*Food Sci. Biotechnol. Vol. 15, No. 3, pp. 392 ~ 398 (2006)*

Food Science and Biotechnology



# Isolation and Characterization of Phytochemical Constituents from Soybean (*Glycine max* L. Merr.)

**Jin Hwan Lee, In-Youl Baek, Nam Suk Kang, Jong Min Ko, Won-Young Han, Hyun-Tae Kim, Ki-Won Oh, Duck-Yong Suh, Tae Joung Ha*, and Ki Hun Park[1]***

*Yeongnam Agricultural Research Institute, National Institute of Crop Science, RDA, Milyang, Gyeongnam 627-803, Korea*
*[1]Division of Applied Life Science (BK21 program), Department of Agricultural Chemistry, Research Institute of Life Science, Gyeongsang National University, Jinju, Gyeongnam 660-701, Korea*

**Abstract** Four flavonoids **1-4** and one phytosterol **5** were isolated from methanol extract of Taekwangkong, one of the soybean cultivars, and the structures of these compounds were fully characterized by physical and spectral analysis. The content of compounds **1-4** as determined by $C_{18}$ reversed phase HPLC (high-performance liquid chromatography) coupled with diode-array detector were 12.1, 624.6, 18.0, and 219.6 μg/g, respectively, and the total phenolic content of this cultivar was measured as 3.7 mg gallic acid equivalent per g dry material (GAE/g). Also, compound **1** showed strong radical scavenging activity in the 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay ($IC_{50}$ = 47.6 μM), five-fold higher than seen in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. These results lead to the conclusion that soybean not only has many phytoestrogens but also has potent antioxidant activity.

**Key words:** phytoestrogen, *Glycine max,* Taekwangkong, HPLC, total phenolic content, ABTS

## Introduction

Phytoestrogens are compounds naturally present in many foods of plant origin and include isoflavones, lignans, dihydrochalcones, and coumestans (1-3). Many studies and reviews have been reported concerning the health properties of phytoestrogens as hormone substitutes with respect to estradiol (4-6). Following the consumption of phytoestrogens, heterocyclic phenols are formed which have a stereochemical structure similar to estrogen, and have the capacity to bind to estrogen receptors (7). Although there are many valuable phytoestrogens found in natural sources and fermented beverages (8-11), Soybean (*Glycine max* L. Merr.) and soy products have important secondary metabolic compounds and phytoestrogens as well as potential beneficial effects on various chronic diseases such as cancer, coronary heart disease, osteoporosis, and menopausal discomfort (12-15). For these reasons and the popularity of soybean cultivars in Korea, research into the identification and determination of phytoestrogens using HPLC from this species is continuing. Also the isolated phytoestrogen exhibits potent antioxidant properties towards 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

Antioxidants are used widely in the production of foods, drugs, and cosmetics (16, 17). They help to maintain the quality of many food products by delaying or inhibiting the oxidation of molecules including lipids by inhibiting the initiation or propagation of oxidative chain reactions (18). The occurrence of oxidation in living organisms is known to cause damage to proteins, lipids, and DNA, resulting in cancer, heart disease, macular degeneration, and accelerated aging (19-21).

In this study, we report the isolation of five phytochemicals including 4′,5,7-trihydroxyisoflavone (**1**), 4′,5,7-trihydroxyisoflavone-7-*O*-β-D-glucopyranoside (**2**), 4′,7-dihydroxyisoflavone (**3**), 4′,7-dihydroxyisoflavone-7-*O*-β-D-glucopyranoside (**4**), and β-sitosterol (**5**) from soybean and their structures as determined by spectroscopic methods. Furthermore, we provide the first reported isolation of compounds **1-4** as characterized by $C_{18}$ reverse phase HPLC as well as the total phenolic contents from this cultivar. We also demonstrate selective ABTS radical scavenging activity compared with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in compound **1** among the isolated soybean compounds.

## Materials and Methods

**Plant material** Taekwangkong (*G. max*) was collected during the period of October 12-15, 2004, in the experimental field of the Yeongnam Agricultural Research Institute, National Institute of Crop Science, Rural Development Administration, Milyang, Korea.

**Reagents** Gallic acid, Folin-Ciocalteau's phenol reagent, ABTS, DPPH, sodium persulfate, butylated hydroxyl anisol (BHA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade water and acetonitrile (EMD Chemicals, Darmstadt, Germany) were used. Standard samples for the calibration curve were obtained from Taekwangkong by isolation and identification.

*Corresponding author:
Ki Hun Park, Tel: +82-55-751-5472; Fax: +82-55-757-0178, E-mail: khpark@gsnu.ac.kr
Tae Joung Ha, Tel: +82-55-350-1239; Fax: +82-55-352-3059, E-mail: taejoung@rda.go.kr
Received January 31, 2006; accepted April 21, 2006
Both corresponding authors contributed equally to the work.

**Instruments** Melting points were measured on a Thomas Scientific capillary melting point apparatus (Electrothermal 9300; London, UK) and were uncorrected. IR spectra were recorded on a Bruker IFS66 (Bruker, Karlsruhe, Germany) infrared Fourier transform spectrophotometer and UV spectra were measured on a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). $^{1}$H- and $^{13}$C-NMR along with 2D-NMR data were obtained on a Bruker AM 500 ($^{1}$H-NMR at 500 MHz, $^{13}$C-NMR at 125 MHz) spectrometer (Bruker) in $CDCl_3$, and DMSO-$d_6$ (Sigma-Aldrich Co). TLC was conducted on precoated Kieselgel $60F_{254}$ plates (E. Merck Co., Darmstadt, Germany) and the spots were detected either by examining the plates under a UV lamp or by treating the plates with a 10% ethanolic solution of phosphomolybdic acid (Wako Pure Chemical Industries, Osaka, Japan) followed by heating at 110°C. HPLC was performed using an Agilent 1100 series (Boeblingen, Germany) quaternary pump (flow speed: 1 mL/min), Agilent 1100 series diode-array detector (260 nm), and LichroCART 125-4 HPLC-Cartridge (Lichrophore 100 RP-18e; Merck KGaA, Darmstadt, Germany) column. EIMS (electron imfact mass spectroscopy) were obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). All the reagent grade chemicals were purchased from sigma (Sigma Chemical Co).

