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Identification of Phenolic Compounds and Antioxidant Effects from the Exudate of Germinating Peanut (*Arachis hypogaea*)

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Abstract Five phenolic compounds **1-5** were isolated for the first time from the exudate of geminating peanut (*Arachis hypogaea*). The structures were fully characterized by analysis of physical and spectral data. All isolated compounds were tested for antioxidant activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and hydroxyl radical. Compounds **2**, **3**, and **5** exhibited a strong scavenging effect on DPPH (**2**: IC_{50} = 10.4 μM, **3**: IC_{50} = 45.2 μM, **5**: IC_{50} = 5.0 μM), and ABTS (**2**: IC_{50} = 9.6 μM, **3**: IC_{50} = 5.5 μM, **5**: IC_{50} = 3.3 μM) radical activity, whereas these compounds had weak hydroxyl radical scavenging activity (IC_{50} > 200 μM). The total phenolic contents of the extracts using *n*-hexane, EtOAc, and *n*-BuOH were found to be 96.4-964.3 mg gallic acid equivalent per g dry material (GAE/g) and *n*-BuOH fraction showed the highest total phenolic content (964.3 mg GAE/g). These studies suggest that the exudate of geminating peanut may possess possible health related benefits to humans.

Keywords: exudate of geminating peanut, phenolic compound, total phenolic content, antioxidant activity, DPPH, ABTS

Introduction

Arachis hypogaea L. (commonly known as the peanut) has become one of the most important crops in many countries during the past few decades and has also shown antioxidant and antimutagen activities (1-5). As lower rates of chronic hemorrhage, bronchitis, oxidation, and cancer with higher intake of peanuts, the bioactive properties of peanuts have been subjected to extensive investigation (4). Although there are various phytochemicals belong to the stilbene, flavanone, and proanthocyanidin families present in peanuts, researchers mainly focused on compounds found in the nuts rather than in the plant itself (6-10). It is well established that various germinated plants contain abundant secondary metabolites possessing biological activity (11-13), but the elucidation of the biologically active substance from the exudate of germinating peanut has not been widely attempted. Thus, the evaluation of the biological function of secondary metabolites in the peanut and its germinating exudates are great importance not only the value of the whole plant as a source of bioactive materials but also that of peanut as dietary supplement. Recently, in the course of our studies on bioactive natural sources, we found that phenolic compounds from the exudate of geminating peanut showed potent antioxidant activity.

Antioxidants can be defined as compounds that delay or prevent the oxidation of lipids or other molecules by inhibiting the initiation or propagation of an oxidizing chain reaction (14). Phenolic compounds are widely distributed in the plant kingdom including food products as secondary metabolic products and nutraceutical importance (15-18), which possess potent antioxidant properties and free radical scavenging capabilities (16, 19). Also, it is well established that phenolic compounds are known to exert various physiological effects in humans, such as preventing oxidative damage of lipid and low-density lipoproteins (LDL) (20), anti-inflammatory and antimutagenetic (21-23), and reducing the risk of coronary heart disease and cancer (24, 25). Typically, phenolic compounds have been tested using *in vitro* assays of antioxidant activity before being tested *in vivo* in a biological system. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays have both been commonly used to measure the total antioxidant status of various biological specimens because of their high reproducibility and easy quality control (26, 27). When an antioxidant is added to the radicals, there is a degree of decolorization owing to the presence of the antioxidant which reverses the formation of the ABTS and DPPH radical. Also, hydroxyl radical plays a prominent role in biological phenomena (28, 29) and undergoes addition reactions with aromatic compounds leading to specific hydroxylated products.

In this study, we isolated five phenolic compounds for the first time from the exudate of germinating peanut and identified their structures through spectral analysis. These isolated compounds were also evaluated for their antioxidant activity using DPPH, ABTS, and hydroxyl radical scavenging assays. We also report that the total phenolic contents of the extracts using *n*-hexane, EtOAc, and *n*-BuOH.

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Materials and Methods

Plant material Peanut was collected during September 12-15, 2004, in the experimental field of the Yeongnam Agricultural Research Institute (YARI), National Institute of Crop Science, Rural Development Administration, Miryang, Korea. Peanut (12.0 kg) was cleaned and soaked in 8.0 L of water for 7 days at room temperature.

