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A New Monoterpene from the Flower Buds of *Buddleja officinalis*

Chul Lee[1], Sora Lee[2], and So-Young Park[1,2]*

[1]*Laboratory of Pharmacognosy, College of Pharmacy, Dankook University, Cheonan 330-714, Korea*
[2]*World Class University, Department of Nanobiomedicine, Dankook Univeristy, Cheonan 330-714, Korea*

Abstract – A new monoterpene, crocusatin M (**1**), was isolated from the flower buds of *Buddleja officinalis*, together with four known monoterpenes, (6*R*)-hydroxy-1,1,5-trimethylcyclohex-4-enone (**2**), (+)-dehydrovomifoliol (**3**), 7-epiloliolide (**4**), and crocusatin D (**5**). Their structures were determined by an extensive analysis of 1D, 2D NMR, HRESI-MS, and CD data as well as by comparison of their spectroscopic data with those of literatures. All isolates were evaluated for inhibitory activities on LPS-induced nitric oxide production in RAW 264.7 cells. Among them, compounds **2** and **3** showed moderate inhibitory effects with IC_{50} values of 63.8 and 24.4 μg/ml, respectively.
Keywords – *Buddleja officinalis*, Monoterpene, Nitric oxide

Introduction

The nitric oxide (NO) radical, synthesized by the inducible nitric oxide synthase (iNOS)-catalyzed oxidation of L-argininin, plays a critical role in a number of physiological and pathological processes in mammals. However, excess production of NO by iNOS in macrophages causes various inflammatory diseases (Alderton *et al*., 2001). Therefore, potent inhibitors of NO production might be regarded as promising anti-inflammatory drugs.

As part of our ongoing discovery for NO inhibitors from medicinal plants, we found the MeOH extract of flower buds of *Buddleja officinalis* Maxim. (Buddlejaceae) possessed the inhibitory effect on NO production in LPS-induced RAW 264.7 cells. *B. officinalis* is widely distributed in America, Africa, and Asia, and it has been used traditionally for the treatment of conjunctivitis and ophthalmologic diseases (Piao *et al*., 2003; Lee *et al*., 2010). Previous phytochemical studies on this plant have led to the isolation of triterpenoidal saponins (Guo *et al*., 2004), phenylethanoids (Tai *et al*., 2009), flavonoids (Piao *et al*., 2003), and iridoids (Tai *et al*., 2011) which are well-known for containing anti-oxidative, and anti-inflammatory activities (Liao *et al*., 1999; Piao *et al*., 2003). In this work, we have described in the isolation and structure determination of monoterpenoids, including their NO inhibitory effects.

*Author for correspondence
So-Young Park, PhD, Pharmacognosy Lab., College of Pharmacy, Dankook University, San29, Anseo-dong, Dongnam-gu, Cheonan-si, Chungnam 330-714, Korea
Tel: +82-41-550-1434; E-mail: soypark23@dankook.ac.kr

Experimental

General experimental procedures – UV and CD spectra were obtained on JASCO UV-550 and JASCO J-715 spectrometers, respectively. NMR spectra were recorded on a Bruker Avance III 700 MHz NMR spectrometer using CD_3OD and DMSO-d_6 as solvents with the tetramethylsilane as an internal standard. Chemical shift are presented in ppm. High resolution electrospray-ionization-time-of-flight (HR-ESI-TOF) and low resolution ESI mass spectra were measured on maXis 4G (Bruker) and LCQ Fleet (Thermoscientific), respectively. Semipreparative HPLC was performed using a Shimadzu Prominence UFLC with UV detector. Open column chromatography was performed using a silica gel 60 (Kiesel gel 60, 70 - 230 mesh, Merck) and RP-18 (Part NO. 5982-5752, Agilent). Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F_{254} (0.24 mm, Merck) plates.

Plant material – The flower buds of *B. officinalis* was purchased from a commercial market (Samhong medicinal herb market, Seoul, Korea) in 2013. One of the authors (S.-Y. Park) performed botanical identification, and a voucher specimen has been deposited in Pharmacognosy laboratory of College of Pharmacy, Dankook University, Korea.

