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Identification and quantification of antioxidants in *Fructus lycii*

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Abstract

Fructus lycii was extracted with 95% ethanol and the flavonoid content was determined to be 1.56 mg quercetin-equivalents/g extract. The following antioxidative activities were determined in the extract: (a) radical-scavenging activity (80 μmol trolox equivalent antioxidant capacity/g), measured by its ability to scavenge 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical, (b) reducing capacity (301 μmol trolox equivalent reducing capacity/g), measured by its ability to directly donate an electron in the reduction of Fe(III) to Fe(II), and (c) chelating activity (2.5 μmol ethylenediamine-tetraaceticacid equivalents/g), measured by its ability to remove Fe(II) ion from complexation with ferrozine. Three flavonol species in the extract were identified and quantitated by reversed-phase HPLC and their structures confirmed by electrospray ionization-mass spectroscopy: kaempferol (135 μg/g), quercetin (296 μg/g) and myricetin (247 μg/g). These results showed that *F. lycii* contains substantial amounts of the three antioxidative activities and is rich in flavonoids. The three flavonols accounted for 43% of total flavonoid content.

Keywords: *Fructus lycii*; *Lycium barbarum*; Antioxidant activity; TEAC; APTS; Reducing capacity; Iron chelating activity; Flavonols; Kaempferol; Quercetin; Myricetin

1. Introduction

The interest in many traditional herbs and plant food supplements, as a source of nutritional antioxidants, is due to our increasing knowledge of the role of antioxidants and free radicals in human heath and disease (for reviews, see Halliwell, 1994; Higdon & Frei, 2003; Scalbert, Manach, Moran, & Remesy, 2005; Willcox, Ash, & Catignani, 2004). Plant materials contain a diverse group of phenolic compounds with antioxidant activity, including flavonoids, lignans and stilbenes, and simple phenolic acids, such as hydroxybenzoic acids and hydroxycinnamic acids (Bravo, 1998). The flavonoid class is the most prominent and the most important plant antioxidant (Higdon & Frei, 2003). Flavonoid sub-classes include flavones, isoflavones, flavanones, flavonols, flavanols (catechins and gallocatechins), chalcones and anthocyanidins. To add to the structural diversity, as much as 90% of flavonoids isolated from plant sources are glycosylated with one or more sugars linked to an OH group (*O*-glycosides) or through carbon bonds (C-glycosides). Glucose, galactose and rhamnose are the predominant neutral sugars found (Bravo, 1998; Hollman & Katan, 1998; Williams & Grayer, 2004).

The antioxidative activities of flavonoids are multifaceted. Most flavonoids possess the ability to scavenge free radicals by acting as hydrogen as well as electron donors. Some flavonoids can also act as antioxidant by direct reaction with radicals to form less reactive products, and some species possess a capacity to chelate transition elements. Metal chelation is an important antioxidant activity as transition ions such as iron and copper can participate in radical production, as pro-oxidants, through the Fenton reaction (Lloyd, Hanna, & Mason, 1997). Some flavonoids are also strong inhibitors of certain metabolic enzymes in the body that generate free radical products such as cyclooxygenase, lipooxygenase, monoamine oxidase, xanthine

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oxidase, inducible nitric oxide synthase and some Phase I and II detoxifying enzymes (Higdon & Frei, 2003; Willcox et al., 2004).

The common structural features of flavonoids are the presence of aromatic rings and hydroxyl group(s), with some species being more potent than vitamin E as hydrogen donors. The hydrogen-donating antioxidative activity depends on the number and position of the hydroxyl group in the flavonoid structure and on other types of substituent that may be electron-withdrawing or electron-donating groups (for reviews on structure–activity relationships, see Heims, Tagliaferro, & Bobilya, 2002; Montoro, Braca, Pizza, & Tommasi, 2005; Rice-Evans, Miller, & Paganga, 1996; Silva et al., 2002). This is due to hydrogen donation dependent on the acidity of the phenolic hydrogen. An increase in hydroxylation of the gallate moiety (Ring B), for example, increases acidity of the *para* substituted hydroxyl hydrogen and hence increases hydrogen-donating ability. This structural feature can be seen in flavonols, such as myricetin, quercetin and kaempferol (Fig. 1). The antioxidant potency increases from kaempferol, quercetin to myricetin, which coincides with the increase hydroxylation pattern of the gallate moiety.

Lycium barbarum and *L. chinese* are two closely related species belonging to the family Solanaceae (Zhu, 1998). They are medicinal plants native to China but are now also widely found in Korea, Japan and also, to a lesser degree, in other Asian countries. The plant grows as a spreading shrub, with tiny branches with violet purple flowers, and produces orange red fruits called *Fructus Lycii*, also known as *Gouqizi* in Chinese. These fruits, which are harvested in summer and autumn, are first dried in the shade and then exposed to the sun for further drying until the skin is hard and dry but the pulp remains soft for storage and consumption. The nutritional interest in *F. Lycii* is due to its prominence in the diet of many Asian communities. It is traditionally consumed as a health food supplement cooked as a broth with poultry and as a medicinal elixir for improving health and vitality in the form of a beverage infused in liquor.

It is well established that *F. lycii* has a number of bioactive properties. Bioactive compounds isolated from this fruit include a cerebroside (Kim, Choi, Kim, Kim, & Lee, 1997), an arabinogalactan-protein (Peng & Tian, 2001), the carotenoids lutein and xeaxanthin (Leung, Li, Tso, & Lam, 2001) and a pro-vitamin C, 2-*O*-(β-D-glucopyranosyl)ascorbic acid (Toyoda-Ono et al., 2004). The cerebroside was found to protect primary cultured rat hepatocytes exposed to galactosamine-induced hepatotoxicity (Kim, Lee, Kim, Lee, & Kim, 2000). Interest in the arabinogalactan-protein was intense. It was shown to inhibit time-and hyperthermia-induced damage in cultured seminiferous epithelium (Wang et al., 2002), the growth of a transplantable sarcoma in rats (Gan, Zhang, Yang, & Xu, 2004), and the proliferation and induction of apoptosis in a human hepatoma cell line (Zhang et al., 2005). Furthermore, the arabinogalactan-protein was found to up-regulate expression of interleukin-2 and tumor necrosis factor-α at both mRNA and protein levels in human peripheral blood mononuclear cells (Gan, Zhang, Liu, & Xu, 2003), reduce blood glucose and serum cholesterol and triglyceride levels in rabbit (Luo, Cai, Yan, Sun, & Corke, 2004), and protect rats against carbon tetrachloride-induced hepatotoxicity (Ha et al., 2005).

