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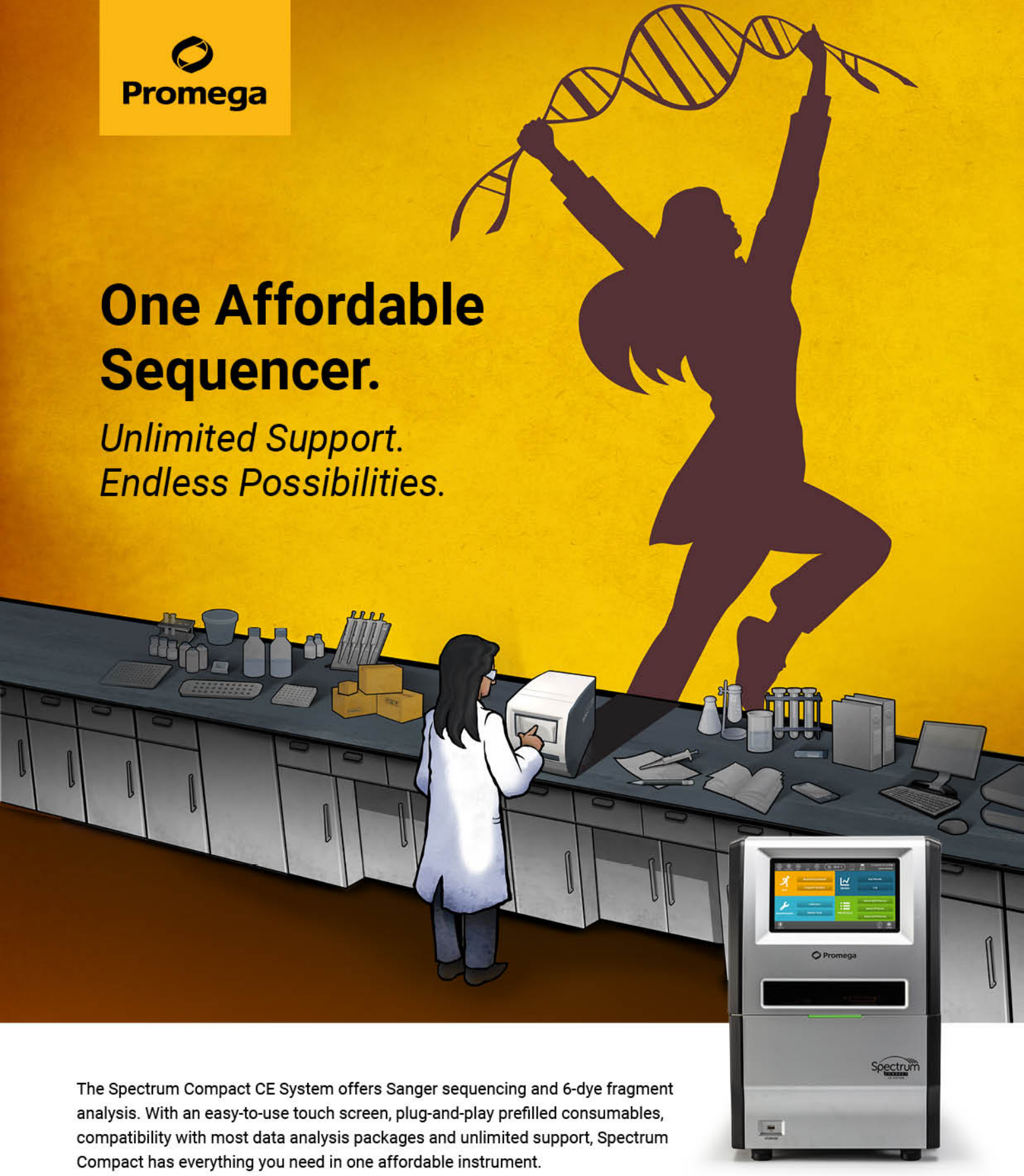

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Flavonoids of Lotus (*Nelumbo nucifera*) Seed Embryos and Their Antioxidant Potential

Mingzhi Zhu, Ting Liu, Chunyun Zhang, and Mingquan Guo

Abstract: Flavonoids from lotus (*Nelumbo nucifera*) seed embryos were fractionated over a macroporous resin chromatography into 2 main fractions (I and II), and subsequently identified by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS^2). Sixteen flavonoids were identified in lotus seed embryos, including 8 flavonoid *C*-glycosides and 8 flavonoid *O*-glycosides, in which the flavonoid *C*-glycosides were the main flavonoids. Among them, 2 flavonoid *O*-glycosides (luteolin 7-*O*-neohesperidoside and kaempferol 7-*O*-glucoside) were identified in lotus seed embryos for the 1st time. For further elucidating the effects of flavonoid *C*-glycosides to the bioactivities of lotus seed embryos, we compared the differences of the flavonoids and their antioxidant activities between leaves and seed embryos of lotus using the same methods. The results showed the antioxidant activity of flavonoids in lotus seed embryos was comparable or higher than that in lotus leaves, whereas the total flavonoid content in seed embryos was lower than lotus leaves which only contained flavonoid *O*-glycosides. The flavonoid *C*-glycosides of lotus seed embryos had higher antioxidant properties than the flavonoid *O*-glycosides presented in lotus leaves. This study suggested that the lotus seed embryos could be promising sources with antioxidant activity and used as dietary supplements for health promotion.

Keywords: flavonoid *C*-glycosides, HPLC-MS, lotus (*Nelumbo nucifera*), seed embryos

Introduction

Lotus (*Nelumbo nucifera*) is a perennial aquatic plant widely distributed throughout Asia, Australia, and North America (Kredy and others 2010). Almost all parts of lotus, including the seed embryos, have long been used as functional foods. The seed embryos have mainly been used for the treatment of nervous disorders, insomnia, and cardiovascular diseases such as hypertension and arrhythmia (National Commission of Chinese Pharmacopoeia 2010). More recently, their anti-ischemic, antioxidant, antiinflammatory, antiarrhythmic, and anti-HIV activities have been revealed (Mukherjee and others 2009). Besides being used as functional food, lotus seed embryos have also been used as Chinese traditional herbal medicines more than 2000 y in Eastern Asia (Mukherjee and others 2009). Increasing attention have been paid on analyzing bioactive components from lotus seed embryos (Zhu and others 2016).

