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Antioxidant Activity and Phenolic Compositions of Lentil (*Lens culinaris* var. Morton) Extract and Its Fractions

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ABSTRACT: Phenolic compounds were extracted from Morton lentils using acidified aqueous acetone. The crude Morton extract (CME) was applied onto a macroresin column and desorbed by aqueous methanol to obtain a semipurified Morton extract (SPME). The SPME was further fractionated over a Sephadex LH-20 column into five main fractions (I−V). The phytochemical contents such as total phenolic content (TPC), total flavonoid content (TFC), and condensed tannin content (CTC) of the CME, SPME, and its fractions were examined by colorimetric methods. Antioxidant activity of extracts and fractions were screened by DPPH scavenging activity, Trolox equivalent antioxidant capacity (TEAC), ferric reduced antioxidant power (FRAP), and oxygen radical absorbing capacity (ORAC) methods. In addition, the compositions of active fractions were determined by HPLC-DAD and HPLC-MS methods. Results showed that the fraction enriched in condensed tannins (fraction V) exhibited significantly higher values of TPC, CTC, and antioxidant activity as compared to the crude extract, SPME, and low molecular weight fractions (I−IV). Eighteen compounds existed in those fractions, and 17 were tentatively identified by UV and MS spectra. HPLC-MS analysis revealed fraction II contained mainly kaempferol glycoside, fractions III and IV mainly contained flavonoid glycosides, and fraction V was composed of condensed tannins. The results suggested that the extract of Morton lentils is a promising source of antioxidant phenolics and may be used as a dietary supplement for health promotion.

KEYWORDS: *Lens culinaris* var. Morton, phenolics, antioxidant activity, fractionation

INTRODUCTION

Legumes, including lentils (*Lens culinaris* L.), peas (*Pisum sativum* L.), chickpeas (*Cicer arietinum* L.), and common beans (*Phaseolus vulgaris* L.), are among the oldest crops cultivated by humans and are important crops consumed in Europe, the Middle East, Africa, and South Asia. Although the global consumption of pulse is in decline, lentils consumption is steadily increasing. The annual production of lentils is reported to be about 4 megatons.[1] Lentils not only are an excellent source of macronutrients such as protein, fatty acids, fibers, and carbohydrates but also contain phytochemicals,[2] which can be categorized into phenolic acids, flavanols, flavonols, soyasaponins, phytic acid, and condensed tannins.[3] Some phytochemicals were thought to be antinutrional factors in the past. However, the functions of phytochemicals in lentils have been reversed nowadays, and they works as a "double-edged sword".[4] Epidemiological studies suggest that lentils confer protection against chronic diseases through a multitude of biological activities including antioxidant and anticancer activities, angiotensin I-converting enzyme inhibition, reduction of blood lipid, and reduction of the risk of cardiovascular diseases.[5,6]

A recent study aiming at comparing the phenolic contents and antioxidant activity of legumes, including peas, lentils, chickpeas, common beans, and soybeans, in our laboratory showed that lentils possessed the highest concentrations of phenolic contents and antioxidant activity, and the higher antioxidant activity was strongly correlated with the phenolic contents.[7] The antioxidant activity of the extract from legumes has been investigated. Amarowicz et al. studied the antioxidant activity of low molecular weight and tannin fractions of adzuki bean using a β-carotene−linoleate model, DPPH scavenging activity, and reducing power.[8] Troszyńska et al. determined the phenolic contents of seed coat of legumes and their Sephadex LH-20 fractions.[9] Karamac et al. extracted and fractionated tannin fractions from tannin-rich plant materials including red lentils and green lentils.[10] However, systematic studies on the fractionation of lentils extracts into different groups and subsequent characterization of their antioxidant activity and phenolic compositions are lacking.

The objectives of this study were (1) to concentrate the phenolic substances in the crude lentils extract by adsorption−desorption and to fractionate substances using gel filtration column into different groups; (2) to determine the antioxidant potential of lentils extracts and its fractions; and (3) to characterize the phenolic compositions of fractions by HPLC and HPLC-MS.

MATERIALS AND METHODS

Materials and Chemicals. Lentils (*L. culinaris* var. Morton) were purchased from Spokane Seed Co. (Spokane, WA). The lentils were ground to powder with an IKA all basic mill (IKA Works Inc., Wilmington, NC) and passed through a 60-mesh sieve. The powders were stored at −20 °C before use. XAD-7 was purchased from Sigma-Aldrich Inc. (St. Louis, MO) and Sephadex LH-20 from Pharmacia LKB Biotech. (Uppsala, Sweden).

Sixteen phenolic acid standards (gallic, protocatechuic, 2,3,4-trihydroxybenzoic, *p*-hydroxybenzoic, gentistic, vanillic, caffeic, chlorogenic,

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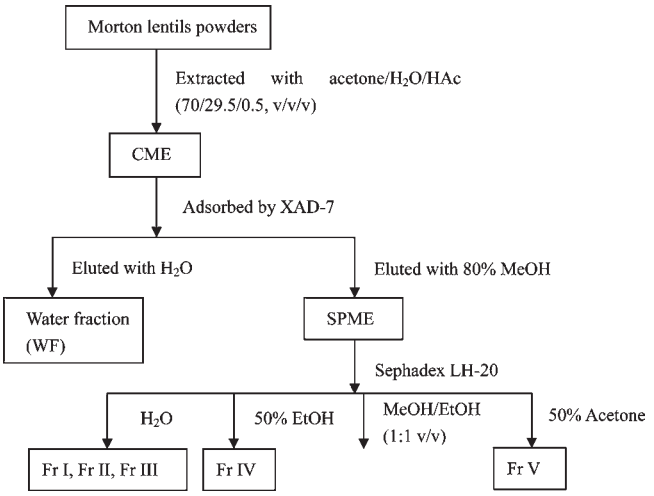

Figure 1. Flow diagram of extraction and fractionation of Morton lentils.

