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ARTICLE

Variability in the Distribution of Phenolic Compounds in Milled Fractions of Chickpea and Horse Gram: Evaluation of Their Antioxidant Properties

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Seed coat, cotyledon and embryonic axe fractions of chickpea *(Cicer arietinum L.)* and horse gram (*Macrotyloma uniflorum* L.) were evaluated for their phenolic composition in relation to antioxidant activities. Compositional analysis of phenolics by HPLC revealed a wide variation in the distribution of flavonols, isoflavones, phenolic acids and anthocyanins among these legume fractions. Although cotyledon fractions of both the legumes were rich in phenolic acids, the concentrations of flavonols such as quercetin, kaempferol, and myricetin were significantly ($p < 0.05$) lower than the embryonic axe and seed coat fractions. Ferulic, chlorogenic, caffeic, and vanillic acids were the principal phenolic acids found in cotyledons. The most striking difference was the predominance of isoflavones in embryonic axe fractions. Although the isoflavone genistein was detected in all three fractions of chickpea, it was present exclusively in the embryonic axe fraction of horse gram at levels greater than daidzein. Furthermore, cyanidin, petunidin, and delphinidin were detected in seed coat and embryonic axe fractions but not in cotyledons. In addition to these three anthocyanins, malvidin was found only in the horse gram seed coat fraction. Seed coat fractions having higher total phenolic indexes were found to be the most active 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavengers (IC_{50} 13.1 to 18.6 μg/mL) followed by embryonic axe and cotyledon fractions (IC_{50} 15.4 to 34.2 μg/mL). Hydrogen peroxide (H_2O_2) scavenging capacities of cotyledons, embryonic axe and seed coats were 12.3, 34.1 and 78.6% for chickpea and 15.1, 56.8 and 92.6% for horse gram, respectively. The multiple antioxidant activity of horse gram and chickpea fractions was evident, as they also possessed reducing power and ferrous ion-chelating potency. These results contributed to the understanding of the relationships between major phenolic compounds and antioxidant activities of legumes and provided useful information for effective utilization of legume-milled fractions as functional food ingredients for promoting health.

KEYWORDS: Horse gram; chickpea; milled fractions; seed coat; embryonic axe; cotyledon; phenolic acids; flavonols; isoflavones; anthocyanins; antioxidants

INTRODUCTION

Phenolic compounds are bioactive secondary plant metabolites that are widely present in commonly consumed foods of plant origin. The three main types of phenolic compounds are flavonoids, phenolic acids and tannins, which act as powerful antioxidants in vitro (*1*). Such antioxidants not only can defend lipids and other compounds contained in plants against undesirable oxidation but also can be used to retard oxidation in various food products as well (*2*). Numerous clinical studies also suggest that phenolic antioxidants possess the ability to prevent some cholesterol-related and oxidative stress-induced diseases, such as cardiovascular disease, obesity, diabetes, and some cancers (*2*−*4*). The main dietary sources of polyphenols are fruits and beverages. In addition, cereals and dry legumes also contribute to polyphenol intake (*3*).

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Chickpea (*Cicer arietinum* L.) and horse gram (*Macrotyloma uniflorum* L.) are common legumes consumed as whole, dehulled splits, canned, boiled, roasted or ground into flour to make a variety of desserts, noodles, snacks and main dishes. These two food legumes are valuable sources of protein, minerals and vitamins, and occupy a very important place in human nutrition in many developing countries (*5*,*6*). Besides nutritional importance, consumption of these legumes has been linked to reduced risk of various diseases such as bronchitis, diabetes, cancer and cardiovascular diseases (*5*, *7*, *8*). These physiological effects of chickpea and horse gram consumption have been associated with the presence of phytochemicals including phenolic compounds, which possess antioxidant properties (*9*, *10*). These legumes are commonly processed by dehulling or milling to improve their cooking and nutritional properties, which results in different types of milled fractions such as cotyledon, embryonic axe and seed coats.

Recently, our group reported variations in the distribution of nutrients and antinutrients in the milled fractions of chickpea and

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horse gram (*11*). The cotyledon fractions are the main reserve for proteins and carbohydrates along with substantial quantities of various antinutritional factors such as proteinaceous enzyme inhibitors, flatulence factors and phytic acid. Phenolic compounds are unevenly distributed in these milled fractions. The seed coat, which acts as a protective barrier for the cotyledon, has the highest concentration of phenolic compounds. In addition, it was also observed that chickpea and horse gram seed coat phenolics showed distinct inhibitory mechanisms against α-amylase and trypsin (*11*). The heterogeneity of phenolics having different structural features in seed coat fractions may have contributed to these distinct modes of inhibition. Moreover, antioxidant and free radical scavenger properties found in whole seeds of chickpea and horse gram were attributed to phenolic compounds (*9*, *10*). However, phenolic compositions including flavonols, isoflavones, phenolic acids, anthocyanins and their distributions in the milled fractions of chickpea and horse gram as related to their contributions to antioxidant activity have not been reported. Therefore, the aims of this study were to elucidate the types of phenolic compounds, to quantify their contents in cotyledon, embryonic axe and seed coat fractions of chickpea and horse gram, and further to investigate their contribution to antioxidant activity of these milled fractions. The distribution of phenolic antioxidant compounds in milled fractions of legumes may provide useful information for food processing because of their potential to serve as functional food ingredients.

MATERIALS AND METHODS

Chemicals. The Folin−Ciocalteu and vanillin reagents, flavonoids (quercetin, kaempferol, and myricetin), and phenolic acid (gallic, *p*-coumaric, ferulic, *p*-hydroxybenzoic, chlorogenic, protocatechuic, sinapic, caffeic, syringic and vanillic acids) standards were purchased from Sigma Chemical Co. (St. Louis, MO). Isoflavonoids (daidzein and genistein) standards were purchased from LC Laboratories (Woburn, MA). The anthocyanin monomeric standards, 3-*O*-*β*-glucopyranosides of cyanidin, petunidin, delphinidin, and malvidin, were provided by Polyphenols Laboratories (Hanabrygene Technology Centre, Sandnes, Norway). Solvents and water used for HPLC analysis were of HPLC grade.