The nutritional value of *F. lycii* as a source of antioxidant was also recognized by many investigators (references above). Several studies have reported the antioxidant activity of *F. lycii* from *L. barbarum* and *L. chinese*. However, a quantitative assessment and an elucidation of the nature of the antioxidative activity are often lacking (Ha et al., 2005; Liu, Ogata, Sato, Unoura, & Onodera, 2001; Nam & Kang, 2004; Wu, Ng, & Lin, 2004; Yim & Ko, 2002).

Kosar, Altintas, Kirimer, and Baser (2003) and Qian, Liu, and Huang (2004) quantitatively determined the antioxidant activity in *F. lycii* from *L. barbarum*, using 1,1′-diphenyl-2-picrylbiphenyl (DPPH) radical. Luo et al. (2004) quantitated the trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) in the same fruit. However, the DPPH, TEAC and ORAC assays measure scavenging of free radicals only. Quantitative determination of other antioxidative activities as an expression of antioxidant activity, such as reducing power and metal chelation, in *F. lycii* has yet to be reported. To date, there are only two reported studies on the antioxidant components in *F. Lycii*, but with contradictory conclusions regarding the presence of the flavonol rutin (Kosar et al., 2003; Qian et al., 2004).

We have an on-going interest in the identification and quantification of antioxidants in traditional medicinal plant materials (Hsu, Coupar, & Ng, 2006). In recent years LC–MS and GC–MS have been applied to unambiguously identify the structures of flavonoids in plant extracts and biological samples with great success (for recent reviews, see Prasain, Wang, & Barnes, 2004; de Rijke et al., 2006). This paper reports the quantification of three antioxidative activities and the identification and quantification of three flavonol antioxidant components in *F. Lycii* from *L. barbarum* by a combination of HPLC and mass spectrometry (MS) methods. The MS was carried out using an LC–MS setup.

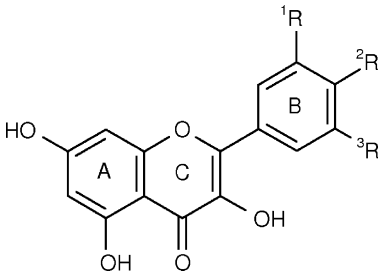

Fig. 1. Structures of some flavonols. Kaempferol: $^{2}R = OH$; $^{1}R = {}^{3}R = H$; quercetin: $^{1}R = {}^{2}R = OH$; $^{3}R = H$; myricetin: $^{1}R = {}^{2}R = {}^{3}R = OH$.

2. Materials and methods

2.1. Chemicals

$AlCl_3$, ascorbic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediamine-tetraaceticacid disodium salt dihydrate ($EDTANa_2 \cdot 2H_2O$), gallic acid, 6-hydroxy-2,5,7-8-tetramethylchromane-2-carboxylic acid (Trolox™), iron(III) chloride hexahydrate, iron(II) chloride, myricetin, quercetin, kaempferol, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*-*p*′-disulfonic acid monosodium salt (ferrozine), potassium ferricyanide (III), potassium persulfate, rutin and 2-thiobarbituric acid were obtained from Sigma–Aldrich Inc. (St Louis, MO, USA). 2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) and trichloroacetic acid were obtained from Fluka Chemical Co. (Buchs, Germany). All other common chemicals and solvents used were of analytical grade and water was of Millipore® HPLC Deionized Water grade.

2.2. Equipment and apparatus

UV absorption measurements were performed with a Cary 3 Bio UV–Visible Spectrophotometer (Variant). Rotary evaporation under vacuum was performed with a BUCHI RotaVapor RE111 rotary evaporator fitted with water jet vacuum system and a BUCHI 461 temperature-controlled water bath. Equipments for HPLC and LC–MS are listed under those sections.

2.3. F. lycii

F. Lycii from *L. barbarum* was purchased from a local herbal supermarket. The fruit was from the Quangdong province of China and was imported from China and distributed by New Eastland Pty. Ltd. Australia (23-24 Kempson Court, Keysborough, Vic. 3173, Australia). The fruit came as whole sun-dried fruit and was packed under vacuum.

2.4. Preparation of F. lycii extract

The imported fruit was oven-dried at 105 °C for 6 h. Thirty grams of the dried fruit were crushed using a mortar and pestle and extracted with 600 ml 95% ethanol at 90 °C with stirring in a hot water bath. The paste disintegrated into small particulate pieces after 10 min. The extraction was continued for 2 h and stood to cool before the extract was filtered using a 200-mesh stainless steel sieve to obtain a slightly turbid bright red-coloured extract. The extract was reduced to a small volume by rotary evaporation under vacuum at 40 °C and then freeze-dried. The freeze-dried extract was dissolved in methanol to 100 mg/ml concentration and kept at −20 °C until used. The yield was 13.8 g (46 %, w/w).

2.5. Determination of total flavonoid content

Total flavonoid content was determined by reaction with $AlCl_3$ and quantitated by absorbance measurement according to Woisky and Salatino (1998). Appropriately diluted extract in ethanol was mixed with 2% $AlCl_3$ solution in ethanol. After 1 h at RT the absorbance was measured at 420 nm. The amounts of flavonoids were determined from a standard absorbance plot, using quercetin as standard, and calculated as quercetin-equivalents (mg/g extract).

2.6. ABTS radical-scavenging activity

2,2′-Azinobis-3 ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging activity was measured by direct absorbance measurement of the radical ($ABTS^{\cdot+}$) according to Nenadis, Wang, Tsimidou, and Zhang (2004). $ABTS^{\cdot+}$ was generated by reacting 5 ml of 7 mM aqueous ABTS solution with 88 μl of a 140 mM potassium persulfate solution (final concentration of potassium persulfate equals 2.45 mM). The solution was stored in the dark for 16 h and then diluted with ethanol to an absorbance of 0.70 ± 0.05 at $\lambda_{734\ nm}$. The reaction mixture (1.00 ml) contained 10 μl of test material (*F. lycii* extract [0–350 μg] or trolox [0–3 μg]) and 990 μl of the $ABTS^{\cdot+}$ solution. The mixture was shaken well and allowed to react at RT for 20 min. The absorbance of the $ABTS^{\cdot+}$ solution was measured at $\lambda_{734\ nm}$ against an ethanol blank. The assay was performed over a range of concentrations and was plotted as a decrease in absorbance *versus* concentration of test material in mg/ml reaction.

