

## Antioxidative Constituents from *Buddleia officinalis*

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Four flavonoids (**1-4**), a phenylethyl glycoside (**5**), and a phenylpropanoid glycoside (**6**) were isolated from the flowers of *Buddleia officinalis* (Loganiaceae). Their structures were determined by chemical and spectral analysis. Among the isolated compounds, luteolin (**1**) and acteoside (**6**) exhibited the most potent antioxidative activity on the NBT superoxide scavenging assay. In addition, compounds **1-6** revealed weak antifungal activity, and no hemolytic activity.

**Key words:** *Buddleia officinalis*, Antioxidant, NBT superoxide scavenging assay, Antifungal activity

### INTRODUCTION

*Buddleia* Flos (Chinese name: Mi Meng Hua) prepared from the flowers and buds of *B. officinalis* (Loganiaceae) is a traditional medicine used for the treatment of conjunctival congestion, clustered nebulae, and antiseptic (Houghton 1984). Previous studies on *Buddleia* species have led to the isolation of various natural products, including triterpenoid glycosides (Ding *et al.*, 1992), phenylethanoids (Pardo *et al.*, 1993), flavonoids (Matsuda *et al.*, 1995), and diterpenes (Houghton *et al.* 1996; Mensah *et al.*, 2000). Concerning the biological studies of *Buddleia* Flos, inhibitory effects on aldose reductase (Matsuda *et al.*, 1995), and antiinflammatory activity were reported (Liao *et al.*, 1999). But, antioxidant, antifungal and hemolytic activity of the constituents of *Buddleia* Flos have not been adequately investigated.

In an ongoing investigation into biologically active compounds from natural products, an ethyl acetate soluble fraction of *B. officinalis* was found to inhibit superoxide radical generation significantly *in vitro*. By means of a bioassay-directed chromatographic separation technique, luteolin (**1**), acacetin-7-O- $\alpha$ -L-rhamnopyranosyl-(1-6)- $\beta$ -D-glucopyranoside (**2**), epigallocatechin (**3**), apigenin-7-O- $\beta$ -D-glucopyranoside (**4**), 2-

phenylethyl- $\beta$ -D-glucopyranoside (**5**), and acteoside (**6**) were isolated. Among them, 2-phenylethyl- $\beta$ -D-glucopyranoside (**3**) have been isolated from this plant for the first time. The antioxidative activities of the isolated compounds were tested by a NBT superoxide scavenging assay according to the established method (Kirby and Schmidt 1997). Among these compounds, luteolin (**1**), and acteoside (**6**) exhibited potent antioxidative activity, while the remaining compounds (**2-5**) showed weak activity. In addition, compounds **1-6** exhibited weak antifungal activity against *C. albicans*, *S. cerevisiae*, and *T. beigeli*, but no hemolytic activity. This paper reports the biological activity of the constituents of *Buddleia* Flos.

### MATERIALS AND METHODS

#### General procedure

NMR spectra were recorded on a Varian Unity Inova 500, and JEOL JNM-LA 300 spectrometer. <sup>1</sup>H-<sup>1</sup>H COSY, DEPT, HMQC, and HMBC NMR spectra were obtained with the usual pulse sequences. FAB/MS were determined on a JMS 700 (JEOL). TLC and column chromatography were carried out on precoated Si Gel F<sub>254</sub> plates (Merck, art. 5715), RP-18 F<sub>254</sub> plates (Merck, art. 15423), and Si gel 60 (Merck, 70-230 and 230-400 mesh).

#### Plant material

The flowers and buds of *B. officinalis* (Loganiaceae)

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were purchased from local Korean herb drug market in Gwangju, Korea, and was authenticated by Department of Pharmacognosy, Chosun University. Voucher specimens were deposited in the Herbarium of College of Pharmacy, Chosun University (881-16).