Preparation of Milled Fractions of Legumes. Chickpea and horse gram seeds were purchased from a local market in Mysore, India. Dehulling and separation of milled fractions from chickpea and horse gram were done as described previously (*11*) using a grain-testing device (Strong-Scott Ltd., Winnipeg, MB, Canada). The separated dehulled cotyledons, embryonic axe and seed coat fractions were milled into flour (60 mesh) using a laboratory coffee grinder. The flour obtained was put into plastic bags under vacuum and stored in the dark, at 4 °C, until used.

Quantification of Total Phenolic, Total Flavonoid, and Condensed Tannin Contents. Total phenolic compounds were determined according to the Folin−Ciocalteu procedure (*12*) by measuring the absorbance at 760 nm, and results are expressed as milligrams of gallic acid equivalents (GAE) per gram of bean flour. Total flavonoid content was determined according to the method described by Jia et al. (*13*) by reading the absorbance at 510 nm. The results are expressed as milligrams of catechin equivalents (CAE) per gram of bean flour. Condensed tannins were determined according to the vanillin-HCl procedure (*14*), and results are expressed in milligrams of (+)-catechin equivalents (CAE) per gram. In order to correct for interference from natural pigments in seed coats, a blank sample was prepared by subjecting the original extract to the same conditions of the reaction, but without vanillin reagent.

Extraction, Hydrolysis, and Determination of Flavonoids and Phenolic Acids. Phenolic acids and flavonoids were determined according to the method of Lozovaya et al. (*15*) with minor modifications. Briefly, phenolic acids and flavonoids from milled fractions (1 g) were extracted with 3 × 10 mL of 80% methanol and centrifuged; the extract was diluted with 6 mL of 2 N HCl and heated at 95 °C for 2 h to hydrolyze the glycosides. The organic components were extracted from the acidic solution with 20 mL of ethyl acetate. The ethyl acetate was removed under vacuum, the residue was resuspended in 2.0 mL of 80% methanol and centrifuged, and supernatant was used for HPLC analysis. Identification and quantification of flavonoids and phenolic acids was achieved using analytical reversed-phase HPLC in a Shimadzu LC-10A system with binary pump coupled to a diode array detector (Shimadzu Corporation, Kyoto, Japan) fitted with LiChrospher 100 RP-18 column (4 × 250 mm, 5 μm; Merck, Darmstad, Germany). Solvent A was water adjusted with acetic acid to pH 2.8, and solvent B was acetonitrile. Injection volume was 20 μL, and flow rate was 1.0 mL/min. For flavonoid elution, the gradient was linear to 10% B in 5 min, 23% B in 31 min, and 35% B in 43 min, after the column was washed with 100% B for 6 min and equilibrated for 6 min at 100% A to start the next sample; total running time was 55 min. Ultraviolet absorbance at 260.6 nm was used to detect quercetin, kaempferol, daidzein, myricetin and genistein. For phenolic acid elution, the gradient was linear to 12% B in 24 min, 20% B in 32 min, and 35% B in 44 min, after the column was washed with 100% B for 6 min and equilibrated 6 min at 100% A to start the next sample; total running time was 56 min. Quantitative determination of the eluted phenolic acids was performed at 320 nm for cinnamic acid derivatives (ferulic, *p*-coumaric, caffeic, chlorogenic and sinapic acids) and 254 nm for benzoic acid derivatives (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and syringic acids).

Extraction, Hydrolysis, and Determination of Anthocyanins. Anthocyanin analysis was carried out according to the method of Romani et al. (*16*) as modified by Espinosa-Alonso et al. (*17*) for common beans (*Phaseolus vulgaris* L.). Anthocyanins were extracted from 500 mg of milled fractions, using 3 × 15 mL of 70% ethanol, adjusted to pH 2.0 with formic acid. Ethanolic extracts were dried under vacuum and dissolved in $H_2O/CH_3CN/MeOH/HCOOH$ (45:22.5:22.5:10) to a 2 mL final volume. The hydrolysis of the anthocyanin standard and the samples was carried out according to the method of Takeoka et al. (*18*). One milliliter of extract was heated with 1.0 mL of 2 N HCl in a boiling water bath for 60 min and cooled. The anthocyanins were extracted twice with ethyl acetate, then dried, redissolved with 10% formic acid, centrifuged, and used for HPLC analysis. Anthocyanin analysis was done using the HPLC system and column previously described. The solvent was made with (A) 5% HCOOH and (B) 5% HCOOH in acetonitrile. The injection volume was 20 μL, and the flow rate 1.0 mL/min. The gradient for anthocyanin elution was linear at 5% B in 0 min, to 10% B in 10 min, and to 18% B in 20 min. UV absorbance at 520 nm was used to detect cyanidin, petunidin, delphinidin, and malvidin.

Identification and Quantification of Flavonoids, Phenolic Acids and Anthocyanins. Calibration was performed by injecting the standards three times at five different concentrations ($R^2 \geq 0.999$). The relative retention time (RT), peak area under the curve, and diode array spectral characteristics of the standards were used to identify the type and quantity of phenolic compounds present in the samples. All flavonoids, phenolic acids and anthocyanins were determined in three independent analyses, and results are expressed as micrograms per gram of sample in dry weight.

DPPH Free Radical Scavenging Activity. Free radical scavenging capacities of the seed coat, embryonic axe and cotyledon fractions of horse gram and chickpeas were determined by reaction with the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical, according to the method adapted from Brand-Williams et al. (*19*). Briefly, an aliquot of samples (0.3 mL, 5−100 μg/mL) was mixed with 2.7 mL of a methanol solution of $DPPH^{\bullet}$ (500 μM). The mixture was shaken vigorously and left to stand at room temperature for 60 min in the dark. The absorbance was measured at 517 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Decreasing the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. The radical scavenging activity of different milled fractions is expressed in terms of IC_{50} (concentration in μg required for a 50% decrease in absorbance of DPPH radical), calculated at 517 nm. A plot of absorbance vs concentration was made to establish the standard curve and calculate IC_{50} values. Catechin and BHT were used as positive controls at 100 μg/mL concentration. Data are reported as means ± SD for three replications.

