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Superoxide Radical Scavengers from the Whole Plant of *Veronica peregrina*

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Abstract – In the course of screening for antioxidant compounds by measuring the radical scavenging effect on 1,1-diphenyl- 2-picrylhydrazyl (DPPH), a total extract from the whole plant of *Veronica peregrina* (Scrophulariaceae) was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of six phenolic compounds including chrysoeriol (**1**), diosmetin (**2**), 4-hydroxybenzoic acid (**3**), apigenin (**4**), caffeic acid methylester (**5**) and protocatechuic acid (**6**). Their structures were elucidated by spectroscopic studies. Compounds **1**-**5** were isolated for the first time from this plant. Compounds **5** and **6** showed significant antioxidative effects in DPPH free radical scavenging and superoxide quenching activity assays.
Keywords – *Veronica peregrina*, Scrophulariaceae, phenolic compounds, DPPH, superoxide quenching activity

Introduction

Veronica peregrina L. (Scrophulariaceae) is an annual or biannual flowering plant, widely distributed in Korea (Lee, 2003; Lee, 1996). The whole plant has been used in chinese folk medicine for the treatment of dysmenorrhea, hemorrhage, fractures and traumatic injuries (Kwak *et al*., 2009). Earlier investigations on the chemical constituents of *V. peregrine* have isolated several flavonoids, phenolic compounds and iridoid glycosides from the whole plant (Kwak *et al*., 2009; Du and Jin, 1996).

In the course of searching for antioxidants from plants by measuring the radical scavenging effect on DPPH (1,1-diphenyl-2-picrylhydrazyl), a total extract of the whole plant of *V. peregrina* was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of six compounds from the active ethyl acetate fraction. In this paper, the isolation and structural characterization of these compounds in addition to their scavenging activities of the stable DPPH free radical as well as their superoxide quenching activities were performed.

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Experimental

General experimental procedures – NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. Sephadex LH-20 was used for column chromatography (Pharmacia, 25 - 100 μm). Prep-HPLC was carried out on a Jaigel GS310 column (Japan). TLC was carried out on Merck precoated silica gel F_{254} plates and silica gel for column chromatography was Kiesel gel 60 (230 - 400 mesh, Merck). Spots were detected under UV and by spraying with 10% H_2SO_4 in ethanol followed by heating at 100 - 120 °C for 3 min. All other chemicals and solvents were of analytical grade and used without further purification. Ascorbic acid and BHA (butylated hydroxyanisole) were obtained from Sigma Chemical Co.

Plant materials – The whole plant of *V. peregrina* was collected in July 2007 at Wanju, Jeonbuk, Korea and identified by Dae Keun Kim, College of Pharmacy, Woosuk University. A voucher specimen was deposited in the herbarium of the College of Pharmacy, Woosuk University (WSU-07-009).

Extraction and isolation – The shade dried and powdered plant of *V. peregrina* (700 g) was extracted three times with MeOH at room temperature. The MeOH extracts were combined and evaporated *in vacuo* at 40 °C.

The resultant methanolic extract (115 g) was successively partitioned as n-hexane, chloroform, ethyl acetate, *n*-butanol and water soluble fractions. Each fraction was tested for their radical-scavenging effect on DPPH (1,1-diphenyl-2-picrylhydrazyl). Among these fractions, the ethyl acetate soluble fraction (12 g) showed the most significant free radical scavenging effect on DPPH. This fraction was subjected to chromatography on silica gel column chromatography ($CHCl_3$-EtOAc-MeOH as the eluents at 10 : 2 : 1 → 4 : 2 : 1)) to give seven fractions (E1-E7). Fraction E1 was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O as the eluents at 90 : 12 : 1) to give five subfractions (E11-E15). Subfraction E11 was purified by HPLC using a GS310 column (MeOH) to give compounds 1 (7 mg) and 2 (4 mg). Subfraction E15 was purified using a Sephadex LH-20 column (MeOH) to give compound 3 (30 mg). Subfraction E12 was chromatographed on a GS310 column (MeOH) and purified using Sephadex LH-20 column (MeOH) to give compounds 4 (8 mg) and 5 (9 mg). Fraction E3 was chromatographed on a silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 90 : 15 : 1) and purified using a Sephadex LH-20 column (MeOH) to give compound 6 (105 mg).

