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RESEARCH ARTICLE

Comparative Analysis of Physicochemicals and Antioxidative Properties in New Red Rice (*Oryza sativa* L. cv. Gunganghongmi)

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Abstract

The main objectives of this study were to investigate physicochemicals and antioxidant activities of new red rice (*Oryza sativa* cv. Gunganghongmi (GH)) by comparing normal brown (Nampyeongbyeo, NB) and reported red rice (Jukjinjubyeo, JB) in Korea. The nutritional constituents, including protein, oil, sugar, fatty acid, GABA, and γ-oryzanol were not significantly different between normal brown and colored rice. However, the ethanol extract of GH showed the highest phenolic content (24.7 ± 1.3 mg g^{-1}). The ethanol extracts of GH showed higher scavenging activities against DPPH (0.2 mg mL^{-1} = 62.1 ± 2.5%) and ABTS (0.2 mg mL^{-1} = 63.2 ± 3.5%) radicals. Moreover, GH more inhibited LPS-induced nitric oxide (NO) production (13.2 ± 1.4 μM) than JB (18.3 ± 2.3 μM) and NB (22.1 ± 1.4 μM) at the same concentration (0.2 mg mL^{-1}) without cytotoxicity. These results suggest that new red rice (GH) would be considered to be new functional rice due to its anti-oxidative effect and high nutrition.

Key words: antioxidant activity, ethanol extract, nutritional constituent, phenolic compound, red rice

Introduction

Nutrient components and phenolic compounds are widely distributed in foods, crops, vegetables, fruits, and medicinal plants (Lee 2010; Yuk et al. 2011). The major nutritional constituents, such as proteins, fats, carbohydrates, amino acids, vitamins, sugars, and minerals have a positive effect on human health (Deshpande 1992). In contrast, phenolic compounds belong to two main groups, flavonoid (flavanes, flavanols, and anthocyanins) and non-flavonoid (phenolic acids, phenolic alcohols, and stilbenes), which also show various biological activities (Lee 2011; Yuk et al. 2011). In particular, the beneficial effects originating from phenolic compounds can be a major determinant of antioxidant activities in many edible sources (Lee et al. 2005). Antioxidants are the chemical substances that reduce or prevent oxidation. Moreover, they have the ability to prevent the damaging effects of free radicals in tissues (Irie et al. 2004) and have many beneficial health effects to protect against cancer, arteriosclerosis, heart disease, and several other diseases (Lee et al. 2005; Nam et al. 2005; Yuk et al. 2011). The possible toxicity of the most-used synthetic antioxidants has lead to obtaining antioxidants from natural sources. For this reason, the food and medicinal industries are interested in various foods (vegetables, crops, and fruits) with high phenolic compound contents to manufacture supplements with preventative and therapeutic properties.

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world, particularly in Asian countries due to its

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nutritional and functional value (Lamberts et al. 2007). It contains abundant nutrient components, such as protein, fatty acids, amino acids, carbohydrates, vitamins, and minerals. Thus, rice is an excellent edible source and a recommended crop for human health (Heinemann et al. 2005). Many studies have demonstrated that phenolic compounds in rice have received unprecedented attention due to their physiological properties, including antioxidant, antimutagenic, and anticancer (Butsat and Siriamornpun 2010; Nam et al. 2005). Moreover, rice has many chemovarietal forms, including white, red, black, brown, purple, and yellow according to kernel color, which is formed by anthocyanins and polyphenols in different layers of the seed coat and pericarp (Chaudhary 2003). In particular, colored rice is documented as a potent antioxidant source (Chaudhary 2003; Yawadio et al. 2007). Among these, red rice has been widely used in functional foods in Japan due to its high polyphenol content (Itani and Ogawa 2004). Previous studies have revealed that the beneficial health effects of this species are due to its biological components (Chaudhary 2003; Itani and Ogawa 2004; Sompong et. al. 2011). Thus, few studies have investigated the phenolic compounds and antioxidant activities in red rice (Jun et al. 2012). Therefore, our study was designed to analyze the antioxidant properties, including radical scavenging activity and nitric oxide inhibitory activity as well as the phenolic compounds from different colored rice.

