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Article

Antioxidative Constituents from the Whole Plant of *Actinostemma lobatum* Maxim

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As part of an ongoing search for natural plants with antioxidant compounds by measuring the radical-scavenging effect on DPPH (1,1-diphenyl- 2-picrylhydrazyl), a total extract of the whole plant of *Actinostemma lobatum* (Cucurbitaceae) was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of eight compounds: actinostemmoside C (1), kaempferol-3-*O*-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (2), quercetin-3-*O*-β-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (3), quercetin-3-*O*-β-D-glucopyranoside (4), kaempferol (5), quercetin (6), lobatoside C (7), and lobatoside B (8). Their structures were elucidated using spectroscopic studies. Compounds 2, 4, 5, and 6 were isolated for the first time from this plant. Compounds 2-4 and 6 showed significant antioxidative effects during the DPPH-free radical-scavenging test and the superoxide quenching activity test.

Key words: Cucurbitaceae, DPPH, superoxide quenching activity

Actinostemma lobatum Maxim. (Cucurbitaceae) is an annual vine that grows in Korea, China, and Japan [Lee, 1996; Lee, 2003]. Earlier investigation of the chemical constituents of *A. lobatum* dealt with dammarane-, baccharane-, and oleanane-type triterpenoids [Fujioka *et al*., 1987; Iwamoto *et al*., 1987; Fujioka *et al*., 1988; 1989a; 1989b; 1992]. Some oleanane triterpene glycosides, including lobatosides B-E isolated from *A. lobatum* showed cytotoxicities against several human cancer cell lines [Fujioka *et al*., 1996]. Furthermore, Kim *et al*. [2008] reported that butanol fraction of *A. lobatum* could exert antithrombotic activity through inhibition of platelet aggregation via GP IIb/IIIa and thromboxane A2 pathways, along with anticoagulatory activity through intrinsic and extrinsic pathways.

In the course of searching for antioxidants from plants by measuring their radical-scavenging effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH), extract of the whole plant of *A. lobatum* was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of eight compounds from the active ethyl acetate and *n*-butanol-soluble fractions. In the present study, the isolation and structural characterization of these compounds, as well as their scavenging activity of the stable DPPH free radicals and superoxide quenching activity were evaluated.

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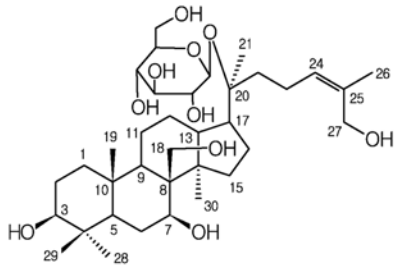
Materials and Methods

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; Fluka, Buchs, Switzeland). 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), 1,1-diphenyl-2-picrylhydrazyl (DPPH), xanthine and xanthine oxidase were obtained from Sigma Chemical Co. (St. Louis, USA).

Plant materials. The whole plant of *A. lobatum* was collected in August 2008 at Wanju, Jeonbuk, Korea, and was identified by Dae Keun Kim, College of Pharmacy, Woosuk University. A voucher specimen was deposited



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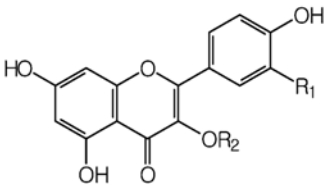
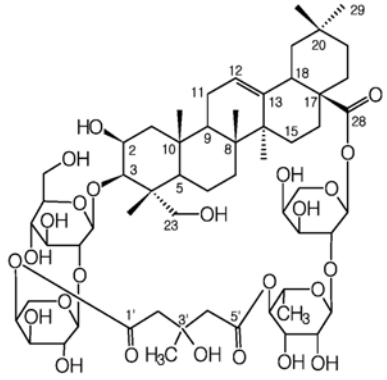


	R_1	R_2
2	H	α-L-Rha (1→6)-β-D-Glc
3	OH	α-L-Rha (1→6)-β-D-Glc
4	OH	β-D-Glc
5	H	H
6	OH	H