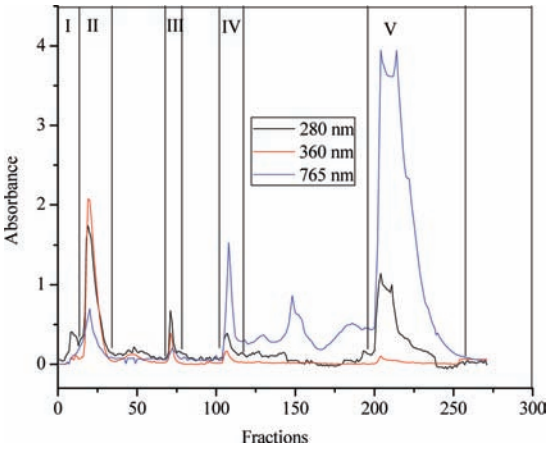

Figure 2. Elution curve of fractionation of semipurified Morton extract over Sephadex LH-20. Ten milliliters per fraction was collected. Each fraction was monitored at 280 and 360 nm, and each fraction was also detected at 765 nm after reaction with Folin−Ciocalteu reagent. Fractions were combined according to their absorbance.

syringic, *p*-coumaric, *m*-coumaric, *o*-coumaric, ferulic, salicylic, sinapic, and *trans*-cinnamic acids), three aldehydes (vanillin, syringealdehyde, and protocatechualdehyde), (+)-catechin, (−)-epicatechin, epigallocatechin, epicatechin gallate, myricetin, luteolin, quercetin, kaempferol, 2-diphenyl-1-picrylhydrazyl radical (DPPH), fluorescein disodium (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), potassium persulfate, trifluoroacetic acid (TFA), Folin−Ciocalteu reagent, sodium carbonate, and 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Sigma-Aldrich Inc. Kaempferol-3-glucoside and quercetin-3-glucoside were purchased from Extrasynthese S.A. (Genay, France). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). HPLC-grade solvents (methanol, B&J Brand), analytical grade acetic acid, and other analytical grade solvents used for extraction were purchased from VWR International (West Chester, PA).

Extraction and Fractionation of Crude Morton Extract (CME). The experimental protocol used for the extraction and fractionation of CME is shown in Figure 1. Morton powders (200 g) were extracted with a solvent mixture (acetone/water/acetic acid 70:29.5:0.5, v/v/v) with a solid to solvent ratio of 1:10 (w/v) and subsequently placed on a magnetic stirrer (Thermolyne, Dubuque, IA) at room temperature for 12 h. The extract was filtered through Whatman no. 1 filter paper in a Buchner funnel. The residues were re-extracted twice under the same conditions, and all of the supernatants were combined and concentrated to a small volume at 40 °C using a rotary evaporator (Labconco Co., Kansas City, MO) under vacuum. Then the CME was obtained by lyophilizing the concentrated extract and stored at −20 °C until use.

Two methods were used consecutively for the fractionation of phenolic compounds from CME: adsorption−desorption over a macroporous XAD-7 resin and Sephadex-LH 20 column chromatography.[11] Four grams of CME was suspended in 20 mL of water by vortexing vigorously. The suspension was centrifuged to remove the insoluble part, and the supernatant was filtered to get a clear solution. The residue was suspended in water twice, and all of the supernatants were combined. The clear solution was poured in a column previously packed with a macroporous resin XAD-7 (column of 20 × 1.6 cm, i.d., bed volume (BV) = 33.5 mL). The solution was pumped down through the column at a speed of 1.8 BV/h. The resin was washed with 2 BV of distilled water to remove the sugars, organic acids, and other water-soluble compounds (water eluate). Eighty percent methanol was used to elute the phenolic compounds at a speed of 3.6 BV/h. The eluate was rotary-evaporated under vacuum to remove solvents and then freeze-dried to yield semipurified Morton extract (SPME). A quantity of 0.3 g of SPME was redissolved in water, and the obtained solution was further fractionated over a Sephadex LH-20 column (35 × 2.6 cm, i.d., BV = 185 mL). The column was eluted successively with H_2O (900 mL), 50% aqueous ethanol (600 mL), ethanol/methanol (1:1, v/v, 400 mL), and 50% aqueous acetone (800 mL) at a flow rate of 2 mL/min, and 270 fractions of 10 mL each were collected. The absorbance of each fraction was determined at 280 and 360 nm, using a Multiskan Spectrum microplate reader (Thermo Electron Corp., Asheville, NC). Moreover, each fraction was reacted with Folin−Ciocalteu reagent, and the absorbance of the obtained solution was determined at 765 nm. The fractions were combined into five fractions (I−V) according to the peaks as shown in Figure 2.

Determination of Phenolic Substances. *Determination of Total Phenolic Content (TPC).* Total phenolics in all samples were determined with the Folin−Ciocalteu assay [12] with minor modifications using gallic acid as a standard phenolic compound. Briefly, the freeze-dried samples (50 mg) were dissolved in 5 mL of 50% methanol, and then 5 μL of appropriately diluted extracts or standard gallic acid solutions (31.25, 62.5, 125, 250, 500, and 1000 μg/mL) was mixed with 195 μL of distilled water in a well of a 96-well plate; 25 μL of Folin−Ciocalteu reagent solution was then added. After 6 min, 75 μL of 7% Na_2CO_3 was added and mixed gently. The reaction mixture was kept in the dark for 2 h, and its absorbance was measured at 765 nm against a blank solution, which was prepared according to the procedure described above except that extract solution was substituted by 5 μL of water, using the microplate reader. TPC was expressed as milligrams of gallic acid equivalents (mg GAE/g).

For determining the absorbance of each fraction at 765 nm after reaction with Folin−Ciocalteu reagent, the procedure described above was employed except that 40 μL of fractions and 160 μL of H_2O were mixed in a well, and the elution curve was obtained by plotting the absorbance at 765 nm against the numbers of fractions.

Determination of Total Flavonoid Content (TFC). Total flavonoids in the extracts were determined using a slightly modified colorimetric method described previously.[13] A 30 μL aliquot of appropriately diluted sample solution was mixed with 180 μL of distilled water in a well of a 96-well plate, and subsequently 10 μL of a 5% $NaNO_2$ solution was added. After 6 min, 20 μL of a 10% $AlCl_3$ solution was added and allowed to stand for 6 min; then 60 μL of a 4% NaOH solution was added to the mixture and left to stand for another 15 min. The absorbance of the mixture was determined at 510 nm versus a prepared water blank using a

Multiskan Spectrum microplate reader. (+)-Catechin was used as standard compound for the quantification of total flavonoids. All values were expressed as milligrams of catechin equivalents per gram of sample (mg CE/g sample).

Determination of Condensed Tannin Content (CTC). The CTC in the extracts and its fractions was determined using the modified vanillin assay.[14] Ten microliters of appropriately diluted sample solution was mixed with 200 μL of 4% vanillin solution (in methanol) in a well of a 96-well plate, and then 100 μL of concentrate HCl was added and mixed. After 15 min, the absorbance of the mixture was determined at 500 nm against a blank solution, which was prepared according to the procedure described above except that extract solution was substituted by 10 μL of water. Concentrations of (+)-catechin ranging from 31.25 to 1000 μg/mL were used as standard compound for the quantification of total condensed tannins. All values were expressed as milligrams of catechin equivalents per gram of sample (mg CE/g).