### Extraction and isolation

*Buddleiae Flos* (600 g) was extracted with MeOH at room temperature to afford 159.7 g of residue. The methanol extract was suspended in water and then partitioned by dichloromethane, ethyl acetate, and *n*-butanol in turn. Three gram of the EtOAc fraction were subjected to column chromatography over a silica gel (300 g, 4.7×53 cm) eluting with a CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (10:1:0.1 → 8:1:0.1 → 5:1:0.1 → 2:1:0.1 → 1:1:0.1 → MeOH only) gradient system. Fractions were combined based on their TLC pattern to yield subfraction designated as E1-E15. Subfraction E3 (127.14 mg), and E10 (126.41 mg) were recrystallized from MeOH to afford compound **2** (35 mg), and **4** (60 mg), respectively. Subfraction E7 (355.01 mg) was further purified by column chromatography over a silica gel (120 g, 2.1×50 cm) eluting with a CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O gradient system to afford four subfractions (E71-E74). Subfraction E72 (14.22 mg), and E73 (285.03 mg) were purified by column chromatography over a silica gel, RP-18, and Sephadex LH 20 to give compound **1** (2.19 mg), and **3** (9.91 mg). Subfraction E9 (138.68 mg) was purified by column chromatography over a silica gel, Sephadex LH 20 eluting with a CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O gradient system, and MeOH, respectively, to afford compound **5** (6.37 mg). In addition, subfraction E13 (644.62 mg) was further purified by column chromatography over a silica gel (100 g, 2.8×44.5 cm) eluting with an EtOAc-MeOH-H<sub>2</sub>O gradient system to give three subfractions (E131-E133). Subfraction E132 (337.55 mg) was finally purified by repeated column chromatography over a silica gel, RP-18, and Sephadex LH 20 to afford compound **6** (48.23 mg).

#### Luteolin (1)

FAB-MS: *m/z* 287.1 [M+H]<sup>+</sup>; <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz): δ 6.64 (1H, s, H-3), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.16 (1H, d, *J* = 2.0 Hz, H-6), 7.37 (1H, d, *J* = 2.0 Hz, H-2'), 6.87 (1H, d, *J* = 8.0 Hz, H-5'), 7.40 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'); <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz): δ 164.58 (C-2), 103.44 (C-3), 182.28 (C-4), 162.14 (C-5), 99.58 (C-6), 165.07 (C-7), 94.58 (C-8), 158.00 (C-9), 104.26 (C-10), 121.99 (C-1'), 113.91 (C-2'), 146.48 (C-3'), 150.64 (C-4'), 116.69 (C-5'), 119.69 (C-6').

#### Acacetin-7-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside (2)

FAB-MS: *m/z* 593.1 [M+H]<sup>+</sup>; <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 300

MHz): δ 6.94 (1H, s, H-3), 6.45 (1H, d, *J* = 2.0 Hz, H-6), 6.78 (1H, d, *J* = 2.0 Hz, H-8), 8.04 (2H, d, *J* = 8.7 Hz, H-2', 6'), 7.14 (2H, d, *J* = 8.7 Hz, H-3', 5'), 3.85 (3H, s, OCH<sub>3</sub>), 3.11-3.66 (sugar protons), 5.05 (1H, d, *J* = 7.0 Hz, H-1''), 4.54 (1H, br.s., H-1'''), 1.07 (3H, d, *J* = 6.0 Hz, H-6'''); <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 75 MHz): δ 163.16 (C-2), 104.02 (C-3), 182.22 (C-4), 161.35 (C-5), 99.87 (C-6), 164.14 (C-7), 94.98 (C-8), 157.19 (C-9), 105.67 (C-10), 122.89 (C-1'), 128.66 (C-2', 6'), 114.91 (C-3', 5'), 162.62 (C-4'), 55.77 (OCH<sub>3</sub>), 100.72 (Glc-1''), 73.27 (C-2''), 76.45 (Glc-3''), 70.94 (C-4''), 75.87 (C-5''), 66.29 (C-6''), 100.14 (C-1'''), 70.55 (C-2'''), 69.79 (C-3'''), 72.25 (C-4'''), 68.53 (C-5'''), 18.00 (C-6''').

#### 2-Phenylethyl-β-D-glucopyranoside (3)

FAB-MS: *m/z* 285.2 [M+H]<sup>+</sup>; <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz): δ 7.16-7.28 (5H, m, H-2,3,4,5,6), 2.85 (2H, t, *J* = 6.5 Hz, H-7), 3.64 (1H, m, H-8), 3.93 (1H, m, H-8), 4.17 (1H, d, *J* = 8.0 Hz, Glc-1'), 3.30-3.50 (sugar protons); <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz): δ 139.42 (C-1), 128.91 (C-2,6), 129.60 (C-3,5), 126.75 (C-4), 36.35 (C-7), 70.16 (C-8), 103.55 (C-1'), 74.13 (C-2'), 77.60 (C-3'), 70.80 (C-4'), 77.48 (C-5'), 61.79 (C-6').