Herein, we report the nutrient contents and anti-oxidative potential properties of Gunganghongmi (GH) brown rice under various extract conditions and antioxidant assay systems. Therefore, the newly measured nutritional, physicochemical, and anti-oxidative effects of GH rice can be used to enhance health promotion and develop new functional rice.

Materials and Methods

Plant material and chemicals

Three different colored rice cultivars, including Nampyeongbyeo (NB, brown rice), Gunganghongmi (GH, red rice), and Jukjinjubyeo (JB, reported red rice) were cultivated at the National Institute of Crop Science (NICS), Rural Development Administration (RDA). These cultivars were grown at the experimental field of the Functional Crop Division, NICS at Miryang during 2011 harvest under the same cultivation conditions. NB, GH, and JB samples were stored in sealed plastic bags at -10°C until they were used. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), butylated hydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), DMSO-d_6, Sephadex LH-20, Folin-Ciocalteu's phenol reagent, lipopolysaccharide (LPS), trifluoroacetic acid (TFA), acetic acid, and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Analytical grade methanol, acetonitrile, and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Silica gel 60 Rp-18 (40 – 63 μm) and TLC aluminum sheets (RP-18 F_{254}) were obtained from Merck (Darmstadt, Germany). RAW246.7 cells (murine macrophage cell line) were obtained from the American Type Culture Collection (Manassas, VA, USA). Sugar, γ-aminobutyric acid (GABA), and γ-oryzanol standards as well as fatty acid standards such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) methyl esters were obtained from Merck (Darmstadt, Germany) for analyses of nutritional constituents. All other reagents were of analytical grade and were purchased from Sigma Chemical.

Instruments

Extracts of grounded red (GH and JB) and normal brown rice (NB) were centrifuged using a Vision apparatus (750 nm, VS-30000MT, Vision, Korea). UV–Vis absorption spectra were measured on a Beckman DU650 spectrophotometer (517 nm, 734 nm, Beckman Coulter, Fullerton, CA, USA). Protein and oil were determined using a B-339 Auto Kjeldahl analyzer (Buchi, Schweiz) and a BUCHI B-811 Extraction System (Buchi, Schweiz). A DS6200 (DONAM Instruments Inc., Seoul, Korea) gas chromatography with a flame ionization detector and a capillary column (0.32 mm i.d. × 25 m HP-FFAP) were used to assess fatty acid composition. GABA contents were determined by Ultra performance Liquid Chromatography (UPLC) and AccQ˙tag Ultra system (Waters, Milford, MA, US).

Determination of protein, oil, and fatty acid contents

Protein was determined according to the AOAC official method (AOAC 1990). The pulverized grains (0.2 g) were digested in a Buchi B-435 digestion system and a Buchi B-412 scrubber with 20 ml of H_2SO_4 and 3.0 g of catalyst ($CuSO_4$:K_2SO_4 = 1:9, w/w). Protein content was determined using Kjeldahl nitrogen in an automated distillation unit (Büchi 339, Switzerland) and was calculated as the percentage of nitrogen, multiplied by 6.25. Oil was measured by the Soxhlet method using a Buchi B-811 extracted system (Kim et al. 2006). Briefly, powdered grains (2.0 g) were added to 200 mL of n-hexane in an extraction thimble and were boiled for 2 h at 105ºC. After cooling to room temperature in a desiccator, the extracted oil was weighed and represented relative to the dry matter of red rice. Fatty acid methyl esters were prepared using the gas chromatographic method by methylation of the extracted fat using H_2O:MeOH:toluene (1:20:10, v/v) (Kim et al. 2006). This constituent was extracted with 2 mL *n*-hexane, and 1 μL was injected into the gas chromatograph in split mode. An SP-2380 capillary column was used for gas chromatography (30 m × 0.25 mm, 0.25 mm, Supelco, Bellefonte, PA, USA) and the temperature was programmed as follows: 150ºC for 1 min and from 150 to 230ºC at 2.5ºC min^{-1} before being held for 2 min. Nitrogen was used as the carrier gas (1.0 mL min^{-1}). The inlet and detector were set to 250ºC. The standards, including C16:0, C18:0, C18:1, C18:2, and C18:3 methyl esters, were calibrat-

ed using the above mentioned procedures. Individual fatty acid methyl esters were identified by comparing the gas chromatography retention time with those of standards and the amount was expressed as a percent of total fatty acids.