Determination of Antioxidant Activity. *DPPH Free Radical Scavenging Activity.* The radical scavenging activity of the extracts and fractions against DPPH free radical was measured using the method of Brand-Williams,[15] slightly modified as follows: 10 μL of appropriately diluted sample or Trolox solution (31.25, 62.5, 125, 250, 500, 750, and 1000 μM) was added to 190 μL of DPPH solution (final concentration was 0.1 mM in methanol) in a well of a 96-well plate. The mixture was shaken gently and left to stand at room temperature in the dark for 30 min. Thereafter, the absorbance at 517 nm was measured against methanol using a Multiskan Spectrum microplate reader. The DPPH radical scavenging activity of extracts was calculated from the standard curve of Trolox and expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol TE/g).

To determine the IC_{50} of samples on DPPH, four concentrations ranging from 0.00625 to 5 mg/mL (concentrations depended on the type of samples) were used. Controls containing methanol instead of samples were made. Ascorbic acid, vitamin E, and Trolox were used as positive controls. The inhibition of the DPPH radical by the samples was calculated according to the following formula: DPPH scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100. The percentage of scavenging activity was plotted against the sample concentration to obtain the IC_{50}, defined as the concentration of sample necessary to cause 50% inhibition.

Determination of Trolox Equivalent Antioxidant Capacity (TEAC). This assay was performed as reported previously with slight modification.[16] In brief, ABTS radical cations were prepared by mixing equal volumes of ABTS (7 mM in H_2O) and potassium persulfate (4.9 mM in H_2O), and the solution was left to stand in the dark for 12−16 h at room temperature; then the above solution was filtered and diluted with 80% ethanol to an absorbance of about 0.7 at 734 nm. Ten microliters of appropriately diluted samples was added to 190 μL of ABTS solution in a well of a 96-well plate, and the absorbance was recorded at 734 nm after 30 min of incubation at room temperature. Trolox was used as standard, and a standard calibration curve was obtained for Trolox at concentrations of 15.63, 31.25, 62.5, 125, 250, and 500 μM. The TEAC of samples was calculated from the standard curve of Trolox and expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol TE/g). The scavenging activity of different concentrations of extracts and fractions against $ABTS^{\bullet+}$ radical were also measured to calculate the IC_{50}, and the procedure was similar to the DPPH scavenging method described above.

FRAP Assay. The ferric reducing antioxidant power assay was performed as previously described by Benzie and Strain.[17] This method was developed to measure the ferric reduction ability of plasma at a low pH. When the ferric 2,4,6-tripyridyl-*s*-triazine complex (Fe^{3+}−TPTZ) is reduced to the ferrous form (Fe^{2+}−TPTZ), an intense blue color is developed. Briefly, the FRAP reagent was prepared by mixing 10 volumes of 250 mM acetate buffer (pH 3.6), with 1 volume of 10 mM TPTZ in 40 mM HCl and with 1 volume of 20 mM $FeCl_3 \cdot 6\,H_2O$. A total of 10 μL of properly diluted samples and 30 μL of distilled water was added to 260 μL of freshly prepared FRAP reagent in a well of a 96-well plate. The mixture was incubated at 37 °C throughout the reaction. After 8 min, the absorbance was read using a Multiskan Spectrum microplate reader at 593 nm against reagent blank. The FRAP value was calculated and expressed as millimoles of Fe^{2+} equivalents per 100 g of sample (mmol Fe^{2+} equiv/100 g) based on a calibration curve plotted using $FeSO_4 \cdot 7H_2O$ as standard at a concentration ranging from 0.125 to 2 mM.

ORAC assay. The determination of ORAC was carried out according to the method of Prior and others[18] with slight modifications. A Fluostar Optima plate reader (BMG Labtech, Durham, NC) equipped with an incubator and two injector pumps, and fluorecsein as the probe and AAPH as the radical generator, was used. The analysis was performed using polystyrene 96-well microplates (flat bottom, Nalge Nunc International, Denmark), in which 20 μL of appropriately diluted extract, blank, or Trolox was mixed with 200 μL of working fluorecsein solution (1.08×10^{-4} mM in phosphate buffer, pH 7.0) and incubated for 10 min at 37 °C in the built-in incubator. Subsequently, 20 μL of AAPH (43.2 mg/mL in PBS buffer) solution was pumped into the microplate to initiate the reaction. The plate was shaken for 30 s, and the fluorecsein was recorded for 60 cycles with 40 s per cycle with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. All of the samples were laid out in a "forward-then-reverse" manner and analyzed in duplicate. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated on the basis of the difference in the area under the fluorescence decay curve between the blank and each sample. The area under the curve (AUC) was calculated as follows: $AUC = [0.5 + (R_2/R_1 + R_3/R_1 + R_3/R_1 + ... + R_n/R_1)] \times CT$, where R_1 was the fluorescence reading at the initiation of the reaction, R_n was the reading of last measurement, and CT was cycle time in minutes. The net AUC was obtained by subtracting the AUC of the blank from that of a sample or standard. The final ORAC values were calculated as Trolox equivalents per gram sample (μmol TE/g) using a standard curve prepared with 6.25−50 μM Trolox.

HPLC-DAD and HPLC-MS Analysis of Phenolic Compounds. HPLC-DAD analysis was performed on an Agilent 1200 series HPLC systems equipped with a G13798 degasser, a G1312A binary pump, a G1329A autosampler, and a G1315D diode array detector (Agilent Technologies, Santa Clara, CA). HPLC separation was achieved using a Zorbax Stablebond Analytical SB-C18 column (250 × 4.6 mm, 5 μm, Agilent Technologies, Rising Sun, MD) at 40 °C. Elution was performed using mobile phase A (0.1% TFA aqueous solution) and mobile phase B (methanol), and samples (20 μL) were eluted at a flow rate of 0.7 mL/min. The UV−vis spectra were scanned from 220 to 600 nm on a DAD with a detection wavelength of 270 nm. The solvent gradient in volumetric ratios was as follows: 5−30% B over 50 min. The solvent gradient was held at 30% B for an additional 15 min and increased to 100% B at 66 min. The solvent gradient was held at 100% B for an additional 10 min to clean up the column, followed by re-equilibration of the column for 5 min with 95% A and 5% B before the next run. Identification of phenolic compounds was made either by comparison of their retention time or UV and MS spectra with available standards or by LC-MS and MS/MS studies with the spectra consistent with published data.[30,31]

LC-MS was performed on an Agilent 6100 ion trap mass spectrometer equipped with an ESI interface (LC-MSD, Trap SL, Agilent Technologies, Santa Clara, CA). The HPLC separation conditions were almost the same as HPLC-DAD except that the TFA in the mobile phase was substituted by acetic acid. The mass spectrometer was operated in both negative and positive ion modes under the following conditions: nebulizer pressure, 20 psi; dry gas (N_2), 8 L/min; dry temperature, 325 °C; capillary voltage, 3500 V. The mass spectra were recorded in the scale from m/z 100 to 2500. The MS/MS spectra of ions of interest were

obtained using the Smart Fragmentation mode: cutoff 15% of precursor mass, start amplitude 30%, end amplitude 200%, and other MS conditions the same as used in LC-MS.