Reagents Gallic acid, Folin-Ciocalteau's phenol reagent, DPPH, ABTS, sodium persulfate, 2-deoxy-D-ribose, butylated hydroxyl anisol (BHA), and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Instruments The purity of all compounds were monitored by thin layer chromatography (TLC; E. Merck Co., Darmstadt, Germany), using commercially available glass-backed plates and visualized under UV at 254 and 366 nm or sprayed with phosphomolybdic acid (PMA) solution. Column chromatography was carried out using 230-400 mesh silica gel (Kieselgel 60, Merck, Germany). Melting points were measured on a Thomas Scientific capillary melting point apparatus (Electrothermal 9300, Essex, UK) and are uncorrected. IR spectra were recorded on a Bruker IFS66 (Bruker, Karlsruhe, Germany) infrared Fourier transform spectrophotometer (KBr) and UV spectra were measured on a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). ^{1}H- and ^{13}C-nuclear magnetic resonance (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 CD_3OD and acetone-d_6. Electron impact mass spectroscopy (EIMS) was obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). All the reagent grade chemicals were purchased from Sigma.

Determination of total phenolic content Total phenolic content was measured according to the modified Folin-Ciocalteau colorimetric method (30, 31). Briefly, each 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. The concentration of total phenolic content in the extracts was calculated using the following linear equation based on the calibration curve: $y = 0.0026x - 0.00045$, $R^2 = 0.999$, where, y was the absorbance and x was the total phenolic contents in mg of gallic acid equivalents (mg GAE/g extract).

DPPH radical scavenging activity Antioxidant activities of crude compounds (or 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.* (32). 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 $[A_c\text{-}A_t/A_c] \times 100$, where A_t and A_c were the absorbance of samples with and without crude compounds (or isolated compounds), respectively.

Trolox equivalent antioxidant capacity (TEAC) scavenging activity The TEAC assay is based on the relative ability of antioxidants to scavenge the radical cation $ABTS^{\bullet+}$ in comparison to a standard (Trolox) (33). 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 is 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 compounds were 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: $E = [(A_c\text{-}A_t)/A_c] \times 100$, where A_t and A_c were absorbance of samples with and without crude compounds (or isolated compounds), respectively. Antioxidant activity was expressed as TEAC values.

Hydroxyl radical scavenging activity Competition between deoxyribose and crude compounds (or isolated compounds) against hydroxyl radical generated from the Fe^{3+}/ascorbic acid/EDTA/H_2O_2 system were measured for determination of hydroxyl radical scavenging activity (34). The reaction mixture consisted of 10 mM KH_2PO_4/KOH buffer (pH 7.4), 16.8 mM 2-deoxyribose, 300 mM $FeCl_3$, 1.2 mM EDTA (ethylenediamine tetraacetic acid), 16.8 mM H_2O_2 and crude compounds (or isolated compounds). The reaction mixture was incubated at 37°C for 2 hr, and then 1 mL of 1.0% TBA (thiobarbituric acid in 50 mM NaOH) and 1 mL of 2.8% TCA (trichloroacetic acid) were added to test tubes and boiled for 20 min. After cooling the mixture, the absorbance was measured at 532 nm. Hydroxyl radical scavenging activity was evacuated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical and the radical scavenging effect was calculated as $[A_c\text{-}A_t/A_c] \times 100$, where A_t and A_c were absorbance of samples with and without crude compounds (or isolated compounds), respectively.

Extraction and isolation Peanut (12.0 kg) was cleaned and soaked in 8.0 L of water for 7 days at room temperature, and then the exudate of germinating water was partitioned with *n*-hexane, EtOAc, and *n*-BuOH to give *n*-hexane- (0.8 g), EtOAc- (1.2 g), and *n*-BuOH-extractable (1.9 g) residues. Subsequent bioactivity-guided fractionations of the EtOAc and *n*-BuOH extracts led to five compounds (Fig. 3). First, the EtOAc phase (1.2 g) was subjected to chromatography on silica gel (2.5×60 cm, 230-400 mesh, 120 g) using $CHCl_3$/acetone [20:1 (300 mL), 15:1 (300 mL), 10:1 (300 mL), 5:1 (300 mL), 1:1 (300 mL)] and $CHCl_3$/MeOH [10:1 (150 mL), 5:1 (150 mL), 1:1 (150 mL)] mixtures to give fraction A (40 mg), fraction B (120 mg), fraction C (65 mg), fraction D (220 mg), fraction E (200 mg), and fraction F (280 mg). Fraction E was applied to a silica gel column (1.8×40 cm, 230-400 mesh, 60 g) and separated with $CHCl_3$/acetone [10:1 (100 mL), 6:1 (80 mL), 3:1 (80 mL), 1:1 (80 mL)] to afford 15 subfractions; subfractions 11-15 were subjected to