Determination of GABA and γ-oryzanol contents

Samples were prepared for the GABA analysis according to a previously reported method with slight modifications (Seo et al. 2011). The distilled water (2 mL) was added to pulverized red and normal brown rice (0.5 g). Then, the vapor-extracted samples were dried, cooled, and centrifuged at 13,000 g for 10 min at 4ºC. The supernatant was measured for concentrations of GABA and amino acids by the AccQ· tag UPLC detection system (Waters, Milford, MA, US). Once the filtrate had been diluted to a suitable concentration, fluorescence derivatization was performed by the AccQ·Tag manufacturer's instruction. The derivatized GABA was detected by a PDA eλ detector (Waters, USA). The chromatography data was analyzed using Empower software (Waters, USA). To determine the concentrations of GABA, a standard solution containing known concentrations of GABA was analyzed with samples in every series of the analysis. γ-Oryzanol content was measured according to the method of Chotimarkorn et al. (2008). The HPLC analysis was performed on an Agilent 1200 series system equipped with a Hypersil ODS column (4.0 × 250 mm, 5 μm, Agilent Technologies, Palo Alto, CA, USA) at 330 nm. The mobile phase used was MeOH:CH_3CN:CH_2Cl_2:CH_3COOH (50:44:3:3) at a flow rate 1.0 mL min^{-1}. g-Oryzanol was identified by the retention time of the standard and calculated by comparing the peak area of the sample with the standard calibration curve.

Determination of total phenolic contents

Total phenolic content in the sample extract was determined using the Folin–Ciocalteau method (Meda et al. 2005). Five gallic acid solutions of 100, 250, 500, 750, and 1000 mg L^{-1} were prepared in deionized water, of which a 500 μL solution was collected and mixed with 250 μL 2 N Folin–Ciocalteau reagent. After mixing and standing at room temperature for 3 min, 500 μL of 25% sodium carbonate solution was added, and the mixture was allowed to stand at room temperature for 1 h. The absorbance of the solution was measured at 750 nm, and the standard curve was obtained by plotting concentration against absorbance. The results are expressed as mg gallic acid equivalents (GAE g^{-1}) extract.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined by a method described previously (Lee 2011). Pulverised grains (10 g) were extracted with 100 mL methanol for 3 days at 4°C. The extract was then filtered through Whatman no. 42 filter paper to remove the sediments, and the supernatants were immediately analyzed for radical scavenging effects. Briefly, sample extracts (0.1 mL) at various concentrations were added to both 0.49 mL of methanol and 0.39 mL of a α, α-Diphenyl-β-picrylhydrazyl (DPPH) methanolic solution (1 mM). The mixtures were vigorously vortexed and incubated for 30 min at room temperature in the dark. Absorbance of the mixtures was determined using a spectrophotometer at 517 nm. BHT was used as the reference. Scavenging activity (%) was calculated with the following formula:

DPPH radical scavenging activity (%) = (1 - absorbance of sample/absorbance control) × 100.

$ABTS^{\cdot+}$ radical scavenging activity

The $ABTS^{\cdot+}$ method is based on the ability of different substances to scavenge 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cations in comparison to a standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). $ABTS^{\cdot+}$ was dissolved in ethanol at a final concentration of 7 mM. The radical cation was produced by mixing the ABTS stock solution with 2.45 mM potassium persulfate. The mixture was incubated for 10 - 14 h until the reaction was complete, and the absorbance was stable. The $ABTS^{\cdot+}$ stock solution was diluted in ethanol to an absorbance of 0.70 at 734 nm for measurement. After adding 0.9 mL of diluted $ABTS^{\cdot+}$ to 0.1 mL of sample (sample preparation: see section 2.4), the absorbance was taken 3 min after the initial mixing (Lee 2011). Scavenging activity (%) was calculated with the following formula:

$ABTS^{\cdot+}$ radical scavenging activity (%) = [(absorbance of control – absorbance of sample)/absorbance of control] × 100.

Cell viability

RAW264.7 cells were grown in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine (2.5 mM), and antibiotics [penicillin (50 unit mL^{-1}) and streptomycin (50 g mL^{-1})] (Gibco)] at 37°C in a 5% CO_2 humidified atmosphere. The rice ethanol extract was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolidium bromide (MTT) based colorimetric method as previously described (Han et al. 2010).

