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Antioxidative, Antimutagenic, and Anticarcinogenic Activities of Rice Bran Extracts in Chemical and Cell Assays

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Ethanol−water (70:30 v/v) extracts from rice brans removed from seeds of two blackish-purple pigmented (Sanhaehyanghyulla and Suwon 415) and one nonpigmented (Chuchung) brown rice cultivars were evaluated for antioxidative, anti-tumor-promoting, and anticarcinogenic activities in chemical assays and in mammalian cells (human leukemia HL-60, marmoset B lymphoblastoid B95-8, and Chinese hamster V79 lung cells) by the following tests: inhibition of xanthine oxidase activity; chelation of ferrous ions; reduction of potassium ferricyanide; scavenging of superoxide anions, hydroxyl radicals, and intracellular peroxides; inhibition of 4-nitroquinoline *N*-oxide-induced mutagenesis; and inhibition of phorbol ester-induced tumor promotion. The extracts from the pigmented rice seeds had generally higher activities in all tests than did the extract from the nonpigmented variety. The results suggest that brans from pigmented rice varieties may provide a source of new natural antioxidants and anticarcinogens and that such rice cultivars with high antioxidative potential also provide a genetic resource for the development of new, improved rice cultivars that may make it possible to enhance both the nutritional and medical value of rice-based diets.

KEYWORDS: Antioxidative; antimutagenic; anticarinogenic; rice bran

INTRODUCTION

It is widely recognized that dietary ingredients have a dual role in relation to some human diseases. They can contribute to both the causes and prevention of diseases such as cancer and atherosclerosis as well as the aging process. One way in which the dietary components can protect against cell proliferation is to destroy certain reactive oxidative species (ROS) that initiate carcinogenesis through oxidative damage of DNA (*1*). Such reactive species include hydroxyl radicals, superoxide anions, singlet oxygen, and nitroso compounds. Biological alkylating agents such as aflatoxin B_1 can also initiate carcinogenesis. Natural antioxidants that can neutralize ROS include cysteine, reduced glutathione, polyphenolic compounds (anthocyanins, flavonoids, phenolic acids), carotenoids (α-carotene, β-carotene, lycopene), ascorbic acid (vitamin C), α-tocopherol (vitamin E), and indole carbinols (*1*−*3*).

For the purpose of this study, we define the following terms: brown rice = nonpigmented rice = commercial variety Chuchung used in home cooking; pigmented black rice = pigmented rice = experimental rice cultivars Sanhaehyanghyulla and Suwon 415. We will use nonpigmented for the brown rice and pigmented for the two pigmented black rice brans.

To place in proper perspective our findings described below on preventive effects of anthocyanin-containing rice bran extracts, we will briefly mention selected studies on beneficial effects of rice antioxidants in cells. Relevant studies associated with rice include the observations that aqueous−ethanol extracts of rice bran exhibited antioxidative properties (*4*); feruloyl-*myo*-inositols present in rice bran inhibited phorbol ester-induced superoxide anion generation in HL-60 cells (*5*); the cyanidin 3-*O*-β-D-glucoside isolated from pigmented rice scavenged superoxide anions but not hydroxyl radicals (*6*); a quinolone alkaloid isolated from the pigmented rice exhibited antioxidative activity (*7*); dietary pigmented rice protected against lipid peroxidation in the rat kidneys (*8*); cyanidin and malvidin isolated from a pigmented rice inhibited the growth of leukemia cells (*9*); pigmented rice suppressed reactive oxygen species in an in vitro assay (*10*); and protocatechuic acid methyl ester isolated from black rice inhibited the enzymatic activity of tyrosinase (*11*).

The main objective of this study was to complement previous studies by evaluating antioxidative, antimutagenic, anti-tumor-promoting, and anticarcinogenic activities in mammalian cells of bran extracts from a brown rice variety used in the home and two pigmented experimental rice cultivars. The results

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further demonstrate the health-promoting potential of the pigmented rice cultivars.

MATERIALS AND METHODS

Materials. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), *tert*-butyl hydroperoxide (*t*-BuOOH), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), xanthine oxidase (XOD), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was purchased from Calbiochem (San Diego, CA). All reagents were of analytical grade and were used without further purification. Cell culture media, Hanks' balanced salt solution (HBSS), and fetal bovine serum (FBS) were the products of Life Technologies (Grand Island, NY). The monoclonal antibody raised against the early diffusible antigen of Epstein−Barr virus (EBV-EA) and fluorescein isothiocyanate- (FITC-) conjugated goat anti-mouse immunoglobulin (IgG) was purchased from Novocastra Laboratories (Newcastle-upon-Tyne, U.K.).

