Food Sci. Biotechnol. Vol. 16, No. 1, pp. 116 ~ 122 (2007)

Food Science and Biotechnology

Isolation and Identification of Antioxidants from Peanut Shells and the Relationship between Structure and Antioxidant Activity

Ji-Hyang Wee, Jae-Hak Moon, Jong-Bang Eun, Jin Ho Chung, Young Gook Kim[1], and Keun-Hyung Park*

Department of Food Science and Technology and Functional Food Research Center, Chonnam National University, Gwangju 500-757, Korea

[1]Dasan Institute of Life and Science, Gwangju 506-812, Korea

Abstract Four compounds with antioxidant activity were isolated from the MeOH extract of peanut shells (pod) and identified as 5,7-dihydroxychromone (**1**), eriodictyol (**2**), 3',4',7-trihydroxyflavanone (**3**), and luteolin (**4**) by electron impact-mass spectrometry (EI-MS) and nuclear magnetic resonance (NMR) analyses. The relationship between antioxidant activity and chemical structure of the isolated compounds with their analogues [(-)-epicatechin, quercetin, taxifolin] was examined by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity and using the 2-deoxy-D-ribose degradation system. The order of antioxidant activity on the basis of DPPH radical-scavenging was quercetin = (-)-epicatechin (6.0 molecules) > taxifolin (4.5 molecules) > **4** (luteolin; 4.0 molecules) > **2** (eriodictyol; 2.5 molecules) > **3** (3',4',7-trihydroxy-flavanone; 2.0 molecules) > **1** (5,7-dihydroxychromone; 0.5 molecules). On the other hand, using the 2-deoxy-D-ribose degradation system, the order of antioxidant activity was quercetin > **4** >> (-)-epicatechin ≥ **2** ≥ taxifolin > **3** > **1**. These compounds from peanut shells may provide defensive measures against oxidative stress and insects in the soil.

Keywords: peanut shell, antioxidant, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, flavonoid, structure-activity relationship

Introduction

Antioxidants are widely used in the manufacture and storage of fat, oil, and fatty foods to minimize rancidity caused by oxidation. Moreover, antioxidants may also be associated with the effective protection from peroxidative damage in living systems and play an important role in the prevention of carcinogenesis, diabetes, and inflammatory disease (1-3). Numerous studies have reported on the effectiveness of antioxidants in fat, oil, and fatty foods. However, the continued use of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), the most widely used artificial antioxidants in food, is being questioned because of their safety (4). Therefore, much attention has been focused on the identification and development of natural antioxidants.

The shell (pod) and seed of the peanut (*Arachis hypogaea*), one of the principal agricultural plants in the world, mature in the ground (5). Therefore, compounds in peanut shells may provide protection against attack by insects and oxidative damage. Recently, many researchers have been investigating the active antioxidant substances in hull extracts from, for example, rice and oat (6, 7). However, there has been no attempt to study the antioxidant properties of compounds found in peanut shells.

In previous papers, we reported the isolation and identification of antimicrobial and antioxidant substances from peanut shells (8-10). As part of our continuing investigation, we now report on the further isolation and identification of antioxidant compounds from peanut shells and the relationship between structure and antioxidant activity of the isolated compounds and flavonoid analogues using two different assay systems.

*Corresponding author: Tel: 82-62-530-2143; Fax: 82-62-530-2149
E-mail: khpark@chonnam.ac.kr
Received October 10, 2006; accepted November 27, 2006

Materials and Methods

Biological materials Peanut shells (pods) were harvested at Gochang, Korea, and air-dried. The moisture levels of the dried peanut shells were 4.80±0.04%.

Chemicals 3,3',4',5,7-Pentahydroxyflavone (quercetin), 3,3',4',5,7-pentahydroxyflavanol [(-)-epicatechin], 3,3',4',5,7-pentahydroxyflavanonol (taxifolin), 3',4',5,7-tetrahydroxy-flavone (luteolin), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and α-tocopherol were purchased from Sigma chemicals (St. Louis, MO, USA). 3',4',5,7-Tetrahydroxyflavanone (eriodictyol) was supplied by Funakoshi (Tokyo, Japan). 2-Deoxy-D-ribose was purchased from Acros Chemical (Organics, NJ, USA). Dimethyl sulfoxide-d_6 (DMSO-d_6) and methanol-d_4 (CD_3OD) were obtained from Merck (Darmstadt, Germany).

Isolation of antioxidants Peanut shells (6.1 kg, dry weight) were extracted with methanol (MeOH, 10 L) and concentrated *in vacuo*. The MeOH extract (145.2 g) was solvent-fractionated into aqueous and organic phases with ethylacetate (EtOAc, 3×5 L) and a buffer solution of 0.2 M glycine/0.2 N HCl (3 L) at pH 3.0 (11). The organic phase was subsequently fractionated with a buffer solution of 0.2 M NaH_2PO_4/0.2 M Na_2HPO_4 (3 L) at pH 8.0. The organic phase was successively solvent-fractionated with a buffer solution of 0.2 M KCl/0.2 M NaOH (3 L) at pH 12.0. The obtained aqueous phase was adjusted to pH 6.0 with 1.0 N HCl, and then extracted with EtOAc (3×5 L, EtOAc-soluble phenolic fraction).

