Structural and Quantitative Analysis of Antioxidant and Low-Density Lipoprotein-Antioxidant Flavonoids from the Grains of Sugary Rice

Dae-Young Lee,¹ Sabina Shrestha,¹ Woo Duck Seo,² Min-Ho Lee,¹ Tae-Sook Jeong,³ Jun Hyun Cho,² You Chun Song,² Hang Won Kang,² Yeong-Deok Rho,¹ and Nam-In Baek¹

¹Department of Oriental Medicinal Materials & Processing, Kyung Hee University, Yongin, Korea.

²Department of Functional Crop, National Institute of Crop Science, Rural Development Administration, Miryang, Korea.

³Korea Research Institute of Bioscience and Biotechnology, Taejeon, Korea.

ABSTRACT Grains of sugary rice were extracted with 80% aqueous methanol, and the concentrated extracts were successively partitioned using ethyl acetate, *n*-butanol, and water. From the *n*-butanol fractions, four flavonoid glycosides were isolated through repeated silica gel, octadecyl silica gel, and Sephadex LH-20 column chromatographies. Based on the nuclear magnetic resonance, mass spectrometry, and infrared spectroscopic data, the chemical structures of the compounds were determined to be taxifolin-7-*O*- β -D-glucopyranoside (**1**), hyperin (**2**), isoquercitrin (**3**), and quercetin gentiobioside (**4**). These compounds were isolated from the grains of sugary rice for the first time. All isolated compounds were tested for antioxidant activity and low-density lipoprotein (LDL)-antioxidative activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and LDL assays. Compound **1** exhibited a strong scavenging effect on DPPH, with a 50% inhibition concentration (IC₅₀) value of 8.1 μ M, and also inhibited LDL oxidation with an IC₅₀ value of 40.0 $\pm$ 20 μ M. A simple and efficient high-performance liquid chromatography/diode array detection method for the simultaneous determination of the four bioactive flavonoids (**1–4**) has been developed and applied to their content determination in the sugary rice. The grains were extracted by 80% methanol, and the contents of **1**, **2**, **3**, and **4** were determined to be 1.12 $\pm$ 0.045, 0.65 $\pm$ 0.011, 0.68 $\pm$ 0.032, and 0.89 $\pm$ 0.021 mg/g, respectively.

KEY WORDS: • antioxidant activity • flavonoid • low-density lipoprotein oxidation • nuclear magnetic resonance • quantitative analysis • sugary rice

INTRODUCTION

DEVELOPMENT OF CROPS with high quality as well as various beneficial functions is necessary to satisfy consumers who are concerned with their health and to boost the competitiveness of agricultural products in the international market.¹ It is expected that the production and distribution of rice with increased physiological functionality could make a great contribution to agricultural industries.² Accordingly, the production of functional rice can contribute to the advancement of agriculture science and technology as well as enhancing the incomes of growers. It is widely recognized that eating habits are highly related to development of diseases such as atherosclerosis, obesity, etc.^{3,4} Consequently, the development of functional rice with increased physiological activity is necessary. Therefore, the Korean National Institute of Crop Science has recently produced a new line of sugary rice (*Dan-mi*) by crossing rice lines such as *Hwayeong-byeo*, *Hwasam-byeo*, and *Seomjin-byeo*.⁵

The health benefits of whole rice have been attributed to its complex carbohydrates, vitamins, minerals, and other phytochemical constituents.⁶ Alternatively, sugary rice is a whole grain with applications in processed food, especially cereal, due to its sucrose and glucose contents and unique taste. However, little is known about the physiological properties and phytochemical components of this new type of rice.⁵ Methanol (MeOH) extracts of common rice grain and rice hull have been used to enhance the stability of ground beef, implying their role as effective antioxidants.⁷ With the exception of sugar content,⁵ the specific antioxidants in sugary rice have not been identified. The primary aims of this study were to evaluate the antioxidant properties of sugary rice and to identify specific compounds responsible for the antioxidant activity. The contents of the active compounds of rice extracts were then correlated with antioxidant activity.

MATERIALS AND METHODS

Plant material

The sugary rice (5 kg) was supplied in January 2009 by the Department of Functional Crop, Korean National Institute of Crop Science, Rural Development Administration in Miryang, Korea (YR55412-7 lines). A voucher specimen

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Address correspondence to: Prof. Nam-In Baek, Department of Oriental Medicinal Materials & Processing, Room 255, Life Sciences Building, Kyung Hee University, Yongin 446-701, Korea, E-mail: nibaek@khu.ac.kr

(number KHU09-0101) was preserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.

