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미국가막사리 지상부의 항산화 성분

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Antioxidant Components of the Aerial Parts of *Bidens frondosa* L.

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Abstract – As a part of an ongoing search for natural plants with antioxidant compounds by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH), a total extract of the aerial parts of *Bidens frondosa* L. (Compositae) was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of five compounds, quercetin-3-*O*-β-D-glucopyranoside (1), luteolin-7-*O*-β-D-glucopyranoside (2), 7,8,3',4'-tertrahydroxy-flavanone (3), okanin-4-*O*-β-D-glucopyranoside (4), and okanin (5). Their structures were elucidated by spectroscopic studies. Compounds 3-5 were isolated for the first time from this plant. Among them, compounds 3 and 5 showed the significant radical scavenging effects on DPPH, and compounds 3 and 5 also showed the potent riboflavin and xanthine originated superoxide quenching activities.

Key words – *Bidens frondosa* L., Compositae, Antioxidant activity, DPPH radical, Superoxide quenching activity

Reactive oxygen species (ROS) include superoxide radical, hydrogen peroxide, and hydroxyl free radical (ROS; $\cdot O_2^-$, $\cdot OH$, H_2O_2), all of which have one or more unpaired electrons that potentially cause damage to cells. All these ROS are potently toxic and mutagenic.[1,2] They are occurred by environmental and biochemical factors caused by the reduction of metabolism, pollutants, chemicals, and photochemistry reaction.[3,4] An imbalance between production of ROS and their elimination in the organisms causes oxidative stress.[5] Oxidative stress has been implicated as a possible factor in the etiology of several human diseases, including cancer, cardiovascular disease, Alzheimer's disease and aging.[6] Antioxidant compounds are the important defense factors against oxidative stress caused by ROS. The synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, have shown toxic and carcinogenicity. Thus, there is a growing interest in finding natural herbal plants that advantage of low in toxicity and high in radical scavenging activity.[7,8]

In the course of screening for antioxidants from Korean natural plants by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH), a methanolic extract of the *Bidens frondosa* L. (Compositae) was found to show a potent antioxidant activity. This genus commonly contains chalcones based on okanin,[9] and there are a few reports of B-ring methylated chalcones in the genus.[10] *B. bipinnata* has been used as a folk medicine with beneficial effects for diarrhea, acute nephritis, stomachache, carcinoma of esophagus and appendicitis.[11,12] It has been reported that several constituents isolated from this plant have anti-inflammatory effects.[13] In this paper, the isolation and structural characterization of isolated compounds, as well as their scavenging activity of the stable DPPH free radicals and superoxide quenching activity were described.

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Materials and Methods

General Experimental Procedures – NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. Sephadex LH-20 was used for column chromatography (25-100 μm; GE Healthcare, Uppsala, Sweden). Prep-HPLC was carried out on a Jaigel GS310 column (Tokyo, Japan). TLC was carried out on Merck (Darmstadt, Germany) 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, butylated hydroxyanisole (BHA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, USA). Absorbance of the resultin solution was measured on a microplate reader (GENios, Tecan, Grödig, Austria).

Plant Materials – The aerial parts of *Bidens frondosa* were collected and air-dried in October 2010 at Wanju, Jeonbuk, Korea. A voucher specimen was deposited in the herbarium of College of Pharmacy, Woosuk University (WSU-10-007).

