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Growth-Inhibitory Effects of Pigmented Rice Bran Extracts and Three Red Bran Fractions against Human Cancer Cells: Relationships with Composition and Antioxidative Activities

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ABSTRACT: We determined the phenolic, anthocyanin, and proanthocyanidin content of three brown, purple, and red rice brans isolated from different rice varieties using HPLC-PDA with the aid of 27 standards of known structure and matching unknown peaks to a spectral library of known compounds. Antioxidative capacities were determined by DPPH and ORAC and cell-inhibiting effects using an MTT assay. Based on the calculated IC_{50} values, the light-brown bran had no effect, the purple bran exhibited a minor effect on leukemia and cervical cancer cells, and the red bran exhibited strong inhibitory effects on leukemia, cervical, and stomach cancer cells. High concentrations of protocatechuic acid and anthocyanins in purple bran and proanthocyanidins in red bran were identified. The red bran was further fractionated on a Sephadex column. Fraction 3 rich in proanthocyanidin oligomers and polymers had the greatest activity. Red bran has the potential to serve as a functional food supplement for human consumption.

KEYWORDS: *brown rice bran, purple rice bran, red rice bran, composition, HPLC-PAD, MTT assay, cancer cells*

INTRODUCTION

About 600 million tons of rice (*Oryza sativa*) are harvested worldwide annually. Rice bran, a byproduct of the rice milling industry, contributes about 10% to the weight of the grain.[1] Because they contain high amounts of structurally different bioactive compounds,[2] brans from colored (pigmented) rice varieties have the potential to serve as a functional food that can promote human health. Reviews by Jariwalla[3] and Rondanelli, Perna, Monteferrario, and Opizzi[1] discuss how rice-bran-rich phytonutrients can help control blood sugar levels, cholesterol metabolism, and prostate health, and prevent the growth of cancer cells, the main theme of the present study.

Rice bran contained both lipophilic antioxidants (tocopherols, tocotrienols, and γ-oryzanol) and phenolics.[2] Tocotrienols, a subgroup of vitamin E analogues, have received a lot of attention for their potential against cancer and other chronic diseases.[4] Concentrations of tocotrienols in bran varied among rice varieties but were not related to bran color.[2] Studies have shown that pigmented rice bran extracts exhibit a number of beneficial bioactivities. These include the observations that black (i.e., purple) rice bran extracts reduced the progression of dietary cholesterol-induced atherosclerotic plaque development and cholesterol plasma levels in rabbits,[5] decreased serum triglycerides and total cholesterol in mice,[6] lowered total plasma cholesterol levels in pigs without affecting pork quality,[7] exhibited antimutagenic and anticarcinogenic activities in chemical and cell assays,[8−10] inhibited tumor promotion in lymphoblastoid B cells,[11] exhibited antiallergic activities in cell assays,[12] exhibited immunomodulating activities in cell culture,[13] protected mice against inflammation,[14] and provided protection against light-induced retinal damage.[15]

Little information is available regarding the bioactivity of red pigmented bran. Chemical analyses show that both red and purple brans have high antioxidant capacities and total phenolic contents, while each contains a unique flavonoid subgroup, specifically anthocyanins in purple and proanthocyanidins in red.[2] Proanthocyanidins are a class of polymeric phenolic compounds consisting mainly of catechin, epicatechin, gallocatechin, and epigallocatechin units. The degree of polymerization and galloylation affects their bioactivity,[16] and proanthocyanidin profiles differed depending on the food sources.[17] In addition, metabolomic and functional genomic analyses reveal large varietal differences in bioactive compounds in rice brans.[18] Thus, more research is needed to determine the variability in health-promoting bioactivity of pigmented rice brans.

Pigmented rice and its bran also exhibit strong antioxidative effects.[2,9,19−24] The reported antioxidative effects seem to be related to the content of several types of phenolic compounds.

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These include simple phenolics such as ferulic and protocatechuic acids as well more complex anthocyanins and proanthocyanidins.

The objective of this study was to compare phenolic, anthocyanin, and proanthocyanidin contents and antioxidative effects of bran extractions from light-brown, purple, and red rice varieties on human cancer and normal cell growth.

■ MATERIALS AND METHODS

Chemicals and Reagents. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), gallic acid, sodium nitrite, aluminum chloride, apigenin, *o*-cresol, 3,5-dimethylphenol, 4-dimethylamino cinnamaldehyde (DMAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), doxorubicin hydrochloride (≥98.0%), epicatechin, eriodictyol, fluorescein disodium, Folin−Ciocalteu reagent, hesperetin, isorhamnetin, kaempferol, luteolin, myricetin, naringenin, protocatechuic acid, quercetin, rutin, caffeic, chlorogenic, *p*-coumaric, 4-hydroxybenzoic acid, and methoxycinnamic, sinapic, syringic, and vanillic acids were obtained from Sigma-Aldrich (St. Louis, MO), ethanolamine, pro B2, (+)-catechin, and *p*-cresol from Fluka (Milwaukee, WI), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) from Wako (Richmond, VA), cyanidin chloride, cyanidin-3-galactoside, cyanidin-3-glucoside, and cyanidin-3-*O*-rutinoside from Sigma-Aldrich (St. Louis, MO), peonidin-3-glucoside from Extrasynthèse (Genay, France), delphinidin chloride, delphinidin-3-glucoside, and peonidin chloride from Chromadex (Irvine, CA), ferulic acid from ICN (Aurora, Ohio), HPLC grade acetic acid, acetone, and methanol from Fisher (Fair Lawn, NJ), HPLC grade ethanol from Pharmco (Brookfield, CT), and HCl (36.5−38%) from J. T. Baker (Phillipsburg, NJ).

Rice Bran Extracts. Three rice cultivars of different bran color [red IITA 119 (IITA), purple IAC 600 (IAC), and light-brown Cocodrie (CCDR)] were selected for this study. IITA from GRIN (PI 458466) (Germplasm Resources Information Network, http://www.ars-grin.gov) is a medium-grain rice originating from Nigeria. IAC is a short-grain rice developed by Candido Bastos of Agronomico Instituto, Campinas, Brazil. This variety has been reported to control hypercholesterolemia in rats.[25] CCDR is a U.S. long-grain rice developed by Linscombe et al.[26] All cultivars were grown at Beaumont, TX, using standard cultural management practices for this region. The IITA variety was harvested in 2009, and the IAC and CCDR varieties were harvested in 2007.

After harvest, the rough rice was stored at −20 °C until use. After removing the hulls from the rough rice, whole grain rice was milled using a Satake One Pass Mill (Pearler, Model SKD), and then the bran isolated after sieving through a 590-μm mesh sieve (E. H. Sargent & Co., Chicago, IL). The bran was defatted twice with hexane at a 1:8 bran-to-solvent ratio with shaking at 250 rpm for 2 h at room temperature (RT). The residual hexane was evaporated at reduced pressure, and the bran was then extracted with 70% ethanol at a 1:10 bran-to-solvent ratio for 2 h at RT with shaking (250 rpm). The supernatant of the extract was collected after centrifugation at 10000*g* at 4 °C for 15 min, then filtered through a 0.45 μm PVDF membrane, ethanol-evaporated at reduced pressure, and lyophilized. The dry extract was stored at −20 °C under nitrogen until use.

Sephadex LH-20 Fractionation of Red Bran Extract. A second batch was extracted as follows: red bran (120 g) was extracted with hexane at a 1:5 bran to solvent ratio with shaking at 200 rpm for 1 h at RT and then centrifuged (7000*g*, 4 °C, 15 min). The hexane layer was discarded, and the hexane extraction was repeated once. After evaporation of hexane from the defatted bran, 70% ethanol was added at a 1:5 bran-to-solvent ratio followed by extraction for 2 h at RT, centrifugation (7000*g*, 4 °C, 15 min), and collection of the supernatant. The extraction was repeated once, and the supernatants were combined. The solvent was evaporated from the pooled 70% ethanol extract, and the residue was then sonicated and loaded onto a Sephadex LH-20 cartridge to separate into three fractions.[27,28] Briefly, 10 g of Sephadex LH-20 resin (Cat. No. LH20100, Sigma-Aldrich) was hydrated in deionized water overnight at 4 °C and packed into a 60-mL filtration tube with polyethylene frit (Supelco, Bellefonte, PA, Cat. No. 57178). After ethanol evaporation, a 10-g bran equivalent extract was loaded onto a LH-20 cartridge and sequentially eluted with 120 mL of 20% methanol, 50% ethanol, and 70% acetone to produce fractions IITA-20M, IITA-50E, and IITA-70A, respectively. Isolates from a total of 12 columns of each fraction were pooled, the organic solvent was evaporated, and then the sample was lyophilized to dryness.