Chrysoeriol (1) – Yellow powder (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.46 (1H, dd, *J* = 8.8, 2.0 Hz, H-6'), 7.44 (1H, d, *J* = 2.0 Hz, H-2'), 6.91 (1H, d, *J* = 8.8 Hz, H-5'), 6.58 (1H, s, H-3), 6.43 (1H, d, *J* = 2.0 Hz, H-8), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 3.94 (3H, s, OCH_3). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 183.8 (C-4), 166.1 (C-7), 165.9 (C-2), 163.2 (C-5), 159.4 (C-9), 152.6 (C-3'), 149.5 (C-4'), 123.5 (C-1'), 121.7 (C-6'), 116.8 (C-5'), 110.6 (C-2'), 104.4 (C-3), 104.2 (C-10), 100.2 (C-6), 95.1 (C-8), 56.6 (OCH_3).

Diosmetin (2) – Yellow powder (MeOH), ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.45 (1H, dd, *J* = 8.8, 2.0 Hz, H-6'), 7.35 (1H, d, *J* = 2.0 Hz, H-2'), 7.04 (1H, d, *J* = 8.8 Hz, H-5'), 6.54 (1H, s, H-3), 6.41 (1H, d, *J* = 2.0 Hz, H-8), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 3.92 (3H, s, OCH_3). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 183.8 (C-4), 166.2 (C-7), 165.9 (C-2), 163.2 (C-5), 159.4 (C-9), 152.6 (C-4'), 148.2 (C-3'), 125.0 (C-1'), 120.0 (C-6'), 113.9 (C-2'), 112.7 (C-5'), 105.3 (C-10), 104.4 (C-3), 100.2 (C-6), 95.1 (C-8), 56.5 (OCH_3).

4-Hydroxybenzoic acid (3) – White powder (MeOH), ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.87 (2H, d, *J* = 8.8 Hz, H-2, 6), 6.81 (2H, d, *J* = 8.8 Hz, H-2, 6). ^{13}C-NMR (100 MHz, CD_3OD, $_C$) 170.1 (COOH), 163.3 (C-4), 133.0 (C-2, 6), 123.0 (C-1), 116.0 (C-3, 5)

Apigenin (4) – Yellow powder (MeOH), ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.84 (2H, d, *J* = 8.8 Hz, H-2', 6'), 6.92 (2H, d, *J* = 8.8 Hz, H-3', 5'), 6.59 (1H, s, H-3), 6.46 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 183.9 (C-4), 174.9 (C-4'), 166.3 (C-2), 166.0 (C-7), 162.7 (C-5), 159.4 (C-9), 129.4 (C-2', 6'), 123.3 (C-1'), 117.0 (C-3', 5'), 103.8 (C-3, 10), 100.1 (C-6), 95.0 (C-8).