Extraction and Isolation – The shade dried plant material (1.1 kg) was extracted three times with MeOH at 50°C and filtered. The extracts were combined and evaporated *in vacuo* at 50°C. The resultant methanolic extract (570 g) was subjected to successive solvent partitioning to give *n*-hexane (19.8 g), methylene chloride (3.4 g), ethyl acetate (6.8 g), *n*-BuOH (19.4 g) and H_2O soluble fractions. Each fraction was tested for the radical scavenging effect on DPPH. Among these fractions, the ethyl acetate fraction showed the most significant free radical scavenging effect on DPPH (Fig. 1). The ethyl acetate soluble extract was subjected to chromatography on a Sephadex LH-20 column and give eight fractions (E1-E6). Fraction E2 (92 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 40:10:1) to give eight subfractions (E21-E28). Subfraction E27 (30 mg) was purified on a JAI-GS310 cloumn (MeOH) to give compound 1 (7 mg). Compound 2 was obtained by recrystallization of fraction E8 (30 mg) from methanol. Fraction E3 (580 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 40:10:1) to give eight subfractions (E31-E38). Subfraction E34 (35 mg) was purified on a JAI-GS310 cloumn (MeOH) to give compound 3 (10 mg). Fraction E4 (210 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 80:15:1) to give five subfractions (E41-E45). Subfraction E43 (35 mg) was further chromatographed on a JAI-GS310 cloumn (MeOH) and purified by Sephadex LH-20 (MeOH) to give compound 4 (25 mg). Fraction E5 (580 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 40:10:1) to give six subfractions (E51-E56). Subfraction E53 (31 mg) was further chromatographed on a JAI-GS310 cloumn (MeOH) to give compound 5 (9 mg).

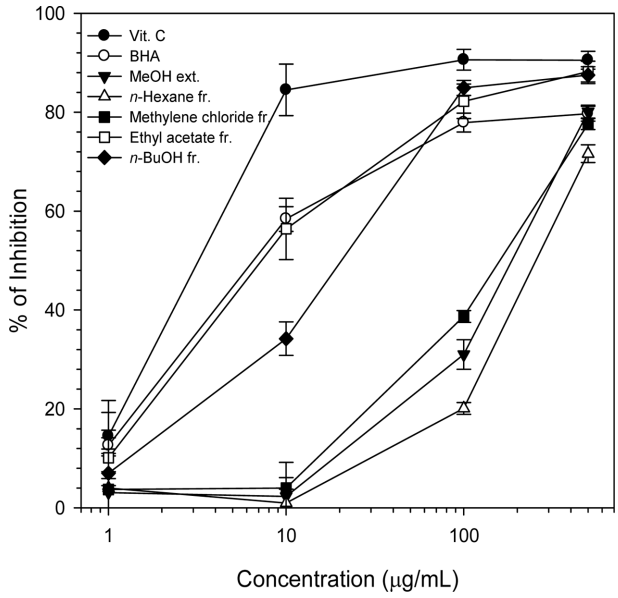

Fig. 1. Scavenging effects of methanol extract and its subsequent fractions from the aerial parts of *Bidens frondosa* on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

Compound 1 (Quercetin-3-*O*-β-D-glucopyranoside, isoquercitrin): Amorphous powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.70 (1H, d, *J*=2.4 Hz, H-2'), 7.56 (1H, dd, *J*=8.8, 2.4 Hz, H-6'), 6.71 (1H, d, *J*=8.8 Hz, H-5'), 6.38 (1H, d, *J*=2.0 Hz, H-8), 6.19 (1H, d, *J*=2.0 Hz, H-6), 5.25 (1H, d, *J*=7.2 Hz, H-1"). ^{13}C-NMR (100 MHz, CD_3OD): Table I.

Compound 2 (Luteolin-7-*O*-β-D-glucopyranoside): Yellowish amorphous solid, ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.46 (1H, dd, *J*=8.4, 2.4 Hz, H-6'), 7.42 (1H, *brs*, H-2'), 6.90 (1H, d, *J*=8.4 Hz, H-5'), 6.79 (1H, d, *J*=2.4 Hz, H-8), 6.76 (1H, s, H-3), 6.45 (1H, d, *J*=2.0 Hz, H-6), 5.09 (1H, d, *J*=7.4 Hz, H-1"). ^{13}C-NMR (100 MHz, DMSO-d_6) (Table I).