Total Phenolics, Flavonoids, Anthocyanins, and Proanthocyanidins and Antioxidant Capacities. Total phenolic (TP) content was analyzed using the Folin−Ciocalteu assay[29] with modification and the total flavonoid (TF) content by the spectrophotometric method described by Zhishen, Mengcheng, and Jianming.[30] Total anthocyanin content of the purple bran extract was the sum of the individual anthocyanins determined by HPLC-PDA according to the method of Min et al.[2] Total proanthocyanidin content was determined using the 4-DMAC method.[27] The DPPH assay of radical scavenging capacity was based on the method of Sánchez-Moreno, Larrauri, and Saura-Calixto.[31] The hydrophilic oxygen radical absorption capacity (ORAC) was determined by the method of Huang, Ou, Hampsch-Woodill, Flanagan, and Prior.[32] Detailed protocols for all these methods are described in Min et al.[2]

Free and Esterified Phenolics. The free and esterified phenolics in three bran extracts were determined using HPLC-PDA. The dried extract was dissolved in DMSO, filtered through a 0.45 μm PTFE membrane (Cat. No. WAT200502, Waters, Milford, MA) and then resolved on Symmetry C18 guard and analytical columns (Part No. WAT054275, 5 μm, 250 × 4.6 mm; Symmetry C18 Guard, Part No. WAT054225). The esterified phenolic acids were determined by the difference between the free phenolics and total phenolics in a hydrolyzed sample.

To hydrolyze the samples, portions of the three bran extracts and the red bran LH-20 fractions in DMSO were treated with 2 N NaOH containing 10 mM EDTA and 1% ascorbic acid for 30 min at 30 °C under nitrogen.[33] The hydrolysates were then acidified to pH 4 with HCl and cleaned up through an OASIS HLB cartridge (500 mg sorbent, Waters). The OASIS HLB cartridge was first activated with 100% methanol and then equilibrated with 6 mL of 0.1% formic acid. The phenolics were eluted with 5 mL of 100% methanol in 0.1% formic acid followed by 5 mL of 95% methanol plus 5% isopropanol in 0.1% formic acid. The two eluents were pooled for HPLC analysis.

The HPLC instrument was equipped with a Waters 2695 Alliance separation module, a Waters 2996 photodiode array detector (PDA), and Empower 2 software for data acquisition. The PDA three-dimensional data were collected over a wavelength range of 210−600 nm. The binary mobile phase consisted of (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile. The gradient program was as follows: 0−2 min, isocratic at 5% B, linearly increased to 16% B in 44 min, 24% B in 16 min, 25% B in 3.4 min, 27% B in 28.6 min, 30% B in 10 min, and then to 100% B in 7 min. The gradient program returned to initial conditions after 1 min isocratic at 100% B and equilibrated for 7 min before the next injection. A PDA spectral library of the 27 standards was generated using Empower 2 software. The unknown peaks were matched to library standards on the basis of the spectral contrast theory of the Empower software. If an unknown peak was matched to a standard's spectra with a spectral contrast angle that was less than 10° and higher than the threshold angle, it was reported as the name of that standard with an asterisk (i.e., standard*). If an unknown peak was matched to a standard with the same retention time and with a spectral contrast angle that was less than 10° and less than the threshold angle, it was identified as that standard. The selection of the 27 standards was primarily based on published results.[34−36]

Proanthocyanidins in IITA-50E and IITA-70A were resolved on a HPLC-Develosil Diol column (100 Å, 250 × 4.6 mm, 5 μm particle size, from Phenomenex, Torrance, CA) and a silica cartridge (Phenomenex) using a Waters 2695 Alliance separation module equipped with fluorescence detector. The column temperature was 35 °C. The binary mobile phase consisted of (A) acetonitrile:acetic acid, 98:2, v/v, and (B) acetonitrile:water:acetic acid, 95:3:2, v/v/v. The linear 70 min gradient was as follows: 0−3 min, 7% B isocratic; 3−60 min, 7.0−37.6% B; 60−63 min, 37.6−100% B; 63−70 min, 100% B isocratic; followed by 7 min

Table 1. Total Flavonoid (TF), Total Phenolic (TP), Total Anthocyanin, and Total Proanthocyanidin Concentrations and DPPH and ORAC Antioxidant Capacities of Brown (CCDR), Purple (IAC), and Red (IITA) Bran Extracts[a]

extract	extract wt (g/100 g bran)	TF (mg of catechin equiv/g extract)	TP (mg of gallic acid equiv/g extract)	total anthocyanins (mg/g extract)	total proanthocyanidins (mg proB2 equiv/g extract)	DPPH (μmol of Trolox equiv/g extract	ORAC (μmol of Trolox equiv/g extract)
CCDR	5.5	10.9 ± 0.2	29.3 ± 0.2	ND	ND	133.9 ± 4.3	723.2 ± 31.2
IAC	4.4	71.1 ± 0.5	188.8 ± 0.3	55.7 ± 2.1	ND	940.4 ± 7.6	3088.4 ± 127.1
IITA (first)	9.3	84.8 ± 2.1	250.8 ± 0.7	ND	66.88 ± 6.23	1272.7 ± 19.6	1929.1 ± 30.2

[a]Values are mean ± SD (n = 2). ND, not detectable.

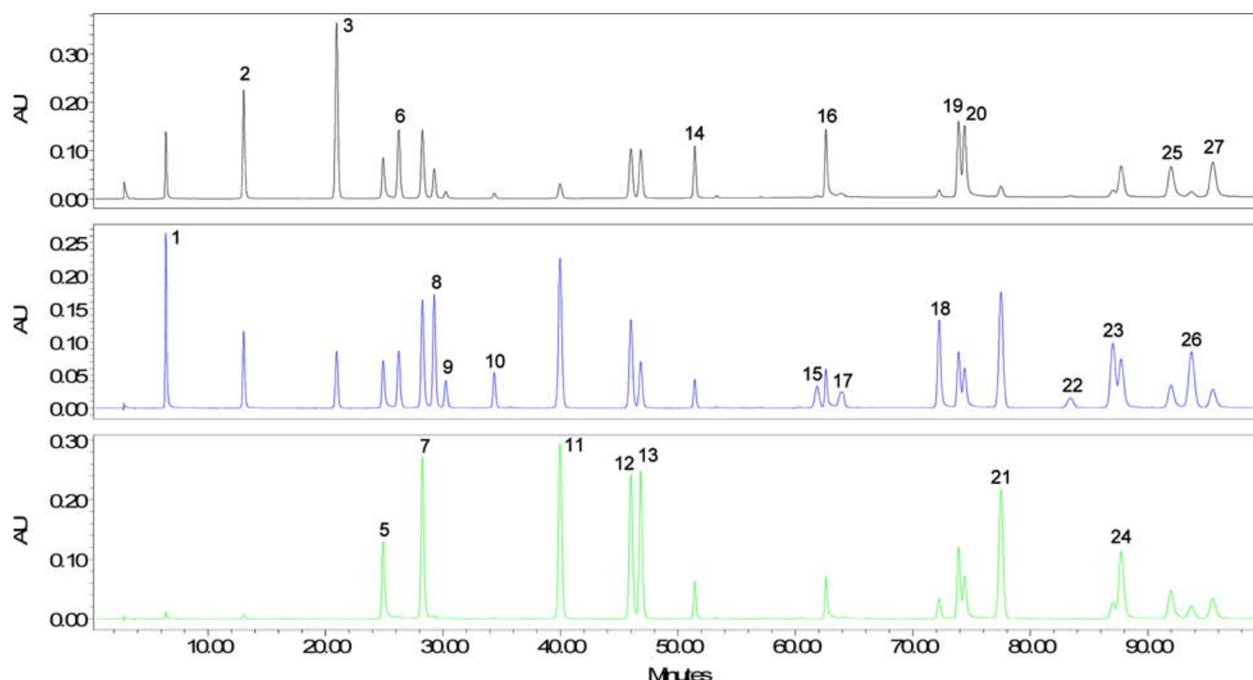

Figure 1. Chromatograms of 27 phenolic standards (1 μg each) resolved on a C18 column. Black trace, 250 nm; blue trace, 280 nm; green trace, 320 nm. Gallic acid (1), protocatechuic acid (2), 4-hydroxybenzoic acid (3), chlorogenic acid (5), vanillic acid (6), caffeic acid (7), syringic acid (8), ProB2 (9), epicatechin (10), *p*-coumaric acid (11), ferulic acid (12), sinapic acid (13), rutin (14), *p*-cresol (15), myricetin (16), *o*-cresol (17), eriodictyol (18), luteolin (19), quercetin (20), methoxycinnamic acid (21), 3,5-dimethylphenol (22), naringenin (23), apigenin (24), kaempferol (25), hesperetin (26), and isorhamnetin (27). Catechin (4) was run separately due to coelution with chlorogenic acid (5).

of returning to initial 7% B.[37] The fluorescence excitation and emission wavelengths were set at 276 nm and at 317 nm, respectively. Monomer to decamer proanthocyanidin content of fractions IITA-50E and IITA-70A were determined using standards [degree of polymerization (DP) 1−10] purified from cocoa (*Theobroma cacao*), a gift from Mars Food US. Polymers of DP above 10 were quantified using decamers as a standard.