**Extraction and isolation** Taekwangkong (8.0 kg) were pulverized and extracted with MeOH (2 L × 5) at room temperature for 7 days. Filtration and concentration gave the resultant brown extract (1.6 kg), which was suspended in $H_2O$ and then partitioned with *n*-hexane, EtOAc, and BuOH to give *n*-hexane- (600 g), EtOAc- (5.1 g), and BuOH-extractable (8.6 g) residues. The EtOAc extract (5.1 g) was chromatographed over silica gel using *n*-hexane:acetone and $CH_2Cl_2$:acetone gradient to give 25 fractions (F1-F25). The F10 (0.8 g) was applied to a silica gel column chromatography with *n*-hexane:acetone (20:1 → 5:1) to afford 40 subfractions (20-28) which were subjected to a silica gel chromatography (*n*-hexane:EtOAc =14:1→5:1) and purified by recrystallization (*n*-hexane: EtOAc) to yield β-sitosterol **5** (45 mg). The F21 (1.4 g) was chromatographed over a silica gel as stationary phase using *n*-hexane:acetone gradient (20:1 → 1:1) as mobile phase to afford 75 subfractions. Subfractions 50-58 were pooled and rechromatographed on silica gel with a *n*-hexane: acetone gradient (8:1 → 1:1) to give compound **1** (9 mg). Subfractions 60-68 were evaporated and submitted to preparative TLC [*n*-hexane:acetone (2:1)] to give compound **3** (11 mg). The BuOH phase was chromato-graphed on silica gel using $CHCl_3$: MeOH mixture to give 9 fractions (1-9). Fraction 6 (940 mg) was repeatedly chromatographed over silica gel using $CHCl_3$:MeOH to give compound **2** (155 mg) and fraction 7 (640 mg) was separately subjected to silica gel column chromatography with the same solvent and further purified through Sephadex LH-20 and eluted with methanol to yield compound **4** (69 mg).

**HPLC apparatus and measurements** The pulverized Taekwangkong (1.0 g) were extracted with 20 mL MeOH overnight in a vortex mixer at room temperature to form the final extract which was then centrifuged. The extracts used for HPLC analysis were passed through a 0.45-μm filter (Millipore, MSI, Westboro, MA, USA) before injection into a reverse phase LichroCART 125-4 HPLC-Cartridge (5 μm, Merck KGaA) and a 10 μL portion of these solutions was injected into the HPLC system (Agilent 1100 series). The mobile phase was water containing 0.1% acetic acid (A) and acetonitrile (B). The gradient was as follows: 0 min, 10% B; 20 min, 20% B; 30 min, 25% B; 40 min, 35% B; 50 min, 40% B; 60 min, 45% B; 70 min, 50% B and then held for 10 min before returning to the initial conditions. The flow rate was 1.0 mL/min and the wavelengths of detection were set at 260 nm.

**Calibration curve preparation and quantification of compounds 1-4** About 10 mg of each isolated compound (**1-4**) was accurately weighed and dissolved in a 10 mL volumetric flask in MeOH to obtain stock solutions. For calibration curves, the stock solution was diluted with MeOH to obtain the concentration sequence. The linear range and the equations of linear regression were obtained through a sequence of 50, 25, 12.5, and 6.25 μg/mL. Mean areas generated from the standard solutions were plotted against concentration to establish calibration equations.

**Total phenolic contents** Total phenolic contents were measured according to the modified Folin-Ciocalteau colorimetric method (22, 23). Briefly, sample (1.0 mL) was mixed with Folin and Ciocalteu's phenol reagent (1.0 mL). After 5 min, 2 mL of 2% $Na_2CO_3$ solution was added to the mixture, and the volume brought up to 10 mL by adding distilled water. After the reaction mixture was kept in the dark for 2 hr, absorbance was measured at 724 nm. A calibration curve was constructed with different concentrations of gallic acid as a standard. Total phenolic contents were determined as mg of gallic acid equivalents (mg GAE/g extract).

**Measurement of Trolox equivalent antioxidant capacity (TEAC)** TEAC assay is based on the relative ability of antioxidants to scavenge the radical cation $ABTS^{\bullet+}$ in comparison to a standard (Trolox) (24, 25). The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The reaction mixture was maintained for 4-8 hr until the mixture was complete and the absorbance stable. $ABTS^{\bullet+}$ solution was diluted with ethanol and the absorbance was read at 734 nm. For the photometric assay, 0.9 mL $ABTS^{\bullet+}$ solution and 0.1 mL of test compound was mixed for 45 sec and the absorbance measured immediately after 1 min at 734 nm. Antioxidant activity of each compound was calculated by determining the decrease in absorbance at different concentrations using the following equation: E = [Ao-Ae/Ao]×100, where Ae and Ao were absorbances of samples with and without isoflavones, respectively. Antioxidant activity was expressed as TEAC values. TEAC values, which express the μM of Trolox having the antioxidant capacity corresponding to 1.0 μM of the test substance.

