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Original paper

Antioxidative Constituents in the Leaves of *Paeonia anomala* Grown in Mongolia

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We successfully identified the main constituents responsible for the high antioxidant properties of the leaves of *Paeonia anomala* (*Paeoniaceae*) for the first time. *P. anomala* is an endemic plant widely found throughout Mongolia, and its air-dried leaves are main ingredients in some kinds of herbal teas. Infusions of *P. anomala* leaves are also used as home remedies for the treatment of liver and kidney ailments by the local population. Three major antioxidative constituents were isolated from the ethyl acetate layer of a *P. anomala* leaf ethanol extract via 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay-guided fractionation, consisting of liquid-liquid partition, two types of open column chromatography and reversed-phase HPLC. Chemical structures of the purified compounds were identified using LC-MS, ^{1}H-NMR, and ^{13}C-NMR as methyl gallate, pentagalloylglucose, and tellimoside. The identified compounds contributed to 35% of the total DPPH radical scavenging activity of the *P. anomala* leaf ethanol extract.

Keywords: *Paeonia anomala* leaf, antioxidant constituent, methyl gallate, pentagalloylglucose, tellimoside

Introduction

Paeonia is the single genus in the family *Paeoniaceae*, which consists of approximately 35 species. Many species of this genus have been used in traditional folk medicine (Wu *et al*., 2010).

Two species of *Paeonia, P. anomala* and *P. albiflora,* grow wild in Mongolia. According to ancient Mongolian prescriptions, the leaves and roots are used for their anti-diuretic and high blood pressure alleviating effects. *P. albiflora* is a rare and endangered medicinal plant in Mongolia. *P. anomala* (peony) is widely distributed from central China to the Kola Peninsula of Russia, including northeast Kazakhstan and northern Mongolia (Hong *et al*., 2004). In Mongolia, the peony grows in the area that falls within the longitudes 110°00'E-34°20'W and from the latitude 46°50'N and northward. Air-dried peony leaves are the main ingredients of some kinds of herbal teas. Moreover, infusions prepared from peony leaves are utilized as home remedies for the treatment of liver and kidney ailments by the local population.

Over the past six decades, approximately 262 compounds have been isolated from different parts of *Paeonia* species, such as the flower, seed, leaf, root, root cortex, and rhizome. Crude extracts prepared from different parts of various *Paeonia* species and their chemical constituents have shown the following biological activities: antioxidant, anti-tumor, anti-mutagenic, anti-pathogenic, anti-coagulative, anti-inflammatory, anti-osteoporotic, anti-atherosclerotic, anti-allergy, hypoglycemic, sedative, and urea-nitrogen decreasing effects (Wu *et al*., 2010; He *et al*., 2010).

Data on the antioxidant activity of *Paeonia* species has been reported. Among 100 plants screened for cosmetic use, *P. suffruticosa* bark was included in 14 plants described as potential sources of antioxidants against lipid peroxidation and DPPH radicals (Kim *et al*., 1997). *P. suffruticosa* exhibited strong DPPH radical scavenging activity, and galloyl glucose was isolated as the active compound. In addition, *P. suffruticosa* showed superoxide dismutase-like activity (Kim *et al*., 1997). Water extracts of *P. lactiflora* and *P. suffruticosa* had significant oxygen radical absorbance capacity (Liao *et al*., 2008). Lee *et al*. (2005) described

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that the ethanol extract of *P. lactiflora* root may be a useful antigenotoxic antioxidant by scavenging DPPH radicals, inhibiting lipid peroxidation and protecting against oxidative DNA damage without exhibiting any pro-oxidant effect. Some bioactive compounds with antioxidant potential such as galloylpaeoniflorin, galloyloxypaeoniflorin, suffruticosides, and pentagalloylglucose were isolated from the root, root cortex, and fruit of *Paeonia* species (Wu *et al*., 2010; He *et al*., 2010).

Among 35 *Paeonia* species, *P. lactiflora*, *P. suffruticosa*, *P. veitchii*, and *P. albiflora* have been the focus of the majority of research to date. However, the biological activities and phytochemicals of some species including *P. anomala* remain poorly understood. Moreover, while most of the literature has focused on the chemical components and bioactivities of the roots of *Paeonia* species, a few works have been devoted to the leaves. For instance, a methanol extract of *P. peregrina* leaves exhibited slight anti-cholinergic and anti-serotonin activities (He *et al*., 2010). *P. suffruticosa* leaves were the source of gallo- and condensed tannins that showed nematicidal activity (Mohamed *et al*., 2000). Quercetin 3-galacto-7-rhamnoside, gallic acid, pentatriacontane, and tetratriacontanol were detected in *P. decora* leaves, whereas foeniculin, quercetin 3-*β*-D-galactopyranoside, quercetin 3-*β*-D-glucopyranoside, and paeoniflorin were isolated from *P. tenuifolia* leaves. Furthermore, three monoterpenoid glycosides (benzoylpaeoniflorin, oxypaeoniflorin, and albiflorin) were identified in *P. lactiflora* var. *trichocarpa* leaf along with its root, flower, and stem (He *et al*., 2010).