Hydrogen Peroxide Scavenging Activity. H_2O_2 scavenging capacities (HPSC) of the seed coat, embryonic axe and cotyledon fractions of legumes were determined according to the procedure of Ruch et al. (*20*). Extracts were dissolved in 3.4 mL of a 0.1 M phosphate buffer (pH 7.4) and mixed with 0.6 mL of 53 mM solution of H_2O_2 prepared in the same buffer solution. Final concentrations of the extracts were 25 μg of phenolics as gallic acid equivalents. Immediately after mixing, the zero time absorbance

was read at 230 nm, and subsequent readings were taken at 10 min intervals over 40 min. For each concentration, a separate blank sample devoid of H_2O_2 was used for background subtraction. Control contained 3.4 mL of phosphate buffer and 0.6 mL of 53 mM H_2O_2. The H_2O_2 scavenging capacities were calculated using the following equation:

$$\text{HPSC}\ (\%) = 100 - [(H_2O_2)\text{sample}/(H_2O_2)\text{control}] \times 100$$

where (H_2O_2) sample is the concentration of H_2O_2 in the sample and (H_2O_2) control is the concentration of H_2O_2 in the control.

Reducing Power Determination. The reducing power of different milled fractions of legumes was determined according to the method of Oyaizu (*21*). Different concentrations of extracts (5−100 μg/mL in 50% methanol) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide $[K_3Fe(CN)_6]$. The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 mL, 10%) was added to the mixture, which was then centrifuged at 4000 rpm for 10 min. The supernatant (2.5 mL) was mixed with an equal quantity of distilled water and 0.5 mL of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a positive control.

Metal Chelating Activity. The chelating of ferrous ions by the phenolic extracts of seed coat, embryonic axe and cotyledon fractions of horse gram and chickpea was estimated by the method of Dinis et al. (*22*). Briefly, the extract (50−300 μg/mL) was added to 0.05 mL of 2 mM $FeCl_2$. The reaction was initiated by the adding 0.2 mL of 5 mM ferrozine; the mixture was vortexed and allowed to stand at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine−Fe^{2+} complex formation (metal chelation) was calculated using the following equation:

$$\text{metal chelating activity} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control without sample, and A_1 is the absorbance of the sample. Ethylenediaminetetraacetic acid (EDTA) was used as a positive control.

Statistical Analysis. Quantitative data are presented as mean values with the respective standard deviation values corresponding to three replicates. Results were processed by one-way analysis of variance (ANOVA). A Duncan multiple range test was used to determine significant differences. Differences at $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Phenolic Indexes of Milled Fractions. Distinctive variations were observed in total phenolic content (TPC), total flavonoid content (TFC), and condensed tannin content (CTC) of chickpea and horse gram milled fractions (**Figure 1**). These morphological parts showed more differences in TPC and CTC, while variations, although statistically significant ($p < 0.05$), were minimal in flavonoids. The seed coat fractions had significantly ($p < 0.05$) higher levels of TPC, TFC and CTC than embryonic axe and cotyledon fractions except embryonic axe fraction of horse gram, which has more flavonoids. Embryonic axe fractions possessed significantly ($p < 0.05$) higher TPC, TFC, CTC values than cotyledon fractions. These results indicate that the phenolic compounds are mostly concentrated in the seed coat fractions and might be easily removed by dehulling.

Highest concentrations of phenolic compounds were found in horse gram seed coat fraction (484.6 mg GAE/g), while its cotyledon fraction had the lowest total amount of polyphenolics (13.8 mg GAE/g). Chickpea fractions also showed similar results, although the levels were much lower (**Figure 1**). The content of phenolic compounds in seed coat fractions of these legumes is in agreement with the values reported for beach pea (*23*). However, TPC of cotyledon fractions are lower than those reported for beach pea (*23*), cowpea, pigeon pea and chickpea (*24*), but higher than the values reported for cool season legumes (*25*). The total flavonoid content of chickpea seed coat fractions (12.6 mg CAE/g) was significantly higher than that of embryonic axe (9.3 mg CAE/g)

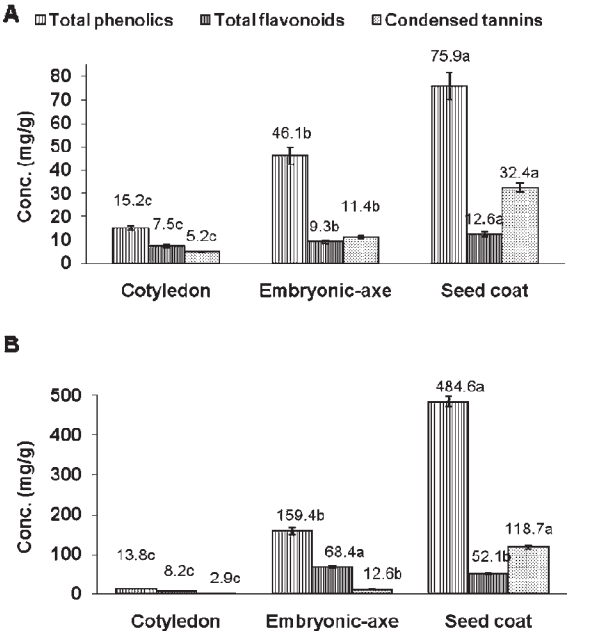

Figure 1. Phenolic indexes in different milled fractions of chickpea (**A**) and horse gram (**B**). Data points marked above the bar are the concentration of phenolics in mg/g on dry weight basis expressed as mean ± standard deviation (n = 3). Values marked with different letters (a, b, c) are significantly different ($p < 0.05$).