2.7. Reducing power

Reducing power was measured by the direct reduction of $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$ and was determined by absorbance measurement of the formation of the Perl's Prussian Blue complex following the addition of excess Fe^{3+}, as described by Yen and Chen (1995). The reaction mixture (1.16 ml) contains 160 μl of test material (*F. lycii* extract (0–1 mg) or trolox (0–60 μg)), 500 μl of 1% (w/v) potassium ferricyanide ($K_3Fe^{3+}(CN^-)_6$) in water and 500 μl of 0.2 M phosphate buffer, pH 6.6. The mixture was then incubated for 20 min at 50 °C and the reaction was terminated by the addition of 500 μl of 10% (w/v) trichloroacetic acid, followed by centrifugation for 10 min at 3000 rpm. Five hundred microliters of the supernatant was mixed with 500 μl water and 100 μl 0.1% (w/v) ferric chloride ($FeCl_3$). The absorbance was measured at $\lambda_{700\ nm}$ against the phosphate buffer blank. The reducing capacity ($RC_{0.5\ AU}$) is arbitrarily defined as the concentration of test material (mg/ml reaction volume) that produces 0.5 AU at $\lambda_{700\ nm}$ and was obtained from a line of best fit of the absorbance data using the linear regression method.

2.8. Ferrous ion chelating activity

Ferrous ion chelating activity was measured by inhibition of the formation of iron(II)–ferrozine complex after treatment of test material with Fe^{2+}, following the method of Decker and Welch (1990). The reaction mixture (1.50 ml) contained 500 μl test material (*F. lycii* extract (0–35 mg) or $Na_2EDTA \cdot 2H_2O$ (0–25 μg)), 100 μl $FeCl_2$ (0.6 mM in water) and 900 μl methanol. The control contained all the reaction reagents except the extract and EDTA. The mixture was shaken well and allowed to react at RT for 5 min. One hundred microliters of ferrozine (5 mM in methanol) was then added, the mixture shaken again, followed by further reaction at RT for 10 min to complex the residual Fe^{2+} ion. The absorbance of the Fe^{2+}–ferrozine complex was measured at $\lambda_{562\ nm}$ against a methanol blank. The chelating effect was calculated as a percentage, using the equation below, and plotted against concentration of test material in mg/ml of reaction mixture. The assay was performed over a range of concentrations (mg/ml reaction volume), such that saturation (complete chelation of free iron not bound to antioxidant) was reached. Because the increase in absorbance was non-linear with increasing concentration, an EC_{50} value (defined as the effective concentration of test material which produces 50% of maximal scavenging effect) was obtained from the plot using a non-linear regression algorithm:

$$\text{Chelating effect } (\%) = \left(1 - \frac{A^{\text{Sample}}_{\lambda_{562\ nm}}}{A^{\text{Control}}_{\lambda_{562\ nm}}}\right) \times 100$$

2.9. Deglycosylation of flavonoids

Flavonoid glycosides were degylcosylated by a quantitative acid hydrolysis procedure, as described by Hertog, Hollman, and Venema (1992). Twenty milligrams of extract was dispersed in 4 ml of 62.5% methanol containing 2 mg/ml of butylated hydroxyanisole (BHA) as a protective antioxidant in a thick screw-cap pyrex® glass tube. The mixture was sonicated for 5 min and 1 ml of 6 M hydrochloric acid was then added. The mixture was bubbled with nitrogen for 60 s to purge dissolved oxygen and then sealed tightly. Hydrolysis was carried out in a 90 °C water bath for 2 h. After hydrolysis, the mixture was cooled and filtered with a 0.22 μm membrane filter disc for HPLC.

2.10. HPLC

The reversed phase HPLC system consisted of a Luna™ (USA) 5 μm particle size C_{18} silica guard column disc (5 mm length × 4.6 mm diameter) and main column (250 mm length × 4.6 mm diameter) connected to a UV detector (Waters™ 486, Tunable Absorbance Detector) and a HPLC pump (Waters™ Model 510). Data acquisition and manipulation were performed with Waters Millennium® software. The injection (sample loop) volume was 100 μl and the column developed using a step gradient of increasing concentration of acetonitrile (ACN) in acidified water (1% acetic acid, v:v) as follows: 10% ACN (0–10 min), 20% ACN (10–30 min), 30% ACN (30–60 min), 40% ACN (60–90 min) and 60% ACN (90–120 min). At the end of a run, the column was washed with 100% acetonitrile (30 min) and reconditioned in 10% ACN (30 min) before further injection. Optimal detection wavelength for the flavonoid chromophore was set at $\lambda_{370\ nm}$.

Spiking of flavonol peaks in the extract was performed with commercially obtained myricetin, quercetin and kaempferol as standard compounds; 0.2 μg of each standard compound was added individually or as a mixture to 100 μl of the deglycosylated extracts and analyzed by HPLC as described above.

The amount of the three flavonol species in the deglycosylated extract was determined from standard calibration plots. Flavonol concentrations of 0.25, 0.5, 1.0, 2.2, 5.0 μg/ml were prepared for each standard (myricetin, quercetin or kaempferol) and subjected to HPLC separately. The injection volume was 100 μl and the column developed using the same step gradient as described above. The calibration curve was plotted as peak area (arbitrary units) obtained from absorbance at $\lambda_{370\ nm}$ against standard (μg) and the data points fitted into a line of best fit by the linear regression method.

2.11. LC–ESI-MS of putative flavonol peaks from HPLC

The structures of the putative flavonol peaks, separated and identified by analytical HPLC and spiking with standards, were confirmed by spectroscopic analysis of each of the recovered peak materials from HPLC by LC–ESI–MS.

