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Antioxidant Properties of *Erigeron annuus* Extract and Its Three Phenolic Constituents

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Abstract The antioxidant activity of the extract of *Erigeron annuus* was assessed by means of two different *in vitro* tests: bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH test) and the scavenging of authentic peroxynitrite in company with peroxynitrite generation from 3-morpholinosydnonimine (SIN-1). In both tests, the 85% aq. MeOH and *n*-BuOH soluble fractions of the crude extract showed a significant scavenging effect on peroxynitrite and DPPH radical in comparison to L-ascorbic acid. And bioassay-guided fractionation of the *n*-BuOH soluble fraction led to the isolation of three compounds: Apigenin (1), quercetin-3-O-glucoside (2), and caffeic acid (3). The structures of the isolated compounds were elucidated on the basis of their spectroscopic data and their antioxidant activities were measured by determining their capacity to scavenge peroxynitrite and the DPPH radical.

Keywords: *Erigeron annuus*, authentic peroxynitrite ($ONOO^-$), 3-morpholinosydnonimine (SIN-1), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH)

INTRODUCTION

Cells must maintain a proper balance between the levels of free radicals and antioxidants to ensure the structural integrity of critical components. When the levels of free radicals exceed those of antioxidants during oxidative stress, sensitive biomolecules such as lipids, proteins and DNA in particular can be damaged [1]. Reactive oxygen species (*e.g.*, hydroxyl radical or hydrogen peroxide) and reactive nitrogen species (*e.g.*, peroxynitrite) have been implicated to be involved in the development of chronic degeneration disease and in the aging process. Biochemical and epidemiological evidences indicate that increased antioxidant defence may lower the risk of such diseases [2,3].

Peroxynitrite is formed in biological systems when superoxide anion and nitric oxide are produced at near equimolar ratio. Although not a free radical by chemical nature, peroxynitrite is a powerful oxidant exhibiting a wide array of tissue damaging effects ranging from lipid peroxidation, inactivation of enzymes and iron channels via protein oxidation and nitration to inhibition of mitochondrial respiration [4]. There are many reports of naturally or synthetic peroxynitrite scavenger including melatonin, deferoxamine, D(-)penicillamine, flavonoids, none the less, isolated compounds from marine natural plants were hardly not reported [5-8]. It is also very important to explore antioxidant from marine natural plants, because most of peroxynitrite scavenger cannot be synthesized by humans and must be taken in the diet.

DPPH is a stable free radical for about 1 h at the room temperature and has strong optical density at the 520 nm wavelength of dark purple. It has been widely used as a substrate to evaluate the antioxidative properties because the color is easily disappeared by donating a hydrogen or an electron from reacting material [9].

Recently, in our laboratory, we screened a number of salt marsh plants extracts for the DPPH radical and peroxynitrite-scavenging activities [10,11]. Of these, *Erigeron annuus* was found to have the most predominant DPPH radical and peroxynitrite-scavenging effects of those screened. *E. annuus* (L.) Pers., a member of the Compositae (Asteraceae), is widely distributed throughout the urban and rural areas of Korea [12-14] and has also been used as a hypoglycemic drug in China [15]. It had been reported that (5-butyl-3-oxo-2,3-dihydrofuran-2-yl)-acetic acid, 3-hydroxy-pyran-4-one and two cinnamic acid derivatives isolated from *E. annuus* act as anti-germination constituents [16]. Sesquiterpenoids, diterpenoids, and cyclopentenone derivatives have also been reported to be present in *E. annuus* [17,18]. However, antioxidant activity of this plant has not been studied. As a part of our search for antioxidants from marine plants, *E. annuus* was selected, and extracted, and then its crude extract was investigated.

In this paper, the antioxidant activities of the fractions from *n*-hexane, 85% aqueous MeOH, *n*-butanol (*n*-BuOH) and water (H_2O) partitioned from the combined solvent extracts of *E. annuus* were evaluated by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and per-

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oxynitrite. The isolation and identification of three known compounds from the active *n*-BuOH fraction of *E. annuus* were also reported.

MATERIALS AND METHODS

Plant Materials

Whole plant of *E. annuus* (1.2 kg) was collected at Daebudo Kyungkido, Korea in September 2002. The collected sample was briefly dried under shade and kept at –25°C until use.

