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Research Article

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Anthocyanin Profiling and Radical Scavenging Activity of Selected Pigmented Rice Varieties

Hiba A. Ali,[1] Il Kyu Cho,[2] Sun-Ju Kim,[3] Se Na Kim,[4]
So Young Kim,[4] Young Sook Cho,[4] Hyung Jin Baek[4] and Jung Bong Kim[4*]

[1]Commission for Biotechnology and Genetic Engineering, National Center for Research, Khartoum, Sudan

[2]Department of Molecular Biosciences and Bioengineering University of Hawaii at Manoa
1955 East-West Road Honolulu, HI 96822-2321, USA

[3]Department of Bio-Environment Chemistry, Chungnam National University,
99 Daehak-Ro, Yuseong-Gu, Daejon 305-764, Korea

[4]National Academy of Agricultural Science, Rural Development Administration, Suwon 441-883, Republic of Korea

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Abstract

BACKGROUND: Anthocyanins have been recognized as health-enhancing substances due to their antioxidant activity, anti-inflammatory, anticancer, and hypoglycemic effects. The objective was to identify anthocyanins-rich rice grains for the development of functional foods and/or functional food colorants

METHODS AND RESULTS: Rice grains of one black and three red-hulled rice varieties were extracted with acidified 80% aqueous methanol. The antioxidant activity of the methanolic extracts was screened on TLC plates and in an *in vitro* assay using DPPH (1, 1-diphenyl-2-picrylhydrazyl) as a free radical source. Red-hulled rice varieties exhibited higher antioxidant activity (88%, 1 mg/mL) than black rice (67%, 1 mg/mL). Among the red-hulled varieties tested, rice variety SSALBYEO54 (901452) was the most active (72%, 0.5 mg/mL). Rice extracted anthocyanin compounds were analyzed by HPLC-DAD-FLD and LC-MS/MS. Red-hulled varieties comprised cyanidin-3-glucoside in addition to ferulic acid esters, apigenin and kaempferol glycosides.

CONCLUSION(s): Anthocyanins identified in the black rice variety were cyanidin-7-O-galactoside, cyanidin-3-O-glucoside, cyanidin-3'-O-glucoside, cyanidin-5-O-glucoside, cyanidin-3, 7-O-diglucoside, cyanidin-3, 5-O-diglucoside and peonidin-4'-O-glucoside. The results of this study show that the black rice (IT212512) and red-hulled rice variety SSALBYEO54 (901452) contain notable antioxidant activity for potential use in nutraceutical or functional food formulations.

Key Words: Anthocyanins, Antioxidant activity, Pigmented rice, Radical scavenging activity

*교신저자(Corresponding author):
Tel: +82-31-299-0511 Fax: +82-31-299-0504
E-mail: jungbkim@korea.kr (김정봉)

Introduction

Rice (*Oryza sativa*) is the most important cereal grain food for a large part of human population worldwide. Among many varieties, the white-hulled is the most common types (>85%). Other types include green, black, and red hull varieties. Black and red varieties are planted mainly in South Asia as well as Italy, Greece, and USA (Martinez-Valverde *et al.* 2001). Cereal grains contain unique free phenolic compounds and their glycosides and a significant amount of insoluble phenolic compounds bound to polysaccharides in the cell wall (Tian *et al.* 2004). Phenolic compounds in grains have

antioxidant properties associated with the health benefits of grains and grain products. About 74% and 69% of the total phenolics in rice and corn, respectively, are in insoluble bound forms, with ferulic acid and *p*-oumaric acid representing the most abundant phenolic compounds (Sosulski *et al.* 1982; Tian *et al.* 2004). Phenolic compounds in grains include derivatives of benzoic and cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds (Shibuya *et al.* 1984; Oste *et al.* 1991; Kroon *et al.* 1999; Zhao *et al.* 2003). Grains contain tocotrienols and tocopherol, and rice contains oryzanols. Kaempferol and apigenin were among the flavonoids reported in rice (Ko *et al.* 2006). Most phenolic compounds in rice are lost when rice bran is removed during processing (Shibuya *et al.* 1984; Oste *et al.* 1991).