Liquid chromatography–electrospray ionization–mass spectroscopy (LC–ESI–MS) analysis was conducted in positive mode electrospray ionisation on a Waters Micromass Q-TOF quadrupole time-of-flight mass spectrometer coupled to a Waters 2795 HPLC (Milford, MA, USA). Chromatographic separation was achieved with a water–acetonitrile gradient (containing 0.05% formic acid) at a flow rate of 0.4 ml/min, using a 50 × 2.1 mm i.d., 4 μm particle size Phenomenex Synergi Hydro C_{18} column (Torrance, CA, USA) maintained at 40 °C. The ionisation of the flavonols was optimized at a capillary voltage of 3.2 kV and cone voltage of 40 eV. The following conditions were maintained during analysis: desolvation temperature of 350 °C, source block temperature of 90 °C, desolvation gas flow of 400 l/h and cone gas flow of 100 l/h. MS and MS–MS spectra were collected in the mass range of 100–1000 mass units and collision-induced dissociation (CID) experiments were conducted with a collision energy of 30 eV.

2.12. Treatment of data

The experimental results for the antioxidative assays were expressed as means ±1 SD of quadruplicate reactions

and all uncertainties were reported to two significant figures (EURACHEM/CITAC Guide (2000) on "Quantifying Uncertainty in Analytical Measurement", in "http://www.measurementuncertainty.org"). Linear regression and non-linear algorithm plots were performed using SigmaPlot® (version 9).

3. Results and discussion

3.1. Extraction of F. lycii and total flavonoids content

Imported *L. barbarum's F. lycii* from the Quangdong province of China was studied for its flavonoid content. *F. lycii* was oven-dried until the weight was steady (6 h). The oven-dried fruit was extracted with hot 95% ethanol for 2 h, which yielded 13.8 g of extract (46%, w/w to dried fruit). Total flavonoid content was determined by reaction with $AlCl_3$ and the Al–flavonoid complex was quantitated by absorbance measurement at $\lambda_{420\ nm}$ and calculated as quercetin equivalents from a quercetin standard plot. The extracts contained 1.56 quercetin-equivalents/g extract of flavonoids, which gave a value of 0.72 mg quercetin-equivalents/g of dried fruit. No flavonoids were detected in the extracted residue, which indicated that all detectable flavonoids were extracted by the ethanol treatment.

3.2. ABTS radical-scavenging activity

Antioxidant activity is defined as the ability of a compound to inhibit oxidative degradation, such as lipid peroxidation (Roginsky & Lissi, 2005). However, the nature of the antioxidant activity could be multifaceted. Hydrogen donation is one such activity and studies on flavonoids, such as catechin, using *tert*-butoxyl radicals in acetonitrile showed that the rate of hydrogen donation is very fast, around $1–3 \times 10^8\ M^{-1}\ s^{-1}$ (Cren-Olive, Hapiot, Pinson, & Rolando, 2002), comparable to that of α-tocopherol (Evans, Scaiano, & Ingold, 1992). The hydrogen-donating activity is derived mainly from the flavonoid's A-ring hydroxyl (Cren-Olive, Hapiot, et al., 2002; Cren-Olive, Lebrun, Hapiot, Pinson, & Rolando, 2000; Cren-Olive, Wierulesky, Maes, & Rolando, 2002).

2,2′-Azinobis-3 ethylbenzothiazoline-6-sulfonic acid radical ($ABTS^{\cdot+}$) is a stable organic radical that has gained general acceptance as the organic radical for use in measuring radical-scavenging activity as an expression of hydrogen-donating antioxidative activity in plant crude extract (Arts, Dallinga, Voss, Haenen, & Bast, 2004; Arts, Dallinga, Voss, Haenen, & Bast, 2003). $ABTS^{\cdot+}$ strong absorbance, maximum at $\lambda_{734\ nm}$, is well removed from interfering UV absorption that is often present in a crude extract. This reliably permits quantitation by absorbance measurement. Because $ABTS^{\cdot+}$ is reduced to a colourless product in the presence of hydrogen-donating antioxidant, the degree of discoloration correlates with the amount of $ABTS^{\cdot+}$ that is scavenged. However, reaction with $ABTS^{\cdot+}$ does not permit a correlation of structure–activity relationships for some flavonoids due to further scavenging of $ABTS^{\cdot+}$ by some reaction products (Arts, Haenen, & Bast, 2004).

The ability of the ethanol extract to donate hydrogen in scavenging $ABTS^{\cdot+}$ was indicated by a decrease in absorbance at $\lambda_{734\ nm}$. The results (Fig. 2a) showed a linear correlation between concentration of extract (from 0 to 0.35 mg/ml reaction tested) and discoloration of the $ABTS^{\cdot+}$ solution. The hydrogen-donating activity of trolox was similarly measured (Fig. 2b), and was also concentration-dependent.

By relating extract activity to pure compound such as trolox, comparison with the literature values is possible. The trolox equivalence antioxidant capacity (TEAC) of the extract can be calculated by comparing the decrease in absorbance to that of trolox on a molar basis (Nenadis et al., 2004). There are a number of methods for this to be done. The most accurate method is by relating the gradient