The EtOAc-soluble phenolic fraction (6.2 g) was subjected to silica gel adsorption column chromatography (Kieselgel 60, 25×250 mm, 70-230 mesh; Merck, 12). Elution was carried out stepwise with an increasing concentration of MeOH in EtOAc (0:10, 1:9, 2:8, 3:7, 4:6, 5:5, v/v, each 600 mL). The active fraction was rechromatographed using silica gel adsorption column chromatography (Merck) with EtOAc in *n*-hexane (6:4, 4:6, 2:8, 0:10, v/v, each 400 mL). The active fraction obtained from the silica gel column was chromatographed on a Sephadex LH-20 (30×800 mm, 25-100 mesh; Phamacia Fine Chemicals, Uppsala, Sweden) column with MeOH/chloroform [($CHCl_3$), 4:1, v/v]. Each active fraction (fraction I-III) was applied to a column of octadecylsilane (ODS, 70-230 mesh, fraction I, 10×100 mm; fraction II, 10×90 mm; fraction III, 10×180 mm; YMC, Kyoto, Japan) and eluted stepwise with an increasing concentration of MeOH in H_2O. The active eluate was subjected to HPLC under the following conditions: ODS column, Senshu-pak (8×250 mm, Tokyo, Japan); detection, UV detector (254 nm, 486 Tunable absorbance detector; Waters, Milford, MA, USA); flow rate, 1.5 mL/min (Model 510 solvent delivery system; Waters) at room temperature; mobile phases, 60% MeOH (fraction I), 40% MeOH (fraction II), and 65% MeOH (fraction III).

Structural elucidation of the active compounds Electron impact-mass spectrometry (EI-MS) was performed using a mass spectrometer (Waters Integrity^{TM} System) under the following conditions: ion source temperature, 200°C; ionizing voltage, 70 eV; scanning mass range, *m/z* 50-500.

Nuclear magnetic resonance (NMR) spectra were measured in DMSO-d_6 (**1**, **4**) and CD_3OD (**2**, **3**) with an ^{unit}INOVA 500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz; Varian, Walnut creek, CA, USA) referenced to tetramethylsilane (TMS, δ=0; **2**, **3**) and the residual proton solvent resonance (DMSO-d_6, δ=2.5 for ^{1}H-NMR; **1**, **4**).

Evaluation of DPPH radical-scavenging activity for the MeOH extract and solvent-fractionation DPPH radical-scavenging activity of the MeOH extract and each fraction after solvent fractionation was measured according to the method of Abe *et al.* (13). The DPPH radical was prepared in EtOH as a 100 μM solution (final concentration, 90.9 μM). This DPPH solution (900 μL) was mixed with different concentrations of peanut shell extract (100 μL in MeOH solution) or MeOH (100 μL, as control). After the reaction was carried out at room temperature for 10 min in the dark, the free radical-scavenging activity of each sample was quantified by measuring decolorization at 517 nm and expressed as the concentration of the samples required to scavenge 50% of the DPPH radicals (SC_{50}).

Evaluation of DPPH radical-scavenging activity of purified antioxidants Each peanut shell fraction was spotted on a silica gel thin layer chromatography plate (TLC, Silica gel 60 F_{254}, 0.25 mm thickness; Merck) and developed with a suitable solvent. DPPH EtOH solution (200 μM) was then sprayed on the plate surface (14). After spraying the DPPH solution, fractions displaying a decolorization of the spot were regarded as possessing antioxidant activity.

Evaluation of the number of DPPH radicals scavenged per molecule of antioxidant The number of free radicals scavenged per molecule of the isolated compounds and their flavonoid analogues was determined using DPPH (15, 16). An EtOH solution (0.5 mL) containing each of the test samples at different concentrations (5-250 μM) was addes into the 1.0 mM DPPH (final concentration, 250 μM) in EtOH (0.5 mL). The total volume of the reaction mixture was then adjusted to 2.0 mL with 100 mM Tris-HCl buffer (pH 7.4). The reaction was carried out at room temperature for 30 min in the dark, after which the free radical-scavenging activity of each flavonoid was quantified by decolorization measured at 517 nm. α-Tocopherol was used as the standard sample. The number of DPPH radicals scavenged by one molecule of each sample was calculated as

Number of DPPH radicals scavenged
= DPPH concentration in the reaction mixture (250 μM)/ initial concentration showing no change of absorbance.

Evaluation of antioxidant activity using 2-deoxy-D-ribose Protection against deoxyribose oxidative degradation was used for the evaluation of hydroxyl radical-scavenging activity of isolated peanut shell compounds and their derivatives according to the modified method of Lopes *et al.* (17). Briefly, each 10 mL reaction mixture contained 20 mM potassium phosphate buffer (pH 7.4), 2.8 mM deoxyribose, and each test compounds (final concentration, 0.1 mM). Each reaction was initiated by the addition of $Fe(NO_3)_3$/ascorbic acid (final concentration, 0.01 mM/1.0 mM) and H_2O_2 at a final concentration of 1.0 mM. The reaction mixture (1.0 mL) was incubated at 37°C for 1 hr. The radical-mediated decomposition of deoxyribose to malonaldehyde (MDA) was assessed by reaction with thiobarbituric acid (TBA) in an acid solution. Trichloroacetic acid (2.8%, w/v) solution and 1.0 mL of 4,6-dihydroxy-2-mercaptopyrimidine (1%, w/v) in a 50 mM NaOH solution were then added. The mixture was heated at 100°C for 10 min. After cooling, 2 mL of BuOH was added. The extent of deoxyribose degradation by the formed hydroxyl radical was measured in the BuOH phase at 532 nm. The amount of deoxyribose degradation expressed as the TBA-reactive substance (TBARS) value was calculated from a calibration curve with tetraethoxypropane as the standard.