Black, blue, and purple grains are currently produced only in small amounts for making specialty foods or for use in ornamentation due to their colorful appearance. Anthocyanins, a group of reddish to purple water-oluble flavonoids, are the primary pigments in these grains (Abel *et al.* 2006). Anthocyanins have been recognized as health-enhancing substances due to their antioxidant activity (Nam *et al.* 2006), anti-inflammatory, anticancer, and hypoglycemic effects (Abel *et al.* 2006). Although an extensive collection of scientific literature on the composition of anthocyanins in fruits and vegetables exists, little is known about anthocyanin composition in grains. Rice plants mainly accumulate two anthocyanin pigments, cyanidin and peonidin, an o-methyl derivative of cyanidin. Incidentally, this combination of anthocyanin pigments appears to be exclusive to indica rice. In certain japonica plants, the presence of malvidin was reported (Reddy *et al.* 1996). Black rice was reported to contain a wide range of anthocyanin species, with cyanidin 3-glucoside as the most common anthocyanin and peonidin 3-glucoside as the second most abundant anthocyanin (Ryu *et al.* 1998). In addition, rice plants seem to accumulate a unique class of pigments called proanthocyanidins, imparting brown color, particularly in the pericarp of rice. These pigments yield cyanidin and peonidin upon hydrolysis (Reddy *et al.* 1996).

In this study, anthocyanin composition was evaluated in four varieties of red-hulled and black-hulled rice varieties. The *in vitro* antioxidant activity of each rice variety was measured. The objective was to identify anthocyanins-rich rice grains for the development of functional foods and/or functional food colorants.

Materials and Methods

Plant Materials

Four rice cultivars that have red or black hulls (Table 1) were newly released by the Plant Genetic Resource (PGR) Evaluation Laboratory's Gene bank of the Rural Development Administration (RDA), Suwon, South Korea. They were composed of weedy collections, introduced and breeding lines and land races.

Reagents

All authentic anthocyanin samples were purchased from Extrasynthese (Genay, France). Stock standard solutions were 1 mg of each anthocyanin dissolved in 1 mL of acidified methanol. HPLC grade acetonitrile (CH_3CN) and methanol (MeOH) were obtained from

Table 1. Identification and chromatographic characteristics detected in selected rice varieties: retention time (R_t), molecular ions $[M+H]^+$ and fragments ions of anthocyanins

Peak	Compounds	R_t (min)	$[M]^+$ (*m/z*)	MS/MS main fragments and intensities (*m/z*)	UV λ_{max} (nm)
1	Cyanidin-3-glucoside	10.8	449	287 (100)	235, 280, 515
2	*Cyanidin-5-glucoside	10.6	449	287 (100)	230, 280, 515
3	Cyanidin-3′-glucoside	13.0	449	287 (100)	220, 260, 285 sh, 515
4	Cyanidin-7-galactoside	8.8	449	287 (100)	225, 280, 515
5	Cyanidin-3,7-diglucoside	9.4	611	449 (5), 287 (100)	225, 280, 515
6	Cyanidin-3,5-diglucoside	5.0	611	449(60), 287 (100)	225, 265, 520
7	Peonidin-4′-glucoside	14.0	463	301(100), 286(100), 257 (100)	225, 280, 330 sh, 515
	Standards				
	Cyanidin	12.1	287	259, 231, 136	225, 280, 515
	Peonidin	27.8	301	286, 257, 230	205, 275, 525

*Anthocyanin identified in all rice varieties studied.

J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Formic acid was provided from Kanto Chemical Co., Inc. (Tokyo, Japan).

Extraction and Hydrolysis

Rice grains were extracted by adding 5 mL acidified methanol (MeOH: 0.1% HCl) to 1 g rice powder. The mixture was allowed to percolate for seven days at 4℃. Rice sample hydrolysis was performed as previously described (Mc Nally *et al.,* 2002) with minor modifications. Rice grains were extracted with 80% MeOH for one day under ultrasonication. Samples were then hydrolyzed by adding an equal volume of 4N HCl, and the mixture was refluxed using a water bath at 100℃ for one hour. The acidic mixture was extracted with ethyl acetate. The solvent was then removed with a rotary evaporator under vacuum. Precolumn purification of the methanolic extracts was achieved by passing them through a reverse phase column (C_{18}, Strata, Phenomenex). Extracts were eluted sequentially using 100% H_2O, 50% MeOH and 100% MeOH. Collected fractions were subjected to RP-HPLC and LC-MS/MS for identification of anthocyanin content.