Statistical Analysis. Data were expressed as the mean ± standard deviation of triplicate measurements. The data were statistically analyzed using statistical software, SAS version 9.1 (SAS institute Inc. Cary, NC). One-way analysis of variance (ANOVA) and Pearson correlation coefficients were conducted, and significant difference was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Extraction, Fractionation, and Phytochemical Contents. Our previous study confirmed that acidic aqueous acetone (acetone/H_2O/HOAc, 70:29.5:0.5, v/v/v) was the best system for extraction phenolics from lentils because the solvent systems gave rise to the highest TPC and antioxidant activity.[19] The yield of CME was 10.1%, which is much higher than those of green lentils and red lentils (5.4 and 5.3%, respectively) reported by Karamac et al.[10] The difference might be attributed to the genotype of lentils and different extraction method. The TPC, TFC, and CTC of the crude Morton extract were 70.0 mg GAE/g, 30.0 mg CE/g, and 61.6 mg CE/g, respectively. Lentils possessed the highest concentrations of TP among apples, cherries, plums, broccoli, cabbages, grapes, dry beans, onions, and potatoes. The TPC in lentils in the present study was 6.93 mg GAE/g lentil powder, which was in the same range as the contents reported by Xu and Chang[7] and higher than that of other fruits and vegetables.

Adsorption on macroporous resin and then desorption by aqueous methanol or ethanol is a popular method for the concentration of polyphenols or recovery natural antioxidants and is extensively used in the food, pharmaceutical, and cosmetic industries. The purification is mainly through the adsorption capacity of resins for compounds with different molecular weights, polarities, or shapes of the molecules in the solution, which leads to differences in affinity for the resins.[20] After purification with resin, the yields of water eluate (WE) and 80% MeOH eluate (semipurified Morton extract, SPME) were 73.1 and 11.0%, respectively. Although the yield of WE was much higher than that of SPME, the TPC (4.9 mg GAE/g) in WE was almost 80 times lower than that of SPME (377.2 mg GAE/g). Therefore, the WE fraction was not taken into account in the following study. Almost all phenolic compounds were adsorbed on the macroporous resin and desorbed by 80% MeOH, which demonstrated that this method was effective in removing organic acids, sugars, and proteins from crude extracts. The TPC, TFC, and CTC in SPME were increased by 5.4-, 6.8-, 5.2-fold compared to those in the crude extract (Table 1).

Adsorption to Sephadex LH-20 in aqueous ethanol and selective debinding with aqueous acetone is an established method for separating tannins from nontannin phenolics.[11] The SPME was further fractionated using a Sephadex LH-20 column, which was eluted successively with H_2O, 50% ethanol, ethanol/methanol (1:1, v/v), and 50% acetone (Figure 1). Five fractions were obtained from this column according to their absorbance at 280, 360, and 765 nm (Figure 2), which represented a total recovery of 59.7% of the SPME applied to the column. Fraction V, eluted with 50% acetone, and fraction II, eluted with water, were the main fractions. The yield of fractions and their TPC, TFC, and CTC are shown in Table 1. Fraction I contained almost no phenolic compounds. The highest TPC was found in fraction V, containing

Table 1. Yield, TPC, TFC, and CTC of Extracts and Fractions of Morton Lentils[a]

	yield (%)	TPC (mg GAE/g)	TFC (mg CE/g)	CTC (mg CE/g)
CME	10.1[b]	70.0 ± 2.2 f	30.0 ± 0.7 d	61.6 ± 2.7 d
SPME	11.0[c]	377.2 ± 8.6 c	202.7 ± 24.3 b	319.5 ± 2.9 c
fraction I	4.7[d]	9.0 ± 0.7 g	0.1 ± 0.0 d	nd
fraction II	10.0[d]	111.8 ± 7.4 e	5.9 ± 1.1 d	nd
fraction III	0.7[d]	262.6 ± 19.6 d	88.7 ± 5.4 c	96.5 ± 9.4 d
fraction IV	3.3[d]	434.4 ± 25.9 b	367.7 ± 21.3 a	546.8 ± 22.1 b
fraction V	41.0[d]	633.3 ± 25.8 a	193.9 ± 5.2 b	744.5 ± 62.2 a

[a] Results are expressed as the mean ± standard deviation ($n = 3$); values with different letters within a column are significantly different ($p < 0.05$). GAE, gallic acid equivalents; CE, catechin equivalents. nd: not detected. [b] Based on Morton lentil powders. [c] Based on CME. [d] Based on SPME.

633.3 mg GAE/g followed by fractions IV, III, and II, which contained 434.4, 262.6, and 111.8 mg GAE/g, respectively. In the case of flavonoids content, fraction IV contained the highest TFC (367.7 mg CE/g), which showed that this fraction, eluted by 50% EtOH, was mainly composed of flavonoids other than phenolic acids or condensed tannins. The CTC, measured using the vanillin/HCl method, was determined among all fractions. Condensed tannins are relatively high molecular weight compounds and can be eluted by acetone with an appropriate polarity. Fractions I and II contained no condensed tannins. The CTC in fraction V was the highest (744.5 mg CE/g), whereas fractions III and IV showed positive color reaction with vanillin/HCl reagent, and it seemed to contain large amounts of condensed tannins. However, the color reaction might be caused by the reaction between catechin or other monomeric flavanols and vanillin/HCl reagent,[21] thus overestimating the content of condensed tannins in fractions III and IV.