and cotyledon fractions (7.5 mg CAE/g) (**Figure 1A**). Similarly, horse gram embryonic axe and seed coat fractions also possessed higher flavonoids than cotyledon fraction. Furthermore, embryonic axe fraction of horse gram has more flavonoids (68.4 mg CAE/g) than seed coat fraction (52.1 mg CAE/g) (**Figure 1B**). The flavonoid content of horse gram seed coat fraction is comparable to the flavonoid content reported for black soybean (*26*). Although substantial quantities of condensed tannins were found in embryonic axe fractions, they are mostly concentrated in the seed coat fractions of these legumes. Cotyledon fractions showed very low quantities of condensed tannins (**Figure 1**). Horse gram seed coat fraction had the highest concentration of CTC (118.7 mg CAE/g), while its cotyledon fraction recorded the lowest CTC (2.9 mg CAE/g). Unlike TFC, similar values of CTC were observed in embryonic axe fractions of chickpea and horse gram (11.4 and 12.6 mg CAE/g, respectively). The CTC values of seed coat fractions of the chickpea (32.4 mg CAE/g) and horse gram (118.7 mg CAE/g) were within the range of phenolic indexes observed in the seed coats of Brazilian and Peruvian bean cultivars (*27*).

Distribution of Flavonoids in Milled Fractions. Distribution of flavonoids in cotyledon, embryonic axe and seed coat fractions of chickpea and horse gram are presented in **Table 1**. Three flavonols (quercetin, kaempferol and myricetin) and two isoflavones (daidzein and genistein) were quantified by reversed-phase HPLC, and their content of each fraction was compared by statistical analysis. Quercetin, kaempferol and myricetin were detected in all three fractions of legumes with varying levels. Furthermore, contents of these flavonols were higher in horse gram fractions than chickpea fractions (**Table 1**). Seed coat fractions contain significantly ($p < 0.05$) higher amounts of quercetin, kaempferol and myricetin than embryonic axe and cotyledon fractions. Although myricetin was present in higher amounts in horse gram seed coat fraction (35.5 μg/g) than embryonic axe fraction (32.9 μg/g), the difference was not statistically significant ($p < 0.05$). In agreement with various researchers (*17*, *27*−*29*), predominant

Table 1. Distribution of Flavonoids in Different Milled Fractions of Chickpea and Horse Gram[a]

	concn (μg/g on dry weight basis)					
	chickpea fractions			horse gram fractions		
flavonoids	cotyledon	embryonic axe	seed coat	cotyledon	embryonic axe	seed coat
quercetin	7.01 ± 0.29 c	48.7 ± 2.0 b	104.9 ± 4.8 a	9.7 ± 0.55 c	113.4 ± 6.0 b	129.5 ± 11.3 a
kaempferol	5.5 ± 0.14 c	30.4 ± 1.4 b	97.9 ± 4.4 a	6.0 ± 0.25 c	67.4 ± 3.7 b	117.2 ± 10.5 a
myricetin	4.4 ± 0.09 c	11.6 ± 0.7 b	28.3 ± 1.6 a	2.4 ± 0.07 b	32.9 ± 3.3 a	35.5 ± 5.2 a
daidzein	3.4 ± 0.00 b	40.3 ± 1.8 a	nd[b]	4.1 ± 0.08 b	22.2 ± 1.3 a	0.94 ± 0.03 c
genistein	3.2 ± 0.07 b	33.8 ± 2.1 a	0.86 ± 0.03 c	nd	44.7 ± 3.22	nd

[a] Data are expressed as mean ± standard deviation of three independent determinations. Means with the same letters (a, b, c) within the same row for each legume do not differ significantly ($P > 0.05$). [b] Below detection limit.

Table 2. Distribution of Phenolic Acids in Different Milled Fractions of Chickpea and Horse Gram[a]

	concn (μg/g on dry weight basis)					
	chickpea fractions			horse gram fractions		
flavonoids	cotyledon	embryonic axe	seed coat	cotyledon	embryonic axe	seed coat
			Benzoic Acid Derivatives			
gallic acid	8.5 ± 0.31 b	22.0 ± 1.6 a	4.1 ± 0.08 c	26.9 ± 1.3 a	19.8 ± 0.8 b	5.5 ± 0.06 c
protocatechuic acid	48.0 ± 2.9 a	28.3 ± 1.8 c	41.6 ± 1.6 b	39.0 ± 2.0 a	11.8 ± 0.4	23.1 ± 1.2 b
p-hydroxybenzoic acid	2.1 ± 0.02 c	44.4 ± 3.1 a	10.5 ± 0.9 b	20.1 ± 0.8 b	13.1 ± 0.5 c	28.8 ± 1.4 a
vanillic acid	57.9 ± 4.3 a	14.7 ± 1.1 b	9.7 ± 0. 6 c	58.4 ± 3.3 a	53.2 ± 5.1 a	42.4 ± 3.6 b
syringic acid	21.9 ± 1.4 a	20.1 ± 1.6 a	20.6 ± 1.2 a	18.4 ± 1.0 a	nd[b]	4.5 ± 0.02 b
subtotal	138.6	129.5	86.5	162.8	97.9	104.3
			Cinnamic Acid Derivatives			
caffeic acid	103.3 ± 6.1 a	23.8 ± 1.6 b	17.7 ± 0.7 c	88.9 ± 5.0 a	61.5 ± 4.9 b	10.6 ± 0.6 c
chlorogenic acid	77.4 ± 5.5 a	13.7 ± 0.9 c	27.3 ± 1.6 b	160.8 ± 9.8 a	26.8 ± 2.1 b	22.5 ± 1.1 b,c
ferullic acid	159.2 ± 12.3 a	60.9 ± 4.2 b	42.2 ± 3.7 c	70.1 ± 5.1 a	31.4 ± 2.4 c	37.5 ± 2.5 b
sinapic acid	12.5 ± 0.8 b	20.7 ± 2.1 a	nd	9.7 ± 0.09 a	nd	3.7 ± 0.02 b
p-coumaric acid	99.4 ± 5.1 a	44.3 ± 3.9 b	17.6 ± 0.9 c	40.9 ± 3.2 a	21.4 ± 1.3 c	24.5 ± 1.8 b
subtotal	451.8	163.4	104.8	370.4	141.1	98.8
total	590.4	292.9	191.3	533.2	239.0	203.1

[a] Data are expressed as mean ± standard deviation of three independent determinations. Means with the same letters (a, b, c) within the same row for each legume do not differ significantly ($P > 0.05$). [b] Below detection limit.

flavonols in embryonic axe and seed coat fractions were quercetin and kaempferol with small amounts of myricetin. The concentrations of these flavonols in cotyledon fractions were significantly lower than in the embryonic axe and seed coat fractions (**Table 1**). This variation in flavonoid distribution also indicates that the majority of compounds contributing to the total phenolic content of the cotyledon fraction are not flavonoids. This observation is consistent with the role of flavonoids as phytoalexins, which are localized to the seed coat layer surrounding seeds and nuts protecting them against bacterial, fungal, and other environmental stresses (*30*).

