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Isolation of carotenoids, flavonoids and polysaccharides from *Lycium barbarum* L. and evaluation of antioxidant activity

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ABSTRACT

A preparative column chromatography method was developed to isolate carotenoids, flavonoids and polysaccharides, from *Lycium barbarum* L., possessing vital biological activity, and their antioxidant activity was evaluated. Carotenoids were isolated by a column containing magnesium oxide and diatomaceous earth (1.5:1, w/w), and β-carotene was eluted with hexane, β-cryptoxanthin and neoxanthin with ethyl acetate and zeaxanthin with ethyl acetate–ethanol (80:20, v/v). Flavonoids and phenolic acids were separated using a Cosmosil 140 C18-OPN column, with phenolic acids being eluted with deionized water and neutral flavonoids with methanol. Polysaccharides were fractionated using a DEAE-Sepharose CL-6B column; neutral polysaccharides were eluted with water and acidic polysaccharides with different concentrations of NaCl. For antioxidant activity, the flavonoid fraction was the most effective in scavenging $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ free radicals, chelating metal ions and reducing power, while the zeaxanthin fraction and polysaccharides showed the most pronounced effect in scavenging hydroxy free radicals and superoxide anions, respectively.

1. Introduction

Lycium barbarum L., a traditional Chinese herb possessing vital biological activities, such as prevention of cancer and age-related macular degeneration (AMD), is widely used in Asian countries. Many functional components in *L. barbarum*, including flavonoids, carotenoids and polysaccharides, have been reported to be closely associated with the health-enhancing effect (Fraser & Bramley, 2004; Marchand, 2002; Sheng et al., 2007; Yang, Zhao, Yang, & Ruan, 2008). Several physiological studies have focused on polysaccharides; however, both carotenoids and flavonoids have been less investigated, especially for their antioxidant activity.

Carotenoids, a group of lipid-soluble compounds responsible for yellow and red colours of many plants and food products, have been demonstrated to be effective in preventing chronic diseases such as cardiovascular disease and skin cancer (Fraser & Bramley, 2004; Kohlmeier & Hastings, 1995). Inbaraj et al. (2008) reported the composition of carotenoids in *L. barbarum*, with zeaxanthin constituting the largest portion (0.1%). This is important for prevention of AMD as both lutein and zeaxanthin are the main retina pigments (Cooper, Eldridge, & Peters, 1999; Snodderly, 1995). Therefore, it would be a great advantage to the health food industry if zeaxanthin in *L. barbarum* could be isolated for possible production of functional foods in the future.

Flavonoids, a class of polyphenol compounds, are also widely distributed in plants, especially fruits and vegetables (Erlund, 2004; Kanaze, Gabrieli, Kokkalou, Georgarakis, & Niopas, 2003). More than 6000 flavonoids have been characterised in nature, but their variety and amount vary because of differences in growing environments, maturity and growth conditions (Erlund, 2004). The physiological activities of flavonoids, such as anti-cancer, anti-inflammation, and anti-atherosclerosis, have been well documented (Arai et al., 2000; Havsteen, 2002). Thus, it would be beneficial to isolate flavonoids from *L. barbarum* for use as a nutritional supplement.

Polysaccharides, composed of 100 or more monosaccharides, are often present in Chinese herbs in large amounts. Polysaccharides can be water-soluble or water-insoluble, with the former types being glucurono β-glucan and β-glucan, and latter xylo-β-glucan, xylomann-β-glucan, hetero-β-glucan and manno-β-glucan (Huie & Di, 2004; Sun, Tang, Gu, & Li, 2005). It has been well established that polysaccharides possess antitumor activity (Sheng et al., 2007), and they can enhance immunity through production of interleukin and antibody (Yang et al., 2008). However, the antioxidant activity of polysaccharides in *L. barbarum* still remains unknown.

In view of the impact of carotenoids, flavonoids and polysaccharides on human health, this study was aimed to develop a preparative chromatography method for isolation of these functional components from *L. barbarum* and to study their antioxidant activity.

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2. Materials and methods

2.1. Materials

A total of 12 kg of *L. barbarum* fruits was purchased from a local drug store in Taipei, Taiwan, and was stored at −20 °C for use. Adsorbents for column chromatography, including magnesium oxide, diatomaceous earth and Cosmosil 140C18-OPN (particle size 140 μm), were obtained from Sigma (St. Louis, MO, USA), J.T. Baker (Phillipsburg, NJ, USA) and Nacalai (Kyoto, Japan), respectively. The ion-exchange resin, DEAE-Sepharose CL-6B, was from GE Amersham Bioscience (Uppsala, Sweden).

Flavonoid and phenolic acids standards, including caffeic acid, gallic acid, chlorogenic acid, *p*-coumaric acid, rutin and kaempferol, with purities of 99%, 98%, 95%, 98%, 95% and 90%, respectively, were from Sigma. Internal standard, taxifolin, with a purity of 85% was also from Sigma. Carotenoid standards, such as all-*trans*-zeaxanthin and all-*trans*-β-cryptoxanthin, were from Extrasynthese Co. (Genay, France), and all-*trans*-β-carotene was from Sigma. Internal standard, β-*apo*-8′-carotenal, was from Fluka Chemical Co. (Buchs, Switzerland). The glucose standard was from Sigma.

The HPLC-grade solvents, including ethyl acetate, methanol, isopropyl alcohol, hexane, acetonitrile, methylene chloride, acetone, acetic acid and toluene, were from Lab-Scan Co. (Gliwice, Poland). Formic acid was from Riedel-de-Haën Co. (Seelze, Germany). Deionized water was made using a Milli-Q water purification system from Millipore Co. (Bedford, MA, USA).

Chemicals, including iron(II) chloride, iron(III) chloride and potassium ferricyanide, were from Showa Chemical Co. (Tokyo, Japan). Ferrozin, trichloroacetic acid (TCA), α-tocopherol, trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), gallic acid, DPPH(2,2-diphenyl-1,picrylhydrazyl), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid), TBA (2-thiobarbituric acid), hydrogen peroxide, NBT (NitroBlue Tetrazolium), NADH (β-nicotinamide adenine dinucleotide), 2-deoxy-D-ribose, PMS (phenazine methosulphate), and proteinase (from *Bacillus licheniformis* Subtilisin A) were from Sigma. Disodium hydrogen phosphate, sodium dihydrogen phosphate, manganese oxide, ascorbic acid, sodium nitrite, aluminium chloride, EDTA (ethylenediaminetetraacetic acid), BHT (butylated hydroxytoluene), iron(II) sulphate heptahydrate and butylated hydroxyanisole were from Nacalai Tesque Co. (Kyoto, Japan). The Folin–Ciocalteu reagent was from Merck (Darmstadt, Germany). Sodium hydroxide was from Riedel-de Haën Co. (Seezle, Germany). Anhydrous sodium sulphate was from Nacalai Tesque Co. Polyacryl cotton was from Applied Separation Co. (Allentown, PA, USA). BSA (bovine serum albumin) was from Sigma. Sulphuric acid, boric acid, methyl red and bromophenol blue were from Panreac Co. (Barcelona, Spain).