Nitric oxide (NO) scavenging activity

The NO scavenging activity of the samples was measured by the method described by (Han et al. 2010) with a slight modification. Briefly, RAW264.7 cells (4 × 10^5 cells $well^{-1}$) in a 24-well plate were treated with or without the rice ethanol extract (0.2 mg mL^{-1}) for 3 h, followed by an incubation with 1 μg mL^{-1} LPS for 24 h. The concentration of nitrite as an indicator of NO production was determined in culture supernatant using the Griess reagent system (Kim et al. 1997) according to the manufacturer°Øs instructions (Promega, Madison, WI, USA).

Statistical analysis and curve fitting

All measurements were repeated three times, and results are shown as mean ± standard deviation of three experi-

ments. Statistical analysis was carried out using Student's *t*-test and Duncan's multiple range test (DMRT). A $P < 0.05$ was accepted as statistically significant. Curve fitting was performed using Sigma Plot 2001 (Systat Software Inc., Chicago, IL, USA).

Results and Discussion

Comparison of six nutritional constituents in GH, JB, and NB rice

There are several results concerning nutritional constituents including protein, oil, fatty acid, GABA, and γ-oryzanol which have been observed in rice (Lee et al. 2009; Sompong et al. 2011). However, many studies have not extensively evaluated the nutritional constituents in rice cultivars containing different color pigments. Therefore, we investigated the nutritional contents, such as protein, lipid, free sugar, fatty acids, GABA, and γ-oryzanol in the red, black, and brown rice. In red rice (GH), the contents of protein and oil were 7.6 and 3.0%, respectively, and were not significantly different from those of the other colored rice cultivars (Table 1). The five fatty acid compositions also showed no remarkable differences in the different colored rice. As illustrated in Table 1, oleic acid exhibited the highest content, ranging from 43.5 to 51.0%. Moreover, linoleic acid (29.5 - 36.4%) was the second major component, followed by palmitic acid (16.1 - 19.8%), and stearic acid (1.1 - 2.6%). The lowest content was observed for linolenic acid at 0.8 - 1.3%. It is well established that nutritional constituents in crops may be affected by factors including cultivar, environmental stressors (temperature, year, light, genetic, germination, and storage), and agronomic conditions (Sompong et al. 2011; Tsukamoto et al. 1995). Based on these results, we suggest that protein, oil, and fatty acids may be not key factors determining red rice quality in comparison with brown and red rice. GABA and γ-oryzanol are also considered significant constituents in rice due to their potential beneficial effects (Lee et al. 2009). As shown in Table 1, their contents in new red rice (GH) were 1.6 and 0.41 mg g^{-1} and brown (NB: 1.5 and 0.38 mg g^{-1}) and normal red rice (JB: 1.5 and 0.37 mg g^{-1}) rice showed similar contents.

Comparison of total phenolic contents in GH, JB, and NB rice

Phenolic compounds including phenolic acids and flavonoids are a group of aromatic secondary metabolites ubiquitously distributed in natural plants, crops, fruits, and vegetables. These compounds are of great interest in the food industry due to their beneficial health effects (Lee et al. 2005; Yuk et al. 2011). In the present study, we evaluated total phenolic contents in GH, JB, and NB rice with ethanol extract. Total phenolic content was measured through gallic acid equivalents (GAE) ($y = 0.0123x + 0.098$, $r^2 = 0.9995$) as described by the colorimetric method of Folin–Ciocalteau with a slight modification (Meda et al. 2005). The most abundant total phenolic content occurred in GH, followed by red rice (JB), and brown rice (NB), and their contents were 24.7 ± 1.3, 17.2 ± 1.5, and 11.3 ± 0.9 mg GAE g^{-1} in ethanol extracts, respectively. Our results reveal that total phenolic contents in the ethanol extract of new red rice (GH) may be responsible for the major portion of antioxidant effects including radical scavenging activities (Choi et al. 2007). Therefore, new red rice (GH) may be an effective antioxidant rice source compared with JB and NB.

