). But except for the characteristic petunidin aglycone ion (m/z 317), no other fragments have been detected. Anthocyanins rarely exist without sugar moiety. So peak 1 maybe the hydrolysate of other petunidin glycosides derivatives. Finally, as a result of limited information, the exact structure of peak 1 could not be identified yet.

Peak 2 was a mixture, which was consisted of compound 1 ($M^+ = m/z$ 641; MS/MS = m/z 479/317) and compound 2 ($M^+ = m/z$ 633; MS/MS = m/z 465/303). Compound 1 shared the same fragment pattern with peak 3, but with different retention time (41.983 and 45.358 min, respectively). The similar mass spectrums suggested that both of them were petunidin glycosides. Taken the elution order into consideration, compound 1 had higher polarity than peak 3, since the elution order was based upon polarity on reversed phase column. As reported, the most common sugars were galactose and glucose in anthocyanins. Meanwhile, Abad-García,

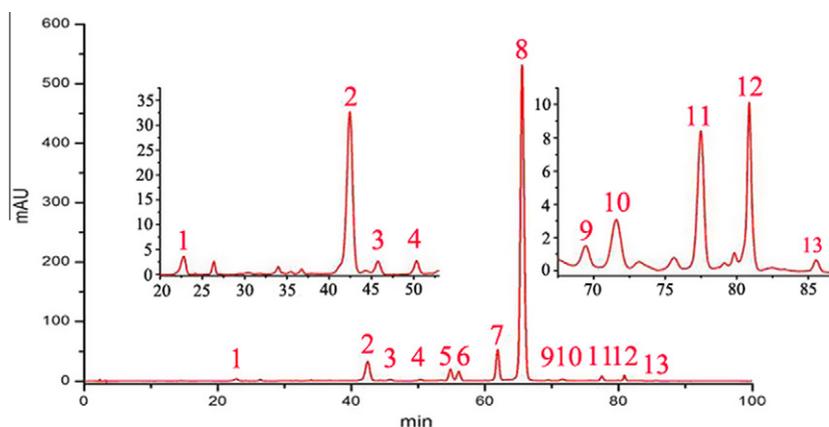


Fig. 2. The detailed chromatogram of sample A at 525 nm, the partially enlarged chromatograms of 20.00–55.00 min and 65.00–90.00 min were displayed in the insert figures. Peak 1, unknown; peak 2, petundin-3-*O*-galactoside-5-*O*-glucoside and a unknown compound; peak 3, petundin-3-*O*-glucoside-5-*O*-glucoside; peak 4, delphinidin-3-*O*-rutinoside (*cis-p*-coumaroyl)-5-*O*-glucoside; peak 5, delphinidin-3-*O*-rutinoside (*trans-p*-coumaroyl)-5-*O*-glucoside; peak 6, petundin-3-*O*-rutinoside (caffeoyl)-5-*O*-glucoside; peak 7, petundin-3-*O*-rutinoside (*cis-p*-coumaroyl)-5-*O*-glucoside; peak 8, petundin-3-*O*-rutinoside (*trans-p*-coumaroyl acid)-5-*O*-glucoside; peak 9, petundin-3-*O*-glucoside (maloyl)-5-*O*-glucoside; peak 10, petundin-3-*O*-rutinoside (feruloyl)-5-*O*-glucoside; peak 11, malvidin-3-*O*-rutinoside (*cis-p*-coumaroyl)-5-*O*-glucoside; peak 12, unknown; peak 13, unknown.

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	Unknown	22.542	281.31 524.90	317	–
2	Petundin-3- <i>O</i> -galactoside-5- <i>O</i> -glucoside	41.933	–	641	479/317
	Unknown	41.933	–	633	465/303
3	Petundin-3- <i>O</i> -glucoside-5- <i>O</i> -glucoside	45.358	275.70 533.64	641	479/317
4	Delphinidin-3- <i>O</i> -rutinoside (<i>cis-p</i> -coumaroyl)-5- <i>O</i> -glucoside	49.942	275.70 530.84	919	757/627/465/303
5	Delphinidin-3- <i>O</i> -rutinoside (<i>trans-p</i> -coumaroyl)-5- <i>O</i> -glucoside	54.392	280.10 529.40	919	757/627/465/303
6	Petundin-3- <i>O</i> -rutinoside (caffeoyl)-5- <i>O</i> -glucoside	55.583	279.50 529.70	949	787/641/479/317
7	Pentundin-3- <i>O</i> -rutinoside (<i>cis-p</i> -coumaroyl)-5- <i>O</i> -glucoside	61.275	278.00 533.80	933	771/641/479/317
8	Pentundin-3- <i>O</i> -rutinoside (<i>trans-p</i> -coumaroyl)-5- <i>O</i> -glucoside	64.625	280.50 531.50	933	771/641/479/317
9	Pentundin-3- <i>O</i> -glucoside (maloyl)-5- <i>O</i> -glucoside	63.633	281.21 532.24	757	641/479/317
10	Pentundin-3- <i>O</i> -glucoside (feruloyl)-5- <i>O</i> -glucoside	70.792	277.10 532.24	963	787/641/479/317
11	Malvidin-3- <i>O</i> -rutinoside (<i>cis-p</i> -coumaroyl)-5- <i>O</i> -glucoside	76.933	281.31 532.24	947	785/493/331
12	Unknown	80.503	282.71 535.05	977	771/479/317
13	Unknown	85.100	257.48 534.82	917	641/479/317