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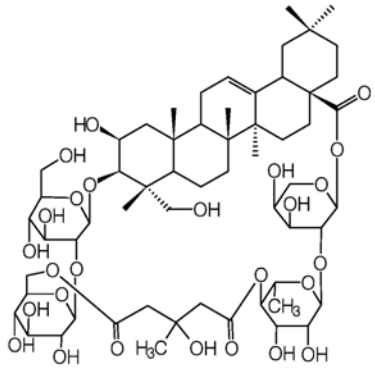

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Fig. 1. Structures of compounds 1-8 isolated from *Actinostemma lobatum*.

in the herbarium of the College of Pharmacy, Woosuk University (WSU-08-005).

Extraction and isolation. The shade-dried and powdered plant of *A. lobatum* (600 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 (95 g) was successively partitioned into *n*-hexane, methylene chloride, ethyl acetate, *n*-butanol, and water-soluble fractions. Each fraction was tested for the radical-scavenging effect on DPPH. Among these fractions, the ethyl acetate-soluble fraction (6 g) showed the most significant free radical-scavenging effect on DPPH. This fraction was subjected to chromatography on a Sephadex LH-20 column (MeOH) to afford six fractions (E1-E6). Fraction E1 was chromatographed on silica gel column chromatography (EtOAc-MeOH, 6:1) to give seven subfractions (E11-E17). Subfraction E16 was purified by HPLC using a GS310 column (MeOH) to give compound **1** (12 mg). Fraction E3 was purified by HPLC using a GS310 column (MeOH) to give compounds **2** (6 mg) and **3** (45 mg). Fraction E4 was applied to silica gel column chromatography using $CHCl_3$-MeOH-H_2O (30:10:1) as an eluent to yield two fractions (E41 and E42), and E42 was purified by Sephadex LH-20 column (MeOH) to give compound **4** (10 mg). Fraction E5 was chromatographed on a silica gel column eluting with ethyl acetate only to give compounds **5** (5 mg). Fraction E6 was crystallized with MeOH to yield compound **6** (8 mg). Another free radical-scavenging effective *n*-butanol-soluble fraction on DPPH was subjected to silica gel column chromatography using $CHCl_3$-MeOH-H_2O (30:10:1) as an eluent to yield eight fractions (B1-B8). Fraction B8 was purified by HPLC using a GS310 column (MeOH) to give compounds **7** (15 mg), **8** (20 mg) and **3** (78 mg), successively.

Actinostemmoside C (1). A colorless amorphous powder (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 5.18 (1H, d, *J*=7.8 Hz, H-1'), 4.41 (1H, d, *J*=8.8 Hz, H-18a), 4.29 (1H, d, *J*=12.0 Hz, H-27a), 4.04 (1H, d, *J*=12.0 Hz, H-27b), 3.98 (1H, d, *J*=8.8 Hz, H-8), 3.56 (1H, dd, *J*=12.0, 5.2 Hz, H-7), 3.25 (1H, t, *J*=8.1 Hz, H-3), 1.66

(3H, s, H-26), 1.20 (3H, s, H-21), 0.91, 0.90, 0.89, 0.72 (each 3H, s, H-19, 28, 29, 30). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 135.2 (C-25), 129.4 (C-24), 98.6 (C-1'), 83.7 (C-20), 79.4 (C-3), 78.5 (C-5'), 77.5 (C-7), 75.6 (C-2'), 71.8 (C-4'), 62.9 (C-6'), 62.5 (C-18), 61.5 (C-27), 55.5 (C-5), 53.0 (C-9), 50.8 (C-14), 47.9 (C-17), 45.3 (C-13), 49.9 (C-8), 41.5 (C-22), 40.6 (C-1), 40.0 (C-4), 38.6 (C-10), 37.0 (C-15), 30.2 (C-6), 28.8 (C-28), 28.7 (C-2), 28.3 (C-16), 26.1 (C-12), 23.7 (C-23), 23.5 (C-11), 22.0 (C-21), 21.6 (C-26), 17.2 (C-30)[a], 17.1 (C-29)[a], 16.6 (C-19)[a]. [a]Values may be interchangeable.