Cell Assay. All human cell lines were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cell culture reagents were obtained from GibcoBRL (Cergy-Pontoise, France). The cells were maintained in an RPMI1640, F-12 (Gibco) or DMEM (WelGENE, Daegu, Korea) medium supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 mg/mL streptomycin, at 37 °C in a 5% CO_2 incubator. For the first round of assays using three colored bran extracts, we evaluated the following cells: normal kidney (293T), normal lung (WI-38), bone marrow erythroid leukemia (HEL), breast adenocarcinoma (MCF-7), stomach adenocarcinoma (AGS), and cervix adenocarcinoma (HeLa). For the second round of assays using a second red rice bran extract and three of its fractions, we evaluated normal liver (Chang), normal lung fibroblast (Hel299), hepatoma (HepG2), lung carcinoma (A549), AGS, and HeLa cells.

Defatted dried bran extract was dissolved in methanol (5 mg/mL, Merck, Darmstadt, Germany). The final concentration of methanol was adjusted to 2% with each cell growth medium (the methanol did not affect cell growth, data not shown). The cancer drug doxorubicin was dissolved in distilled H_2O.

The MTT assay was performed using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Cells were seeded into a 96-well microplate (5×10^3 cells/well) and incubated for 24 h. Next, cells were treated with five concentrations (1, 10, 100, 200, and 300 μg/mL) of bran extract samples for 24 h. The control cells were treated the same as the experimental cells except no bran extract was added. The CCK-8 solution (10 μL) was then added to each well. After 2 h of incubation at 37 °C, the absorbance (A) was read at 450 nm using an ELISA reader (BIO-TEK, VT). Eight wells were used for each concentration. Experiments were repeated three times. The decrease in A measures the extent of decrease in the number of viable cells following exposure to the test substances calculated by the following formula:

$$\% \text{ inhibition of cells} = (A_{\text{control}} - A_{\text{test substance}})/A_{\text{control}} \times 100$$

Statistical Analysis. The IC_{50} values (concentration that inhibits growth of 50% of the cells) were calculated from concentration-dependent data using GraphPad Prism v.5.0 (GraphPad Software, CA). Correlations of IC_{50} values with composition data were calculated by the Pearson Product Moment Correlation ($p < 0.05$), using SigmaPlot11 (Systat Software, San Jose, CA) and PROC CORR of SAS program version 9.2 (SAS, NC).

RESULTS AND DISCUSSION

Phenolics and Antioxidant Capacities of Bran Extracts. Concentrations of total phenolics, flavonoids, anthocyanins,

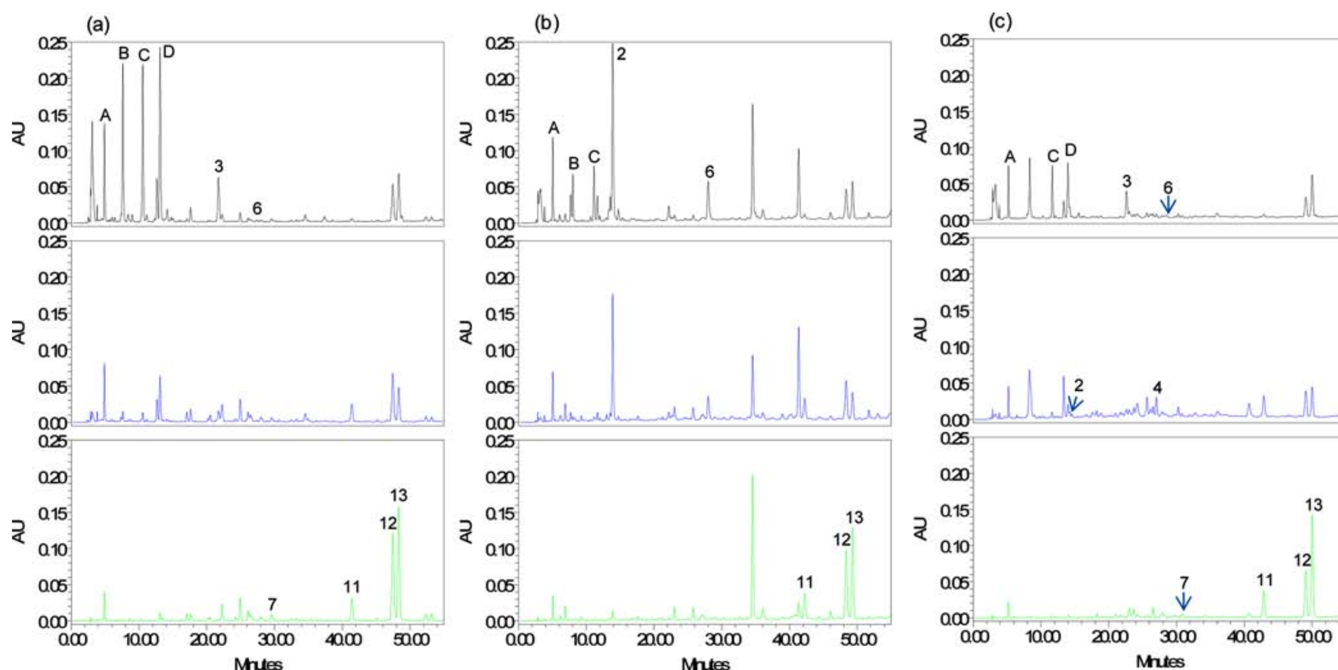

Figure 2. Chromatograms of three bran extracts treated with NaOH, (a) CCDR, (b) IAC, and (c) IITA (first). Quantity of injection was 250 μg each. Black trace, 250 nm; blue trace, 280 nm; green trace, 320 nm. Peaks identified: protocatechuic acid (2), 4-hydroxybenzoic acid (3), catechin (4), vanillic acid (6), caffeic acid (7), *p*-coumaric acid (11), ferulic acid (12), and sinapic acid (13). Peaks labeled by letters were spectra matched among three bran extracts, but not to the standards.

proanthocyanidins, and antioxidant capacities (DPPH and ORAC) in three rice bran extracts (CCDR-light brown, IAC-purple, and IITA-red first) are shown in Table 1. The dark-colored brans had more than a 6-fold higher TF and TP content than the light-brown bran. Anthocyanins were exclusively identified in purple bran. The major anthocyanin was cyanidin-3-glucoside, which accounted for 91.8% of the total. Peonidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-galactoside (4.9, 2.2, and 1.1%, respectively) were also present. Only the red IITA bran contained proanthocyanidins. Based on DPPH values, IITA bran has higher reducing capacities than IAC and CCDR brans. The higher ORAC antioxidant capacity of IAC bran indicates that phytochemicals in purple bran are more effective in scavenging radicals than those in red bran.

Twenty-seven phenolic standards were resolved on a C18 column, and a spectral library was generated (Figure 1) and used to identify phenolics in the three bran extracts (Figure 2) and in the IITA bran LH-20 fractions. With some exceptions, all three bran extracts contained low concentrations of free simple phenolics (benzoic and cinnamic acids). Most of the simple phenolics were present as esters. In IAC bran, free protocatechuic and vanillic acid levels were greater than in the other two brans (Table 2). High concentrations of esterified protocatechuic and vanillic acids were another unique aspect of the phenolic profile of IAC bran. Purple IAC bran contained a 10-fold greater concentration of the benzoic acid subgroup than the light brown and red brans (Table 2 and Figure 2). The 4-hydroxybenzoic acid ester was identified in CCDR and IITA brans. Ferulic acid and sinapic acid were the two major esterified cinnamic acids in all three brans, accounting for more than 84% of the total cinnamic acid subgroup. Caffeic acid and *p*-coumaric acid were the other minor cinnamic acids present. Overall, CCDR bran had the highest total content of cinnamic acids (5.12 mg/g extract), compared with IAC (4.01 mg/g) and IITA (3.92 mg/g). A few major peaks (peaks A−D) with a maximum absorbance at ∼250 nm were matched spectrally to the three bran extracts, but were not matched to the phenolic standard spectra (Figure 2). Based on peak height, the light brown bran CCDR had the highest quantity of these unknown compounds. Based on the data in Figure 2 and Table 1, the more than 6-fold greater concentrations of TF and TP in red and purple than in light brown brans were unlikely to be due to cinnamic and benzoic acids or to other peaks absorbing at 250 nm (peaks A−D in Figure 2).

Anthocyanins and proanthocyanidins of purple and red brans contributed to the high levels of TP and TF and to the antioxidant capacities of purple and red brans. In addition to anthocyanins, the high content of protocatechuic acid in purple bran also contributed to the high TP levels.

Growth Inhibition of Cells by Rice Brans of Different Color. The potential anticancer properties of the extracts of light brown, purple, and red brans were evaluated in two sets of experiments. In the first set, normal human kidney (293T) and lung (WI-38) and four cancer cell lines, HEL, MCF-7, AGS, and HeLa, were treated with five concentrations (1, 10, 100, 200, and 300 μg/mL) of the CCDR (light brown), IAC (purple), and IITA (red) bran extracts. Figure 3 shows the concentration-dependent cell-growth-inhibition plots induced by the three brans against the six cell lines.