2.2. Instrumentation

The HPLC instrument was composed of a column temperature controller (G1316A), a degasser (G1379A), a quaternary pump (G1311A), and a photodiode-array detector (G1315B), all of which were from Agilent Technologies (Palo Alto, CA, USA). An evaporative light scattering detector (ELSD) 800 was from Alltech (Deerfield, IL, USA). The quadrupole LC/MS (model 6130), with multimode ion source (ESI and APCI), was from Agilent Technologies. The preparative column (C26140) was from GE Amersham Biosciences Co. (Uppsala, Sweden).

The YMC C30 reversed-phase column (250 × 4.6 mm I.D., particle size 5 μm) and C30 guard column (6 x 4.6 mm I.D.) were from the Waters Co. (Milford, MA, USA). The Vydac 201TP54 C18 column (250 × 4.6 mm I.D., particle size 5 μm) was from the Vydac Co. (Hesperia, CA, USA). The Cosmosil 5Diol-300-II column (300 × 7.5 mm I.D., particle size 5 μm) was from Nacalai Tesque. The spectrophotometer (Beckman DU 640) was from Beckman (Fullerton, CA, USA). The rotary evaporator (Eyela N-1) was from the Eyela Co. (Tokyo, Japan). The high-speed centrifuge (Sorvall RC5C) was from DuPont (Wilmington, Delaware, USA). The sonicator (model DC 400H) was from Taipei, Taiwan. The homogenizer (model 890-68) was from the Oster Co. (Wisconsin, USA). The shaker (V–U) was from Hsiang-Tai Co. (Taipei, Taiwan).

2.3. Extraction and preparation of carotenoids

A 10 g powdered fruit sample of *L. barbarum* was mixed with 50 ml of 10% anhydrous sodium sulphate solution, after which the mixture was shaken for 3 min, followed by adding 100 ml of hexane–ethanol–acetone–toluene (10:6:7:7, v/v/v/v) and shaking for 1 h. Then the solution was centrifuged at 4000 rpm for 1 min and the supernatant was collected. The residue was repeatedly extracted with 50 ml of hexane until colourless. The supernatants were combined, evaporated to dryness under vacuum, and dissolved in 100 ml of hexane–ethanol–acetone–toluene (10:6:7:7, v/v/v/v). A 5 ml aliquot of 40% methanolic KOH solution was added and saponification was carried out under nitrogen in the dark for 6 h. Next, the extract was evaporated to dryness, and dissolved in 20 ml of methylene chloride. For preparative chromatography, 5 ml of concentrated carotenoid extract was poured into a column (300 × 16 mm I.D.) containing a mixture of 7.5 g magnesium oxide and 5 g diatomaceous earth (1.5:1, w/w) to form a height of about 10 cm, followed by adding anhydrous sodium sulphate to form a layer of approximately 1 cm above the adsorbent. β-Carotene was eluted with 30 ml of 100% hexane, neoxanthin and β-cryptoxanthin with 50 ml of 100% ethyl acetate, and zeaxanthin with 50 ml of ethyl acetate/ethanol (80:20, v/v). The carotenoid composition in each fraction was determined using an HPLC method based on Inbaraj et al. (2008). A binary mobile phase of methanol/acetonitrile/water (81:14:5, v/v/v) (A) and methylene chloride (B) was used with the following gradient elution: 84% A and 16% B initially, decreased to 83% A in 22 min, 45% A in 40 min, 25% A in 55 min and returned to the initial ratio in 60 min. A Waters YMC C30 column was used for separation with detection at 450 nm, column temperature at 25 °C and flow rate at 1 ml/min. The various carotenoids were identified and quantified using an HPLC-MS technique, as described in our previous study (Inbaraj et al., 2008).

2.4. Quantitation of carotenoids

For quantitation, an internal standard, β-*apo*-8′-carotenal, was used for calibration of each standard by taking ten concentrations of all-*trans*-zeaxanthin (0.5, 1, 5, 10, 20, 30, 50, 100, 150 and 170 μg/ml) and seven concentrations each of all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene and mixing with β-*apo*-8′-carotenal for a final concentration of 15 μg/ml. Standard curves were prepared by plotting concentration ratio of carotenoid standard to internal standard against its area ratio and the regression equations were $y = 0.6689x - 0.0484$, $y = 0.7869x - 0.0175$ and $y = 0.9425x - 0.0293$ for all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene, respectively, with the R^2 values being 0.9982, 0.9985 and 0.9949. Because of the unavailability of commercial standards of *cis*-carotenoids, the quantitation of the *cis* isomers was based on the standard curves of their corresponding all-*trans* forms, while neoxanthin was quantified by multiplying concentration of β-*apo*-8′-carotenal by peak area ratio of neoxanthin to β-*apo*-8′-carotenal. For determination of both detection limit (DL) and quantitation limit (QL), three concentrations (0.025, 0.05 and 0.1 μg/ml) of each standard were prepared and analysed by HPLC. Based on S/N ⩾ 3 and S/N ⩾ 10, the DL and

QL were calculated to be 0.05 and 0.15, 0.025 and 0.075, and 0.025 and 0.075 μg/ml, respectively, for all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene.

2.5. Extraction and preparation of flavonoids and phenolic acids

A method, based on Lu (2006), was modified to extract flavonoids and phenolic acids (acidic flavonoids) from *L. barbarum*. A 10 g powder sample of *L. barbarum* fruits was mixed with 100 ml of 80% ethanol, after which the mixture was shaken in a 90 °C water bath for 2 h, followed by centrifuging at 6000 rpm for 25 min, and collecting and filtering supernatant to remove impurities. For preparative chromatography of flavonoids and phenolic acids, a 5 ml of extract (pH 7) was poured into a glass column (300 × 25 mm I.D.) containing 25 g of adsorbent Cosmosil 140C18-OPN, which was preactivated with 100 ml of methanol and 50 ml of deionized water. Then 100 ml of deionized water were added to elute phenolic acids and 40 ml of methanol to elute flavonoids. Next, both fractions were separately collected, evaporated to dryness under vacuum, dissolved in 10 ml of acetonitrile–water (1:1, v/v), filtered through a 0.2 μm membrane filter and 20 μl were injected for HPLC analysis of phenolic acids and flavonoids in each fraction. A ternary solvent system of 0.1% formic acid solution (A), tetrahydrofuran (B) and acetonitrile (C), with the following gradient elution, was used: 98% A and 2% B initially, maintained for 3 min, decreased to 93% A, 2% B and 5% C in 11 min, 76% A, 2% B and 22% C in 20 min, 68% A, 2% B and 30% C in 25 min, 63% A, 2% B and 35% C in 30 min, and returned to 98% A and 2% B in 35 min. The various flavonoids and phenolic acids were identified and quantified, based on an HPLC-DAD-ESI-MS technique developed by Lu (2006).