Instrumental analyses

Optical rotations were measured on a JASCO (Tokyo, Japan) model P-1010 digital polarimeter. Ultraviolet (UV) spectra were measured on a Shimadzu (Kyoto, Japan) model UV-1601 spectrophotometer. Fast atom bombardment (FAB)-mass spectrometry (MS) spectra were recorded on a JEOL (Tokyo) model JMS-700 spectrometer. Infrared (IR) spectra were run on a Perkin Elmer (Beaconsfield, United Kingdom) Spectrum One FT-IR spectrometer. ^1H -Nuclear magnetic resonance (NMR) (400 MHz) and ^{13}C -NMR (100 MHz) spectra were taken on a Varian (Lake Forest, CA, USA) Unity Inova AS 400 FT-NMR spectrometer.

Extraction and isolation procedure

The dried and powdered rice grains were extracted three times with 80% aqueous MeOH (5 L $\times$ 3) at room temperature. The extracts were successively partitioned with water (1 L), ethyl acetate (EtOAc) (1 L $\times$ 3), and *n*-butanol (*n*-BuOH) (0.8 L $\times$ 3). Then the *n*-BuOH fraction (DMB) (30 g) was applied to silica gel (60A, 70–230 mesh ASTM, Merck, Darmstadt, Germany) column (10 $\times$ 60 cm) chromatography, eluted with CHCl_3 -MeOH- H_2O (by volume, 9:3:1 $\rightarrow$ 8:3:1 $\rightarrow$ 7:3:1 $\rightarrow$ 6:5:35:10, each 3 L), and monitored via thin-layer chromatography (TLC) to provide 21 fractions (DMB-1–DMB-21). Fraction DMB-4 (elution volume/total volume [V_e/V_t], 0.21–0.25; 900 mg) was applied to a silica gel column (3 $\times$ 20 cm) and eluted with CHCl_3 -MeOH- H_2O (12:3:1 by volume, 3 L) to provide 10 subfractions (DMB-4-1–DMB-4-10). Subfraction DMB-4-7 (V_e/V_t , 0.78–0.88; 191 mg) was subjected to octadecyl silica gel (ODS) (Merck) column (2.5 $\times$ 7 cm) chromatography and eluted with MeOH- H_2O (1:2 vol/vol, 1 L) to yield compound **1** (22 mg; TLC [RP-18, F_{254}] ratio of fronts [R_f]=0.40; MeOH- H_2O 1:1 vol/vol). Fraction DMB-5 (V_e/V_t , 0.26–0.39; 5.28 g) was subjected to silica gel column (5 $\times$ 20 cm) chromatography and eluted with CHCl_3 -MeOH (1:1 vol/vol, 3 L) to provide 11 subfractions (DMB-5-1–DMB-5-11), one of which, fraction DMB-5-1 (V_e/V_t , 0.01–0.27; 1.59 g), was subjected to ODS column (3 $\times$ 10 cm) chromatography and eluted with MeOH- H_2O (6:1 vol/vol, 1.5 L) to provide eight subfractions (DMB-5-1-1–DMB-5-1-8). Subfraction DMB-5-1-5 (V_e/V_t , 0.64–0.78; 180 mg) was subjected to ODS column (3 $\times$ 7 cm) chromatography and eluted with MeOH- H_2O (1:2 vol/vol, 1.3 L) to give five subfractions (DMB-5-1-5-1–DMB-5-1-5-1-5), which yielded compounds **2** (18 mg; V_e/V_t , 0.01–0.24; TLC [RP-18 F_{254}] R_f =0.50; MeOH- H_2O 2:1 vol/vol) and **3** (20 mg; V_e/V_t , 0.90–0.99; TLC [RP-18 F_{254}] R_f =0.50; MeOH- H_2O 2:1 vol/vol). Fraction DMB-11 (V_e/V_t , 0.68–0.75; 3.42 g) was separated by silica gel column (5 $\times$ 22 cm) chromatography and eluted with CHCl_3 -MeOH (1:3 vol/vol, 3 L) to provide eight subfractions (DMB-11-1–DMB-11-8). DMB-11-4 (V_e/V_t , 0.33–0.39; 48 mg) was subjected to Sephadex LH-20 (Amersham Pharmacia Biotech,

Uppsala, Sweden) column (2 $\times$ 50 cm; 80% MeOH, 300 mL) chromatography to give compound **4** (25 mg, V_e/V_t , 0.20–0.66; TLC [RP-18, F_{254}] R_f =0.6; MeOH- H_2O 2:3 vol/vol).