The light brown bran CCDR extract did not show any cytotoxic effects on 293T (normal), WI-38 (normal), and MCF-7 (breast cancer) cells. Inhibition of cell growth by the purple bran started at a concentration of ∼100 μg/mL. It increased steeply for the HeLa and HEL cells, reaching a maximum of ∼60% at 300 μg/mL. By contrast, inhibition of cell growth induced by the red bran extract (first) started at ∼100 μg/mL and reached a maximum near 100% for the AGS, HEL, and HeLa cells; while the maximum inhibition of the normal cells 293T and WI-38 cells was only ∼20%. The phenolic profiles of the three bran extracts (Tables 1 and 2 and Figure 2) suggest that the

Table 2. Simple Phenolics[a] in the Three Bran Extracts, Free, Esterified and Total (Sum of Free and Esterified)

			free (mg/g extract)			esterified (mg/g extract)			total (mg/g extract)		
phenolic subgroup	phenolics	λ^b	CCDR (brown)	IAC (purple)	IITA (red)[c]	CCDR (brown)	IAC (purple)	IITA (red)	CCDR (brown)	IAC (purple)	IITA (red)
benzoic acid	protocatechuic acid	250	ND	1.547 ± 0.020	0.097 ± 0.051	ND	4.230 ± 0.078	0.071 ± 0.047	ND	5.777 ± 0.098	0.168 ± 0.004
	4-hydroxybenzoic acid	250	ND	ND	ND	0.673 ± 0.010	0.000 ± 0.000	0.427 ± 0.015	0.673 ± 0.010	ND	0.427 ± 0.015
	vanillic acid	250	ND	0.535 ± 0.073	ND	0.034 ± 0.002	1.033 ± 0.037	0.072 ± 0.003	0.034 ± 0.002	1.568 ± 0.036	0.072 ± 0.003
total benzoic acids				2.082 ± 0.092	0.097 ± 0.051	0.707 ± 0.012	5.263 ± 0.041	0.570 ± 0.065	0.707 ± 0.012	7.345 ± 0.134	0.668 ± 0.014
	caffeic acid	320	ND	ND	ND	0.157 ± 0.014	0.000 ± 0.000	0.111 ± 0.003	0.157 ± 0.014	ND	0.111 ± 0.003
	p-coumaric acid	320	ND	ND	ND	0.424 ± 0.001	0.517 ± 0.049	0.511 ± 0.002	0.424 ± 0.001	0.517 ± 0.049	0.511 ± 0.002
cinnamic acid	ferulic acid	320	0.061 ± 0.002	0.070 ± 0.015	0.077 ± 0.014	1.935 ± 0.005	1.523 ± 0.082	0.978 ± 0.025	1.995 ± 0.004	1.593 ± 0.067	1.055 ± 0.011
	sinapic acid	320	ND	ND	ND	2.544 ± 0.017	2.039 ± 0.082	2.266 ± 0.016	2.544 ± 0.017	2.039 ± 0.082	2.266 ± 0.016
total cinnamic acids			0.061 ± 0.002	0.070 ± 0.015	0.077 ± 0.014	5.060 ± 0.003	4.079 ± 0.214	3.866 ± 0.042	5.121 ± 0.001	4.149 ± 0.198	3.943 ± 0.028

[a]Values are mean ± SD (n = 2). [b]Absorbance wavelength (nm) used for quantitation. [c]First IIT red bran extract.

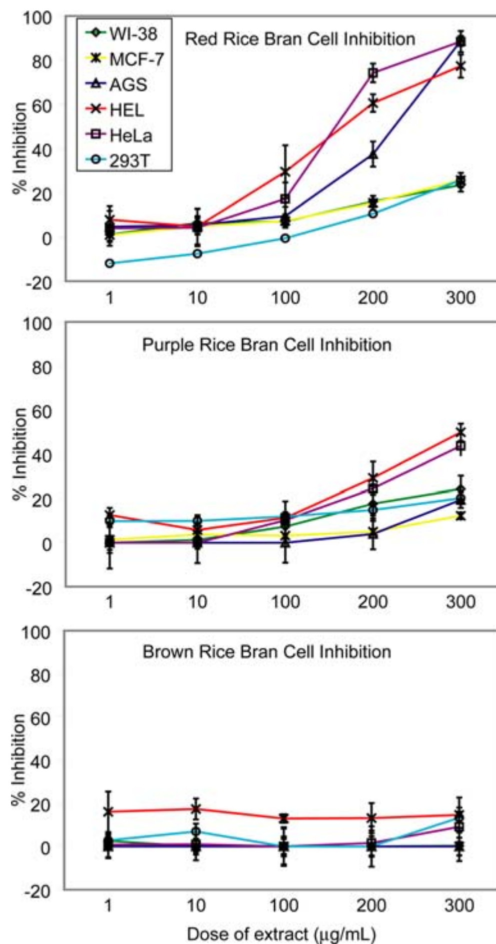

Figure 3. Growth-inhibiting effects by the MTT assay of light brown (CCDR), purple (IAC), and red (IITA first extract) bran extracts against six cell lines (WI-38, MCF-7, AGS, HEL, HeLa, and 293T). Error bars represent ± SD, n = 3.

anticancer activity of red bran can be attributed primarily to proanthocyanidins. The cytotoxic effect of purple bran extract seems to be due to both anthocyanins and protocatechuic acid. Due to the drastic differences of phenolic subgroups present among the three color bran extracts, the correlation between antioxidant capacity and anticancer activity was not attempted. Studies have demonstrated that the mechanisms of anticancer properties of these phytochemicals are not solely due to their antioxidative ability to quench free radicals but rather are modulated via signal transduction pathways and gene expression of enzymes involved in the metastatic cascade of cancer cells.[38,39] Chen and others[38] showed that an anthocyanin-rich fraction from a black (purple) rice bran suppressed the tumor growth in hepatoma cell xenografted nude mice, and that the purified cyaniding 3-glucoside showed antimetastatic effects in vitro. In another study, Yin and others[39] reported that, at 2−8 µmol/L, protocatechuic acid showed a concentration-dependent increase of apoptotic effects on cancer cells. At 300 µg/mL, the concentration of free and total protocatechuic acid of IAC (2.98 and 11.1 µmol/L) is within range of the apoptotic effect reported by Yin and others.[39]

Phenolics in Red Bran Fractions. In an effort to further identify compound(s) of red bran contributing to the suppression of cancer cell growth, a second red IITA bran extract was further fractionated through Sephadex. Individual

Table 3. Extract Weights and Total Flavonoid (TF), Total Phenolic (TP), and Total Proanthocyanidin Concentrations of IITA Red Bran Extract and Its Three LH20 Fractions (IITA-20M, IITA-50E, and IITA-70A)[a]

red bran and fractions	extract wt (g/100 g bran)	% extract (w/w)	TF (mg catechin equiv/g extract or fraction)	TP (mg gallic acid equiv/ g extract or fraction)	total proanthocyanidins (mg proB2 equiv/ g extract or fraction)
IITA (second)	7.588	100.00	92.70 ± 2.79	228.97 ± 0.81	69.82 ± 2.68
IITA-20M	5.815	76.63	13.93 ± 0.36	33.98 ± 0.02	5.84 ± 0.14
IITA-50E	0.444	5.85	239.33 ± 7.48	510.60 ± 3.43	148.19 ± 7.06
IITA-70A	1.328	17.51	359.52 ± 9.50	992.43 ± 3.63	406 ± 6.17

[a]Values are mean ± SD (n = 2).

phenolic profiles of the second IITA red bran extract and of the three LH-20 fractions are shown in Tables 3 and 4. Approximately 7.6 g of extract per 100 g of red bran were obtained, and 76.6% of the extract weight was collected in the IITA-20M fraction. This fraction had the lowest concentration of TP, TF, and total proanthocyanidins (Table 3). Table 4 shows the concentration of phenolics that were identified by being matched with either standards or standard spectra. Among the phenolic compounds identified in IITA-20M (total = 5.1 mg of phenolics/g fraction), 24.2%, 70.2%, 1.5%, and 4.1% were benzoic acids, cinnamic acids, flavonoids, and proanthocyanidins, respectively (Table 4). The sum of phenolics in IITA-50E was 48.0 mg/g fraction, in which 5.1%, 41.9%, 4.1%, and 48.9% were benzoic acids, cinnamic acids, flavonoids, and proanthocyanidins, respectively. For IITA-70A (94.5 mg of phenolics/g fraction), most of the phenolic compounds were proanthocyanidins (94.6%). IITA-50E had the highest concentrations of benzoic acid and cinnamic acid subgroups, followed by IITA-20M and IITA-70A. Numerous peaks in both the IITA-50E and IITA-70A fractions were matched to the spectra of catechin, epicatechin, and proB2. A few peaks were not identified in the IITA-50E and IITA-70A fractions (Figure 4).

Analyses of proanthocyanidins based on their degree of polymerization (DP) were done on a HPLC-diol column with fluorescence detection (Figure 5). The IITA-50E and IITA-70A fractions differed significantly in their composition and content of proanthocyanidins (Table 5). Monomers to trimers of lower molecular weight proanthocyanidins account for 38.7% of the total (28.3 mg/g) in IITA-50E fractions. The IITA-70A fraction with a total of 314.8 mg/g was composed of dimers to 14-mers and polymers (DP >14) (Table 5). Proanthocyanidins in both fractions were most likely not homogeneous within each DP fraction (Figures 4 and 5).