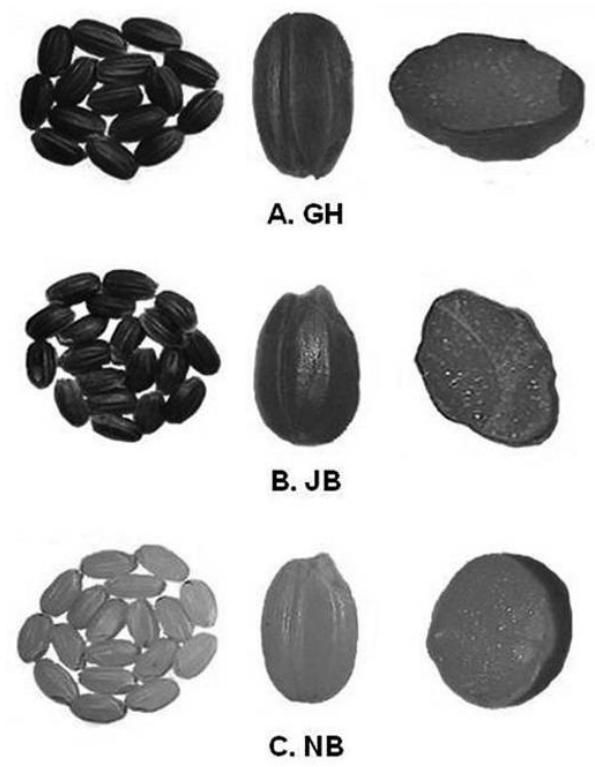

Fig. 1. Micrographs of whole grain (left, middle) and cross-section (right) of new red rice (A), reported red rice (B) and normal brown rice (C).

Scavenging effects of DPPH and ABTS radicals

The DPPH and ABTS radical methods are commonly used to measure the antioxidant effects of various materials due to

Table 1. Comparative analyses concerning the nutritional content in different colored rice cultivars

Rice varieties	Color	Nutritional component contents[1)]								
		Protein (%)	Oil (%)	Fatty acid composition (%)					GABA (mg g^{-1})	γ-oryzanol (mg g^{-1})
				C16;0	C18;0	C18;1	C18;2	C18;3		
GH	Red	7.6 ± 0.3	3.0 ± 0.2	19.8 ± 1.5	1.1 ± 0.0	33.4± 2.2	45.7 ± 2.3	0.9 ± 0.0	1.6 ± 0.1	0.41 ± 0.09
JB	Red	7.8 ± 0.4	3.1 ± 0.1	16.1 ± 1.4	2.6 ± 0.1	29.5± 1.2	51.0 ± 2.3	0.8 ± 0.0	1.5 ± 0.1	0.37 ± 0.04
NB	Brown	7.8 ± 0.6	2.8 ± 0.2	17.1 ± 1.9	1.8 ± 0.2	36.4± 1.9	43.5 ± 2.7	1.3 ± 0.1	1.5 ± 0.1	0.38 ± 0.05

their reproducibility and simple quality control (Choi et al. 2007). The DPPH radical exhibits scavenging activity for hydrogen donating antioxidants and the ABTS radical shows scavenging activity for hydrogen donating and chain breaking antioxidants through many components in the rice extract as reported previously (Choi et al. 2007). The scavenging activities on the free radical ABTS, generated by potassium persulfate, were compared with a standard amount of Trolox (Choi et al. 2007). These two assays estimate the percentage inhibition of radical formation. To determine the antioxidant activities of red rice (GH, JB) and normal brown (NB) rice, we carried out extractions with ethyl acetate (EtOAc), ethanol (EtOH), and methanol (MeOH). Table 2 shows the DPPH and ABTS radical scavenging activities of the various rice crude extracts. In the EtOH fraction, GH showed the highest DPPH (62.1 ± 2.5 at 0.2 mg mL^{-1}) and ABTS (63.2 ± 3.5 at 0.2 mg mL^{-1}) radical scavenging activities, whereas the EtOAc fraction showed the lowest DPPH (54.2 ± 1.2 at 0.2 mg mL^{-1}) and ABTS (40.2 ± 1.6 at 0.2 mg mL^{-1}) radical scavenging activities. In the two antioxidant assays, the EtOH fraction of GH rice exhibited the strongest free radical scavenging activities against DPPH and ABTS compared to the other red rice (JB) and normal brown rice (NB) crude extracts. These results were in agreement with those of total phenolic content. All results suggest that these variations in free radical scavenging activities of rice (GH, JB, and NB) extracts may have been due to differences in total phenolic contents. Consequently, new red rice (GH) may be great importance to enhance not only functional material but also dietary supplements because of possibly as primary antioxidants.