Antioxidant Activity of Extracts and Fractions. The antioxidant activity cannot be evaluated by only a single method due to the complex nature of phytochemicals, and the antioxidant activity determination is reaction-mechanism dependent. Therefore, it is important to employ multiple assays to evaluate the antioxidant activity of plant extracts or phytochemicals.[22] Numerous antioxidant methods have been developed to evaluate antioxidant activity and to explain how antioxidants function. Of these, total antioxidant activity, reducing power, DPPH assay, and ORAC are the most commonly accepted assays to evaluate the antioxidant activity of a food matrix. Therefore, a series of assays including DPPH scavenging activity, $ABTS^{\bullet+}$ scavenging activity, FRAP, and ORAC was used for the determination of the antioxidant activity of extracts and fractions of Morton lentils.

DPPH radical is a stable organic free radical. When accepting an electron or hydrogen in the presence of a hydrogen-donating antioxidant, it can be reduced to a nonradical form, DPPH-H. Because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used for screening the antiradical activities of fruit and vegetable juices or extracts.[22] The DPPH scavenging activity of all extracts and fractions was expressed as Trolox equivalents and IC_{50}, which was defined as the necessary concentration at which the radicals generated by the reaction systems were scavenged by 50%. The results are shown in Figure 3A and Table 2. All extracts and fractions except for fractions I and II possessed good DPPH radical scavenging activity. Fraction V presented the highest DPPH scavenging activity (5031.6 μmol TE/g), followed by SPME (3541.3 μmol TE/g),

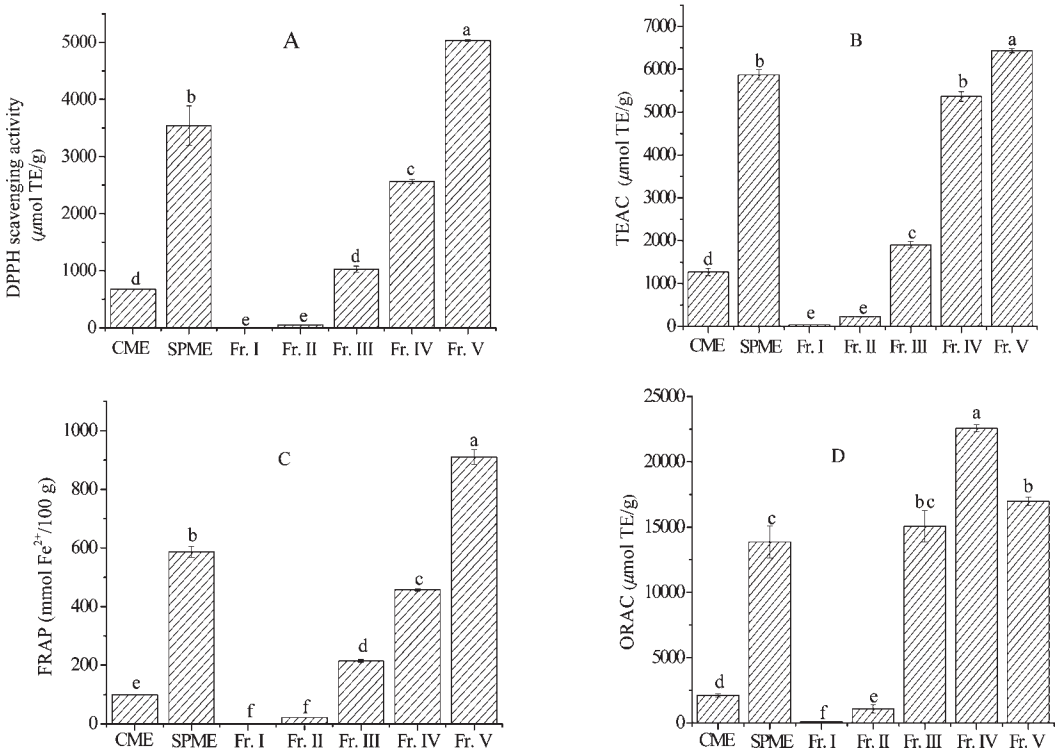

Figure 3. Antioxidant activity of extracts and fractions of Morton lentils: (A) DPPH scavenging activity; (B) TEAC; (C) FRAP; (D) ORAC. Results are expressed as the mean ± standard deviation (n = 3). Bars sharing different letters were significantly different (p < 0.05).

Table 2. IC_{50} Values of Extracts and Fractions of Morton Lentils against DPPH and ABTS Free Radicals[a]

	IC_{50} (mg/mL)	
	DPPH	ABTS
CME	0.781 ± 0.011 a	0.282 ± 0.002 b
SPME	0.144 ± 0.001 cd	0.053 ± 0.001 c
fraction I	>5	>5
fraction II	>5	1.171 ± 0.275 a
fraction III	0.643 ± 0.183 b	0.116 ± 0.011 bc
fraction IV	0.213 ± 0.006 c	0.044 ± 0.002 c
fraction V	0.095 ± 0.002 d	0.031 ± 0.001 c
ascorbic acid	0.086 ± 0.006 d	0.062 ± 0.001 c
α-tocopherol	0.158 ± 0.003 cd	0.124 ± 0.024 bc
Trolox	0.164 ± 0.003 cd	0.092 ± 0.002 c

[a] Results were expressed as means ± standard deviation (n = 3), values with different letters within a column were significantly different (p < 0.05).

fraction IV (2562.8 μmol TE/g), fraction III (1027.0 μmol TE/g), and finally CME (677.8 μmol TE/g). With regard to the IC_{50} values, it was not possible to determine the IC_{50} of fracitons I and II at the range of concentrations tested in the present study. At the highest concentration (5 mg/mL), only 10.4 and 26.8% of DPPH radicals were scavenged by fractions I and II, respectively. The DPPH scavenging activity was in the following order: ascorbic acid > fraction V > SPME > vitamin E > Trolox > fraction IV > fraction III > CME. Ascorbic acid, a strong antioxidant, exhibited the highest DPPH scavenging activity as anticipated. In the case of fractions, fraction V, which had the highest levels of total phenolics, had the lowest IC_{50} value, similar to that of ascorbic acid, and its radical scavenging activity surpassed those of α-tocopherol and Trolox.