Chemicals

The 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, 2,6-Di-tert-butyl-4-methoxyphenol (butylatedhydroxyanisol: BHA), 2,6-Di-tert-butyl-4-methylphenol (butylatedhydroxytoluene: BHT), DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid), and 3-morpholinosydnonimine (SIN-1) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The Dihydrorhodamine 123 (DHR 123) and peroxynitrite were of the highest quality commercially available and were purchased from Molecular Probes (Eugene, Oregon, USA), and Cayman (Ann Arbor, MI, USA), respectively.

General Experimental Procedures

Thin layer chromatography (TLC) was carried out on a RP-18 F_{254s} plate (Merck) and spots were detected using 10% ethanolic H_2SO_4 reagent. ^1H and ^{13}C NMR analyses were performed with a Varian NMR 300 spectrometer (300 MHz for ^1H and 75.5 MHz for ^{13}C). Chemical shifts were referenced to the respective residual solvent peaks, recorded in values, and expressed in ppm. The solvent used was CD_3OD (Cambridge Isotope Laboratories, Inc., USA, deuterium degree 99.8%). HMQC, and HMBC spectra were recorded using pulsed field gradients. The multiplicities of the ^1H NMR signals were indicated as s (singlet), d (doublet), and m (multiplet). FAB-MS was measured on a Concept-1S (Kratos Co., Manchester, UK) mass spectrometer.

Extraction, Fractionation, and Isolation

The dried whole plants of *E. annuus* were extracted successively three times with each of CH_2Cl_2 and MeOH, respectively. The total filtrate was concentrated to dryness *in vacuo* at 40°C to render the MeOH (20.6 g) and CH_2Cl_2 (16.3 g) extracts, respectively. The two extracts were combined and suspended in H_2O and then partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 (19.9 g) fraction was further partitioned with *n*-hexane and 85% aqueous MeOH and the H_2O fraction successively fractionated with *n*-BuOH and H_2O. This resulted in 4 fractions, *i.e.*, the *n*-hexane (8.9 g), 85% aqueous MeOH (9.1 g), *n*-BuOH (6.6 g), and H_2O (10.1 g) fractions (scheme 1). A MeOH soluble portion of the *n*-BuOH (2.3 g) fraction was subjected to Sephadex LH-20 column chromatography using MeOH as the eluting solvent to yield 18 subfractions (subfr. 1~18). The 8 subfraction (53.3 mg) was further separated by preparative-TLC (PTLC) on a silica gel plate $CHCl_3$ and MeOH (10:1) as the solvent system to give the compound **1** (apigenin: 7 mg). Subfractions 6 and 4 were also further separated by RP-PTLC on a C_{18} plate with 40% aqueous AcCN to give the compounds **2** (Quercetin-glucoside: 3.8 mg) and **3** (Caffeic acid: 9.2 mg), respectively.

Apigenin (1)

Yellowish powder; FAB-MS *m/z* 269 $[M-H]^+$; ^1H NMR (300 MHz, CD_3OD) 6.51 (1H, s, H-3), 5.90 (1H, d, J = 1.9 Hz, H-6), 6.14 (1H, d, J = 1.9 Hz, H-8), 6.83 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 7.78 (2H, d, J = 8.8 Hz, H-2′ and H-6′) ; ^{13}C NMR (75.5 MHz, CD_3OD) 164.7 (C-2), 102.6 (C-3), 182.6 (C-4), 158.1 (C-5), 99.0 (C-6), 165.0 (C-7), 93.9 (C-8), 161.5 (C-9), 102.6 (C-10), 122.0 (C-1′), 128.2 (C-2′), 115.8 (C-3′), 161.9 (C-4′), 115.8 (C-5′), 128.2 (C-6′).

Quercetin-3-O-glucoside (2)