Physical properties and ^1H -NMR data of compounds **1–4**

Compound **1** was a brown amorphous powder (MeOH) with the following properties: melting point, 178–180°C; UV (MeOH) λ_{max} =269, 364 nm; $[\alpha]_D^{25}$ =+34.5° (c =0.1, MeOH); IR (KBr) ν_{max} , 3400, 1650, 1570, 1520 cm^{-1} ; positive FAB-MS m/z 467 [$\text{M}+\text{H}$]; ^1H -NMR (400 MHz, CD_3OD , δ), 7.38 (1H, d, J =2.0 Hz, H-2'), 7.08 (1H, dd, J =8.0, 2.0 Hz, H-6'), 6.88 (1H, d, J =8.0 Hz, H-5'), 6.16 (1H, d, J =2.0 Hz, H-8), 6.08 (1H, d, J =2.0 Hz, H-6), 4.97 (1H, d, J =7.8 Hz, H-1''), 4.90 (1H, d, J =11.2 Hz, H-2), 4.46 (1H, d, J =11.2 Hz, H-3), 3.72 (1H, dd, J =12.0, 2.4 Hz, H-6a''), 3.20–3.64 (4H, m, H-2'', 3'', 4'', 5''), 3.52 (1H, dd, J =12.0, 5.6 Hz, H-6b''); ^{13}C -NMR (100 MHz, CD_3OD , δ) (Table 1).

Compound **2** was a yellow powder (MeOH); melting point, 235–238°C; UV (MeOH) λ_{max} =261, 365 nm; $[\alpha]_D^{25}$ =−12.5° (c =0.9, MeOH); IR (KBr) ν_{max} , 3401, 2918, 1646, 1606, 1506 cm^{-1} ; negative FAB-MS m/z 463 [$\text{M}-\text{H}$]; ^1H -NMR (400 MHz, CD_3OD , δ), 7.84 (1H, d, J =2.4 Hz, H-2'), 7.56 (1H, dd, J =2.4, 8.6 Hz, H-6'), 6.84 (1H, d, J =8.6 Hz, H-5'),

TABLE 1. ^{13}C -NUCLEAR MAGNETIC RESONANCE (100 MHz) CHEMICAL SHIFT OF COMPOUNDS **1–4** (IN METHANOL- D_4) ISOLATED FROM GRAINS OF SUGARY RICE

Carbon number	Compound			
	1	2	3	4
2	85.2	158.5	158.1	159.0
3	73.6	135.3	135.4	135.0
4	199.1	179.1	179.1	179.6
5	164.4	162.7	162.7	163.0
6	98.2	99.7	99.7	99.8
7	166.9	165.7	165.7	166.0
8	96.9	94.6	94.6	94.7
9	163.9	158.2	158.2	158.4
10	101.1	105.5	105.4	105.1
1'	129.4	122.8	122.6	122.8
2'	115.8	115.9	115.8	116.2
3'	148.6	145.6	145.5	146.1
4'	148.1	149.7	149.6	149.8
5'	115.8	117.7	117.4	117.4
6'	122.0	122.8	123.0	123.1
1''	103.3	105.3	104.3	104.5
2''	74.5	73.1	75.6	75.6
3''	77.6	75.0	78.2	78.2
4''	71.0	71.1	71.1	71.2
5''	78.1	77.1	78.0	77.7
6''	62.2	61.9	62.5	66.2
1'''				104.1
2'''				73.1
3'''				77.1
4'''				70.0
5'''				77.6
6'''				62.4

6.34 (1H, d, $J=2.0$ Hz, H-8), 6.16 (1H, d, $J=2.0$ Hz, H-6), 5.13 (1H, d, $J=8.0$ Hz, H-1''), 3.85 (dd, $J=3.2, 2.0$ Hz, H-4''), 3.82 (dd, $J=8.0, 7.8$ Hz, H-2''), 3.65 (dd, $J=11.0, 3.2$, H-6''a), 3.56 (dd, $J=11.0, 5.5$ Hz, H-6''b), 3.58 (dd, $J=7.8, 2.0$ Hz, H-3''), δ 3.48 (m, H-5''); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD , δ) (Table 1).