silica gel column (1.2×30 cm, 230-400 mesh, 30 g) chromatography with $CHCl_3$/acetone (8:1 → 1:2) to yield compound **3** (14 mg). Fraction F was subjected to silica gel column (2.0×40 cm, 230-400 mesh, 75 g) chromatography with $CHCl_3$/MeOH (50:1 → 6:1) and then purified by a second flash silica gel column (1.5×30 cm, 230-400 mesh, 45 g) using a gradient of $CHCl_3$/MeOH [40:1 (80 mL), 30:1 (80 mL), 20:1 (80 mL), 10:1 (80 mL), 5:1 (80 mL)] to yield compounds **1** (9 mg) and **4** (15 mg). The BuOH phase was separated on silica gel (2.8×60 cm, 230-400 mesh, 190 g) using a gradient of $CHCl_3$/MeOH [20:1 (250 mL), 15:1 (200 mL), 10:1 (200 mL), 6:1 (200 mL), 3:1 (200 mL), 1:1 (200 mL)] to give fractions A-F. Fraction D (90 mg) was repeatedly chromatographed over silica gel (1.0×30 cm, 230-400 mesh, 15 g) using $CHCl_3$/MeOH [10:1 (30 mL), 6:1 (30 mL), 3:1 (30 mL), 1:1 (30 mL)] to give compound **2** (16 mg) and fraction E (75 mg) was separately subjected to silica gel column (1.0×30 cm, 230-400 mesh, 12 g) chromatography with the same solvent [12:1 (45 mL), 10:1 (45 mL), 8:1 (45 mL), 4:1 (45 mL), 2:1 (45 mL)] used for **2** and then purified by a second flash silica gel column using a gradient of $CHCl_3$/MeOH to yield compound **5** (17 mg).

Data analysis and curve fitting Each assay was conducted in triplicate 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 (35).

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

Results and Discussion

Total phenolic content The amount of total phenolics were determined by *n*-hexane, EtOAc, and *n*-BuOH fraction and ranged from 96.4 to 964.3 mg GAE/g of the exudate germinating extracts of peanut. The highest total phenolic levels were detected in *n*-BuOH fraction (964.3 mg GAE/g), followed by EtOAc fraction (943.7 mg GAE/g) and hexane fraction (96.4 mg GAE/g) (Fig. 2). These results suggested that higher extraction yields of phenolic compounds were obtained with increasing polarity of the solvent and potent antioxidant activity (36, 37).

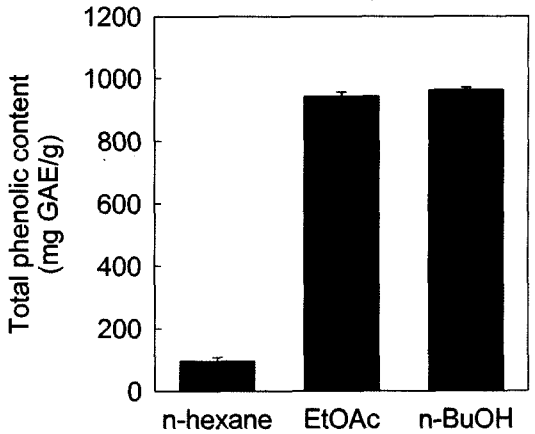

Fig. 2. Total phenolic contents of solvent fractions from the exudate of germinating peanut. All values are mean±SD (n = 3).

Identification of active compounds In connection with our studies on the screening of antioxidants from various natural sources, we found that two fractions exhibited significant antioxidant activities against DPPH (EtOAc: IC_{50} = 17 μg/mL, *n*-BuOH: IC_{50} = 9 μg/mL), ABTS (EtOAc: IC_{50} = 5 μg/mL, *n*-BuOH: IC_{50} = 4 μg/mL), and hydroxyl (EtOAc: IC_{50} > 800 μg/mL, *n*-BuOH: IC_{50} > 800 μg/mL) radicals (Fig. 3). Subsequently, silica gel chromatography and bioassay-guided fractions of the EtOAc and *n*-BuOH