Apart from alkaloids that have been well reported from lotus seed embryos, flavonoids have attracted enough attention due to their health benefits in treating some diseases, including neurodegenerative diseases, type II diabetes, and cardiovascular diseases (Perveen and others 2015). However, systematic studies on flavonoids composition of the lotus seed embryos are scarce, unlike for their leaves, flowers fruits, and other tissues, which have been reported to be rich in flavonoid *O*-glycosides (Chen and others 2012). Li and others (2014) 1st identified flavonoid *C*-glycosides in lotus seed embryos by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS^2), and the flavonoid *C*-glycosides were also identified for the 1st time in lotus. Flavonoid *C*-glycosides received less attention as secondary plant metabolites compared with their well-understood *O*-glycosyl cousins (Courts and Williamson 2015). The *C*-glucosyl bond between the flavonoid carbon skeleton and the saccharide moiety are stable to hydrolytic effect of acidic and enzymatic treatments. This leads to big differences in the bioactivity and pharmacokinetics of these flavonoid *C*-glycosides (Courts and Williamson 2015; Xiao and others 2016). Flavonoid *C*-glycosides have multiple pharmacological benefits including antioxidant, hepatoprotective, antiviral, antiinflammatory, and anticancer activities (Courts and Williamson 2015). Further research into the flavonoid *C*-glycosides of lotus could explore more important applications in functional food industry. In addition, lotus has been widely cultivated throughout Asia and northern Australia, especially in China. It is estimated that annual harvest of dry lotus seeds in China has reached 15000 tons (Guo 2009). To explore other essential applications of lotus seed embryos in food and pharmaceutical industries, it is necessary to thoroughly investigate the constituents and activity of lotus seed embryos. Zhao and others (2014) measured the concentration of some flavonoids and alkaloids in lotus seed rhizomes from Korea, China, Vietnam, and Thailand, and evaluated the antioxidant activities of these lotus seed rhizomes. However, only 4 flavonoids in lotus seed rhizomes were measured. It is necessary to profile the flavonoids in louts seed rhizomes, especially including flavonoid *C*-glycosides.

Consequently, the total flavonoids from lotus seed embryos were fractionated by macroporous resin chromatography, and then subjected to chemical analysis with HPLC-ultraviolet detection (HPLC-UV) and HPLC-MS^2, followed by a subsequent antioxidant activity assay to explore the correlations between chemical components of lotus seed embryos and their nutritional or pharmaceutical activities.

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Materials and Methods

Chemicals and materials

Eight flavonoid standards, apigenin 6-*C*-arabinosyl-8-*C*-glucoside (isoschaftoside), apigenin 6-*C*-glucosyl-8-*C*-arabinoside (schaftoside), apigenin 8-*C*-glucoside, luteolin 6-*C*-glucoside (isoorientin), luteolin 8-*C*-glucoside (orientin), quercetin 3-*O*-glucoside (isoquercitrin), rutin, and quercetin 3-*O*-galactoside (hyperoside) were purchased from Shanghai Tauto Biotech (Shanghai, China). Luteolin7-*O*-neohesperidoside and isorhamnetin 3-*O*-rutinoside were isolated from lotus seed embryos by our team, identified by ^{1}H NMR and ^{13}C NMR, and used as standards for structure identification of LC-MS. Acetonitrile and formic acid (chromatographic grade) were purchased from Sigma-Aldrich Corp. (Shanghai, China). 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), α,α-diphenyl-β-picrylhydrazyl (DPPH), 1,3,5-tri(2-pyridyl)-2.4.6-triazine (TPTZ), butylated-hydroxy-toluene (BHT), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Corp. D101 macroporous resin was produced by Nan Kai Univ. (Tianjin, China). Distilled water was obtained from a Milli-Q System (Millipore, Billerica, Mass., U.S.A.). Other analytical grade chemicals were purchased from China Medicine Group and Shanghai Chemical Reagent Corp. (Shanghai, China). The fresh lotus seed embryos were harvested from Guangchang (Jiangxi, China) in August 2013. The lotus seed embryos were lyophilized, and then powdered before stored at −40 °C.

Extraction and fractionation of crude extract from lotus seed embryos

The lotus seed embryo powers (100 g) were ultrasonically extracted with 1000 mL of 70% ethanol for 40 min. The extraction process was repeated 3 times, and all of the supernatants were combined and concentrated under vacuum to afford the dark-green residues. The residues were re-dissolved in 50 mL H_2O and continuously extracted with petroleum ether (B.P. 60 to 90 °C) to remove low polar components. The residual fraction after petroleum ether extraction was poured in a D101 macroporous adsorption resin column. The column was eluted with distilled water to remove polar compounds including sugars and amino acids. Then the column was washed successively with 30%, 50%, 70%, and 90% ethanol and designated fractions I-IV, respectively. The eluents were evaporated under reduced pressure to remove solvents, and then subjected to both chromatography and bioactivity analysis. The experiment was repeated 3 times.

Method validation

Flavonoids in lotus seed embryos were quantified semiquantitatively by linear regression of rutin at 350 nm. Six concentrations of the rutin solution were measured in triplicate. The limits of detection (LOD) and quantification (LOQ) were calculated, based on signal-to-noise ratios (S/N) of 3/1 and 10/1, respectively. Relative standard deviations (RSDs) values were calculated to express the intra- and interday precisions. The standard solution was examined for 6 replicates within 1 d to validate the intraday precision, although standard solution was analyzed in duplicate for 3 consecutive days to determine the interday precision. Flavonoids in lotus seed embryos are presented as mg/100 g DW (dry weight).