The TEAC assay is one of the popular indirect methods of determining the antioxidant activity of compounds or extracts. $ABTS^{\bullet+}$, a cation free radical soluble in both water and organic media, is produced by reacting ABTS solution with potassium persulfate or metmyoglobin. In the absence of antioxidants, the $ABTS^{\bullet+}$ radical is rather stable, but it reacts energetically with an H atom donor and is converted into a noncolored form of ABTS.[23] The TEAC, expressed as Trolox equivalents, is illustrated in Figure 3B. Fraction V had the highest TEAC value (6436.5 mg TE/g), whereas fraction I exhibited the lowest value (41.5 mg TE/g). Interestingly, the Trolox equivalents of extract and fractions determined by TEAC were almost 2 times higher than that of corresponding samples determined by DPPH assay. Antioxidant compounds scavenging ABTS radical at a higher level compared to DPPH radical were also reported by Sachindra et al.[23] In terms of IC_{50}, fraction V had the lowest value, which demonstrated that fraction V possessed the strongest radical scavenging activity against $ABTS^{\bullet+}$. Moreover, the $ABTS^{\bullet+}$ scavenging activity of fraction V was even higher than that of ascorbic acid. The IC_{50} calculated from the regression equation showed the following order: fraction V > fraction IV > SPME > ascorbic acid > fraction III > vitamin E > Trolox > CME > fraction II. However, the differences in IC_{50} among fraction V, fraction IV, SPME, ascorbic acid, Trolox, vitamin E, and fraction III were not significant. The ABTS method was more sensitive than the DPPH assay when measuring the antioxidant activity of water-soluble proteins and peptides, partly due to the differences of the radical's solubility and diffusivity in the reaction medium.[24]

With regard to the ferric reducing capacity of the extracts and fractions, the trend was almost the same as those of the DPPH

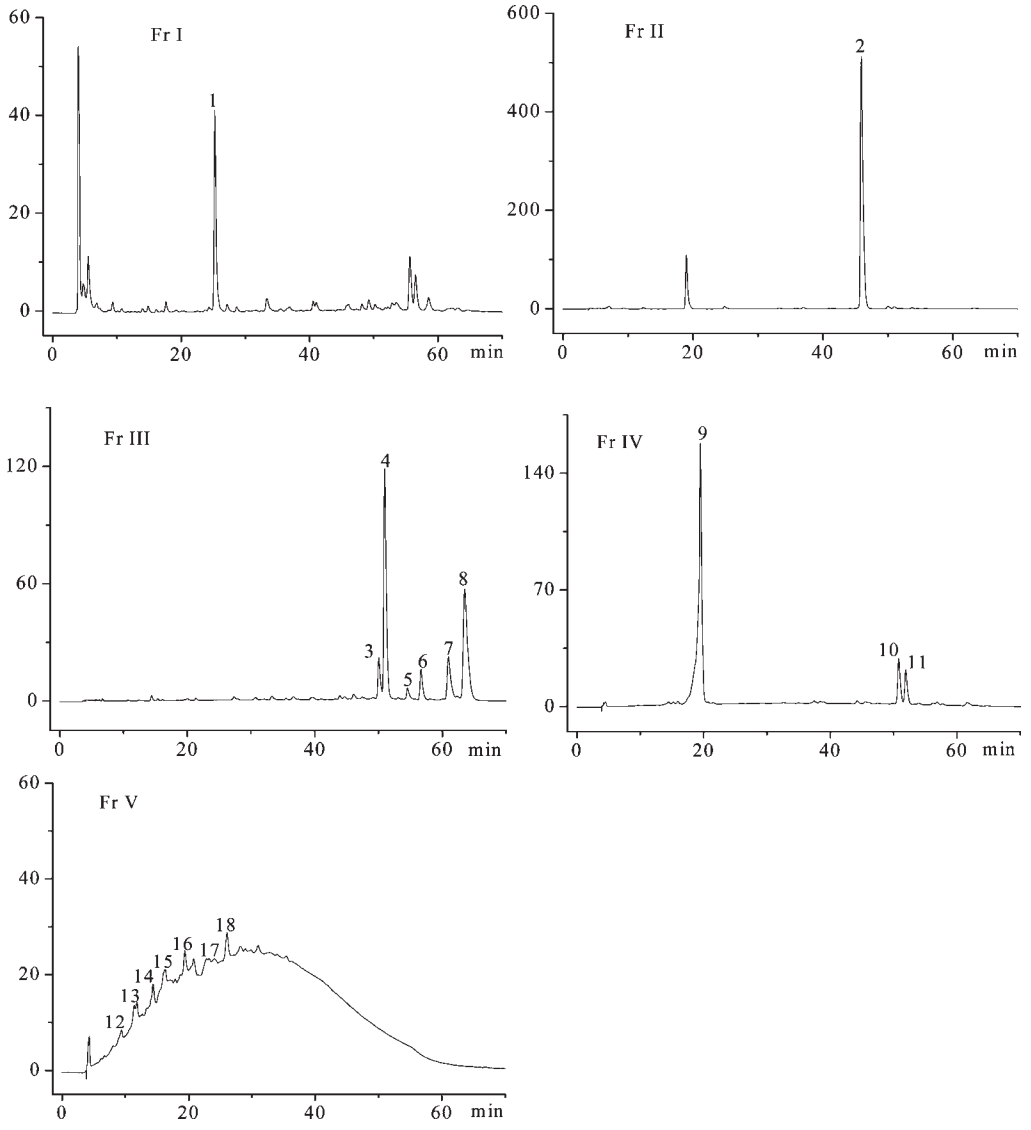

Figure 4. HPLC profiles of phenolic compounds in five fractions of Morton lentils recorded at 270 nm.

and TEAC assays. The FRAP value is presented in Figure 3C. Fraction V possessed the highest reducing power (910.8 mmol Fe^{2+} equiv/100 g), followed by SPME (586.7 mmol Fe^{2+} equiv/100 g), fraction IV (457.5 mmol Fe^{2+} equiv/100 g), fraction III (214.0 mmol Fe^{2+} equiv/100 g), CME (99.6 mmol Fe^{2+} equiv/100 g), and fraction II (21.8 mmol Fe^{2+} equiv/100 g). Fraction I exhibited almost no reducing power.

Finally, the ORAC values of the extracts and fractions were determined. ORAC is perhaps the most commonly used antioxidant activity assay; it depends on the free radical damage to a fluorescent probe to result in a downward change of fluorescent intensity. Antioxidants can compete with free radicals, thus leading to the inhibition of decay of fluorescent probe. The ORAC assay can provide information on a sample's ability to scavenge the peroxyl radical through a hydrogen atom transfer mechanism.[18] In the case of ORAC, the trend was a little different from that of free radical scavenging activity and ferric reducing power. Fraction V, which possessed the highest free radical scavenging activity and reducing power, had a little lower ORAC than that of fraction IV. This observation was in accordance with the conclusion that the correlation coefficient between CTC and ORAC was relatively lower than that between TFC, TPC, and ORAC.[12] The order of ORAC of the extracts and fractions was as follows: fraction IV > fraction V > fraction III > SPME > fraction II > CME > fraction I (Figure 3D).