Extraction and isolation – Dried and pulverized buds

(3 kg) were extracted with 100 % MeOH (24 L, 3 times) at room temperature. The MeOH filtrate was evaporated *in vacuo* to yield the MeOH extract (301 g). The extract was suspended in distilled water, and then partitioned sequentially into *n*-hexane (55 g), CH_2Cl_2 (15 g), EtOAc (19 g), and *n*-BuOH (61 g). The CH_2Cl_2 soluble fraction (15 g) was chromatographed over a silica gel column chromatography (CC) to yield 8 subfractions (BOC1-BOC8), eluting with a step-gradient solvent system (100% CH_2Cl_2 to 100% MeOH). The subfraction BOC2 and BOC3 were individually subjected over a RP-18 CC (Biotage MPLC, 10 to 100% MeOH, gradient) to give 6 and 7 subfractions (BOC21-BOC26, and BOC31-BOC37), respectively. The subfraction BOC21 was purified with a RP-18 preparative HPLC (15 to 45% ACN, gradient) to yield compounds **2** (6 mg), **3** (4 mg), and **4** (5 mg). Compounds **1** (6 mg), and **5** (3 mg) were obtained by a RP-18 preparative HPLC (15 to 36% ACN, gradient) from the subfraction BOC31.

Crocusatin M (1) – Colorless oil; $[\alpha]_D^{25}$ –45.5° (*c* 0.08, MeOH); UV (MeOH) λ_{max} 243.0 (3.70) nm; CD (*c* 0.06, MeOH): $[\theta]_{323.4}$ +0.42, $[\theta]_{241.2}$ –4.45, $[\theta]_{218}$ +2.96; HRESI-MS *m/z* 185.1177 $[M+H]^+$ (calcd for $C_{10}H_{18}O_3$, 185.1178); ^1H NMR (700 MHz, CD_3OD), and ^{13}C NMR (177 MHz, CD_3OD), see Table 1.

(6*R*)-Hydroxy-1,1,5-trimethylcyclohex-4-enone (2) – Colorless oil; ESI-MS *m/z* 155 $[M+H]^+$; ^1H NMR (700 MHz, CD_3OD) δ_H: 5.81 (1H, s, H-4), 3.99 (1H, s, H-6), 2.33 (1H, d, *J* = 16.1 Hz, H_a-2), 2.22 (1H, d, *J* = 16.1 Hz, H_b-2), 2.04 (3H, s, H_3-10), 1.05 (3H, s, H_3-9), 0.98 (3H, s, H_3-8); ^{13}C NMR (177 MHz, CD_3OD) δ_C: 200.5 (C-3), 164.1 (C-5), 124.9 (C-4), 75.7 (C-6), 48.6 (C-2), 38.1 (C-1), 25.8 (C-9), 20.8 (C-8), 20.2 (C-10).

(+)-Dehydrovomifoliol (3) – Colorless oil; ESI-MS *m/z* 223 $[M+H]^+$; ^1H NMR (700 MHz, CD_3OD) δ_H: 7.00 (1H, d, *J* = 15.4 Hz, H-7), 6.42 (1H, d, *J* = 15.4 Hz, H-8), 5.93 (1H, s, H-4), 2.60 (1H, d, *J* = 16.8 Hz, H_a-2), 2.30 (3H, s, H_3-10), 2.26 (1H, d, *J* = 16.8 Hz, H_b-2), 1.89 (3H, s, H_3-13), 1.05 (3H, s, H_3-12), 1.01 (3H, s, H_3-11); ^{13}C NMR (177 MHz, CD_3OD) δ_C: 199.2 (C-3), 199.0 (C-9), 163.3 (C-5), 146.9 (C-7), 130.3 (C-8), 126.6 (C-4), 78.6 (C-6), 49.2 (C-2), 41.3 (C-1), 26.3 (C-10), 23.4 (C-12), 22.1 (C-11), 17.8 (C-13).