HPLC-UV and HPLC-MS analysis of flavonoids

HPLC-UV analysis was performed on a Thermo Accela 1250 series U-HPLC system (Thermo Fisher Scientific, San Jose, Calif., U.S.A.) coupled with a UV detector. Separation was achieved using a Waters Sunfire C18 column (150 × 4.6 mm, 3.5 μm) at 30 °C. In the solvent system, eluent A was distilled water containing 0.5% (v/v) formic acid, and eluent B was acetonitrile containing 0.1% (v/v) formic acid. The gradient condition was as follows: 88% A in 10 min, 88% A to 80% A in 32 min, 80% A to 70% A in 13 min, 70% A to 40% A in 8 min, 40 % A to 88% A in 1 min followed by 1 min at 88% A. The flow rate was 0.6 mL/min and chromatograms were acquired at 350 nm.

LC-MS was performed on a Thermo Accela 600 series HPLC system equipped with a triple-stage quadropole mass spectrometer (TSQ Quantum Access MAX,Thermo Fisher Scientific, San Jose, Calif., U.S.A.). Mass spectra were acquired using negative ion mode with the following conditions: The spray voltage was set at 3 kV. The capillary temperature and vaporizer temperature were at 350 and 300 °C, respectively. Sheath gas (N_2) and auxiliary gas (N_2) pressures were set at 40 and 10 arbitrary units, respectively. The scan range was from 150 to 1500 amu. The data-dependent mode was used to obtain the MS^2 spectra.

Determination of antioxidant activity of flavonoids

Three assays including DPPH scavenging activity, ABTS free radical scavenging activity, and ferric reducing/antioxidant power (FRAP) assay were used for the determination of the antioxidant activity of flavonoids in lotus seed embryos.

DPPH free radical scavenging activity

DPPH free radical scavenging activity was assessed by the method described by Zhu and others (2015). Ten microliters of appropriately diluted samples (diluted with methanol) or Trolox solution (31.25 to 1000 μM) were reacted with 190 μL of DPPH methanol solution (final concentration was 0.1 mM) in the dark for 30 min. The absorbance was acquired at 517 nm by a multifunctional micro-plate reader by triplicate measurements (Tecan Infinite M200 PRO, TECAN, Switzerland). Methanol was used for the baseline correction, and BHT was used as positive control. Trolox was used for calibration, and the results were expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol TE/g).

ABTS free radical scavenging activity

ABTS free radical scavenging activity was measured using the method of Zhu and others (2015). The $ABTS^+$ cation solution was generated by the reaction of potassium persulfate (4.9 mM in H_2O) with an equal volume of ABTS (7 mM in H_{2O}) in the dark for 12 to 16 h. The $ABTS^+$ cation solution was diluted with 80% ethanol until it reached an absorbance of 0.700 ± 0.005 at 734 nm. 10 μL of properly diluted samples (diluted with ethanol) or Trolox solution (31.25 to 500 μM) were incubated with 190 μL of diluted $ABTS^+$ cation solution in the dark for 30 min. The absorbance was acquired at 734 nm by triplicate measurements. Ethanol was used as the blank, and BHT was used as positive control. Trolox was used for calibration, and the results were expressed as micromoles of TE per gram of sample (μmol TE/g).

FRAP assay

FRAP assay was performed as reported by Zhu and others (2015). Fe_3^+–TPTZ complex solution was prepared with 20 mM $FeCl_3$•$6H_2O$ solution, 10 mM TPTZ solution in 40 mM HCl, and 300 mM acetate buffer (3.1 g $C_2H_3NaO_2$•$3H_2O$ and 16 mL $C_2H_4O_2$, pH 3.6) at a ratio of 1: 1: 10 (v/v/v). Ten microliters of appropriately diluted samples were mixed with 30 μL

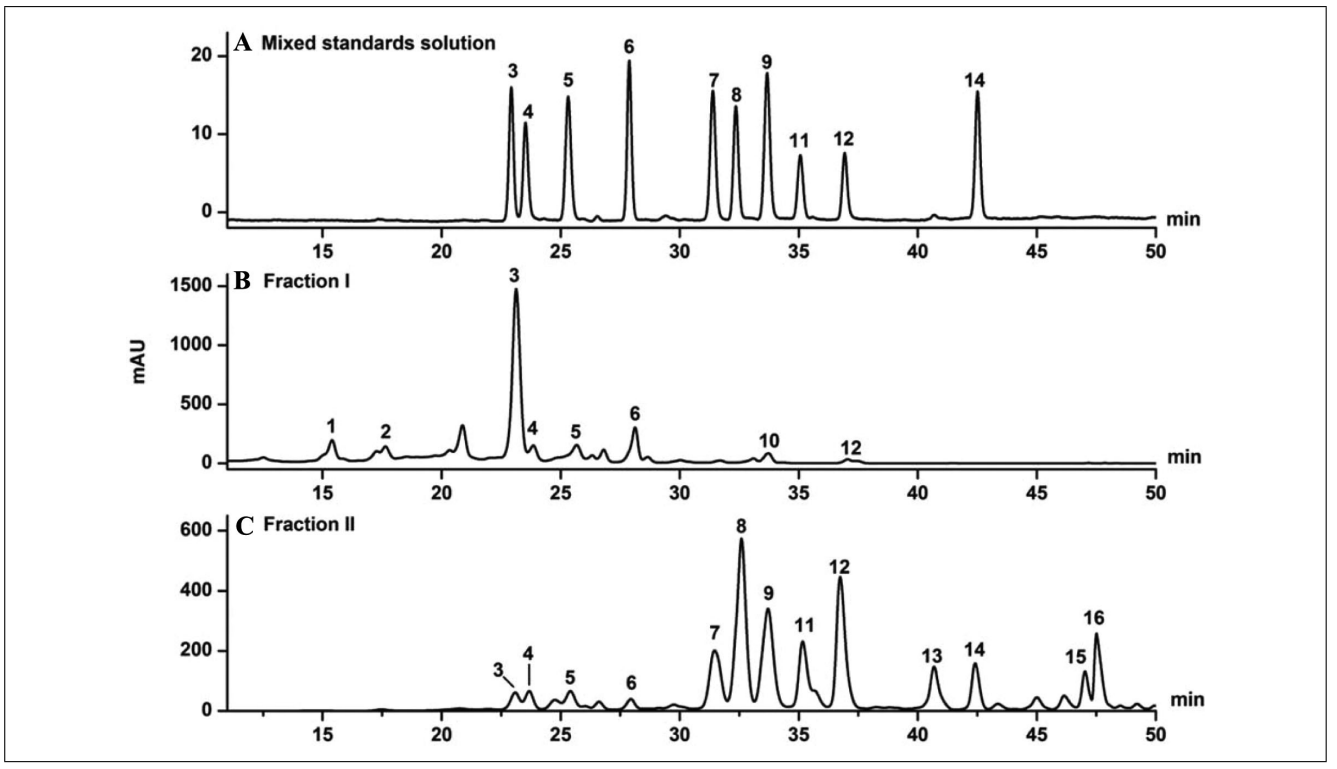

Figure 1–HPLC-UV chromatogram of 10 flavonoids standards (A), fractions I (B), and II (C) from lotus seed embryos recorded at 350 nm.