Growth Inhibition of Cancer Cells by Red Bran Fractions. We compared the cancer cell growth inhibition of extracts of the first red bran mentioned above and this second red bran and its three fractions against normal human liver (Chang) and normal lung (Hel299) and four human cancer cell lines (HepG2, hepatoma; A549, lung; AGS, gastric; HeLa, cervix) at five concentrations (10, 25, 50, 100, and 200 μg/mL). Figure 6 shows plots of concentration-dependent cell growth inhibition induced by both red bran extracts and the LH-20 fractions of the second red bran against the six cell lines. The reciprocal ($1/IC_{50}$) values of the cell growth inhibitions are plotted in Figure 7. Because the greater the $1/IC_{50}$ value the greater the inhibition, the figure shows that the first red bran extract and the second red bran extract (used to prepare the three LH-20 fractions) showed nearly identical inhibitions of the six cell lines, as did the first fraction (IITA-20M). By contrast, compared to the whole red bran extracts, inhibition of the AGS stomach cancer cells induced by the IITA-50E fraction increased about 3-fold. Inhibition of the AGS, HepG2, and HeLa cells caused by the IITA-70A fraction increased about 6-, 4-, and 3-fold, respectively. These results show that the IITA-70A fraction contains the highest concentration of anticancer compounds.

The red bran extract did not show cytotoxic effects either on the normal lung cell Hel299, at 200 μg/mL, or on Chang normal liver cells at 100 μg/mL. Some cytotoxicity was however apparent on Chang cells at 200 μg/mL (Figure 6). Higher cytotoxic responses of Chang than of Hel299 cells have been reported in a related study with tea compounds.[40]

Among the three fractions tested, the IITA-70A fraction had the highest anticancer activity on all the cancer cells. At high concentrations, this fraction also had a cytotoxic effect on both normal Chang and Hel299 cells. At low concentration (50 μg/mL), IITA-70A induced 74% AGS cancer cell death; whereas it had no cytotoxic effect on normal lung Hel299 cells, but killed 52% of normal Chang cells. Fraction IITA-50E, at 100 μg/mL, induced death in 70% AGS, 26% Chang, and 0% Hel299 cells. The red bran extracts and IITA-20M and IITA-50E fractions also induced normal Hel299 cell growth in a dose-dependent manner.

Relationship of Composition of Red Bran Fractions to Anticancer Bioactivity. As mentioned above, the IITA-70A fraction had the strongest anticancer activity. Based on the chromatogram from the C18 column, >94% (w/w) of the identified compounds were proanthocyanidins (Table 4), and only a few minor peaks represent unknown compounds (Figure 4b). The anticancer activity most likely derives from the proanthocyanidins (DP 2−14 and polymers DP >14). Fraction IITA-50E had strong activity against AGS. The two phenolic subgroups that are found in high concentration in the IITA-50E fraction are cinnamic acids and proanthocyanidins, with high levels of monomers and dimers. The lack of activity of the light brown bran CCDR extract, which had a higher cinnamic acid concentration than the red and purple bran extracts, and the Pearson correlation analysis of the phenolics and IC_{50} values of red bran fractions discussed below suggest that the benzoic and cinnamic acids in red bran were unlikely to contribute to the observed anticancer cell effects.

If no other compounds contribute to the antiproliferative activity of proanthocyanidins in both IITA-50E and IITA-70A fractions, then proanthocyanidins in IITA-50E seem to have greater efficacy than those in IITA-70A. The IC_{50}s of IITA-50E against AGS, HepG2, and HeLa were 69.2, 133.6, and 193.7 μg/mL, respectively; while the proanthocyanidin concentration in IITA-50E was 28.3 ng/μg. With a proanthocyanidin concentration of 314.8 ng/μg in fraction IITA-70A, the IC_{50}s of IITA-70A against AGS, HepG2, and HeLa cells were 33.9, 86.7, and 63.4 μg/mL, respectively.

As for the potential compounds in fractions of IITA-20M and IITA-50E that might have induced growth of Hel299 normal lung cells, the common phenolic subgroups in both of these two

Table 4. Concentrations of Phenolics[a] (mg/g extract) in NaOH-Treated IITA (Second) Extract and Its LH20 Fractions

phenolic subgroup	phenolics[b]	retention time (min)	λ[c]	IITA (second)	IITA-20M	IITA-50E	IITA-70A
benzoic acid	gallic acid	8.70	280	0.120 ± 0.013			0.299 ± 0.077
	protocatechuic acid	7.49	250	0.068 ± 0.032	0.084 ± 0.035		
		13.65	250	**0.168 ± 0.004**	**0.144 ± 0.022**		
	4-hydroxybenzoic benzoic acid	8.53	250		0.013 ± 0.005		
		13.19	250	0.630 ± 0.015	0.509 ± 0.004		
		21.75	250	**0.427 ± 0.015**	**0.431 ± 0.002**	**1.609 ± 0.049**	0.469 ± 0.047
	vanillic acid	13.61	250			0.447 ± 0.007	
		27.35	250	**0.075 ± 0.007**		**0.229 ± 0.003**	
	syringic acid	**30.58**	280	**0.038 ± 0.002**		**0.148 ± 0.001**	
	p-cresol	11.99	280				0.361 ± 0.010
total benzoic acid spectra				1.527 ± 0.011	1.228 ± 0.122	2.466 ± 0.045	1.130 ± 0.134
cinnamic acid	caffeic acid	**29.64**	320	**0.165 ± 0.080**	**0.101 ± 0.006**	**0.323 ± 0.014**	
		51.79	320			0.134 ± 0.031	
		53.22	320		0.071 ± 0.001	0.161 ± 0.002	
	p-coumaric acid	26.31	320		0.053 ± 0.002	0.118 ± 0.000	
		26.73	320			0.036 ± 0.001	
		27.92	320	0.038 ± 0.004			
		28.08	320	0.011 ± 0.000			
		41.45	320	**0.510 ± 0.003**	**0.231 ± 0.047**	**3.198 ± 0.014**	**0.640 ± 0.0197**
		71.69	320			0.031 ± 0.011	
	ferulic acid	**47.46**	320	**1.049 ± 0.003**	**0.932 ± 0.006**	**5.066 ± 0.006**	**0.155 ± 0.005**
		55.85	320			0.031 ± 0.011	
	sinapic acid	**48.33**	320	**2.257 ± 0.002**	**2.052 ± 0.010**	**10.853 ± 0.017**	**0.121 ± 0.005**
	methoxycinnamic acid	26.59	320		0.078 ± 0.002	0.117 ± 0.000	
		28.17	320	0.030 ± 0.012		0.082 ± 0.002	
total cinnamic acid spectra				4.059 ± 0.000	3.566 ± 0.027	20.115 ± 0.022	0.468 ± 0.662
flavanones	naringenin	39.53	280			0.936 ± 0.011	2.851 ± 0.032
		58.60	280			0.040 ± 0.006	0.092 ± 0.003
	eriodictyol/hesperetin	52.23	280			0.298 ± 0.008	
flavones	apigenin	45.20	320	0.078 ± 0.001	0.078 ± 0.012		
		49.55	320			0.088 ± 0.001	
		59.21	320			0.073 ± 0.000	
		60.25	320			0.116 ± 0.006	
	luteolin	54.89	320	0.184 ± 0.043		0.324 ± 0.106	
flavonols	isorhamnetin	75.25	370				0.149 ± 0.001
total flavonoid spectra				0.291 ± 0.003	0.078 ± 0.012	1.955 ± 0.115	3.091 ± 0.035
proanthocyanidins	catechin	6.22	280	0.219 ± 0.023			0.731 ± 0.012
		8.15	280	2.516 ± 0.079		2.226 ± 0.289	7.588 ± 0.602
		17.03	280	0.843 ± 0.018			
		24.34	280	2.566 ± 0.033		1.735 ± 0.329	
		25.84	280	**2.275 ± 0.103**		**5.168 ± 0.333**	**11.583 ± 0.374**
		36.06	280				1.005 ± 0.190
		44.98	280				1.795 ± 0.032
	total catechin spectrum			8.420 ± 0.016	0.000 ± 0.000	9.801 ± 0.951	22.701 ± 0.082
	epicatechin	6.11	280			0.268 ± 0.004	
		7.98	280	4.653 ± 0.421		6.080 ± 0.027	11.403 ± 0.259
		26.16	280				0.434 ± 0.058
	total epicatechin spectrum			4.653 ± 0.421	0.000 ± 0.000	6.331 ± 0.024	11.837 ± 0.201
	proB2	12.68	280				0.258 ± 0.011
		14.75	280				0.550 ± 0.036
		15.68	280				2.539 ± 0.211
		16.15	280				0.419 ± 0.115
		16.90	280			0.761 ± 0.014	4.753 ± 0.166
		17.80	280				0.106 ± 0.018
		18.77	280			0.191 ± 0.038	1.846 ± 0.004
		19.12	280	0.147 ± 0.015		0.275 ± 0.077	1.258 ± 0.032
		20.71	280	0.204 ± 0.030			3.563 ± 0.406
		21.43	280			0.583 ± 0.224	2.495 ± 0.425
		21.97	280				2.405 ± 0.558
		23.15	280			3.672 ± 0.356	9.434 ± 0.838