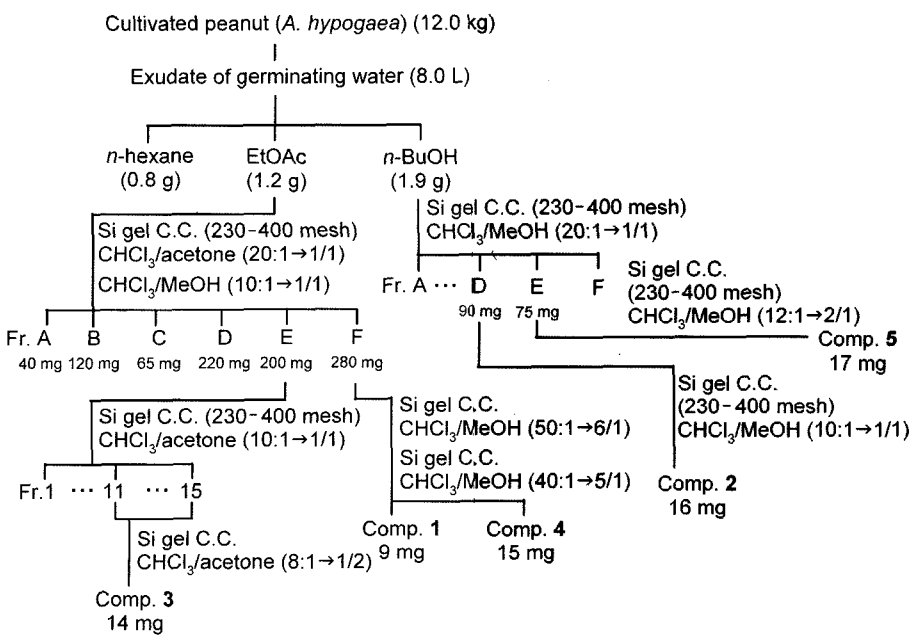

Fig. 1. Isolation of antioxidant metabolites from peanut (*Arachis hypogaea*) germinating exudates.

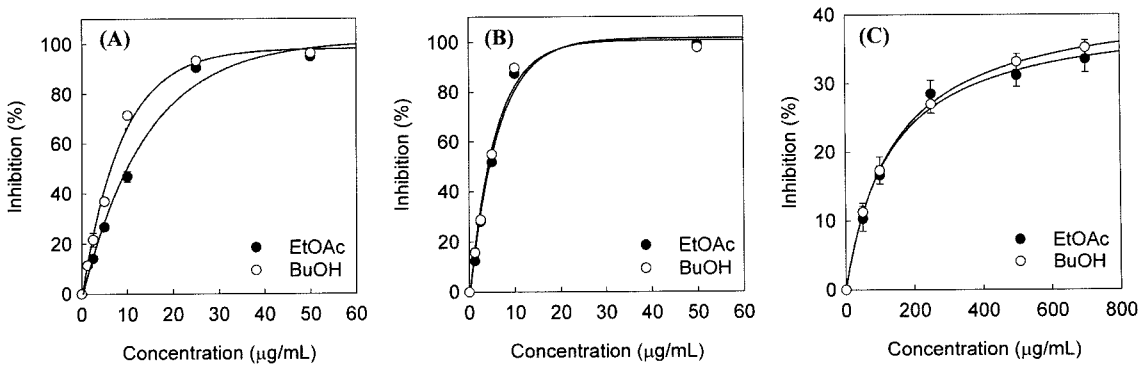

Fig. 3. Antioxidant effects of solvent fractions from the exudate of germinating peanut. A, DPPH radical scavenging activity; B, ABTS radical scavenging activity; C, Hydroxyl radical scavenging activity. All values are mean±SD (n = 3).

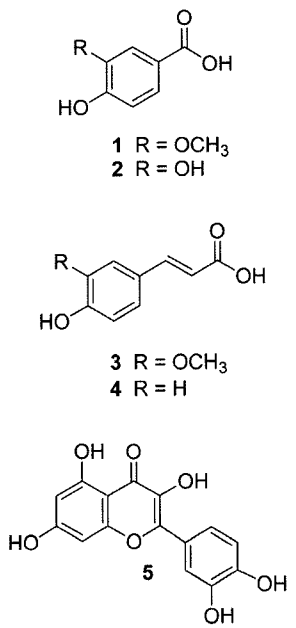

Fig. 4. Structures of isolated compounds 1-5.

layers of peanut germinative extractions yielded five phenolic acids, which were analyzed as physical and spectroscopic data to determine their structures (Fig. 4).

Compound (1): amorphous white powder; mp 297-299°C; EIMS *m/z* (relative intensity) 168 (M^+, 70), 151 (100), 152 (25); IR (KBr) ν_{max} 3414, 1653, 157/cm; UV λ_{max} 305, 262 nm (MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 3.91 (3H, s, OCH_3), 6.86 (1H, d, J = 8.7 Hz, H-5), 7.57 (1H, d, J = 1.9 Hz, H-2), and 7.58 (1H, dd, J = 1.9, 8.7 Hz, H-6). ^{13}C NMR (125 MHz, CD_3OD): see Table 1.

Compound (2): slightly yellow powder; mp 255-257°C; EIMS *m/z* (relative intensity) 154 (M^+ 83), 137 (100), 109 (38), 81 (15), 63 (30); IR (KBr) ν_{max} 3340, 1655/cm; UV λ_{max} 338, 272 nm (MeOH); ^{1}H NMR (300 MHz, CD_3OD) δ 6.89 (1H, d, J = 11.0 Hz, H-5), 7.52 (1H, dd, J = 2.1, 11.0 Hz, H-6), and 7.53 (1H, d, J = 2.1 Hz, H-2). ^{13}C NMR (75 MHz, CD_3OD): see Table 1.