Results and Discussion

Purification and isolation of active antioxidant substances from peanut shell The antioxidant activity of MeOH extract (145.2 g) of peanut shells (6.1 kg dry wt) was measured on the basis of the sample concentration required for 50% scavenging (SC_{50}) of the DPPH radical (final concentration, 90.9 μM). The SC_{50} value of the MeOH extract was 10 μg. Interestingly, the MeOH extract had higher activity than α-tocopherol (14 μg), a natural antioxidant used as a control compound. Therefore, the MeOH extract was solvent-fractionated with EtOAc and a buffer solution to purify active antioxidant compounds. The EtOAc-soluble acidic and phenolic fractions showed SC_{50} value of 90 and 8 μg, respectively. However, the

EtOAc-soluble neutral fraction did not exhibit any antioxidant activity.

In previous reports (8, 9), we have already described the isolation and the identification of 3-methoxy-4-hydroxybenzoic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid as active antioxidant and antimicrobial substances from the EtOAc-soluble acidic fraction. The current study describes the elucidation of structure and the characterization of active antioxidant compounds isolated from the EtOAc-soluble phenolic fraction.

The EtOAc-soluble phenolic fraction (6.2 g) was subjected to silica gel adsorption column chromatography (stepwise elution with an increasing concentration of MeOH in EtOAc). The active fraction (EtOAc/MeOH, 100:0, v/v, 4.2 g) was repurified by repeated silica gel adsorption column chromatography (EtOAc/*n*-hexane). The eluate (640 mg) with *n*-hexane/EtOAc (50:50-40:60, v/v) was found to have antioxidant activity and was subjected to Sephadex LH-20 column chromatography (MeOH/$CHCl_3$, 4:1, v/v). The active components were found in three fractions with a Ve/Vt (elution volume/column bed volume) of 1.08-1.18 (fraction I, 133.0 mg), 1.24-1.30 (fraction II, 19.7 mg), and 1.40-1.60 (fraction III, 277.2 mg). Each active fraction was then further purified using ODS column chromatography (H_2O/MeOH). The active fractions [60-80% MeOH eluate (from fraction I, 23.4 mg; fraction I-a), 40-50% MeOH eluate (from fraction II, 14.9 mg; fraction II-a), 60-90% MeOH eluate (from fraction III, 53.6 mg; fraction III-a)] were successively purified by reversed-HPLC [ODS column, 60% MeOH (fraction I-a), 40% MeOH (fraction II-a), 65% MeOH (fraction III-a)] to obtain four active compounds [**1** (from fraction I-a, t_R 10.0 min, 16.0 mg), yellow amorphous powder; **2** (from fraction II-a, t_R 14.3 min, 3.4 mg), white amorphous powder; **3** (from fraction II-a, t_R 32.7 min, 2.5 mg), yellow amorphous powder; **4** (from fraction III-a, t_R 13.7 min, 2.4 mg) yellow amorphous powder].

Identification of isolated compounds The molecular weight of **1** was determined through its EI-MS spectrum, in which the molecular ion was found at *m/z* 178 $[M]^+$ together with other fragment ions at *m/z* 152 $[M-C_2H_2]^+$, 124 $[M-C_3H_2O]^+$, and 69 $[M-C_6H_4O_2]^+$. The ^{1}H-NMR spectrum (DMSO-d_6, Table 1) of **1** showed signals corresponding to 4 sp^2 carbon protons (δ 8.17-6.19, 4H), including two pairs of equivalent proton signals [δ 6.19 and 6.35 (d, *J*=2.0 Hz, H-6 and 8); 6.27 and 8.17 (d, *J*=6.0 Hz, H-3 and 2)]. In addition, 1H proton signal detected at δ 12.69 (sec) suggested the presence of a carbonyl group and a hydroxyl group in the 4 and 5 positions of the chromone ring, respectively. The ^{13}C-NMR spectrum (DMSO-d_6, Table 2) showed 9 signals comprising 9 sp^2 carbons (δ 94.7-182.0) including one carbonyl carbon (δ 182.0). From EI-MS, ^{1}H- and ^{13}C-NMR spectroscopic data, **1** was suggested to be a chromone structure. The important correlations between ^{1}H and ^{13}C in the HMBC spectrum (data not shown) as well as the splitting patterns in the ^{1}H-NMR spectrum revealed the complete linkages of carbons and protons. On the basis of these results, the structure of **1** was identified with certainty as 5,7-dihydroxychromone.

