Phenolic Antioxidants in Beans and Their Effects on Inhibition of Radical-Induced DNA Damage

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ABSTRACT: Four bean varieties with different hull colors (white, brown, red, and black) were extracted with 80% acetone and evaluated for their antioxidant potential. Red, brown, and black bean extracts were able to effectively retain supercoiled DNA against 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH)-induced radical damage at the concentration levels (5, 10, 50, and 100 ppm) tested. Delphinidin, cyanidin, and procyanidins B2, C1, C2, and X were identified in red, brown, and black bean hull extracts, whereas white bean hull extract contained only a small amount of flavonoids. Vanillic, caffeic, *p*-coumaric, ferulic, and sinapic acids were the main phenolic acids identified in bean hull extracts.

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KEY WORDS: Anthocyanins, antioxidants, beans, DNA, flavonoids, phenolic acids, procyanidins.

Beans, traditionally a staple food in many Latin American countries, have received attention as health-promoting and functional food ingredients. A number of epidemiological studies have shown a protective effect for vegetables and fruits against myriad of diseases such as coronary heart disease, cancer, and degenerative diseases, including the process of aging (1). Various *in vitro* and *in vivo* studies have shown that naturally occurring plant bioactives inhibit various stages of cancer initiation and tumor promotion (1). In both developed and developing countries, the prevalence of diabetes and cardiovascular diseases is on the rise, especially in young individuals. Nutrition has been identified as a significant factor in the prevention of such diseases (2). Beans contain a number of bioactives that exert metabolic effects on humans when consumed on a regular basis. The effect of these substances may be positive, negative, or both (3). Beans contain a number of nutrients and nonnutritive bioactive components such as lectins, protease inhibitors, galactosides of sucrose, phytates, flavonoids, and other phenolic compounds that may be protective against cancer (4). Prevention of DNA damage has been identified as one of the properties of bioactives with cardioprotective effects (4,5). Pulses (seeds of leguminous plants) that are rich in nonnutritive substances are believed to prevent DNA damage and thereby reduce the risk of cancer (4).

The objective of this study was to investigate the antioxidant potential and structural characteristics of active components of extracts of bean varieties with different hull colors. Special emphasis was placed on the effect of bean extract on the prevention of DNA damage.

MATERIALS AND METHODS

Red, brown, black, and white beans were obtained from a local grocery store in Singapore. Ethidium bromide, catechin, diethylenetriaminepentaacetic acid, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), EDTA, ferrous ammonium sulfate, DNA of pBR 322 (*Escherichia coli* strain RRI), and xylene cyanol were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). Standards of cyanidin and delphinidin chlorides were obtained from Extrasynthese (Genay, France). Standards for procyanidins were a gift from Professor Jan Oszmianski, Agricultural University, Wroclaw, Poland.

Preparation of crude phenolic extracts. Phenolic compounds present in defatted bean samples (10 g) were extracted with 80% acetone (180 mL) under reflux in a thermostated water bath at 60°C for 1 h. The resulting slurries were centrifuged for 5 min at 4000 × *g* (ICE Centra M5; International Equipment Co., Needham Heights, MA), and supernatants were collected. The residue was re-extracted with 80% acetone for 30 min, and the supernatants were collected again. The combined supernatants were desolventized *in vacuo* at 40°C, and the resulting concentrated solutions were lyophilized for 72 h at –47°C and 35×10^{-3} mbar (Freezone 6, Model 77530; Labconco Co., Kansas City, MO). The hulls were separated by air classification, as described elsewhere (6), and used for subsequent solvent extraction.

Determination of total phenolic content. Extracts were dissolved in methanol to obtain a concentration of 0.50 mg/mL of whole seed extracts or 0.20 mg/mL of hull extracts. The total phenolic content was determined according to an improved version of the procedure explained by Singleton and Rossi (7). Briefly, 1 mL of Folin–Ciocalteu's phenol reagent was added to centrifuge tubes containing 1 mL of methanolic extracts. Contents were mixed thoroughly, and 8.0 mL of sodium carbonate (75 g/L) was added to each tube. To the mixture, 10 mL of distilled water was added and mixed thoroughly. Tubes were then allowed to stand for 2 h at ambient temperature and subsequently centrifuged for 5 min at 4000 × *g*. Absorbance of the supernatant was read at 765 nm. A blank sample for each extract was used for background subtraction. The content of total phenolics in each extract was determined using a standard

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curve prepared for (+)-catechin. Total extracted phenolics were expressed as milligrams of (+)-catechin equivalents per gram of extract.