**Measurement of scavenging activity of the DPPH radical** Antioxidant activities of the isolated compounds

were measured on the basis of the scavenging activity of the stable DPPH free radical following the method described by Braca *et al.* (26). Various concentrations of the compounds were added to a concentration of 0.15 mM in EtOH, and the mixture was shaken vigorously. Absorbance at 517 nm was determined after 30 min, and the radical scavenging effect was calculated as [Ao-Ae/Ao]×100, where Ae and Ao were the absorbance of samples with and without isolated compounds, respectively.

**Data analysis and curve fitting** Each assay was conducted in triplicate in separate experiments. The data analysis was performed by using Sigma Plot 2001. The inhibitory concentration leading to 50% activity loss ($IC_{50}$) was obtained by fitting experimental data to the logistic curve by the equation as follows (27) where [I] is the inhibitor concentration of the remaining free radical.

Activity (%) = $100[1/(1 + ([I]/ IC_{50})]$

## Results and Discussion

The pulverized Taekwangkong (8.0 kg) was extracted with MeOH. After filtration and concentration the resulting extracts were suspended in $H_2O$ and successively partitioned with *n*-hexane, EtOAc, and BuOH, which yielded *n*-hexane-, EtOAc-, and *n*-BuOH-extractable residues. Through various chromatographic purifications of EtOAc and *n*-BuOH fraction, five compounds (**1-5**) were isolated and analyzed by physical and spectral data to determine their structures (Fig. 1).

**Compound (1)**: amorphous yellow powder; mp 297-299 °C; EIMS *m/z* 180; IR (KBr) $\nu_{max}$ 3414, 1653, 1570/cm; UV $_{max}$ nm 305, 262 (MeOH); $^1$H NMR (500 MHz, DMSO-$d_6$) δ 6.12 (1H, d, *J* = 2.1 Hz, H-6), 6.28 (1H, d, *J* = 2.1 Hz, H-8), 6.99 (2H, dd, *J* = 6.7, 1.8 Hz, H-3′ and H-5′), 7.28 (2H, dd, *J* = 6.7, 1.8 Hz, H-2′ and H-6′), 8.20 (1H, s, H-2), and 12.8 (1H, s, 5-OH). $^{13}$C NMR (125 MHz, DMSO-$d_6$): see Table 1.

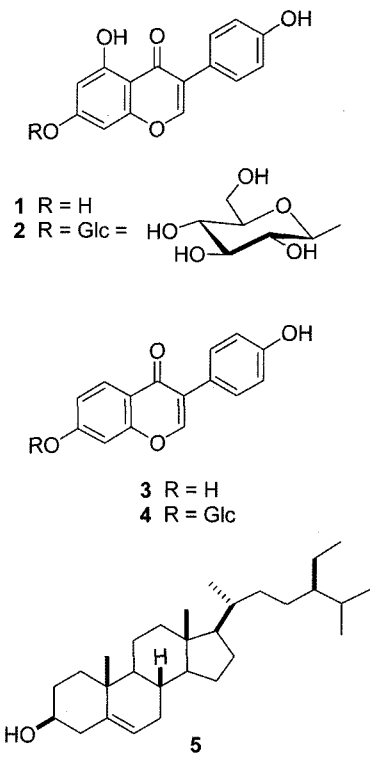


**Fig. 1. Structures of isolated compounds 1-5.**

**Compound (2)**: slightly yellow powder; mp 255-257°C; EIMS *m/z* 432; $[\alpha]_{20}^{D}$ -25.7 (*c* 0.8, MeOH); IR (KBr) $\nu_{max}$ 3340, 1655/cm; UV $\lambda_{max}$ nm 338, 272 (MeOH); $^1$H NMR (500 MHz, DMSO-$d_6$) δ 3.19 (1H, dd, *J* = 9.9, 4.8 Hz, H-4), 3.29 (1H, dd, *J* = 13.4, 7.4 Hz, H-2), 3.32 (1H, dd, *J* = 13.4, 4.8 Hz, H-3″), 3.45 (1H, m, H-5″), 3.48 (1H, dd, *J* = 11.4, 5.5 Hz, H-6″β), 3.73 (1H, dd, *J* = 11.4, 4.8 Hz, H-6″α), 5.06 (1H, d, *J* = 7.4 Hz, H-1″), 6.49 (1H, d, *J* = 2.1 Hz, H-6), 6.72 (1H, d, *J* = 2.1 Hz, H-8), 6.84 (2H, d, *J* = 6.6 Hz, H-3′ and H-5′), 7.41 (2H, d, *J* = 6.6 Hz, H-2′ and H-6′), 8.41 (1H, s, H-2), 9.53 (1H, s, 4′-OH), and 12.9 (1H, s, 5-OH). $^{13}$C NMR (125 MHz, DMSO-$d_6$): see Table 1.

**Compound (3)**: yellow needles; mp 317-320°C; EIMS *m/z* 254; IR (KBr) $\nu_{max}$ 3412, 1645/cm; UV $\lambda_{max}$ nm 303, 259, 249, 238 (MeOH); $^1$H NMR (500 MHz, DMSO-$d_6$) δ 6.85 (1H, d, *J* = 2.1 Hz, H-8), 6.93 (1H, dd, *J* = 8.8, 2.1Hz, H-6), 6.99 (2H, d, *J* = 8.6 Hz, H-3′ and H-5′), 7.38 (2H, d, *J* = 8.6 Hz, H-2′ and H-6′), 7.96 (1H, d, *J* = 8.8 Hz, H-5), and 8.28 (1H, s, H-2). $^{13}$C NMR (125 MHz, DMSO-$d_6$): see Table 1.