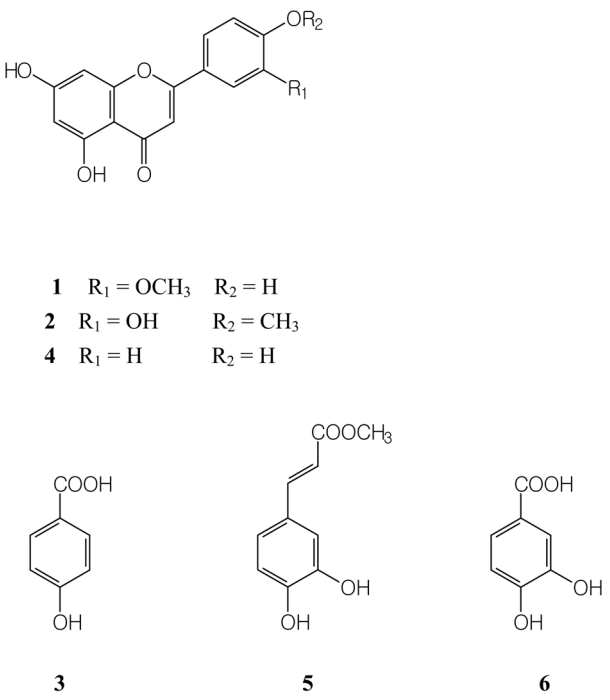

Fig. 1. Structures of compounds **1 - 6** isolated from *V. peregrina.*

Caffeic acid methylester (5) – Pale yellow powder (MeOH), ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.53 (1H, d, *J* = 15.6 Hz, H-7), 7.05 (1H, d, *J* = 2.0 Hz, H-2), 7.03 (1H, dd, *J* = 8.8, 2.0 Hz, H-6), 6.92 (1H, d, *J* = 8.8 Hz, H-5), 6.26 (1H, d, *J* = 15.6 Hz, H-8), 3.87 (3H, s, OCH_3). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 171.0 (COO), 151.4 (C-4), 148.0 (C-3), 146.4 (C-7), 129.0 (C-1), 122.6 (C-6), 116.9 (C-2), 114.7 (C-5), 112.5 (C-8), 56.4 (OCH_3).

Protocatechuic acid (6) – Colorless powder (MeOH), ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.44 (1H, d, *J* = 2.0 Hz, H-2), 7.42 (1H, dd, *J* = 8.8, 2.0 Hz, H-6), 6.79 (1H, d, *J* = 8.8 Hz, H-5). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 170.3 (COO), 151.5 (C-4), 146.0 (C-3), 123.9 (C-6), 123.1 (C-1), 117.7 (C-5), 115.8 (C-2).

DPPH radical scavenging effect – Ethanol solutions of test samples at various concentrations (0.1 - 100 μg/mL) were added to a solution of DPPH in methanol (0.2 mM) in 96 well plates. After storing these mixtures for 30 minutes at room temperature, the remaining amounts of DPPH were determined by colorimetry at 520 nm on a

microplate reader (Yoshida *et al*., 1989). The radical scavenging activity of each compound was expressed as a ratio DPPH absorbance in the absence or presence of compounds. The mean values were obtained from triplicate experiments.

Superoxide quenching activity – Superoxide quenching activities of test samples were measured photochemically, using an assay system consisting of methionine, riboflavin, and nitrobluetetrazolium (NBT) (Choi *et al*., 2001; Ginnopolitis and Ries, 1977). The reaction mixture was composed of 0.13 μM riboflavin, 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, PBS buffer (pH 7.4) and various concentrations of test samples. The sample was placed in a light storage box and replaced every 5 min for 15 min. The temperature within the light storage box was 20 ± 1 ºC during illumination. The light intensity at the sample level was 5,500 lux. During illumination, NBT was reduced to blue formazan crystals and was measured by the absorbance at 560 nm. The inhibition of formazan formation was considered to be superoxide quenching activity.

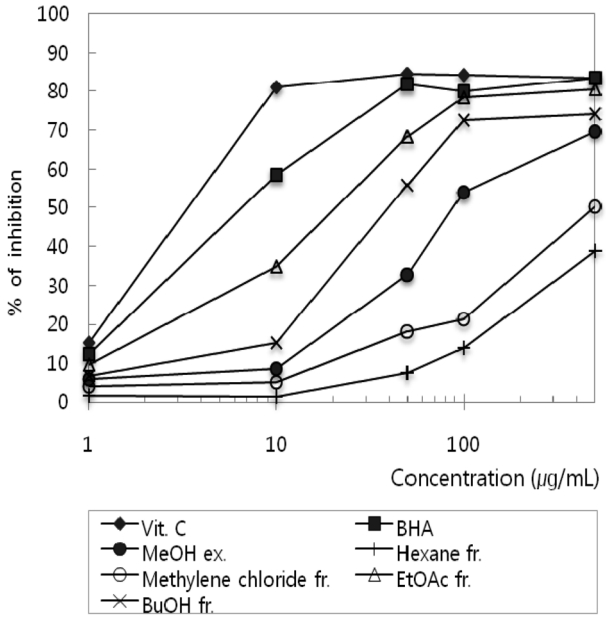

Fig. 2. DPPH radical scavenging effects of the fractions from the whole plant of *V. peregrina*.

