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Antioxidants and Antioxidant Activity of Several Pigmented Rice Brans

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This study investigated the antioxidant content and activity of phenolic acids, anthocyanins, α-tocopherol and γ-oryzanol in pigmented rice (black and red rice) brans. After methanolic extraction, the DPPH free radical scavenging activity and antioxidant activity were measured. The pigmented rice bran extract had a greater reducing power than a normal rice bran extract from a long grain white rice. All bran extracts were highly effective in inhibiting linoleic acid peroxidation (60−85%). High-performance liquid chromatography (HPLC) analysis of antioxidants in rice bran found that γ-oryzanol (39−63%) and phenolic acids (33−43%) were the major antioxidants in all bran samples, and black rice bran also contained anthocyanins 18−26%. HPLC analysis of anthocyanins showed that pigmented bran was rich in cyanidin-3-glucoside (58−95%). Ferulic acid was the dominant phenolic acid in the rice bran samples. Black rice bran contained gallic, hydroxybenzoic, and protocatechuic acids in higher contents than red rice bran and normal rice bran. Furthermore, the addition of 5% black rice bran to wheat flour used for making bread produced a marked increase in the free radical scavenging and antioxidant activity compared to a control bread.

KEYWORDS: Antioxidant; pigmented rice bran; black rice bran; red rice bran

INTRODUCTION

Over the past 20 years a significant amount of research has been directed toward the study of rice because its several key components exhibit antioxidant properties that could provide a source of natural antioxidant in the prevention of colon cancer, digestive cancers, breast cancer and prostate cancer (*1*). Rice is consumed by over half of the world population and is the second-largest cultivated crop worldwide (*2*). However, throughout history milled rice (white rice) has been the major form of consumed rice while the remaining part of the whole rice grain has been discarded or used as animal feed.

Rice bran is the most abundant and underutilized coproduct produced in the milling process. Research conducted in last two decades has shown that rice bran contains a unique complex of naturally occurring antioxidant compounds (*3*). Bidlack (*4*) has shown that rice bran may contain over 100 different antioxidants. Rice bran is potentially a valuable source of natural antioxidants such as tocopherols, tocotrienols and oryzanols (*5*). Increased concern over the safety of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) has increased the interest in finding effective and economical natural antioxidants, and the compounds in rice are a potential source to replace synthetic antioxidants. Studies by Hettiarachchy (*6*) have shown that rice bran antioxidants at 500 ppm provided the same level of antioxidant activity as a mixture of BHA/BHT at 200 ppm. However, antioxidants in rice bran are present in small quantities, and more effective extraction methods will need to be investigated.

Phenolic acid is another group of antioxidants found in cereal grain. In rice, phenolic acids can be classified into 2 types as bound and free phenolic acids. Zhou et al. (*7*) reported that rice bran contained 70−90% of phenolic acids in whole grain. In rice, γ-oryzanol is a mixture of ferulic acid esters of triterpene alcohol and sterol. It is an oil soluble antioxidant and is found in abundance in rice bran oil because of its high concentration. Many studies have shown the properties of lowering total cholesterol and LDL-cholesterol efficiency of γ-oryzanol (*8*, *9*). Another oil soluble antioxidant in rice bran is α-tocopherol which possesses a hydroxychromane ring and a terpenoid side chain located at position 2 of the ring. α-Tocopherol prevents oxidation of body lipids such as organelle membranes by donating a hydrogen atom from the hydroxyl on the ring system to a free radical (*10*). Pigmented rice also contains anthocyanins in pericarp. Anthocyanins are responsible for cyanic color of pigmented rice and are regarded as important nutraceuticals mainly due to their antioxidant effect, which provide a potential to prevent various diseases associated with oxidative stress (*11*, *12*). Ferulic acid, γ-oryzanol and vitamin E are also found in pigmented rice bran -(*13*). Pigmented rice bran is a type of rice which may provide additional benefits to human health.

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Antioxidant activities have been reported from extracts of wheat and corn bran (*14*, *15*) with the chemical antioxidants coming from phenolic acids. For rice bran, few studies have been reported on pigmented rice bran where antioxidant activities could come from various chemicals including γ-oryzanol and anthocyanins because of their higher concentrations. However, there have been no reports of the identification and quantification of the antioxidants of pigmented rice bran.

The first objective of this study was to analyze pigmented (black and red) rice bran and brown rice bran for antioxidant components and their level of activities. The second objective was to compare fortified rice bran breads with a normal wheat bread (control) for differences in levels of bioactive compounds content and their antioxidant properties.