Supercoiled strand DNA scission by peroxyl and hydroxyl radicals. Plasmid supercoiled DNA (pBR 322) was dissolved in 10 mM PBS (pH 7.4, 0.15 mM sodium chloride). DNA (20 ng/μL) was mixed with catechin or bean extracts dissolved in the same PBS solution to obtain final concentrations of 5, 10, 50, and 100 ppm. Peroxyl radical was generated using AAPH (dissolved in PBS; pH 7.4, 0.15 mM sodium chloride) to obtain a final concentration of 1 mM AAPH and mixed with the DNA and the extract mixture at a total volume of 12 μL. The reactants were incubated at 37°C for 1 h in the dark (8). Upon completion of incubation, the loading dye (3 μL) (consisting of 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose in distilled water) was added to the sample and loaded onto a 0.7% (wt/vol) agarose gel prepared in Tris-acetic acid-EDTA buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5). Horizontal gel electrophoresis was performed at 32 V for 8 h. DNA strands were stained with 0.5 μg/mL ethidium bromide and visualized under UV light. Images were analyzed using AlphaEase™ stand-alone software (Alpha Innotech Co., San Leandro, CA). The protective effect of extracts and catechin was calculated based on the following equation:

$$\text{DNA retention},\% = \left(\frac{\text{supercoiled DNA content in sample}}{\text{supercoiled DNA contenin control}}\right)\times 100\% \quad [1]$$

Identification of active compounds in bean extracts. (i) Analysis of anthocyanidins. A 10-mg quantity of sample was accurately weighed into 8 mL thick-walled screw-top glass tubes and sealed with a Teflon-lined screw cap; 3 mL of acidified butanol (950 mL of *n*-butanol mixed with 50 mL of concentrated HCl) and 0.1 mL of iron reagent (0.5 g of ferrous ammonium sulfate dissolved in 25 mL of 2 M HCl) were then added and vortexed. The samples so prepared were kept in a boiling water bath for 50 min. The samples were then cooled, transferred to 10-mL volumetric flasks and adjusted to 10 mL with acidified butanol (9). The samples were passed through a 0.45 μM filter, and filtrates were collected and mixed with 250 μL methanol. Samples (20 μL) were injected onto a Shimadzu HPLC system (Mandel Scientific Co., Guelph, Canada) (two LC 10AD pumps, SPD M10A diode array detector, SCL-10A system controller, CTO 10AS column oven). The conditions for separation were as follows: column LUNA (5 μm C18; 4.6 × 250 mm; Phenomenex, Macclesfield, United Kingdom); mobile phase, 4% aqueous phosphoric acid/acetonitrile (80:20, vol/vol); flow rate, 1 mL/min; oven temperature, 25°C; and detection at 525 nm (10).

(ii) Analysis of procyanidins. Analysis of procyanidins was carried out according to the procedure explained by Oszmianski and Moutounet (11). A 20-mg quantity of extract was dissolved in 10 mL of methanol, and a 20 μL amount of the methanolic solution of the extract was injected onto a Shimadzu HPLC system. The HPLC system and the column used for proantho- cyanidin analysis were the same as those used for anthocyanidin analysis. The samples were separated in a gradient system consisting of A and B solvent systems using gradient B from 0 to 100% over 50 min. The parameters were: A, 5% (vol/vol) acetonitrile in 5% (vol/vol) acetic acid and B, 40% acetontrile in 5% acetic acid (vol/vol).

(iii) Analysis of phenolic acids. A ~50 mg quantity of each bean extract was dissolved in 10 mL of 2 M NaOH and hydrolyzed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 with 6 M HCl, free phenolic acids were extracted five times into 15 mL diethyl ether using a separatory funnel. The ether extract was then evaporated to dryness under vacuum at 30°C. The dry residue was subsequently dissolved in 2 mL of methanol and filtered through a 0.45-μm nylon filter (12). The sample so obtained was injected onto the Shimadzu HPLC system described earlier. The conditions for separation were as follows: prepacked LiChrospher 100 RP-18 column (5 μm, 4.0 × 250 mm; Merck, Darmstadt, Germany) reversed phase: water/acetontrile/acetic acid (88:10:2, by vol); flow rate 1 mL/min; oven temperature 25°C; injection volume 20 μL; detection at 260 nm (13).