We previously reported a screening study on the antioxidant activity of leaves from eight plants growing wild in Mongolia (Enkhtuya *et al*., 2014). From the results, we found that peony leaf was the most effective candidate for further study. In this paper, we aimed to elucidate the most active antioxidative constituents in peony leaf.

Material and Methods

General Spectrophotometric measurements were carried out using a UV-Vis spectrophotometer UV mini 1240 (Shimadzu, Kyoto, Japan). The HPLC system was comprised of an L-7100 pump (Hitachi, Tokyo, Japan), L-7405 UV detector (Hitachi), and D-2500 Chromato-integrator recorder (Hitachi). ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR spectra were recorded in methanol-d_4 with a JNM-ECA 500 instrument (JEOL, Tokyo, Japan) at 500 MHz for ^{1}H and 125 MHz for ^{13}C, using tetramethylsilane as an internal standard. Letters (br.)s, d, t, q, and m represent (broad)singlet, doublet, triplet, quartet, and multiplet, respectively, and coupling constants are expressed in Hz. LC-MS data were recorded with a Bruker HCT-KOC mass spectrometer (Berlin, Germany) by using the direct injection method. Optical rotation was measured with a HORIBA SEPA-200 spectropolarimeter (Kyoto, Japan).

Chemicals All chemicals were of the highest commercial grade and were used without further purification.

Plant material Fresh peony leaves were collected in Khuvsgul province of Mongolia in the beginning of August, 2014. The plant was identified by its vernacular name and validated at the Institute of Biology, Mongolian Academy of Science. Well-grown and healthy leaves were collected without petioles, air-dried at room temperature in the shade to constant weight, powdered using a laboratory mill, and then sifted through a mesh 0.5 − 1 mm in size.

Preparation of crude extract To isolate and identify the antioxidative constituents, powdered peony leaf (50 g) was extracted with 50% (v/v) ethanol/water (1000 mL) with constant mixing by magnetic stirrer for 2 h at room temperature. The suspension was centrifuged using a refrigerated centrifuge (18PR-52; Hitachi) at 6,800 × g for 20 min at 4℃, and the supernatant was stocked. This procedure was repeated twice and the supernatants were pooled, vacuum filtered through No. 5C filter paper (Advantec Toyo, Tokyo, Japan), evaporated under reduced pressure at 37℃ to remove the solvent, and then freeze-dried.

DPPH radical scavenging assay The scavenging activity of samples, including the peony leaf ethanol extract, its fractions, and reference compounds, against DPPH radicals was determined as described by Adedapo *et al*. (2009) with minor modifications. A 2-mL aliquot of 0.135 mM DPPH (Wako Pure Chemical Industries, Osaka, Japan) in methanol was mixed with 100 μL of sample diluted with methanol. After 30 min in the dark at room temperature, absorbance of the reaction mixture was measured at 517 nm. The DPPH radical solution was freshly prepared daily and kept in the dark during measurements. Results were reported as IC_{50} values, which is the amount of antioxidant necessary to scavenge the initial DPPH radical concentration by 50%. To investigate the IC_{50} values, the dose-response relationship was studied and the capacity to scavenge DPPH radicals was expressed as a percentage according to the following equation:

$$\text{DPPH scavenging capacity (\%)} = \left(1 - \frac{\text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}}\right) \cdot 100 \quad \cdots\cdots \text{Eq. 1}$$

Known standard antioxidants, namely L (+)-ascorbic acid (Wako Pure Chemical Industries), quercetin hydrate (Tokyo Chemical Industry, Tokyo, Japan), butylated hydroxytoluene (BHT, Nacalai Tesque, Kyoto, Japan), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich, St. Louis, MO), were used as reference compounds.

Isolation of antioxidative compounds from peony leaf ethanol extract The initial fractionation of the freeze-dried ethanol extract obtained from peony leaf involved liquid-liquid partitioning. The ethanol extract (21.26 g) was suspended in 600 mL of distilled water, and then successively extracted with hexane (420 mL × 3, 0.16 g), diethyl ether (420 mL × 3, 2.41 g), ethyl acetate (420 mL × 3, 5.35 g), and water-saturated butanol (420 mL × 3, 5.23 g). The total amount of the remaining aqueous layer was 7.82 g. The resulting five fractions were evaporated under reduced pressure at