Reverse Phase HPLC - Photodiode Array Detector (PDA) and Fluorescence Detector FLD

Anthocyanin content in the pigmented rice varieties was estimated on a Shimadzu Prominence high performance liquid chromatograph system equipped with a diode array detector (SPD - M20A Prominence) and a fluorescence detector (FLD) (RF-10AXL Shimadzu) (Shimadzu, Kyoto, Japan). Samples were passed through a 0.45 μm pore syringe-driven filter prior to injection. A 20-μL aliquot of sample solution was injected and separated on a C_{18} reverse phase analytical column (4 μm, 4.6 250 μm, Phenomenex, Torrance, CA, USA). UV detection was performed at 520 nm and 360 nm for anthocyanins and flavonoids or other phenolic acids, respectively; λ_{ex} = 330 nm and λ_{em} 440 nm were used for fluorescence detection.

The column was eluted with a gradient mobile phase consisting of solvent A [95% water containing 5% acetonitrile (MeCN) and 0.025% formic acid] and solvent B [50% MeCN and 0.025% formic acid] at a flow rate of 1 mL/min. Gradient elution was performed as follows: 0 min (90% A, 10% B), 15 min (65% A, 35% B), 30 min (100% B), 40 min (90% A, 10% B). Column temperature was set at 35℃. Anthocyanin identification was based on the congruence of retention times (R_t) and UV-visible spectra with those of pure authentic standards.

Identification of anthocyanins by LC ion trap mass spectrometric analysis (LC-MS/MS)

Mass spectra were recorded on a Shimadzu HPLC - Finnigan LCQ Deca XP MAX ion trap mass spectrometer (San Jose, CA, USA). Rice grain extracts were separated into fractions on a reverse-phase column (LC-18; 4 μm, polar-RP80A, 250 2.00 mm, 4 micron; Phenomenex, USA) at 35℃ using a flow rate of 200 mL/min. The column was eluted with a gradient mobile phase consisting of 0.035% formic acid in 5% acetonitrile (phase A) and 0.025% formic acid in 50% acetonitrile using the following gradient program: 0 min (85% A, 15% B), 40 min (35% A, 65% B), 45 min (35% A, 65% B), 50 min (85% A, 15% B).

The mass spectrometer was operated with the ESI positive ion mode. For the condition of positive ion mode, capillary temperature was set to 275℃ and the spray voltage was set to 5000 V. Nitrogen was used as sheath gas, and the flow was set to 20 U. Helium was used as collision gas at 0.9 mTorr. Fragmentation of compounds studied and neutral loss scan were investigated with a scan range from *m/z* 110 to 1000 at collision energy of 40 eV. LC-MS confirmation of anthocyanin identity was carried out by comparison with authentic sample peaks and reported data.

Antioxidant Activity

The extracts of pigmented rice varieties were screened for their antioxidant activity using 1, 1-diphenyl-2-picrylhydrazyle (DPPH) as a free radical source. Rice extracts were separated using Merck RP-18 F_{254} precoated plates and MeOH: H_2O: acetic acid (4:4:2) as the developing system. Developed chromatograms were then sprayed using 5% DPPH in ethanol. Similar TLC plates were sprayed with natural product reagent for detection of flavonoids and other phenolic acids. Active compounds developed a yellow color over the deep purple background of DPPH. Extracts showing positively active compounds were subjected to further *in vitro* antioxidant activity assay by employing the methodology reported by (Shyur *et al.* 2005) with minor modifications. Assays were performed in 3 ml reaction mixtures containing 2.0 ml 0.1 mM DPPH-ethanol solution, 0.9 ml 50 mM Tris-HCl buffer (pH 7.4), and 0.1 ml MeOH (as control) or test plant extracts at different concentrations of 0.06, 0.1, 0.2, 0.5, and 1 mg/ml. After 30

min incubation at room temperature, absorbance of the reaction mixtures at 517 nm was recorded. The inhibitory effect on radical production of DPPH was calculated as a percentage according to the following formula:

Inhibition (%) = [(Absorbance$_{control}$ − Absorbance$_{sample}$) / Absorbance$_{control}$] 100. IC_{50} represents the level where 50% of the radicals were scavenged by test samples.