#### Aapigenin (4)

FAB-MS: *m/z* 271.2 [M+H]<sup>+</sup>; <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 300 MHz): δ 6.76 (1H, s, H-3), 6.46 (1H, d, *J* = 2.1 Hz, H-8), 6.17 (1H, d, *J* = 2.1 Hz, H-6), 7.91 (2H, d, *J* = 8.7 Hz, H-2', 6'), 6.91 (2H, d, *J* = 8.7 Hz, H-3', 5'); <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 75 MHz): δ 163.88 (C-2), 102.99 (C-3), 181.89 (C-4), 161.38 (C-5), 99.07 (C-6), 164.58 (C-7), 94.17 (C-8), 157.51 (C-9), 103.78 (C-10), 121.34 (C-1'), 128.64 (C-2', 6'), 116.14 (C-3', 5'), 161.63 (C-4').

#### Apigenin-7-O-β-D-glucopyranoside (5)

FAB-MS: *m/z* 433.4 [M+H]<sup>+</sup>; <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz): δ 6.85 (1H, s, H-3), 6.82 (1H, d, *J* = 2.0 Hz, H-8), 6.43 (H, d, *J* = 2.0 Hz, H-6), 7.94 (2H, d, *J* = 8.5 Hz, H-2', 6'), 6.93 (2H, d, *J* = 8.5 Hz, H-3', 5'), 5.05 (1H, d, *J* = 7.5 Hz, H-1''), 3.16-3.88 (sugar protons); <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz): δ 164.49 (C-2), 103.25 (C-3), 182.19 (C-4), 161.72 (C-5), 99.72 (C-6), 163.15 (C-7), 95.04 (C-8), 157.14 (C-9), 105.53 (C-10), 121.11 (C-1'), 128.81 (C-2',6'), 116.23 (C-3',5'), 161.30 (C-4'), 100.11 (C-1''), 73.30 (C-2''), 76.63 (C-3''), 69.76 (C-4''), 77.37 (C-5''), 60.80 (C-6'').

#### Acteoside (6)

FAB-MS: *m/z* 625.2 [M+H]<sup>+</sup>; <sup>1</sup>H-NMR (CD<sub>3</sub>OD, 500 MHz): δ 6.70 (1H, d, *J* = 2.0 Hz, H-2), 6.67 (1H, d, *J* = 8.0 Hz, H-5), 6.56 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 2.79 (2H, m, H-7), 3.71 (1H, dd, *J* = 16.0, 8.0 Hz, H-8), 4.02 (1H, dd, *J* = 16.0, 8.0 Hz, H-8), 4.37 (1H, d, *J* = 8.0 Hz, H-1'), 3.39

(1H, dd  $J = 9.3, 8.0$  Hz, H-2'), 3.81 (1H, t,  $J = 9.3$  Hz, H-3'), 4.92 (1H, t,  $J = 9.3$  Hz, H-4'), 3.52 (1H, t,  $J = 9.3$  Hz, H-5') 3.52 (1H, m, H-6'), 3.60 (1H, m, H-6'), 5.19 (1H, d,  $J = 1.5$  Hz, H-1''), 3.93 (1H, d,  $J = 1.5$  Hz, H-2''), 3.58 (2H, m, H-3'', 5''), 3.30 (1H, m, H-4''), 1.09 (3H, d,  $J = 6.5$  Hz, H-6''), 7.05 (1H, d,  $J = 2.0$  Hz, H-2'''), 6.78 (1H, d,  $J = 8.0$  Hz, H-5'''), 6.95 (1H, dd,  $J = 8.0, 2.0$  Hz, H-6'''), 7.59 (1H, d,  $J = 13.0$  Hz, H-7'''), 6.27 (1H, d,  $J = 16.0$  Hz, H-8''');  $^{13}\text{C}$ -NMF ( $\text{CD}_3\text{OD}$ , 125 MHz):  $\delta$  131.46 (C-1), 117.10 (C-2), 146.12 (C-3), 144.67 (C-4), 116.50 (C-5), 121.25 (C-6), 36.51 (C-7), 72.25 (C-8), 104.20 (C-1'), 76.20 (C-2'), 81.64 (C-3'), 70.57 (C-4'), 76.03 (C-5'), 62.36 (C-6'), 103.02 (C-1''), 72.34 (C-2''), 72.04 (C-3''), 73.78 (C-4''), 70.4 (C-5''), 18.44 (C-6'') 127.65 (C-1'''), 115.21 (C-2'''), 146.83 (C-3'''), 149.79 (C-4'''), 116.30 (C-5'''), 123.21 (C-6'''), 148.01 (C-7'''), 114.69 (C-8'''), 168.28 (C=O).