Compound 3 (7,8,3'4'-tetrahydroxyflavanone3): Amorphous yellow powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.29 (1H, d, *J*=8.8 Hz, H-5), 6.98 (1H, d, *J*=2.0 Hz, H-2'), 6.86 (1H, dd, *J*=8.0, 2.0 Hz, H-6'), 6.78 (1H, d, *J*=8.0, H-5'), 6.52 (1H, d, *J*=8.8 Hz, H-6), 5.37 (1H, dd, *J*=12.8, 2.8 Hz, H-2), 3.08 (1H, dd, *J*=16.8, 12.8 Hz, H-3α), 2.74 (1H, dd, *J*=16.8,

Table I. ^{13}C-NMR spectral data of compounds **1-5**

C	**1**[a]	**2**[b]	**3**[a]	**4**[a]	**5**[a]
1				128.2	128.4
2	159.0	164.5	81.6	115.9	115.1
3	135.6	103.1	45.1	146.9	146.9
4	179.5	181.9	194.0	150.2	149.9
5	163.1	161.1	119.3	116.6	115.8
6	100.0	99.5	110.9	124.0	123.6
7	166.1	163.0	154.1	-	-
8	95.0	94.7	134.0	-	-
9	158.5	156.4	152.7	-	-
10	105.7	105.3	115.7	-	-
1'	123.1	121.2	131.9	117.3	116.6
2'	116.0	113.5	115.0	153.8	153.3
3'	146.0	145.8	146.5	135.7	133.8
4'	150.0	150.2	146.9	151.6	150.0
5'	117.5	116.0	116.2	108.0	108.5
6'	123.2	119.2	119.6	122.6	123.2
1"	104.3	99.9		102.5	-
2"	75.7	73.1		74.7	-
3"	78.1	77.2		77.5	-
4"	71.2	69.5		71.3	-
5"	78.4	76.4		78.4	-
6"	62.6	60.6		62.4	-
C=O				194.6	194.1
α				118.1	118.4
β				146.8	146.0

[a]Recorded at 100 MHz in CD_3OD
[b]Recorded at 100 MHz in DMSO-d_6

2.8 Hz, H-3β). ^{13}C-NMR (100 MHz, CD_3OD) (Table I).

Compound 4 (Okanin-4'-*O*-β-D-glucopyranoside): Yellowish powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.52 (1H, d, *J*=15.6 Hz, H-β), 7.58 (1H, d, *J*=9.2 Hz, H-6'), 7.24 (1H, d, *J*=5.6 Hz, H-α), 7.18 (1H, d, *J*=2.0 Hz, H-2), 7.10 (1H, dd, *J*=8.0, 2.0 Hz, H-6), 6.82 (1H, d, *J* = 9.2 Hz, H-5') 6.81 (1H, d, *J*=7.6 Hz, H-5), 4.98 (1H, d, *J*=7.2 Hz, H-1"). ^{13}C-NMR (100 MHz, CD_3OD) (Table I).

Compound 5 (Okanin): Amorphous orange powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.75 (1H, d, *J*=15.6 Hz, H-β), 7.52 (1H, d, *J*=8.2 Hz, H-6'), 7.53 (1H, d, *J*=14.8 Hz, H-α), 7.17 (1H, d, *J*=2.0 Hz, H-2), 7.11 (1H, dd, *J*=8.0, 2.0 Hz, H-6), 6.81 (1H, d, *J*=8.0, H-5), 6.46 (1H, d, *J*=8.8 Hz, H-5'). ^{13}C-NMR (100 MHz, CD_3OD) (Table I).

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 ethanol (1.5×10^{-4} M) in 96-well plates. After storing these mixtures for 30 min at room temperature, the remaining amounts of DPPH were determined by colorimetry at 520 nm on a microplate reader.[14] The radical scavenging activity of each compound was expressed by the ratio of the lowering of the DPPH solution in the absence of compounds. The mean values were obtained from triplicate experiments.

Riboflavin-originated Superoxide Quenching Activity – Superoxide quenching activities of test samples were measured photochemically, using an assay system consisting of methionine, riboflavin, and nitrobluetetrazolium (NBT).[15,16] The reaction mixture was composed of 2.6 μM riboflavin, 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 0.05 M sodium phosphate (pH 7.8), and various concentrations of test samples. The sample was randomly placed in a lighted storage box, and the sample was randomly replaced every 5 min for 15 min. During the light illumination, the temperature of the lighted storage box was 20±1°C. The light intensity at the sample level was 5,500 lux. During the light illumination, NBT was reduced to blue formazan formation that was measured by the absorbance at 560 nm. The inhibition of blue formazan formation was taken as a superoxide quenching activity.