MATERIALS AND METHODS

Materials. Normal rice (California long grain rice) was obtained from Lundberg Family Farms (Richvale, CA). Aromatic red rice and black rice no. 1 (black japonica rice) were obtained from SunWest Foods, Inc. (Davis, CA) and two black rice samples, black rice no. 2 (black japonica rice) and black rice no. 3 (a Hong Kong type of black rice), were obtained from Lundberg Family Farms. All pigmented rice samples were milled with a laboratory rice miller, 2 times at levels 3 and 4, respectively. All samples were stored immediately at −18 °C after milling until analysis.

Evaluation of Antioxidant Activity. (*1*) *Sample Preparation and Extraction of Samples*. Rice bran samples were finely ground and defatted twice with hexane (1:20 w/v) for 30 min. The defatted rice bran was extracted with 100% methanol (1:20 w/v) with an electrical shaker overnight at room temperature and then filtered through Whatman No. 1 filter paper. The extracts were stored at −18 °C until used for further analysis. All analysis was performed within 2 weeks after extraction.

For analysis of the bread samples, the crumb was defatted and then was extracted with methanol under the same conditions as used for the rice bran samples.

(*2*) *Determination of DPPH Radical Scavenging Activity*. The free radical scavenging capacity of each bran extract was measured following a previously reported procedure using the stable 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH•) (*16*). Briefly, different dilutions of the extracts were prepared. An aliquot of 1.0 mL of a diluted extract was vigorously mixed with 1.0 mL of freshly prepared 0.004% DPPH in methanol and held in the dark for 30 min at room temperature. The absorbance was then read at 517 nm against blanks. DPPH free radical-scavenging ability was calculated by using the following formula:

$$\text{scavenging ability (\%)} = [\text{absorbance(517 nm of control)} - \text{absorbance(517 nm of sample)}/\text{absorbance(517 nm of control)}] \times 100$$

The scavenging activity of rice bran extracts was expressed as 50% effective concentration, EC_{50} (mg/mL), and was obtained by interpolation from linear regression analysis. BHT and α-tocopherol were used for comparison.

(*3*) *Determination of Reducing Power*. The reducing power of the extracts was determined by the method of Yen and Duh (*17*) with some modifications. An aliquot (2.5 mL) of an extract was mixed with sodium phosphate buffer (2.5 mL, 2 M, pH 6.6) and potassium fericyanide $[K_3Fe(CN)_6]$ (0.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (10%, 5 mL) was added to the mixture, which was then centrifuged at 6000*g* for 10 min to stop the reaction. An aliquot (5 mL) from the upper layer of the solution was mixed with deionized water (5 mL) and ferric chloride solution (1 mL, 1%). The absorbance at 700 nm was then measured. Higher absorbance of the reaction mixture indicated higher reducing power.

(*4*) *Determination of Lipid Peroxidation Inhibition*. Lipid peroxidation inhibition of rice bran extract was measured according to the method reported by Lingnert et al. (*18*). Briefly, linoleic acid (5 mM) was emulsified with the aid of an equal amount of Tween 20 in sodium phosphate buffer (0.1 M, pH 7). An aliquot (4 mL) of linoleic acid dispersion was mixed with 200 μL of rice bran extract in a test tube. The tubes were placed in darkness at 37 °C for 8 h to accelerate the oxidation, and 6 mL of 60% methanol was then added. The progress of autoxidation was monitored by UV absorbance at 234 nm (A_{max} of conjugated diene peroxides from linoleic oxidation). The absorbance at 234 nm was measured against blanks. Controls without antioxidant were run in parallel. BHT and α-tocopherol were used for comparison. The inhibition of lipid peroxidation was calculated according to the following equation:

$$\text{\% inhibition of lipid peroxidation} = [\text{absorbance(234 nm of control)} - \text{absorbance(234 nm of sample)}/\text{absorbance(234 nm of control)}] \times 100$$

Quantitative Analysis of Antioxidants in Rice Bran. (*1*) *Chemicals*. Standard α-tocopherol, ferulic acid, 2,2-diphenyl-1-picryhydrazyl (DPPH•), butylated hydroxytoluene (BHT), Folin−Ciocalteu reagent, *p*-coumaric acid, sinapic acid and *p*-hydroxybenzoic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile, formic acid, trifluoroacetic acid (TFA), protocatechuic acid and gallic acid were purchased from Acros Organics (New Jersey, USA). HPLC grade of methanol was purchased from Fisher Chemical (New Jersey, USA). γ -Oryzanol was purchased from Wako Chemicals USA, Inc. (Virginia, USA). Cyanidin-3-glucoside and peonidin-3-glucoside were purchased from ChromaDex Inc. (Calfornia, USA).