Rice Extracts. Two pigmented rice cultivars (Suwon 415 and Sanghaehyanghyulla) and one nonpigmented cooking rice (Chuchung) were harvested at the experimental rice field of National Crop Experiment Station, RDA (Suwon, Korea). The rice seeds were dehulled, degermed, polished in a laboratory mill, and then passed through a 60-mesh sieve, resulting in a uniform fraction of rice bran. The pigments and other compounds in the hull were extracted by shaking overnight at room temperature with 10 times the sample weight of 70%−30% ethanol−water. The filtrate was passed through Whatman no. 1 filter paper and the solvent was then removed from the extract by rotary evaporation at room temperature.

Mammalian Cell Cultures. Human promyelocytic leukemia cell line (HL-60) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA), and cultured in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS) with 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. To induce myeloid differentiation, cells were seeded at a density of 5×10^5 cells/mL and were then cultivated for 5 days in RPMI 1640 medium containing 1.24% dimethyl sulfoxide (DMSO). The characteristics of mature cells were determined by cell sizing together with expression of CD11b in plasma membrane. The B lymphoblastoid cell line B95-8, established from EBV-infected marmoset B cells, and V79 strain of Chinese hamster lung cells were also purchased from ATCC and cultivated in RPMI 1640 medium and Eagle's minimum essential medium (MEM), both supplemented with 10% FBS.

Chemical Tests. *Inhibition of Xanthine Oxidase Activity by Bran Extracts.* Enzyme activity of xanthine oxidase was determined spectrophotometrically by measuring uric acid formation at 295 nm with hypoxanthine as substrate (*12*). The assay system consisted of 0.2 mL of reaction medium containing 50 mM sodium phosphate buffer (pH 7.4), 0.1 unit/mL xanthine oxidase, and 0.5 mM hypoxanthine. Following preincubation of the rice extracts with the enzyme for 10 min, the reaction was started by addition of hypoxanthine. The blank solution used as a reference contained all reagents except the enzyme.

Ferrous Ion Binding by Bran Extracts. The capacity of the extracts to bind Fe^{2+} ions was adapted from the method reported by Carter (*13*).

Ferricyanide Test of Reducing Power of Bran Extracts. The reducing power of each extract was determined according to the method of Oyaizu (*14*) with some modifications as follows: Extracts (0.16−3.3 mg/mL) were diluted with 0.7 mL of 50 mM phosphate buffer (pH 6.6). The dilute sample was then mixed with 0.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] and the mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (10%; 0.5 mL) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Aliquots (0.5 mL) from the upper layer of the solution were mixed with the same volume of deionized water and 0.1% $FeCl_3$ (0.1 mL). The absorbance at 700 nm was then measured on a UV/vis spectrophotometer (V-550, Jasco).

Superoxide Anion Scavenging Activity by Bran Extracts Measured by Electron Spin Resonance Spectroscopy. The effect of rice bran extracts on generation of superoxide anions (O_2^-) in the xanthine/xanthine oxidase (HPX/XOD) reaction was performed by an electron spin resonance (ESR) spin-trapping technique with 5,5-dimehtyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap agent according to the method described by Mitsuta et al. (*15*). The aqueous extracts, in 50 μL of 0.1 M phosphate buffer solution (pH 7.4), were mixed with 50 μL of 2 mM hypoxanthine, 35 mL of 5.5 mM diethylenetriaminepentaacetic acid (DETAPAC), 15 μL of 9.2 mM DMPO, and 0.4 unit of xanthine oxidase. Electron spin resonance (ESR) spectra were then recorded on an ESR spectrometer (TE-200, JEOL) under the following instrumental conditions: modulation amplitude, 0.1 mT; recording range, 4 mT; recording time, 1 min; time constant, 0.1 s; microwave power, 1.8 mW; and microwave frequency, 9.40432. The superoxide anion scavenging ability was expressed as the percentage reduction of the peak heights (representing the DMPO−O_2 spin trap adduct) as compared to the peak heights measured in the absence of the rice extracts.

Hydroxyl Radical Scavenging Activity by Bran Extracts Measured by Electron Spin Resonance Spectroscopy. Hydroxyl radical (HO•) scavenging activity was determined by quantification of DMPO−OH spin trap adducts resulting from the reaction of DMPO with the radicals generated by the Fenton reaction, as described by Rosen and Rauckman (*16*). ESR spectra were recorded after each extract, in 50 μL of 0.1 M phosphate buffer solution (pH 7.4), was mixed with 50 μL of 0.3 M DMPO, 50 μL of 10 mM $FeSO_4$, and 50 μL of 10 mM H_2O_2. Conditions used in the ESR experiments were the same as those described above. The percentage of scavenging by extracts was calculated from the ratio of the peak heights of ESR spectra observed with DMPO−OH spin trap adducts compared to the peak heights measured without extracts.