Xanthine-originated Superoxide Scavenging Activity – Superoxide radicals were generated by xanthine/xanthine oxidase and measured by previously reported method.[17] In brief, test samples were mixed with 20 mM phosphate buffer (pH 7.8) containing 0.48 mM NBT and 1.6 mM xanthine. After 5 min, xanthine oxidase (0.05 U/mL) 100 μL was added. The absorbance of reaction mixture was read at 570 nm after 30 min incubation at 37°C. Superoxide radical scavenging activity was expressed by the degree of NBT reduction of a test group in comparison to that of control.

Results and Discussion

In the course of our ongoing search for antioxidants from Korean natural plants, the ethyl acetate soluble fraction of methanolic extract of the aerial parts of *B. frondosa* was found to show scavenging activity on DPPH radical (Fig. 1). Subsequent activity-guided fractionation of the ethyl acetate soluble fraction led to the isolation of five phenolic compounds (Fig. 2).

In the ^{1}H-NMR spectrum of compound 1, the typical flavonoid signals were observed. Singlet signals at δ 6.38 (1H, d, *J*=2.0 Hz, H-8) and 6.19 (1H, d, *J*=2.0 Hz, H-6) revealed 5,7-dihydoxyl groups of A-ring in the flavonoid skeleton. A double doublet signal at δ 7.56 (1H, dd, *J*=8.8, 2.4 Hz, H-6′) and two doublets at 6.71 (1H, d, *J*=8.8 Hz, H-5′) and 7.70 (1H, d, *J*=2.4 Hz, H-2′) showed 3',4'-dihydroxy functional structure corresponding to aromatic B-ring. Therefore, the aglycone of the compound 1 was assignable to quercetin. The glucose proton signals were also detected with an anomeric proton signal at δ 5.25 (1H, d, *J*=7.2 Hz, H-1"). The relative large coupling constant of anomeric proton signal indicated the β configuration of glycoside linkages.[18] The ^{13}C-NMR spectrum showed 21 carbon signals including 15 carbon signals of aglycone and 6 peaks of monosaccharide. On the basis of these observations and the comparison of the data with those previously published, the structure of compound 1 was identified as quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin).[19-21]

In the ^{1}H-NMR of compound 2, two *meta*-coupled aromatic signals were detected at δ 6.79 (1H, d, *J*=2.0 Hz, H-8) and 6.45 (1H, d, *J*=2.0 Hz, H-6). Furthermore, typical 1,3,4-