(*2*) *Determination of Anthocyanin Components*. Anthocyanins of rice bran samples were determined according to the method described by Kim et al. (*19*), with some modifications. Briefly, 3 g of the defatted rice bran was extracted twice by mixing with 30 mL of methanol acidified with 1.0 N HCl (85:15 v/v) and shaking at 4 °C for 24 h. The crude extracts were filtered with Whatman No. 1 filter paper. The extracts were centrifuged at 12000*g* and 5 °C for 20 min. The extracts were kept in a refrigerator at 4 °C for 2 days to precipitate large molecules and then centrifuged at 12000*g* and 5 °C for 20 min. The upper layer was concentrated and filtered through 0.45 μL syringe filter before being injected to HPLC.

The HPLC pumps (LC-10AT, Shimadzu) and column were connected with a dual wavelength UV/vis detector (SPD-10A, Shimadzu) which was used for analysis. A TosoHaas Super-ODS, C18 2 μm 4.6 × 100 mm column was used to separate the anthocyanin components. The mixture of water, methanol and formic acid (75:20:5 v/v) was used as a mobile phase with isocratic elution at 0.5 mL/min flow rate. The UV/vis detector was set at 530 nm, and the sample loop was 5 μL. The authentic standards were used to identify and quantify the separated anthocyanin components of the bran via external standard method.

(*3*) *Determination of α-Tocopherol and γ-Oryzanol Content*. α-Tocopherol and γ-oryzanol in the bran extract were determined according to method of Aguilar-Garcia et al. (*20*), with some modifications. Rice bran samples (100 mg) were extracted twice with 6 mL of methanol and centrifuged 10 min at 825*g*. The supernatant was combined and then evaporated to 4 mL, then made up to exactly 5.0 mL with HPLC grade methanol in a volumetric flask. This solution was filtered through a 0.45 μL syringe filter before being subjected to HPLC analysis.

α-Tocopherol and γ-oryzanol were analyzed by HPLC using a Shimadzu (LC-10AT) HPLC equipped with UV/vis detector. The C18 column (Inertsil ODS-3, 5 μm, 250 × 4.6 mm) was used to separate these compounds. The mobile phase was a mixture of methanol and acetonitrile (15:85 v/v) at a flow rate of 2 mL/min with isocratic mode. The sample loop was set at 20 μL. The UV/vis detector was set at 292 and 325 nm for α-tocopherol and γ-oryzanol, respectively.

(*4*) *Determination of Total and Bound Phenolic Acids*. Total and bound phenolic acids were determined according to the method reported by Tian et al. (*21*), with some modifications. For total phenolic acids determination, 2 g of rice bran samples were extracted with hexane (4 × 50 mL) to remove fat. The residue was hydrolyzed with 1 M NaOH (2 × 100 mL, 2 h each). The supernatants were pooled and acidified with 4 N HCl to pH 1 and then were extracted (4 times) with ethyl acetate (200 mL each). The ethyl acetate fractions were evaporated to dryness, and then were dissolved with methanol (5 mL, 15%) and analyzed by HPLC. For bound phenolic acid determination, defatted rice bran was extracted with 70% ethanol to remove free phenolic acids before hydrolysis, and the determination was performed by following the same method as for total phenolic acids determination, mentioned above.

The extracts were separated by using HPLC with the C18 column (5 μm, 4.6 × 250 mm) and UV/vis detector. The mobile phase was a mixture

Table 1. Antioxidant Contents (μg/g) of Rice Bran Samples

	bran samples				
antioxidants[a]	normal	red	black no. 1	black no. 2	black no. 3
anthocyanin		188 ± 8	2562 ± 34	1135 ± 43	1658 ± 20
	(0%)	(5.2%)	(25.8%)	(17.7%)	(20.1%)
phenolic acids	2101 ± 175	1526 ± 103	3289 ± 116	2772 ± 102	3183 ± 229
	(35.9%)	(42.5%)	(33.1%)	(43.2%)	(38.5%)
α-tocopherol	71 ± 8	16 ± 4	24 ± 3	27 ± 3	25 ± 4
	(1.2%)	(0.5%)	(0.2%)	(0.4%)	(0.3%)
γ-oryzanol	3681 ± 30	1859 ± 121	4057 ± 464	2483 ± 170	3390 ± 225
	(62.9%)	(51.8%)	(40.9%)	(38.7%)	(41.1%)
total	5853	3589	9932	6417	8256
	(100%)	(100%)	(100%)	(100%)	(100%)

[a] Numbers in parentheses are percentage of each antioxidant in rice bran samples.

of acetonitrile (B) and pure water with TFA (0.1%) at a flow rate of 1.5 mL/min. Gradient elution was performed as follows: 0−15 min, linear gradient from 5 to 9% solvent B; from 15 to 30 min 9% solvent B; 30−37 min, linear gradient from 9 to 13% solvent B; from 37 to 55 min, linear gradient from 13 to 18% solvent B; from 55 to 60, linear gradient from 18 to 20% solvent B. The detector was set at 250 nm to detect hydroxybenzoic acid and at 325 nm to detect hydroxycinammic acid. Comparing their retention times with authentic compounds by using an external standard method identified the separated phenolic acids.