Kaempferol-3-*O*-α-L-rhamnopyranosyl (1→6)-β-D-glucoypyranoside (2). A yellow amorphous powder (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.99 (2H, d, *J*=8.8 Hz, H-2', 6'), 6.88 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.30 (1H, d, *J*=1.8 Hz, H-8), 6.10 (1H, d, *J*=1.8 Hz, H-6), 5.15 (1H, d, *J*=7.0 Hz, H-1"), 4.48 (1H, d, *J*=1.8 Hz 1.4, H-1'"), 1.10 (3H, d, *J*=6.0 Hz, H-6'"). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) See Table 1.

Quercetin-3-*O*-α-L-rhamnopyranosyl (1→6)-β-D-glucoypyranoside (3). A yellow amorphous powder (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.66 (1H, d, *J*=2.0 Hz, H-2'), 7.62 (1H, dd, *J*=8.8, 2.0 Hz, H-6'), 6.86 (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), 5.10 (1H, d, *J*=8.0 Hz, H-1"), 4.52 (1H, s, H-1'"), 1.11 (3H, d, *J*=6.4 Hz, H-6'"). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) See Table 1.

Quercetin-3-*O*-β-D-glucoypyranoside (4). A yellow amorphous powder (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.59 (1H, d, *J*=2.0 Hz, H-2'), 7.58 (1H, dd, *J*=8.8, 2.0 Hz, H-6'), 6.84 (1H, d, *J*=8.8 Hz, H-5'), 6.37 (1H, d, *J*=2.0 Hz, H-8), 6.18 (1H, d, *J*=2.0 Hz, H-6), 5.17 (1H, d, *J*=8.0 Hz, H-1"). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) See Table 1.

Kaempferol (5). A yellow amorphous powder (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.99 (2H, d, *J*=8.8 Hz, H-2', 6'), 6.81 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.29 (1H, d, *J*=1.8 Hz, H-8), 6.08 (1H, d, *J*=1.8 Hz, H-6). ^{13}C-NMR (100 MHz, CD_3OD, δ_C) See Table 1.

Quercetin (6). A yellow amorphous powder (MeOH); ^{1}H-NMR (400 MHz, DMSO-d_6, δ_H) 12.5 (1H, s, 5-OH), 7.68 (1H, d, *J*=2.0 Hz, H-2'), 7.55 (1H, dd, *J*=8.8, 2.0 Hz, H-6'), 6.88 (1H, d, *J*=8.8 Hz, H-5'), 6.41(1H, d, *J*=2.0 Hz, H-8), 6.19 (1H, d, *J*=2.0 Hz, H-6). ^{13}C-NMR (100 MHz, DMSO-d_6, δ_C) See Table 1.

Lobatoside C (7). A white amorphous powder (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 5.42 (1H, brs, 28Ara-1), 5.28 (1H, brs, 28Rha-1), 5.24 (1H, brs, H-12), 5.06 (1H, t, *J*=9.0 Hz, 3Rha-5), 1.54 (3H, s, H-6'), 1.27 (3H, s, H-25), 1.15, 1.14, 1.00, 0.93, 0.92, 0.72 (each 3H, s, H-24, 26, 27, 29, 30, 28Rha-6), ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 177.8 (C-28), 172.2 (C-5')[a], 172.0 (C-1')[a], 145.0 (C-13), 124.2 (C-12), 104.4 (C-3Ara-1), 103.9 (C-3Glc-1), 100.7 (C-28Rha-1), 94.9 (C-28Ara-1), 84.2 (C-3), 80.2 (C-3Glc-2), 79.0 (C-3Glc-3), 77.9 (C-3Glc-5), 75.5 (C-28Rha-4), 75.0 (C-28Ara-2), 73.9 (C-3Ara-2), 73.0 (C-3Ara-3), 72.7 (C-3Ara-4), 71.5 (C-3Glc-4), 71.3 (C-28Ara-3), 70.9 (C-28Rha-3), 70.5 (C-3'), 68.5 (C-28Rha-5), 68.2 (C-28Ara-4), 65.1 (C-23), 65.0 (C-28Ara-5), 64.9 (C-3Ara-5), 62.7 (C-3Glc-1), 48.3 (C-9), 47.9 (C-4'), 47.2 (C-5), 47.1 (C-17), 47.0 (C-2'), 44.6 (C-1), 44.0 (C-4), 43.0 (C-14), 42.6 (C-18), 41.0 (C-8), 38.1 (C-10), 35.1 (C-21), 33.9 (C-29), 33.3 (C-7), 31.8 (C-22), 29.9 (C-20), 29.8 (C-15), 27.0 (C-6'), 26.1 (C-27), 24.9 (C-11), 24.5 (C-30), 23.7 (C-16), 19.7 (C-6), 18.6 (C-28Rha-6), 18.3 (C-25), 18.2 (C-26), 16.1 (C-24). [a]Values may be interchangeable.