2.6. Quantitation of phenolic acids and flavonoids

Both phenolic acids and flavonoids were quantified using an internal standard, taxifolin, which was dissolved in acetonitrile–water at a concentration of 200 μg/ml. Seven concentrations of 1, 5, 7, 10, 15, 17 and 20 μg/ml, for chlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin and kaempferol, were separately prepared in acetonitrile–water (1:1, v/v). Similarly, seven concentrations of 5, 7, 10, 15, 17, 20 and 25 μg/ml were prepared for rutin in acetonitrile–water (1:1, v/v). All the seven concentrations of each phenolic acid and flavonoid standard were then mixed with internal standard taxifolin for a final concentration at 20 μg/ml and 20 μl were injected into the HPLC system. By plotting concentration ratio (phenolic acid or flavonoid standard vs internal standard) against its area ratio, the calibration curve of each standard was prepared and the regression equations determined were $y = 2.734x - 0.035$, $y = 1.350x - 0.051$, $y = 0.716x - 0.052$, $y = 12.00x - 0.092$ and $y = 2.810x + 0.027$ for chlorogenic acid, caffeic acid, *p*-coumaric acid, rutin and kaempferol, respectively, with R^2 values of all being higher than 0.99. The amounts of phenolic acids or flavonoids in *L. barbarum* fruits were quantified using the following formula:

$$\text{phenolic acid or flavonoid } (\mu g/g) = \frac{[(A/RRF)/Ai] \times Ci \times \text{volume of extract} \div \text{recovery}}{Ws}$$

where relative response factor (RRF) = (A/Ai) ÷ (C/Ci), A = peak area of phenolic acid or flavonoid, Ai = peak area of internal standard, C = concentration of phenolic acid or flavonoid (μg/ml), Ci = concentration of internal standard (μg/ml) and Ws = weight of sample (g). Because of the unavailability of commercial standards, caffeoylquinic acid, quercetin-diglycoside and kaempferol-3-*O*-rutinoside were quantified using the standard curves of chlorogenic acid, rutin and kaempferol, respectively. For determination of DL and QL, four concentrations of 0.05, 0.1, 0.5 and 1.0 μg/ml for each phenolic acid and flavonoid standard were prepared, and 20 μl were injected into the HPLC system three times. Based on the criteria described for carotenoids, the DL and QL for chlorogenic acid, caffeic acid, *p*-coumaric acid, rutin and kaempferol were determined to be 0.1 and 0.3, 0.1 and 0.3, 0.05 and 0.15, 0.5 and 1.5, and 1.0 and 3.0 μg/ml, respectively.

2.7. Determination of total phenolic compounds in the phenolic acid fraction

A method, based on Kao and Chen (2006), was used and the amount of total phenolic compounds was expressed as gallic acid equivalents. Six concentrations of 50, 100, 200, 250, 300 and 350 μg/ml of gallic acid standard were prepared and 50 μl of each were collected, followed by adding 200 μl of Folin–Ciocalteau reagent, mixing thoroughly, standing at room temperature for 5 min, adding 1000 μl of 15% sodium carbonate solution, reacting at room temperature for 60 min and measuring the absorbance at 750 nm. The gallic acid standard curve was obtained by plotting concentration against absorbance and the regression equation was used to determine the amount of total phenolic compound for a 50 μl sample from acidic flavonoid (phenolic acid) fractions.

2.8. Determination of total flavonoids in the flavonoid fraction

A method, based on Kao and Chen (2006), was used for determination and the total amount of flavonoids was expressed as catechin equivalents. One mg of catechin standard was dissolved in 1 ml of ethanol/water (3:7, v/v) and 5 concentrations of 1, 5, 10, 25, and 50 μg/ml of catechin standard were prepared. A 500 μl solution of each was collected and mixed with 75 μl of 5% sodium nitrite solution, followed by mixing thoroughly, standing at room temperature for 5 min, adding 150 μl of 10% aluminium chloride solution, standing for a further 5 min, adding 500 μl of 1 N sodium hydroxide solution and measuring the absorbance at 510 nm. The catechin standard curve was prepared by plotting concentration against absorbance and the regression equation was used to determine the total content of flavonoids for a 500 μl sample from the neutral flavonoid fraction.

2.9. Extraction and preparation of polysaccharides

A 10 g fruit sample of *L. barbarum* was mixed with 100 ml of deionized water and homogenised for 1 min, followed by heating in boiling water (100 °C) for 30 min and centrifuging at 6000 rpm for 25 min. After filtering through a filter paper to remove impurities, the crude extract was concentrated under vacuum at 40 °C and diluted to 50 ml with deionized water. The crude extract of polysaccharide (CE) was used for the subsequent antioxidant activity study. Then 250 ml of 95% ethanolic solution was added for precipitation overnight, and the supernatant was removed after centrifugation. The precipitate was vacuum-dried at 40 °C to obtain crude polysaccharide (CP) and ground into powder, which was used for the antioxidant activity study. 0.2 g of dried polysaccharide was collected and dissolved in 40 ml of 50 mM phosphoric acid-buffered solution (pH 8), after which the mixture was heated in a 60 °C water bath for 5 min and 1 ml of 2.5 U/ml of proteinase (Type III from *Bacillus* L.) was added to react at 60 °C (pH 8) for 4 h. This condition was selected as a high degree of protein hydrolysis could be attained. Next, 40 ml of 5% trichloroacetic acid were added to terminate the reaction, followed by cooling for 30 min and centrifuging at 10,000*g* for 15 min to collect the supernatant. Five millilitres of filtrate were poured into a glass column (30 × 2.4 cm) containing 5 g of DEAE-Sepharose CL-6B, which

was previously equilibrated with 150 ml of deionized water and the neutral fraction of polysaccharide (LBPN) was eluted with 200 ml of deionized water and the acidic fractions of polysaccharides LBPa1, LBPa2 and LBPa3 were eluted with 200, 100 and 100 ml of 0.1, 0.26 and 0.6 M of sodium hydroxide, respectively. All of the LBPN, LBPa1, LBPa2 and LBPa3 were used for antioxidant activity study.