(*5*) *Bread Baking Method*. Breads were made using an automatic bread maker machine (model 5891, Sunbeam Programmable Bread Maker, USA). The program for normal white bread was chosen for bread making. This program adopts the following sequential process: first kneading (5 min), rest (5 min), second kneading (20 min), first rising (15 min), third kneading (10 s), second rising (30 min), and baking (50 min). A formula was adapted from results from preliminary trials. Rice bran was substituted with wheat flour for 5%.

(*6*) *Determination of Total Phenolic Content in Breads*. Total phenolic content of rice bran breads was determined by using the Folin−Ciocalteu reagent, according to the method reported by Goffman and Berman (*22*) with some modifications. Briefly, 200 mg of crumb was extracted with 5 mL of methanol overnight (vortex 2 times on first and final) and then was centrifuged at 4000*g* for 5 min. Four milliliters of the supernatant was filtered by a 1 μm syringe filter. The extract was diluted with deionized water. Folin−Ciocalteu reagent (500 μL) and ethanolamine (1 mL, 0.5 M) were added to 1.2 mL of the diluted solution, mixed and left standing at room temperature for 30 min. The absorbance at 600 nm was measured. The results were expressed as mg of gallic acid equivalents per g of sample.

RESULTS AND DISCUSSION

Antioxidant Contents. The contents of anthocyanins, phenolic acids, α-tocopherol and γ-oryzanol in rice bran were determined by using HPLC analysis, **Table 1**. Among the rice brans, the total antioxidant contents, ranked in descending order, were black bran no. 1, black bran no. 3, black bran no. 2, normal bran and red bran. The range of anthocyanins in the black rice brans (1.13−2.56 mg/g) was similar to that reported in the seed coat of black soy bean (0.22−1.87 mg/g) (*23*) and purple wheat bran (1.16 mg/g) (*24*). Total phenolic acids found in rice bran samples were in the range of 1.53−3.29 mg/g, which was slightly lower than reported in wheat bran (3.36−3.97 mg/g) (*25*). Rice bran samples contained γ-oryzanol in the range 1.86−4.06 mg/g. γ-Oryzanol is not present in common cereals at a high level, with the exception of rice. α-Tocopherol is an important natural antioxidant in rice bran. Rice bran contains vitamin E up to 150 μg/g (*26*). The content of α-tocopherol in the rice bran samples had a range of 16−71 μg/g. Among the tocopherols, α-tocopherol has the greatest vitamin E activity due to its high level of antioxidant activity (*27*). According to a previous study by Aguilar-Garcia et al. (*20*), this study showed lower α-tocopherol content in rice bran, which may be due to the difference of genotype or sample preparation method. However, these results showed that the amount of γ-oryzanol was about 50 times higher than that of α-tocopherol.

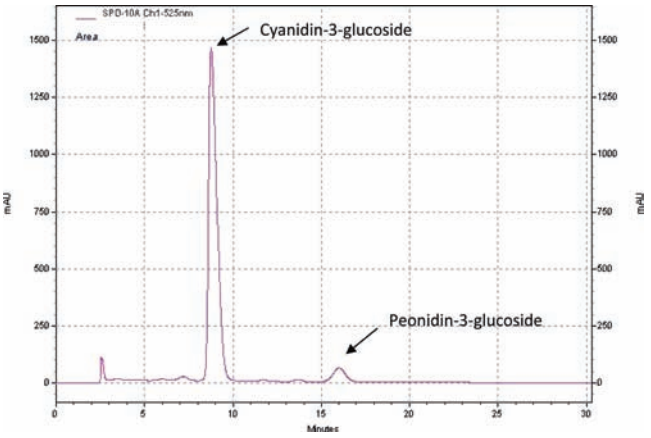

Figure 1. Chromatogram of anthocyanin compounds in pigmented rice bran.