Compound **3** was a yellow powder (MeOH): melting point, 230–232°C; UV (MeOH) λ_{max} , 256, 366 nm; $[\alpha]^{25}_{\text{D}} = -54.2^\circ$ ($c=0.1$, MeOH); IR (KBr) ν_{max} , 3400, 2919, 1656, 1606, 1508 cm^{-1} ; negative FAB-MS m/z 463 $[\text{M}-\text{H}]^-$; $^1\text{H-NMR}$ (400 MHz, CD_3OD , δ), 7.70 (1H, d, $J=2.4$ Hz, H-2'), 7.55 (1H, dd, $J=8.4, 2.4$ Hz, H-6'), 6.85 (1H, d, $J=8.4$ Hz, H-5'), 6.34 (1H, d, $J=2.0$ Hz, H-8), 6.16 (1H, d, $J=2.0$ Hz, H-6), 5.22 (1H, d, $J=7.2$ Hz, H-1''), 3.22–3.51 (4H, m, H-2'', 3'', 4'', 5''), 3.71 (1H, dd, $J=12.0, 2.4$ Hz, H-6a''), 3.58 (1H, dd, $J=12.0, 5.2$ Hz, H-6b''); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD , δ) (Table 1).

Compound **4** was a yellow amorphous powder (MeOH): melting point, 210–212°C; UV (MeOH) λ_{max} , 267, 348 nm; $[\alpha]^{25}_{\text{D}} = -34.0^\circ$ ($c=0.50$, MeOH); IR (CaF₂ window) ν_{max} , 3450, 2944, 1702, 1650, 1616 cm^{-1} ; positive FAB-MS m/z 627 $[\text{M}+\text{H}]^+$; $^1\text{H-NMR}$ (400 MHz, CD_3OD , δ), 7.60 (1H, d, $J=2.4$ Hz, H-2'), 7.50 (1H, d, $J=8.4, 2.4$ Hz, H-6'), 6.88 (1H, d, $J=8.4$, H-5'), 6.37 (1H, d, $J=2.0$ Hz, H-8), 6.19 (1H, d, $J=2.0$ Hz, H-6), 5.36 (1H, d, $J=7.6$ Hz, H-1''), 4.68 (1H, d, $J=7.8$ Hz, H-1'''), 3.18–3.88 (disaccharide moieties, overlapped); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD , δ) (Table 1).

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

Antioxidant activities of isolated compounds were measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical following the method described by Lee *et al.*⁸

Low-density lipoprotein isolation and oxidation assay

Plasma was obtained from fasted healthy normolipidemic volunteers. The low-density lipoprotein (LDL) was isolated by a standard procedure with a slight modification.⁹ The thiobarbituric acid–reactive substances assay of Buege and Aust¹⁰ was used with a slight modification. Consequently, an LDL solution (250 μL , 50–100 μg of protein) in 10 mM phosphate-buffered saline (pH 7.4) was supplemented with 10 μM CuSO₄. The oxidation was performed in a screw-capped 5-mL glass vial at 37°C in a shaking water bath. After a 4-h incubation, the reaction was terminated by addition of 1 mL of 20% trichloroacetic acid. Following precipitation, 1 mL of 0.67% thiobarbituric acid in 0.05 *N* NaOH was added and vortex-mixed, and the final mixture was heated for 5 min at 95°C, cooled on ice, and centrifuged for 2 min at 1000 *g*. The optical density of the malondialdehyde produced was measured at 532 nm. Calibration was completed with a malondialdehyde standard prepared from tetramethoxypropane.

Extraction and high-performance liquid chromatography conditions

The samples were homogenized to powder using a mill and passed through a 40-mesh sieve. Fine powder was

weighed (1 g), extracted in 5 mL of 80% MeOH, and ultrasonically extracted for 1 h at 30°C. The extract was then evaporated under reduced pressure, and the residues were successively partitioned with water (3 mL) and *n*-BuOH (3 mL). The solution was filtered through a syringe filter (pore size, 0.22 μm) and injected directly into the high-performance liquid chromatography (HPLC) system. HPLC was performed on a 1200 RRLC SL+ system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary solvent delivery system and an autosampler. Chromatographic separations were performed on a Zorbax RRHT C₁₈ column (2.7 $\times$ 100 mm i.d.; particle size, 1.5 μm ; Agilent Technologies). The column oven was maintained at 40°C, and the mobile phases consisted of solvent A (0.1% [vol/vol] formic acid in water) and solvent B (0.1% [vol/vol] formic acid in acetonitrile). Optimized liquid chromatography elution conditions were as follows: 0–6 min, 20% B; 6–8 min, 25% B; 8–10 min, 30% B; and 10–15 min, 100% B. The flow rate was 0.5 mL/min. A 5- μL aliquot was injected onto the column using the autosampler.