Cell Assays. *Inhibition of Lipid Peroxidation of Rabbit Erythrocyte Membranes by Bran Extracts.* Preparation of erythrocyte membrane ghosts and subsequent determination of the antioxidant activity of each extract based on the chemically induced lipid peroxidation was performed according to the method described by Tsuda et al. (*17*) with some modifications as follows. Rabbit blood (100 mL) collected by cardiac puncture was subjected to an isotonic buffer (10 mM phosphate/152 mM KCl, pH 7.4) followed by centrifugation at 1500*g* for 20 min to a pellet of erythrocyte membrane ghosts. The reaction mixture, consisting of 0.9 mL of rabbit erythrocyte membrane ghost containing 1.8 mg of protein, 50 μL of 24 mM *tert*-butylhydroperoxide, and 3 mg of the test extracts in the same phosphate buffer solution (50 μL) was incubated for 30 min at 37 °C with constant shaking. Trichloroacetic acid (0.67%; 0.25 mL) was then added to stop the reaction. The resulting chromophore, which indicates the extent of inhibition of peroxidation, was measured at 535 nm after the solution was colored with thiobarbituric acid.

Intracellular Peroxide Scavenging by Bran Extracts in Leukemia HL-60 Cells. The scavenging of reactive oxygen species (ROS) by the rice bran extracts in a cell milieu was performed according to the method described by Lin et al. (*18*). The intracellular peroxide level was determined by a nonfluorescence probe (dichlorofluorescein diacetate, DCF-DA), which emits fluorescence following oxidation by hydrogen peroxide produced during an oxidative respiratory burst. Specifically, human leukemia HL-60 cells (1×10^6 cells/mL) were suspended in Hanks' balanced salt solution (HBSS) with 50 μM DCF-DA and the appropriate concentration of the test material at 37 °C for 30 min. For stimulation, cells were incubated for another 30 min with the addition of 100 ng/mL of the tumor-promoting phorbol ester (TPA). Flow cytometric analysis was then performed on the FACSvantage instrument (Becton Dickinson). The fluorescence profiles of $>1 \times 10^4$ cells were collected and analyzed for the cells actively producing peroxides.

Cytotoxicity and Antimutagenicity of Bran Extracts in Chinese Hamster V79 Cells. As the first step to determine antimutagenicity, the cytotoxic effects of the rice bran extracts were examined by determining the colony-forming activity of cells. Triplicate inocula of 5×10^2 cells were incubated in 100-mm Petri dishes in normal medium for 24 h, rinsed two times with HBSS, and treated with the rice extracts at various concentrations for 3 h. After treatments with rice bran extracts, cell were rinsed with HBSS and incubated in normal medium for 7 days at 37 °C. The cell colonies formed in Petri dishes were fixed with methanol and stained with Giemsa solution. Colonies containing more than 50 cells were scored under a microscope, and the colony-forming ability was calculated from the average number of

colonies as a percentage of the number of cells initially inoculated. The effect of rice bran extracts on cell survival is expressed as surviving fractions of the colony-forming ability in untreated control cultures.

The antimutagenicity of the rice extracts in mammalian cells was determined by the replating method described by Kuroda (*19*) with some modifications. The inocula (2×10^5 Chinese hamster strain V79 lung cells in 100-mm culture dishes) were incubated for 24 h at 37 °C and then treated with a series of concentrations of the mutagen 4-nitroquinoline oxide (4-NQO) for 3 h. The cells were then rinsed twice with Hanks' balanced salt solution (HBSS) and incubated for 7 days in normal medium to allow expression of mutagenicity. The cells were then dissociated by treatment with trypsin, followed by replating 2×10^5 cells in five 100-mm dishes in medium containing 5 μg/mL 6-thiaguanine (6TG) for 8 days. The 6TG-resistant mutant colonies formed were fixed, stained, and scored for the number of colony-forming units. In parallel experiments, triplicate samples of 5×10^2 cells each in 100-mm dishes were incubated in normal medium for 7 days. The colony-forming ability of replated cells was determined as described above. The number of induced mutants was calculated by subtracting the number of colonies in untreated control cultures from those in treated cultures. The induced mutation frequency (IMF) was expressed as the number of induced mutants per 10^5 colony-forming cells.

Anti-Tumor-Promoting Activities of Bran Extracts in Lymphoblastoid B95-8 Cells. To determine the anti-tumor-promoting capacities of the rice extracts, lymphoblastoid B95-8 cells were cultivated for 48 h at a density of 1×10^6 cells/mL in RPMI 1640 medium containing 4% FBS, and appropriate amounts of the rice extracts with or without 50 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and 3 nM sodium butyrate, as described (*20*). After cultivation, cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100, as described (*21*). Chemically fixed and permeabilized cells were then incubated with the monoclonal antibody to EBV-EA (100-fold dilution) on ice for 20 min. After washing with phosphate-buffered saline (PBS), the cells were further incubated with FITC-labeled anti-mouse IgG as a secondary antibody (1000-fold dilution) on ice for 20 min. The washes were repeated to remove the fluorescence-labeled secondary antibody before the immune-stained cells were finally suspended in PBS containing 1% bovine serum albumin (BSA). Flow cytometric analysis of the stained cells was performed on a FACSvantage instrument (Becton-Dickinson). The fluorescence profiles of more than 5×10^4 cells were used to calculate mean fluorescence values of the cell populations for evaluating the expression level of EBV-EA.