Results and Discussion

Rice samples were hydrolyzed to identify and/or confirm their anthocyanin content. Peak identification of rice sample components was made based on comparison of the experimental values with those of chemical standards and published information concerning anthocyanins and flavonoids in general. It has been found that sugars with a D-configuration, namely glucose, galactose, xylose and glucuronic acid, are usually linked to aglycone by β bonds while α linkages occur in L-arabinose and L-rhamnose (Robards *et al.* 1997). Rules governing the chromatographic behavior of anthocyanins on a reversed-phase column were established by Goiffon (Goiffon *et al.* 1991). The overall polarity and stereochemistry of the compound are key factors. In particular, the following factors are well documented: substitution of the anthocyanidin B-ring, the position, nature and number of sugar moieties attached to the anthocyanidin, and the extent of sugar acylation. Thus, substitution of the B-ring gives the elution order delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin with hydroxyl groups decreasing and methoxyl groups increasing retention (Robards *et al.* 1997).

Reverse phase HPLC-DAD-FLD of black rice revealed the presence of at least seven peaks characteristic of anthocyanins (520 nm), five of which (R_t 7.7, 8.5, 8.7, 9.0, and 10.1 min) were expected to be conjugated at the 3-position, which was previously reported (Rijke *et al.* 2006). Two peaks at 3.4 and 11.7 min gave a prominent absorbance using HPLC-FLD, confirming a hydroxyl group at the 3-position (Rijke *et al.* 2006). Red-hulled rice samples 28, 29 and 34 showed identical chromatograms using HPLC-DAD (not shown) and LC-MS (360 nm). RP-HPLC-DAD confirmed the presence of one anthocyanin (520 nm) with a conjugation at the 3-position, as indicated by using FLD.

LC-MS chromatograms (520 nm) of black rice revealed that four compound peaks (R_t 8.8, 10.1, 10.8, and 13.0 min) comprise a similar ESI/MS peak at *m/z* 449. Glucose fragmentation $[M+H-162]^+$ was the main peak in positive ion mode analysis giving rise to a product ion of *m/z* 287. According to the fragmentation pattern of the product ion and after comparison with already established data (Montoro *et al.* 2006), these compounds were confirmed to contain the same anthocyanidin, namely cyanidin. These compounds are probably isomers because they gave similar mass spectra, had the same molecular weight (e.g., compounds 1, 2, 3 & 4 showed the same ion), had different retention times. In addition the intensity of the aglycone product ion depends on the attachment position of the sugar and showed the following order: 5 > 3 > 3′ = 5 > 4′ > 7 (Cuyckens *et al.* 2004).

Compound 1 (R_t, 10.8 min) is the major compound in black rice and is the only anthocyanin in the three red-hulled rice varieties (28, 29, 34) analyzed. LC-MS chromatograms of the hydrolyzed rice extracts (samples 28, 29 and 34) measured at 520 nm. They showed a similar peak at 12.1 min (R_t) with an ESI/MS peak at *m/z* 287, similar in fragmentation to the standard cyanidin under the same LC-MS/MS conditions (Table 1). Moreover, except for the prominent Y^+ peak (*m/z* 287), neither fragmentation of the glucose moiety nor product ions > *m/z* > 150 were observed, suggesting that compound 1 is cyanidin-3-glycoside (Fig. 2). Cyanidin glycosides containing a sugar group at the 3-position are commonly distributed in a wide variety of colored rice (Abel *et al.* 2006).

Compound 2 (R_t 10.6 min) possessed the same mass ion (*m/z* 449) and the most prominent aglycone ion (Y^+)

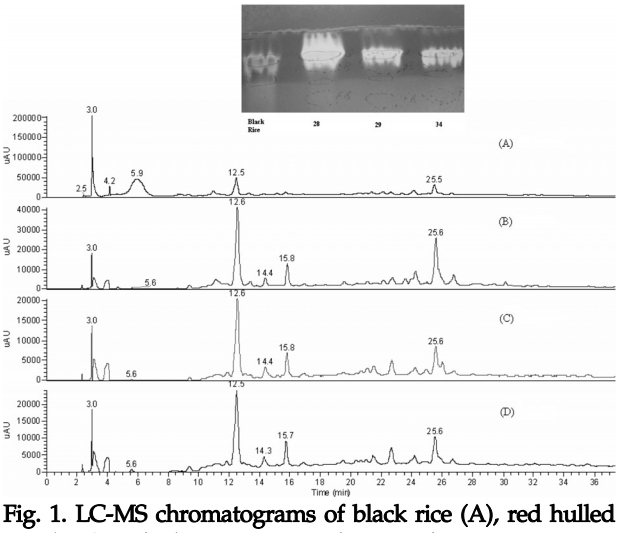
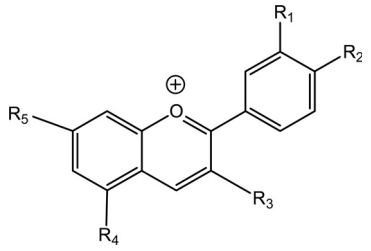