Table 4. continued

phenolic subgroup	phenolics[b]	retention time (min)	λ[c]	IITA (second)	IITA-20M	IITA-50E	IITA-70A
		24.33	280			0.285 ± 0.167	1.638 ± 0.003
		24.47	280				3.650 ± 0.340
		24.95	280	1.146 ± 0.054			4.178 ± 0.629
		27.04	280				1.062 ± 0.041
		27.69	280				1.082 ± 0.023
		28.65	280				1.764 ± 0.033
		28.90	280		0.209 ± 0.016	0.111 ± 0.134	0.568 ± 0.053
		29.57	280	1.127 ± 0.036			1.364 ± 0.013
		31.40	280	0.621 ± 0.008		0.608 ± 0.015	3.274 ± 0.133
		32.59	280				0.810 ± 0.159
		33.80	280	0.153 ± 0.0063		0.179 ± 0.011	1.028 ± 0.295
		34.79	280				1.184 ± 0.315
		35.86	280	0.318 ± 0.07			1.771 ± 0.122
		42.48	280				0.256 ± 0.016
		42.93	280				0.433 ± 0.031
		44.80	280				0.565 ± 0.062
		53.51	280				0.542 ± 0.057
	total proB2 spectrum			3.716 ± 0.010	0.209 ± 0.016	7.356 ± 0.977	54.796 ± 4.573
total proanthocyanidin spectra				16.789 ± 0.415	0.209 ± 0.016	23.489 ± 1.904	89.334 ± 4.291
total phenolic spectra				22.666 ± 0.423	5.081 ± 0.176	48.025 ± 2.042	94.47012 ± 4.16274

[a]Data are mean ± SD (n = 2). Spaces with no values indicate compounds are not detectable. [b]Peaks that matched with the spectrum of a standard are grouped under that standard. The bold font indicates the peak with that retention time has a perfect match with the standard, both retention time and spectrum. [c]Absorbance wavelength (nm) used for quantitation.

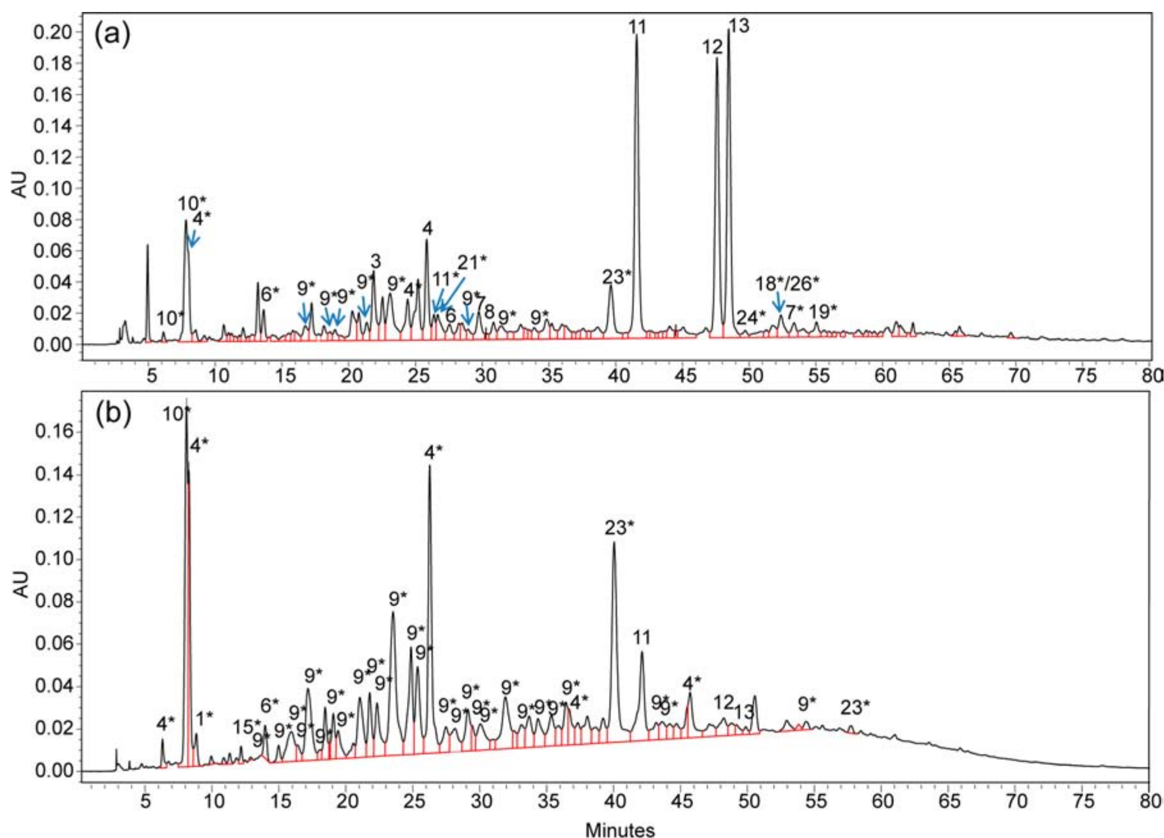

Figure 4. Chromatograms of NaOH-treated IITA-50E (a) and IITA-70A (b) fractions of second IITA red bran extract (λ = 280 nm). Quantity of injection was 250 μg. Each fraction was collected through LH20 cartridge with 50% ethanol and 70% acetone, respectively, after elution with 20% methanol. Gallic acid (1), 4-hydroxybenzoic acid (3), catechin (4), vanillic acid (6), caffeic acid (7), syringic acid (8), ProB2 proanthocyanidins (9), epicatechin (10), p-coumaric acid (11), ferulic acid (12), sinapic acid (13), p-cresol (15), eriodictyol (18), luteolin (19), naringenin (23), apigenin (24), hesperetin (26). Number with an asterisk (*) indicates that the peak matched with the spectrum of the standard.

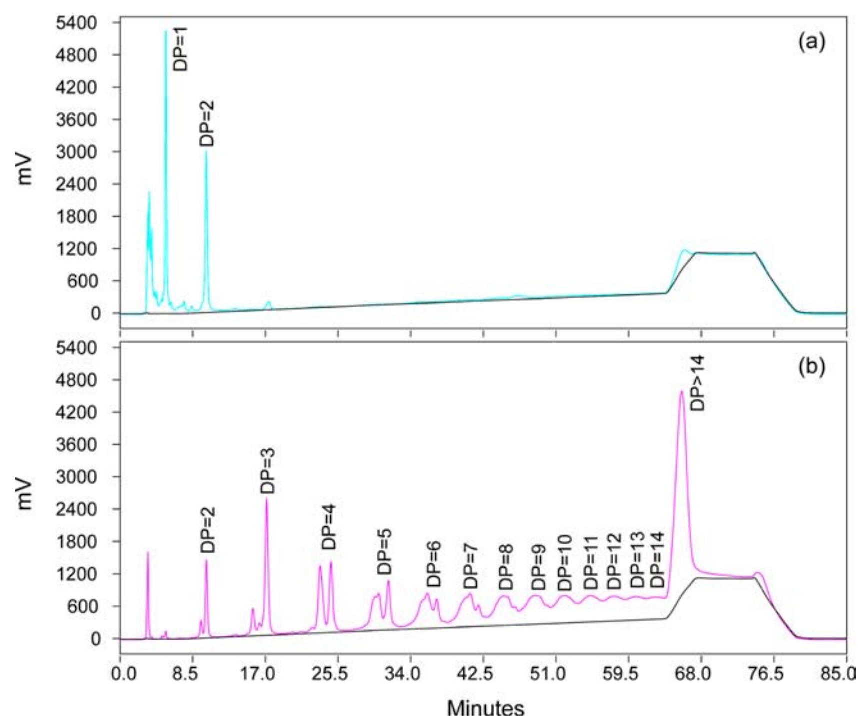

Figure 5. Fluorescence traces of proanthocyanidins in red bran IITA-50E fraction (a), and IITA-70A fraction (b) resolved on a HPLC-diol column (ex = 276 nm, em = 317 nm). A 12.5 μg quantity of each fraction was run through a HPLC-diol column. Black trace is the solvent baseline. The degree of polymerization (DP) of proanthocyanidins was determined based on procyanidin standards (monomer to decamer) purified from cocoa (*Theobroma cacao*).