In parallel experiments, cell viability on the same batch of cells was measured by flow cytometric analysis to determine the ratio of viable cells resistant to incorporation of propidium iodide (PI) into the nuclei (*22*).

Statistical Analysis. Statistical analysis was accomplished with Statistical Analysis System software package on triplicate test data. Significant differences between means were determined by Anova procedure test, and a P value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Antioxidative Activities: General Aspects. Hydrogen peroxide (H_2O_2) can produce reactive hydroxyl radicals ($^{\bullet}OH$) in cells by the two reactions shown in eqs 1 and 2 (*23*, *24*):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad \text{(Fenton reaction)} \quad (1)$$

$$O_2^{-\bullet} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2 \quad \text{(Harber-Weiss reaction)} \quad (2)$$

$$AH\ (\text{antioxidant}) + {}^{\bullet}OH\ (\text{hydroxyl radical}) \rightarrow H_2O + {}^{\bullet}A\ (\text{antioxidant radical}) \quad (3)$$

The first reaction involves oxidation of ferrous iron (Fe^{2+}) by hydrogen peroxide (H_2O_2) to the ferric (Fe^{3+}) form, hydroxyl radicals ($^{\bullet}OH$), and hydroxide ions (OH^-). The second involves metal ion-catalyzed oxidation of superoxide anion radicals ($O_2^{-\bullet}$) by hydrogen peroxide to hydroxyl radicals, hydroxide ions, and oxygen. Chelation of ferrous iron (Fe^{2+}) with antioxidants can inhibit hydroxyl radical formation from the Fenton reaction (*25*, *26*). Antioxidants can minimize damaging effects of hydroxyl radicals by removing their free electrons to form H_2O and antioxidant radicals ($^{\bullet}A$), as illustrated above in eq 3. The free electron on $^{\bullet}A$ is less reactive (has less energy) than that on $^{\bullet}OH$ because its charge is delocalized (dissipated) via interactions with the electron clouds of the double bonds of the aromatic rings of phenolic and/or the conjugated system of other antioxidants (*27*). The free radical $^{\bullet}A$ is therefore less cell damaging than is $^{\bullet}OH$. The antioxidative free radical $^{\bullet}A$ can dimerize to form the dimeric form A−A and/or polymerize to the polymer $(A)_n$, where n equals the number of monomeric units.

Table 1. Inhibition of Xanthine Oxidase Activity by Rice Bran Extracts[a]

rice bran (μg/mL)	xanthine oxidase inhibition (%)		
	Suwon 415 (pigmented)	Chuchung (nonpigmented)	Sanghaehyanghyulla (pigmented)
0.1	12.124 ± 1.419	–5.682 ± 0.909	–14.996 ± 0.115
0.2	11.697 ± 4.424	2.841 ± 0.568	–11.324 ± 0.230
0.5	10.209 ± 1.436	–8.977 ± 0.341	–11.477 ± 0.265
1.0	18.477 ± 1.396	–14.545 ± 1.364	–16.373 ± 0.115
2.0	30.790 ± 5.262	–7.159 ± 0.114	–5.815 ± 1.377
5.0	73.097 ± 0.307	45.227 ± 0.682	6.121 ± 1.148

[a] Values are expressed as mean ± SD ($n = 3$).

Chemical Assays. The in vitro xanthine oxidase inhibition and ferrous ion chelation tests were carried out to rule out the possibility that the scavenging activity of the pigmented rice brans toward superoxide anions and hydroxyl radicals is the result of suppression of generation of the two reactive species and not from real scavenging.

Inhibition of Xanthine Oxidase Activity by Bran Extracts. Since xanthine oxidase induces the formation of ROS in cells, another measure of antioxidative activities is to determine the inhibition of the enzyme by potential antioxidants (*17*, *28*). **Table 1** shows inhibitory activities by three rice bran extracts as a function of concentration ranging from 0.1 to 5.0 μg/mL. The data show that concentration-dependent significant inhibition occurred with bran from Suwon 415 rice ($IC_{50} = 3.1$ μg/mL). Surprisingly, the brans from nonpigmened Chuchung and pigmented Sanghaehyanghyulla rice cultivars did not inhibit the enzyme at concentrations up to 2.0 μg/mL. At 5.0 μg/mL, the inhibition by Chuchung bran was ~45% and that by Sanghaehyanghyulla bran was ~6%. We do not know the reasons for these differences. The results suggest that inhibition of xanthine oxidase as a mechanism of antioxidative activity can vary widely among rice cultivars. The inhibitory effect is probably the result of chelation of rice bran constituents to molybdenum, which is part of the active site of xanthine oxidase (*29*).