Results and Discussion

The ethyl acetate soluble fraction of the initial methanolic extract of the whole plant of *V. peregrina* was found to show scavenging activity for DPPH radical (Fig. 2). Subsequent activity-guided fractionation of the ethyl acetate soluble fraction led to the isolation of three flavonoid compounds and three phenolic compounds.

Compounds **1** and **2** have similar patterns in their NMR spectra. Compounds **1** and **2** were obtained as yellow powders from MeOH. ^{1}H-NMR spectra of **1** and **2** showed the typical pattern of a coupling group of 1,3,4-trisubstituted benzene ring at δ_H 7.46 (1H, dd, J = 8.8, 2.0 Hz, H-6'), 7.44 (1H, d, J = 2.0 Hz, H-2') and 6.91 (1H, d, J = 8.8 Hz, H-5') of **1**, and δ_H 7.45 (1H, dd, J = 8.8, 2.0 Hz, H-6'), 7.35 (1H, d, J = 2.0 Hz, H-2') and 7.04 (1H, d, J = 8.8 Hz, H-5') of **2**. A methoxy peak was observed at δ_H 3.94 of **1** and 3.92 of **2**, respectively. The main differences between compounds **1** and **2** were chemical shifts of H-2' and H-5' of aromatic B-ring. In the ^{13}C-NMR spectra of compounds **1** and **2**, twenty-one carbon signals were observed, which included a carbonyl group at δ_C 183.8 of **1** and **2**. From these results, compounds **1** and **2** were deduced to be flavones compounds bearing a 1,3,4-trisubstituted aromatic B-ring. The structures of **1** and **2** were determined to be chrysoeriol (**1**) and diosmetin (**2**) on the basis of the above evidence and comparison with published literature (Wagner *et al*., 1976; Qian *et al*, 2006). Compound **3** was obtained as a white powder from MeOH. The ^{1}H-NMR spectrum of **3** showed two ortho-coupled doublets each of two protons with a J value of 8.8 Hz at δ_H 7.87 (2H, d, H-2, 6) and 6.81 (2H, d, H-3, 5), indicating the presence of a 1,4-disubstituted aromatic ring. In the ^{13}C-NMR spectrum of **3**, a carbonyl carbon peak (δ_C 170.1, COO) was observed. From these results, compound **3** was determined to be 4-hydroxybenzoic acid on the basis of the above evidence, together with a comparison to published data (Pyo *et al*., 2002). Compound **4** was obtained as a yellow powder from MeOH. The ^{1}H-NMR spectrum of **4** showed two ortho-coupled doublets each of two protons with a J value of 8.8 Hz at δ_H 7.84 (2H, d, H-2', 6') and 6.92 (2H, d, H-3', 5'), indicating the presence of a 1,4-disubstituted aromatic ring and two doublet protons at δ_H 6.46 and 6.20 (each 1H, d, J = 2.0 Hz), together with one singlet proton at δ_H 6.59 (1H, s) were observed in the olefinic area. In the ^{13}C-NMR spectrum of **4**, a carbonyl carbon signal at δ_C 184.2 was observed. From these results, compound **4** was determined to be apigenin by the direct comparison of the above data with those published in the literature (Kim and Choi, 1993). The ^{1}H-NMR spectrum of **5** showed the typical pattern of a coupling group of the 1,3,4-trisubstituted benzene ring at δ_H 7.05 (1H, d, J = 2.0 Hz, H-2), 7.03 (1H, dd, J = 8.8, 2.0 Hz, H-6) and 6.92 (1H, d, J = 8.8 Hz, H-5). Further, two olefinic protons having a *trans*-configuration were observed at δ_H 7.53