Compound (3): yellow needles; mp 317-320°C; EIMS *m/z* (relative intensity) 194 (M^+, 100), 178, (20), 177 (5); IR (KBr) ν_{max} 3412, 1645/cm; UV λ_{max} 303, 259, 249, 238 nm (MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 3.88 (3H, s, OCH_3), 6.31 (1H, d, J = 15.9 Hz, H-8), 6.82 (1H, d, J = 8.2 Hz, H-5), 7.05 (1H, dd, J = 1.9, 8.2 Hz, H-6), 7.15 (1H, d, J = 1.9 Hz, H-2), and 7.60 (1H, d, J = 15.9 Hz, H-7). ^{13}C NMR (125 MHz, CD_3OD): see Table 1.

Compound (4): slightly yellow powder; mp 210-212°C; EIMS *m/z* (relative intensity) 164 (M^+, 100), 147 (15), 119 (5); IR (KBr) ν_{max} 3420, 1635/cm; UV λ_{max} 330, 280 nm (MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 6.29 (1H, d, J = 15.9 Hz, H-8), 6.83 (2H, d, J = 14.3 Hz, H-3 and H-5), 7.45 (2H, d, J = 14.3 Hz, H-2 and H-6), and 7.62 (1H, d, J = 15.9 Hz, H-7). ^{13}C NMR (125 MHz, CD_3OD): see Table 1.

Table 1. ^{13}C-NMR of compounds 1-5 at 125 MHz (ppm, m)[1)]

Position	Compound				
	1	2	3	4	5
1	122.1 (s)	121.7 (s)	128.2 (s)	125.9 (s)	
2	112.8 (d)	116.4 (d)	112.2 (d)	129.7 (d)	147.9 (s)
3	147.7 (s)	144.6 (s)	149.8 (s)	115.4 (d)	137.4 (s)
4	151.7 (s)	150.1 (s)	150.9 (s)	159.7 (s)	177.3 (s)
5	114.8 (d)	114.5 (d)	116.9 (d)	115.4 (d)	162.6 (s)
6	124.3 (d)	122.8 (d)	124.4 (d)	129.7 (d)	99.5 (d)
7	169.0 (s)	169.2 (s)	147.4 (d)	145.3 (d)	165.7 (s)
8			116.3 (d)	114.2 (d)	94.7 (d)
9			171.4 (s)	169.7 (s)	158.3 (s)
10					104.6 (s)
1					124.3 (s)
2					116.5 (d)
3					146.4 (s)
4					149.0 (s)
5					116.2 (d)
6					121.9 (d)
OCH_3	55.4 (q)		56.9 (q)		

[1)]The chemical shifts of compounds **1-4** were determined in CD_3OD and the chemical shifts of compound **5** was determined in CD_3OD and acetone-d_6.

Compound (5): yellow powder; mp 282-285°C; EIMS *m/z* (relative intensity) 302 (M^+, 100), 273 (8), 153 (10), 137 (15); IR (KBr) ν_{max} 3400, 1640/cm; UV λ_{max} 382, 353, 274 nm (MeOH); ^{1}H NMR (500 MHz, CD_3OD and acetone-d_6) δ 6.21 (1H, d, J = 2.0 Hz, H-6), 6.43 (1H, d, J = 2.0 Hz, H-8), 6.92 (1H, d, J = 8.5 Hz, H-5), 7.67 (1H, dd, J = 8.5, 2.2 Hz, H-6), and 7.77 (1H, d, J = 2.2 Hz, H-2). ^{13}C NMR (125 MHz, CD_3OD and acetone-d_6): see Table 1.