Table 1. ^{13}C-NMR spectral data of compounds 2-6 (100 MHz)

Compound position	**2** δ_c	**3** δ_c	**4** δ	**5** δ_c	**6** δ_c
2	158.3	159.4	158.4	147.9	146.8
3	135.4	135.8	135.4	137.0	135.7
4	179.0	179.5	179.3	177.2	175.8
5	162.8	163.0	163.0	162.4	160.7
6	100.0	100.2	100.0	99.2	98.1
7	165.8	166.2	166.3	165.4	163.9
8	95.0	95.1	94.8	94.4	93.3
9	159.2	158.6	158.8	158.1	156.1
10	105.6	105.8	105.6	104.5	103.0
1'	122.6	123.7	123.0	123.7	121.9
2'	132.3	116.2	116.0	130.6	115.0
3'	116.1	146.0	146.0	116.2	145.0
4'	161.4	149.9	149.9	160.4	147.7
5'	116.1	117.9	117.3	116.2	115.6
6'	132.3	123.3	123.3	130.6	119.9
1"	104.6	104.9	104.4		
2"	75.7	76.0	75.5		
3"	78.2	78.4	78.2		
4"	71.4	71.6	71.2		
5"	77.2	77.4	78.4		
6"	68.6	68.8	62.6		
1'"	102.3	102.6			
2'"	72.0	72.3			
3'"	72.3	72.5			
4'"	74.0	74.2			
5'"	69.7	69.9			
6'"	18.0	18.2			

Compounds **2-5** were dissolved in CD_3OD, and compound **6** in DMSO-d_6.

Lobatoside B (8). A colorless needle (MeOH); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 5.39 (1H, brs, 28Rha-1), 5.31 (1H, brs, H-12), 5.24 (1H, d, *J*=9.0 Hz, 28Ara-1),

5.03 (1H, t, *J*=9.0 Hz, 28Rha-4), 1.47, 1.38, 1.28, 1.20, 0.93, 0.91, 0.87, 0.73 (each 3H, s, H-6', 24, 25, 26, 27, 29, 30, 28Rha-6), ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 176.9 (C-28), 172.7 (C-5')[a], 172.6 (C-1')[a], 145.2 (C-13), 123.7 (C-12), 106.1 (C-3Glc'-1), 103.9 (C-3Glc-1), 102.6 (C-28Rha-1), 95.3 (C-28Ara-1), 85.3 (C-3Glc-2), 84.2 (C-3), 78.5 (C-3Glc-3), 77.7 (C-3Glc-5), 77.5 (C-3Glc'-3), 77.1 (C-3Glc'-2), 76.9 (C-28Rha-3), 76.0 (C-28Rha-4), 75.9 (C-28Ara-3), 75.7 (C-28Ara-2), 74.3 (C-3Glc'-5), 72.9 (C-28Rha-2), 71.0 (C-28Ara-4)[a], 70.9 (C-3Glc'-4)[a], 70.8 (C-3'), 70.7 (C-3Glc-4)[a], 70.6 (C-2)[a], 68.5 (C-28Ara-5)[b], 68.2 (C-28Rha-5)[b], 65.1 (C-3Glc'-6), 64.7 (C-23), 62.4 (C-3Glc-6), 48.6 (C-5), 48.5 (C-9), 48.3 (C-17), 48.0 (C-4'), 46.7 (C-19), 46.1 (C-2'), 44.7 (C-1), 43.1 (C-14), 41.8 (C-18), 41.0 (C-8), 37.3 (C-10), 34.2 (C-21), 33.8 (C-7), 33.7 (C-29), 32.9 (C-22), 31.8 (C-20), 31.6 (C-15), 27.7 (C-6'), 26.6 (C-27), 25.0 (C-11), 24.9 (C-30), 24.4 (C-16), 19.4 (C-28Rha-6), 18.5 (C-6), 18.1 (C-26), 18.0 (C-25), 15.6 (C-24). [a,b]Values may be interchangeable.