Berrueta, Garmón-Lobato, Gallo, and Vicente (2009) concluded that for glycosylated polyphenols, *O*-galactoside structure elutes before *O*-glucoside structure. And if the anthocyanin contains two hexoses, the two hexoses would likely link to different positions of the aglycone, most likely at the 3- and 5-position, according to the study of Wu et al. (2005). Considering the preceding studies and MS data, compound **1** was tentatively identified as petundin-3-*O*-galactoside-5-*O*-glucoside, and peak 3 was tentatively identified as petundin-3-*O*-glucoside-5-*O*-glucoside. Compound **2** was delphinidin derivative, and the fragment ion of m/z 465 indicated it linked with a hexose. But as to the unusual fragment ion of m/z 633, limit information about it has been reported. Consequently, the exact chemical structure of compound **2** could not be determined.

Acylation was another common existing form of anthocyanins besides glycosylation. In the case, coumaric acid (146 Da) was

the major acylated group in *L. ruthenicum*. Meanwhile, in addition to coumaric acid, three other organic acids have been detected in anthocyanins, involving caffeic acid (162 Da), malic acid (116 Da) and ferulic acid (176 Da).

Peaks 4, 5, 7, 8 and 11 were identified as coumaric acid acylated anthocyanins by MS spectra. Two pairs of isomers, with the only difference of coumaric acid configuration (*cis* and *trans*), have been detected (peaks 4 and 5; peaks 7 and 8). Peaks 4 and 5 had the same molecular ions $[M]^+$: 919 m/z . The aglycon ion (303 m/z) indicated that both of them were delphinidin derivatives. In addition to the acylated group, the fragmentation pattern (MS/MS = m/z 919/757/627/465/303) indicated that delphinidin was attached with two hexose and one pentose. While, as for the elution order, previous researches have concluded that the *cis-p*-coumaroyl derivatives had higher polarity than its *trans* configuration, and eluted earlier (Downey & Rochfort, 2008; George, Figueiredo, Toki, Tatsuzawa,

Saito, & Brouillard, 2001). Compared the MS data with the previous reports (Giusti, Rodríguez-Saona, Griffin, & Wrolstad, 1999), peaks 4 and 5 were tentatively identified as delphinidin-3-*O*-rutinoside (*cis-p*-coumaroyl)-5-*O*-glucoside, delphinidin-3-*O*-rutinoside (*trans-p*-coumaroyl)-5-*O*-glucoside. For peaks 7 and 8, the molecular ion $[M]^+$: 933 was in good agreement 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. And by the guideline used above, peaks 7 and 8 were identified as petunidin-3-*O*-rutinoside (*cis-p*-coumaroyl)-5-*O*-glucoside and petunidin-3-*O*-rutinoside (*trans-p*-coumaroyl)-5-*O*-glucoside. For peak 11, 331 m/z was detected, which indicated it was malvidin derivatives. Its fragmentation pattern was similar with the four anthocyanins above. So peak 11 was tentatively identified as malvidin-3-*O*-rutinoside (*cis-p*-coumaroyl)-5-*O*-glucoside.

Acylation would decrease the polarity of anthocyanins, and extend their retention time in HPLC. In view of the retention time, elution order and MS data, peaks 6, 9 and 10 could be identified as acylated anthocyanins. Peak 6 ($M^+ = m/z$ 949) and 10 ($M^+ = m/z$ 963) shared the similar fragmentation pattern: the molecular ion fragmented to four same production ions MS/MS = m/z 787/641/479/317. The only difference between the two compounds was that they were attached with different organic acid. By comparing the MS data and elution order with those reported by Wu & Prior (2005), peaks 6 and 10 were identified as petunidin-3-*O*-rutinoside (caffeoyl)-5-*O*-glucoside and petunidin-3-*O*-rutinoside (feruloyl)-5-*O*-glucoside. Peak 9 had a smaller molecular ion ($M^+ = m/z$ 757) compared with other acylated anthocyanins. MS/MS information (m/z 641/479/317) indicated that only two sugar moieties linked to the aglycone, which causing its molecular mass lower than other anthocyanins contained three sugars. The acylated group was identified as malic acid according to the neutral molecular loss (116 Da). Finally, peak 9 was identified as petundin-3-*O*-glucoside (maloyl)-5-*O*-glucoside.