Fraction V exhibited high antioxidant activity in all assays. Considering the relatively high yield of fraction V, the antioxidant activity of Morton lentils may be attributed largely to the condensed tannins. However, other phytochemicals such as catechin and flavonols also contributed to the total antioxidative capacity of Morton lentils. Our results are consistent with those of Amarowicz[8] and Alasalvar,[25] who reported that high molecular weight or condensed tannin-rich fractions from adzuki bean and hazelnut skin exhibited the highest radical scavenging activity and antioxidant activity. Phenolic hydroxyl groups attached to the flavanol skeleton[26] and the presence of an interflavonoid link[27] might play important roles in the higher radical scavenging activity and antioxidant activity of condensed tannins.

Phenolic Compound Profiles of Fractions by HPLC-MS. The SPME was separated over Sephadex LH-20 into five main fractions, which could be analyzed by HPLC-MS to identify the phenolic compound profiles through UV spectra and mass

Table 3. HPLC Retention Times, UV and ESI/MS Data, and Tentative Identification of Phenolic Compounds in Fractions of Morton Lentils

			MS			
fraction	peak	t_R (min)	negative	positive	UV (λ_{max}, nm)	tentative identification
I	1	25.2	446.9	159.3, 265.2, 467.2	267	not identified
II	2	45.9	901, 285.3		265, 348	kaempferol tetraglycoside
III	3	50.0	755.7	757.5, 287.9	269, 348	kaempferol triglycoside
	4	51.0	755.5	757.5, 287.9	269, 348	kaempferol triglycoside
	5	54.6	725.3	727.4, 287.5	272, 347	kaempferol triglycoside
	6	56.7	597.4, (579, 561, 507, 489, 477, 447, 387, 357)[a]	599.4	230, 288, 329 sh	3′,5′-di-*C*-β-glucopyranosyl phloretin
	7	61.0	523.8, 1047.7	547.5, 1049.6	267, 321, 351 sh	flavonoid derivative
	8	63.5	523.7, 1047.7	547.5, 1049.7	270, 319, 351 sh	flavonoid derivative
IV	9	19.5	451.2, 903.6	291.2, 453.1	279	catechin-3-*O*-glucoside
	10	50.8	609.8	611.1, 449.2, 287.2	268, 349	kaempferol-dihexoside
	11	51.9	609.3	611.1, 449.2, 287.2	268, 349	kaempferol-dihexoside
V	12	9.0	897.5		277	prodelphinidin trimer
	13	11.5	882.3		278	digallate procyanidin dimer
	14	14.1	880.9, 1169.4		278	digallate procyanidin trimer
	15	16.0	881.7, 1169.1		278	digallate procyanidin trimer
	16	19.3	1153.4		278	procyanidin tetramer
	17	24.3	593.8, 880.9, 1169.2		277	prodelphinidin tetramer
	18	26.2	1153.3		278	procyanidin tetramer

[a] Data in parentheses represent the fragment ions in MS/MS.

spectra. The HPLC chromatographic profiles of the fractions recorded at 270 nm are shown in Figure 4, and the UV spectra and mass spectra, as well as the tentative identification of phenolic compounds, are summarized in Table 3.

An unidentified compound was detected in fraction I. It yielded $[M + H]^+$ at m/z at 467, $[M - H - H_2O]^-$ at m/z 447, and positive fragment ions at m/z 265 and 159.

A predominant compound was presented in fraction II. Peak 2 exhibited absorption maxima at 265 and 348 nm, which are considered to be the typical UV spectra of flavone derivatives. It had pseudomolecular ions at m/z 901 $[M - H]^-$ and fragment ions at m/z 285 $[\text{aglycone} - H]^-$, which suggested the aglycone was kaempferol. This compound was tentatively identified as kaempferol tetraglycoside, which included two units of hexose and two units of deoxyhexose, as reported by Taylor et al.[28] Kaempferol glycosides existed in lentils very commonly, and many kinds of kaempferol glycosides have been reported in the literature.[28,29]

Six major peaks (3−8) were detected in fraction III. All compounds showed similar UV spectra with λ_{max} between 318 and 350 nm and at around 270 nm. Peaks 3 and 4 possessed the same UV and MS spectra. They had pseudomolecular ions at m/z 755 $[M - H]^-$ and 757 $[M + H]^+$, which further yielded fragment ions at m/z 287 $[\text{aglycone} + H]^+$. Compared with peak 2, the loss of 146 amu, which corresponded to the loss of a deoxyhexose sugar, was observed. Thus, both compounds were tentatively identified as the isomers of kaempferol triglycoside, which included two units of hexose and one unit of deoxyhexose. Peak 5 showed pseudomolecular ions at m/z 725 $[M - H]^-$and 727 $[M + H]^+$ and fragment ions at m/z 287 $[\text{aglycone} + H]^+$. Compared with peaks 3 and 4, the loss of 30 amu was due to the substitution of one hexose by one pentose. Peak 5 was tentatively identified as kaempferol triglycoside including one hexose, one deoxyhexose, and one pentose unit. The UV spectra of peak 6 exhibited absorption maxima at 230 and 288 nm, along with a shoulder at 329 nm, which is similar to the spectrum of dihydroxyflavonoid. The negative and positive ESI-MS spectra showed pseudomolecular ions at m/z 597 $[M - H]^-$ and 599 $[M + H]^+$, respectively. MS/MS analysis provided the typical fragment ions of 6,8-di-*C*-hexosyl flavones, namely, 579 $[M - H - 18]^-$, 561 $[M - H - 18 - 18]^-$, 507 $[M - H - 90]^-$, 477 $[M - H - 120]^-$, 387 $[M - H - 90 - 120]^-$, and 357 $[M - H - 120 - 120]^-$. These ions are indicative for *C*-glycosylflavonoids.[30] The ions at m/z 357 $[\text{aglycone} + 83]$ and 387 $[\text{aglycone} + 113]$ suggested the aglycone was tetrahydroxydihydrochalcone (MW = 274). The UV and mass spectra were consistent with those reported previously.[31] Therefore, peak 6 was identified as 3′,5′-di-*C*-β-glucopyranosyl phloretin. To our best knowledge, this is the first report of a *C*-glycosylflavonoid from legumes. Peaks 7 and 8 were identified as isomers of flavonoid derivatives. It showed a pseudomolecular ion at m/z 523 $[M - H]^-$, a molecular complex at m/z 1047 $[2M - H]^-$, a sodium adduct at m/z 547 $[M + Na]^+$, and a molecular complex at m/z 1049 $[2M + H]^+$.