The molecular weight of **2** was determined through its EI-MS spectrum, in which the molecular ion was found at *m/z* 288 $[M]^+$ together with other fragment ions at *m/z* 179 $[M-C_6H_5O_2]^+$, 166 $[M-C_6H_4O_3]^+$, 153 $[M-C_8H_8O_2]^+$, 136 $[M-C_7H_4O_4]^+$, 123 $[M-C_9H_8O_3]^+$, and 110 $[M-C_9H_7O_4]^+$. The ^{13}C-NMR spectrum (Table 2) showed 15 signals including 12 sp^2 aromatic carbons (δ 167.7-95.1), a carbonyl carbon (δ 196.5, C-4), and 2 sp^3 carbons [δ 79.3 (C-2), 42.9 (C-3)]. The ^{1}H-NMR spectrum (Table 1) showed 8 signals corresponding to 5 sp^2 carbon protons (δ 5.87-6.92) and 3 sp^3 carbon protons [δ 5.28 (1H, dd, *J*=3.0, 12.9 Hz, H-2), 2.69 (dd, *J*=3.0, 17.1 Hz, H-3a), 3.06 (dd, *J*=12.9, 17.1 Hz, H-3b)]. Two proton signals at δ 5.87 and 5.89 possessed the same coupling constant (*J*=2.0 Hz) ascribable to the flavonoid A ring. In addition, a series of three proton signals at δ 6.92 (1H, br. s, H-2') and 6.79 [2H, m, two 1H portion signals (H-5', 6') were overlapped] were assigned to the flavonoid B ring. The coupling constant values and splitting patterns of the sp^3 carbon protons showed the presence of contiguous protonated carbons including methine and geminal methylene protons of the AB type (δ 2.69 and 3.06) corresponding to the flavonoid C ring. Based on these data, **2** was likely to be

Table 1. ^{1}H-NMR data for compounds 1-4 (500 MHz)

Position	δ_H (mult., *J*)			
	1 (DMSO-d_6)	**2** (CD_3OD)	**3** (CD_3OD)	**4** (DMSO-d_6)
2	8.17 (1H, d, 6.0)	5.28 (1H, dd, 12.9, 3.0)	5.32 (1H, dd, 13.0, 3.0)	-
3	6.27 (1H, d, 6.0)	3a: 2.69 (1H, dd, 17.1, 3.0) 3b: 3.06 (1H, dd, 17.1, 12.9)	3a: 2.69 (1H, dd, 16.9, 3.0) 3b: 3.01 (1H, dd, 16.9, 13.0)	6.66 (1H, s)
5	12.69 (1H, s, -OH)	-	7.72 (1H, d, 8.3)	12.96 (1H, s, -OH)
6	6.19 (1H, d, 2.0)	5.87 (1H, d, 2.0)	6.49 (1H, dd, 8.3, 2.5)	6.18 (1H, br. s)
8	6.35 (1H, d, 2.0)	5.89 (1H, d, 2.0)	6.35 (1H, d, 2.5)	6.43 (1H, br. s)
10	-	-	-	-
2'	-	6.92 (1H, br. s)	6.93 (1H, d, 1.5)	7.39 (1H, br. s)
5'	-	6.79 (2H, m, overlapped with H-6')	6.80 (2H, m, overlapped with H-6')	6.87 (1H, d, 8.5)
6'	-	6.79 (2H, m, overlapped with H-5')	6.80 (2H, m, overlapped with H-5')	7.40 (1H, d, 8.5)

Table 2. ^{13}C-NMR data for compounds 1-3 (125 MHz)

Position	1 (DMSO-d_6)	2 (CD_3OD)	3 (CD_3OD)
2	158.1	79.3	81.1
3	111.2	42.9	45.1
4	182.0	196.5	193.6
5	162.3	164.2	129.9
6	99.7	96.0	112.0
7	165.2	167.7	165.6
8	94.7	95.1	103.9
9	158.5	163.6	167.3
10	105.5	102.1	114.9
1'	-	130.6	132.1
2'	-	113.5	114.7
3'	-	145.3	146.6
4'	-	145.7	146.9
5'	-	115.1	116.3
6'	-	118.2	119.3

flavanone. The complete identification of **2** was established by HMBC analysis (data not shown). Consequently, the structure of **2** could be identified with certainty as 3',4',5,7-tetrahydroxyflavanone (eriodictyol).

The molecular weight of **3** was determined through its EI-MS spectrum, in which the molecular ion was found at *m/z* 272 $[M]^+$ together with other fragment ions at 163 $[M-C_6H_5O_2]^+$, 137 $[M-C_7H_4O_3]^+$, 108 $[M-C_9H_7O_3]^+$. The ^{1}H-NMR spectrum (Table 1) was closely related to that of **2**, except for the proton signals [δ 6.49 (1H, dd, *J*=2.5, 8.3 Hz, H-6), 7.72 (1H, d, *J*=8.3 Hz, H-5)] corresponding to the A ring of flavanone. The signal splitting and coupling constant values of the A ring indicated that it has three substituent groups with vicinal methine protons. The ^{13}C-NMR spectrum (Table 2) also exhibited signals suitable for the 15 carbon atoms of flavanone. The important correlation between ^{1}H and ^{13}C in HMBC spectrum (data not shown) revealed the complete linkages of carbons and protons. On the basis of these assignments, the structure of **3** was unambiguously concluded to be 3',4',7-trihydroxy-flavanone.