Two anthocyanins of low polarity, peaks 12 ($M^+ = m/z$ 977) and 13 ($M^+ = m/z$ 917) eluted lastly (respectively 80.508 and 85.100 min). Their fragmentation patterns (Table 1) and retention time indicated that both of them were acylated anthocyanins. But, due to lack of relative reports and standard compounds for comparing, the two anthocyanins have not been identified in present study.

3.3. Composition and content of anthocyanins

The composition and quantitative analysis of major anthocyanins are presented in Fig. 3. It was notable that peak 8

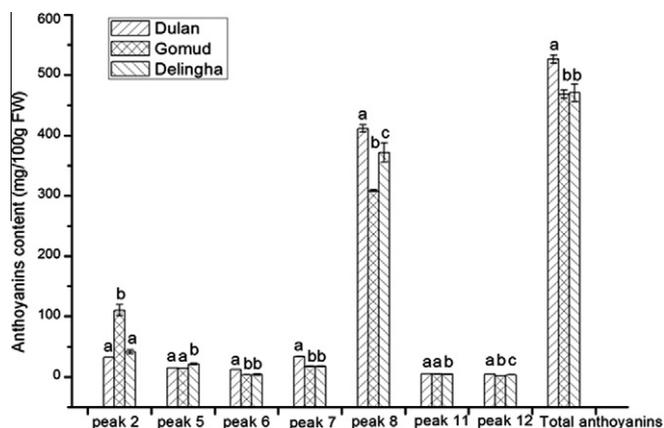


Fig. 3. Major anthocyanins content (mg/100 g FW) in different samples (mean \pm SD, $n = 3$). Bars with different letters in common are significant different ($p < 0.05$).

(petunidin-3-*O*-rutinoside (*trans-p*-coumaroyl)-5-*O*-glucoside) accounted for almost 80% of the total anthocyanins, which was seldom in the common edible berry to our knowledge (Wu et al., 2006). Furthermore, most of the anthocyanins were acylated. According to the research of Sadilova, Stintzing, and Carle (2006) acylated anthocyanins are more stable than nonacylated anthocyanins. We speculated that the unique geography (high altitude) and harsh weather (cold, dry and strong sunshine) dramatically influenced the anthocyanins composition of *L. ruthenicum*, and in turn that the abundant acylated anthocyanins content could prevent plant from UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage. It was very important for plant to survive in such an ultraviolet rich area (Dixon & Paiva, 1995; Gould, Davies, & Winefield, 2008).

In terms of total anthocyanins content, *L. ruthenicum* from Dulan was better than the others. Probably, the environment of Dulan is more suitable for *L. ruthenicum*. But the main causes have not been investigated yet, since the remarkable discrepancies could come from different factors such as soil component, geographic condition and genetic difference.

3.4. Total polyphenol content

A wide range of methanol-soluble compounds were typically present in plant extract, such as amino acids, monosaccharide, organic acids can also be found. Furthermore, the TPC assay is affected by several interfering substance by amino acids, monosaccharide and organic acids. In our study, the SPE procedure was applied to remove the interfering compounds from the fruit extract. Since different phenolic acids obtain different reaction rate with Folin–Ciocalteu reagents, the results were obtained when the absorbance was constant during the assay to guarantee fully evaluation of the TPC presented in the extracts. Results displayed in Fig. 4 show significant differences between various samples under One-Way ANOVA analysis ($p < 0.05$). TPC for each sample ranged from 698.14 ± 27.15 (Dulan) to 1311.10 ± 80.05 (Delingha) mg per 100 g FW, which was much higher than those of bayberry fruits (360.3–446.1 mg per 100 g FW), a delicious common fruit in China (Fang, Zhang, & Wang, 2007) and black berry (261.9–929.6 mg per 100 g FW) (Sellappan, Akoh, & Krewer, 2002). The TPC of each sample increased in the order Dulan < Gomud < Delingha. The high TPC indicated that the *L. ruthenicum* fruit had potential antioxidant activity. As reported, polyphenol as effective antioxidant are involved in many diseases related to free radicals (Kong, Chia, Goh,