2.10. Quantitation of polysaccharides

The amounts of polysaccharide in CE, CP, LBPN, LBPa1, LBPa2 and LBPa3 were determined using the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Briefly, 0.2 ml of polysaccharide solution was mixed with 0.2 ml of 5% phenol solution, followed by adding 1 ml of concentrated sulphuric acid and shaking the mixture for 30 min. The absorbance was measured at 490 nm and used to quantify polysaccharide, based on the standard curve of glucose, which was prepared by plotting six concentrations (10–100 μg/ml) against their absorbances. The polysaccharides in each fraction were further diluted to adjust concentration within the linear range of the standard curve.

2.11. Scavenging of DPPH free radical

The method of Kao and Chen (2006) was used. One ml of flavonoids, polysaccharides, carotenoids, Vit E, ascorbic acid, EDTA or BHA solutions was mixed with a 0.2 ml of 1 mM DPPH· solution (in ethanol) separately, after which each solution was mixed thoroughly and then stood in the dark for 30 min, and the absorbance was measured at 517 nm. The scavenging effect (%) was determined using the formula described by Kao and Chen (2006).

2.12. Trolox equivalent antioxidant capacity (TEAC)

The method of Kao and Chen (2006) was used. A 20 ml solution of 1 mM $ABTS^{\cdot+}$ was filtered through a filter paper containing 2 g of manganese dioxide, after which the filtrate was passed through a 0.2 μm PVDC syringe filter to obtain a blue–green solution. Then the solution was diluted with 5 mM phosphate-buffered saline (pH 7.7) and the absorbance was 1.00 ± 0.02 at 734 nm. For preparation of the trolox standard curve, one ml of $ABTS^{\cdot+}$ solution was mixed with 0.1 ml of trolox standard solutions (50, 100, 150, 200, 300 and 400 μM) prepared in 5 mM of phosphate-buffered saline (pH 7.7), after which each solution was mixed thoroughly for 30 s and then stood for a further 30 s, and the absorbance was measured at 734 nm. The trolox standard curve was obtained by plotting concentration against absorbance, and the regression equation was automatically calculated. Next, 0.1 ml of flavonoids, carotenoids, polysaccharides, α-tocopherol, ascorbic acid, EDTA or BHA was mixed with 1 ml of $ABTS^{\cdot+}$ solution separately, and each solution was mixed thoroughly for 30 s and stood for a further 30 s, after which the absorbance was measured at 734 nm. Then the relative trolox concentration was obtained, based on the regression equation, and the higher the trolox concentration, the better was the antioxidant activity.

2.13. Reducing power

The method of Kao and Chen (2006) was used. One ml solutions of carotenoids, polysaccharides, flavonoids, α-tocopherol, ascorbic acid, EDTA or BHA were mixed with 0.5 ml of 0.2 M phosphate-buffered solution (pH 6.6) and 0.5 ml of 1% potassium ferricyanide, separately, after which each mixture was heated in a 50 °C water bath for 20 min and cooled immediately, followed by adding 0.5 ml of 10% trichloroacetic acid. After mixing thoroughly, one ml of supernatant was collected and treated with 1 ml of deionized water and 1 ml of 0.1% ferrous chloride. The solution was mixed thoroughly for 10 min and the absorbance was measured at 700 nm. The higher the absorbance, the better was the reducing power.

2.14. Chelating of ferrous ion

The method of Kao and Chen (2006) was used. One ml solutions of carotenoids, polysaccharides, flavonoids, α-tocopheol, ascorbic acid, EDTA or BHA were mixed with 3.7 ml of methanol and 0.1 ml of ferrous chloride solution (2 mM), separately, followed by mixing thoroughly for 30 min, and 0.2 ml of ferrozine solution (5 mM) was added. The mixture was reacted at room temperature for 10 min, and the absorbance was measured at 562 nm. A lower absorbance indicated a better ferrous ion-chelating ability. The chelating effect was calculated by using the formula described by Kao and Chen (2006).

2.15. Scavenging ability of hydroxyl free radical

A method based on Ghiselli, Nardini, Baldi, and Scaccini (1998) and Ren et al. (2008) was used. A 200 μl solution of flavonoids, carotenoids, polysaccharides, α-tocopherol, ascorbic acid, EDTA or BHA was mixed with 500 μl each of phosphate-buffered solution (pH 7.4), EDTA-ferrous ion solution (10 mM) and α-deoxyribose solution (10 mM), after which each solution was mixed thoroughly and 100 μl of hydrogen peroxide (10 mM) were added and the whole shaken for a few seconds. The mixture was settled at 37 °C for 15 min, and 500 μl each of 2.8% trichloroacetic acid and 1% TBA (thiobarbituric acid) reagent were added. After heating at 100 °C for 10 min, the absorbance was measured at 532 nm. A lower absorbance indicated a better hydroxyl radical-scavenging ability. The scavenging effect was determined using the formula described by Ren et al. (2008).

2.16. Scavenging ability of superoxide anion

The method of Li, Zhou, and Han (2006) was used. A 0.2 ml solution of flavonoids, carotenoids, polysaccharides, α-tocopherol, ascorbic acid, EDTA or BHA was mixed with 0.4 ml of 150 μM nitro blue tetrazolium (NBT) in phosphate-buffered solution (pH 7.4) and 0.4 ml of 470 μM β-nicotinamide adenine dinucleotide (β-NADH), followed by adding 0.05 ml of 60 μM phenazine methosulphate (PMS), shaking for few seconds, standing at room temperature for 5 min and measuring the absorbance at 560 nm. A lower absorbance indicated a better superoxide anion-scavenging ability. The scavenging effect was calculated by using the formula described by Li et al. (2006).

2.17. Statistical analysis

All the treatments were performed in duplicate and the data were analysed by variance (ANOVA) and Duncan's multiple range test for statistical significance ($\alpha = 0.05$) by using a SAS software system (2004).