The molecular weight of **4** was determined through its EI-MS spectrum, in which the molecular ion was found at *m/z* 286 $[M]^+$ together with other fragment ions at *m/z* 153 $[M-C_8H_6O_2]^+$ and 134 $[M-C_7H_4O_4]^+$. The ^{1}H-NMR spectrum (Table 1) of **4** was also closely related to that of **2**. The ^{1}H-NMR spectrum of **4** showed one more sp^2 carbon signal (δ 6.66, 1H, s, H-3) instead of sp^3 carbon signals in comparison with **2**. This suggested that **4** is a flavone. Two proton signals at δ 6.18 (1H, H-6) and 6.43 (1H, H-8) possessing the same signal pattern (br. s) ascribable to the A ring and a series of three proton signals at δ 7.39 (1H, br. s, H-2'), 6.87 (1H, d, *J*=8.5 Hz, H-5'), and 7.40 (1H, d, *J*=8.5 Hz, H-6') assigned to the B ring showed it to be the known compound luteolin. Therefore, in order to confirm the proposed structure **4**, we carried out ^{1}H-NMR analysis of commercial standard luteolin (data not shown) in a similar manner to that for **4**, and then the ^{1}H-NMR spectrum was compared with that of **4**. The spectra were superimposed and **4** were confirmed to be luteolin.

The four active antioxidative compounds (Fig. 1) isolated from the EtOAc-soluble phenolic fraction of the MeOH extract of peanut shells were identified as 5,7-dihydroxy-chromone (**1**), eriodicryol (**2**), 3',4',7-trihydroxyflavanone (**3**), and luteolin (**4**). Compound **1** has been reported to inhibit velvet leaf (*Abutilon theophrasti*) seed germination (18) and depress aflatoxin production by *Aspergillus parasiticus* (19). Compound **3** has been identified from *Dalbergia odorifera*, *Gliricidia sepium*, and *Cassia nomame* (20-22). Compounds **2** and **4** are also widely distributed in many plants, and they have various activities such as antioxidant, antiinflammatory,

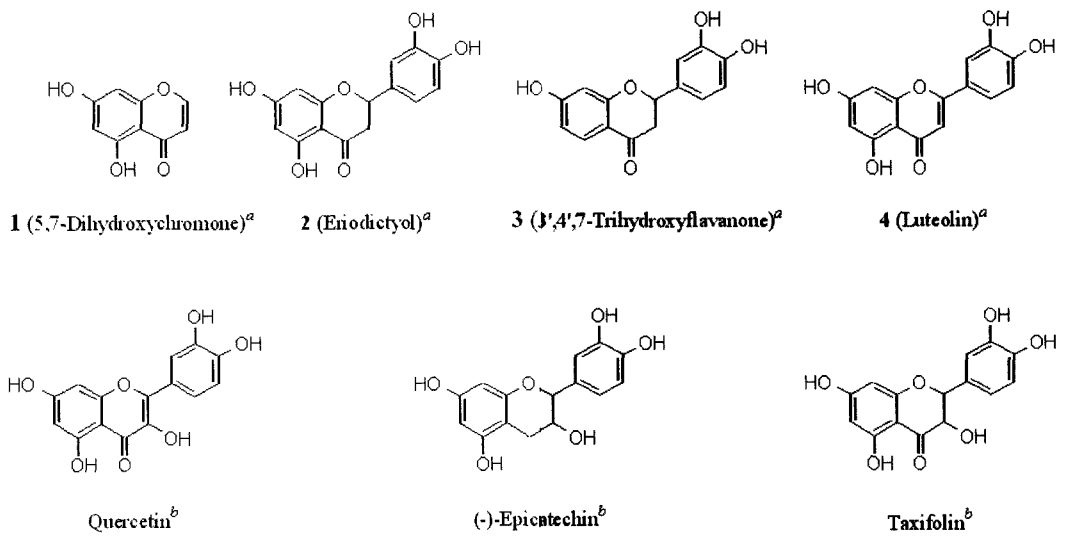

Fig. 1. Structures of compounds 1-4[a] identified from MeOH extract of peanut shells and flavonoid analogues[b] used for evaluation of structure-activity relationships.

antimicrobial, and cancer-preventive activities (23-26). However, the isolation and identification of compounds **1**-**4** from peanut shells is being reported for the first time as far as we know.

DPPH radical-scavenging activity of isolated compounds and their analogues The number of molecules of DPPH radical scavenged by one molecule of each of the four antioxidative compounds isolated from peanut shells was calculated with the assumption that one molecule of α-tocopherol scavenges two molecules of DPPH radical (Table 3). The DPPH radical-scavenging activity of **4** (4.0 molecules) was significantly higher than that of **2** (2.5 molecules) or **3** (2.0 molecules), lacking the double bond between C-2 and C-3, and the hydroxyl group at C-5, respectively. However, **1** showed considerably lower activity (0.5 molecule) in comparison with **2**-**4**. The structure of **1** is consistent with the partial structure of the A and C rings in **4**. The radical-scavenging activities and structural relationship of **1** and **4** (4.0 molecules) indicate that the catechol structure (*o*-dihydroxyl compounds) of the flavonoid B ring is one of the important factors for the action of free radical-scavenging. It has already been reported that the *o*-dihydroxyl structure in the B ring is essential for the free radical-trapping and metal-chelating effects of flavonoids (27-30). Therefore, it is likely that the lower radical-scavenging ability of **1** is due to the lack of the B ring.