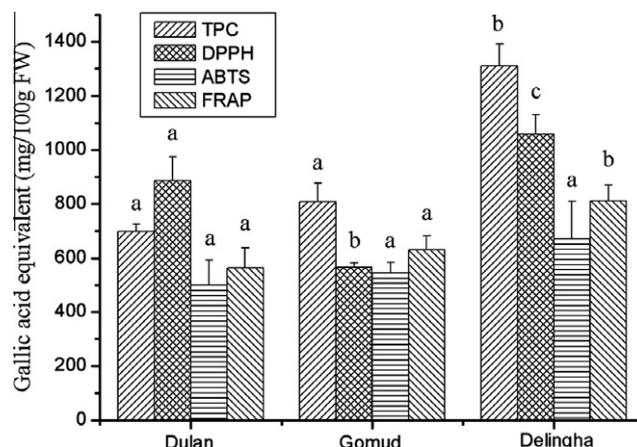


Fig. 4. TPC (mg/100 g FW) (mean \pm SD, $n = 3$) and antioxidant capacities (DPPH, ABTS and FRAP) of each sample (mean \pm SD, $n = 3$). Bars with different letters in common are significant different ($p < 0.05$).

Table 2

Correlation coefficients of the content of anthocyanins, total polyphenol content, DPPH, ABTS and FRAP assay.

	DPPH	ABTS	FRAP
Anthocyanins	0.222	−0.503	−0.598
TPC	0.625	0.619	0.838

Chia, & Brouillard, 2003). Furthermore, its health promoting effects may be attributed to the abundant polyphenol.

3.5. Antioxidant activity

Antioxidant assay results were displayed in Fig. 4. DPPH, ABTS⁺ and FRAP results were expressed as GA equivalents mg per 100 g FW. The results showed that sample from Delingha obtained the highest antioxidant activity (1059.1 ± 72.08 mg per 100 g FW) in DPPH assay, while Gomud obtained the lowest antioxidant activity (565.74 ± 15.73 mg per 100 g FW). Significant differences have been detected between three samples ($p < 0.05$). The consequences of ABTS⁺ and FRAP were similar, both showed that sample from Delingha (671.77 ± 137.01 mg per 100 g FW for ABTS⁺; 811.96 ± 58.58 mg/100 g FW for FRAP) possessed the maximal antioxidant activity, and followed by Gomud (545.74 ± 37.39 mg per 100 g FW for ABTS⁺; 631.63 ± 49.82 mg per 100 g FW for FRAP) and Dulan (500.04 ± 91.78 mg per 100 g FW for ABTS⁺; 563.28 ± 74.11 mg per 100 g FW for FRAP). Polyphenolic compounds, including flavonoids and phenolic acids, are responsible for antioxidant activity in plants extract. Plants with higher TPC exerted stronger antioxidant activity (Rice Evans & Miller, 1996). The antioxidant activity results were in accordance with the results of above section.

3.6. Correlation coefficient between anthocyanins, TPC and DPPH, ABTS and FRAP assays

Correlations coefficients between anthocyanins, TPC and DPPH, ABTS and FRAP assays are displayed in Table 2. A positive correlation between TPC and DPPH ($r = 0.625$), ABTS ($r = 0.619$) and FRAP ($r = 0.838$) were observed. As discussed in the above section, plants with higher TPC possessed stronger antioxidant activity. Although anthocyanins belong to polyphenol, the negative correlation between anthocyanins and ABTS ($r = -0.503$) and FRAP ($r = -0.598$) have been noticed. In present study, there were very limited evidences to elucidate the cause. The unexpected results might indicate that acylation substitution may decrease the antioxidant activity, or could be attributed to other phenols in the solution. According to the previous report, anthocyanins with *o*-dihydroxyl substitution are the most susceptible to oxidation such as cyanidin, delphinidin and petunidin, while pelargonidin, peonidin and malvidin oxidation were uneasy because they are not *o*-dihydroxyl substituted (Castaneda-Ovando et al., 2009). Petunidin was a potent antioxidant, but the main anthocyanins in *L. ruthenicum* were acylated petunidin derivatives. Giusti and Wrolstad (2003) reported acylation substitution would improve the anthocyanins stability by keeping it from the nucleophilic molecule attack. But whether acylation could decrease the antioxidant activity of petunidin is still uncertain. Relying on preparative chromatography to obtain the pure major anthocyanins of *L. ruthenicum* was another part of our research (still carrying on). Whether acylation would decrease the antioxidant activity of petunidin glycosides could be clarified by comparing the antioxidant activity of nonacylated petunidin with the acylated one.

4. Conclusions

The present study provided the chemical basis for setting up the anthocyanins fingerprint of *L. ruthenicum*. And in view of the abundant anthocyanins, pleasing colour and potent antioxidant activity of *L. ruthenicum*, it could be considered as natural pigment source and functional food. Furthermore, we only take a glimpse into *L. ruthenicum* chemical composition. For further understanding its biological effects, more investigations are needed to be carried out. In conclusion, this study is valuable for further full development of *L. ruthenicum*.

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