7-Epiloliolide (4) – Colorless oil; ESI-MS *m/z* 197 $[M+H]^+$; ^1H NMR (700 MHz, CD_3OD) δ_H: 5.74 (1H, s, H-3), 4.21 (1H, m, H-7), 2.42 (1H, dt, *J* = 14.7, 2.8 Hz, H_a-8),1.98 (1H, dt, *J* = 14.0, 2.8 Hz, H_b-8), 1.76 (3H, s, H_3-12), 1.74 (1H, dd, *J* = 14.7, 3.5 Hz, H_a-6), 1.53 (1H, dd, *J* = 14.7, 3.5 Hz, H_b-6), 1.46 (3H, s, H_3-11), 1.27 (3H, s, H_3-10); ^{13}C NMR (177 MHz, CD_3OD) δ_C: 184.3 (C-4), 173.1 (C-2), 111.9 (C-3), 87.6 (C-9), 65.8 (C-7), 46.6 (C-6), 45.0 (C-8), 35.8 (C-5), 29.6 (C-11), 26.1 (C-10), 25.6 (C-12).

Crocusatin D (5) – Colorless oil; ESI-MS *m/z* 185 $[M+H]^+$; ^1H NMR (700 MHz, CD_3OD) δ_H: 4.33 (1H, dd, *J* = 14.0, 5.6 Hz, H-3), 4.27 (1H, d, *J* = 11.9 Hz, H_a-7), 4.22 (1H, d, *J* = 11.9 Hz, H_b-7), 2.02 (1H, dd, *J* = 12.6, 5.6 Hz, H_a-2), 1.97 (3H, s, H_3-10), 1.76 (1H, t, *J* = 12.6 Hz, H_b-2), 1.26 (3H, s, H_3-9), 1.22 (3H, s, H_3-8); ^{13}C NMR (177 MHz, CD_3OD) δ_C: 200.7 (C-4), 160.8 (C-6), 130.7 (C-5), 69.1 (C-3), 57.8 (C-7), 45.8 (C-1), 35.4 (C-2), 28.2 (C-9), 24.3 (C-8), 10.4 (C-10).

Cell cultures – RAW 264.7 mouse macrophage cells were purchased from Korean Cell Line Bank. The cells were routinely cultured in RPMI medium supplemented with 10% fetal bovine serum (Equitech-Bio, USA) at 37 ºC under 5% CO_2. These cells were utilized for experiments during the exponential growth phase.

Table 1. ^1H and ^{13}C-NMR data of compounds **1**[a]

Carbon No.	^1H	^{13}C
1		39.9
2	2.92 (1H, d, *J* = 17.5 Hz), 2.08 (1H, d, *J* = 17.5 Hz)	49.6
3		200.5
4	5.92 (1H, s)	126.9
5		166.3
6		77.5
7	3.76 (1H, d, *J* = 11.2 Hz), 3.64 (1H, d, *J* = 11.2 Hz)	64.5
8	1.01 (3H, s)	23.8
9	1.11 (3H, s)	22.7
10	2.02 (3H, d, *J* = 1.4 Hz)	18.6

[a] TMS was used as an internal standard; chemical shifts (δ) are expressed in ppm; *J* values are given in parentheses; ^1H (700) and ^{13}C (177) MHz in CD_3OD.

Cytotoxicity test – The cytotoxic effects of extracts, fractions or isolated pure compounds were tested using MTT assay. Briefly, RAW 264.7 cells were plated on a 96 well plate and incubated overnight. Following morning, cells were treated with samples for 24 h. Then, cells were incubated with MTT (5 mg/ml) for 3 h and followed by lysis buffer for overnight. Absorbance was measured at 540 nm using a microplate reader (Molecular Device *E*-max). All determinations were carried out in triplicates and repeated at least three times.

Determination of NO production – The NO production was determined using Griess reagents (Sigma Aldrich). The cells pre-treated with samples for 1 h were incubated with LPS for additional 24 h. Then, the supernatants were transferred to Eppendorf tubes and centrifuged at 12,000 rpm for 15 min in order to remove the cell debris. The same amount of Griess reagent and supernatant was mixed and incubated at 37 °C for 20 min in the dark. The absorbance was determined at 540 nm using a microplate reader.