Major antioxidants found in normal rice bran were γ-oryzanol and phenolic acids, which were 62.9% and 35.9% of total antioxidant content, respectively, while α-tocopherol was found as only 1.2% of the total antioxidants. The anthocyanins were not found in normal bran. γ-Oryzanol and phenolic acids were the major antioxidants for red rice bran containing 51.8% and 42.5% of total antioxidant content, respectively. Red bran contained a small amount of anthocyanins and α-tocopherol, which were 5.2% and 0.5% of total antioxidant content, respectively. For black rice bran, 17.7−25.8% of total antioxidant was anthocyanins which are responsible for the black color of the bran. In addition, black rice bran showed high content of phenolic acids and γ-oryzanol, the percentages of which were 33.1−43.2% and 38.7−41.1% of total antioxidant content, respectively. These results showed that γ-oryzanol (1.86−4.06 mg/g) and phenolic acids (1.53−3.29 mg/g) were the major antioxidants in all bran samples. α-Tocopherol was found at the lowest level among all rice bran samples, compared with the other antioxidants. Xu et al. (*28*) reported that γ-oryzanol is a more potent antioxidant than α-tocopherol in reduction of cholesterol oxidation.

Antioxidant Composition. (*a*) *Anthocyanins*. HPLC analysis was used to separate anthocyanins in the pigmented rice brans into two major compounds, **Figure 1** and **Table 2**. Anthocyanins were not found in normal rice bran but were found in all pigmented rice bran samples. The two principal anthocyanins found in pigmented rice bran were cyanidin-3-glucoside and peonidin-3-glucoside. Cyanidin-3-glucoside was the most predominant

anthocyanin in pigmented rice bran with a value of 90−95% of total anthocyanin content, except in the black rice bran no. 2, which had a content of 58% of the total anthocyanins. Cyanidin-3-glucoside and peonidin-3-glucoside have been reported as the major anthocyanins found in ten pigmented rice varieties (*29*). The content depended on rice variety. Escribano-Bailón et al. (*30*) reported that cyanidin-3-glucoside was the major anthocyanin in pigmented rice, found in a range of 80−100% of total anthocyanins. Further, it was reported that cyanidin-3-glucoside was a stronger antioxidant than peonidin-3-glucoside in both food and biological model systems (*31*−*33*). Cyanidin-3-glucoside and peonidin-3-glucodide were the major anthocyanins found in black rice (*Oryza sativa* L. *japonica*) (*13*).

(*b*) *Phenolic Acids*. The major portion of phenolic acids existed in insoluble (bound) form, **Table 3**. The phenolic acids in all rice bran samples were dominated by ferulic acid with lesser amounts of gallic, protocatechuic, hydroxybenzoic, *p*-coumaric and sinapic acids (**Figure 2**). The results showed that in rice bran most of the ferulic acid was in the bound form. Several studies have reported the occurrence of bound ferulic acid in cereal (*34*,*35*). This observation was confirmed in the present study. Ferulic acid was reported to be the major phenolic compound in corn, wheat, oats and rice grains (*1*). The percentage of bound ferulic acid in black rice bran ranged from 70.8 to 76.4%. Black rice bran showed higher contents of gallic, hydroxybenzoic and protocatechuic acids as compared to normal and red rice bran (**Table 3**). Phenolic acids from durum wheat bran were reported to be partially responsible for the total antioxidant activity of the bran extract (*14*).

Anitoxidant activity of methanolic extracts. (*a*) *DPPH Radical Scavenging Activity*. DPPH is a stable free radical, and when it encounters proton-radical scavengers, the maximum absorbance at 517 nm fades rapidly. The antioxidant effect is proportional to the disappearance of DPPH in test samples. Based on this principle, the radical scavenging effect of each rice bran extract was measured, and the results are expressed as EC_{50}, which is the amount of an antioxidant that causes a decrease in an initial DPPH concentration by 50% (*16*).

The bran extracts of 5 rice varieties of different colors were examined and compared for their free radical scavenging activities against DPPH$^{\bullet}$ radical (**Figure 3**). With regard to EC_{50} value, the highest radical scavenging activity was found in the extract of black bran no. 1 (0.10 mg/mL) and the lowest activity was found in normal rice bran (0.56 mg/mL). The order of the DPPH radical scavenging activity was black bran no. 1 > black bran no. 3 > black bran no. 2 > red bran > normal bran. All pigmented rice bran showed higher radical scavenging activity than the normal rice bran. The activity of black bran no. 1 was approximately 6 times higher than that of normal bran. This can be due to the high concentration of anthocyanins particularly the cyanidin-3-glucoside in black rice bran no. 1 (**Table 2**). However, DPPH free radical scavenging of all extracts was less than that of BHT and α-tocopherol. The varied radical scavenging activity of the methanolic extract may depend on the amount and type of antioxidants in rice bran. However, the results showed that, to reach a similar EC_{50} effect of BHT, the amounts required for black bran no. 1 and 3 extracts were less than that required for normal bran extract.