The HPLC chromatogram of fraction IV showed three main peaks. Peak 9 had the molecular weight of 452 as manifested by the pseudomolecular ions at m/z 451 $[M - H]^-$ and 453 $[M + H]^+$, as well as a molecular complex at m/z 903 $[2M - H]^-$. In addition, a fragment ion at m/z 291 $[M + H - 162]^+$ due to the loss of glucose was observed. On the basis of UV and MS spectra, this compound was tentatively identified as catechin-3-*O*-glucoside.

Table 4. Pearson Correlation Coefficients (R^2) among the Antioxidant Activity and Phytochemical Contents[a]

	TFC	CTC	DPPH	TEAC	FRAP	ORAC
TPC	0.6215*	0.9094**	0.8993*	0.8768**	0.9293**	0.7605*
TFC		0.6706*	0.5055	0.7388*	0.5016	0.8344**
CTC			0.8637**	0.8496**	0.8834**	0.6440*
DPPH				0.9273**	0.9948**	0.5348
TEAC					0.9103**	0.6984*
FRAP						0.5616*

[a] *, correlation is significant at the 0.05 level (two-tailed); **, correlation is significant at the 0.01 level (two-tailed) (n = 21).

This compound was also detected in the seed coat of lentils from Spain.[32] Peaks 10 and 11 had almost the same UV and MS spectra. They had pseudomolecular ions at m/z 609 $[M - H]^-$ and 611 $[M + H]^+$, which further yielded fragment ions at m/z 449 $[M + H - 162]^+$ and 287 $[M + H - 162 - 162]^+$. The loss of 162 amu corresponded to the loss of a hexose sugar, and both compounds were tentatively identified as isomers of kaempferol-dihexoside.

The HPLC chromatogram of fraction V was completely different from the above fractions. All peaks had the λ_{max} about 277 nm, and a broad hump, characteristics for oligomers and polymers.[33] In the mass spectrum of peak 12, a negative ion at m/z 897 $[M - H]^-$ was observed. It was tentatively identified as prodelphinidin trimer with two units of (epi)gallocatechin and one unit of (epi)catechin. Peak 13 was tentatively identified as digallate procyanidin dimer, which exhibited a pseudomolecular ion at m/z 882 $[M - H]^-$, corresponding to two units of (epi)catechin gallate. In the case of peaks 14 and 15, a pseudomolecular ion at m/z 1169 $[M - H]^-$ appeared, and they were tentatively identified as isomers of digallate procyanidin trimer with two units of (epi)catechin gallate and one unit of (epi)catechin. A fragment ion at m/z 882 $[M - H]^-$ was due to the loss of one unit of (epi)catechin (288 amu). Peaks 16 and 18 were tentatively identified as isomers of procyanidin tetramer with four units of (epi)catechin, because a pseudomolecular ion at m/z 1153 $[M - H]^-$ appeared in the MS spectra. Peak 17, with a pseudomolecular ion at m/z 1169 $[M - H]^-$, as well as fragment ions at m/z 881 $[M - H]^-$ and m/z 593 $[M - H]^-$ corresponded to the loss of (epi)catechin. Thus, peak 17 was tentatively identified as prodelphinidin tetramer with three units of (epi)catechin and one unit of (epi)gallocatechin.

Correlations among Antioxidant Activity and Phytochemical Contents. Many previous studies reported that the antioxidant capacity of plant extracts could be attributed to the total phenolic content.[7,19,34] To analyze the correlative relationships among total antioxidant activity (DPPH, ABTS, FRAP, and ORAC) and TPC, as well as TFC and CTC, a Pearson correlation analysis was conducted, and the results are shown in Table 4.

Significant correlations were found among all phytochemical contents. The strongest correlation was found between CTC and TPC (R^2 = 0.91, $p < 0.01$), which suggested that condensed tannins could contribute a lot to the total phenolic content in lentils.

Comparison of coefficients among antioxidant activity and phytochemical contents revealed that significant correlations ($p < 0.05$ or $p < 0.01$) existed between various parameters except for between DPPH and TFC and between FRAP and TFC. That is, with regard to DPPH scavenging activity, a good correlation was found with TPC (R^2 = 0.89) and an inferior one with CTC (R^2 = 0.86). TEAC correlated well with TPC (R^2 = 0.87), followed with CTC (R^2 = 0.84) and TFC (R^2 = 0.73). For FRAP, the correlation coefficient was 0.92 and 0.88 with TPC, and CTC, respectively. For ORAC, the highest correlation was found with TFC (R^2 = 0.83), whereas the correlation between ORAC and CTC was the lowest (R^2 = 0.64). The strongest free radical scavenging activity and reducing power were detected in fraction V, which contained the highest TPC and CTC. These results suggested that total phenolics, especially condensed tannins, were mostly responsible for the antioxidant activity and reducing power of Morton lentils.

The antioxidant activity assays (DPPH, TEAC, FRAP, and ORAC) all showed significant correlations, suggesting that all antioxidant activity assays were reliable and interchangeable. For example, DPPH correlated well with FRAP (R^2 = 0.99, $p < 0.01$), TEAC (R^2 = 0.92, $p < 0.01$), and ORAC (R^2 = 0.53), whereas TEAC correlated well with FRAP (R^2 = 0.91, $p < 0.01$) and ORAC (R^2 = 0.69, $p < 0.05$) and FRAP correlated significantly with ORAC (R^2 = 0.56, $p < 0.05$). The correlation coefficients among the ORAC assay and other assays (<0.7) were generally lower than that among DPPH, TEAC, and FRAP assays (>0.9), which was in agreement with that by a regression analysis. The ORAC assay takes into account the kinetic action of antioxidants, which might explain the discrepancy between the results obtained with the ORAC assay and those obtained with other colorimetric assays.

In summary, a semipurified Morton extract was obtained by adsorption−desorption on macroporous resin, and it was further fractionated into five fractions over Sephedex LH-20. Phenolic compounds identified in those fractions comprised a range of low molecular weight flavonol glycosides, flavanol glycosides, C-glycosyl dihydrochalcone, and high molercular weight condensed tannins. Fraction V, composed mainly of condensed tannins, exhibited the highest antioxidant activity in different assays used for antioxidant efficacies and possessed the highest total phenolic content. The present study also indicated that the antioxidant activity of the Morton lentils extract and its fractions correlated well with their phenolic contents. On the basis of their antioxidant activity, the Moton lentils extract and its condensed tannins fraction might be developed for the food industry as a dietary supplement for health promotion. Further research is required to determine the antioxidant activity of Morton lentils extract in vivo.

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