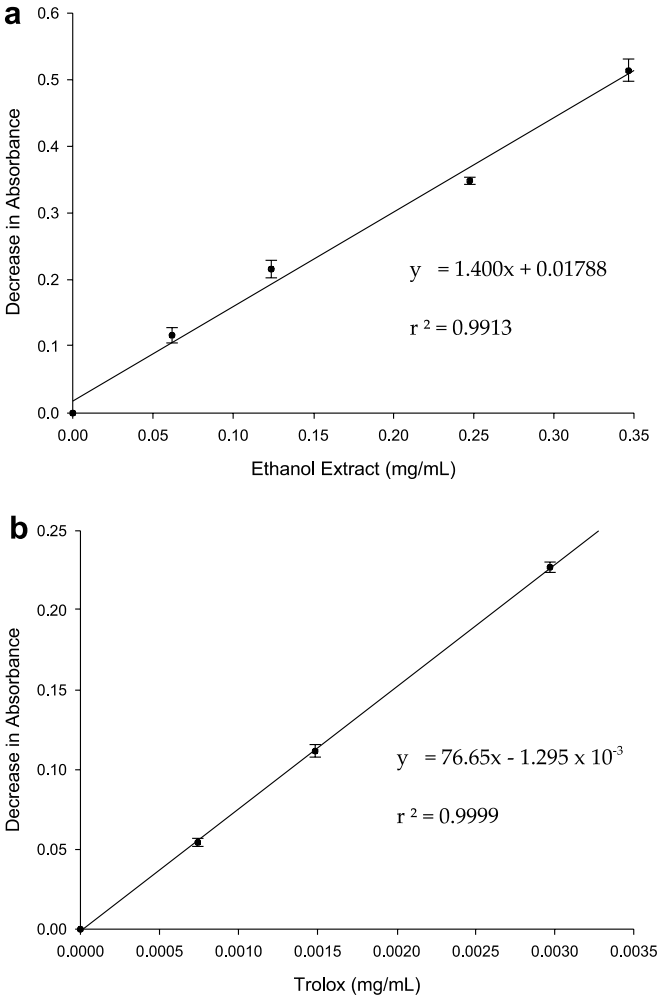

Fig. 2. $ABTS^{\cdot+}$-scavenging activity of: (a) *Fructus lycii* ethanol extract and (b) trolox. The decreases in absorbance values were directly plotted as the means of replicate determinations ± SD ($n = 4$) against test material concentration in mg/ml of reaction volume. The data were fitted into a line of best fit using the linear regression method.

Table 1
Nature of antioxidative activities in *Fructus lycii*

Hydrogen-donating activity	80 μmol TEAC/g of extract
	36 μmol TEAC/g of dried fruit
Electron-donating activity	301 μmol TERC/g of extract
	138 μmol TERC/g of dried fruit
Iron(II)-chelating activity	2.5 μmol EDTA-E/g of extract
	1.1 μmol EDTA-E/g of dried fruit

Values in mass were obtained and calculated from data contained in Figs. 2–4 and were converted to μmoles using the molecular weights of the pure compounds.

of the activity/concentration plot of the extract to that of trolox, that is, by the ratio of the linear coefficient of the extract to the linear coefficient of the trolox standard plot (Martinez-Tome et al., 2004). This method takes a range of values in generating the two gradients for comparison.

The results showed that the extract contained 80 μmol TEAC/g extract or 36 μmol TEAC/g dried fruit (Table 1). This value is more than 3.6 times the TEAC value obtained by Luo et al. (2004) for an 80% methanol extract at 35 °C for 24 h of the same fruit, but originated from another region in China.

3.3. Reducing power

The ability of a compound to donate electron in an oxidation-reduction reaction can also be used as a measure of antioxidative activity, and is referred as its reducing capacity (or power). This is because reduction of a free radical converts it to a less reactive or unreactive product. Flavonoids are also electron donors and electron donation is mainly derived from the flavonoid's B-ring (Cren-Olive, Hapiot, et al., 2002; Cren-Olive et al., 2000; Cren-Olive, Teissier, Duriez, & Rolando, 2003; Cren-Olive, Wierulesky, et al., 2002).

The reducing capacity of a compound or crude extract can be measured by the direct reduction of $Fe^{3+}(CN^{-})_6$ to $Fe^{2+}(CN^{-})_6$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian Blue complex, $Fe_4^{3+}[Fe^{2+}(CN^{-})_6]^3$, which has a strong absorbance maximum at $\lambda_{649\ nm}$ Again, the visible λ_{max} is favourable for a crude extract. An increase in absorbance of the reaction mixture would indicate an increase in reducing capacity due to an increase in the formation of the complex.

As shown in Fig. 3a, the extract had strong electron-donating capability, and hence reducing capacity. Absorbance increased linearly with concentration of extract. Since the electron acceptor ($Fe^{3+}(CN^{-})_6$) was added in large excess in the assay method, the range of concentrations (0–1.0 mg/ml reaction) was chosen such that the absorbance did not exceed 1.5 U, as higher absorbance decreases the accuracy due to the logarithmic relation between absorbance and transmittance. From the absorbance plot, an arbitrary point at $RC_{0.5\ AU}$ (reducing capacity at 0.5 absorbance unit) can be obtained to indicate the

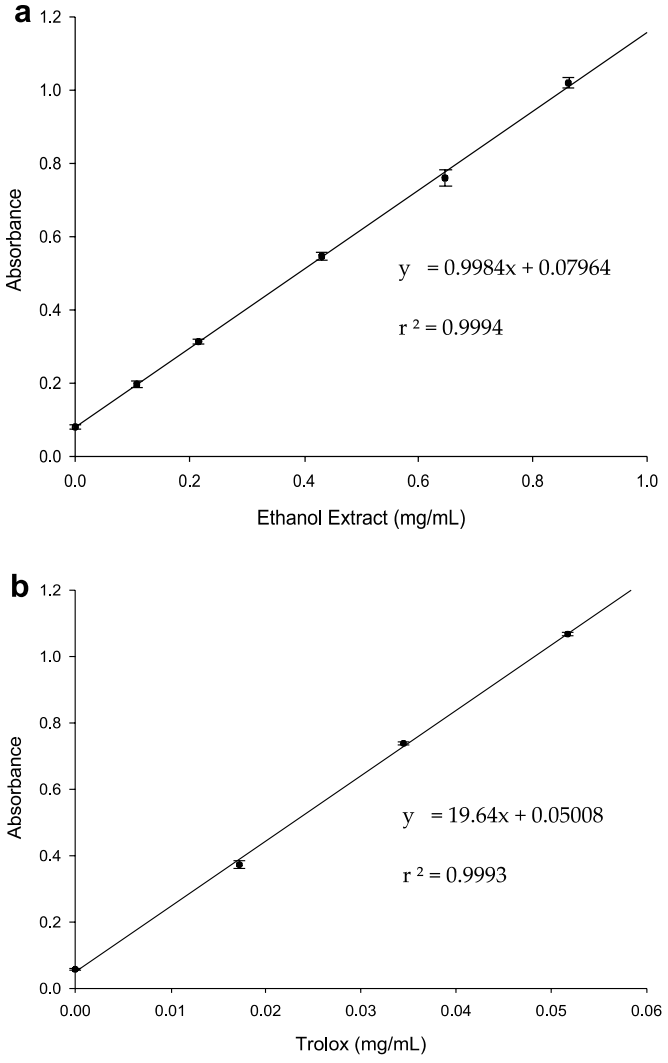

Fig. 3. Reducing capacity of: (a) *Fructus lycii* ethanol extract and (b) trolox. The increases in absorbance values were directly plotted as the means of replicate determinations ± SD ($n = 4$) against test material concentration in mg/ml of reaction volume. The data were fitted into a line of best fit using the linear regression method.

potency of a test substance. It is a convenient point for comparison with an electron-donating antioxidant, such as trolox (Fig. 3b), to obtain a trolox equivalent reducing capacity (TERC) value. The $RC_{0.5\ AU}$ values were 0.43 mg/ml of extract and 0.023 mg/ml of trolox. The TERC value for the extract was calculated to be 301 μmol TERC/g of extract or 138 μmol TERC/g of dried fruit (Table 1).