The structures of **2** and **3** differ only in the presence or absence of a hydroxyl group in the C-5 position. However, the difference in the radical-scavenging capacities was not so remarkable. It is likely that the hydroxyl group at the C-5 position has little effect on the antioxidant activity of flavonoids. However, compound **4**, which only differs from **2** in having a double bond between C-2 and C-3, showed higher DPPH radical-scavenging activity than **2**. It has been suggested that the C ring is one of the important factors determining the antioxidant activity of flavonoids. Therefore, the relationship between structure and antioxidant activity toward DPPH radicals was investigated using flavonoid analogues [(-)-epicatechin, quercetin, taxifolin, including **2** (eriodictyol) and **4** (luteolin)] (Fig. 1), which have common structural units in the A (5,7-dihydroxyl group) and B rings (catechol structure) but differ only in the C ring. Bors *et al.* (27) have proposed that the C-2,3 double bond in conjunction with 4-oxo function is responsible for electron delocalization, and that the additional presence of C-3 and C-5 hydroxyl groups provides the maximal radical-scavenging potential and strongest radical absorption factors. However, the DPPH radical-scavenging capability of (-)-epicatechin (6.0 molecules), which does not satisfy the proposed structures, was identical to that of quercetin (6.0 molecules), which satisfies all of the proposed structures. In addition, the activity of taxifolin (4.5 molecules), lacking only the C-2,3 double bond in comparison with quercetin, was lower than that of quercetin.

Table 3. Number of DPPH radicals scavenged by one molecule of antioxidant

Compound	DPPH radical-scavenging number[1)]
α-Tocopherol	2.0
5,7-Dihydroxychromone (**1**)	0.5
Eriodictyol (**2**)	2.5
3',4',7-Trihydroxyflavanone (**3**)	2.0
Luteolin (**4**)	4.0
Quercetin	6.0
Taxifolin	4.5
(-)-Epicatechin	6.0

[1)]Number of DPPH radicals trapped by 1 molecule of α-tocopherol was assumed to be 2.0.

Taxifolin (4.5 molecules) showed an even lower activity than (-)-epicatechin (6.0 molecules), which lacks both the C-2,3 double bond and 4-oxo group. Terao *et al.* (28) have reported that the peroxyl radical-scavenging activity of (-)-epicatechin in the phosphatidylcholine unilamellar liposome model system initiated by a water-soluble radical generator [2,2'-azobis(2-amidinopropane)hydrochloride] was lower than that of quercetin, and this may be due to the lower electron-donating ability of (-)-epicatechin relative to quercetin. On the other hand, in a study addressing the relationship between polyphenyl structure and DPPH radical-scavenging speed using ^{13}C-NMR analysis, Sawai *et al.* (31, 32) showed that the radical-scavenging activity (speed) of *o*-dihydroxyl phenolic compounds with a conjugated olefinic double bond (e.g., quercetin) was superior to that of compounds without this bond (e.g., taxifolin), whereas the ability of *o*-dihydroxyl phenolic compounds possessing a conjugated carbonyl bond (protocatechuate) was inferior to that of compounds lacking this bond (catechol). Therefore, the relative antioxidant activity of flavonoids seems to be dependent on the environment where the oxidation happens.

It has been demonstrated that antioxidants are gradually converted to secondary products and oxidative dimers in a reaction system involving phenolic compounds with DPPH radicals (13, 33-37). This suggests that antioxidant activity in DPPH radical-scavenging systems may depend on the electron-donating ability of the native starting antioxidants as well as the secondary products and intermediates produced after and/or during the radical-scavenging reaction. Therefore, the reaction conditions such as the ratio of antioxidant to DPPH, kind of solvent, light and darkness, temperature, pH, etc. may act as important factors affecting antioxidant capacity.

Antioxidant activity of isolated compounds and their analogues regarding 2-deoxy-D-ribose degradation The relative extent of the inhibition of free radical-mediated 2-deoxy-D-ribose degradation will give an indication of hydroxyl radical-scavenging potential and/or iron chelating propensity. Compound **1** showed the lowest activity not only in the DPPH reaction system, but also in this reaction system (Fig. 2). On the other hand, **4** (luteolin) showed the highest activity of the four compounds isolated from peanut shell MeOH extract. Moreover, the protective effect of **4** against 2-deoxy-D-ribose degradation was akin to that of quercetin, although the activity of **4** was significantly lower than that of quercetin which satisfies important determinants for radical-scavenging in the