**Compound (4)**: slightly yellow powder; mp 230-232°C; EIMS *m/z* 416; $[\alpha]_{D}^{20}$ -35.4 (*c* 0.6, MeOH); IR (KBr) $\nu_{max}$ 3420, 1635/cm; UV $\lambda_{max}$ nm 330, 280 (MeOH); $^1$H NMR (500 MHz, DMSO-$d_6$) 3.30 (2H, m, H-4″ and H-2″), 3.47 (3H, m, H-3″, H-5″, and H-6″β), 3.73 (1H, dd, *J* = 14.2, 7.4 Hz, H-6″α), 5.12 (1H, m, H-1″), 6.82 (2H, d, *J* = 8.6 Hz, H-3′ and H-5′), 7.15 (1H, dd, *J* = 8.6, 2.1 Hz, H-6′), 7.24 (1H, d, *J* = 2.1 Hz, H-8), 7.41 (2H, d, *J* = 8.6 Hz, H-2′ and H-6′), 8.04 (1H, d, *J* = 8.9 Hz, H-5), 8.39 (1H, s, H-2), and 9.56 (1H, s, 4′-OH). $^{13}$C NMR (125 MHz, DMSO-$d_6$): see Table 1.

**Compound (5)**: whiter powder; mp 140-142°C; EIMS *m/z* 414; $[\alpha]_{D}^{20}$ -3.9 (*c* 0.62, $CHCl_3$); IR (KBr) $\nu_{max}$ 3400, 1640/cm; $^1$H NMR (500 MHz, $CDCl_3$) δ 0.61 (3H, s, H-18), 0.75 (3H, d, *J* = 6.7 Hz, H-26), 0.76 (3H, d, *J* = 7.0 Hz, H-27), 0.77 (3H, t, *J* = 9.3 Hz, H-29), 0.83-0.89 (1H, m, H-24), 0.83-0.86 (1H, m, H-9), 0.85 (3H, d, *J* = 6.4 Hz, H-21), 0.89-0.93 (1H, m, H-14), 0.94 (3H, s, H-19), 0.91-1.01 (1H, m, H-15α), 0.95-1.05 (1H, m, H-1α), 0.99-1.08 (1H, m, H-17), 1.06-1.12 (2H, m, H-23), 1.06-1.12 (1H, m, H-12α), 1.10 (2H, m, H-22), 1.15-1.21 (2H, m, H-28), 1.16-1.19 (1H, m, H-16α), 1.18 (1H, m, H-25), 1.24-1.30 (1H, m, H-20), 1.37-1.41 (1H, m, H-8), 1.37-1.44 (2H, m, H-11), 1.41-1.47 (1H, m, H-7α), 1.48-1.53 (1H, m, H-15β), 1.75-1.81 (1H, m, H-1β), 1.75-1.79 (2H, m, H-2), 1.77 (1H, m, H-16β), 1.84-1.90 (1H, m, H-7β), 1.88-1.95 (1H, m, H-12β), 2.17 (1H, ddd, *J* = 11.4, 4.9, 2.0 Hz, H-4α), 2.21 (1H, ddd, *J* = 13.1, 4.9, 2.0 Hz, H-4β), 3.41-3.45 (1H, m, H-3), 5.28 (1H, dd, *J* = 2.6, 5.2 Hz, H-6), $^{13}$C NMR (125 MHz, $CDCl_3$): d 12.3 (C-18), 12.4 (C-29), 19.2 (C-21), 19.4 (C-26), 19.8 (C-19), 20.2 (C-27), 21.5 (C-11), 23.5 (C-28), 24.7 (C-15), 26.5 (C-23), 28.6 (C-16), 29.6 (C-25), 32.1 (C-2), 32.3 (C-8/7), 34.4 (C-22), 36.5 (C-20), 36.9 (C-10), 37.7 (C-1), 40.2 (C-12), 42.7 (C-4/13), 46.3

**Table 1.** $^{13}$C-NMR of compounds 1-4 at 125 MHz (ppm, m)[1)]

| Position | Compounds | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 1 | | | | |
| 2 | 154.3 (d) | 154.9 (d) | 153.2 (d) | 153.8 (d) |
| 3 | 122.7 (s) | 121.4 (s) | 123.9 (s) | 122.8 (s) |
| 4 | 180.6 (s) | 180.9 (s) | 175.1 (s) | 175.2 (s) |
| 4a | 104.8 (s) | 106.5 (s) | 117.0 (s) | 118.9 (s) |
| 5 | 162.4 (s) | 162.0 (s) | 127.7 (d) | 127.4 (d) |
| 6 | 99.3 (d) | 100.0 (d) | 115.6 (d) | 116.0 (d) |
| 7 | 164.7 (s) | 163.4 (s) | 163.1 (s) | 161.9 (s) |
| 8 | 94.0 (d) | 95.0 (d) | 102.5 (d) | 100.5 (d) |
| 8a | 158.0 (s) | 157.9 (s) | 157.9 (s) | 157.7 (s) |
| 1′ | 121.6 (s) | 123.0 (s) | 123.0 (s) | 124.2 (s) |
| 2′and 6′ | 130.5 (d) | 130.5 (d) | 130.5 (d) | 130.6 (d) |
| 4′ | 157.8 (s) | 157.6 (s) | 157.6 (s) | 157.5 (s) |
| 3′and 5′ | 115.4 (d) | 115.5 (d) | 115.4 (d) | 115.6 (d) |
| 1″ | | 100.4 (d) | | 104.5 (d) |
| 2″ | | 73.6 (d) | | 73.6 (d) |
| 3″ | | 76.9 (d) | | 76.9 (d) |
| 4″ | | 70.1 (d) | | 70.1 (d) |
| 5″ | | 77.6 (d) | | 77.7 (d) |
| 6″ | | 61.1 (t) | | 61.1 (t) |

[1)]Chemical shifts of compounds **1-4** were determined in DMSO-$d_6$.