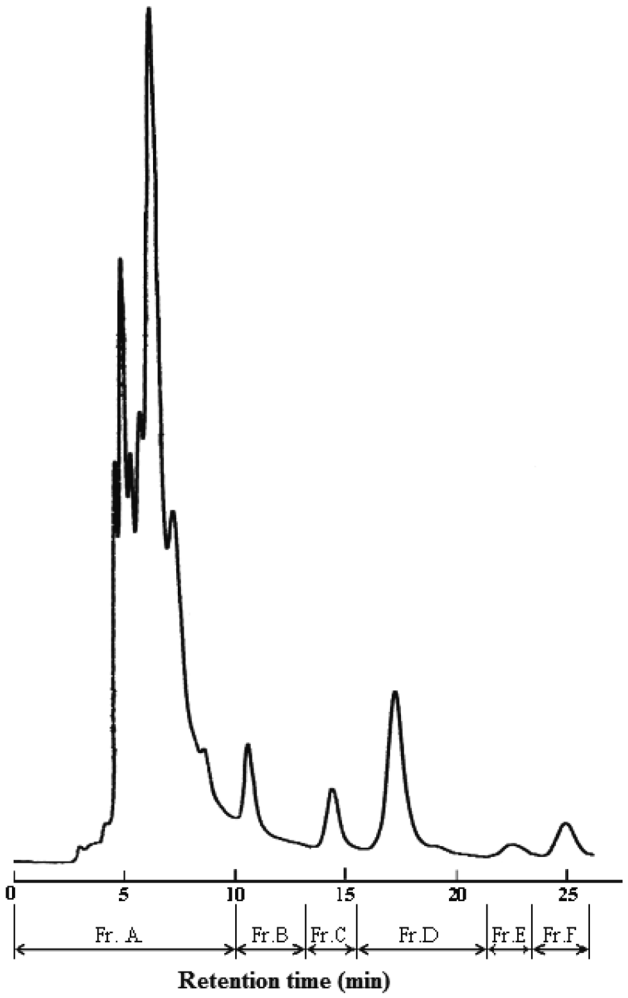

Fig. 1. HPLC chromatogram of the 50% methanol fraction from ODS column. Column- COSMOSIL $5C_{18}$-MS-II, 250 × 10 mm; column temperature- 30℃; mobile phase- 45% methanol/water; flow rate- 3 mL/min; detection- 254 nm; sample injection volume- 300 µL

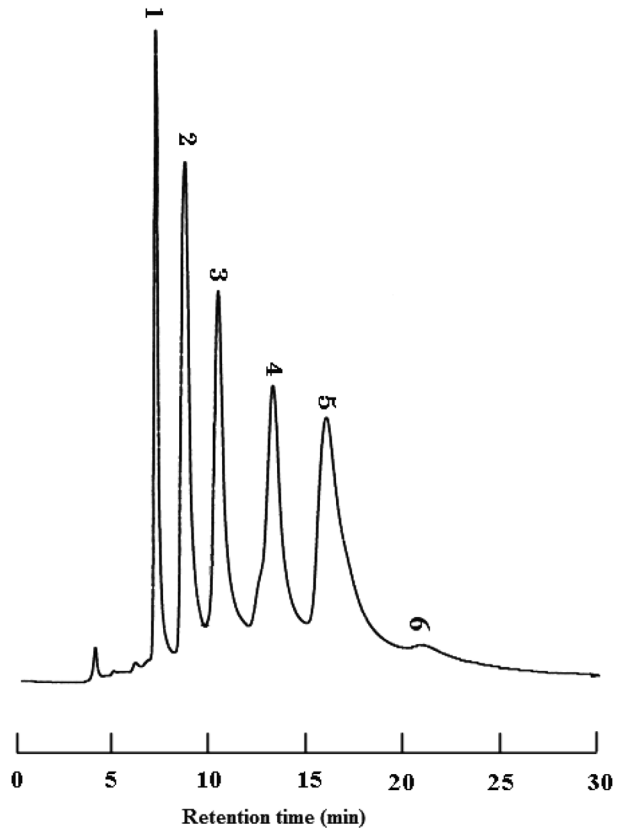

Fig. 2. HPLC chromatogram of the fraction A. Column- COSMOSIL $5C_{18}$-MS-II, 250 × 10 mm; column temperature- 30℃; mobile phase- 40% methanol/water; flow rate- 3 mL/min; detection- 254 nm; sample injection volume- 300 µL

37℃ to dryness and subjected to the DPPH assay. The most active ethyl acetate fraction (5.00 g) was poured onto a silica gel open column (360 mm × 40 mm i.d., Wako gel C-300, 45 − 75 µm; Wako Pure Chemical Industries) and eluted with hexane containing increasing proportions of ethyl acetate: 30% ethyl acetate (0.03 g), 50% ethyl acetate (0.11 g), 70% ethyl acetate (0.14 g), and 100% ethyl acetate (4.00 g), consecutively. The remaining compounds in the column were eluted with 100% methanol (0.70 g). The most potent 100% ethyl acetate fraction (3.51 g) was then subjected to a Chromatorex ODS open column (360 mm × 40 mm i.d., 100 − 200 mesh; Fuji Silysia Chemical, Aichi, Japan) and eluted with ten bed volumes of 30% methanol/water (0.41 g), 50% methanol/water (2.35 g), 70% methanol/water (0.03 g), and 100% methanol (0.07 g). Next, the eluate from 50% methanol was repeatedly separated into six fractions, A (*Rt*=0 − 10 min), B (*Rt*=10 − 13.2 min), C (*Rt*=13.2 − 15.5 min), D (*Rt*=15.5 − 21 min), E (*Rt*=21 − 23.3 min), and F (*Rt*=23.3 − 26 min), by reversed-phase HPLC (COSMOSIL $5C_{18}$-MS-II column, 250 × 10 mm i.d., Nacalai Tesque), eluting with 45% methanol/water at a flow rate of 3 mL/min and UV detection at 254 nm (Fig. 1). The DPPH radical scavenging activity of all obtained fractions was assessed. Fraction A was divided into seven sub-fractions, A-1 (*Rt*=0 − 6.9 min), A-2 (*Rt*=6.9 − 8 min), A-3 (*Rt*=8 − 9.9 min), A-4 (*Rt*=9.9 − 12.1 min), A-5 (*Rt*=12.1 − 15 min), A-6 (*Rt*=15 − 20 min), and A-7 (*Rt*=20 − 24 min), by HPLC performed using the same column at 3 mL/min flow rate, eluting with 40% methanol/water (Fig. 2). Compounds **1**, **2**, and **3** were isolated at *Rt*=7.3 min, *Rt*=8.8 min and *Rt*=10.5 min, respectively, from fraction A. All samples were adequately diluted with the corresponding eluent and filtered through a 0.45 µm membrane filter (Millipore, Tokyo, Japan) prior to injection to HPLC.