Table 5. Proanthocyanidin Concentrations (mg/g)[a], Identified by Their Degree of Polymerization (DP), in Two Red Bran Fractions, IITA-50E and IITA-70A

DP of oligomer	IITA-50E	IITA-70A
1	2.8 ± 0.5	0.1 ± 0.1
2	6.4 ± 0.2	3.0 ± 0.3
3	1.7 ± 0.3	13.5 ± 1.3
4	0.8 ± 0.5	16.8 ± 1.8
5	0.9 ± 0.5	15.6 ± 1.8
6	1.5 ± 0.4	17.3 ± 1.8
7	1.9 ± 0.4	19.3 ± 2.1
8	2.8 ± 0.3	16.4 ± 1.8
9	2.1 ± 0.4	20.8 ± 1.9
10−14	4.7 ± 1.6	98.2 ± 10.7
polymer (>14)	2.7 ± 0.0	93.8 ± 12.0
total	28.3 ± 4.0	314.8 ± 11.5

[a]Data are mean ± SD (n = 2).

fractions are benzoic and cinnamic acids. Compared to IITA-20M, IITA-50E contains 1.9-fold and 5.6-fold higher amounts of these acids, respectively. Since IITA-70A did not induce Hel299 cell growth, proanthocyanidins of DP 2 to polymers that were found in this fraction were most likely not responsible for the growth.

Statistical Correlations of IC$_{50}$s and Phenolic Contents of Red Bran Fractions. An IC$_{50}$ was determined for each of the four red bran extracts (the crude extract and the three LH20 fractions) for each cell line. These four IC$_{50}$s were used to correlate with the content of phenolics in these extracts.

IC$_{50}$s of the four red bran extracts (crude and the fractions) were highly correlated with total flavonoid content for AGS (r = −0.954), HepG2 (r = −0.920), and HeLa (r = −0.945), respectively, with total phenolic content for AGS, HepG2, and HeLa (r = −0.910, −0.935, and −0.972, respectively), and with total proanthocyanidins for HepG2 and HeLa (r = −0.953 and −0.988, respectively). The strong negative correlations indicate that higher contents of total flavonoids, total phenolics, and total proanthocyanidins result in greater growth inhibition of the different cancer cell lines.

Correlations were also performed between the IC$_{50}$s and each of the sums of phenolic subgroup contents (benzoics, cinnamics, flavonoids, catechins, epicatechins, proB2s, and proanthocyanidins) (Table 4) in the four red bran extracts (crude and the three LH20 fractions) for each cell line. IC$_{50}$s of the extracts for AGS were correlated with epicatechins (r = −0.948, P = 0.05). IC$_{50}$s of the extracts for HepG2 were correlated with flavonoids, proB2s, and proanthocyanidins (r = −0.962, −0.956, and −0.950, P = 0.038, 0.044, and 0.050, respectively). IC$_{50}$s of the extracts for HeLa cells were correlated with catechins (r = −0.986, P = 0.014), epicatechins (r = −0.968, P = 0.032), proB2s (r = −0.973, P = 0.027), and proanthocyanidins (sum of catechins, epicatechins, and proB2s) (r = −0.995, P = 0.005). None of the IC$_{50}$s of the four extracts were correlated with the contents of benzoics and cinnamics for any cell line (P > 0.1).

Sensitivity of Cells to Doxorubicin Hydrochloride. Cells were also treated with four concentrations of the medical cancer drug doxorubicin. The relative percentage of surviving cells was then compared with untreated cells determined by the MTT assay. The approximate IC$_{50}$ values of doxorubicin were 2.05, 5.15, 1.45, 0.63, and 1.23 μM for 293T, WI-38, AGS, HEL, and HeLa cells, respectively. On a weight basis, the activity of doxorubicin was about 50 to 100 times greater than that of the red bran extracts.

In conclusion, compositional, antioxidative, and cancer-cell inhibiting data of brown, purple, and red rice brans indicate

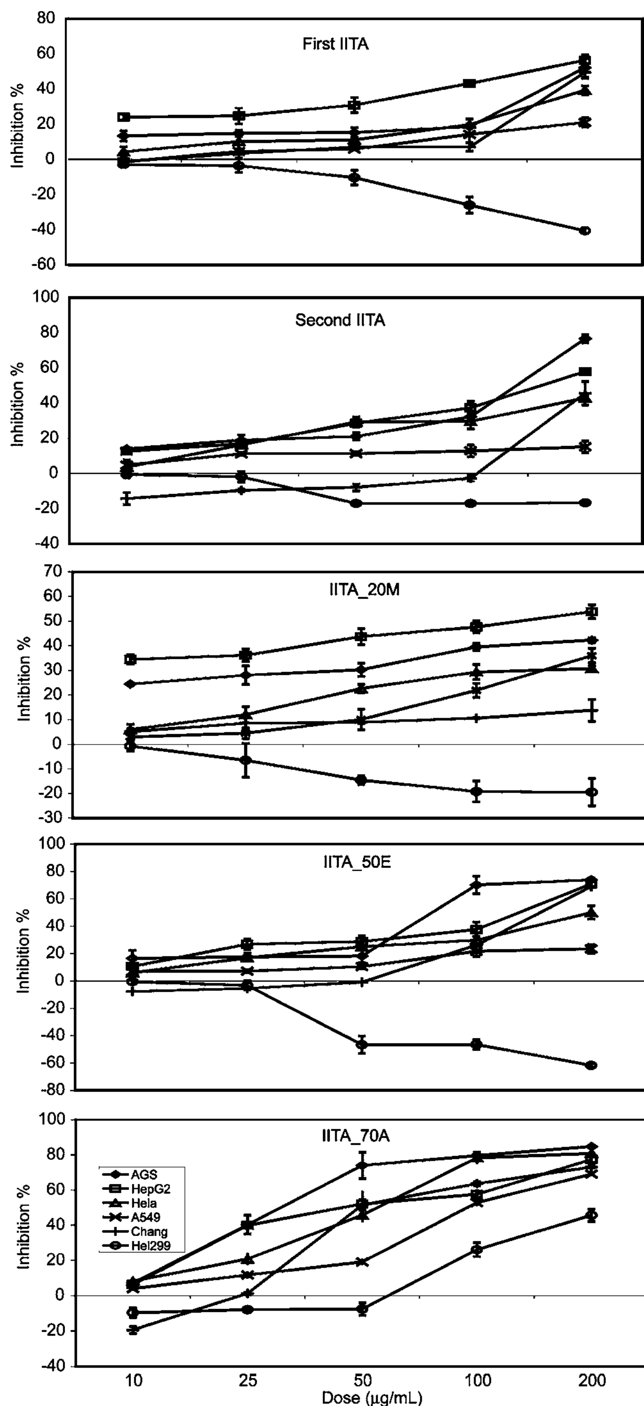

Figure 6. Growth-inhibitory effects by the MTT assay of two IITA red bran extracts (first and second) and three LH-20 fractions of the second IITA red bran extract (IITA-20M, IITA-50E, and IITA-70A) against six cell lines (AGS, HepG2, HeLa, A549, Chang, and Hel299). Error bars represent ± SD, $n = 3$.

strong correlations between total flavonoids and total phenolic content, antioxidative activities, and inhibition of cervical, leukemia, liver, and stomach cancer cells. One red bran fraction rich in proanthocyanidins exhibited exceptionally high activity against the cancer cells. These findings suggest that consumers could benefit from eating foods with added red rice bran. Red-bran-compatible foods could include grain-based products such as bread, cereals, and cookies.

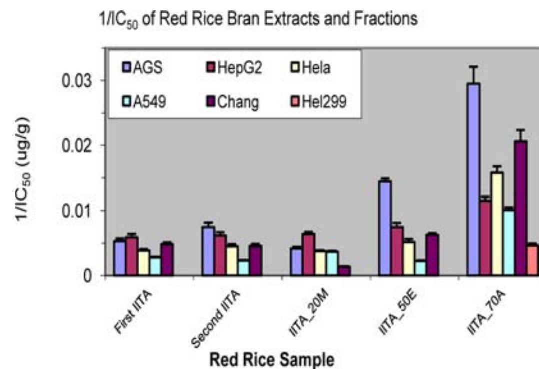

Figure 7. Growth-inhibitory activities ($1/IC_{50}$ values) of first and second red rice bran extracts (IITA) and three fractions (IITA-20M, IITA-50E, and IITA-70A) of the second extract against six cell lines. Error bars represent + SD, $n = 3$.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Rondanelli, M.; Perna, S.; Monteferrario, F.; Opizzi, A. Update on the therapeutic qualities of the rice bran in the treatment of dyslipidemia and chemo-prevention. *Recent Prog. Med.* **2011**, *102*, 310−313.

(2) Min, B.; McClung, A. M.; Chen, M.-H. Phytochemicals and antioxidant capacities in rice brans of different color. *J. Food Sci.* **2011**, *76*, C117−C126.

(3) Jariwalla, R. J. Rice-bran products: phytonutrients with potential applications in preventive and clinical medicine. *Drugs Exp. Clin. Res.* **2001**, *27*, 17−26.

(4) Aggarwal, B. B.; Sundaram, C.; Prasad, S.; Kannappan, R. Tocotrienols, the vitamin E of the 21st century: Its potential against cancer and other chronic diseases. *Biochem. Pharmacol.* **2010**, *80*, 1613−1631.

(5) Ling, W. H.; Cheng, Q. X.; Ma, J.; Wang, T. Red and black rice decrease atherosclerotic plaque formation and increase antioxidant status in rabbits. *J. Nutr.* **2001**, *131*, 1421−1426.