Structural identification of five isolated compounds were carried out by interpretation of several spectroscopic and physical sources, which were readily identified as 4-hydroxy-3-methoxybenzoic acid (**1**), 3,4-dihydroxybenzoic acid (**2**), 4-hydroxy-3-methoxycinnamic acid (**3**), 4-hydroxy-cinnamic acid (**4**), and quercetin (**5**). Compound **1** was obtained as a white powder and in the EIMS, the molecular ion peak showed as *m/z* 180. IR spectrum showed absorption bands at 3410 and 1680/cm, which indicated the presence of hydroxyl and carbonyl moiety, respectively. The ^{1}H-NMR spectrum of compound **1** showed an ABX-type aromatic proton system appearing at δ 7.57 (H-2, d, J = 1.9 Hz), 7.58 (H-6, dd, J = 1.9, 8.7 Hz), and 6.86 (H-5, d, J = 8.7 Hz) as well as one methoxy at δ 3.91. The ^{13}C-NMR and DEPT spectrum showed the presence of eight carbon as three methines [δ 112.8 (C-2), 114.8 (C-5), and 124.3 (C-6)], one methoxy (δ 55.4), and four quaternary carbons [δ 122.1 (C-1), 147.7 (C-3), 151.7 (C-4), and 169.0 (C-7)]. The ^{1}H-^{1}H COSY spectrum of **1** showed one spin-system for this compound. Thus, H-5 at δ 6.86 was correlated to H-6 at δ 7.58, also unassigned connectivities carbonyl group and quaternary carbons were determined on the basis of HMBC correlations. The connectivity between C-1 and H-2/H-5, C-2 and H-6, C-3 and H-2, C-4 and H-6, C-7 and H-2 were determined on the basis of HMBC correlations (Fig. 5). Additionally, methoxy group should be linked to the C-3 of the benzene ring, as indicated by the HMBC correlations of OCH_3 with C-2 and C-3. Therefore, compound **1** was identified as 4-hydroxy-3-methoxybenzoic acid (**1**). Compound **2** was isolated as a white powder and its mass spectrum showed a molecular ion peak at *m/z* 154. IR spectrum showed strong hydroxyl and carbonyl group absorption bands at 3305 and 1680/cm, respectively. The ^{1}H- and ^{13}C-NMR spectra of compound **2** were almost the same as those for except for methoxy group of compound **1**. From these data, the identity **2** was established as 3,4-dihydroxy-benzoid acid (**2**). Compound **3** was obtained yellow amorphous powder, with a molecular ion peak at *m/z* 194, as revealed by EIMS. UV spectrum showed absorption maximum at 330 nm and IR spectrum showed the presence of hydroxyl (3390/cm) and carbonyl (1690/cm) respectively. The exact structures were inferred from a detailed analysis of ^{1}H- and ^{13}C-NMR spectrum, together with 2D-NMR experiments. The ^{1}H-NMR spectrum showed three aromatic protons [δ 7.15 (d, J =1.9 Hz, H-2), δ 7.05 (dd, J = 1.9, 8.2 Hz, H-5), and δ 6.82 (d, J = 8.2 Hz, H-6)], methoxy group δ 3.88 (s, 3-OCH_3) and two doublets at δ 7.60 (H-7) and 6.31 (H-8) which on the basis of the observed large proton-proton coupling constant (J = 15.9 Hz) were assigned to a pair of *trans*-olefinic protons. The ^{13}C-NMR and DEPT spectrum showed the presence of ten carbon as one carbonyl (δ 171.4, C-9), one methoxy (δ 56.9, OCH_3), five methines [δ 112.2 (C-2), δ 116.3 (C-8), δ 116.9 (C-6), δ 124.4 (C-5), and d 147.4 (C-7)], and three quaternary carbons [δ 128.2 (C-1), δ 149.8 (C-3), and δ 150.9 (C-4)]. Also, the ^{1}H-^{1}H COSY spectrum showed correlation peak H-5–H-6, H-2–H-6, and H-7–H-8. Unassigned connectivities of carbonyl and quaternary carbons were determined on the basis of HMBC correlations. The HMBC spectrum showed cross peaks C-1, C-4, and C-9 with H-2/H-5, H2/H-6, and H-7/H-8 (Fig. 5). Additionally, methoxy group also should be linked to the C-3 of the benzene ring, as indicated by the HMBC correlations of OCH_3 with C-2 and C-3, while the HMBC cross peaks C-1–H-7/H-8 and C-2–H-7 indicated the attachment of propenoic acid group at the position C-1 of benzene ring. Thus, based on these data, the identity of compound **3** was 4-hydroxy-3-methoxycinnamic acid (**3**). Compound **4** showed a major ion peak at *m/z* 164 and UV and IR spectrum were very similar to compound **3**. The ^{1}H-NMR of **4** showed *ortho*-coupled doublets of two protons each at δ 6.83 (d, J = 14.3 Hz, H-3 and H-5) and δ 7.45 (d, J = 14.3 Hz, H-2 and H-6), two *trans*-olefinic protons δ 6.29 (1H, d, J = 15.9 Hz, H-8) and 7.62 (1H, d, J = 15.9 Hz, H-7). The ^{13}C-NMR and DEPT spectrum showed the presence of nine carbons and these spectrums were almost identical to compound **3** except for the methoxy group present compound **3**. These data indicated that the identity of compound **4** was 4-hydroxycinnamic acid (**4**). Compound **5** was obtained as yellow powder and a molecular ion peak at *m/z* 302. The IR spectrum showed the presence of hydroxyl (3425/cm), aromatic C=C (1550/cm), and carbonyl (1668/cm). The ^{1}H- and ^{13}C-NMR data with DEPT experiments showed the presence of fifteen carbon atoms as five methins [δ 99.5 (C-6), 94.7 (C-8), 116.2 (C-2), 116.5 (C-5), 121.9 (C-6)], one carbonyl δ 177.3 (C-4), and nine quaternary carbons [δ 137.4 (C-3), 147.9 (C-2), 162.6 (C-5), 165.7 (C-7), 158.3 (C-9), 104.6 (C-10), 124.3 (C-1), 146.4 (C-3), 149.0 (C-4)]. The ^{1}H-NMR data showed evidence for five aromatic protons [δ 6.21 (1H, d, J = 2.0 Hz, H-6), 6.43 (1H, d, J = 2.0 Hz, H-8), 6.92 (1H, d, J =