*Compound **1** (methyl gallate, methyl 3,4,5-trihydroxybenzoate)* LC-MS ESI (Positive) *m/z*: 185 $[M + H]^+$, ESI (Negative) *m/z*: 153 $[M - OCH_3]^-$; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 7.02 (2H, s, H-2 and 6), 3.92 (3H, s, -OCH_3); ^{13}C-NMR (CD_3OD, 125 MHz): δ_C 167.7 (C=O, C-7), 145.15 (C, C-3 and 5), 138.45 (C, C-4), 120.07 (C, C-1), 108.69 (CH, C-2 and 6), 51.0 (CH_3, -OCH_3), (calculated for $C_8H_8O_5$).

*Compound **2** (pentagalloylglucose, 1,2,3,4,6-penta-O-galloyl-β-D-glucopyra-nose)* $[\alpha]_D^{20}$ +22.5° (c 0.4, acetone); LC-MS ESI (Positive) *m/z*: 963 $[M + Na]^+$, ESI (Negative) *m/z*: 793 $[M - Gall]^-$; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 7.11 (2H, s, 6-Gall-H-2 and -6), 7.05 (2H, s, 1-Gall-H-2 and -6), 6.98 (2H, s, 4-Gall-H-2 and -6), 6.95 (2H, s, 2-Gall-H-2 and -6), 6.90 (2H, s, 3-Gall-H-2

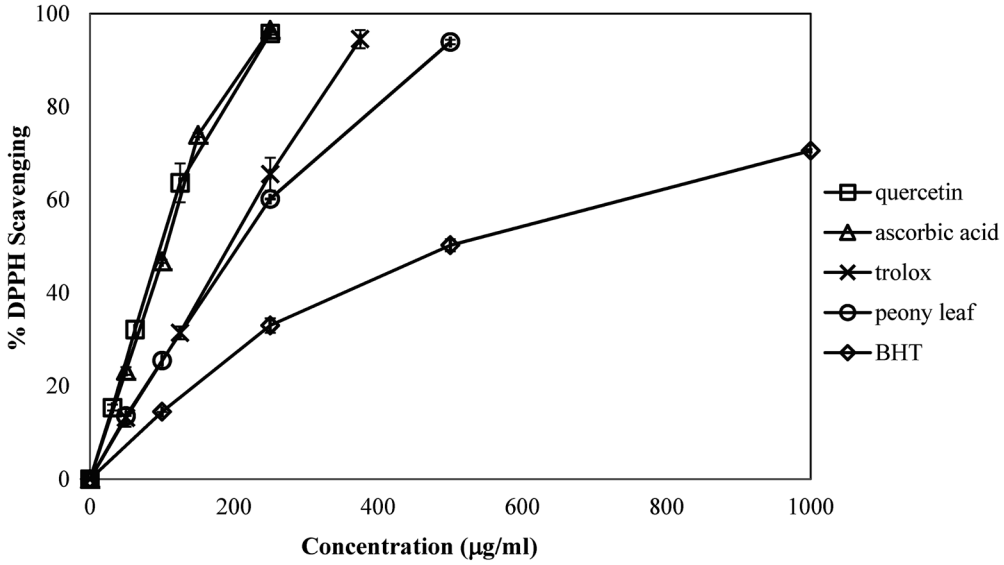

Fig. 3. Dose-response curve of DPPH radical scavenging activity for the peony leaf ethanol extract and references. Values= mean ± standard deviation (n=3).

and -6), 6.24 (1H, d, *J* = 8.1 Hz, H-1), 5.91 (1H, dd, *J* = 9.7 and 9.7 Hz, H-3), 5.61 (1H, dd, *J* = 9.7 and 9.7 Hz, H-4), 5.58 (1H, dd, *J* = 9.7 and 8.0 Hz, H-2), 4.51 (1H, d, *J* = 11.0 Hz, H-6_a), 4.41 (1H, dd, *J* = 9.7 and 4.0 Hz, H-5), 4.39 (1H, dd, *J* = 11.5 and 4.0 Hz, H-6_b); ^{13}C-NMR (CD_3OD, 125 MHz): δ_C 164.94, 165.62, 16 5.73, 166.01, 166.65 (C=O, Gall-C-7), 144.96, 145.06, 145.11, 145.14, 145.23 (C, Gall-C-3, Gall-C-5), 139.01 (C, Gall-C-4), 118.39, 118.87, 118.90, 119.72 (C, Gall-C-1), 109.06, 109.17, 109.34 (CH, Gall-C-2, Gall-C-6), 92.51 (CH, C-1), 73.07 (CH, C-5), 72.80 (CH, C-3), 70.88 (CH, C-2), 68.47 (CH, C-4), 61.79 (CH_2, C-6), (calculated for $C_{41}H_{32}O_{26}$).