Chelation of Ferrous Ions by Bran Extracts. Because chelation of ferrous ions by bran antioxidants can inhibit hydroxyl radical formation (*25*, *26*), we also examined the extent of chelation of ferrous sulfate by the three brans. **Figure 1** shows that at 0.5 mg/mL the three bran extracts did not differ in their chelating abilities. However, at 5.0 mg/mL, the extent of complexation by the extract from the pigmented Sanghaehyanghyulla cultivar was significantly greater than those observed with the two other cultivars. These observations suggest that the nature of the biologically active constituents of the Sang-

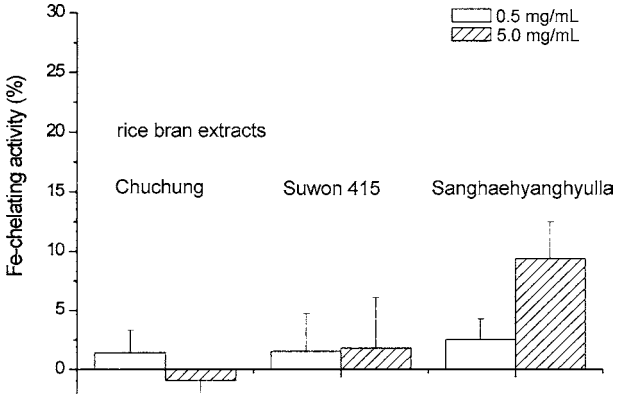

Figure 1. Ferrous ion chelating activities of bran extracts from three rice cultivars.

haehyanghyulla rice bran may be different from those present in the Chuchung and Suwon 415 brans. See also below section entitled Conclusions.

Antioxidative Power of Bran Extracts Measured by Reduction of Ferricyanide. Another test of antioxidative activity uses a colorimetric assay to measure the reduction of potassium ferricyanide (Fe^{3+}) to the ferrocyanide (Fe^{2+}) state (*14*). **Table 2** lists the extent of reduction in terms of absorbance values at 700 nm for a series of bran extracts ranging in concentration from 0.083 to 3.3 mg/mL. The data show that, for the five concentrations tested, the reducing power of the bran extract from the cooking rice variety Chuchung increased from 0.23 to 1.88 absorbance units. The corresponding increase for the brans from the two pigmented rice cultivars was much greater. It ranged from 0.57 to 3.14 for the Suwon 415 cultivar and from 0.43 to 3.06 for the Sanghaehyanghyulla cultivar. This test reinforces the greater antioxidative activity of the brans from pigmented rices compared to the bran from the nonpigmented variety.

Inhibition of Superoxide Anions by Rice Bran Extracts. **Figure 2** illustrates dose−response plots of the inhibition of formation of superoxide anions by three rice bran extracts. The maximum inhibition with the extract from the nonpigmented brown rice at a concentration of 6 mg/mL was 59.2%. The calculated IC_{50} value (concentration that inhibits 50% of the scavenging activity) was 2.09 mg/mL. The corresponding values for the Suwon 415 pigmented bran were 89.8% and 0.25 mg/mL, and for the pigmented bran from the Sanghaehyanghyulla rice, 85.7% and 0.56 mg/mL, respectively. Since the lower the IC_{50} value the greater the activity, these results indicate that scavenging activity of Sanghaehyanghyulla rice was about 4 times greater and that of Suwon 415 rice 8 times greater than the activity of Chuchung rice. Further studies are needed to demonstrate whether these differences are due to the differences in the nature and concentrations in pigmented anthocyanin antioxidative compounds among the three varieties or to other factors.

Inhibition of Hydroxyl Radicals by Rice Bran Extracts. **Figure 3** illustrates dose−response plots of the scavenging of hydroxyl

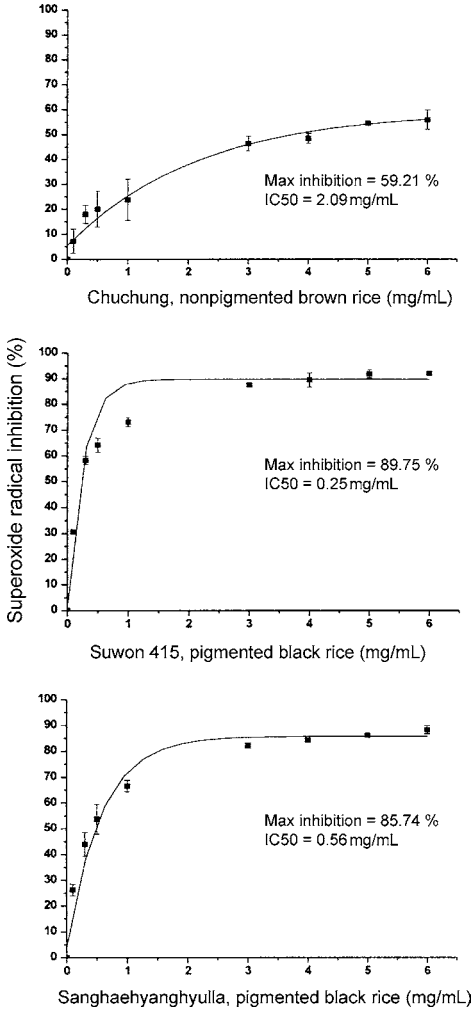

Figure 2. Superoxide anion-scavenging activities of three bran extracts from nonpigmented Chuchung and pigmented Suwon 415 and Sanghaehyanghyulla rice cultivars.