(6) Xia, M.; Ling, W. H.; Ma, J.; Kitts, D. D.; Zawistowski, J. Supplementation of diets with the black rice pigment fraction attenuates atherosclerotic plaque formation in apolipoprotein deficient mice. *J. Nutr.* **2003**, *133*, 744−751.

(7) Kil, D. V.; Ryu, S. N.; Piao, L. G.; Kong, C. S.; Han, S. J.; Kim, Y. Y. Effect of feeding cyanidin 3-glucoside (C3G) high black rice bran on nutrient digestibility, blood measurements, growth performance and pork quality of pigs. *Asian-Australas. J. Anim. Sci.* **2006**, *19*, 1790−1798.

(8) Punyatong, M.; Pongpiachan, P.; Pongpiachan, P.; Karladee, D.; Mankhetkorn, S. Cytotoxicity of crude proanthocyanidin extract from purple glutinous rice bran (*Oryza sativa* L.) (Kum Doi Saket) compared

with cyanidin 3-glucoside on X63 myeloma cancer cell lines. *Kasetsart J. (Nat. Sci.)* **2008**, *42*, 676−681.

(9) Nam, S. H.; Choi, S. P.; Kang, M. Y.; Kozukue, N.; Friedman, M. Antioxidative, antimutagenic, and anticarcinogenic activities of rice bran extracts in chemical and cell assays. *J. Agric. Food Chem.* **2005**, *53*, 816−822.

(10) Miyazawa, M.; Oshima, T.; Tokura, M.; Hisama, M. Suppression of chemical mutagens-induced SOS response by phenolic acids from black rice bran using *Salmonella typhimurium* TA1535/pSK1002 umu test. *J. Oleo Sci.* **2003**, *52*, 471−481.

(11) Nam, S. H.; Choi, S. P.; Kang, M. Y.; Koh, H. J.; Kozukue, N.; Friedman, M. Bran extracts from pigmented rice seeds inhibit tumor promotion in lymphoblastoid B cells by phorbol ester. *Food Chem. Toxicol.* **2005**, *43*, 741−745.

(12) Choi, S. P.; Kang, M. Y.; Koh, H. J.; Nam, S. H.; Friedman, M. Antiallergic activities of pigmented rice bran extracts in cell assays. *J. Food Sci.* **2007**, *72*, S719−726.

(13) Okai, Y.; Okada, T.; Higashi-Okai, K.; Kasahara, E.; Inoue, M.; Yamashita, U. Immunomodulating activities in bran extracts of Japanese red, black and brown rices. *J. UOEH* **2009**, *31*, 231−242.

(14) Choi, S. P.; Kim, S. P.; Kang, M. Y.; Nam, S. H.; Friedman, M. Protective effects of black rice bran against chemically-induced inflammation of mouse skin. *J. Agric. Food Chem.* **2010**, *58*, 10007−10015.

(15) Tanaka, J.; Nakanishi, T.; Ogawa, K.; Tsuruma, K.; Shimazawa, M.; Shimoda, H.; Hara, H. Purple rice extract and anthocyanidins of the constituents protect against light-induced retinal damage *in vitro* and *in vivo*. *J. Agric. Food Chem.* **2011**, *59*, 528−536.

(16) Lizarraga, D.; Lozano, C.; Briedé, J. J.; Van Delft, J. H.; Touriño, S.; Centelles, J. J.; Torres, J. L.; Cascante, M. The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS J.* **2007**, *274*, 4802−4811.

(17) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **2004**, *134*, 613−617.

(18) Heuberger, A. L.; Lewis, M. R.; Chen, M. H.; Brick, M. A.; Leach, J. E.; Ryan, E. P. Metabolomic and functional genomic analyses reveal varietal differences in bioactive compounds of cooked rice. *PLoS One* **2010**, *5*, e12915.

(19) Nam, S. H.; Choi, S. P.; Kang, M. Y.; Koh, H. J.; Kozukue, N.; Friedman, M. Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chem.* **2006**, *94*, 613−620.

(20) Finocchiaro, F.; Ferrari, B.; Gianinetti, A.; Dall'Asta, C.; Galaverna, G.; Scazzina, F.; Pellegrini, N. Characterization of antioxidant compounds of red and white rice and changes in total antioxidant capacity during processing. *Mol. Nutr. Food Res.* **2007**, *51*, 1006−1019.

(21) Shen, Y.; Jin, L.; Xiao, P.; Lu, Y.; Bao, J. Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. *J. Cereal Sci.* **2009**, *49*, 106−111.

(22) Min, B.; Chen, M.-H.; Green, B. W. Antioxidant activities of purple rice bran extract and its effect on the quality of low-NaCl, phosphate-free patties made from channel catfish (*Ictalurus punctatus*) belly flap meat. *J. Food Sci.* **2009**, *74*, C268−C277.

(23) Muntana, N.; Prasong, S. Study on total phenolic contents and their antioxidant activities of Thai white, red and black rice bran extracts. *Pak. J. Biol. Sci.* **2010**, *13*, 170−174.

(24) Laokuldilok, T.; Shoemaker, C. F.; Jongkaewwattana, S.; Tulyathan, V. Antioxidants and antioxidant activity of several pigmented rice brans. *J. Agric. Food Chem.* **2011**, *59*, 193−199.

(25) Salgado, J. M.; Oliveira, A. G.; Mansi, D. N.; Donado-Pestana, C. M.; Bastos, C. R.; Marcondes, F. K. The role of black rice (*Oryza sativa* L.) in the control of hypercholesterolemia in rats. *J. Med. Food* **2010**, *13*, 1355−1362.

(26) Linscombe, S. D.; Jodari, F.; Bollich, P. K.; Groth, D. E.; White, L. M.; Chu, Q. R.; Dunand, R. T.; Sanders, D. E. Registration of 'Cocodrie' rice. *Crop Sci.* **2000**, *40*, 294.

(27) Prior, R. L.; Fan, E.; Ji, H.; Howell, A.; Nio, C.; Paynef, M. J.; Reed, J. Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *J. Sci. Food Agric.* **2010**, *90*, 1473−1478.

(28) Gu, L.; Kelm, M.; Hammerstone, J. F.; Beecher, G.; Cunningham, D.; Vannozzi, S.; Prior, R. L. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *J. Agric. Food Chem.* **2002**, *50*, 4852−4860.

(29) Singleton, V. L.; Orthofer, R.; Lamuela-Raventós, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin−Ciocalteu reagent. *Methods Enzymol.* **1998**, *299*, 152−178.

(30) Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555−559.

(31) Sánchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **1998**, *76*, 270−276.

(32) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* **2002**, *50*, 4437−4444.

(33) Nardini, M.; Cirillo, E.; Natella, F.; Mencarelli, D.; Comisso, A.; Scaccini, C. Detection of bound phenolic acids: Prevention by ascorbic acid and ethylenediaminetetraacetic acid of degradation of phenolic acids during alkaline hydrolysis. *Food Chem.* **2002**, *79*, 119−124.

(34) USDA database for the flavonoid content of selected foods. Release 2.1. 2007. Web site: http://www.ars.usda.gov/nutrientdata.

(35) Hudson, E. A.; Dinh, P. A.; Kokubun, T.; Simmonds, M. S. J.; Gescher, A. Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol., Biomarkers Prev.* **2000**, *9*, 1163−1170.

(36) Kim, M.-J.; Hyun, J.-N.; Kim, J.-A.; Park, J.-C.; Kim, M.-Y.; Kim, J.-G.; Lee, S.-J.; Chun, S.-C.; Chung, I.-M. Relationship between phenolic compounds, anthocyanins content and antioxidant activity in colored barley germplasm. *J. Agric. Food Chem.* **2007**, *55*, 4802−4809.

(37) Robbins, R. J.; Leonczak, J.; Johnson, J. C.; Li, J.; Kwik-Uribe, C.; Prior, R. L.; Gu, L. Method performance and multi-laboratory assessment of a normal phase high pressure liquid chromatography-fluorescence detection method for the quantitation of flavanols and procyanidins in cocoa and chocolate containing samples. *J. Chromatogr., A* **2009**, *1216*, 4831−4840.

(38) Chen, P.-N.; Kuo, W.-H.; Chiang, C.-L.; Chiou, H.-L.; Hsieh, Y.-S.; Chu, S.-C. Black rice anthocyanins inhibit cancer cells invasion via repressions of MMPs and u-PA expression. *Chem.-Biol. Interact.* **2006**, *163*, 218−229.

(39) Yin, M.-C.; Lin, C.-C.; Wu, H.-C.; Tsao, S.-M.; Hsu, C.-K. Apoptotic effects of protocatechuic acid in human breast, lung, liver, cervix, and prostate cancer cells: Potential mechanisms of action. *J. Agric. Food Chem.* **2009**, *57*, 6468−6473.

(40) Friedman, M.; Mackey, B. E.; Kim, H. J.; Lee, I. S.; Lee, K. R.; Lee, S. U.; Kozukue, E.; Kozukue, N. Structure-activity relationships of tea compounds against human cancer cells. *J. Agric. Food Chem.* **2007**, *55*, 243−253.