*Compound **3** (tellimoside, quercetin 3-O-(6''-O-galloyl)-β-D-glucopyranoside)* $[\alpha]_D^{20}$ +18.2° (c 0.4, acetone); LC-MS ESI (Positive) *m/z*: 639 $[M + Na]^+$, 655 $[M + K]^+$, ESI (Negative) *m/z*: 303 $[M - Gall - Glu + H_2O]^-$; ^{1}H-NMR ($CD_3OD$, 500 MHz): δ_H 7.54 (1H, dd, *J* = 8.5 and 2.0 Hz, H-6'), 7.52 (1H, brs, H-2'), 6.93 (2H, s, H-2''', H-6'''), 6.70 (1H, d, *J* = 8.5 Hz, H-5'), 6.32 (1H, brs, H-8), 6.16 (1H, brs, H-6), 5.19 (1H, d, *J* = 7.5, H-1''), 4.33 (1H, dd, *J* = 5.0 and 7.0 Hz, H-6''), 4.25 (1H, dd, *J* = 1.5 and 12.0 Hz, H-6''), 3.49 (1H, m, H-2''), 3.43-3.48 (3H, m, H-3'', H-4'', H-5''); ^{13}C-NMR (CD_3OD, 125 MHz): δ_C 179.6 (C=O, C-4), 168.5 (C=O, C-7'''), 166.2 (C, C-7), 162.8 (C, C-5), 159.6 (C, C-2), 158.6 (C, C-9), 150.0 (C, C-4'), 146.6 (C, C-3''', 5'''), 146.1 (C, C-3'), 140.0 (C, C-4'''), 135.6 (C, C), 123.8 (CH, C-6'), 123.3 (C, C-1'), 121.5 (C, C-1'''), 117.5 (CH, C-2'), 116.2 (CH, C-5'), 110.4 (CH, C-2''', 6'''), 105.8 (C, C-10), 104.5 (CH, C-1''), 100.2 (CH, C-6), 95.1 (CH, C-8), 78.3 (CH, C-3''), 76.1 (CH, C-5''), 75.9 (CH, C-2''), 71.7 (CH, C-4''), 64.5 (CH_2, C-6''), (calculated for $C_{28}H_{24}O_{16}$).

Quantification of identified antioxidative compounds Quantitative analysis of the identified constituents from peony leaf was performed by HPLC. A COSMOSIL 5C_{18}-MS-II (150 mm × 4.6 mm i.d.) column was used. The samples were eluted using 40% methanol/water as the mobile phase at a flow rate of 1 mL/min. The column temperature was maintained at 30℃ and the injection volume was 10 μL. Peaks of the compounds were monitored at 254 nm. The known antioxidative constituents present in peony leaf were identified by comparing their retention times with reference compounds. Pentagalloylglucose and tellimoside purified from this study were used as authentic samples. A calibration curve was plotted using peak areas from chromatograms against a known concentration of the reference. Results were expressed as mg/g of the ethanol extract on a dry weight basis.

Statistics All results are expressed as mean ± standard deviation of at least three determinations. A *p*-value < 0.05 was considered as statistically significant.

Results and Discussion

According to our previous results, the 50% ethanol extract of peony leaf showed remarkable scavenging activity against DPPH radicals, ABTS radical cations and superoxide anions, and exerted significant ferric reducing antioxidant power. The extract also contained a considerable amount of phenolics (Enkhtuya *et al*., 2014).

DPPH radical scavenging activity The IC_{50} value of the peony leaf ethanol extract against DPPH free radicals was determined using the dose-response relationship in comparison to ascorbic acid, quercetin, Trolox, and BHT (Fig. 3). In this assay condition, the ethanol extract was more effective in DPPH radical scavenging (IC_{50}=206 ± 2 μg/mL) than BHT (IC_{50}=499 ± 3 μg/mL), whereas it was less effective than quercetin (IC_{50}=98 ± 4 μg/mL) and ascorbic acid (IC_{50}=106 ± 1 μg/mL). Moreover, the ethanol extract showed similar potency to Trolox, with an IC_{50} of 191 ± 3 μg/mL.