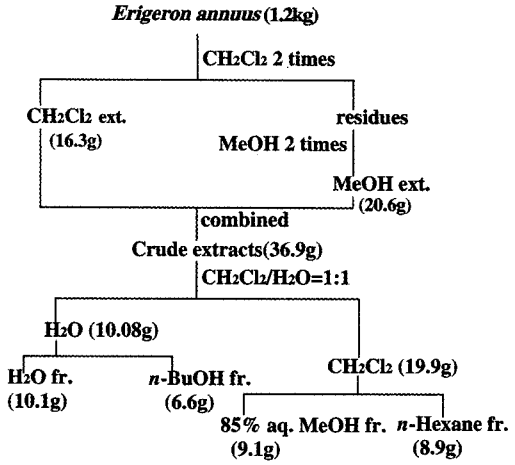
Yellowish powder; FAB-MS *m/z* 463 $[M-H]^+$; ^1H NMR (300 MHz, CD_3OD) 6.20 (1H, d, J = 1.9 Hz, H-6), 6.39 (1H, d, J = 1.9 Hz, H-8), 6.85 (1H, d, J = 8.5 Hz, H-5′), 7.57 (1H, dd, J = 2.2, 2.2 Hz, H-6′), 7.69 (1H, d, J = 2.2 Hz, H-2′), 5.24 (1H, d, J = 7.4 Hz, H-1′) ^{13}C NMR (75.5 MHz, CD_3OD) 158.8 (C-2), 135.4 (C-3), 179.2 (C-4), 162.8 (C-5), 99.8 (C-6), 165.8 (C-7), 94.6 (C-8), 158.3 (C-9), 105.6 (C-10), 122.9 (C-1′), 115.9 (C-2′), 145.7 (C-3′), 149.7 (C-4′), 117.4 (C-5′), 123.1 (C-6′), 104.1 (C-1″), 75.7 (C-2″), 78.3 (C-3″), 71.1 (C-4″), 78.0 (C-5″), 64.3 (C-6″).

Caffeic acid

Yellowish powder; FAB-MS *m/z* 179 $[M-H]^+$; ^1H NMR (300 MHz, CD_3OD) 6.21 (1H, d, J = 15.7 Hz, H-8), 6.75 (1H, dd, J = 2.8 Hz, 3.0 Hz, H-5), 6.89 (1H dd, J = 2.2 Hz, 1.9 Hz, H-6), 7.01 (1H, d, J = 1.9 Hz, H-2), 7.46 (1H, d, J = 15.7 Hz, H-7); ^{13}C NMR (75.5 MHz, CD_3OD) 126.8 (C-1), 113.8 (C-2), 145.5 (C-3), 147.9 (C-4), 115.3 (C-5), 121.5 (C-6), 144.8 (C-7), 115.7 (C-8), 170.7 (C-9).

Determination of the Scavenging Effect on the DPPH Radical

The DPPH radical scavenging effect was evaluated according to the method employed by Blois [9]. To 1.0 mL of DPPH methanol solution (1.5×10^{-1} M), 4.0 mL of MeOH solution of a sample at various concentrations was added. After mixing gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The scavenging activity of the sample was determined by comparing its ab-



Scheme 1. Extraction and fractionation procedure of *Erigeron annuus*.

sorbance with that of the blank (100%) which contained only DPPH and solvent.

Measurement of Peroxynitrite Scavenging Activity

The peroxynitrite ($ONOO^-$) scavenging ability was measured by monitoring the oxidation of dihydrorhodamine 123 using modified version of the method of Kooy *et al.* [19]. The peroxynitrite reacts with DHR 123, makes oxidized DHR 123 form and its conversed chemical structure is capable of emitting fluorescence. A stock solution of DHR 123 (5 mM) purged with nitrogen was prepared in advance and stored at 80°C. A working solution of DHR 123 (final concentration, 5 μM) was diluted from the stock solution and placed on ice in the dark immediately prior to the measurement. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4) and 5 mM potassium chloride with 100 μM (f.c.) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. The $ONOO^-$ scavenging ability, based on the oxidation of DHR 123, was determined with a microplate fluorescence spectrophotometer, FL 500 (Bio-Tek Instruments, Inc., USA) using the wavelengths of 485 nm and 530 nm for excitation and emission, respectively, at room temperature. The background and final fluorescent intensities were measured 5 min after treatment with or without SIN-1 (f.c. 10 μM) or authentic $ONOO^-$ (f.c. 10 μM) in 0.3 N sodium hydroxide. The oxidation of DHR 123 due to decomposition of the SIN-1 gradually proceeded whereas the authentic $ONOO^-$ rapidly oxidized DHR 123 with the final fluorescent intensity being stable over time. Penicillamine was used as a positive control.

RESULTS AND DISCUSSION

This study investigated the general antioxidant effects of the *n*-hexane-, 85% aq. MeOH-, *n*-BuOH-, and H_2O fractions from the crude extract of *E. annuus*. Also, the isolation of the active components from the active fraction was conducted by using a bioassay-directed method.

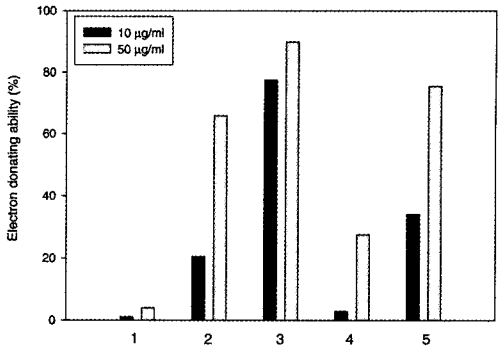


Fig. 1. DPPH radical scavenging activities of several fractions from *Erigeron annuus* crude extract. 1. *n*-hexane; 2. 85% aq. MeOH; 3. *n*-BuOH; 4. H_2O; 5. BHA.