(C-24), 50.6 (C-9), 56.5 (C-17), 57.2 (C-14), 77.1 (C-3), 122.1 (C-6), and 141.2 (C-5).

**Identification of isolated compounds** Structural identification of five compounds was carried out by the interpretation of several spectral data. Isolated compounds were readily identified as 4′,5,7-trihydroxyisoflavone (**1**), 4′,5,7-trihydroxyisoflavone-7-*O*-β-D-glucopyranoside (**2**), 4′,7-dihydroxyiso-flavone (**3**), 4′,7-dihydroxyisoflavone-7-*O*-β-D-glucopyranoside (**4**), and β-sitosterol (**5**). Compound **1** was obtained as a yellow powder and in the EIMS, the molecular ion peak showed at *m/z* 270. UV spectrum analysis showed an absorption maximum at 305 nm and IR spectrum analysis showed strong hydroxyl and carbonyl group absorption bands at 3414 and 1653/cm respectively. The $^1$H-NMR spectrum of compound **1** showed signals for two symmetric protons of the B-ring [δ 7.28 (2H, dd, $J$ = 6.7, 1.8 Hz, H-2′ and H-6′), 6.99 (2H, dd, $J$ = 6.7, 1.8 Hz, H-3′ and H-5′)], two aromatic protons of the A-ring [δ 6.12 (1H, d, $J$ = 2.1 Hz, H-6), 6.28 (1H, d, $J$ = 2.1 Hz, H-8)], and one olefinic proton [δ 8.20 (1H, s, H-2)]. Also, a characteristic hydrogen bonded proton signal of the hydroxyl group was detected at δ 12.8 (1H, s, C-5-OH). Hence, this inferred that compound **1** has an isoflavone skeleton. The HMBC (heteronuclear multiple bond connectivity spectroscopy) spectrum of **1** showed correlations of H-2 with C-3, C-1′, C-4, and C-8a, and H-2′/6′ with C-3′, C-1′, C-4′, and H-6 with C-4a, and H-8 with C-4a (Fig. 2). Thus, based on all the above obtained spectral data, compound **1** was identified as 4′,5,7-trihydroxyisoflavone. Compound **2** is a slightly yellow powder and its EIMS showed a major ion peak at *m/z* 432. UV spectrum analysis showed an absorption maximum at 338 nm and IR spectrum analysis showed strong hydroxyl and carbonyl group absorption bands at 3340 and 1655/cm, respectively. The $^1$H-NMR spectrum of compound **2** were almost the same as those for **1**, except for the sugar moiety. Sugar conformation was assumed to be β-configuration form from the chemical shift and coupling constant value of this anomeric proton signal at 5.06 (1H, d, $J$ = 7.4 Hz, H-1). The signal at δ 100.4, 77.6, 76.9, 73.6, 70.1, and 61.1 in the $^{13}$C-NMR spectrum, and 3.19 (1H, dd, $J$ = 4.8, 9.9 Hz, H-4), 3.29 (1H, dd, $J$ = 7.4, 13.4 Hz, H-2), 3.32 (1H, dd, $J$ = 4.8, 13.4 Hz, H-3), 3.45 (1H, m, H-5) and 5.06 (1H, d, $J$ = 7.4 Hz, H-1″) in the $^1$H spectrum suggested the presence of a β-glucopyranose group. All data mentioned above indicate that the structure of compound **2** is 4′,5,7-trihydroxyisoflavone-7-*O*-β-D-glucopyranoside. The characteristics of compounds **3** and **4** were essentially the same as those for **1** and **2** except for the $^1$H spectrums [**3**: 6.93 (1H, dd, $J$ = 8.8, 2.1Hz, H-6), 7.96 (1H, d, $J$ = 8.8 Hz, H-5) **4**: 7.15 (1H, dd, $J$ = 8.6, 2.1 Hz, H-6), 8.04 (1H, d, $J$ = 8.9 Hz, H-5)] and $^{13}$C spectrums [**3**: 127.7 (C-5), 115.6 (C-6), **4**: 127.4 (C-5), 116 (C-6)]. These results suggested that compounds **3** and **4** are 4′,7-dihydroxyisoflavone and 4′,7-dihydroxyisoflavone-7-*O*-β-D-glucopyranoside, respectively. Compound **5** was obtained as a white powder and had a molecular ion peak at *m/z* 414. IR spectrum analysis showed peak absorption bands of 3400 and 1640/cm. The $^1$H-NMR spectrum showed evidence for six methyl protons [δ 0.61 (3H, s, H-18), 0.75 (3H, d, $J$ = 6.7 Hz, H-26), 0.76 (3H, d, $J$ = 7.0 Hz, H-27), 0.77 (3H, t, $J$ = 9.3 Hz, H-29), 0.85 (3H, d, $J$ = 6.4 Hz, H-21), and 0.94 (3H, s, H-19)], eight methine protons [δ 0.83-0.86 (1H, m, H-9), 0.83-0.89 (1H, m, H-24), 0.89-0.93 (1H, m, H-14), 0.99-1.08 (1H, m, H-17), 1.24-1.30 (1H, m, H-20), 1.18 (1H, m, H-25), 1.37-1.41 (1H, m, H-8), 3.41-3.45 (1H, m, H-3)], one olefinic proton [δ 5.28 (1H, dd, $J$ = 2.6, 5.2 Hz, H-6)], and eleven methylene protons [δ 0.95-1.05 (1H, m, H-1α), 1.75-1.81 (1H, m, H-1β), 1.75-1.79 (2H, m, H-2), 2.17(1H, ddd, $J$ = 2.0, 4.9, 11.4 Hz, H-4α), 2.21 (1H, ddd, $J$ = 2.0, 4.9, 13.1 Hz, H-4β), 1.41-1.47 (1H, m, H-7α), 1.84-1.90 (1H, m, H-7β), 1.37-1.44 (2H, m, H-11), 1.06-1.12 (1H, m, H-12α), 1.88-1.95 (1H, m, H-12β), 0.91-1.01 (1H, m, H-15α), 1.48-1.53 (1H, m, H-15β), 1.16-1.19 (1H, m, H-16α), 1.77 (1H, m, H-16β), 1.10 (2H, m, H-22), 1.06-1.12 (2H, m, H-23), 1.15-1.21 (2H, m, H-28)]. The methyl proton signal at δ 0.94 (3H, s, H-19) correlated with the methine carbon signal δ (50.6, C-9) in the HMBC, and the methine proton signal at δ 0.83-0.86 (1H, m, H-9) was coupled with the methylene proton signals at δ 1.37-1.44 (2H, m, H-11) in the $^1$H-$^1$H COSY. Both of the methyl proton signals at δ 0.61 (3H, s, H-18) and 0.85 (3H, d, $J$ = 6.4 Hz, H-21) correlated with the carbon signal at δ (56.5, C-17) and the methine proton signal at 0.99-1.08 (1H, m, H-17), which was assigned by the HMQC (heteronuclear multiple quantum coherence spectroscopy), was coupled with the methylene proton signals at δ 1.12 (2H, m, H-16). Thus,