(*b*) *Reducing Power*. The bran extract samples showed significant differences in reducing power (**Figure 4**) as measured by the formation of Perl's Prussian blue color at 700 nm. Pigmented rice bran extracts showed higher reducing power than the normal rice bran. The highest reducing power was found in black rice bran no. 1 extract whereas the lowest was found in normal rice bran extract. All rice bran extracts showed higher reducing power than α-tocopherol and BHT. The reducing power patterns of the bran extracts were in agreement with DPPH radical scavenging activity.

It appears that the antioxidants in the pigmented rice bran extracts are electron donors which are capable of reacting with free radicals and convert them to stable compounds. Thus the radical chain reaction is terminated (*36*).

Table 2. Anthocyanin Composition (μg/g) of Rice Bran Samples

rice bran samples	cyanidin-3-glucoside	peonidin-3-glucoside	total	cyanidin:peonidin ratio
normal[a]				
red	179.0 ± 7.7	9.1 ± 1.4	188.1	95:5
black no. 1	2316.7 ± 34.4	245.7 ± 6.8	2562.4	90:10
black no. 2	662.2 ± 43.4	472.3 ± 7.3	1134.5	58:42
black no. 3	1515.8 ± 20.3	141.7 ± 4.2	1657.5	91:9

[a] Anthocyanins were not found in normal rice bran.

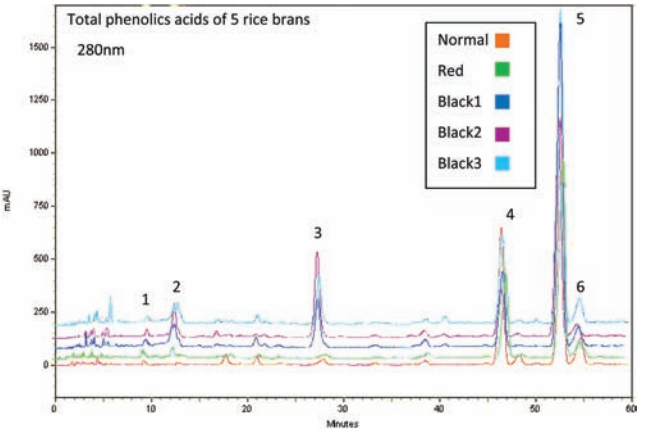

Figure 2. Chromatogram of total phenolic acids in pigmented rice brans (1 = gallic acid, 2 = protocatechuic acid, 3 = hydroxybenzoic acid, 4 = *p*-coumaric acid, 5=ferulic acid and 6=sinapic acid).

Table 3. The Content of Insoluble and Total Phenolic Acids in Rice Bran

	normal		red		black no. 1		black no. 2		black no. 3	
bran fraction (μg/g)	insoluble	total	insoluble	total	insoluble	total	insoluble	total	insoluble	total
gallic acid	20.9 ± 3	25.1 ± 2	16.9 ± 4	39.0 ± 4	13.7 ± 5	161.1 ± 12	40.6 ± 6	85.3 ± 5	18.0 ± 3	101.0 ± 10
protocatechuic acid	7.2 ± 3	13.8 ± 10	60.4 ± 3	81.8 ± 7	245.0 ± 14	539.3 ± 41	158.8 ± 9	453.4 ± 20	123.2 ± 14	538.5 ± 67
hydroxybenzoic acid	55.0 ± 4	68.9 ± 9	40.0 ± 4	52.5 ± 6	330.7 ± 16	443.3 ± 29	525.2 ± 41	789.5 ± 46	277.1 ± 24	433.6 ± 34
p-coumaric acid	404.8 ± 43	420.5 ± 49	237.1 ± 22	277.7 ± 17	207.2 ± 10	230.0 ± 20	139.2 ± 3	148.4 ± 5	200.3 ± 21	253.8 ± 33
ferulic acid	1262.2 ± 121	1314.5 ± 110	943.9 ± 54	1115.1 ± 75	1532.5 ± 76	1663.5 ± 101	948.5 ± 34	1140.0 ± 50	1438.8 ± 82	1570.2 ± 79
sinapic acid	224.0 ± 18	258.7 ± 23	183.4 ± 12	209.8 ± 27	183.4 ± 10	252.1 ± 20	150.2 ± 11	155.2 ± 16	259.1 ± 13	285.9 ± 10
sum	1974.2	2101.3	1481.7	1775.9	2512.6	3289.4	1962.5	2771.9	2316.4	3182.9
% insoluble	94.0		83.4		76.4		70.8		72.8	