Calibration of compounds 1–4

Pure flavonoids **1–4** were dissolved in MeOH and diluted into appropriate concentration ranges for the construction of calibration curves. Duplicate injections were made at five concentration levels. The calibration curve of each standard was constructed by plotting the peak area versus injection amount. The amounts of flavonoids **1–4** in the samples were calculated from the corresponding curves.

Limits of detection and quantification

The limit of detection (LOD) values were calculated according to the expression $3.3\sigma/S$, where σ is the SD of the response and S is the slope of the calibration curve. The limit of quantification (LOQ) values were established by using the expression $10\sigma/S$. LOD and LOQ were experimentally verified by injections of flavonoids **1–4** at the LOD and LOQ concentrations.

RESULTS AND DISCUSSION

To search for bioactive compounds in sugary rice, the grains were extracted with 80% MeOH and partitioned using several solvents. When the MeOH extract of sugary rice was developed using silica gel TLC, the spots exhibited not only UV absorbance at 254 and 365 nm but also a yellow color when sprayed with a 10% H₂SO₄ solution and heated, indicating the presence of flavonoids. The MeOH extract was fractionated into an EtOAc layer, an *n*-BuOH layer, and a H₂O layer through solvent fractionation. The repeated silica gel, ODS, and Sephadex LH-20 column chromatographies for the *n*-BuOH fraction produced four flavonoids, compounds **1–4** (Fig. 1). Structural identifications of these compounds were carried out through the interpretation of extensive spectroscopic data and comparison with the data described in the literature.

Compound **1** was isolated as a brown amorphous powder from MeOH and exhibited a pseudomolecular ion peak at

FIG. 1. Chemical structures of compounds 1–4 isolated from grains of sugary rice. Glc, β -D-glucopyranoside; Gal, β -D-galactopyranoside.

m/z 467 $[M+H]^+$ in the positive FAB-MS spectrum. The IR spectrum revealed a bonded OH signal at 3400 cm^{-1} and an aromatic group signal at 1650 cm^{-1} . The ^1H -NMR and ^{13}C -NMR spectra of compound 1 disclosed resonances for two oxygenated methine protons [δ 4.90 (d, $J=11.2\text{ Hz}$, H-2), δ 4.46 (d, $J=11.2\text{ Hz}$, H-3)], and 21 carbons were characteristic of the presence of a taxifolin [δ 85.2 (C-2), δ 73.6 (C-3)] and a monosaccharide moiety (see Table 1), which led to its identification as taxifolin-7- O - β -D-glucopyranoside. This was confirmed by comparison of spectrometry data with those of an authentic sample and the values reported in the literature.¹¹

Compound 3 was isolated as a yellow powder and exhibited a pseudomolecular ion peak $[M-H]^-$ at m/z 463 in the negative FAB-MS spectrum. IR absorption bands at 3400 cm^{-1} and 1656 cm^{-1} were characteristic of hydroxyl and aromatic groups, respectively. The NMR spectra of compound 3 were almost the same as those of compound 1 except for two oxygenated olefin quaternary carbons (δ 158.1, 135.4) of the C-ring at the 2 and 3 positions. Accordingly, compound 3 was suggested to be an isoquercitrin, a well-known flavonol glycoside. This was confirmed by comparison of the spectroscopic data including IR and MS spectra and ^1H - and ^{13}C -NMR data with those of an authentic sample and values reported in the literature.¹²

Compound 2 was isolated as a yellow powder and exhibited a pseudomolecular ion peak $[M-H]^-$ at m/z 463 in the negative FAB-MS spectrum. IR absorption bands at 3401 cm^{-1} and 1646 cm^{-1} were characteristics of hydroxyl and aromatic groups, respectively. The NMR spectra of compound 2 were almost the same as those of compound 3 with the exception of a monosaccharide moiety. ^{13}C -NMR signals of a sugar moiety were observed at δ 105.3 (C-1''), δ 77.1 (C-5''), δ 75.0 (C-3''), δ 73.1 (C-2''), δ 71.1 (C-4''), and δ 61.9 (C-6''), suggesting the presence of a galactopyranoside. Thus, the structure of compound 2 was identified as quercetin-3- O - β -D-galactopyranoside, hyperin.¹³