### NBT superoxide scavenging assay

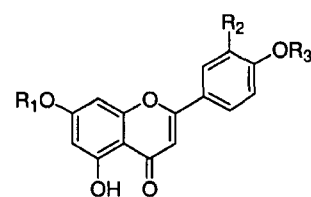
The NBT superoxide scavenging assay was carried out using a slight modification of the established method (Kirby and Schmidt 1997). The reaction mixture, which was equilibrated at 25°C, contained 20  $\mu\text{L}$  of a 15 mM  $\text{Na}_2\text{EDTA}$  solution in a buffer (50 mM  $\text{KH}_2\text{PO}_4/\text{KOH}$  pH 7.4 *in d.w.*), 50  $\mu\text{L}$  of 0.6 mM NBT in a buffer, 30  $\mu\text{L}$  of a 3 mM hypoxanthine in 50 mM KOH solution, 50  $\mu\text{L}$  of xanthine oxidase solution in a buffer (1 units in 10 mL buffer) and 100  $\mu\text{L}$  of the sample. The plate reader (Molecular Devices Vmax) took readings every 20 s for 5 min at 570 nm. The control was 100  $\mu\text{L}$  of 5% DMSO solution instead of the sample. Results were expressed as relative percentage inhibition to control, given by [(rate of control - rate of sample reaction)/rate of control]  $\times 100$ . Allopurinol was used as a reference compound.

### Antifungal activity

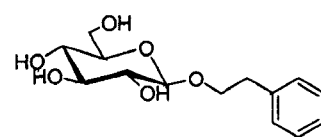
*Candida albicans* (TIMM 1768), *Saccharomyces cerevisiae* (KCTC 7296), and *Trichosporon beigelii* (KCTC 7707) were grown at 28°C in YPD (Yeast extract: Peptone: Dextrose, 10 g: 20 g: 20 g per liter) medium. The fungal cells were seeded in the wells of a 96-microtiter plate in YPD media at a density of  $2 \times 10^3$  cells (100  $\mu\text{L}$  per well). Ten  $\mu\text{L}$  of the serially diluted compound solution was added to each well and the cell suspension was incubated for 24 h at 28°C. Ten  $\mu\text{L}$  of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution [5 mg/mL MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each well, and the plates were incubated for a further at 37°C. 30  $\mu\text{L}$  of 20% (w/v) SDS solution containing 0.02 M HCl was then added, and the plates were incubated at for 16 h 37°C to dissolve the formazan crystals that had formed (Jahn *et al.*, 1995). The turbidity of each well was measured at 570 nm using an ELISA reader (Molecular Devices Emax, California, USA).

### Hemolytic activity

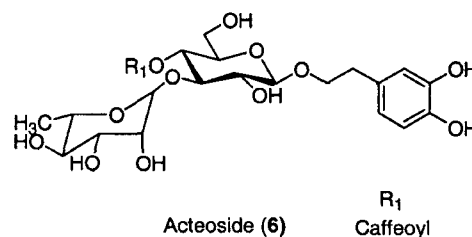
The hemolytic activities of the isolated compounds were evaluated by determining the release of hemoglobin from 4% suspension of fresh human erythrocytes at 414 nm (Blonde and Houghten 1992). Human erythrocyte cells were centrifuged and washed three times with phosphate-buffered saline (PBS: 35 mM phosphate buffer/0.15 M NaCl, pH 7.0). One hundred  $\mu\text{L}$  of human red blood cells suspended in 8% (v/v) in PBS were inoculated into 96-well plates. One Hundred  $\mu\text{L}$  of the sample solution was then added to each well. The plates were incubated at 37°C for 1 h, and then centrifuged at 150 $\times$ g for 5 min. One hundred  $\mu\text{L}$  aliquots of the supernatant were transferred to the flat-bottom 96 well microtiter plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader (Molecular Devices Emax, Sunnyvale, CA, USA). Hemolysis rates of 0% and 100% were determined in PBS and 0.1% Triton-x100, respectively. The hemolysis percentage was calculated using the following equation: % hemolysis = [(Abs<sub>414 nm</sub> in the sample solution - Abs<sub>414 nm</sub> in PBS)/(Abs<sub>414 nm</sub> in 0.1% Triton-x100 - Abs<sub>414 nm</sub> in PBS)]  $\times 100$ .