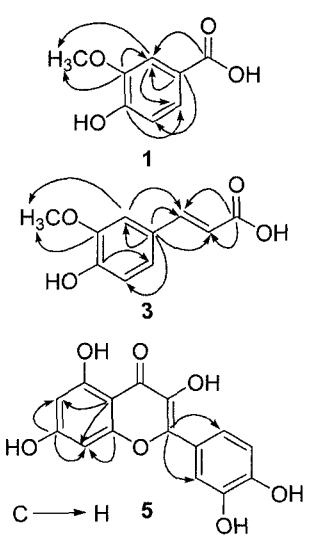

Fig. 5. Important HMBC correlations in compounds 1, 3, and 5.

8.5 Hz, H-5), 7.77 (1H, d, J = 2.2 Hz, H-2), 7.67 (1H, dd, J = 8.5 and 2.2 Hz, H-6)]. Namely, the ^{1}H-NMR spectra of compound **5** showed two *meta*-coupled doublets ascribable to H-8 and H-6 of A-ring in the flavonoid skeleton, and a *meta*-coupled doublet, an *ortho*, *meta*-coupled doublet-doublet and an *ortho*-coupled doublet attributable to H-2, H-6, and H-5 of B-ring, respectively. The connectivity of C-2 and C-9 with H-2/H-6 and H-8 as well as C-10 and C-7 with H-6/H-8 were determined on the basis of HMBC correlations (Fig. 5). These data indicate that compound **5** was quercetin.

Radical scavenging activity of isolated compounds (1-5)

Antioxidant activities of isolated compounds (**1-5**) were tested against three radical sources using UV-VIS spectrophotometry. Although DPPH and ABTS assays are not biologically relevant, both assays were performed as preliminary mean to estimate the direct free radical scavenging abilities of the phenolic compounds. These compounds were also applied to the hydroxyl radical which was regarded as the most reactive oxidant species that may induce severe damage to adjacent biomolecules (38). The radical scavenging activity of phenolic compounds (**1-5**) on the DPPH radical, which can be measured as decolorizing activity following the trapping of the unpaired electron of DPPH, were examined first.

Compounds **2**, **3**, and **5** showed potent antioxidant activities (Table 2), while compounds **1** and **4** did not show any observable activity up to 100 μM and 10±3% inhibition at 200 μM. As shown in Fig. 6, active compounds **2**, **3**, **5**, and BHA, which was used as a positive control, were carried out at dose-dependant inhibitory concentration in DPPH assays.

Compound **5** exhibited the most potent DPPH radical scavenging activities followed by compound **2** with an IC_{50} of 5.0 and 10.4 μM, respectively. Thus, 3,4-dihydroxyl group seems to be important in eliciting potent DPPH radical scavenging activity. The additional methoxyl group at position C-3 in benzene ring and *p*-hydroxyl benzene, as in compounds **1** and **4**, reduces greatly radical scavenging capacity. The data obtained were also compared with those of BHA used as a reference compound. Its IC_{50} was obtained as 31.8 μM (Table 2). The antioxidant activity of **2** and **5** against DPPH radical was 3- and 6-fold more potent than that BHA, respectively. 4-Hydroxycinnamic acid (**4**) did not show radical scavenging activity up to 200 μM, indicating that vicinal diol group is required to elicit the DPPH radical scavenging activity. This *o*-dihydroxyl group in benzene ring structure conferred higher stability in the radical form and participated in electron delocalization. These findings were consistent with those reported in the literature (39, 40). On the basis of DPPH radical scavenging activities, the scavenging ability of the phenolic compounds (**1-5**) against ABTS radical was then evaluated. The formation of the ABTS radical cation takes place almost instantaneously after additing potassium persulfate to an ABTS solution. Compounds **2-5** showed scavenging activities except for compound **1** (IC_{50} > 200 μM). Moreover, quercetin (**5**), 4-hydroxy-3-methoxy-cinnamic acid (**3**) and 3,4-dihydroxybenzoid acid (**2**) showed strong ABTS radical scavenging activity, whereas 4-hydroxycinnamic acid (**4**) showed little scavenging activity. As shown in Fig. 7, isolated compounds **2-5** and Trolox, which was used as a positive control, were tested for a dose-dependant inhibitory effect on ABTS radical. Compounds **2**, **3**, **4**, and **5** showed potent ABTS radical scavenging activities with IC_{50} values of 9.6, 5.5, 21.4, and 3.3 μM, respectively. However, compound **1** did not show any observable activity up to 200 μM. The data obtained were also compared with those of Trolox as a reference compound. Its IC_{50} was obtained as 11.7 μM (Table 2). The antioxidant activity of **3** and **5** against ABTS radical are 2- and 4-fold more potent than that Trolox, respectively. These results also showed that compounds **2** and **5** include the hydroxyl groups in vicinal diol would be effective in ABTS radical scavenging activity. Interestingly, compounds **3** and **4** were more effective scavengers against ABTS radical (**3**: IC_{50} = 5.5 μM and **4**: IC_{50} = 21.4 μM) than DPPH radical (**3**: IC_{50} = 45.2 μM and **4**: IC_{50} > 200 μM) (Fig. 7).