Statistical analysis. Experiments were carried out in triplicate, and results are reported as mean and SD. The significant differences among the values were determined at $P < 0.05$ using ANOVA followed by Tukey's multiple range test.

RESULTS AND DISCUSSION

Total phenolic content. Preliminary studies in this work and literature data indicated the presence of catechin and catechin-related compounds in beans. Therefore, the total phenolic contents were expressed as catechin equivalents. In all four bean types, hulls contained a higher amount of total phenolics when compared with those of their whole seed counterparts. Thus, the phenolic compounds are mainly concentrated in the seed coats. Total phenolic contents of extracts of red, brown, black, and white bean hulls were 223.5, 253.2, 270.0, and 6.7 mg catechin equivalents per gram of extract, respectively, whereas those of red, brown, black, and white whole bean extracts were 93.6, 91.4, 44.0, and 4.9 mg catechin equivalents per gram of extract, respectively. As white bean extracts did not contain any considerable amount of total phenolics, they were not studied any further.

DNA double-strand scission. DNA damage is often measured as single strand-breaks, double strand-breaks, or chromosomal aberrations (14). In the present study, the bean extracts were evaluated for their capacity to inhibit peroxyl radical-induced DNA supercoiled strand scission.

The DNA retention capacities of red whole bean extract (RWE), brown whole bean extract (BWE), and catechin were not significantly ($P > 0.05$) different from one another against AAPH-derived peroxyl radical damage at a 5 ppm level whereas that of black bean hull extract (LWE) was different and low. At other concentrations (10, 50, and 100 ppm as catechin equivalents), all three extracts and catechin showed very high and similar ($P > 0.05$) DNA retention (Table 1). Radicals cleave supercoiled plasmid DNA to a nicked circular or, at higher concentrations of radicals, to a linear DNA form. The presence of peroxyl radical resulted in a dramatic scission of supercoiled DNA. This was clearly seen in the wells where the

TABLE 1
Retention Percentage of Supercoiled DNA (form I) by Bean Extracts and Catechin at Different Concentrations (5–100 ppm as catechin equivalents) in Peroxyl Radical-Induced DNA Strand Scission Studies[a]

Extract	5	10	50	100
RWE	89.5 ± 4.5[a,b]	96.6 ± 2.5[a]	97.5 ± 3.9[a]	100 ± 0.4[a]
BWE	92.6 ± 3.9[b]	97.2 ± 0.8[b]	97.3 ± 4.8[a]	100 ± 0.6[a]
LWE	86.4 ± 2.1[a]	94.0 ± 3.6[a]	98.5 ± 5.1[a]	100 ± 0.5[a]
Catechin	94.5 ± 2.9[b]	98.2 ± 0.7[b]	99.0 ± 2.6[a]	100 ± 0.1[a]

[a]Results reported are mean values of three determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another. Abbreviations: RWE, red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract.

reaction mixture did not contain any antioxidant (Fig. 1). The radical concentration used in the present study was not sufficient to destroy the nicked circular DNA, which might be more difficult to destroy than supercoiled DNA. This is clearly seen by gel electrophoresis (lane 2; Figs. 1A–C). As the concentration of antioxidative extracts was increased, the protective effect against nicking of supercoiled DNA also increased. Peroxyl radicals are more stable than other oxygen radicals (15) and have the ability to diffuse relatively far from the site of their generation before they react with a target molecule (16). It is well documented that frequently associated oxidative stress occurring in biological systems is attributed to peroxyl radicals. In the absence of any antioxidant, the peroxyl radical abstracts a hydrogen atom from the nearby DNA to generate new radicals, which in turn evoke a free radical chain reaction leading to the destruction of DNA molecules.