3. Results and discussion

3.1. Composition of functional components in fractions of L. barbarum

The HPLC chromatograms of different carotenoid fractions, as well as phenolic acid and flavonoid fractions isolated from *L. barbarum* by preparative column chromatography, are shown in Figs. 1 and 2, respectively. Table 1 shows contents of functional components in different fractions of *L. barbarum* extract. In the

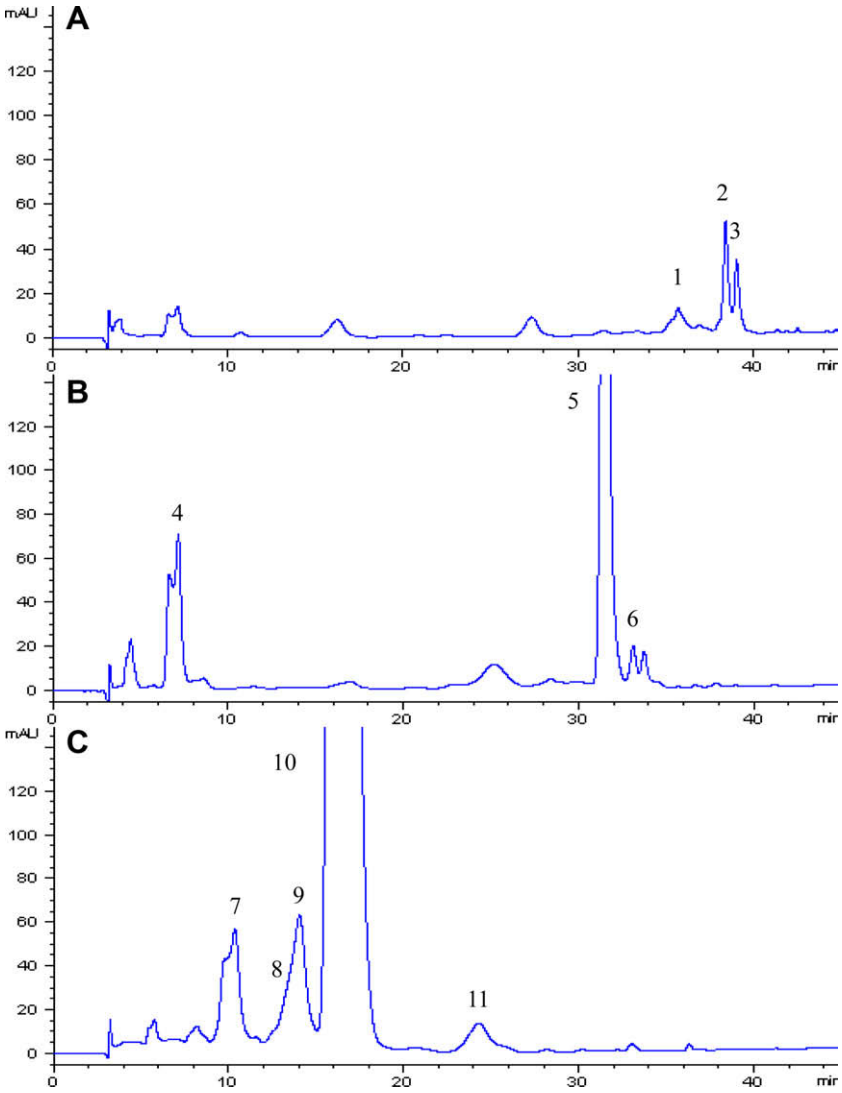

Fig. 1. HPLC chromatograms of three different carotenoid fractions isolated from *L. barbarum* fruits. (A) β-Carotene fraction: peak 1, 13- or 13′-*cis*-β-carotene; peak 2, all-*trans*-β-carotene; peak 3, 9- or 9′-*cis*-β-carotene. (B) Neoxanthin and β-cryptoxanthin fraction: peak 4, neoxanthin; peak 5, all-*trans*-β-cryptoxanthin; peak 6, 9- or 9′-*cis*-β-cryptoxanthin. (C) Zeaxanthin fraction: peak 7, 9- or 9′-*cis*-zeaxanthin; peak 8, 13- or 13′-*cis*-zeaxanthin; peak 9, 15- or 15′-*cis*-zeaxanthin; peak 10, all-*trans*-zeaxanthin; peak 11, 9- or 9′-*cis*-zeaxanthin.

carotenoid fraction, all-*trans*-zeaxanthin and its *cis* isomers constituted the largest portion (1403 μg/g), followed by neoxanthin and cryptoxanthin fraction (72.1 μg/g) and β-carotene fraction (35.9 μg/g). In total, 10 carotenoids were identified in *L. barbarum* extract, including all-*trans* forms of zeaxanthin, β-cryptoxanthin and β-carotene, as well as their *cis* isomers and neoxanthin. In the flavonoid fraction, 3 neutral flavonoids and 4 phenolic acids were present, with the former containing quercetin-diglycoside (66.0 μg/g), rutin (42.0 μg/g) and kaempferol-3-*O*-rutinoside (11.3 μg/g), and the latter containing chlorogenic acid (12.4 μg/g), caffeoylquinic acid (0.34 μg/g), caffeic acid (3.73 μg/g) and *p*-coumaric acid (6.06 μg/g). Obviously the neutral flavonoid was present in a much larger amount than the phenolic acids, on the basis of HPLC analysis. For the polysaccharide fraction, a level of 25.6 μg/g was shown for the neutral polysaccharide, whereas 26.9 μg/g was found for the acidic polysaccharide, including LPBa1 (9.26 μg/g), LPBa2 (9.26 μg/g) and LPBa3 (8.41 μg/g). As several peaks in the flavonoid fraction remained unidentified on the HPLC chromatogram, the total amounts of neutral flavonoid and phenolic acids were also determined using the spectrophotometric method described in the preceding section to avoid quantitation error. This was also used as a basis for calculation of concentration for subsequent antioxidant study. The total amount of flavonoid in the flavonoid fraction, based on catechin equivalents, was shown to be 116 μg/g whereas the total phenolic compounds, based on gallic acid equivalents, was 340 μg/g, which was substantially greater than that by HPLC analysis. Nevertheless, this difference may be also due to overestimation of phenolic content by the Folin–Ciocalteu method, as the reagent added can also react with non-phenolic reducing compounds, such as organic acids, sugars and amino acids. It is also possible that different phenolic compounds may respond differently to Folin–Ciocalteu reagent. For instance, while catechin, caffeic acid, rutin and gallic acid show similar absorption behaviours with the reagent, several flavonoids may show a low absorption, resulting in underestimation of total phenolic content. Likewise, both carotenoid extract and zeaxanthin fraction were selected for antioxidant activity study as the former contained 10 carotenoids and the latter constituted the largest portion, with the total amount in Table 1 being used for concentration calculation, so also were the neutral