On the other hand, in the hydroxyl radical scavenging effects, isolated compounds **1-5** showed weak scavenging activities (IC_{50} > 200 μM). In our three antioxidant assays, catecholic phenolic compounds **2** and **5** exhibited stronger free radical scavenging activities against DPPH and ABTS than compounds **3** and **4** (Fig. 8). It was observed that *ortho*-dihydroxylated phenolic compounds showed a

Table 2. Antioxidant activities of isolated compounds 1-5 on DPPH, ABTS, and hydroxyl radicals

Compound	IC_{50} (μM)[1)]		
	DPPH	ABTS	Hydroxyl
1	>200	> 200	>200
2	10.4	9.6	>200
3	45.2	5.5	>200
4	>200	21.4	>200
5	5.0	3.3	>200
BHA/ Trolox	31.8 (BHA)	11.7 (Trolox)	20.5 (Trolox)

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

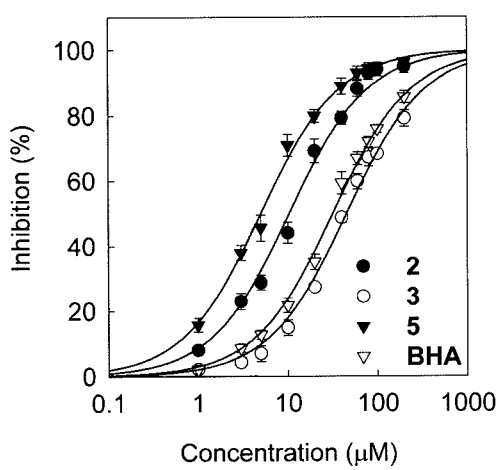

Fig. 6. DPPH radical scavenging activities of compounds 2, 3, 5, and BHA.

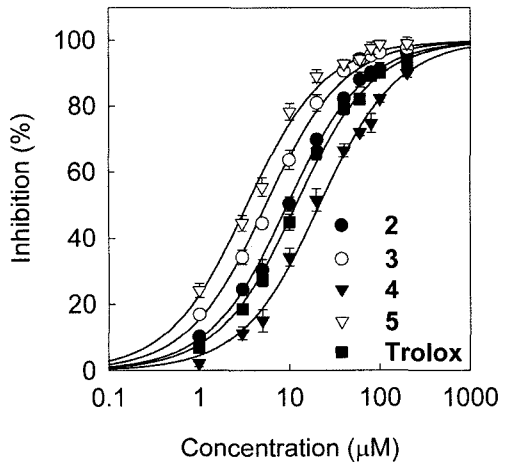

Fig. 7. ABTS radical scavenging activities of compounds 2-5 and Trolox.

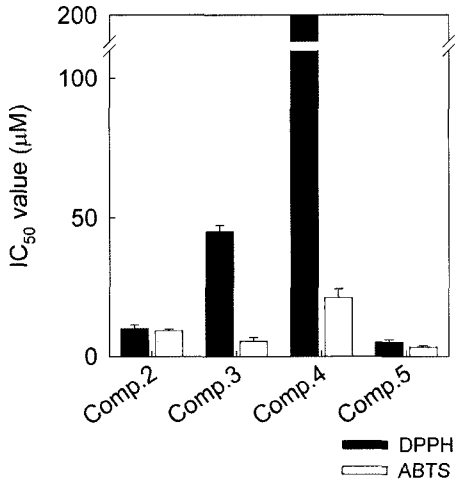

Fig. 8. IC_{50} values of compounds 2-5 on DPPH and ABTS radical.

markedly higher ability to scavenge DPPH and ABTS radicals, in comparison with the non-*ortho*-dihydroxylated phenolic compounds.

In conclusion, there is no doubt that the peanut is the most important product of nut. In comparison with the nut, the germinating exudates are almost completely neglected in commercial terms, although it is shown potent antioxidant activities and there are considerable potential for its exploitation. We isolated and identified five phenolic compounds were isolated for the first time by antioxidant-guided fractionation from the exudate of geminating peanut. Among them, three compounds **2**, **3**, and **5** showed potent antioxidant activities in DPPH as well as ABTS assays. However, all 5 compounds demonstrated only weak hydroxyl radical scavenging activity. The results obtained here indicate that further evaluation of the bioactive properties of these compounds is needed.

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