This is a substantial amount of reducing capacity. If we took account of reducing capacity contributed by 2-*O*-(β-D-glucopyranosyl) ascorbic acid, which Toyoda-Ono et al. (2004) found, and determined in *F. lycii* to be 50 μmol/g dried fruit, and that the reducing capacity of ascorbic is greater than that of trolox (Kim, Lee, Lee, & Lee, 2002), this would indicate that at least 88 μmol TERC/g of dried fruit was derived from other antioxidant components, such as flavonoids.

3.4. Ferrous ion-chelating activity

Most iron is bound to proteins, such as transferring, or held in storage as ferritin in a low a redox state in the body (Rang, Dale, & Ritter, 1996). This is to prevent its participation in the Fenton reaction, which is a transition metal-catalyzed decomposition of hydrogen or lipid peroxide into the highly reactive and biologically damaging hydroxyl radical (Halliwell, 1997). With some diets or disease states, overloading of iron in the body can occur and requires the consumption of exogenous chelators to prevent the build-up of free iron circulating in the body. Such exogenous chelators can be obtained from certain antioxidants in food. An antioxidant's ability to chelate Fe^{2+} or other transition elements, such as copper, is thus an important antioxidant property to measure.

One measurement of the metal-chelating activity of an antioxidant is based on absorbance measurement of iron (II)–ferrozine complex after prior treatment of an iron (II) solution with test material. Ferrozine forms a complex with free Fe^{2+} but not with Fe^{2+} bound to other chelators; thus a decrease in the amount of ferrozine–Fe^{2+} complex formed after treatment indicates the presence of antioxidant chelators. The ferrozine–Fe^{2+} complex produced a red chromophore with absorbance that can be measured at $\lambda_{562\ nm}$. A significant drawback of this complexation reaction, in measuring the presence of antioxidant chelator, is that the reaction is affected by both the antioxidant–Fe^{2+} and of ferrozine–Fe^{2+} complex formation constants and the competition between the two chelators for binding to iron. Thus a weak antioxidant iron chelator would be seriously underestimated in quantitative determination. From a nutritional point of view, it is not yet possible to access the role of a weak antioxidant iron chelator in preventing the Fenton reaction *in vivo*. Nonetheless, this reaction serves as a convenient assay to access iron chelating activity of antioxidant.

The results of the chelating assay were plotted as percentage chelating effect against concentration of test material (mg/ml of reaction volume). The ethanol extract showed Fe^{2+}-chelating activity, exhibiting non-linear concentration-dependent reaction kinetics (Fig. 4a. This merely reflects the complex nature of the extract containing a number of different iron chelators, with different affinities for iron, in competition with ferrozine for binding. Nonetheless, the chelating effect was saturable at high concentration of extract. To evaluate the potency of the extract, the activity was compared to EDTA, a hexadentate metal ion chelator (Heimbach et al., 2000) with a binding constant for Fe^{2+} of $4.9 \times 10^8\ M^{-1}$ (Kolayli, Ocak, Kucuk, & Abbasoglu, 2004). The reaction kinetics were linear with EDTA at a concentration below the saturating level of EDTA (Fig. 4b).

Due to the non-linear nature of the reaction in the crude extract, EC_{50} values were determined from the plot of chelating effect (%) against concentration of test material for comparison. This method is more accurate than comparison at a single fixed point. The results obtained were 10 mg/ml of extract and 5.6 μg/ml of $Na_2EDTA \cdot 2\ H_2O$. Again, the EC_{50} values merely served as a convenient point for comparison with a metal chelator such as EDTA. The activity of the extract in terms of EDTA equivalence (EDTA-E) was calculated from the EC_{50} values to be 2.5 μmol EDTA-E/g extract or 1.1 μmol EDTA-E/g of dried fruit (Table 1).

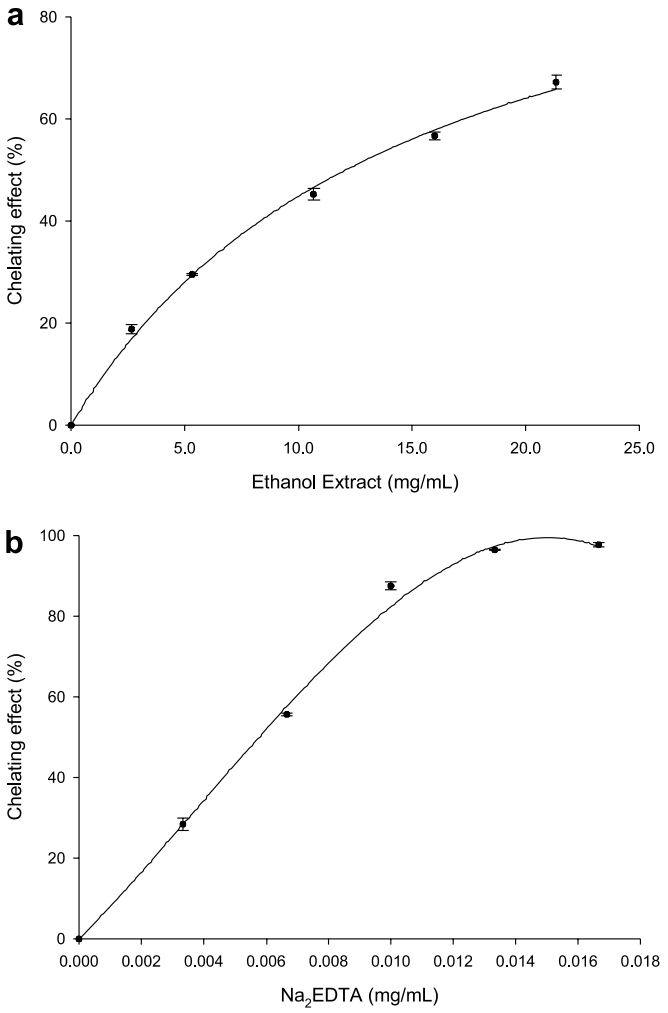

Fig. 4. Ferrous iron-chelating activity of: (a) *Fructus lycii* ethanol extract and (b) EDTA. The absorbance values were converted to chelating effect (%) and data were plotted as the means of replicate determinations ± SD ($n = 4$) against test material concentration in mg/ml of reaction volume. EDTA was added as $Na_2EDTA \cdot 2H_2O$. The data were fitted by a non-linear regression algorithm.