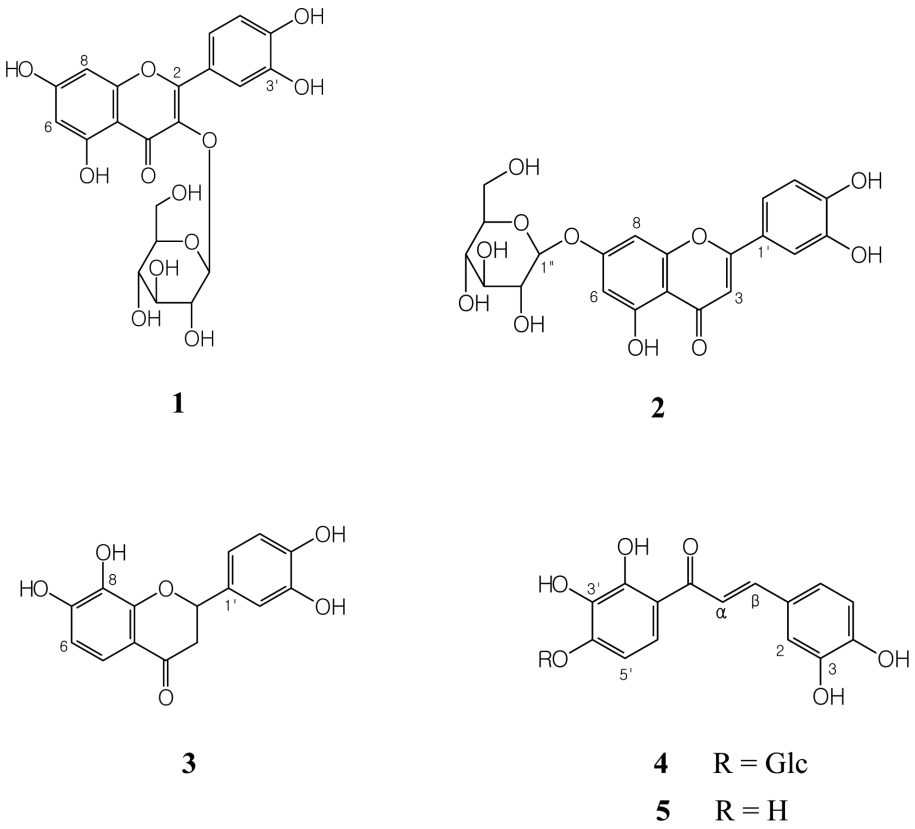

Fig. 2. Structures of compounds 1-5 isolated from *Bidens frondosa.*

trisubstituted benzene signals appeared at δ 7.46 (1H, dd, *J*=8.4, 2.4 Hz, H-6'), 7.42 (1H, d, *brs*, H-2'), and 6.90 (1H, d, *J*=8.4 Hz, H-5'). The signal at δ 6.76 (1H, s, H-3) was characteristic to the C-3 position found in a flavone skeleton. An anomeric proton of the sugar was detected at δ 5.09 (1H, d, *J*=7.4 Hz, H-1"). In the ^{13}C-NMR spectrum of compound 2, chemical shift of the ketone (C-4) was at δ 181.9, indicating a characteristic of flavone flavonoid. The structure of 2 was determined to be luteolin-7-*O*-β-D-glucopyranoside on the basis of the above evidences, together with a comparison of the above data with those published in the literature.[22] Compound 3 showed positive results toward $FeCl_3$ reagent. In ^{1}H-NMR, compound 3 displayed two doublets at δ 6.98 (1H, d, *J*=2.0 Hz, H-2') and 6.78 (1H, d, *J*=8.0 Hz, H-5'), and one double doublet at δ 6.86 (1H, dd, *J*=8.0, 2.0 Hz, H-6') characteristic of a 1,2,4-trisubstituted benzene ring, and two doublets at δ 7.29 (1H, d, *J*=8.8, H-5) and 6.52 (1H, d, *J*=8.8 Hz, H-6) of A-ring. Further, the ^{1}H-NMR spectrum of 3 displayed three double doublets at δ 5.37 (1H, dd, *J*=12.8, 2.8 Hz, H-2), 3.08 (1H, dd, *J*=16.8, 12.8 Hz, H-3α), and 2.74 (1H, dd, *J*=16.8, 2.8 Hz, H-3β) characteristic of C-ring of flavanone. The combination of the substitution patterns of the A, B and C rings suggested that the compound 3 could be 7,8,3',4'-tetrahydroxy flavonoid. In the ^{13}C-NMR spectrum, compound 3 showed 15 carbon signals including a carbonyl signal at δ 194.0. On the basis of these observation and the comparision of the data with those previously published, the structure of compound 3 was identified as 7,8,3',4'-tetrahydroxyflavanone.[23]

Compound 4 showed positive results toward $FeCl_3$ reagent. ^{1}H-NMR spectrum of compound 4 displayed two doublets at δ 7.18 (1H, d, *J*=2.0 Hz, H-2) and 6.81 (1H, d, *J*=8.0 Hz, H-5), and one double doublet at δ 7.10 (1H, dd, *J*=8.0, 2.0 Hz, H-6) characteristic of a 1,2,4-trisubstituted benzene ring, and two doublets at δ 7.58 (1H, d, *J*=9.2, H-6') and 6.82 (1H, d, *J*=9.2 Hz, H-5') of A-ring. The ^{1}H-NMR spectrum of 4 displayed two olefinic protons having trans-configuration were observed at δ 7.24 (1H, d, *J*=15.6 Hz, H-α) and 7.52 (1H, d, *J*=15.6 Hz, H-β). Further, the ^{1}H-NMR spectrum of 4 displayed a doublet at δ 4.98 (1H, d, *J*=7.2 Hz, H-1") for the anomeric proton which suggest that there should be a glycoside group. In the ^{13}C-NMR spectrum, compound 4 showed 21 carbon signals including a carbonyl signal at δ 194.6. On the basis of these observation and the comparison of the data with those previously published, the structure of compound 4 was identified as okanin-4'-*O*-β-D-glucopyranoside.[24]