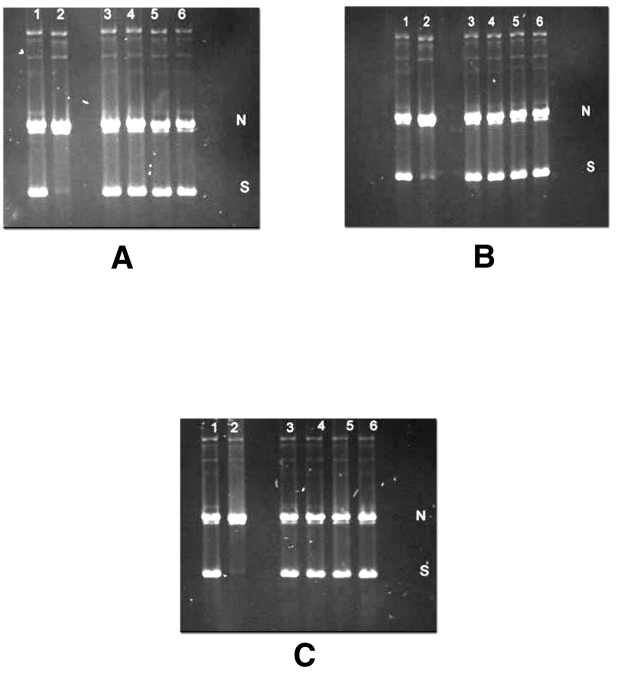

FIG. 1. Effect of bean extracts in preventing peroxyl radical-induced DNA scission. Lane 1 = DNA + PBS; lane 2 = DNA + 1 mM AAPH; lane 3 = DNA + 1 mM AAPH + 5 ppm of extract; lane 4 = DNA + 1 mM AAPH + 10 ppm of extract; lane 5 = DNA + 1 mM AAPH + 50 ppm of extract; lane 6 = DNA + 1 mM AAPH + 100 ppm of extract. AAPH = 2,2′-azobis(2-methylpropionamidine) dihydrochloride; S, supercoiled DNA; N, nicked DNA. (A) Red whole seed extract; (B) brown whole seed extract; (C) black whole seed extract.

Identification of active compounds. HPLC analysis of bean extracts showed the presence of different anthocyanidins, procyanidins, and phenolic acids. Each compound was tentatively identified by its retention time and comparison with standards under identical conditions. The anthocyanidins identified in bean hull extracts were delphinidin and cyanidin (Fig. 2A). The HPLC chromatogram for delphinidin and cyanidin identified in red bean hulls is shown in Figure 3. Red and brown bean extracts contained both delphinidin and cyanidin, whereas black bean extract contained only cyanidin. White bean extracts did not show the presence of either of the two anthocyanidins. Black bean extracts contained the highest amount of cyanidin (122 mg/g extract) followed by brown bean extract (85 mg/g extract), whereas red bean extracts contained the lowest content (41 mg/g extract) of anthocyanidin (Table 2). Red bean extracts contained a significantly higher ($P < 0.05$) amount of delphinidin (9.5 mg/g extract) compared with brown bean extract (5.6 mg/g). The anthocyanidins are the natural pigments responsible for the red, blue, or purple color of many plant products. The major antioxidant activity of anthocyanin pigments in beans can be ascribed to the reducing power of the *o*-dihydroxy structure in the B ring in cyanidin and delphinidin. Rice-Evans *et al.* (17) noted that the presence of a third hydroxyl group in the B ring did not enhance the scavenging effectiveness of delphinidin compared with cyanidin against aqueous phase radicals.

The procyanidins B_2, C_1, and C_2 identified in bean extracts are depicted in Figure 2B. Procyanidin X is unidentified and expressed as equivalents of procyanidin B_3 (based on their close retention times and similar UV spectra). The HPLC chromatogram for procyanidins B_2, C_1, C_2, and X detected in red bean hulls are shown in Figure 4. Procyanidin C_2 is the sum of two compounds, C_2 and another compound, which collectively yielded a broad peak. Red bean extract contained all the procyanidins X, B_2, C_1, and C_2, whereas brown bean extract contained only X and C_2. Black bean extracts contained B_2, C_1, and C_2, and white bean extract contained none of these procyanidins. Table 2 presents the contents of anthocyanin and procyanidin in all four bean hull extracts.