Isolation and purification guided by DPPH assay The peony leaf ethanol extract was fractionated by liquid-liquid partitioning, two types of open column chromatography, and multi-steps of

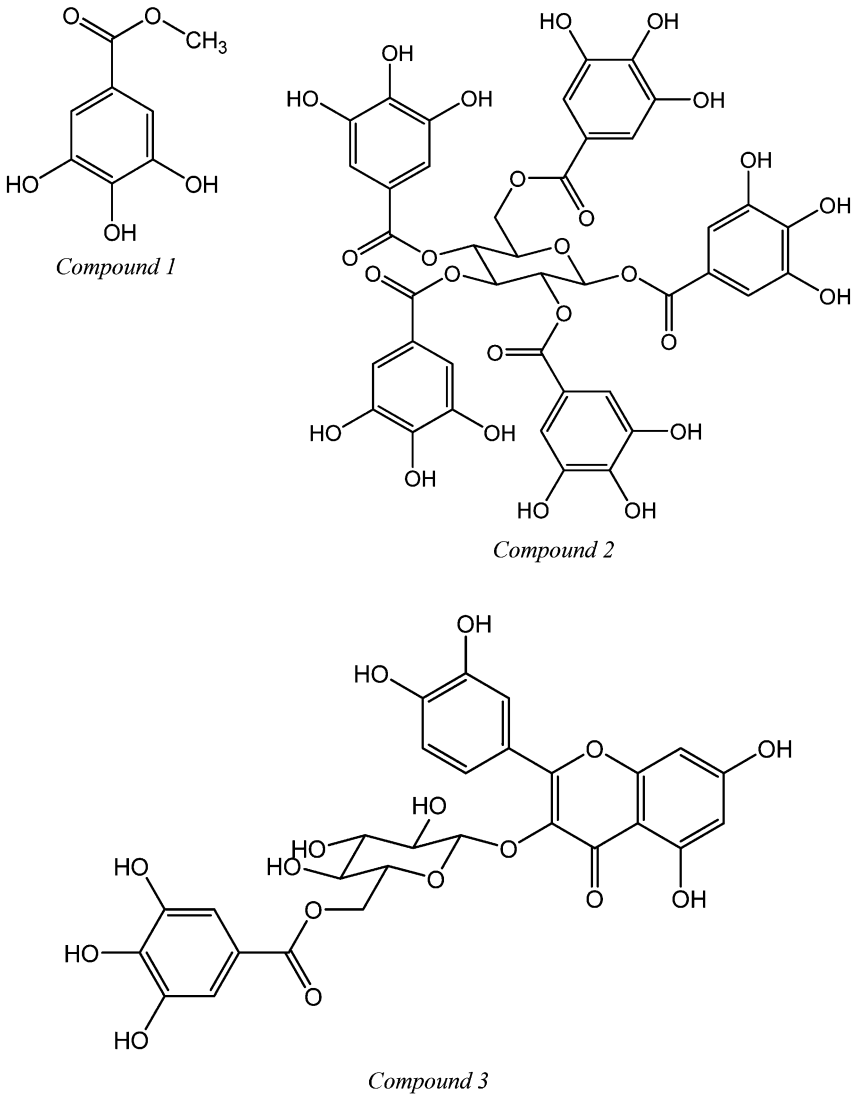

Fig. 4. Chemical structures of the antioxidative compounds identified in peony leaf. Compound 1- methyl gallate; Compound 2- pentagalloylglucose; Compound 3- tellimoside

reversed-phase HPLC and monitoring by DPPH assay, leading to the isolation of three antioxidative compounds. The most active fraction was chosen for further fractionation.

Firstly, the ethanol extract was sequentially partitioned using organic solvents with different polarities to generate hexane, diethyl ether, ethyl acetate, butanol, and water fractions. Among these five fractions, the ethyl acetate fraction (IC_{50}=89 ± 0 μg/mL) showed the highest activity followed by diethyl ether (IC_{50}=172 ± 3 μg/mL), butanol (IC_{50}=748 ± 14 μg/mL), and water (IC_{50} > 2 mg/mL) fractions. The hexane fraction did not show any activity against DPPH radicals. Antioxidant capacities of the ethyl acetate and diethyl ether fractions were higher than that of the original ethanol extract (IC_{50}=206 ± 2 μg/mL).

The most active ethyl acetate fraction was then separated on an open silica gel column, eluting with increasing proportions of ethyl acetate (30 − 100%) in hexane and finally with 100% methanol. The 100% ethyl acetate fraction (IC_{50}=105 ± 1 μg/mL) exhibited potent free radical scavenging activity in a concentration-dependent manner, followed by 70% ethyl acetate (IC_{50}=115 ± 3 μg/mL), 100% methanol (IC_{50}=129 ± 2 μg/mL), and 50% ethyl acetate (IC_{50}=367 ± 1 μg/mL) fractions. The 30% ethyl acetate fraction showed almost no potency against DPPH radicals. Indeed, the potential to scavenge DPPH radicals increased with later elution.

Next, the most active 100% ethyl acetate fraction was applied to a Chromatorex ODS open column and eluted with 30%, 50%, 70%, and 100% methanol/water. The 50% methanol fraction was the most effective DPPH radical scavenger. The IC_{50} value for this fraction was 52 ± 1 μg/mL. A slightly weaker activity was observed for the 30% methanol fraction (IC_{50}=79 ± 1 μg/mL). High IC_{50} values were observed for 70% methanol (IC_{50}=213 ± 5 μg/mL) and 100% methanol (IC_{50} > 1 mg/mL) fractions.