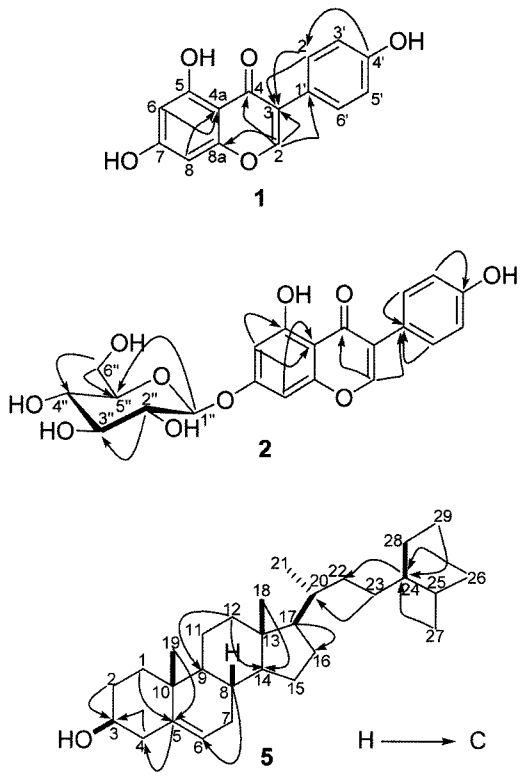


**Fig. 2. Important HMBC correlations in compounds 1, 2, and 5.**

these assignments and analysis of HMBC spectra allowed the unequivocal assignment of all carbons (Fig. 2). All data mentioned above indicate that the structure of compound **5** was β-sitosterol.

**Determination of isolated compounds 1-4** Isolated compounds **1-4** were investigated by quantitative analysis using HPLC, and these compounds were identified by physical and spectroscopic data with the use of authentic standards. The concentrations of **1-4** were calculated on the basis of peak areas in the chromatogram and the

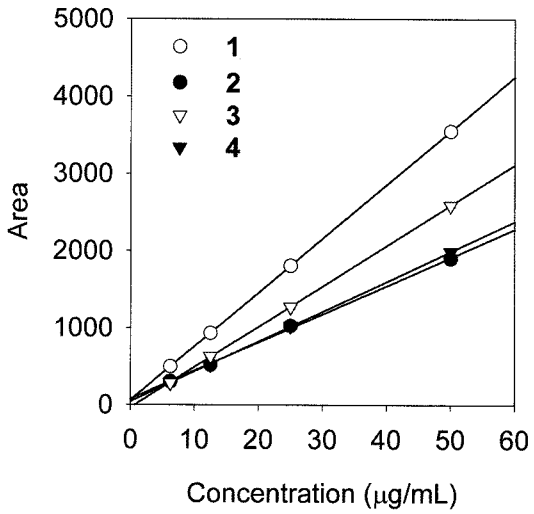


**Fig. 3. Calibration curves of isolated compounds 1-4.**

**Table 2. Calibration curves of the isolated compounds 1-4**

| Compound | Equation of linear regression | Correlation coefficient ($R^2$) |
|---|---|---|
| 1 | $y = 69.85x + 60.70$ | 0.999 |
| 2 | $y = 36.70x + 78.40$ | 0.998 |
| 3 | $y = 52.28x - 26.35$ | 0.999 |
| 4 | $y = 39.13x + 36.00$ | 0.999 |

plotting standard concentration was obtained at four concentrations (50, 25, 12.5, and 6.25 μg/mL) (Fig. 3). A high linearity ($R^2$>0.997) was obtained for each curve (Table 2).

Figure 4A displays HPLC profiles of the methanol extract from Taekwangkong. Although major peaks and other minor peaks were not completely identified, four peaks corresponding to the isolated compounds **1-4** were determined by $C_{18}$ reversed phase HPLC coupled with a diode-array detector and retention times for the isolated compounds were as follows: compound **1** ($t_R$=33.9 min), compound **2** ($t_R$=15.1 min), compound **3** ($t_R$=24.8 min), and compound **4** ($t_R$=9.4 min) (Fig. 4B).

The content of compounds **1-4** (μg/g) in Taekwangkong cultivar were as follows: compound **1**: 12.1 μg/g, compound **2**: 624.6 μg/g, compound **3**: 18.0 μg/g, and compound **4**: 219.6 μg/g. These results demonstrate that the soybean cultivar Taekwangkong is rich in flavonoids, especially compounds **2** and **4** which are present mainly in the form of glycosides (Fig. 4).