Compound 4 was obtained as a yellow amorphous powder and showed a pseudomolecular ion peak $[M+H]^+$ at m/z 627 in the positive FAB-MS spectrum. The IR spectrum showed the bonded OH signal at 3450 cm^{-1} and the aromatic group signal at 1616 and 1650 cm^{-1} . The ^1H -NMR spectrum signals clearly indicated the signals due to a 1',3',4'-

trisubstitution of ring B [δ 7.60 (d, $J=2.4\text{ Hz}$), δ 7.50 (dd, $J=8.4, 2.4\text{ Hz}$), δ 6.88 (d, $J=8.4\text{ Hz}$)], and a typical 5,7-disubstituted A-ring [δ 6.37 (d, $J=2.0\text{ Hz}$, H-8), δ 6.19 (d, $J=2.0\text{ Hz}$, H-6)] on the flavonol skeleton. Also, two hemiacetal protons appeared at 5.36 (d, $J=7.6\text{ Hz}$, H-1'') and 4.68 (d, $J=7.8\text{ Hz}$, H-1'''), and there were oxygenated methine and methylene proton signals indicating the presence of two sugar moieties having β -configurations of the anomer carbons. The chemical shift on the ^{13}C -NMR spectrum and multiplicity of each carbon (distortionless enhancement by polarization transfer) indicated 27 carbon signals and the presence of a quercetin moiety with a disaccharide moiety (see Table 1). In the outer glycoside moiety, an anomeric carbon signal at δ_C 104.1 (C-1''') and oxygenated methine and methylene carbon signals such as δ_C 77.1 (C-3'''), 77.6 (C-5'''), 73.1 (C-2'''), 70.0 (C-4'''), and 62.4 (C-6''') suggested the presence of a β -glucopyranosyl unit. In addition, an inner glycoside moiety, an anomeric carbon signal at δ_C

FIG. 2. 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activities of compounds 1–4 and the reference compound butylated hydroxyanisole (BHA).

FIG. 3. Inhibitory activities of compound **1** and the reference compound butylated hydroxytoluene (BHT) on low-density lipoprotein oxidation in the thiobarbituric acid-reactive substances assay.

104.5 (C-1''), and oxygenated methine and methylene carbon signals such as δ_C 78.2 (C-3''), 77.7 (C-5''), 75.6 (C-2''), 71.2 (C-4''), and 66.2 (C-6'') were similar those of a β -glucopyranosyl group with the exception of the chemical shift of the oxygenated methylene signal (C-6''). The ob-

servation was confirmed by the downfield shifts of the carbon (δ_C 66.2) signal owing to the glycosidation effect. In the heteronuclear multiple-bond correlation spectrum, the anomeric proton signal of the outer glucopyranosyl moiety at 4.68 (1H, d, $J=7.8$ Hz, H-1'') showed correlations with an oxygenated methylene carbon signal of the inner glucopyranosyl moiety at δ_C 66.2 (C-6''), confirming the disaccharide to be a β -gentiobioside, β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranose. The connection between the glucopyranosyl unit (C-1'') and the C-3 of the aglycone was verified by the cross-peak between the anomeric proton signals at δ 5.36 (H-1'') and the oxygenated olefin quaternary carbon signal at δ_C 135.0 (C-3) in the heteronuclear multiple-bond correlation spectrum. Compound **4** was thus identified as quercetin-3-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-glucopyranose, quercetin gentiobioside, and was confirmed by comparison of spectroscopic data with those in the literature.¹⁴ All of these flavonoids were isolated from the grains of sugary rice for the first time (Fig. 1).