3.5. Identification of flavonols in F. lycii

Kosar et al. (2003) putatively identified a number of phenolics but noted the absence of rutin in an ethanol extract of *L. barbarum's F. lycii* from Turkey, by comparing retention times and UV spectrum with standards using HPLC and a photodiode array detector. Qian et al. (2004) reported the putative identification of a number of flavonoids but concluded that rutin was the major flavonoid in a similar extract of *L. barbarum's F. lycii* from China,

also by comparison of retention times in HPLC. Given the incomplete resolution of the extract complex components in HPLC, a more reliable method is required for unambiguous identification and further structural elucidation.

We employed a reversed phase HPLC using a C_{18} column to separate the antioxidant components in the extract. The antioxidant components of interest in this study were the flavonols myricetin, quercetin and kaempferol. As such, the detection wavelength for the HPLC was selected at 370 nm, the λ_{max} for the flavonol chromophore. The column was developed with a step gradient of increasing acetonitrile concentration in water containing 1% acetic acid as an organic acid modifier. To achieve the desired resolution and quantification, the flavonols were deglycosylated by a quantitative deglycosylation method (Hertog et al., 1992). In terms of nutritional relevance, quantification of the aglycone is valid since dietary flavonoid glycosides are deglycosylated during the process of absorption from the lumen of the intestine into the systemic circulation (Manach & Donovan, 2004; Walle, 2004).

Fig. 5 shows a HPLC chromatogram of the deglycosylated extract. The myricetin, quercetin and kaempferol peaks were putatively identified in the extract by spiking with individual flavonol standards in separate chromatography. Fig. 6 shows a HPLC chromatogram of the myricetin, quercetin and kaempferol peaks spiked with the three standard compounds injected together. The fact that the peak co-eluted with standard compound indicates the identity of that peak.

We confirmed the structures of the flavonols by recovering the three HPLC peak materials separately and analysis by positive mode electrospray ionization mass spectrometry (Fig. 6). The MS–MS spectra of the CID experiments (Fig. 7) showed that the expected molecular ions were observed for kaempferol ($C_{15}H_{10}O_6$; MH^+ 287.0556, observed 287.049), quercetin ($C_{15}H_{10}O_7$; MH^+ 303.0505, observed 303.052) and myricetin ($C_{15}H_{10}O_8$; MH^+ 319.0454, observed 319.042). The following fragments were observed for kaempferol, quercetin and myricetin, respectively, in their CID spectra (Fig. 7) using the nomenclature of Ma, Li, Van den Heuvel, & Claeys (1997): $^{0,2}B^+$, 121.030, 137.026 and 153.019; $^{1,3}A^+$, 153.014, 153.021 and 153.019 (strong signal due to additional $^{0,2}B^+$ fragment; $^{0,2}A^+$, 165.015, 165.018 and 165.016; $[M+H-46]^+$, 213.048, 229.052 and 245.046. These fragmentation patterns were consistent with the assigned flavonol structures (Ma et al., 1997; March & Miao, 2004).

The order of elution of the flavonols from HPLC can be explained by their structures and polarities (Fig. 1). Myricetin, having three hydroxyl groups, eluted first, due to its higher polarity, followed by quercetin with two hydroxyl groups while kaempferol, having only one hydroxyl group, eluted last. BHA, added as a protective antioxidant in the acid hydrolysis process, eluted at retention time 153 min where it did not interfere with the chromatography.

The identification of quercetin in *F. lycii* in our study supported the report by Qian et al. (2004) since quercetin is derived from rutin after deglycosylation. Nonetheless, Qian et al. (2004) failed to identify kaempferol and myricetin (or their glycosylated forms) in their extract due to incomplete resolution of the components in their HPLC and the limitation of comparing retention times in identification.

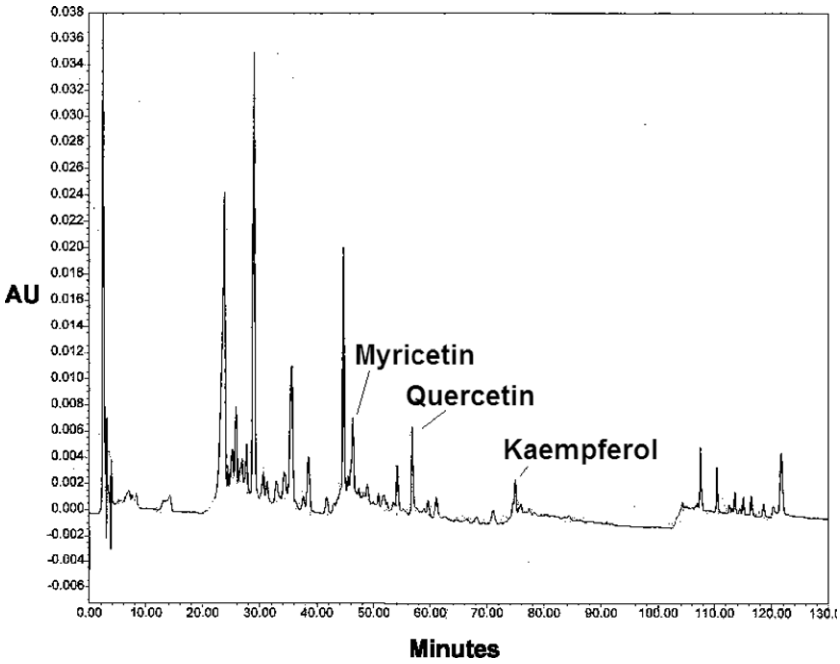

Fig. 5. HPLC Chromatogram of *Fructus lycii* ethanol extract. Four hundred micrograms of deglycosylated extract were injected and the column was developed with a step gradient of increasing acetonitrile concentration.