**Total phenolic contents** The total phenolic contents in the methanol extracts of the taekwangkong were expressed as mg/g of gallic acid. A calibration curve was constructed with different concentrations of gallic acid (200, 100, 50, 25, and 12.5 μg/mL) as a standard. The linear regression equation of this curve and coefficient of determination ($R^2$) were calculated as $y = 0.0026x - 0.00045$, $R^2$=0.999. These results show that the total phenolic content of the methanol extract of Taekwangkong was 3.7 mg GAE/g.

**Radical scavenging activity of isolated compounds (1-4)** The systems ABTS and DPPH are both commonly used to measure the total antioxidant status of various biological specimens because of their reproducibility and ease of quality control (28, 29). Therefore, ABTS and DPPH radicals were chosen to test the antioxidant activities of the isolated compounds **1-4**. For the measurement of anti-oxidant activity, the UV/Vis spectrophotometry method was used to detect ABTS and DPPH radicals. The change in absorbance produced by reduced DPPH was used to evaluate the ability of isolated compounds to act as free radical scavengers. Compounds **1-4** were not capable of reacting with DPPH at a concentration of 200 μM, showing less than 10% scavenging activity. ABTS radical scavenging activity was measured immediately after the addition of potassium persulfate to an ABTS solution. Compound **1** suppressed the absorbance of the ABTS radical with $IC_{50}$ values of 47.6 μM, and Trolox used as a positive control exhibited $IC_{50}$ values of 13.5 μM. The scavenging activity of compound **1** against ABTS was five-fold higher compared to its DPPH radical scavenging

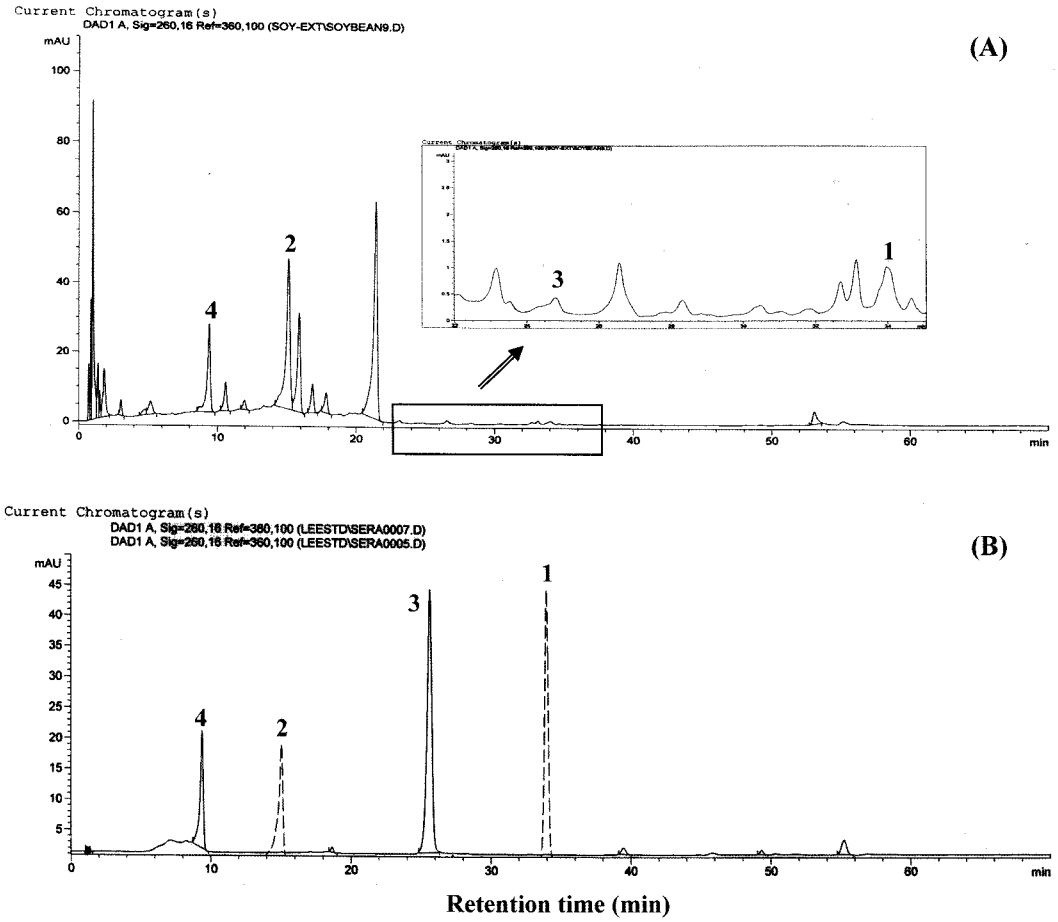


**Fig. 4. Characteristic HPLC chromatograms:** (A) Profile of MeOH extract of Taekwangkong cultivar; (B) Profile of the isolated compounds 1-4.

**Table 3. Antioxidant activities of isolated compounds 1-4 on ABTS and DPPH radicals**

| Compound | $IC_{50}$ (mM)[1)] | |
|---|---|---|
| | ABTS | DPPH |
| 1 | 47.6 ± 2.38 | >200 |
| 2 | >200 | >200 |
| 3 | >200 | >200 |
| 4 | >200 | >200 |
| BHA/ Trolox | 13.5 ± 2.25 (Trolox) | 33.4 ± 2.15 (BHA) |

[1)]Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations.

activity (Table 3). Interestingly, glycosylation of the hydroxyl in C-7 (**2**, **4**) or hydration in C-5 (**3**) compounds lead to a large decrease in scavenging activity as shown by an increase in $IC_{50}$ to 200 μM. Thus, the 5-hydroxyl isoflavone (**1**) seems to be important in eliciting potent ABTS radical scavenging activity. These results demonstrate that the 5-hydroxylated group in the A-ring might be important for antioxidant properties exhibited in the ABTS radical system.