Compound 5 showed positive results toward $FeCl_3$ reagent. NMR spectra of compound 5 showed very similar of compound 4 except glycoside group. ^{1}H-NMR spectrum of compound 5 displayed two doublets at δ 7.18 (1H, d, *J*=2.0 Hz, H-2) and 6.81 (1H, d, *J*=8.0 Hz, H-5) and one double doublet at δ 7.10 (1H, dd, *J*=8.0, 2.0 Hz, H-6) characteristic of a 1,2,4-trisubstituted benzene ring, and two doublets at δ 7.58 (1H, d, *J*=9.2, H-6') and 6.46 (1H, d, *J*=9.2 Hz, H-5') of A-ring. The ^{1}H-NMR spectrum of 5 displayed two olefinic protons having trans-configuration were observed at δ 7.52 (1H, d, *J*=15.6 Hz, H-α) and 7.72 (1H, d, *J*=15.6 Hz, H-β). In the ^{13}C-NMR spectrum, compound 5 showed 15 carbon signals including a carbonyl signal at δ 194.1. On the basis of these observations and the comparison of the data with those previously published, the structure of compound 5 was identified as okanin.[24]

The DPPH radical scavenging effects of each solvent fraction from *B. frondosa* are shown in Fig. 1. The radical scavenging effects of the five compounds isolated from the EtOAc soluble fraction of *B. frondosa* are shown in Fig. 3. Among these compounds, two compounds 3 (IC_{50} value, 3.75 μg/mL) and 5 (IC_{50} value, 3.90 μg/mL) exhibited stronger scavenging effects on DPPH free radical than did vitamin C (IC_{50} value, 3.32 μg/mL).

To verify additional antioxidant activities of the isolated compounds 1-5, superoxide quenching activities were measured. In the present study, superoxide was found to originated from riboflavin and xanthine. Inhibition of blue

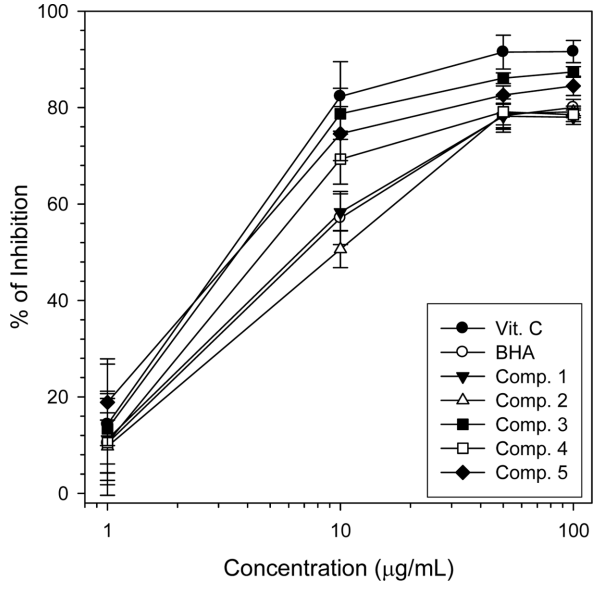

Fig. 3. Scavenging effects of compounds 1-5 from *Bidens frondosa* on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