Among the Chromatorex ODS fractions, the 50% methanol fraction was separated into six fractions by reversed-phase HPLC according to the retention time (Fig. 1). Among these six fractions (A, 0 − 10 min; B, 10 − 13.2 min; C, 13.2 − 15.5 min; D, 15.5 − 21 min; E, 21 − 23.3 min; F, 23.3 − 26 min), fraction A showed the

highest activity against DPPH radicals (IC_{50}=71 ± 2 μg/mL). Lower activities were observed with late eluting fractions as follows: B, 96 ± 2 μg/mL; C, 103 ± 1 μg/mL; D, 154 ± 3 μg/mL; E, 211 ± 5 μg/mL; F, 385 ± 5 μg/mL. These results suggested that the DPPH radical scavenging activity of the HPLC fractions decreased with decreasing polarity. Thus, the peony leaf may contain polar antioxidative compounds. Finally, the most active fraction A was further separated using 40% methanol/water as the mobile phase to afford individual compounds (Fig. 2). Compounds **1**, **2**, and **3** were isolated at *Rt*=7.3 min, *Rt*=8.8 min and *Rt*=10.5 min, respectively, from fraction A.

Identification of the purified compounds According to ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & 135, ^{1}H-^{1}H COSY, HMQC, and HMBC spectra of each purified compound, the chemical structure was identified and compared with the 1D- and 2D-NMR spectral data published in the literature. Chemical structures of the identified compounds in peony leaf are shown in Fig. 4.

Compound **1**, a pale pink amorphous powder, was identified as methyl gallate (methyl 3,4,5-trihydroxybenzoate; gallicin), which is a simple phenolic compound broadly found throughout the plant kingdom. The ^{1}H- and ^{13}C-NMR, and LC-MS data of this compound were identical to that previously published (Kane *et al*., 1988). Methyl gallate, along with many other phenolic compounds, was isolated from multiple plants as an antioxidant, anti-tumor agent, lipid peroxidation inhibitor, enzyme inhibitor, anti-viral agent, anti-microbial agent, anti-protozoal agent, and inhibitor of nitric oxide production by various researchers (Chaubal *et al*., 2005). Moreover, methyl gallate complexes metal ions, including iron and copper (Kane *et al*., 1988), and also protects mammalian and bacterial cells from cytotoxicity induced by hydroperoxides (Chaubal *et al*., 2005). Lee *et al*. (2005) described that the ethanol extracts of *P. lactiflora* root and its active substitutes, gallic acid and methyl gallate, exhibited significant DPPH radical scavenging activity and had an inhibitory effect on lipid peroxidation, as well as a protective effect against oxidative DNA damage. Furthermore, methyl gallate was detected in the root cortex of *P. decomposita* and *P. delavayi*, and *P. suffruticosa* fruit and root cortex (He *et al*., 2010; Xu *et al*., 2006). A methanol extract of *P. suffruticosa* showed potent inhibitory activity against rat intestinal sucrose, and methyl gallate was identified as the active principle (Chaubal *et al*., 2005).

Compound **2** was obtained as a pale purple amorphous powder. According to ^{1}H- and ^{13}C-NMR, and LC-MS data, which were identical to the literature (Khanbabaee *et al*., 1997; Cho *et al*., 2010; Jeon *et al*., 2006), this compound was identified as pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose), a hydrolysable tannin. The distribution of pentagalloylglucose in 70 kinds of plants was observed (Zhang *et al*., 2009). In the genus *Paeonia,* to date it has been reported in *P. lactiflora* root, *P. lactiflora* var. *trichocarpa* root, *P. suffruticosa* root cortex, *P. decomposita* root cortex, *P. delavayi* root cortex and *P. suffruticosa* fresh leaf (He *et al*., 2010; Xu *et al*., 2006). Pentagalloylglucose was isolated from *P. lactiflora* root as a constituent with urea-nitrogen decreasing effects (Wu *et al*., 2010). Both methyl gallate and pentagalloylglucose isolated from the ethyl acetate fraction of *P. suffruticosa* root bark showed potent anti-microbial activity (Chaubal *et al*., 2005). Furthermore, pentagalloylglucose has been shown to exert various biological effects, such as antioxidant (He *et al*., 2010; Zhang *et al*., 2009), anti-platelet (Jeon *et al*., 2006), *a*-glucosidase inhibitory (Cannell *et al*., 1988), anti-cancer, anti-diabetic, anti-mutagenic, anti-inflammatory, anti-allergy, anti-virus and so on (Zhang *et al*., 2009).