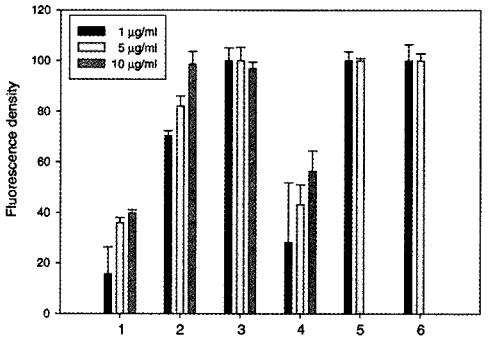


Fig. 2. Scavenging activities of several fractions from *Erigeron annuus* crude extract on authentic peroxynitrite ($ONOO^-$). 1. *n*-hexane; 2. 85% aq. MeOH; 3. *n*-BuOH; 4. H_2O; 5. L-ascorbic acid; 6. penicillamine.

As shown in Fig. 1, the scavenging activity of each fraction of the *n*-hexane-, 85% aq. MeOH-, *n*-BuOH-, and H_2O fractions on the DPPH radical were 4.0, 65.8, 89.8, and 27.6% at 50 μg/mL and 0.0, 20.6, 77.6, and 3.0% at 10 μg/mL concentrations, respectively. The inhibitory activities of the 85% aq. MeOH and *n*-BuOH fraction on DPPH were higher than those of the other fractions. In particular, the *n*-BuOH fraction showed a better than that of the butylatedhydroxyanisole (BHA), which is widely known as a synthetic antioxidant. However, the *n*-hexane and H_2O fractions showed only very weak activities.

For the $ONOO^-$ scavenging activity, the four solvent fractions were tested. They exhibited a good antioxidant activity in a dose dependent manner, and the activity was comparable to that of penicillamine and L-ascorbic acid. Especially, the *n*-BuOH fraction showed 99.99% inhibition at the 1 μg/mL (Fig. 2). Each fraction showed a highly similar scavenging effect on both authentic peroxynitrite and SIN-1 producing a superoxide anion and nitric oxide simultaneously (Fig. 2 and Fig. 3). These results clearly indicate that the *n*-BuOH fraction has a significant scavenging activity on both DPPH and peroxynitrite.

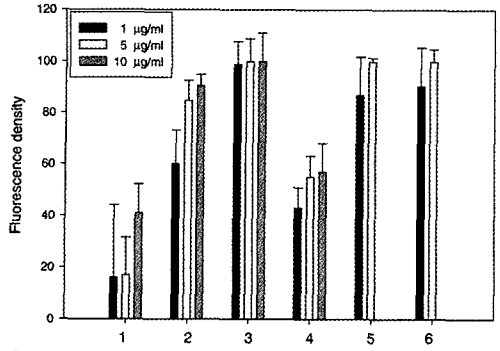
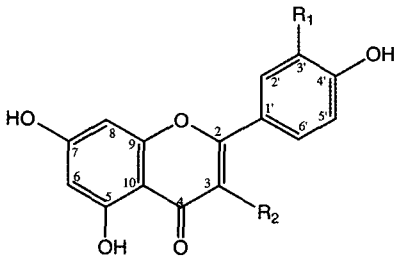


Fig. 3. Scavenging activities of several fractions from *Erigeron annuus* crude extract on peroxynitrite from SIN-1. 1. *n*-hexane; 2. 85% aq. MeOH; 3. *n*-BuOH; 4. H_2O; 5. L-ascorbic acid; 6. penicillamine.



1: $R_1 = R_2 = H$
2: $R_1 = OH$, $R_2 = Glc$

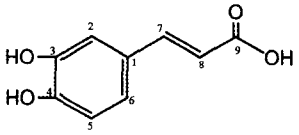


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Fig. 4. Chemical structures of the active compounds isolated from *Erigeron annuus*.