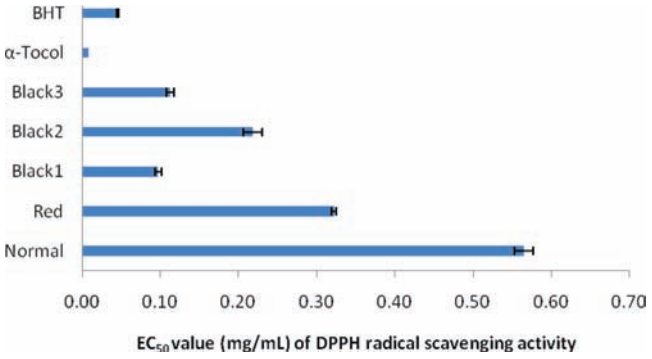

Figure 3. EC_{50} value of DPPH radical scavenging activity of methanolic pigmented rice bran extracts (shorter bar shows higher radical scavenging activity).

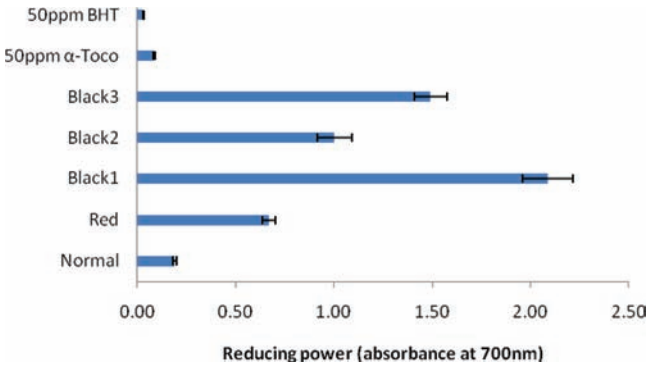

Figure 4. Reducing power (absorbance at 700 nm) of methanolic pigmented rice bran extracts.

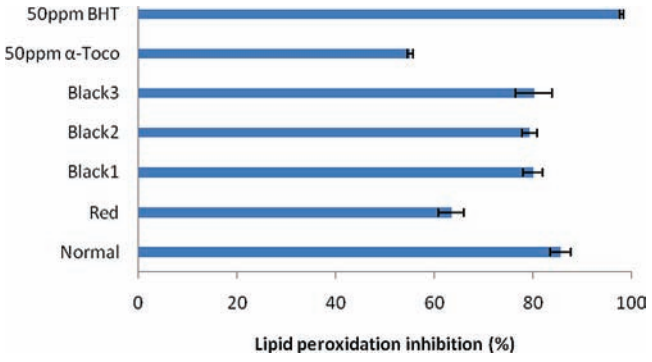

Figure 5. Lipid peroxidation inhibition (%) of methanolic rice bran extracts.

(*c*) *Lipid Peroxidation Inhibition*. Linoleic acid−buffer emulsion (oil-in-water emulsion) was exposed to the air at 37 °C to induce lipid peroxidation. Pigmented rice bran extracts showed lower lipid peroxidation inhibition than the normal rice bran extract. All black brans had similar lipid peroxidation inhibition (79.2−80.1%), **Figure 5**. The lowest inhibition was observed in red bran extract (63.4%) while the highest was in normal bran (85.5%). However, all rice bran extracts showed stronger lipid peroxidation inhibition than α-tocopherol alone (55.1%).

α-Tocopherol is well recognized for its effectiveness to inhibit lipid oxidation (*37*). The effectiveness of an antioxidant at scavenging lipid free radicals depends on its physical location such as lipid droplet in the emulsion. In oil-in-water emulsion, the location can be in the bulk of the lipid droplets and/or at the lipid−water interface (*38*). Nonpolar antioxidants such as α-tocopherol are more effective in emulsions because they are retained in the oil droplets and/or accumulated at the oil−water interface, the location where interaction between hydroperoxides at the droplet surface and pro-oxidants originating in the aqueous occurs. In this study, the normal rice bran contained more α-tocopherol (71 μg/g) than the pigmented rice bran (16−27 μg/g) (**Table 1**) and it may have attributed to the higher inhibition of lipid oxidation of normal rice bran extract compared to the pigment rice varieties.