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 min 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 by the ratio of the lowering of the DPPH solution in the absence of compounds. The mean values were obtained from triplicate experiments.

Riboflavin and superoxide quenching activity. Superoxide quenching activities of test samples were measured photochemically, using an assay system consisting of methionine, riboflavin, and nitrobluetetrazolium (NBT) [Ginnopolitis and Ries, 1977; Choi *et al*., 2001]. 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 samples were randomly placed in a light storage box and replaced randomly every 5 min for 15 min. The temperature within the light storage box was 20±1°C during the light illumination. The light intensity at the sample level was 5,500 lux. During the light illumination, NBT reduced to blue formazane formation was measured at 560 nm. The inhibition of blue formazane formation was taken as superoxide-quenching activity.

Xanthine and superoxide-scavenging assays. Superoxide radicals were generated by xanthine/xanthine oxidase and measured by the method of Thuong *et al*. [2009]. 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 (of mixing?), 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.

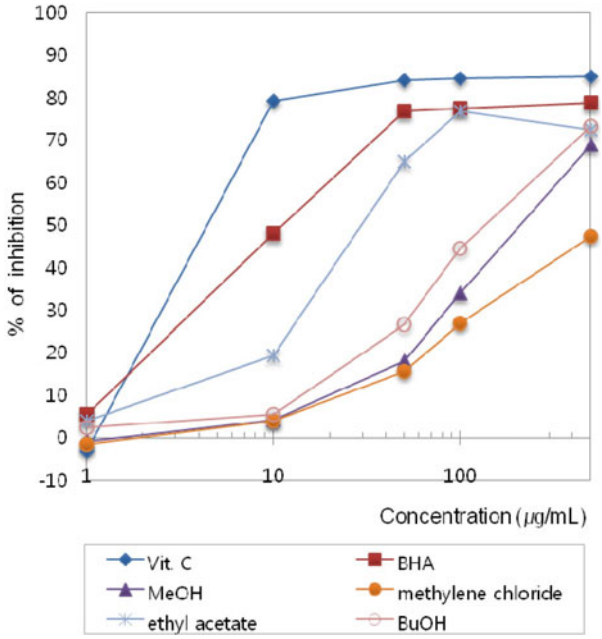

Fig. 2. Radical-scavenging effects of the fractions from the whole plant of *Actinostemma lobatum* on DPPH radicals.

Results and Discussion

In the course of screening for antioxidative components from Korean natural plants, the ethyl acetate and *n*-butanol-soluble fractions of methanolic extract of the whole plant of *A. lobatum* was found to show scavenging activity on DPPH radical (Fig. 2) [Lee *et al*., 2008; Kim, 2009; Lee *et al*., 2009]. Subsequent activity-guided chromatogrphy of the ethyl acetate and *n*-butanol-soluble fractions led to the isolation of five flavonoids and three triterpene compounds.