The phenolic acids of bean hull extracts were extracted and quantified (Table 3). Figure 2C depicts the chemical structures of phenolic acids identified in the bean extracts. Of the four

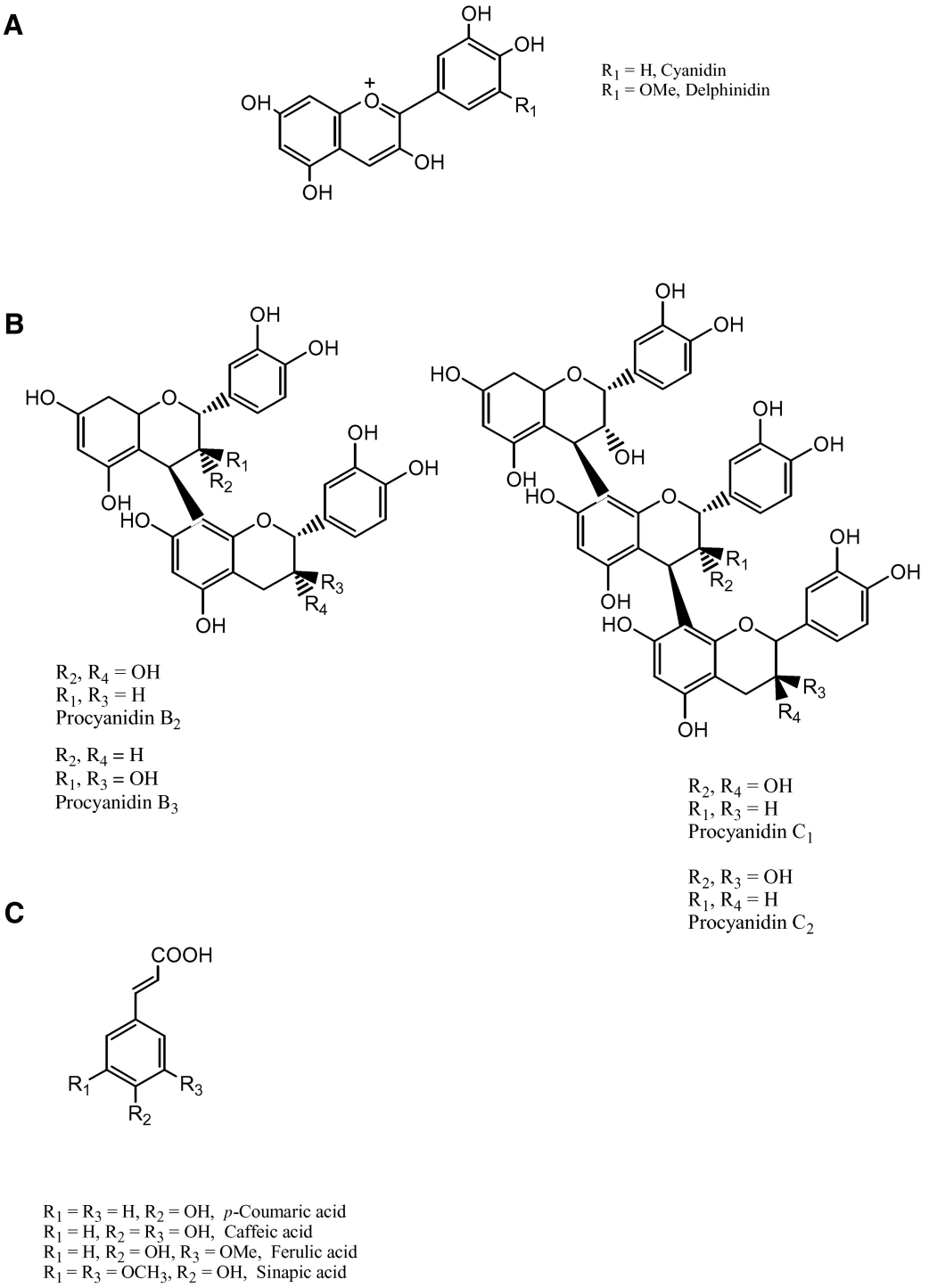

FIG. 2. Chemical structures of (A) anthocyanidins, (B) procyanidins, and (C) phenolic acids identified and quantified in bean extracts. The exact stereochemistry of procyanidin B_3 has not been shown.

types of beans examined, only black beans contained vanillic acid (254 μg/g extract), but it did not contain any caffeic acid. Black beans contained a substantial amount of *p*-coumaric acid and had a high content of ferulic and sinapic acids. Red beans contained the highest amount of *p*-coumaric acid compared with the other beans. None of the other bean types contained caffeic acid. Interestingly, white bean extract contained a significantly ($P < 0.05$) higher level of sinapic and ferulic acids compared with other beans with substantial amounts of *p*-coumaric acid. Among the most widely distributed phenylpropanoids in plant