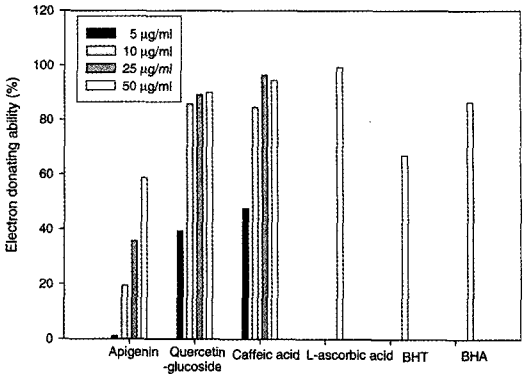

Fig. 5. DPPH radical scavenging activities of the compounds (**1**-**3**) isolated from *Erigeron annuus*.

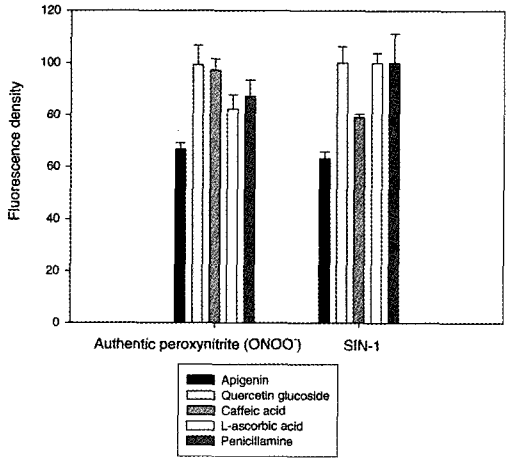

Fig. 6. Scavenging activities of the compounds (**1**-**3**) isolated from *Erigeron annuus* on authentic peroxynitrite and peroxynitrite from SIN-1 at the 1 μg/mL concentration.

The *n*-BuOH fraction was subjected to further separation and, after successive column chromatography, two known flavonoids and one phenolic acid were isolated. These compounds were readily analyzed by extensive 2-D NMR experiments including ^1H, ^1H-COSY, HMQC, and HMBC and by comparison with the reported spectroscopic data and identified as apigenin (**1**), quercetin-3-*O*-glucoside (**2**), and caffeic acid (**3**) [20-23] (Fig. 4).

The antioxidant activities of the three compounds isolated from the *n*-BuOH fraction was shown in Fig. 5 and Fig. 6. Compounds **2** and **3** at 10 μg/mL exhibited a strong antioxidant activity on DPPH as 85.7 and 84.6%, respectively. These are comparable to that of L-ascorbic acid, positive control, at a concentration of 50 μg/mL. Compounds **2** and **3** also exhibited a strong inhibition activity of 99.3 and 97.3% on authentic $ONOO^-$ and 100%, 79.2% on SIN-1 at 1 μg/mL. The activity was comparable with that of L-ascorbic acid and penicillamine respectively. Compound **1** didn't show any activity on DPPH at a 10 μg/mL but showed a moderate activities on authentic $ONOO^-$ and SIN-1 at 1 μg/mL as 66.8 and 63.3%, respectively.

According to the previous reports, these types of compounds revealed a potential ability to prevent the $ONOO^-$ mediated nitration of protein-bound and free tyrosine and to inhibit the $ONOO^-$ mediated oxidation of dihydrorhodamine 123 and DNA [24-26]. It has also been reported that the antioxidant activities of the phenolic compounds are primarily determined by the number of phenolic hydroxyl groups. The hydroxyl groups are the active centers of the molecules in terms of their furnishing hydrogen atoms for the scavenging function. Many other phenolic phytochemicals work as antioxidants in a similar manner. Some other's work by chelating metal ions, which facilitate the formation of the hydroxy radical from reactive species such as H_2O_2 [27-31]. Indeed, quercetin-3-*O*-glucoside that has four phenolic hydroxy groups was a much better scavenger of the DPPH and $ONOO^-$ than apigenin and caffeic acid which have only two or three hydroxy groups. In addition, the activity of

caffeic acid was noticeably superior to apigenin, suggesting that the ortho-dihydroxy functional group on the phenolic ring is one of the important factors for the antioxidant activity. The antioxidant effects of quercerin-3-*O*-glucoside and caffeic acid are consistent with those of other phenolic phytochemicals, which have been reported to scavenge the H_2O_2, hydroxy radical, superoxide anion, and peroxynitrite [33,34].

The present results indicate that the antioxidant activity of the halophyte *E. annuus* is partly attributable to the phenolic derivatives such as compounds **1-3**, contained in *E. annuus*. To the best of our knowledge, this is the first report on the isolation of three active compounds from *E. annuus* and on their antioxidant activities in DPPH and peroxynitrite assay systems. Investigations on the further antioxidative constituents of *E. annuus* are currently in progress.

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