NO production inhibitory activity

Nitric oxide (NO) and reactive oxygen species (ROS) are responsible for oxidative injury to living organisms (Han et al. 2010). In particular, NO is a reactive metabolite generated by nitric oxide synthase (NOS). Activated macrophases release NO, a toxic radical species that causes DNA damage, normal cell apoptosis, and leads to metabolic diseases such as cancer and cardiovascular disease (Moncada et al. 1991). To determine whether or not red rice (GH, JB) and normal brown rice (NB) could inhibit lipopolysaccharide (LPS)-induced NO generation in RAW264.7 cells, we investigated NO production using Griess reaction assay. We first evaluated the toxicity of a rice extract to RAW264.7 cells has been measured by the MTT assay (Han et al. 2010). As shown in Fig. 2A, the ethanol extracts of three different rice cultivars were not toxic to RAW264.7 cell over 48 h at a concentration of 0.2 mg mL^{-1}. As shown in Fig. 2B, exposure of the cells to LPS (1μg mL^{-1}) increased the NO content (22.1 ± 1.4 μM) of the cells by more than 9 fold. However, upon treatment with the red rice (GH, JB) and normal brown rice (NB) extracts, the NO contents were significantly reduced. In particular, the GH rice extract showed the highest inhibition of LPS-induced NO production (13.2 ± 1.4 μM) without cytotoxic effect. It is possible that the phenolic compounds in this variety may be responsible for the scavenging effect concerning NO as shown previously (Han et al. 2010). Based on our results, new red rice (GH) may possess the potential to prevent oxidative stress and inflammation-related diseases compared with other colored rice.

In conclusion, the results of this study demonstrated that a red rice methanol extract had potent DPPH and ABTS radical scavenging activities as well as NO inhibitory effect when compared with brown and red rice. Total phenolic compound was also highest in this extract. Therefore, our results suggest that red rice may be utilized as an effective antioxidant source due to its radical scavenging activities and phenolic compounds. This research may be considered an improvement in the food situation because of the few studies on the crop industry concerning red rice in Korea.

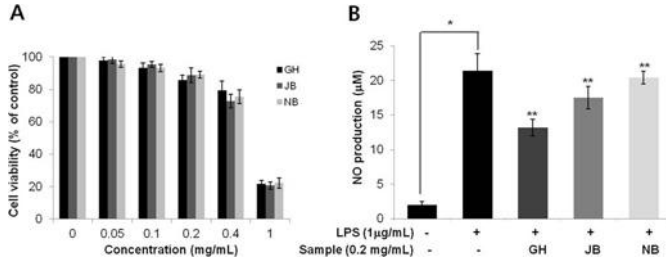

Fig. 2. Comparison of cell viabilities and NO radical scavenging activity of red rice (GH, JB) and brown rice (NB). (A) Cell viabilities of the ethanol extracts in GH, JB, and NB rice; (B) LPS-induced NO radical scavenging activity of ethanol extract from GH, JB, and NB rice.

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Table 2. Total phenolic contents and antioxidant effects[1)] of solvent fractions from red rice (GH, JB) and normal embryo brown rice (NB).

Rice extracts	Total phenolic contents (mg GA[2)] g^{-1})	DPPH radical scavenging assay[3)] (%)			ABTS radical scavenging assay[3)] (%)		
		EtOAC	EtOH	MeOH	EtOAC	EtOH	MeOH
GH	24.7 ± 1.3	54.2 ± 1.2[1)]	62.1 ± 2.5	58.2 ± 2.3	40.2 ± 1.6	63.2 ± 3.5	51.6 ± 1.2
JB	17.2 ± 1.5	50.4 ± 1.3	50.6 ± 3.5	48.6 ± 2.8	31.5 ± 1.5	52.2 ± 2.6	32.7 ± 1.1
NB	11.3 ± 0.9	43.9 ± 1.6	48.5 ± 2.7	40.2 ± 3.2	19.5 ± 1.3	32.1 ± 1.4	22.5 ± 1.1

1) Data presented indicate the mean ± SD (n = 3) of each sample.
2) Gallic acid (GA) was used as a standard for measuring of the total phenolic content.
3) Concentration of all rice extracts was 0.2 mg mL^{-1}.

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