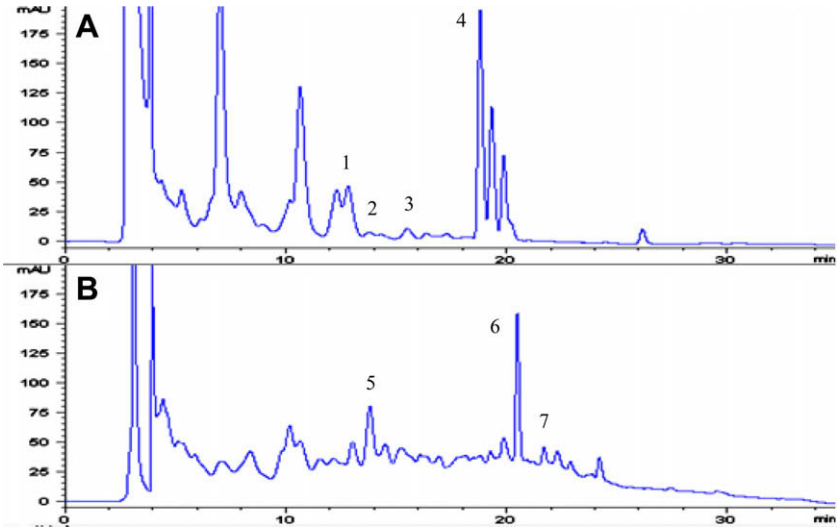

Fig. 2. HPLC chromatograms of phenolic acid (A) and flavonoid (B) fractions isolated from *L. barbarum* fruits. (A) phenolic acid fraction: peak 1, chlorogenic acid; peak 2, caffeoylquinic acid; peak 3, caffeic acid; peak 4, *p*-coumaric acid. (B) flavonoid fraction: peak 5, quercetin-diglycoside; peak 6, rutin; peak 7 kaempferol-3-*O*-rutinoside.

Table 1
Contents of functional components in different fractions of *L. barbarum* extract.

Fraction	Components	Content (mg/g)[a]
Carotenoid		
β-Carotene fraction	13- or 13'-*cis*-β-carotene	8.97 ± 0.00
	All-*trans*-β-carotene	16.6 ± 0.35
	9- or 9'-*cis*-β-carotene	10.3 ± 0.24
Neoxanthin and cryptoxanthin fraction	Neoxanthin	13.2 ± 0.04
	All-*trans*-β-cryptoxanthin	53.3 ± 0.73
	9- or 9'-*cis*-β-cryptoxanthin	5.61 ± 0.03
Zeaxanthin fraction	9- or 9'-*cis*-zeaxanthin	39.3 ± 5.61
	13- or 13'-*cis*-zeaxanthin	4.85 ± 0.80
	15- or 15'-*cis*-zeaxanthin	32.8 ± 2.65
	All-*trans*-zeaxanthin	1326 ± 14.5
Flavonoid		
Neutral fraction	Quercetin-diglycoside	66.0 ± 0.53
	Rutin	42.0 ± 0.39
	Kaempferol-3-*O*-rutinoside	11.3 ± 0.34
Acidic fraction	Chlorogenic acid	12.4 ± 0.67
	Caffeoylquinic acid	0.34 ± 0.30
	Caffeic acid	3.73 ± 0.26
	p-Coumaric acid	6.06 ± 0.16
Polysaccharide		
		Content (mg/g)
Crude fraction	CP[b]	57.2 ± 0.29
Neutral fraction	LPBN[c]	25.6 ± 1.32
Acidic fraction	LPBa1[d]	9.26 ± 0.06
	LPBa2[d]	9.26 ± 0.57
	LPBa3[d]	8.41 ± 1.49
Crude extract	CE[e]	580 ± 20.0

[a] Means of duplicate analyses ± standard deviation.
[b] Crude polysaccharide.
[c] Neutral polysaccharide.
[d] Acidic polysaccharides with different molecular weights.
[e] Crude extract of polysaccharide.

polysaccharide (LPBN), acidic polysaccharide (LPBa1, LPBa2 and LPBa3), crude polysaccharide (CP) and crude extract of polysaccharide (CE).

3.2. DPPH free radical-scavenging activity

Fig. 3A shows the DPPH free radical-scavenging activities of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA. With the exception of EDTA, most treatments were effective in scavenging DPPH free radicals, with Vit C showing the most pronounced effect (96.8%), followed by acidic flavonoids (Fla-A, 96.6%), Vit E (94.1%), BHA (93.7%), rutin (87.7%), LBPa3 (84.9%), neutral flavonoids (82.5%), carotenoid extract (Caro-extract, 70.6%), LBPa2 (69.9%), crude polysaccharide extract (CE, 66.6%), crude polysaccharide (CP, 58.2%), LBPa1 (52.5%), LBPN (38.1%), zeaxanthin standard (Zea-std, 25.2%) and zeaxanthin fraction (19.5%). This outcome clearly revealed a better antioxidative effect of acidic polysaccharides compared to neutral polysaccharides, which should be due to the ability of galacturonic acid present in the former to chelate metal ion and in turn scavenge DPPH radical. A lower DPPH free radical-scavenging activity occurred for both the zeaxanthin fraction and the zeaxanthin standard than for the carotenoid extract, which may be accounted for by the presence of some other lipophilic antioxidant components in the carotenoid extract. In addition, the presence of the β-ionone ring in zeaxanthin may decrease the resonance effect of π electrons because of steric hindrance to lower the free radical-scavenging activity (Jiménez-Escrig, Jiménez-Jiménez, Sánchez-Moreno, & Saura-Calixto, 2000). Moreover, the absorbance measured at 517 nm may interfere with absorption of the chromophore in the zeaxanthin fraction and zeaxanthin standard. Like the acidic polysaccharide, the acidic flavonoids also showed a larger DPPH free radical-scavenging activity than did the rutin standard and the neutral flavonoid.

3.3. TEAC value

The TEAC values of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA are shown in Fig. 3B. The TEAC value was used to assess the scavenging ability of $ABTS^{\cdot+}$ free radicals with the water-soluble vitamin E (trolox) as reference standard for comparison. Of the various treatments, BHA, Vit C, neutral flavonoids, rutin and acidic flavonoids were the most efficient in scavenging $ABTS^{\cdot+}$, which were equivalent to levels of trolox at 367, 366, 365, 364 and 331 μM, respectively. This phenomenon is similar to a report by Soobrattee, Neergheen, Luximon-Ramma, Aruoma, and Bahorun (2005), who observed the ability of flavonoids and phenolic acids, in scavenging $ABTS^{\cdot+}$, to follow the order: procyanidin > flavanol > flavonol > hydroxycinnamic acid > simple phenolic acids. Obviously, the high antioxidant activity of flavonoids can be attributed to hydroxy groups in the