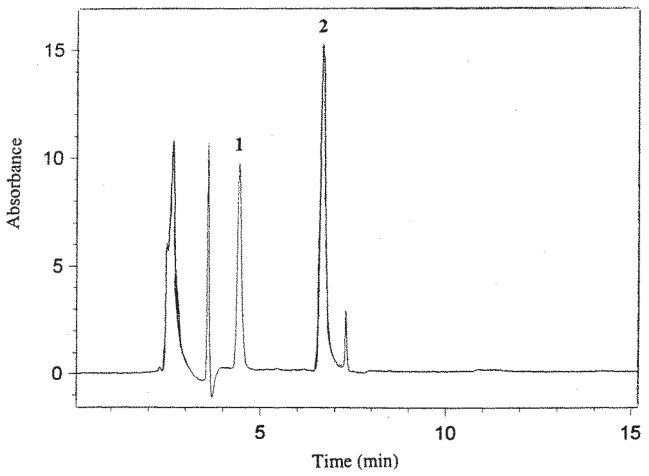

FIG. 3. HPLC profiles of hydrolyzed red bean hull extracts at 525 nm. Peaks: 1, delphinidin; 2, cyanidin.

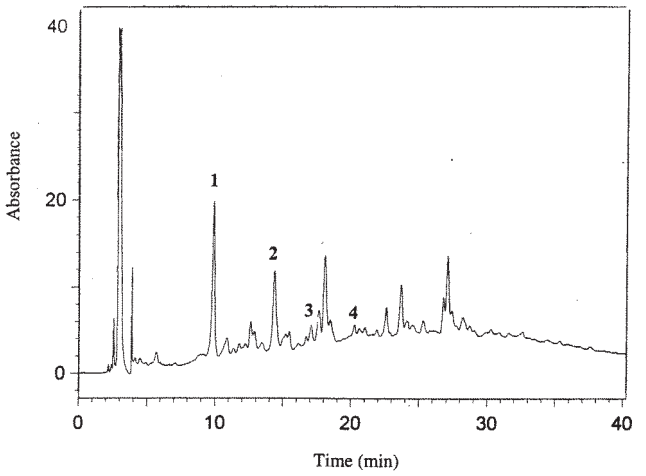

FIG. 4. HPLC profiles of hydrolyzed red bean hull extracts at 525 nm. Peaks: 1, procyanidin X; 2, procyanidin C_2; 3, procyanidin B_2; and 4, procyandin C_1.

TABLE 2
Anthocyanidin and Procyanidin Contents of Bean Extracts[a] in mg/g Extract

Extract	Delphinidin	Cyanidin	Procyanidin X	Procyanidin B_2	Procyanidin C_1	Procyanidin C_2
RHE	9.5 ± 1.2[b]	41.0 ± 2.1[a]	45.1 ± 1.4[a]	2.1 ± 0.4[a]	14.0 ± 0.9[a]	51.0 ± 2.1[b]
BHE	5.6 ± 0.5[a]	85.0 ± 2.1[b]	120.1 ± 1.1[b]	ND	ND	42 ± 2.1[a]
LHE	ND	122.3 ± 4.2[c]	ND	9.2 ± 0.6[b]	14.3 ± 0.3[a]	192.0 ± 4.0[c]
WHE	ND	ND	ND	ND	ND	ND

[a]Results reported are mean values (mg/g extract) of three determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another. Abbreviations: ND, not detectable; RHE, red bean hull extract; BHE, brown bean hull extract; LHE, black bean hull extract; WHE, white bean hull extract.

TABLE 3
Phenolic Acids Contents of Bean Extract[a]

Extract	Vanillic	Caffeic	*p*-Coumaric	Ferulic	Sinapic
RHE	ND	155.1 ± 3.2[a]	1206.0 ± 11[d]	139.8 ± 4.3[b]	283.0 ± 3.1[c]
BHE	ND	ND	209.2 ± 4.2[c]	119.9 ± 1.2[a]	87.2 ± 2.8[a]
LHE	254.2 ± 2.1[a]	ND	96.2 ± 3.6[a]	146.3 ± 2.3[c]	220.6 ± 3.3[b]
WHE	ND	ND	152.4 ± 4.2[b]	1217.0 ± 8.9[d]	603.4 ± 2.1[d]

[a]Results reported are mean values (μg/g extract) of three determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another. For abbreviations see Table 2.

tissues are the hydroxycinnamic acids, including coumaric, caffeic, and ferulic acids produced *via* the shikimate pathway from L-phenylalanine or L-tyrosine (17).