of distilled water, and then the mixtures were incubated with 260 μL of Fe_3^+–TPTZ complex solution at 37 °C for 10 min. The absorbance was acquired at 593 nm by triplicate measurements. The calibration curve was plotted using $FeSO_4 \cdot 7H_2O$ (0.125 to 2 mM). The results were expressed as millimoles of Fe_2^+ equivalents per 100 g of sample (mmol Fe_2^+ equiv/100 g), and Trolox was used as positive control.

Statistical analysis

Data analysis was performed using SPSS 22. Mean ± standard deviations (SD) value of each sample was calculated by 3 independent experiments. Differences at $P < 0.05$ were considered statistically significant. Differences between fractions of lotus were evaluated using a least significant difference (LSD) analysis of variance.

Results and Discussion

Enrichment and identification of flavonoids

Extraction with 70% ethanol yielded 29.2 g of extract from 100 g of lotus seed embryos. After removing low polar components by liquid/liquid partitioning with petroleum ether, the crude extract (29 g) was then fractionated with D101 macroporous resins chromatography by eluting with 30%, 50%, 70%, and 90% ethanol to yield 4 fractions (I-IV), that is: 3.8 (fraction I), 0.9 (fraction II), 1.4 (fraction III), and 1.4 g (fraction IV), respectively. The chromatographic peaks concentrated on the fractions I and II in the LC profiles, whereas no chromatographic peaks were found in fractions III and IV. Thus an effective fractionation of total flavonoids from lotus seed embryos was accomplished by D101 macroporous resins chromatography. Figure 1 showed the chromatographic profiles of fractions I and II recorded at 350 nm, and 16 peaks were well detected. The LC and MS^2 data including retention times and mass spectra (NI and NI-MS^2) were listed in Table 1.

Flavonoid *C*-glycosides

Natural flavonoids often exist as glycoside forms having 2 types of glycosidic bonds, *O*- and *C*-glycosides (Ferreres and others 2007). The *C*-glycoside is difficult to fragment (Ferreres and others 2011) and the sugar is characterized by the loss sequence of the rhamnosyl residues (−104 and −74 Da), the pentosyl residues (−90 and −60 Da), and hexose residues (−120 and −90 Da) giving rise to $^{0,2}X^-$ ion and $^{0,3}X^-$ ion, respectively (Ferreres and others 2003; Cuyckens and Claeys 2004; Marin and others 2004; Vukics and Guttman 2010). According to the rules described above, peaks 1 to 7 and peak 10 were tentatively identified as flavonoid *C*-glycosides. Peak 1 showed the deprotonated molecular ion ($[M-H]^-$) at *m/z* 593, with its fragments at *m/z* 503 ($^{0,3}X_0^-$, $[M-H-90]^-$), 473 ($^{0,2}X_0^-$, $[M-H-120]^-$), 383 ($[M-H-210]^-$), and 353 ($[(M-H)-240]^-$). The MS^2 of peak 1 were in agreement with di-*C*-hexosyl flavones, suggesting the presence of an apigenin (M_w 270), and 2 hexose residues (M_w 324; Barreca and others 2011). Peak 1 was thus tentatively identified as apigenin 6,8-*C*-di-glucoside (vicenin-2). For peak 2, the precursor ion at *m/z* 579 ($[M-H]^-$) and pattern of fragmentation [519 ($^{0,3}X_0^-$, $[M-H-60]^-$), 489 ($[M-H-90]^-$), 459 ($^{0,2}X_0^-$, $[M-H-120]^-$), 399 ($[M-H-180]^-$), and 369 ($[(M-H)-210]^-$] were observed as typical di-*C*-hexosyl-pentosyl flavones with 1 aglycone: luteolin (M_w 286), and 2 sugar moieties (1 hexose residue [M_w 162], and 1 pentose residue [M_w 132]; Ferreres and others 2003). In addition, the sugar substituent linked at C-6 position of aglycone gave preferential fragmentation rather than that linked to C-8 (Ferreres and others 2003). For peak 2, the relative abundance of the $[M-H-120]^-$ ion (Table 1)

Table 1–Identification of flavonoids in fractions I and II from lotus seed embryos by LC-MS^2.

Peak no.	Rt (min)[a]	NI-MS (*m/z*)	MS/MS data (% base peak, *m/z*)	Identification	Reference
1	15.4	593	503(5) 473(30) 383(65) 353(100)	Apigenin 6,8-di-*C*-glucoside (Vicenin-2)	Barreca and others 2011
2	17.6	579	519(6) 489(34) 459(9) 399(100) 369(90)	Luteolin 6-*C*-glucosyl-8-*C*-arabinoside	Omar and others 2011
3	23.1	563	503(11) 473 (20) 383(77) 353(100)	Apigenin 6-*C*-glucosyl-8-*C*-arabinoside (Schaftoside)	Standard
4	23.7	447	357(71) 327(100)	Luteolin 6-*C*-glucoside (Isoorientin)	Standard
5	25.7	447	357(17) 327(100)	Luteolin 8-*C*-glucoside (Orientin)	Standard
6	28.1	563	503(5) 473(15) 383(45) 353(84)	Apigenin 6-*C*-arabinosyl-8-*C*-glucoside (Isoschaftoside)	Standard
7	31.5	431	341(9) 311(100) 283(33)	Apigenin 8-*C*-glucoside	Standard
8	32.6	609	300(100) 301(42)	Rutin	Standard
9	33.7	463	300(100) 301(35) 271(12)	Quercetin 3-*O*-galactoside (Hyperoside)	Standard
10	33.8	577	503(6) 457(38) 383(77) 353(80)	Apigenin 6-*C*-glucosyl-8-*C*-rhamnoside	Benayad and others 2014
11	35.2	463	300(100) 301(36)	Quercetin 3-*O*-glucoside (Isoquercitrin)	Standard
12	36.7	593	285(44) 284(22)	Luteolin 7-*O*-neohesperidoside	Standard
13	40.7	593	285(100) 284(83)	Kaempferol 3-*O*-robinobioside	Chen and others 2012
14	42.4	623	315(100) 314(22) 300(16)	Isorhamnetin 3-*O*-rutinoside	Standard
15	47.0	447	285(100)	Kaempferol 7-*O*-glucoside	Chen and others 2012
16	47.5	607	299(100) 284(34) 255(11)	Diosmetin 7-*O*-rutinoside (Diosmin)	Li and others 2007