Results and Discussion

Repeated column chromatographic separation of the CH_2Cl_2-soluble extract of the flower buds of *B. officinalis* led to isolation of a new monoterpene, crocusatin M (**1**), along with four known monoterpenes (Fig. 1). The known compounds identified as (6*R*)-hydroxy-1,1,5-trimethylcyclohex-4-enone (**2**) (Li and Wu, 2002), (+)-dehydrovomifoliol (**3**) (Kim *et al*., 2004), 7-epiloliolide (**4**) (Borkosky *et al*., 1996), and crocusatin D (**5**) (Li and Wu, 2002), by comparison of their spectroscopic data with those of literatures.

Compound **1** was isolated as colorless oil. The molecular formula was determined as $C_{10}H_{17}O_3$ from quasi-molecular ion peak at *m/z* 185.1177 $[M + H]^+$ (calcd for $C_{10}H_{18}O_3$, 185.1178). This formula indicated 3 degrees of unsaturation, and the UV spectrum at 243.0 nm implied a characteristic absorption band for a conjugated carbonyl group in the molecule (Li and Wu, 2002). The ^1H NMR spectrum of **1** exhibited characteristic proton signals due to a *gem*-dimethyl group at δ_H 1.11 (3H, s) and 1.01 (3H, s), a vinyl methyl proton at δ_H 2.02 (3H, d, $J = 1.4$ Hz), two oxygenated methylene protons at δ_H 3.76 (1H, d, $J = 11.2$ Hz), and 3.64 (1H, d, $J = 11.2$ Hz), two methylene protons 2.92 (1H, d, $J = 17.5$ Hz), and 2.08 (1H, d, $J = 17.5$ Hz), and a vinyl proton at δ_H 5.92 (1H, s). The ^{13}C and DEPT spectra of **1** showed 10 distinct carbon signals including three methyl carbons at δ_C 23.8, 22.7, and 18.6, two methylene carbons at δ_C 64.5, and 49.6, a methine carbon at δ_C 126.9, and four quaternary carbons at δ_C 200.5, 166.3, 77.5, and 39.9. These spectral data suggested that **1** was closely comparable to those of crocusatin C except for the additional hydroxyl group at C-6 instead of the proton signal of crocusatin C (H-6), which is a monocyclic monoterpene isolated from *Crocus sativus* (Li and Wu, 2002). The large coupling constant of H-7 ($J = 11.2$ Hz), the quaternary carbon signal at δ_C 77.5, and the observed HMBC correlation between C-6 (δ_C 77.5) and H-7 (δ_H 3.76 and 3.64) clearly supported this structure (Fig. 2). The relative stereochemistry of **1** was deduced on the basis of NOE experiment (Fig. 3). The key NOE correlations between H_2-7/H-9, H_2-7/H-2α, H-2α/H-9, and H-2β/H-8 were observed, corresponding to a α-quasi-axial orientation of H_2-7 (Machida *et al*., 1998). Furthermore, the CD spectrum of **1** showed the Cotton effects at 323.4 (Δε +0.42), 241.2 (Δε –4.45), and 218.0 (Δε +2.96) nm (Straubinger *et al*., 1997; Kim *et al*., 2004), indicating the chirality of C-6 was *R* configuration (Fig. 4). Therefore, compound **1** was elucidated as (6*R*)-6-hydroxy-6-hydroxymethyl-1,1,5-trimethylcyclohex-4-enone, named as crocusatin M.

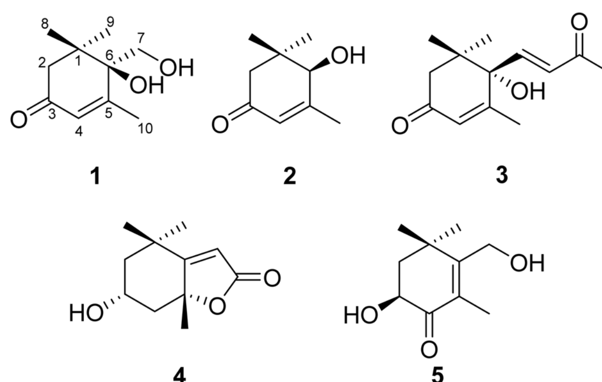

Fig. 1. Chemical structures of isolated compounds **1** - **5**.