Fig. 1. LC-MS chromatograms of black rice (A), red hulled rice (B, C and D) at 360 nm and antioxidant activity using DPPH•(E).

suggesting that glucosidation is found at the 5-position (Cuyckens *et al.* 2004); thus, compound 2 is cyanidin-5-glucoside (Fig. 2). Among these isomers compound 3 exhibited the longest retention time (13.0 min) and the intensity of the sugar fragmentation ion (Y^+) is in agreement with glycosides in positions 3′ or 5′ (Cuyckens *et al.* 2004). Accordingly, compound 3 was assigned as cyanidin-3′-glucoside (Fig. 2).

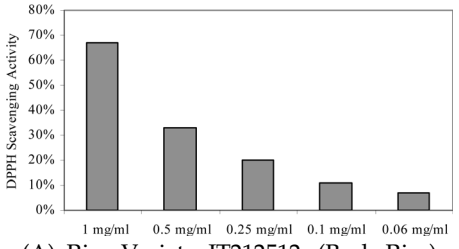
Compound 4 had the shortest retention time (R_t 8.8 min) among other isomers with the same precursor ion (*m/z* 449). According to published data for isomeric compounds that differ in the structure of saccharide residues, glucosides have a longer retention time than galactosides (Robards *et al.* 1997; Cuyckens *et al.* 2004; Liang *et al.* 2005). The aglycone ion of compound 4 (*m/z* 287) was not observed in the M^3 spectrum typical of flavonoids with glycosidation in the 7-position (Cuyckens *et al.* 2004). Additionally, the sugar fragmentation of compound 4 was different than the other isomers; therefore, compound 4 was assigned as cyanidin-7-galactoside (Fig. 2).

In LC-MS of black rice three more peaks (R_t 5.0, 9.4, and 14.0 min) were observed, and the analysis at 520 nm showed UV maxima characteristic of anthocyanins between 510-530 nm, as reported by Robards and Antolovich (Robards *et al.* 1997). Two of these compounds, at 9.4 and 5.0 min, had a similar $[M+1]^+$ of *m/z* 611, and are referred to as compounds 5 and 6, respectively (Fig. 3b). The $[M+1]^+$ ion MS-MS spectrum of the diglycosides of the two compounds showed an ion at *m/z* 449, which indicates a 162 mass unit loss and an ion of *m/z* 287. The loss of 162 mass units is often indicative of a hexose sugar. A difference between the two masses 449 and 287 (i.e. 162) suggests a further loss of a hexose. The *m/z* 287 and UV maxima (520 nm) is indicative of the base component of anthocyanins, that is cyanidin. Furthermore, the ion at *m/z* 449 in compound 5 had a weak intensity (<5%) suggesting that one of the glucose moieties occupies the 7-O-position, and the other occupies the 3-O-position (March *et al.* 2006). Compound 6 had a more abundant ion at m/z 449 (>60 %) suggesting it is a 3,5–diglucoside. This was confirmed by the fact that disaccharide moieties in positions 3 and 7 increase the polarity to a lesser extent than the presence of the same two monosaccharides in positions 3 and 5 (Escribano-Bail´on *et al.* 2004). Finally the MS^3 spectrum of the m/z 287 ion resulted in a fragmentation spectrum in which the main ions matched the fragmentation spectrum of cyanidin (Montoro

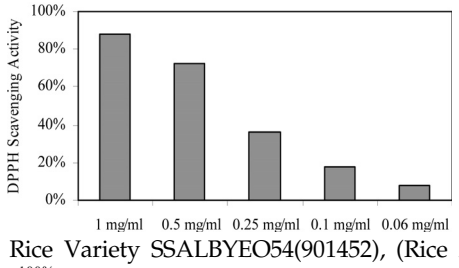


Compounds	R_1	R_2	R_3	R_4	R_5
1	OH	OH	Glucose	OH	OH
2	OH	OH	OH	OH	Glucose
3	Glucose	OH	OH	OH	OH
4	OH	OH	OH	OH	Galactose
5	OH	OH	Glucose	OH	Glucose
6	OH	OH	Glucose	Glucose	OH
7	OMe	Glucose	OH	OH	OH