[a]Rt, retention time on C_{18} column.

was higher than the $[M-H-60]^-$ ion indicating that the hexose was located at C-6, although the pentose at C-8. Thus, peak 2 was tentatively identified as luteolin 6-*C*-glucosyl-8-*C*-arabinoside (Omar and others 2011). Peak 10 corresponded to a precursor ion at *m/z* 577 ($[M-H]^-$), with its fragmentation ions at *m/z* 503 ($^{0,3}X_0^-$, $[M-H-74]^-$), 457 ($^{0,2}X_0^-$, $[M-H-20]^-$), 383 ($[M-H-180]^-$), and 353 ($[\{M-H\}-210]^-$). These data were in agreement with di-*C*-hexosyl-pentosyl flavones containing 1 aglycone (apigenin) and 2 sugar moieties (1 hexose residue and 1 deoxyhexose residue [M_w 146]; Marin and others 2004). Furthermore, the $[M-H-120]^-$ ion showed higher intensity than $[M-H-74]^-$ ion, which implies that the hexose was located at C-6, although the deoxyhexose was located at C-8. Thus, peak 10 could be tentatively identified as apigenin 6-*C*-glucosyl-8-*C*-rhamnoside (Benayad and others 2014). In addition, peaks 3 to 7 were identified by comparing the LC-MS^2 with the corresponding standard as follows: apigenin 6-*C*-glucosyl-8-*C*-arabinoside (schaftoside), luteolin 6-*C*-glucoside (isoorientin), luteolin 8-*C*-glucoside (orientin), apigenin 6-*C*-arabinosyl-8-*C*-glucoside (isoschaftoside), and apigenin 8-*C*-glucoside.

Flavonoid *O*-glycosides

Compared with flavonoid *C*-glycosides, the fragmentation of flavonoid *O*-glycosides is characterized by losing of the sugar moiety (Ablajan and others 2006; Liu and others 2006). The typical fragments of deprotonated aglycone ion (Y_0^-) or radical aglycone ion ($[Y_0-H]^-$.) were yielded by the loss of sugar units in MS^2 (Ferreres and others 2004; Ablajan and others 2006). Peaks 8, 9, and 11 were tentatively identified as quercetin *O*-glycosides by their characteristic fragment ions at *m/z* 301 (Y_0^-) and 300 ($[Y_0-H]^-$; Du and others 2012). Peaks 8, 9, and 11 were further identified as rutin, quercetin 3-*O*-galactoside (hyperoside), and quercetin 3-*O*-glucoside (isoquercitrin) by comparing their LC-MS^2 with the corresponding standards.

Peaks 12 and 13 exhibited the same precursor ion at *m/z* 593 ($[M-H]^-$) with the similar dominant fragment ions at *m/z* 285 (Y_0^-) corresponding to the deprotonated kaempferol or luteolin, which is also indicative of a neutral loss of 308 Da corresponding to the loss of 2 sugar moieties (1 hexose residue and 1 deoxyhexose residue). They were thus identified as isomers of kaempferol *O*-diglycosides or luteolin *O*-diglycosides. By comparing their LC-MS^2 with the corresponding standard isolated by our team, peak 12 was identified as luteolin 7-*O*-neohesperidoside, which was 1st found in lotus seed embryos. Peak 13 was tentatively identified as kaempferol 3-*O*-robinobioside, which was reported in lotus seed embryos (Chen and others 2012). Peak 14 exhibited the precursor ion at *m/z* 623 ($[M-H]^-$) with the fragment ion at *m/z* 315 (Y_0^-), corresponding to the deprotonate isorhamnetin. Peak 14 was thus assigned as isorhamnetin 3-*O*-rutinoside by comparing its LC-MS^2 spectra with corresponding standard isolated by our team. As for peak 15, the precursor ion was observed at *m/z* 447 ($[M-H]^-$), and the predominant fragment ion at *m/z* 285 corresponded to the deprotonated kaempferol (Y_0^-). For flavonol mono-*O*-glycosides, glycosylation is notably different depending on the glycosylation place. Glycosylation was conjugated at the 7-position if the relative abundance of $[Y_0-H]^-$. ion was lower than that of the Y_0^- ion, and opposite abundance trend was observed when glycosylation happened to 3-position (Ablajan and others 2006). The presence of the high relative abundance of Y_0^- ion indicated that glycosylation happened to 7-position. Thus, peak 15 was identified as kaempferol 7-*O*-glucoside (Chen and others 2012), which was 1st reported in lotus seed embryos. The precursor ion of peak 16 was observed at *m/z* 607 ($[M-H]^-$), and its characteristic fragment ion at *m/z* 299 (Y_0^-), corresponding to deprotonated diosmetin. Peak 16 was tentatively identified as diosmetin7-*O*-rutinoside, which was reported in lotus seed embryos in previous study (Li and others 2014).