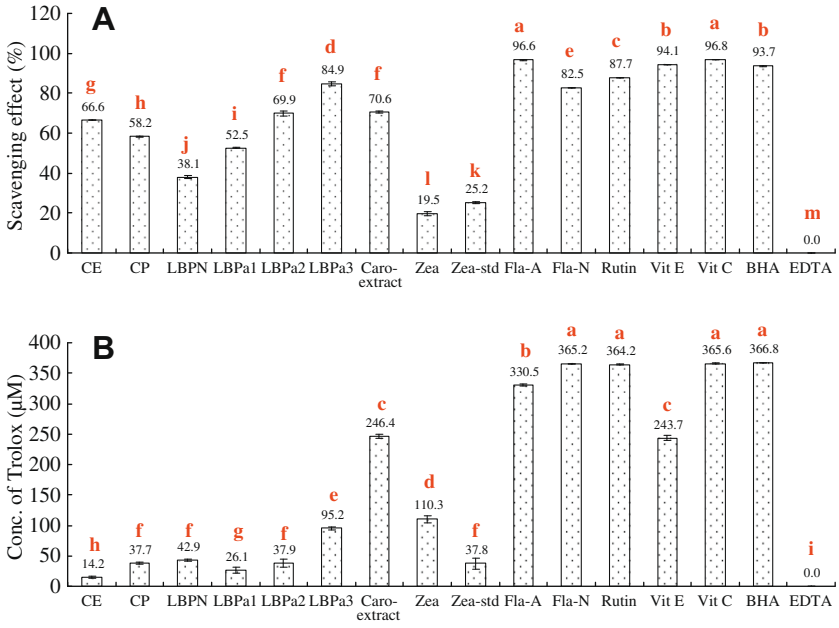

Fig. 3. DPPH free radical-scavenging activity (A) and TEAC values (B) of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA. LBPN: neutral polysaccharide of Lycium fruit; LBPa1: acidic polysaccharide of Lycium fruit (eluted with 0.1 M NaCl); LBPa2: acidic polysaccharide of Lycium fruit (eluted with 0.26 M NaCl); LBPa3: acidic polysaccharide of Lycium fruit (eluted with 0.6 M NaCl); CE: crude extract of polysaccharide; CP: crude polysaccharide; Caro-extract: carotenoids extract; Zea: zeaxanthin fraction; Zea-std; zeaxanthin standard; Fla-A: flavonoids (acidic group); Fla-N: flavonoids (neutral group); Rutin: rutin standard. Data with different letters are significantly different at $P < 0.05$.

A- and B-rings, and the larger the number of hydroxy groups, the higher is the capacity to scavenge free radicals (Kao & Chen, 2006). Both carotenoid extract and Vit E showed similar effects in TEAC values with 246 and 244 μM, respectively, whereas lower TEAC values of 110 and 37.8 μM were found for the zeaxanthin fraction and zeaxanthin standard. As mentioned above, the presence of some other antioxidant components in the carotenoid extract should be responsible for this effect. However, all the polysaccharides showed low TEAC values, ranging from 14.2 μM for CE to 95.2 μM for LBPa3, which should be due to the absence of phenolic-type structures in polysaccharides.

3.4. Reducing power

Fig. 4A shows the reducing powers of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA. A peak reducing power was observed for acidic flavonoids, with ABS at 2.79, followed by neutral flavonoid (1.80), Vit C (1.00), BHA (0.84), rutin (0.54) and Vit E (0.31). Yet, a weak reducing power was found for the other treatments, ranging from 0.05 in ABS for CE to 0.22 for LBPN. Moran, Klucas, Grayer, Abain, and Becana (1997) studied the prooxidant and antioxidant properties of iron complex with phenolic compounds from soybean nodules and reported the hydroxy groups at C-3′ and C-4′ of the B-ring to be more active in reducing iron concentration, while a hydroxy group at C-3 and keto group at C-4 was less active, with the keto group at C-4 and the hydroxy group at C-5 being inactive. However, no activity was observed for the keto group at C-4 and hydroxy group at C-3. In our experiment, the neutral flavonoid was shown to contain rutin, quercetin-diglycoside and kaempferol-3-*O*-rutinoside, all of which belong to the flavonols and the presence of a catechol group in the B-ring should contribute to the reducing power.

3.5. Chelating of ferrous ion

Fig. 4B shows the ferrous ion-chelating ability of carotenoids, flavonoids, polysaccharides, α-tocopherol. Vit C, BHA and EDTA. As expected, EDTA possessed the maximum chelating activity of ferrous ion (99.9%), followed by LBPa2 (95.3%), acidic flavonoid (94.4%) and neutral flavonoid (86.4%). As described previously, the acidic groups in LBPa2 should contribute to the metal ion-chelating ability, so also should the hydroxy groups at C-3′ and C-4′ of the B-ring, as well as at C-5 of the A-ring and keto group at the C-ring of flavonoids (Kao & Chen, 2006). In contrast, a poor ferrous ion-chelating effect was observed for the carotenoid extract (1.8%), zeaxanthin fraction (9.1%) and zeaxanthin standard (0%), which should be caused by absence of the phenolic-type structure. Surprisingly, ascorbic acid (Vit C), a reducing agent containing a carboxyl group, and rutin, a flavonoid containing 4 hydroxy groups, both exhibited a low metal ion-chelating activity, 3.4% for the former and 4.1% for the latter. It was assumed that ascorbic acid may undergo degradation amid the presence of iron(II) chloride in the system used for determination of chelated ferrous ion (Kao & Chen, 2006). Comparatively, both acidic flavonoid and neutral flavonoid showed a much greater metal ion-chelating ability than did rutin, as some other functional components, e.g. polyphenol compounds in the former, may play a significant role. Similar to reducing power, a poor ferrous ion-chelating effect occurred for LBPN (5.0%) and CE (0%), whereas moderate chelating activities were found for CP (38.8%), LBPa3 (42.9%) and LBPa1 (47.0%).

3.6. Scavenging activity of superoxide anion

The superoxide anion-scavenging activities of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA are shown in Fig. 5A, characterised by a poor superoxide anion-scavenging activity for most treatments, with the exception of rutin and LBPa3, which amounted to 28.3% and 14.1%, respectively. It has been well documented that, for flavonoids, the keto group at C-4 and hydroxy group at C-3 or C-5 of the C-ring were effective in scavenging superoxide anion, and the greater the number of hydroxy groups, the better is the scavenging activity (Das & Pereira, 1990; van Ackerm et al., 1995). In addition, the hydroxy groups at C-3′ and C-4′ of the B-ring in rutin should also be responsible for this effect