radicals by the three rice bran extracts. The maximum inhibition with the extract from the nonpigmented brown rice Chuchung at a concentration of 6 mg/mL was 79.3%. The calculated IC_{50} value was 2.66 mg/mL. The corresponding values for the Suwon 415 pigmented bran were 81% and 0.59 mg/mL, respectively. For the pigmented bran from the Sanghaehyanghyulla rice cultivar, the values were 71.2% and 1.35 mg/mL, respectively. These results indicate that the hydroxyl radical scavenging activity of Suwon 415 rice was about 5 times greater and that of Sanghaehyanghyulla rice 2 times greater than the activity of Chuchung rice. Comparison of the data in **Figures 2** and **3** shows that all three rice brans are more efficient scavengers of superoxide anions than of hydroxyl radicals. The order of activities is Suwon 415 > Sanghaehyanghyulla > Chuchung for both superoxide anions and hydroxyl radicals.

Table 2. Concentration Dependence of Reducing Power of Rice Bran Extracts Measured by the Ferricyanide Reduction Colorimetric Test[a]

	absorbance at 700 nm				
rice cultivar	0.083 mg/mL	0.16 mg/mL	0.83 mg/mL	1.6 mg/mL	3.3 mg/mL
Chuchung	0.233 ± 0.031	0.276 ± 0.027	0.669 ± 0.058	1.124 ± 0.077	1.883 ± 0.073
Suwon 415	0.569 ± 0.028	0.910 ± 0.045	2.579 ± 0.107	2.899 ± 0.013	3.141 ± 0.067
Sanghaehyanghyulla	0.434 ± 0.033	0.590 ± 0.022	1.675 ± 0.057	2.636 ± 0.072	3.064 ± 0.018

[a] Values are expressed as mean ± SD ($n = 3$).

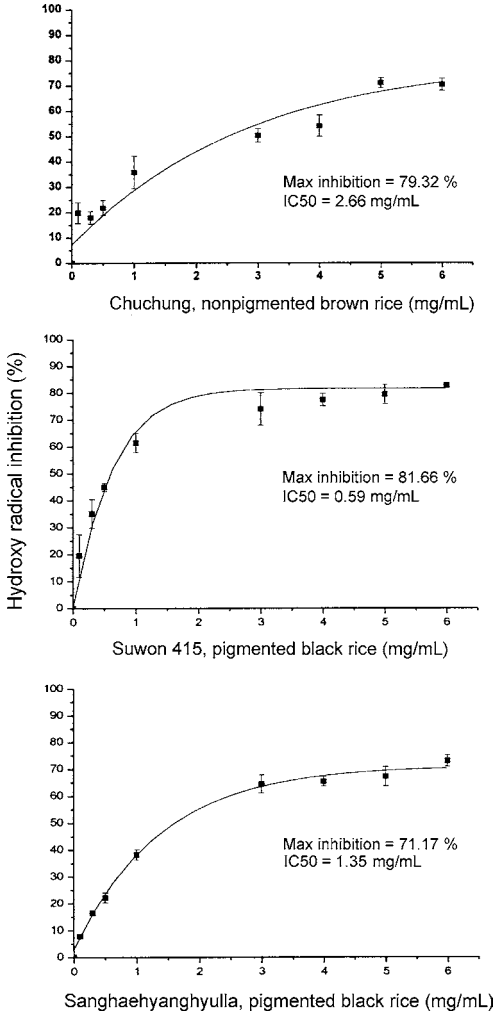

Figure 3. Hydroxyl radical-scavenging activities of three bran extracts from nonpigmented Chuchung and pigmented Suwon 415 and Sanghaehyanghyulla rice cultivars.

Table 3. Antioxidative Activity in the Rabbit Membrane Lipid Peroxidation Assay of Nonpigmented and Pigmented Rice Bran Extracts[a]

rice cultivar	erythrocyte ghost membrane lipid peroxidation assay	
	absorbance at 535 nm	inhibition (%)
control[a]	2.326 ± 0.076[b]	0
Chuchung, nonpigmented	2.013 ± 0.077	13.43
Suwon 415, pigmented	0.988 ± 0.042	57.52
Sanghaehyanghyulla, pigmented	0.895 ± 0.152	61.50

[a] Values expressed as mean ± SD ($n = 3$). [b] Control treatment without bran extract.