In this study, 16 flavonoids were identified in lotus seed embryos, including 8 flavonoid *C*-glycosides and 8 flavonoid *O*-glycosides. Among them, 2 flavonoid *O*-glycosides (luteolin

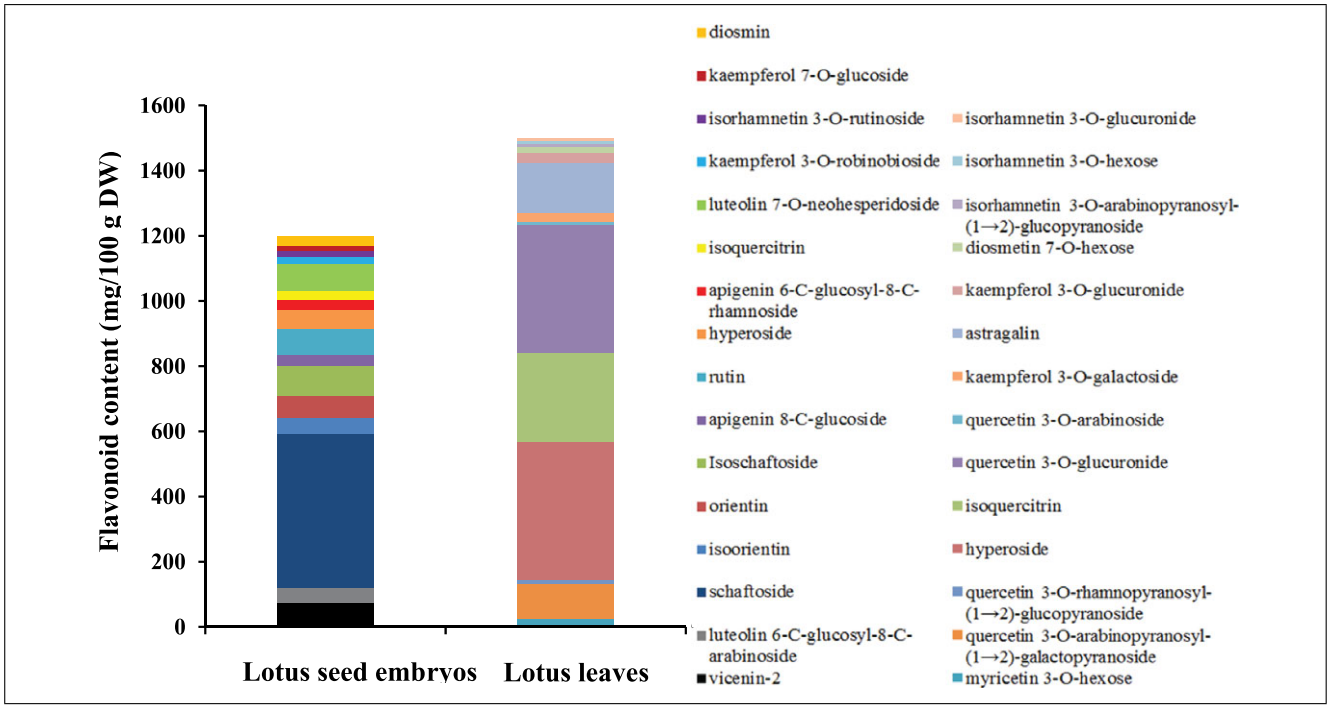

Figure 2–The flavonoid contents in lotus seed embryos and leaves.

7-*O*-neohesperidoside and kaempferol 7-*O*-glucoside) were identified in lotus seed embryos for the 1st time.

Flavonoid content in lotus seed embryos

The calibration curve showed good linearity ($r^2 = 0.9997$) over a relatively wide concentration range (2 to 250 μg/mL). The LOD and LOQ for rutin were 0.21 and 0.69 μg/mL respectively, indicating a high sensitivity of the method with these chromatographic conditions. RSDs values of intraday and interday precisions were 0.54% to 1.80% and 0.35% to 1.88%, respectively, suggesting high repeatability and intermediate precision.

As showed in Figure 2, the total flavonoid content was 1.2 /100 g DW in lotus seed embryos. The flavonoid *C*-glycosides were the main flavonoid in lotus seed embryos accounting more than 70% of the total flavonoid content, in which schaftoside (474.1 mg/100 g DW) was the dominant one accounting more than 35% of the total flavonoid content. Other flavonoid *C*-glycosides, vicenin-2 (72.9 mg/100 g DW), luteolin 6-*C*-glucosyl-8-*C*-arabinoside (46.0 mg/100 g DW), isoorientin (49.5 mg/100 g DW), orientin (67.3 mg/100 g DW), isoschaftoside (91.0 mg/100 g DW), apigenin 8-*C*-glucoside (33.1 mg/100 g DW), and apigenin 6-*C*-glucosyl-8-*C*-rhamnoside (30.5 mg/100 g DW), were also the major components in lotus seed embryos. Compared with flavonoid *C*-glycosides, lotus seed embryos had the low level of flavonoid *O*-glycosides. Five *O*-aglycones, including quercetin, luteolin, kaempferol, isorhamnetin, and diosmetin, were presented in lotus seed embryos, in which quercetin derivatives were the dominant compounds. These quercetin derivatives included rutin (82.1 mg/100 g DW), hyperoside (57.1 mg/100 g DW), and isoquercitrin (27.5 mg/100 g DW). Lotus seed embryos also had a high level of luteolin 7-*O*-neohesperidoside (83.7 mg/100 g DW), followed by kaempferol 3-*O*-robinobioside (21.9 mg/100 g DW), isorhamnetin 3-*O*-rutinoside (17.5 mg/100 g DW), kaempferol 7-*O*-glucoside (14.5 mg/100 g DW), and diosmin (29.1 mg/100 g DW). Furthermore, flavonoids identified in lotus seed embryos differed greatly with the remaining tissue including the lotus leaves, seed coats, flower stamens, and flower petals The flavonoid *C*-glycosides only exist in lotus seed embryos (Du and others 2012; Li and others 2014). Compared with flavonoid *O*-glycosides, the flavonoid *C*-glycosides have received limited attention (Veitch and Grayer 2011). Nonetheless, interest in flavonoid *C*-glycosides has greatly increased in recent years due to their tremendous benefits for human health, including the antioxidant, anticancer, antitumor, hepatoprotective, antidiabetic, antiinflammatory, and memory ameliorating efforts (Courts and Williamson 2015; Xiao and others 2016). Researchers have identified that flavonoid *C*-multiglycosides are absorbed in the intestine as the original structure, which lead the fundamental differences in the pharmacokinetics and bioactivity with *O*-glycosides (Angelino and others 2013). Thus flavonoid *C*-glycosides in lotus need further exploitation.