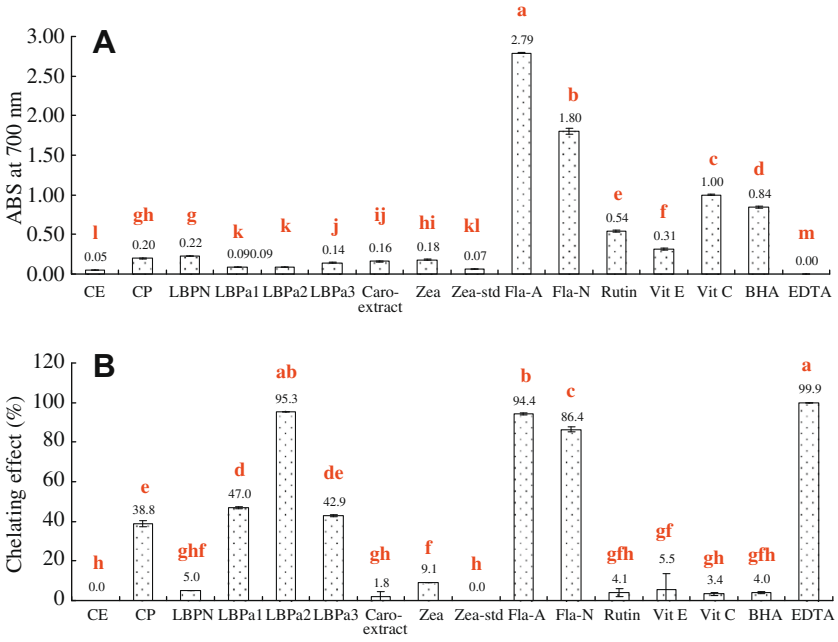

Fig. 4. Reducing power (A) and metal ion-chelating ability (B) of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA. The expansion of abbreviations and statistical significance are the same as given for Fig. 3.

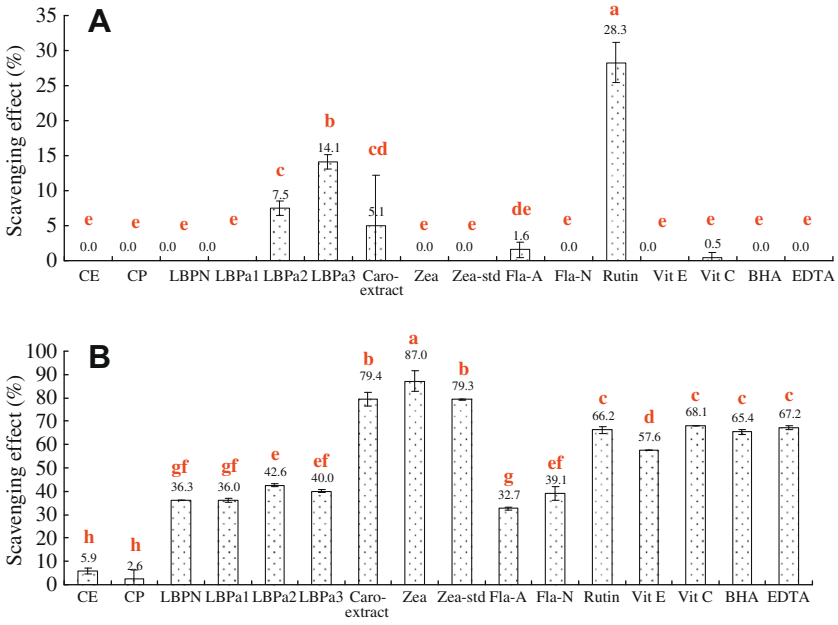

Fig. 5. Superoxide anion-(A) and hydroxyl radical-(B) scavenging activity of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA. The expansion of abbreviations and statistical significance are the same as given for Fig. 3.

because of the hydrogen donation ability (Zhishen, Mengcheng, & Jianming, 1999). Conversely, a low content of rutin in acidic flavonoids and neutral flavonoids may minimise the superoxide anion-scavenging activity. But for the various treatments of polysaccharides and carotenoids, the weak superoxide anion-scavenging activity could be due to a lack of phenolic-type structure.

3.7. Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging activities of carotenoids, flavonoids, polysaccharides, α-tocopherol, ascorbic acid, BHA and EDTA are shown in Fig. 5B. Of all the treatments, carotenoids showed the most distinct effect in scavenging hydroxyl radicals, which equalled 87.0, 79.4 and 79.3% for the zeaxanthin fraction, carotenoid extract and zeaxanthin standard, respectively. The long chain of conjugated double bonds in carotenoids has been demonstrated to play a vital role in scavenging hydroxyl radicals (Trevithick-Sutton, Foote, Collins, & Trevithick, 2006). A similar phenomenon was observed by Trevithick-Sutton et al. (2006), who used an electron spin resonance spectrophotometer to study the hydroxyl radical-scavenging activity of various carotenoids, with zeaxanthin possessing the maximum inhibition effect, followed by β-carotene, lycopene and

lutein. It may be inferred that the non-conjugated double bond within the cyclic ring is more susceptible to bonding with free radicals. However, for acidic flavonoids and neutral flavonoids, only moderate inhibition effects of 32.7% and 39.1% were shown, respectively, which were much lower than the rutin standard (66.2%). As explained above, the hydroxyl groups at C-3′ and C-4′ of the B-ring in rutin should contribute to this effect. For the control treatments, significant scavenging effects of 57.6%, 68.1%, 65.4% and 67.2% were found for Vit E, Vit C, BHA and EDTA, respectively, which were substantially higher than those of flavonoids and polysaccharides. Both CP and CE exhibited poor scavenging activities of 2.6% and 5.9%, respectively, whereas moderate inhibitions occurred for LBPN (36.3%), LBPa1 (36.0%), LBPa2 (42.6%) and LBPa3 (40.0%). This outcome revealed that polysaccharides, purified through a column, possessed a stronger free radical-scavenging activity than those without purification. Furthermore, the galacturonic acid content in acidic polysaccharide should be an imperative factor in enhancing antioxidative activity. This phenomenon was observed by Asker, Mahmoud, and Ibrahim (2007), who isolated acidic polysaccharide from *Bacillus polymyxa* NRC-A and reported the antioxidant activity of polysaccharides to be closely associated with the amount of galacturonic acid. That is, the higher the content of galacturonic acid, the better was the antioxidant activity.

In conclusion, carotenoids in *L. barbarum* L. were extracted with hexane–ethanol–acetone–toluene (10:7:6:7, v/v/v/v), followed by 80% ethanol for flavonoids and phenolic acids, and 100 °C boiling water for polysaccharides. For preparative chromatography, carotenoids were separated by a column containing magnesium oxide and diatomaceous earth at 1:5:1 (w/w), while flavonoids and phenolic acids were separated with a Cosmosil 140C18-OPN column and polysaccharides with a Sepharose CL-6B column. The flavonoid fraction showed the most pronounced effect in scavenging $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ free radicals, chelating metal ions and reducing power, whereas the zeaxanthin and polysaccharide fractions were the most effective in scavenging hydroxy free radicals and superoxide radical anions, respectively. The results of this study may provide a basis for possible production of functional foods in the future with *L. barbarum* as a raw material.

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