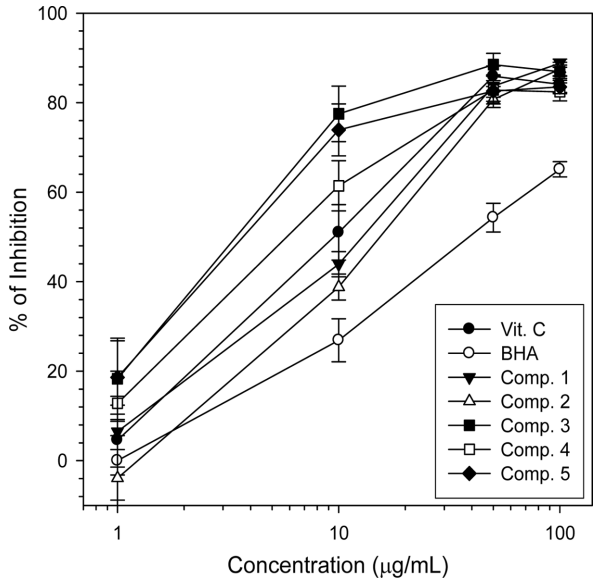

Fig. 4. Riboflavin-originated superoxide quenching activities of compounds 1-5 from *Bidens frondosa.*

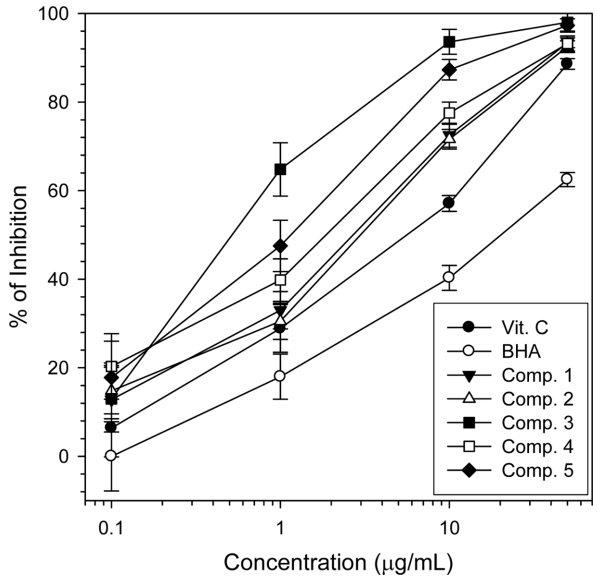

Fig. 5. Xanthine-originated superoxide scavenging activities of compounds 1-5 from *Bidens frondosa.*

formazane formation was taken as a riboflavin-originated superoxide quenching activity. The superoxide quenching activities of five compounds isolated from the EtOAc soluble fraction of *B. frondosa* are shown in Fig. 4. Among five isolated compounds, compounds 3, 4 and 5 exhibited more potent activities (IC_{50} value, each 3.34, 5.62 and 3.71 μg/mL, respectively) than vitamin C (positive control, IC_{50} value, 9.71 μg/mL). Compounds 1 and 2 exhibited weaker quenching activities on riboflavin-originated superoxide than that of the positive control, vitamin C.

The superoxide anion derived from oxidation of xanthine causes the oxidation of NBT into water soluble formazan. The decrease of absorbance at 570 nm with antioxidants represents the quenching of the superoxide anion reaction mixture. The xanthine-originated superoxide quenching activities of five compounds isolated from the EtOAc soluble fraction of *B. frondosa* are shown in Fig. 5. Compounds 1-5 exhibited higher activities with IC_{50} values of 2.42 (1), 2.89 (2), 0.46 (3), 1.86 (4), and 1.13 (5) μg/mL, respectively than that of vitamin C, which was used as a positive control (IC_{50} value, 5.74 μg/mL).

Conclusions

To the best of our knowledge, compounds 3-5 were isolated for the first time from this plant in the present study. Among these compounds, compounds 3 and 5 exhibited stronger scavenging effects on the DPPH free radicals than other isolated compounds, and also compounds 3 and 5 showed the most potent quenching activities against both the riboflavin-originated superoxide and the xanthine-originated superoxide. Results of the present study indicate that the isolated compounds 1-5 from the EtOAc soluble fraction of *B. frondosa* may be useful to treat the various oxidative damages.

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