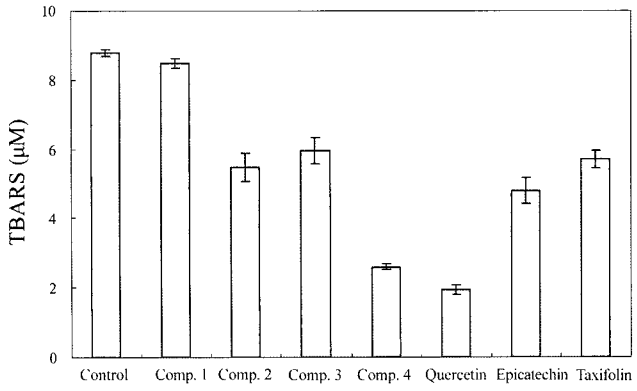

Fig. 2. Inhibition effect of compounds 1-4 identified from MeOH extract of peanut shells and flavonoid analogues used for the evaluation of structure-activity relationships regarding ferric ion-induced 2-deoxy-D-ribose degradation. Data are shown as the mean±SD (n=3).

structure of flavonoids proposed by Bors *et al.* (27).

The order of antioxidant effectiveness for all of the compounds isolated from peanut shells tested in this system is quercetin > **4** (luteolin) >> (-)-epicatechin ≥ **2** (eriodictyol) ≥ taxifolin > **3** > **1**. Interestingly, the order of antioxidant activity was different from that obtained with the DPPH reaction system. For example, **4** (luteolin, 4.0 molecules) and taxifolin (4.5 molecules) showed a comparatively similar level of DPPH radical-scavenging activity. However, in the 2-deoxy-D-ribose degradation system, **4** demonstrated two-fold greater activity than that of taxifolin. This is thought to be due to the chelation of iron ions acting as an initiator of chain-propagating radical reactions, which is one of the important factors for antioxidant activity in the 2-deoxy-D-ribose degradation system. That is, compound **4** and taxifolin are different with regard to such features as stereochemistry as well as the presence or absence of the C-3 hydroxyl group. Therefore, the difference in stereochemistry may affect the chelating effect occurring between the C-3 hydroxyl and C-4 oxo groups. It has been suggested that the contribution of the C-2,3 double bond toward antioxidative activity in the 2-deoxy-D-ribose degradation system may perhaps be higher than that of the C-3 hydroxyl group.

Flavonoids are present mostly in the form of glycosides in plants. However, all of the active antioxidant compounds isolated from peanut shells (pod) in this experiment were identified as aglycone forms. The pod shielding aerial parts (seeds) of the peanuts mature in the ground. Therefore, the constituents contained in the pod may exist in such more non-hydrophilic forms as aglycone because they should not be eluted in humid conditions but are retained for protection against harmful microorganisms or insects existing in soil.

Acknowledgments

This study was financially supported by Chonnam National University in the program, Post-Doc. 2004.

References

1. Halliwell B. Free radicals, antioxidants, and human disease: Curiosity, cause, or consequency. Lancet 344: 721-724 (1994)
2. Gutteridge JMC, Halliwell B. Antioxidants in Nutrition, Health, and Disease. Oxford University Press, Oxford, UK. pp. 1-62 (1994)
3. Halliwell BH, Gutteridge JMC, Arouoma OI. The deoxyribose method: a simple 'test-tube' assay for determination of rate constants for reactions of hydroxyl radicals. Anal. Biochem. 165: 215-219 (1987)
4. Branen AL. Toxicological and biochemistry of butylated hydroxy-toluene, butylated hydroxyanisole. J. Am. Oil Chem. Soc. 52: 59-63 (1975)
5. Ensminger AH, Ensminger ME, Konlande JE, Robson JRK. Foods and Nutrition Encyclopedia. Vol. 2, 1st ed. Pegus Press, CA, USA. p. 1727 (1989)
6. Cho JY, Moon JH, Seong KY, Park KH. Antimicrobial activity of 4-hydroxybenzoic acid and *trans* 4-hydroxycinnamic acid isolated and identified from rice hull. Biosci. Biotech. Bioch. 62: 2273-2276 (1998)
7. Xang Y, White PJ. Identification and function of antioxidants from oat groats and hulls. J. Am. Oil Chem. Soc. 74: 303-307 (1997)
8. Wee JH, Park KH. Identification of 3-methoxy-4-hydroxybenzoic acid and 4-hydroxybenzoic acid with antioxidative and antimicrobial activity from *Arachis hypogaea* shell. Korean J. Biotechnol. Bioeng. 15: 464-468 (2000)
9. Wee JH, Park KH. Isolation of 4-hydroxycinnamic acid, 3-methoxy4-hydroxycinnamic acid, and 3,4-dihydroxybenzoic acid with antioxidative and antimicrobial activity from *Arachis hypogaea* shell. Food Sci. Biotechnol. 10: 551-556 (2001)
10. Wee JH, Moon JH, Park KH. Isolation and identification of pratensein with antimicrobial activity from the peanut shells. Korean J. Food Sci. Technol. 36: 643-647 (2004)
11. Jang MY, Cho JY, Cho JI, Moon JH, Park KH. Isolation of compounds with antioxidative activity from quickly fermented soy-based foods. Food Sci. Biotechnol. 15: 214-219 (2006)
12. Cho JY, Kawazoe K, Moon JH, Park KH, Murakami K, Takaishi Y. Chemical constituents from the fruit peels of *Fortunella japonica*. Food Sci. Biotechnol. 14: 599-603 (2005)
13. Abe N, Nemoto A, Tsuchiya Y, Hojo H, Hirota A. Studies of the 1,1-diphenyl-2-picrylhydrazyl radical scavenging mechanism for a 2-pyrone compound. Biosci. Biotech. Bioch. 64: 306-333 (2000)
14. Takao T, Kitatani F, Sakata K. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. Biosci. Biotech. Bioch. 58: 1780-1783 (1994)
15. Yamamoto N, Moon JH, Tsushida T, Nagao A, Terao J. Inhibitory effect of quercetin metabolites and their related derivatives on copper ion-induced lipid peroxidation in human low-density lipoprotein. Arch. Biochem. Biophys. 372: 347-354 (1999)
16. Kim SJ, Cho JY, Wee JH, Jang MY, Kim C, Rim YS, Shin SC, Ma SJ, Moon JH, Park KH. Isolation and characterization of antioxidative compounds from the aerial parts of *Angelica keiskei*. Food Sci. Biotechnol. 14: 58-63 (2005)
17. Lopes GKB, Schulman HM, Hermes-Lima M. Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. Biochim. Biophys. Acta 1472: 142-152 (1999)
18. Spencer GF. A convenient synthesis of 5,7-dihydroxychromone. OPPI Briefs 23: 390-392 (1991)
19. Delucca AJ, Palmgren MS, Daigle DJ. Depression of aflatoxin production by flavonoid-type compounds from peanut shells. Phytopathology 77: 1560-1563 (1987)
20. Chan SC, Chang YS, Wang JP, Chen SC, Kuo SC. Three new flavonoids and antiallergic, anti-inflammatory constituents from the heartwood of *Dalbergia odorifera*. Planta Med. 64: 153-158 (1998)
21. Jurd L, Manners GD. Isoflavone, Isoflavan, and flavonoid constituents of *Gliricidia speium*. J. Agr. Food Chem. 25: 723-726 (1977)
22. Kitanaka S, Takido M. Demethyltorosaflavones C and D from *Cassia nomame*. Phytochemistry 31: 2927-2929 (1992)
23. Miyake Y, Yamamoto K, Morimitsu Y, Osawa T. Isolation of *C*-