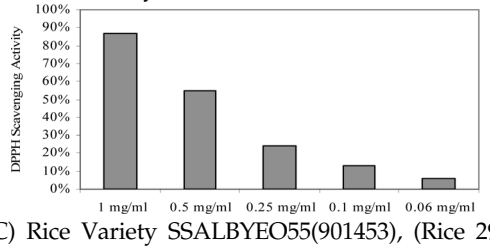
Fig. 2. Anthocyanins identified in selected rice varieties.



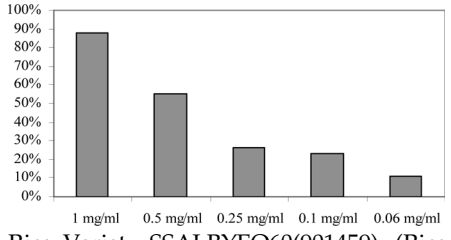
(A) Rice Variety IT212512, (Back Rice)



(B) Rice Variety SSALBYEO54(901452), (Rice 28)



(C) Rice Variety SSALBYEO55(901453), (Rice 29)



(D) Rice Variety SSALBYEO60(901459), (Rice 34)

Fig. 3. DPPH• scavenging activities extracts of black rice (A) and red hulled rice (B, C and D) at various concentrations.

et al. 2006). Accordingly, compound 5 was assigned as cyanidin-3, 7-diglucoside, and compound 6 was assigned as cyanidin 3, 5-diglucoside, (Fig. 2).

Compound 7 had a retention time of 14.0 min and $[M+1]^+$ ion of *m/z* 463. The positive base peak at *m/z* 301 represented the molecular ion of peonidin after the loss of one hexose sugar (162 mass units). This compound exhibited a pronounced signal using HPLC-FLD, indicating the presence of a hydroxyl group at the 3-O-position. Fragmentation of the sugar moiety and the existence of *m/z* 301 product ions at low intensity (>10%) suggest the glucose molecule is located at the 4′-O-positon (March *et al.* 2006). The MS^3 spectrum of the *m/z* 301 ion resulted in a fragmentation spectrum in which the main ions matched the fragmentation spectrum of peonidin (Montoro *et al.* 2006). Hence, compound 7 was assigned as peonidin-4′-glucoside.

Antioxidant data of rice varieties studied at different concentrations (0.06−1 mg/mL) are presented in Fig. 3. At a concentration of 1 mg/mL red-hulled rice varieties (samples 28, 29, and 34) had similar activity (85%) which was greater than black rice activity (60%) at the same concentration. Antioxidant TLC screening using 5% DPPH as a free radical source (Fig. 1) showed that all rice extracts possessed an active compound (R_f 0.8), which also showed a blue color typical of phenolic acids when sprayed with natural product reagent. Analyzing the MS-MS fragmentation of the peak (R_t 12.5) of this active compound (536 *m/z*) revealed it is a ferulic acid derivative (Fig. 1). The antioxidant activity of ferulic acid and its derivatives was reported by Kikuzaki *et al.* (2002). The antioxidant data also showed that at a concentration of 0.5 mg/mL (IC_{50}) rice sample 28 was the most active (72%) among red-hulled varieties examined (Fig. 3). Apigenin and kaempferol glycosides were also detected in red rice variety 28 which contributed to its antioxidant activity.

Anthocyanins are well known as health-enhancing substances and have been found in many types of vegetables, fruits and grains. The present study showed a diversity of anthocyanins in selected varieties of black and red rice grains. Such diversity in anthocyanin composition would help in the selection process for the development of anthocyanin-rich rice grain products. The data suggest that black rice has potential for development as a grain-based functional food or natural colorant on the basis of anthocyanin content and composition. Our results demonstrated the presence of anthocyanins in the black rice variety examined, in particular, cyanidin and peonidin derivatives. The anthocyanins contributed to marked antioxidant activities in scavenging of DPPH free radical *in vitro.* Antioxidant activity in red-hulled rice varieties was mainly due to ferulic acid derivatives. These data suggest that the black rice and red rice varieties examined here have some health benefits associated with the relief of oxidative stress.

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