Compounds **1**, **7**, and **8** were readily elucidated as actinostemmoside C, lobatoside C and lobatoside B, respectively, by comparing with reported spectroscopic data of this plant [Iwamoto *et al*., 1987; Fujioka *et al*., 1989a; 1989b]. Compounds **2-6** showed positive reactions on Mg-HCl tests, and similar patterns in their NMR spectra. Compounds **2-6** were deduced to be flavonoids. The structure characterization of these compounds was carried out by interpretating their spectral data and comparing them with the data reported in the literature. Compounds **2-6** were identified as kaempferol-3-*O*-α-L-

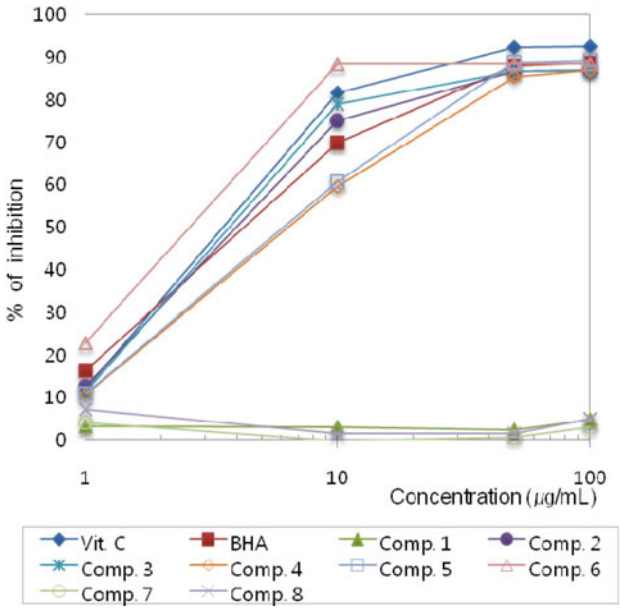

Fig. 3. Radical-scavenging effects of the isolated compounds from the whole plant of *Actinostemma lobatum* on DPPH radicals.

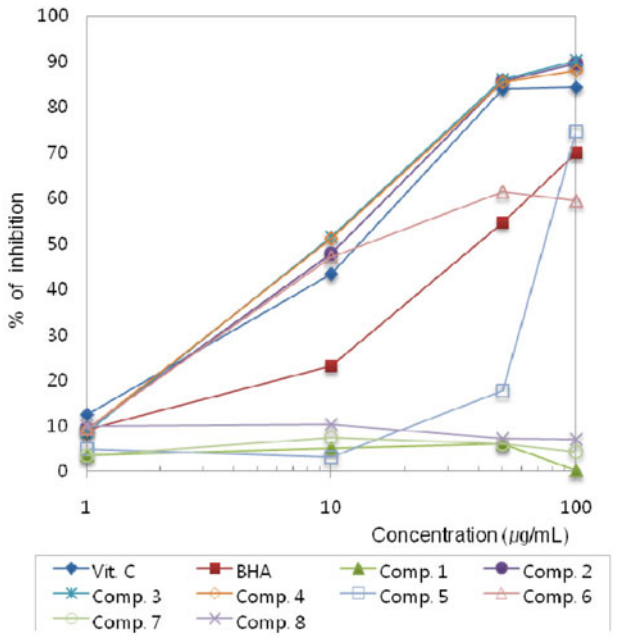

Fig. 4. Riboflavin-originated superoxide-quenching activities of the isolated compounds from the whole plant of *Actinostemma lobatum*.