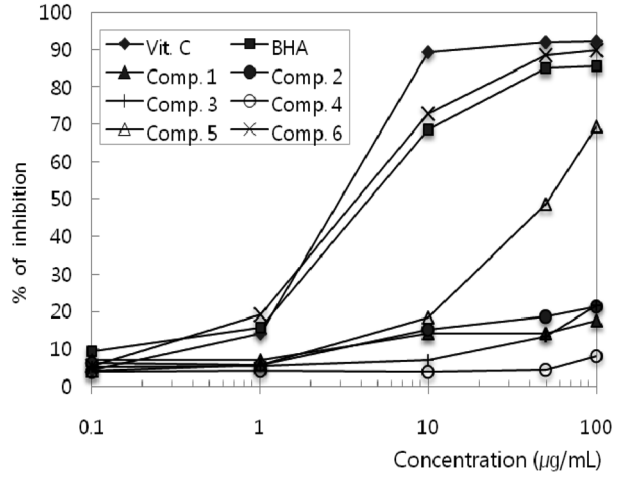

Fig. 3. DPPH radical scavenging effects of the isolated compounds from the whole plant of *V. peregrine*.

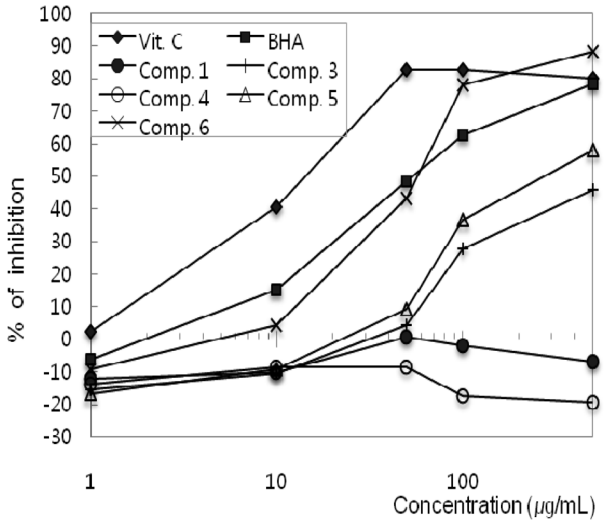

Fig. 4. Riboflavin originated superoxide quenching activities of the isolated compounds from the whole plant of *V. peregrina*.

(1H, d, J = 15.6 Hz, H-7) and 6.26 (1H, d, J = 15.6 Hz, H-8). At δ_H 3.87, a methoxy peak was observed. In the ^{13}C-NMR spectrum of **5**, a carbonyl carbon at δ 171.0 signal was observed. On the basis of these observations and a comparison to previously published (Pyo *et al*., 2002), the structure of compound **5** was identified as caffeic acid methyl ester. The ^{1}H-NMR spectrum of **6** showed the typical pattern of a coupling group of a 1,3,4-trisubstituted benzene ring at δ_H 7.44 (1H, d, J = 2.0 Hz, H-2), 7.42 (1H, dd, J = 8.8, 2.0 Hz, H-6) and 6.79 (1H, d, J = 8.8 Hz, H-5). In the ^{13}C-NMR spectrum of **6**, a carbonyl carbon at δ 170.3 signal was observed. On the basis of these observations and the comparison of the data with those previously published (Yamanaka *et al*., 1995), the structure of compound **6** was identified as proto-catechuic acid.

The radical scavenging effects of six these compounds obtained from *V. peregrina* are shown in Fig. 3. The positive control, vitamin C had a DPPH radical scavenging effect with an established IC_{50} value of 3.1 μg/mL. Compounds **5** and **6** dose-dependently exhibited scavenging activities on DPPH with IC_{50} values of 50.0 and 3.3 μg/mL, respectively. However, compounds **1** - **4** had no scavenging activities compared to ascorbic acid and BHA. Fig. **4** shows the superoxide quenching activities of the isolated compounds **1** - **6**, as measured by the riboflavin-NBT-light system. Among six compounds, **6** was found to be the most potent superoxide radical scavenger (Fig. 4).

It has been reported that increases in free radicals causes aging and age-related diseases (Spiteller, 2010). Further, free radical reactions can produce deleterious modifications in membranes, proteins, enzymes and DNA, increasing the risk of diseases such as cancer, Alzheimer's, Parkinson's, arthritis, asthma, diabetes and degenerative eye disease (Wu *et al*., 2006).

The results from free radical scavenging systems revealed that the ethyl acetate soluble fraction of the whole plant of *V. peregrina* and compound **6** had significant antioxidant activities. Therefore compound **6** may be useful for protection against oxidative damage.

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