As we known, leaves and seed embryos are the major edible parts of lotus as functional foods (Zhu and others 2015, 2016). However, the lotus seed embryos were rich in flavonoid *C*-glycosides, especially the flavonoid *C*-multiglycosides, while lotus leaves only contained flavonoid *O*-glycosides (Zhu and others 2015). The difference of flavonoid types may lead to the change of bioactivities between the leaves and seed embryos of lotus. For further elucidating the effects of flavonoid *C*-glycosides to the bioactivities of lotus seed embryos, we compared the differences of the flavonoids and their antioxidant activity between leaves and seed embryos of lotus. The analytical methods of the flavonoids constituents and their antioxidant activity in lotus leaves were the same as the methods used in this study. Flavonoids in lotus leaves were almost enriched in fraction II, and 14 flavonoid *O*-glycosides were identified in lotus leaves in our previous research (Zhu and others 2015). These flavonoid *O*-glycosides are as follows: myricetin 3-*O*-hexose, quercetin 3-*O*-arabinopyranosyl-(1→2)-galactopyranoside, quercetin 3-*O*-rhamnopyranosyl-(1→2)-glucopyranoside, hyperoside, isoquercitrin, quercetin 3-*O*

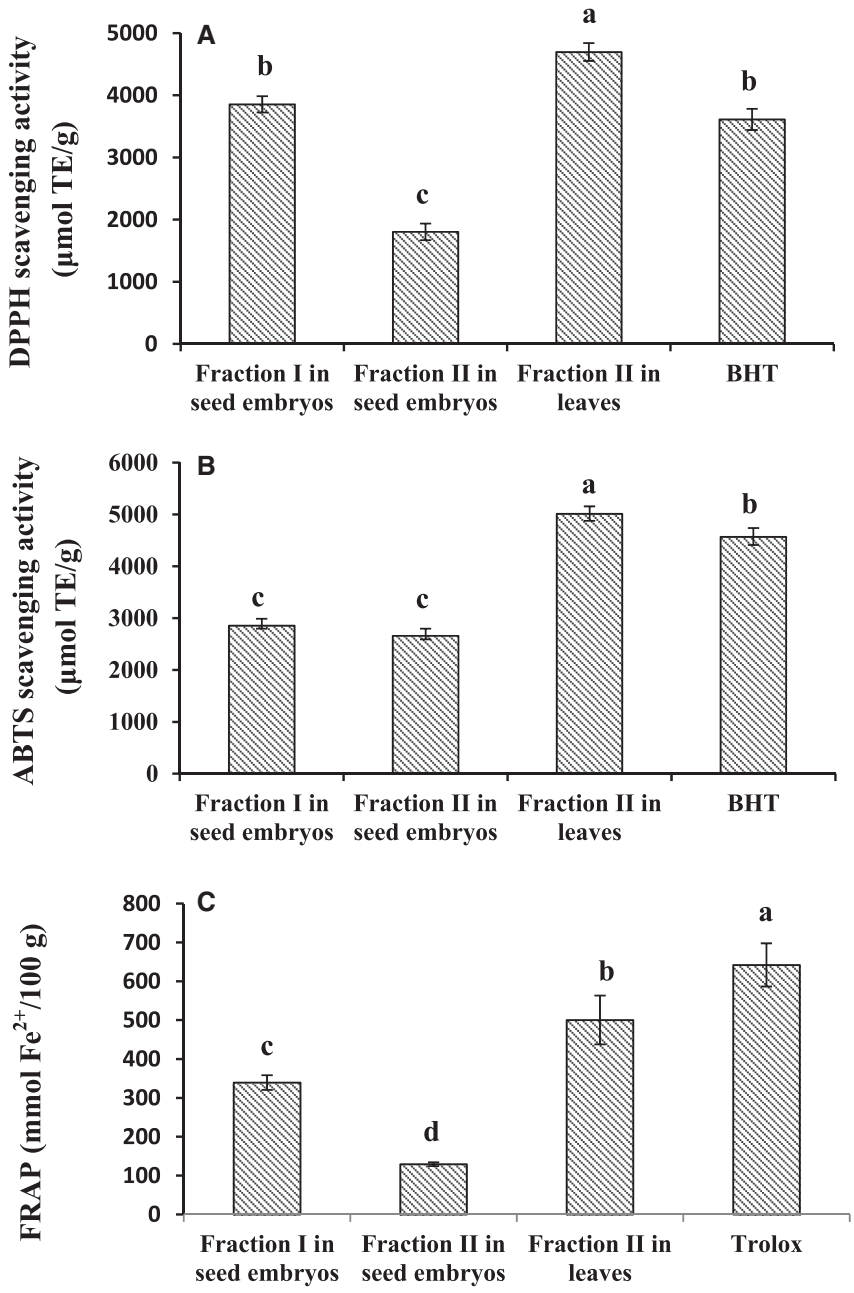

Figure 3–The antioxidant activities of lotus seeds embryos and leaves: (A) DPPH, (B) ABTS, and (C) FRAP. Different lowercase letters show significant differences of the mean by LSD test ($P < 0.05$).