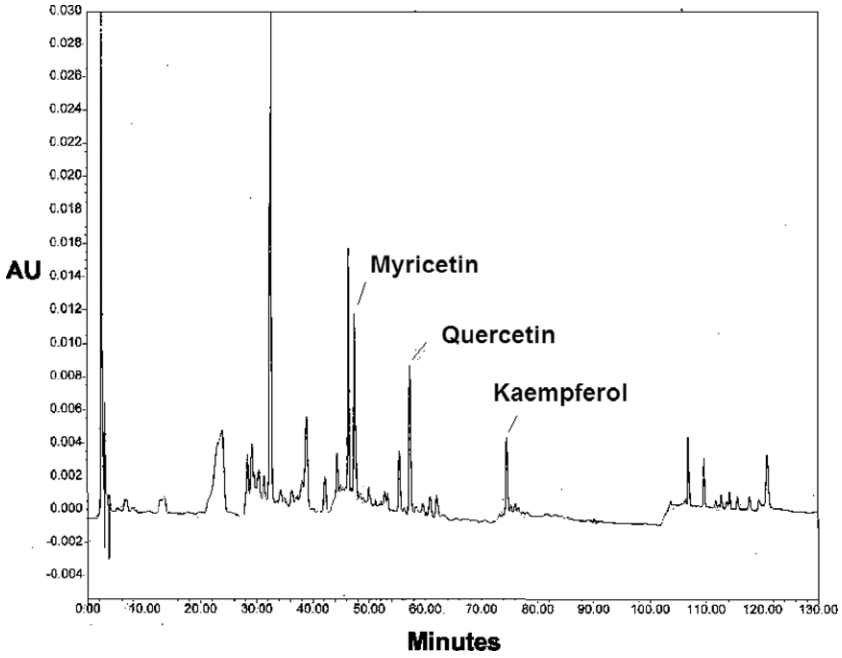

Fig. 6. Co-elution of putative myricetin, quercetin and kaempferol peaks in *Fructus lycii* ethanol extract with standard compounds from HPLC. Four hundred micrograms of deglycosylated extract were co-injected with 0.2 μg each of standard myricetin, quercetin and kaempferol to spike the putative peaks such that each peak area increased approximately 2-fold.

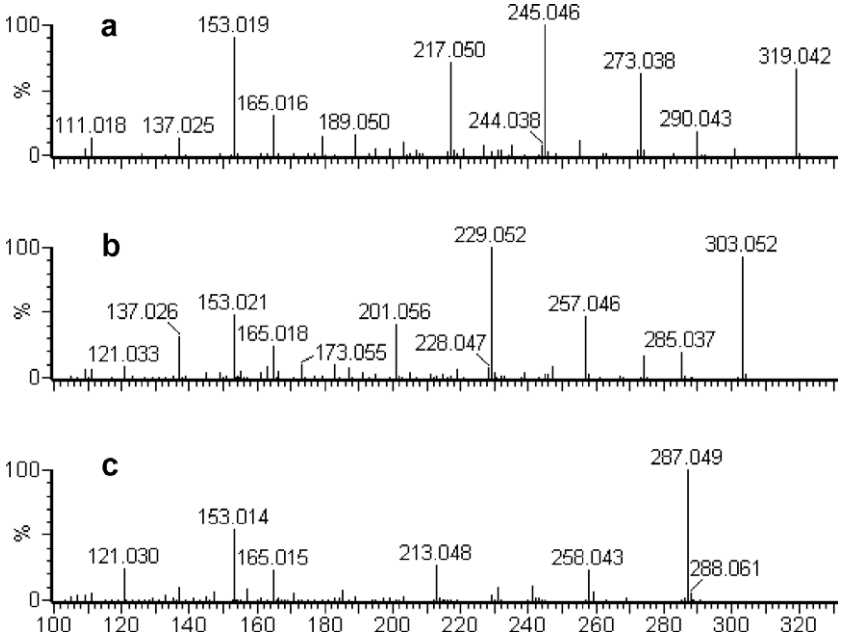

Fig. 7. CID MS–MS spectra of: (a) myricetin, (b) quercetin and (c) kaempferol peak materials isolated from analytical HPLC. Spectra were obtained at collision energy of 30 eV in positive mode electrospray ionization mass spectroscopy and MS was performed in LC–MS mode.

3.6. Quantification of flavonols in F. lycii

A calibration curve, relating mass (μg) to absorbance peak area (arbitrary units) was obtained by HPLC for each of the flavonol standards, myricetin, quercetin and kaempferol. The calibration curve produced a linear relationship between mass and peak area over a range of 0.025–0.100 μg of flavonol with r^2 equal to 0.9970 or better (data not shown).

The amounts of myricetin, quercetin and kaempferol in the extract were obtained from peak area of the individual peak and the flavonol standard calibration plot. The results (Table 2) showed that the fruit contained 247 μg myricetin, 296 μg quercetin and 135 μg kaempferol per g of extract (or

Table 2
Identification and quantification of flavonols in *Fructus lycii*

Flavonols	Myricetin	Quercetin	Kaempferol
Extract (μg flavonol/g)	247	296	135
Dried fruit (μg flavonol/g)	114	136	62

The amounts of myricetin, quercetin and kaempferol were calculated from peak areas of HPLC chromatograms of individual peaks in extract and standard calibration plot. The identities of the flavonols were confirmed by LC–ESI-MS (Fig. 7).

114 μg myricetin, 136 μg quercetin and 62 μg kaempferol per g of dried fruit). Since the fruit contained 1.56 mg flavonoids/g of extract as quercetin equivalents, the three flavonols account for 43% of total flavonoid content.

4. Conclusion

Our study quantitatively evaluated the nature of the antioxidant activity in *F. Lycii* from *L. barbarum* that is traditionally consumed in China and in other overseas Chinese communities as a health food supplement and a medicinal elixir. A hot 95% ethanol extract of the fruit extracted all detectable flavonoids. The extract contained 80 μmol TEAC/g of hydrogen-donating, 301 μmol TERC/g of electron- donating and 2.5 μmol EDTA-E/g of metal-chelating antioxidative activities. In addition, we identified and quantitated three flavonol species, myricetin, quercetin and kaempferol, in the extract by HPLC, spiking with standards and electrospray ionization mass spectroscopy. We found a substantial amount of flavonoids in the extract (1.56 mg quercetin-equivalents/g) in which the three flavonol species predominated (247 μg myricetin, 296 μg quercetin and 135 μg kaempferol/g).

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