Assays in Cells. *Antioxidative Activities of Bran Extracts Measured by Inhibition of Lipid Peroxidation of Rabbit Erythrocyte Cell Membranes.* **Table 3** shows that bran extracts from the nonpigmented Chunchung brown rice variety inhibited 13.4% of lipid peroxidation in the rabbit erythrocyte cell membrane test. By contrast, extracts of the pigmented rice cultivars Suwon 415 and Sanghaehyanghyulla inhibited 57.5% and 61.5%, respectively. The striking difference of antioxidative activities is presumably due to the pigments in the pigmented varieties.

Scavenging of Hydroxyl Radicals in Leukemia Cells by Bran Extracts. **Figure 4** shows the effect of concentrations of rice bran extracts on the inhibition of hydrogen peroxide-generated free-radical scavenging in human leukemia HL-60 cells stimulated with the phorbol ester. The trends illustrated in this figure show that the free-radical-inhibitory activity at ~80 μg/mL of bran extract was 100% for Sanghaehyanghyulla rice, ~65% for Suwon 415 rice, and ~36% for Chuchung rice. The graph also shows that the relative activities of three brans seem to change with concentration in the range 0−120 μg/mL. These beneficial effects of the pigmented rice brans in a cancer cell ex vivo system generally parallel the in vitro antioxidative and scavenging activities described above.

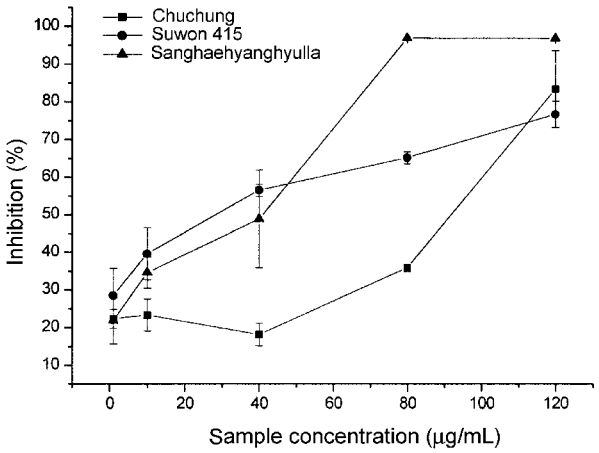

Figure 4. Hydroxyl radical inhibitory activities of bran extracts from nonpigmented Chuchung and pigmented Suwon 415 and Sanghaehyanghyulla rice cultivars in leukemia HL-60 cells.

Table 4. Cytotoxicity and Mutagenicity Tests of Rice Bran Extracts in Chinese Hamster V79 Cells

concn of bran (μg/mL)	surviving fraction of cells	
	Suwon 415	Chuchung
0	1.000	1.000
20	1.038	1.003
40	1.051[a]	1.009[a]
60	0.950	0.845
80	0.958	0.737
100	0.966	0.497
120	0.944	0.280
140	0.972	0.219
160	0.980	0.136
180	0.913	0.109
200	0.900	0.083

[a] Induced mutation frequency per 10^5 surviving cells was 0.000.

Cytotoxicity of Rice Bran Extracts in Chinese Hamster Cells. First, we attempted to establish the doses of the rice extract showing no cytotoxicity. Studies with the results shown in **Table 4** show that, in the concentration range 0−200 μg/mL, the bran extract from the pigmented Suwon 415 rice was not cytotoxic to Chinese hamster cells. However, extract from the Chuchung rice was cytotoxic at concentrations of 60 μg/mL or higher, killing about 92% of the cells at a concentration of 200 μg/mL.

Antimutagenic Activities of Rice Bran Extracts. Mutations are hereditary changes produced in the genetic information encoded in the deoxyribonucleic acid (DNA) in cells (*30*). Because chemically induced mutagenesis often leads to carcinogenesis, it was of interest to find out whether rice bran extracts can protect cells against a direct-acting mutagen. To explore this possibility, we evaluated the antimutagenic potential of extracts from the Chuchung control rice variety and from

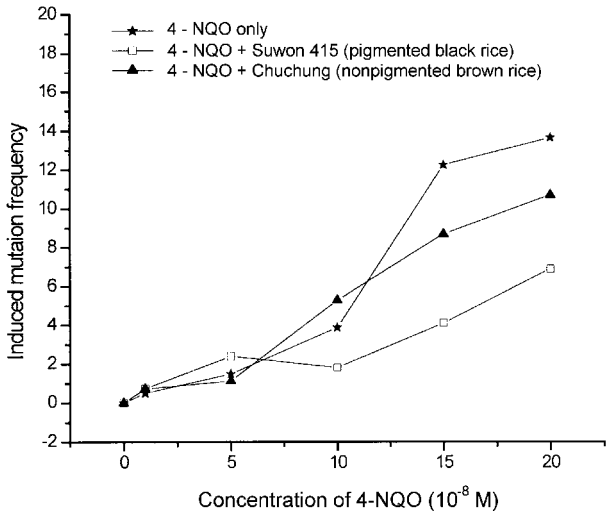

Figure 5. Effect of bran extracts from nonpigmented Chuchung rice and pigmented Suwon 415 rice cultivars on the mutation frequency induced by 4-nitroquinoline *N*-oxide (4-NQO) in cultured Chinese hamster V79 cells.

the Suwon 415 variety, shown earlier to have a high antioxidative potential.