The radical scavenging activities of flavonoid compounds **1–4** on the DPPH radical, which was tested for decolorizing activity following the trapping of the unpaired electron of DPPH, were examined. All compounds showed potent antioxidant activities (**1**, 50% inhibition concentration [IC₅₀]=8.1 μ M; **2**, IC₅₀=44.1 μ M; **3**, IC₅₀=43.8 μ M; **4**, IC₅₀=48.2 μ M). As shown in Figure 2, all compounds and butylated hydroxyanisole (IC₅₀=21.1 μ M), which was used as a positive control, were assessed at dose-dependent inhibitory concentrations in the DPPH assays. The DPPH

FIG. 4. High-performance liquid chromatography/diode array detection (DAD) analysis of grains of sugary rice: (A) high-performance liquid chromatography separation of a standard mixture of compounds **1–4** isolated from grains of sugary rice and (B) high-performance liquid chromatogram of the *n*-butanol fraction of grains of sugary rice at 347 nm. For peak identification, see Figure 1. Sig, signal; STD, standard.

TABLE 2. CALIBRATION CURVE AND LIMITS OF DETECTION AND QUANTIFICATION DATA OF COMPOUNDS 1–4 BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/DIODE ARRAY DETECTION

Compound	Calibration curve ^a	r ²	Concentration (μg/mL)		
			Linear range	LOD	LOQ
1	y = 9.1813x + 0.0054	0.9999	10.00–300.00	5.28	15.99
2	y = 3.4759x + 0.0120	0.9998	5.00–150.00	3.72	11.27
3	y = 3.6734x + 0.0766	0.9999	5.00–150.00	3.78	11.46
4	y = 5.5175x + 0.4844	0.9999	10.00–300.00	4.09	12.40

^ay, peak area; x, concentration of the standard (in μg/mL).

LOD, limit of detection; LOQ, limit of quantification.

radical scavenging activity of compound **1** was significantly higher than those of the other quercetin glycosides, **2–4**. The antioxidative potential of the aglycone moiety having a free hydroxyl group at the C-3 position was greater than that with C-3-substituted derivatives. This result indicates that the free hydroxyl group at C-3 of flavanol is one of the key factors for the free radical scavenging activity of this molecule. The quercetin-3-*O*-glycosides showed DPPH radical scavenging activities similar to one another. It is likely that DPPH radical scavenging activity does not depend on the kind of sugar linked to the C-3 of the flavanol. For example, Rice-Evans *et al.*¹⁵ reported that glycosylation of flavonoids reduces their antioxidant activities when they are compared with the corresponding aglycones. Furthermore, the DPPH radical scavenging activity of compound **4** was also nearly similar to those of compounds **2** and **3**, although they differ in being mono- and disaccharide.

The inhibitory activity of compound **1** on LDL oxidation (IC₅₀ = 40.0 ± 2.0 μM) was inhibited in a dose-dependent manner and had equivalent potency with the positive control, butylated hydroxytoluene (IC₅₀ = 2.5 ± 0.1 μM), a well-known inhibitor, whereas the other flavonoids did not show any observable activity up to 100 μM (Fig. 3). LDL oxidation has been proposed as an important step in the formation of atherosclerotic lesions.¹⁶ Evidence to support this hypothesis is based in part on observational studies that have demonstrated associations between oxidized LDL cholesterol and the presence of atherosclerotic lesions¹⁷ and the progression of carotid artery atherosclerosis.¹⁸

The contents of the flavonoids of rice extracts were then correlated to antioxidant activity. For this reason, quantitative analysis of flavonoids **1–4** from sugary rice extracts was determined using HPLC/diode array detection analysis. The chromatographic conditions were optimized with the aim of obtaining chromatograms with a good resolution of adjacent peaks within a short analysis time (Fig. 4). The results of the calibration curves were determined on the basis of the peak areas in the HPLC/diode array detection chromatogram. The calibration curves showed excellent linearity covering the tested range described above with correlation coefficients of 0.9998 for all four compounds. All calibration data, as well as LOD and LOQ, are presented in Table 2. The LODs of the four flavonoids extract from sugary rice ranged from 3.72 to 5.28 μg/mL. The LOQs of the four flavonoid extracts from

sugary rice ranged from 11.27 to 15.99 μg/mL. It was determined that the contents of bioactive flavonoids **1–4** in the MeOH extract were 1.12 ± 0.04, 0.65 ± 0.01, 0.68 ± 0.03, and 0.89 ± 0.02 mg/g (wt/wt), respectively. Furthermore, the content of taxifolin-7-*O*-β-D-glucopyranoside (**1**) with our extraction and determination method was the most abundant compared with those of the other three flavonoids. In view of the contents and antioxidant activities of flavonoids, sugary rice extracts could afford health benefits by preventing unwanted free radical-induced oxidative reactions and atherosclerosis.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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