Colored beans are a main source of phenolic and polyphenolic compounds compared with white beans. Delphinidin, cyanidin, procyanidin, and phenolic acids were identified as the major phenolic compounds present in the bean hulls. Bean antioxidants exhibited strong protection against *in vitro* AAPH radical-induced DNA strand scission. Beans, especially the colored ones, can be considered an important source of natural antioxidants, hence, a food with potential health benefits.

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REFERENCES

1 Wittenberg, L.W., Inhibition of Carcinogenesis by Minor Dietary Constituents, *Cancer Res. 52* (Suppl.):2085S–2091S (1992).
2. Rizkalla, S.W., F. Bellisle, and G. Slama, Health Benefits of Low Glycemic Index Foods, Such as Pulses, in Diabetic Patients and Healthy Individuals, *Br. J. Nutr. 88* (Suppl. 3):S255–S262 (2002).
3. Champ, M.M.J., Non-nutrient Bioactive Substances of Pulses, *Br. J. Nutr. 88* (Suppl. 3):S307–S319 (2002).
4. Mathers, J.C., Pulses and Carcinogenesis: Potential for the Prevention of Colon, Breast and Other Cancers, *Br. J. Nutr. 88*, (Suppl. 3):S273–S279 (2002).
5. Singh, D.K., and S.M. Lippmann, Cancer Chemoprevention Part 1: Retinoids and Carotenoids and Other Classic Antioxidants, *Oncology 12*:1643–1682 (1998).
6. Shahidi, F., U.D. Chavan, M. Naczk, and R. Amarowicz, Nutrient Distribution and Phenolic Antioxidants in Air Classified Fractions of Beach Pea (*Lathyrus maritimus* L.), *J. Agric. Food Chem. 49*:926–933 (2001).

7. Singleton, V.L., and J.A. Rossi, Colorimetry of Total Phenolics with Phosphomolybdic Phosphotungstic Acid Reagents, *Am. J. Enol. Vitic. 16*:144–158 (1965).
8. Hu, C., Y. Zhang, and D.D. Kitts, Evaluation of Antioxidant and Prooxidant Activities of Bamboo *Phyllostachys nigra* Var. *Henonis* Leaf Extract *in vitro*, *J. Agric. Food Chem. 48*:3170–3176 (2000).
9. Porter, L.J., L.N. Hrstich, and B.G. Chan, The Conversion of Procyanidins and Delphinidins to Cyanidin and Delphindin, *Phytochemistry 25*:223–230 (1986).
10. Hong, V., and R. Wrolstad, Characterization of Anthocyanin-Containing Colorants and Fruit Juices by HPLC/Photodiode Array Detection, *J. Am. Oil Chem. Soc. 38*:698–708 (1990).
11. Oszmianski, J., and M. Moutounet, Color Product of Reaction Between Malvinidin Glucoside, Flavonols and Acetaldehyde, *Pol. J. Food Nutr. Sci. 463*:73–80 (1996).
12. Amarowicz, R., and S. Weidner, Content of Phenolic Acids in Rye Caryopses Determined Using HPLC/Photodiode Array Detection, *Czech. J. Food Sci. 19*:210–215 (2001).
13. Amarowicz, R., and F. Shahidi, Chromatographic Separation of Glucopyranyl Sinapate from Canola Meal, *J. Am. Oil Chem. Soc. 71*, 551–560 (1994).
14. Breimer, L.H., Molecular Mechanisms of Oxygen Radical Carcinogenesis and Mutagenesis: The Role of DNA Base Damage, *Mol. Carcinog. 3*:188–197 (1990).
15. Ross, A.B., and B.H.J. Beilski, Sources of Compilations of Rate Constants for Oxygen Radicals in Solution, *Methods Enzymol. 186*:113–116 (1990).
16. Morrero, R., and L.J. Marnett, The Role of Organic Peroxy Radicals in Carcinogenesis, in *DNA and Free Radicals*, edited by B. Halliwell and O.I Aruoma, Ellis Horwood, New York, 1993, pp. 145–146.
17. Rice-Evans, C., N. Miller, and G. Paganga, Structure–Antioxidant Relationship of Flavonoids and Phenolic Acids, *Free Rad. Biol. Med. 20*:933–956 (1996).

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