Even though chickpea and horse gram are not recognized as isoflavone sources, remarkably high amounts of isoflavones were found in embryonic axe fractions of these legumes (**Table 1**). Soybean is the only legume seed with high isoflavone content, with values for genistein of 950 μg/g and for daidzein of 600 μg/g (*31*). However, the presence of daidzein and genistein in ungerminated (low levels) and germinated (high levels) common been cultivars were recently reported (*17*, *28*). Chickpea and horse gram embryonic axe fractions have higher concentrations of daidzein (40.3 and 22.2 μg/g, respectively) and genistein (33.8 and 44.7 μg/g, respectively) than cotyledon and seed coat fractions. Although genistein was detected in all three fractions of chickpea, it was present exclusively in the embryonic axe fraction of horse gram at levels greater than daidzein (**Table 1**). Hypocotyl (described here as embryonic axe) of soybean also contained the highest concentration of isoflavones (14.0−17.5 mg/g) compared to small quantity of total isoflavones found in cotyledon (2.9 to 3.6 μg/g) (*32*, *33*) and seed coat (0.1−0.2 mg/g) (*32*). Our results are in agreement with these reports, although the concentrations of total isoflavones are much lower in the embryonic axe fractions (74.1 and 66.9 μg/g in chickpea and horse gram, respectively) than reported values for soybean. Very low levels of isoflavones were found in cotyledon and seed coat fractions. Daidzein was not found in the seed coat fraction of chickpea, but a small quantity of it was detected in the horse gram seed coat fraction (0.94 μg/g). These isoflavones seem to be concentrated in the embryonic axe (hypocotyls) fractions of legumes and may represent additional metabolites of particular importance to the embryonic axe.

The isoflavones genistein and daidzein are compounds known as phytoestrogens that have been implicated in the reduction of risk factors for cardiovascular disease and cancer, particularly breast and prostate cancer (*34*, *35*). In addition to these hormonal effects these isoflavones exhibit antioxidant activity (*35*). Additionally, genistein is a specific inhibitor of protein tyrosine kinases and DNA topoisomerases I and II and arrests cell growth by interfering with the signal transduction pathways (*35*). The presence of isoflavones at higher concentrations in embryonic axe fractions of chickpea and horse gram make these legume fractions alternative sources of phytoestrogens.

Distribution of Phenolic Acids in Milled Fractions. The results of the phenolic acid content of chickpea and horse gram milled fractions are presented in **Table 2**. Five benzoic acid derivatives (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and syringic

Table 3. Distribution of Anthocyanins in Different Milled Fractions of Chickpea and Horse Gram[a]

	concn (μg/g on dry weight basis)					
	chickpea fractions			horse gram fractions		
anthocyanins	cotyledon	embryonic axe	seed coat	cotyledon	embryonic axe	seed coat
cyanidin	nd[b]	12.4 ± 0.9 b	431.2 ± 26.2 a	nd	41.6 ± 2.4	175.8 ± 13.4 a
petunidin	nd	10.2 ± 0.6 b	47.8 ± 3.1 a	nd	nd	28.4 ± 1.6
delphinidin	nd	3.0 ± 0.02 b	34.3 ± 2.9 a	nd	21.4 ± 1.7 b	408.7 ± 29.4 a
malvidin	nd	nd	nd	nd	nd	9.7 ± 0.8
total		25.6	513.3		63.0	622.6

[a] Data are expressed as mean ± standard deviation of three independent determinations. Means with the same letters (a, b, c) within the same row for each legume do not differ significantly ($P > 0.05$). [b] Below detection limit.

acids) and five cinnamic acid derivatives (caffeic, chlorogenic, ferulic, sinapic and *p*-coumaric) were identified in these milled fractions. The total content of phenolic acids was highest in chickpea and horse gram cotyledon fractions (590.4 and 533.2 μg/g, respectively) followed by embryonic axe and seed coat fractions (**Table 2**). Further, there were several variations in the individual phenolic acid composition of the milled fractions. Cinnamic acid derivatives were the major phenolic acids found in cotyledon and embryonic axe fractions of both the legumes and seed coat fraction of chickpea, while benzoic acid derivatives dominated in the seed coat fraction of horse gram (**Table 2**).

Ferulic acid was the most abundant phenolic acid present in all three chickpea fractions, whereas gallic and *p*-hydroxybenzoic acids were minor in cotyledon and seed coat fractions. Besides ferulic acid, protocatechuic, caffeic, chlorogenic and *p*-coumaric acids were other major phenolic acids detected in all three fractions. The cotyledon fraction possessed significantly ($p < 0.05$) higher contents of protocatechuic, vanillic, caffeic, chlorogenic, ferulic and *p*-coumaric acids than embryonic axe and seed coat fractions, while embryonic axe fraction possessed significantly ($p < 0.05$) higher contents of gallic, *p*-hydroxybenzoic, vanillic, caffeic, ferulic and *p*-coumaric acids than seed coats. The contents of gallic, *p*-hydroxybenzoic and sinapic acids were higher in embryonic axe fraction than cotyledon and seed coat fractions. There was no statistically significant difference ($p < 0.05$) in the content of syringic acid in all three fractions. The contents of protocatechuic and chlorogenic acids were higher in seed coat than embryonic axe fraction. Although a low level of sinapic acid was extracted from cotyledon (12.5 μg/g) and embryonic axe fraction (20.7 μg/g), it was not detected in seed coat fraction.