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Luteolin (1)	H	OH	H
Acacetin-7-O- $\alpha$ -L-rhamnopyranosyl (1-6)- $\beta$ -D-glucopyranoside (2)	Glc(6-1)Rha	H	CH <sub>3</sub>
Apigenin (4)	H	H	H
Apigenin-7-O- $\beta$ -D-glucopyranoside (5)	Glc	H	H



2-Phenylethyl- $\beta$ -D-glucopyranoside (3)



Acteoside (6) Caffeoyl

Fig. 1. Chemical structures of compounds (1-6) isolated from *B. officinalis*

## RESULTS AND DISCUSSION

Bioassay-guided chromatographic separation of an EtOAc soluble fraction of *B. officinalis* led to the isolation of compounds **1-6** (Fig. 1). Chemical structures of compounds **1-6** were identified as luteolin (Matsuda *et al.*, 1995), acacetin-7-O- $\alpha$ -L-rhamnopyranosyl-(1-6)- $\beta$ -D-glucopyranoside (Matsuda *et al.*, 1995), 2-phenylethyl- $\beta$ -D-glucopyranoside (Fan *et al.*, 1999; Fan *et al.*, 2001), apigenin (Matsuda *et al.*, 1995), apigenin-7-O- $\beta$ -D-glucopyranoside (Matsuda *et al.*, 1995), and acteoside (Kim *et al.*, 2001; Zimin and Zhongjian 1991), respectively, by comparing the UV,  $^1\text{H-NMR}$ , and  $^{13}\text{C-NMR}$  with those of reported in literature.

The antioxidative activity of isolates was summarized in Table I. Among them, luteolin (**1**), and acteoside (**6**) exhibited potent antioxidative activity, while **2-5** showed negligible activity. The  $\text{IC}_{50}$  values for luteolin (**1**), and acteoside (**6**) were 9.1, and 6.0  $\mu\text{g/mL}$  respectively. The antioxidative activities of **1** and **6** were comparable to allopurinol, which was used as a positive control. These results suggest that the *ortho*-dihydroxy (catechol) structure play an important role in the antioxidative function as exhibited in flavonoids (Pietta 2000; Akdemir *et al.*, 2001).

**Table I.** Antioxidative activities of compounds **1-6** isolated from *B. officinalis*.

Compounds	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )
Luteolin ( <b>1</b> )	9.1
Acacetin-7-O- $\alpha$ -L-rhamnopyranosyl-( <b>1-6</b> ) - $\beta$ -D-glucopyranoside ( <b>2</b> )	>120
2-Phenylethyl- $\beta$ -D-glucopyranoside ( <b>3</b> )	>120
Apigenin ( <b>4</b> )	>120
Apigenin-7- $\alpha$ - $\beta$ -D-glucopyranoside ( <b>5</b> )	>120
Acteoside ( <b>6</b> )	6.0
Allopurinol*	1.7

\*Used as a positive control

**Table II.** Antifungal activities of compounds **1-6** isolated from *B. officinalis*

Compounds	Fungal strains MIC ( $\mu\text{g/mL}$ )		
	<i>C. albicans</i>	<i>S. cerevisiae</i>	<i>T. beigeli</i>
<b>1</b>	>50	>50	>50
<b>2</b>	>50	>50	>50
<b>3</b>	>50	>50	50
<b>4</b>	>50	>50	50
<b>5</b>	>50	>50	50
<b>6</b>	>50	>50	50
melittin*	3.125	3.125	6.25

\*Used as a positive control

Antifungal activity of compounds **1-6** was examined against *C. albicans*, *S. cerevisiae*, and *T. beigeli*. As shown in Table II, compounds **1-6** revealed weak antifungal activity. Minimum inhibitory concentration (MIC) of compounds **1-6** were 50 or more than 50  $\mu\text{g/mL}$ . In addition, the hemolysis percentage was measured against human erythrocyte cells at various concentrations of compounds **1-6**. Compounds **1-6** showed no hemolytic activity at 100  $\mu\text{g/mL}$  concentration (data not shown). These results demonstrate that luteolin (**1**) and acteoside (**6**) could be mainly responsible for the potent antioxidative effect of an ethyl acetate soluble fraction of *B. officinalis*. Moreover, luteolin (**1**) and acteoside (**6**), which exhibited potent antioxidative activity, and no hemolytic activity, might be suitable for development as a leading natural antioxidant.

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