The data show that, at a concentration of 40 μg/mL, neither extract induced mutagenicity in cells, as measured by the induced mutation frequency (**Table 4**). The data also show that, at the tested concentrations (40 μg/mL), antimutagenic and other beneficial effects of the bran from both Suwon 415 and Chuchung rice varieties are not accompanied by cytotoxicity.

We also attempted to elucidate the possible mode of action of the pigmented rice Suwon 415 on 4-nitroquinoline *N*-oxide-(4-NQO-) induced mutagenesis. The following experiment describes dose dependence of the inhibitory effects of the two rice brans on 4-NQO-induced mutagenic events in V79 cells. At a concentration of 4×10^{-8} M 4-NQO or higher, bran extracts from both the nonpigmented Chuchung and pigmented Suwon 415 rice cultivars exhibited antimutagenic activities in the cultured Chinese hamster cells (**Figure 5**). The protective effect of the Suwon 415 rice bran was about twice that of the Chuchung variety. These observations suggest that brans may have anticarcinogenic properties due to their ability to repair DNA damage in cells.

Anti-Tumor-Promoting Activity of Rice Bran Extracts. Flow cytometry was used to quantitatively evaluate anti-tumor-promoting activity by means of an in vitro short-term assay system measuring inhibition of Epstein−Barr virus early-antigen activation (EBV-EA) induced by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA). This assay measures both anti-tumor-promoting activity and cytotoxicity of target substances in the same batch of cells. Using this procedure, we determined the anti-tumor-promoting activity of bran extracts.

Figure 6 shows that anti-tumor-promoting activity of Chuchung bran in lymphoblastoid B95-8 cells was ~15% at 40 μg/mL and ~30% at 200 μg/mL. The corresponding inhibitory activities of Suwon 415 bran at the two concentrations were ~55% and ~60%, respectively. Thus, at 40 μg/mL, the anti-tumor-promoting activity of the pigmented bran was about 4 times greater than that of the brown variety. **Figure 6** also shows that there was no significant difference in cytotoxicity between the Suwon 415 and Chuchung brans, as measured by cell viability. This observation suggests that phytochemicals highly active in suppression of chemically induced-tumor promotion may be enriched in the Suwon 415 pigmented rice bran.

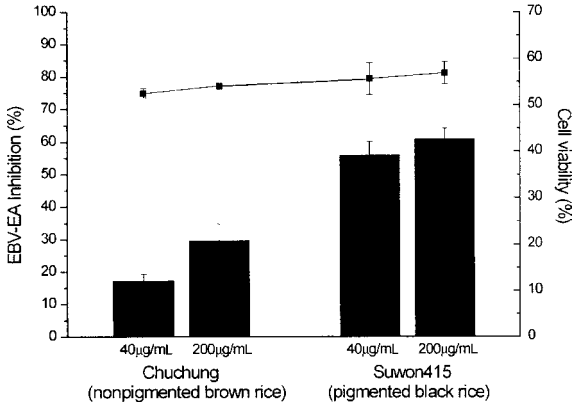

Figure 6. Inhibition of phorbol ester-induced tumor promotion by nonpigmented Chuchung and pigmented Suwon 415 rice bran extracts in lymphoblastoid B95-8 cells.

Conclusions. A panel of chemical and cell culture studies demonstrated that the antioxidative and anticarcinogenic potentials of brans from pigmented rice varieties are greater than those of bran from a brown variety. The data also show that the two pigmented rice varieties differ in their abilities to scavenge reactive oxygen species. Thus, with respect to possible mechanisms of the scavenging effects, it appears that Suwon 415 rice bran inhibited superoxide anions by competitive inhibition of xanthine oxidase and hydroxyl radical scavenging through a direct quenching mechanism, not through chelation of ferrous ions. On the other hand, Sanhaehyanghyulla rice bran scavenged superoxide anions without any direct effect on xanthine oxidase activity or hydroxyl radicals through chelation of ferrous ions. We do not know the reasons for these differences. The present study also suggests that the antimutagenic activity of Suwon 415 pigmented rice is due to its ability to repair cellular DNA, not to adsorption or complexation of the added mutagen.

We anticipate that the bran of pigmented rice cultivars will provide a useful genetic resource for the development of improved rice cultivars to be used in diets designed to minimize chronic diseases and the aging process. Antioxidative rice brans and their biologically active components also merit testing for possible antibiotic activities against human pathogenic bacteria (*31*).

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