In conclusion, five compounds were isolated from methanol extract of Taekwangkong, and these compounds were characterized using physical and spectroscopic data. The content of compounds **1-4** was determined by $C_{18}$ reversed phase HPLC analysis in addition to determining the content of total phenolics in this cultivar. Furthermore, the 5-hydroxylated group on isoflavone **1** showed stronger antioxidant activity in the ABTS radical system than other isolated compounds **2-4**.

## Acknowledgments

This study was supported by a Post Doctoral Course Program of Yeongnam Agricultural Research Institute (YARI), National Institute of Crop Science, Rural Development Administration, Republic of Korea.

## References

1. Miksicek RJ. Commonly occurring plant flavonoids have estrogenic activity. Mol. Pharmacol. 44: 37-43 (1993)
2. Miksicek RJ. Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. J. Steroid Biochem. 49: 153-160 (1994)
3. Miksicek RJ. Estrogenic flavonoids: structural requirements for biological activity. P. Soc. Exp. Biol. Med. 208: 44-50 (1995)
4. Cornwell T, Cohick W, Raskin I. Dietary phytoestrogens and health. Phytochemistry 65: 995-1016 (2004)
5. Liu J, Burdette JE, Xu H, Gu C, Bhat KPL, van Breemen RB, Booth N, Constantinou AI, Pezzuto JM, Fong HHS, Farnsworth

NR, Bolton JL. Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. J. Agric. Food Chem. 49: 2472-2479 (2001)
6. Franke AA, Custer LJ, Cerna CM, Narala KK. Quantitation of phytoestrogens in legumes by HPLC. J. Agric. Food Chem. 42: 1905-1913 (1994)
7. Messina M, Messina V. Soyfoods, soybean isoflavones, and bone health: a brief overview. J. Renal Nutr. 10: 63-68 (2000)
8. Davis SR, Dalais FS, Simpson AL. Murkies, phytoestrogens in health and disease. Recent Prog. Horm. Res. 54: 185-211 (1999)
9. Ghem BD, Mcandrews JM, Chien PY, Jameson JL. Reservatrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. P. Natl. Acad. Sci. USA 94: 14138-14143 (1997)
10. Price K, Fenwick G. Naturally occurring oestrogens on foods-a review. Food Addit. Contam. 2: 73-106 (1985)
11. Rosenblum ER, Stauber RE, Van Thiel DH, Campbell IM, Gavaler JS. Assessment of the estrogenic activity of phytoestrogens isolated from bourbon and beer, alcohol. Clin. Exp. Res. 17: 1207-1209 (1993)
12. Tham DM, Gardner CD, Haskell WL. Potential health benefits of dietary phytoestrogens: a review of clinical, epidemiological, and mechanistic evidence. J. Clin. Endocr. Metab. 83: 2223-2235 (1998)
13. Jacobsen BK, Knutsen SF, Fraser GE. Does high soy milk intake reduce prostate cancer incidence, the adventist health study (United States). Cancer Cause Control 9: 553-557 (1998)
14. Messina M. Soyfoods and soybean phyto-oestrogens (isoflavones) as possible alternatives to hormone replacement therapy (HRT). Eur. J. Cancer 36: S71-S72 (2000)
15. Adlercreutz H. Phytoestrogens and breast cancer. J. Steroid Biochem. 83: 113-118 (2002)
16. Di Carlo G, Mascolo N, Izzo AA, Capasso F. Flavonoids: Old and new aspects of a class of natural therapeutic drugs. Life Science 65: 337-353 (1999)
17. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem. Pharmacol. 32: 1141-1148 (1983)
18. Javanmardi JC, Locke SE, Vivanco JM. Antioxidant activity and total phenolic content of Iranian Ocimum accessions. Food Chem. 83: 547-550 (2003)
19. Breimer LH. Ionizing radiation-induced mutagenesis. Brit. J. Cancer 57: 6-18 (1998)
20. McCord JM. The evolution of free radicals and oxidative stress. Am. J. Med. 108: 652-659 (2000)
21. Gibson GE, Zhang H. Interactions of oxidative stress with thiamine homeostasis promote neurodegeneration. Neurochem. Int. 40: 493-504 (2002)
22. Cheung LM, Cheung PCK, Ooi VEC. Antioxidant activity and total phenolics of edible mushroom extracts. Food Chem. 81: 249-255 (2003)
23. Park YK, Lee WY, Park SY, Ahn JK, Han MS. Antioxidant activity and total phenolic content of *Callistemon citrinus* extracts. Food Sci. Biotechnol. 14: 212-215 (2005)
24. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying improved ABTS radical cation decolorization assay. Free Radical Bio. Med. 26: 1231-1237 (1999)
25. Choi YM, Ku JB, Chang HB, Lee JS. Antioxidant activities and total phenolics of ethanol extracts from several edible mushrooms produced in korea. Food Sci. Biotechnol. 14: 700-703 (2005)
26. Braca A, De Tommasi N, Di Bari L, Pizza C, Politi M, Morelli I. Antioxidant principles from *Bauhinia terapotensis*. J. Nat. Prod. 64: 892-895 (2001)
27. Coperland RA. Enzyme: A practical introduction to structure, mechanism, and data analysis. Wiley-VCH, New York, NY, USA. pp. 266-332 (2000)
28. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensm. -Wiss. Technol. 28: 25-30 (1995)
29. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying improved ABTS radical cation decolorization assay. Free Radical Bio. Med. 26: 1231-1237 (1999)