Chlorogenic acid was the predominant phenolic acid present in the cotyledon fraction of horse gram (160.8 μg/g), while caffeic and vanillic acids were the major phenolic acids in the embryonic axe fraction (**Table 2**). Moreover, vanillic and ferulic acids were other dominant phenolic acids detected in all three fractions. The contents of gallic, protocatechuic, vanillic, syringic, caffeic, chlorogenic, ferulic, sinapic and *p*-coumaric acids were significantly ($p < 0.05$) higher in cotyledon fraction than embryonic axe and seed coat fractions, while embryonic axe fraction possessed significantly ($p < 0.05$) higher contents of gallic, vanillic, caffeic and chlorogenic acids than seed coats. Vanillic, *p*-hydroxybenzoic and ferulic acids were the principal phenolic acids in the seed coat fraction. Although syringic and sinapic acids were detected in measurable amounts in cotyledon and seed coat fractions, they were not detected in embryonic axe fraction.

A comparison of our results with those of literature is difficult because the concentrations of phenolic acids in different morphological parts of chickpea and horse gram have not been sufficiently reported. However, our results were consistent with the previous report that dehulled parts (embryo + cotyledon) of black soybeans contained gallic, vanillic, caffeic, chlorogenic, *p*-coumaric and ferullic acids and seed coats of black soybeans contained vanillic, caffeic and chlorogenic acids (*26*, *36*). Ranilla et al. (*27*) also reported that cotyledons of Brazilian and Peruvian bean cultivars were rich in phenolic acids, such as ferulic, sinapic, chlorogenic, and other hydroxycinnamic acids although in lower amounts compared to our current investigation.

In plants, phenolic acids have been associated with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy (*37*). Phenolic acids also play key roles in color, sensory qualities, and organoleptic (flavor, astringency, and hardness), nutritional and antioxidant properties of foods (*38*, *39*). Additionally, they are used as food preservatives in the food industry (*2*). The role of phenolic acids as dietary antioxidants had received the most attention in recent literature (*1*). These phenolic acids also possess other biological activities. Caffeic acid, one of the most prominent cinnamic acids of cotyledon and embryonic axe fractions of chickpea and horse gram, is known to selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma, and allergic reactions (*40*). Chlorogenic acid, which is predominantly present in the cotyledon fraction of horse gram, is thought to possess cancer chemopreventive, antioxidant and anti-inflammatory properties (*41*, *42*). Ferulic acid, which is abundantly present in cotyledon fractions, is a known antioxidant, being an effective scavenger of free radicals (*43*). It also inhibits chemical carcinogenesis and tumor promotion and exhibits anti-inflammatory properties (*44*). These investigations describing the role of phenolic acids in food quality and their potential healthful dietary impact have reenergized the interest in the development of functional foods enriched with phenolic acids. The cotyledon and embryonic axe fractions of chickpea and horse gram, which contain the highest amounts of phenolic acids, may be used as ingredients in functional foods to derive the health benefits associated with these phenolic acids.

Distribution of Anthocyanins in Milled Fractions. The anthocyanin contents of different milled fractions of chickpea and horse gram are presented in **Table 3**. Cyanidin, petunidin and delphinidin were detected in seed coat and embryonic axe fractions of chickpea and horse gram. In addition to these three anthocyanins, malvidin was exclusively found in the horse gram seed coat fraction. However, anthocyanins were not detected in cotyledon fractions of both the legumes. Although petunidin was present in small amounts (10.2 μg/g) in chickpea embryonic axe fraction, it was not found in horse gram embryonic axe fraction. The contents of cyanindin, petunidin and delphinidin were significantly higher in seed coat fraction than embryonic axe fractions. The total anthocyanin contents were higher in horse gram seed coat (622.6 μg/g) and embryonic axe (63.0 μg/g) fractions than corresponding fractions of chickpea (513.3 and 25.6 μg/g, respectively). These results are in accordance with those published by Ranilla et al. (*27*) for the seed coats of Brazilian bean cultivars (red bean group), who obtained in the range of 160 to

Table 4. Free Radical and H_2O_2 Scavenging Capacities of Phenolics in Different Milled Fractions of Chickpea and Horse Gram[a]

legume	milled fraction	DPPH [IC_{50}] (μg/mL)	H_2O_2[b] (%)
chickpea	cotyledons	34.2 ± 1.8 a	12.3 ± 0.4 c
	embryonic axe	26.4 ± 1.2 b	34.1 ± 1.3 b
	seed coat	18.6 ± 2.1 c	78.6 ± 3.1 a
horse gram	cotyledons	26.3 ± 0.6 a	15.1 ± 0.3 c
	embryonic axe	15.4 ± 1.1 b	56.8 ± 1.8 b
	seed coat	13.1 ± 2.6 b	92.6 ± 2.4 a

[a] Data are expressed as mean ± standard deviation ($n = 3$). Means followed by different letters within the same column for each legume are significantly different ($p < 0.05$). [b] Concentration of phenolics used was 25 μg gallic acid equivalents.

480 μg/g sample, applying HPLC assays for total anthocyanin quantification. Similar to black soybean seed coat (*26*, *36*), the anthocyanin present in the highest concentration in chickpea seed coat fraction was cyanidin (431.2 μg/g), accounting for nearly 84% of the total anthocyanin concentration (**Table 3**). However, in horse gram seed coat fraction, the predominant anthocyanin was delphinidin (408.7 μg/g), followed by cyanidin (175.8 μg/g), petunidin (28.4 μg/g) and malvidin (9.7 μg/g). Delphinidin was also found to be the main anthocyanin in wild and weedy Mexican common bean collection followed by petunidin, cyanidin, malvidin, pelargonidin, and peonidin (*17*).