Table 4. Antioxidant Content of Fortified Rice Bran Bread

bread samples	total phenolic (mg of gallic acid equiv/g)	anthocyanin (μg/g)[a] cyanidin-3-glucoside	anthocyanin (μg/g)[a] peonidin-3-glucoside	γ-oryzanol (μg/g)[a]
0% bran	2.55 ± 0.53	nd	nd	nd
5% normal bran	2.98 ± 0.12	nd	nd	74.72 ± 6.90 (18.14%)
5% red bran	2.89 ± 0.06	nd	nd	59.07 ± 4.38 (15.82%)
5% black bran	3.07 ± 0.08	11.82 ± 1.03 (82.0%)	nd	71.82 ± 7.29 (33.78%)

[a] The numbers in parentheses show % lost after baking; nd = not detectable.

Table 5. Antioxidant Activity of Methanolic Extracts of Fortified Rice Bran Bread Samples

bread samples	DPPH radical scavenging (EC_{50}; mg/mL)	reducing power (mg/mL BHT equiv)	% lipid peroxidation inhibition
0% bran	nd[a]	158.65 ± 31.76	no effect
5% normal bran	40.32 ± 1.32	191.49 ± 35.54	8.36 ± 0.66
5% red bran	41.60 ± 1.54	182.57 ± 39.32	7.41 ± 0.54
5% black bran	18.65 ± 0.94	219.05 ± 39.05	31.72 ± 0.94

[a] Not detectable.

APPLICATIONS IN FOOD

Fortification of Rice Bran in Bread. Bread made with the addition of 5% normal, red or black rice bran no. 1 (which had the highest antioxidant contents among the 3 black rice brans) to wheat flour with was evaluated for antioxidant content and activity. Addition of normal, red and black rice bran increased the total phenolic contents to 16.8%, 13.3% and 20.4%, respectively, compared to the wheat bread, **Table 4**. After baking, black bran bread lost 82.0% and 100% of cyanidin-3-glucoside and peonidin-3-glucoside, respectively, and the red bran bread lost all of its anthocyanins. Cyanidin-3-glucoside was very stable against heat at acidic pH (pH 2), and stability decreased as pH increased to 9 (*39*). Hiemori et al. (*40*) reported that the degradation of rice cyanidin-3-glucoside was about 80% after pressure cooking. γ-Oryzanol was more stable in baking as compared to anthocyanins (**Table 4**). γ-Oryzanol was highly retained in the baked breads and lost only 18.14%, 15.82% and 33.78% for normal, red and black bran bread, respectively.

The methanolic extracts of bread were determined for the DPPH radical scavenging activity. The DPPH radical scavenging activity of control bread was too low to measure, while the rice bran substituted bread showed significant antioxidant activity (**Table 5**). Regarding the EC_{50} value, 5% black bran bread extract showed the highest radical scavenging activity, while lower activity was found in the extracts of 5% normal and red bran bread. Because of the similar level of γ-oryzanol in normal and black bran bread (**Table 4**), thus cyanidin-3-glucoside may contribute to the high antioxidant activity of black bran bread.

Reducing power of methanolic extracts of the fortified rice bran bread is shown in **Table 5**. The highest reducing power was found in 5% black bran bread. The 5% black bran bread extract showed 38.1% higher reducing power than the control bread (0% bran) while fortification with 5% normal and red bran increased the reducing power 20.7% and 15.1%, respectively. The results suggested that antioxidants which remained in the rice bran bread were electron donors.

The antioxidant activity of rice bran bread samples was confirmed by the measurement of lipid peroxidation inhibition in linoleic acid emulsion model (**Table 5**). The extract of control bread showed no inhibition effect against conjugated diene formation during incubation for 8 h, while all rice bran bread extracts showed oxidation inhibition, ranging from 7.41 to 31.72%. The lipid peroxidation inhibition of 5% red bran bread extract (7.41%) was slightly lower than that of 5% normal bran bread (8.36%). The results indicated that incorporation of black rice bran into bread gives it high added value and provides an additional source of antioxidant in the diet.

Conclusions. The results of this study show that black rice bran has the highest antioxidant activity which can be attributed to the high anthocyanins, phenolic acids and γ-oryzanol. Most of the phenolic acids were bound phenolic acids. Ferulic acid was the dominant phenolic acid in rice bran samples. Black rice bran contained more gallic, hydroxybenzoic and protocatechuic acids content than red and normal bran. Two major anthocyanins in black rice were cyanidin-3-glucoside and peonidin-3-glucoside.

The fortification of rice bran in bread increased total phenolics, anthocyanin and γ-oryzanol contents. The methanolic extract of pigmented rice breads had stronger DPPH radical scavenging activity, reducing power and lipid peroxidation inhibition than the control bread (0% bran). Our study suggested that black rice bran contains several compounds, including cyanidin-3-glucoside, that possess antioxidant activities and could be a valuable source of natural antioxidants for use in food supplements as this test with bread suggests.

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