glucosylflavone from lemon peel and antioxidative activity of flavonoid compounds in lemon fruit. J. Agr. Food Chem. 45: 4619-4623 (1997)
24. Miyake Y, Yamamoto K, Osawa T. Metabolism of antioxidant in lemon fruits (*Citrus limon* B_{URM}. f.) by human intestinal bacteria. J. Agr. Food Chem. 45: 3738-3742 (1997)
25. Yamamoto H, Ogawa T. Antimicrobial activity of perilla seed polyphenols against oral pathogenic bacteria. Biosci. Biotech. Bioch. 66: 921-924 (2002)
26. Ueda H, Yamazaki C, Yamazaki M. Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*. Biol. Pharm. Bull. 25: 1197-1202 (2002)
27. Bors W, Heller W, Michel C, Saran N. Flavonoids as antioxidants determination of radical-scavenging efficiences. Method Enzymol. 186: 343-355 (1990)
28. Terao J, Piskula M, Yao Q. Protective effect of epicatechin, epicatechin gallate and quercetin on lipid peroxidation in phospholipid bilayers. Arch. Biochem. Biophys. 308: 278-284 (1994)
29. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships. Free Radical Bio. Med. 22: 749-760 (1997)
30. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Bio. Med. 20: 933-956 (1996)
31. Sawai Y, Moon JH, Sakata K, Watanabe N. Effect of structure on radical-scavenging abilities and antioxidative activities of tea polyphenols: NMR analytical approach using 1,1-diphenyl-2-picryl-hydrazyl radicals. J. Agr. Food Chem. 53: 3598-3604 (2005)
32. Sawai Y, Moon JH. NMR analytical approach to clarify the molecular mechanism of the antioxidative and radical-scavenging activities of antioxidants in tea using 1,1-diphenyl-2-picrylhydrazyl. J. Agr. Food Chem. 48: 6247-6253 (2000)
33. Saito S, Okamoto Y, Kawabata J, Kasai T. Quinone hemiacetal formation from protocatechuic acid during the DPPH radical scavenging reaction. Biosci. Biotech. Bioch. 67: 1578-1579 (2003)
34. Saito S, Kawabata J. Synergistic effects of thiols and amines on antiradical efficiency of protocatechuic acid. J. Agr. Food Chem. 52: 8163-8168 (2004)
35. Kawabata J, Okamoto Y, Kodama A, Makimoto T, Kasai T. Oxidative dimers produced from protocatechuic and gallic esters in the DPPH radical scavenging reaction. J. Agr. Food Chem. 50: 5468-5471 (2002)
36. Abe N, Murata T, Yamamoto K, Hirota A. Bisorbibetanone, a novel oxidized sorbicillin dimer, with 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity from a fungus. Tetrahedron Lett. 40: 5203-5206 (1999)
37. Abe N, Murata T, Hirota A. Novel oxidized sorbicillin dimer with 1,1-diphenyl-2-picrylhydrazyl-radical scavenging activity from a fungus. Biosci. Biotech. Bioch. 62: 2120-2126 (1998)