The benefit of anthocyanin consumption to health is well-known, and the U.S. intake is ≈180−215 mg per day, provided mostly by blue and red fruits, vegetables, and also red wine (*45*). However, these same sources are not available in all developing countries. Therefore, the seed coat fractions of legumes, which are rich sources of anthocyanins, may find applications as functional food ingredients to incorporate higher quantities of anthocyanins in the basic diet (*45*).

Antioxidant Properties. *DPPH Radical Scavenging Capacity*. The DPPH assay has been widely used to test the free radical scavenging ability of various natural products because it is the simplest method that measures the ability of antioxidants to intercept free radicals. The radical scavenging capacities (IC_{50} values) of phenolic extracts of cotyledon, embryonic axe and seed coat fractions of chickpea and horse gram determined at 517 nm are presented in **Table 4**. All these extracts showed concentration dependent radical scavenging activity; the activity increased as the concentration increased for each extract. Among the milled fractions, horse gram seed coat (IC_{50}: 13.1 μg/mL) and embryonic axe (IC_{50}: 15.4 μg/mL) fractions showed the strongest radical scavenging activity (**Table 4**). The radical scavenging activity of chickpea seed coat extracts (18.6 μg/mL) was significantly ($p < 0.05$) greater than that of cotyledon (34.2 μg/mL) and embryonic axe (26.4 μg/mL) fractions. However, in horse gram, antiradical activity of seed coat extract was higher than that of cotyledon (26.3 μg/mL), which is not significantly different ($p < 0.05$) from embryonic axe fraction. Further, embryonic axe fractions of both the legumes showed significantly higher radical scavenging activity than cotyledon fractions. Soybean germ (*46*) and seed coat fractions (*26*) with higher isoflavone content and total phenolic indexes, respectively were reported to have higher antioxidant capacity than cotyledons. Further, lentils and dark peas also showed more antioxidant activity in seed coat fractions than cotyledon fractions (*47*). Therefore, the higher antioxidant activities of chickpea and horse gram seed coat fractions could be attributed to the higher phenolic indexes (**Figure 1**) and flavonol content (**Table 1**). However, in embryonic axe fractions, higher contents of isoflavone and flavonols (**Table 1**) may have contributed to higher radical scavenging activities.

Hydrogen Peroxide Scavenging Capacity. The H_2O_2 scavenging capacity of milled fractions of legumes (after 10 min of the assay) is shown in **Table 4**. Similar to DPPH radical scavenging activity, seed coat fractions of legumes showed strong H_2O_2 scavenging ability. At 25 μg, horse gram seed coat fraction showed strongest effect by scavenging 92.6% of the H_2O_2 in the medium. All three horse gram fractions presented higher H_2O_2 scavenging capacity than the corresponding chickpea fractions. The H_2O_2 scavenging activity was ranked in the order seed coat > embryonic axe > cotyledon for both legumes. These results show that all three fractions of legumes are quite effective in scavenging H_2O_2, with seed coat fractions being more effective than embryonic axe and cotyledon fractions. H_2O_2 is a weak oxidizing agent that can inactivate some enzymes directly, usually by oxidation of their essential thiol (−SH) groups. However, the ability of H_2O_2 in producing other reactive oxygen species (ROS) such as hydroxyl radical cannot be ignored. H_2O_2 is found to be toxic to cells at 10 to 100 mM levels and to cross biological membranes rapidly to form hydroxyl radicals (*48*). Furthermore, DNA is an important target to be damaged when H_2O_2 is added to mammalian cells (*49*). Any biological system generating superoxide radical can produce H_2O_2 by dismutation reactions, unless of course other molecules such as cytochrome *c* intercept all the superoxide radicals. There are also several enzymes that produce H_2O_2 without the intermediacy of free superoxide radicals, which include glycollate oxidase (*50*), D-amino acid oxidase (*51*) and urate oxidase (*52*). H_2O_2 scavenging ability of dietary phenolics may represent a biochemical rationale or mechanism for delivering some of the health benefits attributed to a diet rich in phenolics. Therefore, seed coat fractions of legumes, which contain the highest amounts of phenolic compounds and possess H_2O_2 scavenging ability, may be used as ingredients in functional foods.

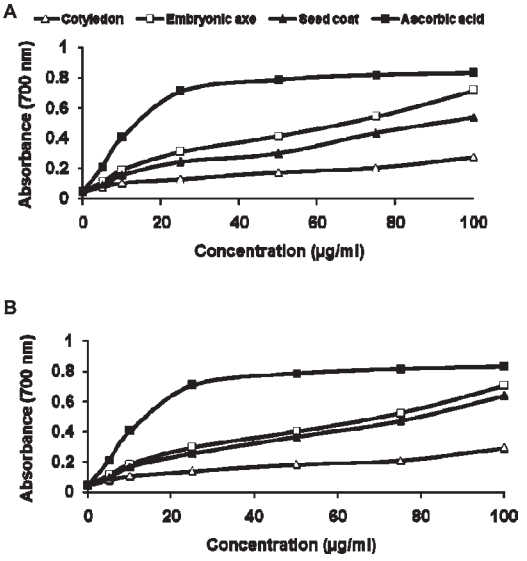

Figure 2. Ferric reducing antioxidant power (FRAP) of chickpea (**A**) and horse gram (**B**) milled fractions. Ascorbic acid was used as a positive control in the concentration range 5−100 μg/mL. The data points represent means of three independent determinations.