-glucuronide, quercetin 3-*O*-arabinoside, kaempferol 3-*O*-galactoside, astragalin, kaempferol 3-*O*-glucuronide, diosmetin 7-*O*-hexose, isorhamnetin 3-*O*-arabinopyranosyl-(1→2)-glucopyranoside, isorhamnetin 3-*O*-hexose, and isorhamnetin 3-*O*-glucuronide. Furthermore, the flavonoids in lotus leaves were quantified in this study. As shown in Figure 2, the total flavonoid content was 1.50 /100 g DW in lotus leaves, being above that in lotus seed embryos. The quercetin derivatives were the dominant compounds (> 80%) in lotus leaves, in which quercetin 3-*O*-arabinopyranosyl-(1→2)-galactopyranoside (104.9 mg/100 g DW), hyperoside (422.0 mg/100 g DW), isoquercitrin (274.6 mg/100 g DW), and quercetin 3-*O*-glucuronide (393.4 mg/100 g DW) were the major ones. Other quercetin derivatives, quercetin 3-*O*-rhamnopyranosyl-(1→2)-glucopyranoside (13.1 mg/100 g DW), and quercetin 3-*O*-arabinoside (7.7 mg/100 g DW), had the low level in lotus leaves. Lotus leaves also had high contents of kaempferol derivatives, including the astragalin (150.8 mg/100 g DW), kaempferol 3-*O*-galactoside (29.5 mg/100 g DW) and kaempferol 3-*O*-glucuronide (33.6 mg/100 g DW). Other flavonoids had the low level in lotus leaves, including myricetin 3-*O*-hexose (26.7 mg/100 g DW), diosmetin 7-*O*-hexose (16.2 mg/100 g DW), isorhamnetin 3-*O*-arabinopyranosyl-(1→2)-glucopyranoside (10.0 mg/100 g DW), isorhamnetin 3-*O*-hexose (9.8 mg/100 g DW), and isorhamnetin 3-*O*-glucuronide (8.9 mg/100 g DW).

Antioxidant activity of flavonoids in lotus seed embryos

To assess antioxidant potential, a series of methods based on different mechanistic principles need be used in parallel due to the highly reaction-mechanism dependent of the antioxidant assays

(Lopez-Alarcon and Denicola 2013). In this study, we determined the free radical scavenging capacities of flavonoids from lotus seed embryos using DPPH, ABTS, and FRAP assays, the most common assays used to evaluate the antioxidant potential (Niki 2010). The antioxidant activity of flavonoids in lotus leaves were also measured as the same as the methods used in this study to further elucidate the effects of flavonoid *C*-glycosides to the bioactivities of lotus seed embryos (Zhu and others 2015). Results of these evaluations were expressed as TE. As showed in Figure 3, fractions I and II in seed embryos showed appreciable free radical scavenging activities in the DPPH assay. The activity of fraction I in lotus seed embryos (3854.3 μmol TE/g) was comparable with that of BHT (3612.3 μmol TE/g, positive control), whereas fraction II in lotus seed embryos (1803.0 μmol TE/g) exhibited a lower activity. Meanwhile, the activity of fraction II in lotus leaves (4695.3 μmol TE/g) was lower than that of the sum of the fractions I and II in lotus seed embryos, whereas the total flavonoid content of fraction II in lotus leaves were higher than that of the sum of fractions I and II in lotus seed embryos. For scavenging activity patterns in the ABTS assay, the activities of fraction I (2854.8 μmol TE/g) and fraction II (2660.3 μmol TE/g) in seed embryos were nearly half of BHT (4567.0 μmol TE/g). Similar to the DPPH assay, the activity of the sum of the fractions I and II in lotus seed embryos were slightly higher than that of fraction II in lotus leaves (5012.3 μmol TE/g). With regard to the ferric reducing capacity of flavonoids in seed embryos and leaves, the trends were similar with the DPPH and ABTS assay. The activity of the sum of the fractions I (339.3 mmol Fe^{2+}/100 g) and II (129.3 mmol Fe^{2+}/100 g) in lotus seed embryos was comparable to that of fraction II in lotus leaves.

The results demonstrated that both fractions I and II from lotus seed embryos possessed antioxidant potential. Furthermore, the overall trend was that the antioxidant activities of the sum of fractions I and II in lotus seed embryos were comparable or higher than that of fraction II in lotus leaves, whereas the total flavonoid content of the sum of fractions I and II in lotus seed embryos were lower than of fraction II in lotus leaves. This result may be correlated with flavonoid components in lotus seed embryos and leaves. As described above, the flavonoid *C*-glycosides were the major flavonoid in lotus seed embryos, whereas lotus leaves only contained flavonoid *O*-glycosides. A common view is that glycosylation usually affects the antioxidant capacity of the aglycones, and their solubility and stability (Plaza and others 2014). However, the effect of glycosylation has not well been documented, especially the *C*-glycosides. In the present research, the flavonoid *C*-glycosides of lotus seed embryos had higher antioxidant potential than the flavonoid *O*-glycosides presented in lotus leaves. Certainly, direct antioxidant properties measured *in vitro* may lack significant correlation with *in vitro* antioxidant capacity (Mladenka and others 2010). The antioxidant potential of flavonoids in lotus seed embryos needs further investigation *in vivo*.

Conclusions

To explore more sources of natural flavonoids and the correlations between their chemical structures and bioactivities, the flavonoids from lotus seed embryos were fractionated over a macroporous resin chromatography into 2 main fractions (I and II), subsequently identified by HPLC- MS^2, and tested on their antioxidant activity. Sixteen flavonoids were identified in lotus seed embryos, including 8 flavonoid *C*-glycosides and 8 flavonoid *O*-glycosides, in which the flavonoid *C*-glycosides were the main flavonoid. Among them, 2 flavonoid *O*-glycosides (luteolin 7-*O*-neohesperidoside, kaempferol 7-*O*-glucoside) were identified in lotus seed embryos for the 1st time. Moreover, the flavonoid *C*-glycosides identified in this work are not commonly found in most plants, which are valuable for further investigations on their effects on human health. For further elucidating the effects of flavonoid *C*-glycosides to the bioactivities of lotus seed embryos, we compared the differences of the flavonoids and their antioxidant activity between leaves and seed embryos of lotus using the same methods. The results showed the antioxidant activities of flavonoids in lotus seed embryos were comparable or higher than that in lotus leaves, although the total flavonoid content in seed embryos were lower than lotus leaves which only contained flavonoid *O*-glycosides. The flavonoid *C*-glycosides of lotus seed embryos had higher antioxidant property than the flavonoid *O*-glycosides presented in lotus leaves. This study suggested that the lotus seed embryos as valuable sources for natural flavonoids could be further explored and utilized in food and pharmaceutical industries.

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Authors' Contributions

M.G. and M.Z. conceived and designed the experiments; M.Z., and T.L. performed the experiments or helped with the data analysis; M.Z., C.Z., and M.G. wrote and revised the paper. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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