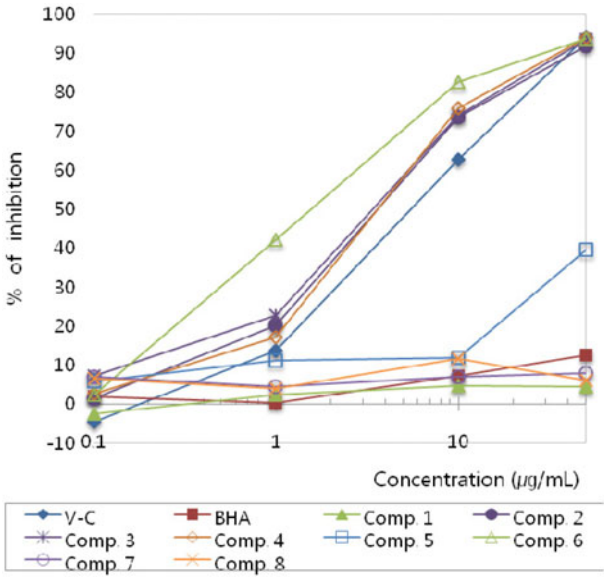

Fig. 5. Xanthine-originated superoxide-quenching activities of the isolated compounds from the whole plant of *Actinostemma lobatum*.

rhamnopyranosyl (1→6)-β-D-glucoypyranoside (**2**) [Han *et al*., 2004], quercetin-3-*O*-α-L-rhamnopyranosyl (1→6)-β-D-glucoypyranoside, rutin (**3**) (Han *al*., 2004), quercetin-3-*O*-β-D-glucoypyranoside, isoquercitrin (**4**) [Han *al*., 2004], kaempferol (**5**) [Budzianowski, 1991], and quercetin (**6**) [Park *et al*., 1996]. Compounds **2**, **4**, **5**, and **6** were isolated for the first time from this plant.

The radical-scavenging effects of eight compounds obtained from *A. lobatum* are shown in Fig. 3. The positive control vitamin C showed the DPPH radical-scavenging effect with the IC_{50} value of 3.3 μg/mL. Compounds **2**, **3**, and **6** exhibited scavenging activities that were dose-dependent on DPPH, with IC_{50} values of 4.0, 3.7, and 2.5 μg/mL, respectively. However, compounds **1**, **7**, and **8** showed no activities in comparison with reference antioxidants such as ascorbic acid and BHA. Fig. 4 shows the superoxide quenching activities of the isolated compounds **1-8**, as measured using the riboflavin-NBT-light system. The positive control vitamin C showed the superoxide-quenching activity with the IC_{50} value of 13.4 μg/mL. In contrast, compounds **2**, **3**, **4**, and **6** exhibited superoxide-quenching activities that were dose-dependent on the riboflavin-NBT-light system, with IC_{50} values of 11.8, 1.05, 1.09 and 18.3 μg/mL, respectively. Compounds **1**, **7**, and **8** showed no activities in comparison with reference antioxidants (Fig. 4). Fig. 5 shows the superoxide-quenching activities of the isolated compounds **1-8**, as measured using xanthine-NBT-xanthine oxidase systems. Compound **6** was found to be a potent scavenger of superoxide radicals generated in this system. In the xanthine-NBT-xanthine oxidase system, compounds **2**, **3**, **4**, and **6** exhibited the formation of the blue formazan in a dose-dependent manner with IC_{50} values of 3.7, 3.2, 3.6, and 1.6 μg/mL, respectively (vitamin C, positive control, IC_{50} value, 5.3 μg/mL) (Fig. 5). The superoxide-quenching activities of compounds **2, 3, 4** and **6** were higher than that of vitamin C, used as a positive control. However, compounds **1**, **7,** and **8** showed no activities in comparison with the reference

antioxidant, ascorbic acid.

The present study showed that, among the isolated compounds, compounds **3** and **6** had potent DPPH radical-scavenging and xanthine-originated superoxide-quenching activities. Antioxidative flavonoids isolated from *A. lobatum* are known as DPPH scavengers; however, little information is available on the riboflavin- and xanthine-originated superoxide-quenching activities [Kang *et al*., 2005]. Triterpene compounds **1**, **7**, and **8** showed no activities. Compound **3** was isolated in relatively higher amount than other constituents from ethyl acetate and butanol-soluble fractions. Therefore. it could be deduced that DPPH radical-scavenging and superoxide-quenching activities of methanolic extract, especially butanol-soluble fraction, of *A. lobatum* were mainly due to compound **3**. In conclusion, the methanolic extract of *A. lobatum* including compounds **2-4** and **6** could be a good source of natural antioxidants.

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