Ferric Reducing Antioxidant Power (*FRAP*). **Figure 2** illustrates the reductive capabilities of seed coat, embryonic axe and cotyledon fractions of legume extracts compared with ascorbic acid. For the measurements of the reductive ability, we investigated the $Fe^{3+}-Fe^{2+}$ transformation in the presence of phenolic extracts of legume-milled fractions using the method of Oyaizu (*21*). Previous reports have shown a direct correlation between antioxidant activity and reducing power of mung bean seed coats (*53*), wheat and barley fractions (*54*) and separated parts

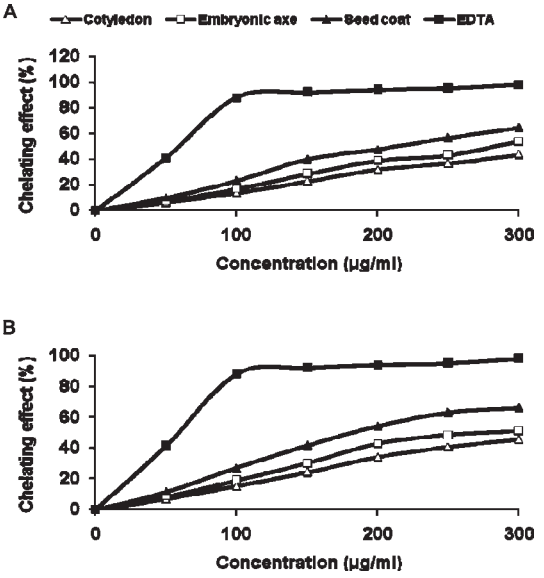

Figure 3. Metal chelating activity of chickpea (**A**) and horse gram (**B**) milled fractions. EDTA was used as a positive control in the concentration range 50−300 μg/mL. The data points represent mean of three independent determinations.

of soybean (*26*). FRAP activity of milled fractions increased with increasing amount of phenolic extracts. However, the FRAP activity of ascorbic acid was relatively more pronounced than that of phenolic extracts of milled fractions. Although wide variations were observed among the milled fractions, both chickpea (**Figure 2A**) and horse gram (**Figure 2B**) fractions exhibited the same trend in the FRAP activity. Embryonic axe fractions possessed significantly higher FRAP activities than seed coat and cotyledon fractions. Reducing power of different milled fractions of chickpea and horse gram are as follows: embryonic axe > seed coat > cotyledon. These differences were statistically significant ($p < 0.005$).

The reducing properties are generally associated with the presence of reductones (*55*), which have been shown to exert antioxidant action by breaking the free radical chain through the donation of a hydrogen atom (*56*). Reductones have also been reported to react with certain precursors of peroxide, thus preventing peroxide formation. Since the FRAP reaction involves a single electron transfer mechanism (*57*), functional groups in predominant phenolic compounds (such as isoflavones) in embryonic axe fractions may be more active in transferring electron. However, the antioxidant activity of oxidants has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (*58*).

Chelating Activity on Metal Ions. **Figure 3** shows the chelating effect of seed coat, embryonic axe and cotyledon fractions of chickpea (**Figure 3A**) and horse gram (**Figure 3B**) on ferrous ions. Ferrozine can quantitatively complex with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result being that the red color of the complexes decreases. Measurement of the rate of color reduction therefore allows estimation of the chelating activity of the coexisting chelator (*59*). In this assay, phenolic extracts of legume milled fractions interfered with the formation of the ferrous and ferrozine complex, suggesting chelating activity and capture of ferrous ion before ferrozine. The chelating effect increased with increased concentrations of phenolic extracts of legume fractions. Similar to free-radical and H_2O_2 scavenging activities, seed coat fractions showed higher metal-chelating activities than embryonic axe and cotyledon fractions. Seed coat extracts of chickpea at 300 μg/mL showed 64.5% chelating activity (**Figure 3A**), whereas horse gram seed coat extracts at the same concentrations showed 66% chelating activity on ferrous ion (**Figure 3B**). Phenolic extracts of embryonic axe fractions exhibited significantly ($p < 0.005$) greater metal-chelating activities than cotyledon fractions. Iron is essential for life because it is required for oxygen transport, respiration and the activity of many enzymes. However, iron is an extremely reactive metal and will catalyze oxidative changes in lipid, protein, and other cellular components (*60*). In addition, the liposome peroxidation and oxidative damage of protein model systems were induced by a Fenton reaction in which ferrous ions catalyze the composition of H_2O_2 to hydroxyl anion and hydroxyl radical with the production of ferric ion. As shown in **Figure 3**, the chelating capacity of all three milled fractions of legumes on ferrous ions is relatively lower when compared with that of EDTA; it may be significant because phenolic compounds of legumes minimize the concentration of metal in the Fenton reaction.

Antioxidant Activities in Relation to the Distribution of Phenolic Compounds. Evaluation of the contribution of phenolic compounds to the overall antioxidant activities revealed that TPC, TFC, CTC, flavonols and anthocyanins were the major contributors to the antiradical (DPPH) and H_2O_2 scavenging activities (**Table 4**) in addition to metal chelating activities (**Figure 3**) in seed coat fractions, while main contributors to these activities in cotyledon fraction were phenolic acids. In embryonic axe fractions, isoflavones were the major compounds that would contribute significantly to FRAP values (**Figure 3**). The seed coat fractions of legumes appear to be good sources of natural antioxidants. These seed coat phenolics may protect unstable polyunsaturated fatty acids against oxidation in some processed foods, when used as food additives. Furthermore, isoflavones, which are predominant in embryonic axe fractions, showed higher reductive capabilities, which could be used not only as ingredients in functional food products but also as dietary supplements for deriving many health benefits in the areas of heart disease risk reduction, cancer prevention, bone health and menopause symptom reduction (*34*, *35*). In addition, phenolic compounds, especially phenolic acids, flavonols and anthocyanins, occurring in cereals and legumes, also exhibit technological functions (*61*). Therefore, cotyledon, embryonic axe and seed coat fractions of legumes are expected to have different functional properties (*62*) and can be used as functionality-based food ingredients.

In conclusion, this study revealed the distribution of phenolic compounds in different milled fractions of chickpea and horse gram and their distinct antioxidant and antiradical activities. Furthermore, these results contributed to the understanding of the relationships between major phenolic compounds and antioxidant activities of legumes and provided useful information for effective utilization of legume-milled fractions as functional food ingredients for promoting health. However, physiological significance of dietary antioxidants depends on their mechanism of absorption and biotransformation, thus warranting further investigations on the bioavailability of the phenolic compounds. Furthermore, elucidating the mechanisms of action of these compounds in several physiological processes including cellular signal transduction, cell proliferation and differentiation, apoptosis, and inflammation may yield important insights into their prophylactic uses.

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