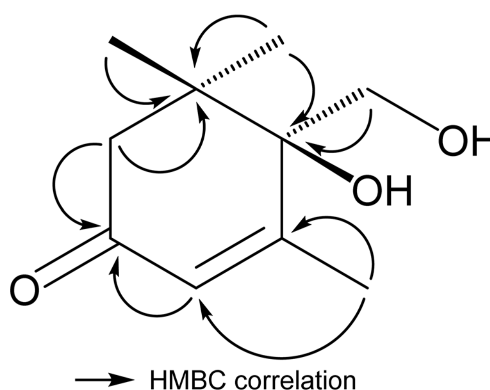

Fig. 2. The HMBC correlations of compound **1**.

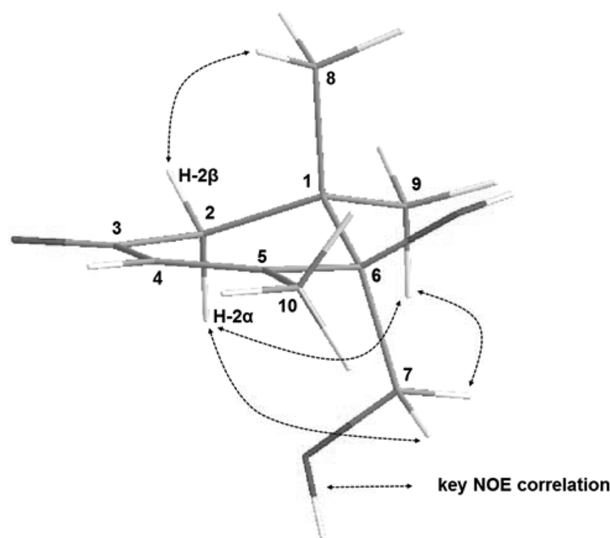

Fig. 3. The key NOE correlation of compound **1**.

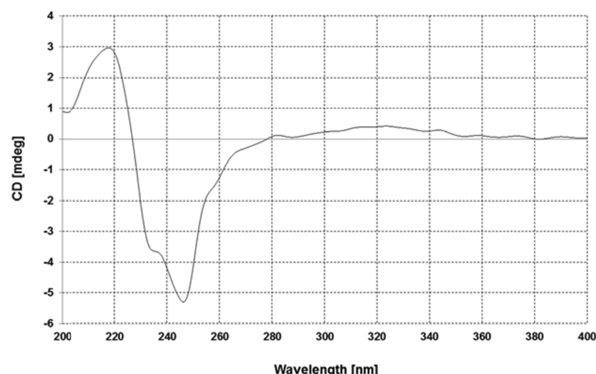

Fig. 4. CD spectrum of compound **1**.

Table 2. Inhibitory effects of compounds **1** - **5** against LPS-induced NO production

Compounds	IC_{50} (μg/ml)[a]
1	> 100
2	63.8
3	24.4
4	–[b]
5	> 100
Caffeic acid[c]	27.6

[a)] Concentration required to achieve 50% inhibition of NO production
[b)] Cell cytotoxicity was found
[c)] A positive control.

All isolates (**1** - **5**) were evaluated for their inhibitory effects on NO production in LPS-stimulated RAW 264.7 cells (Table 2). Compounds **2** and **3** exhibited the inhibitory effects on NO production with IC_{50} values of 63.8 and 24.4 μg/ml, respectively. The inhibitory effect of compound **3** is comparable to that of caffeic acid, the positive control used for this assay (IC_{50} 27.6 μg/ml). However, compounds **1** and **5** had no significant effect (IC_{50} > 100 μg/ml) and compound **4** was cytotoxic to RAW 264.7 cells with the tested concentration.

In summary, five monoterpenes including one new monoterpene, crocusatin M, were isolated from the flower buds of *B. officinalis*, and their structures were elucidated based on the comparison of their spectroscopic data with those of literatures. Their inhibitory effects on NO production were also evaluated in LPS-stimulated RAW 264.7 cells. Compounds **2** and **3** showed the moderate inhibitory effect on NO production. Taken together, our findings suggest that compounds **2** and **3** are nitric oxide inhibitors.

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