Compound **3**, a yellow powder, was assigned as quercetin 3-*O*-(6"-*O*-galloyl)-*β*-D-glucopyranoside (tellimoside), a galloylated flavonoid glycoside. The ^{1}H- and ^{13}C-NMR, and LC-MS data of this compound were identical to those of tellimoside in the literature (Masuda *et al*., 2001; Smolarz, 2002; Sohretoglu *et al*., 2009a). The large coupling constants (*J*) for the anomeric proton signals at 5.19 (d, *J*=7.5 Hz) of this compound indicated that the glucose moiety was a *β*-anomer. In the genus *Paeonia*, tellimoside has been identified in the yellow flowers of several species of tree peony (a woody shrub of the section *Moutan*) (Li *et al*., 2009). This compound showed free radical scavenging activity (Masuda *et al*., 2001) and lipid peroxidation inhibiting activity (Sohretoglu *et al*., 2009a, 2009b; Zielinska *et al*., 2001).

As described above, compounds **1**, **2**, and **3** were already known. However, this study was the first to demonstrate the presence of these compounds in peony leaf as antioxidant constituents.

Peaks 4, 5, and 6 in Fig. 2 may be supermolecules consisting of methyl gallate and pentagalloylglucose based on NMR and HPLC analyses. In ^{13}C-NMR spectra, all divided peaks 4, 5, and 6 showed 30 carbon signals of the same chemical shifts as methyl gallate and pentagalloylglucose. Based on the ratio of the integrated value for the signals of methyl group protons (about 3.90 ppm) in methyl gallate and of anomeric protons (about 6.24 ppm) in pentagalloylglucose (peak 4, 6:3) in the ^{1}H-NMR, peak 4 contained 2 mol of methyl gallate and 3 mol of pentagalloylglucose. When the isolated peak 4 was analyzed by HPLC, peaks 5 and 6 were detected, in addition to itself, methyl gallate and pentagalloylglucose. Similarly, peak 5 (ratio of the integral value, 24:9) and peak 6 (ratio of the integral value, 6:1) consisted of 8 mol of methyl gallate and 9 mol of pentagalloylglucose, and of 2 mol of methyl gallate and 1 mol of pentagalloylglucose, respectively.

DPPH radical scavenging activity of the identified compounds The identified compounds were assayed using the DPPH assay, and their activities were compared with standard antioxidants. The IC_{50} values of methyl gallate, pentagalloylglucose, and tellimoside isolated from peony leaf against DPPH radicals were 69 ± 1 μg/mL (373 ± 5 μM), 85 ± 1 μg/mL (91 ± 1 μM), and 113 ± 2 μg/mL (184 ± 4 μM), respectively. Both methyl gallate and pentagalloylglucose showed more potent DPPH radical scavenging

Table 1. Contribution of the identified compounds to total antioxidant activity of the peony leaf ethanol extract

	DPPH radical scavenging activity (μmol TE/mg)	Content (mg/g ethanol extract on dry weight)	Contribution (%)
Methyl gallate	14 ± 0	8 ± 0	7
Pentagalloylglucose	11 ± 0	19 ± 0	12
Tellimoside	7 ± 0	37 ± 0	16
Total	-	-	35

Values are reported as mean ± standard deviation (n=3).

activity than all standards, namely quercetin (IC_{50}=98 ± 4 μg/mL), ascorbic acid (IC_{50}=106 ± 1 μg/mL), Trolox (IC_{50}=191 ± 3 μg/mL), and BHT (IC_{50}=499 ± 3 μg/mL), assayed in this study. Tellimoside was more effective against DPPH radicals than Trolox and BHT, whereas it was comparable to quercetin and ascorbic acid. When the highly reactive hydroxyl group located on C3 with the adjacent C2-3 double bond is removed and substituted with other chemical groups such as sugar moieties, the antioxidant capacity of quercetin drops drastically. Notably, the activity-lowering effect of glucosidation at C3 of quercetin is cancelled out by the presence of the galloyl group, known for its antioxidative properties (Rice-Evans *et al*., 1996).

Quantitative analysis of identified compounds and their contribution to antioxidant capacity The content and antioxidant activity of the identified compounds were determined, and their contribution to the total antioxidant capacity of peony leaf was calculated. The contribution was calculated as a percentage of the whole antioxidant activity of the ethanol extract. The results are summarized in Table 1. The antioxidant activity of the identified compounds was assessed by DPPH radical scavenging assay as Trolox equivalent (μmol TE/mg). Quantitative analysis of the identified constituents was performed by HPLC, and the amounts were expressed as mg of compound per g of ethanol extract on a dry weight basis. Pentagalloylglucose and tellimoside purified in this study were used as authentic samples for HPLC quantitative analysis. The content of these three compounds varied from 8 to 37 mg/g ethanol extract on a dry weight basis. The identified three compounds contributed to 35% of the total DPPH radical scavenging activity of the peony leaf ethanol extract.

Conclusion

We identified three main constituents in peony leaf, namely methyl gallate, pentagalloylglucose, and tellimoside, which showed potent DPPH radical scavenging activity, for the first time. These three compounds are considered to be major antioxidative constituents in peony leaf, as they contributed more than 30% of the total DPPH radical scavenging activity of the extract. Due to its various biological activities, including antioxidant effects, peony leaf is suitable for use in functional foods, health drinks, neutraceuticals, and as a primary ingredient in herbal teas. Therefore, this work contributes to the development of traditional folk medicines and the functional food industry as well as the growth of value-added products.

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