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Sesquiterpene-Neolignans from the Stem Bark of *Magnolia obovata* and Their Cytotoxic Activity

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Abstract – Three sesquiterpene-lignans, eudeshonokiol B (**1**), eudesobovatol B (**2**), and clovanemagnolol (**3**), were isolated from the stem bark of *Magnolia obovata*, together with magnolol (**4**), honokiol (**5**), and obovatol (**6**) on the basis of spectroscopic and physicochemical analyses including 2D NMR and Mass. Compounds **1** - **3** were belongs to a unique class of natural products made up of a sesquiterpene and biphenyl-type neolignan *via* an ether bond. All the isolated compounds were tested *in vitro* for their cytotoxic activity against the HeLa, A549, and HCT116 cancer cell lines. Compounds **1** - **6** showed the cytotoxic activity against tested cancer cell lines, with IC_{50} values ranging from 7.1 to 14.4 μg/mL.

Keywords – *Magnolia obovata*, Magnoliaceae, sesquiterpene-neolignan, lignan, cytotoxic activity

Introduction

The stem bark of *Magnolia obovata* Thunb. (Magnoliaceae) has been used as traditional medicine for the treatment of gastrointestinal disorders, anxiety, and allergic diseases, including bronchial asthma, in Korea, China, and Japan (Fujita *et al.*, 1972). Previous chemical studies have revealed a variety of neolignans, sesquiterpenes, sesquiterpene-neolignans, phenylpropanoids, and alkaloids. These compounds were shown to display muscle relaxation (Watanabe *et al.*, 1975), central depressant effect (Watanabe *et al.*, 1983), anti-gastriculcer (Watanabe, 1986), vasorelaxant (Yamahara *et al.*, 1986), antiallergic (Hamasaki *et al.*, 1999), antibacterial (Namba *et al.*, 1982; Bae *et al.*, 1998), and neurotrophic activities (Fukuyama *et al.*, 1992). In the course of our continuing study on cytotoxic compounds from natural sources, the constituents of *M. obovata* have been investigated. This paper deals with the isolation and structure elucidation of neolignans and sesquiterpene-neolignans from *M. obovata*, as well as cytotoxic activity against the HeLa (cervical epitheloid carcinoma), A549 (human nonsmall lung), and HCT116 (human colorectal carcinoma) cancer cell lines.

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Material and Method

General experimental procedures – Melting points were measured by using an Electrothermal apparatus. Optical rotation was determined on a JASCO DIP-100 KUY polarimeter. UV spectra were obtained with a Beckman Du-650 UV-vis recording spectrophotometer. IR spectra were recorded on a Jasco Report-100 infrared spectrometer. Mass were carried out with a JEOL JMS-700 Mstation mass spectrometer. ^{1}H-NMR (300 and 400 MHz) and ^{13}C-NMR (75 and 100 MHz) were recorded on Bruker DRX300 and JEOL 400 spectrometers. Two-dimensional (2D) NMR spectra (^{1}H-^{1}H COSY, HMQC, and HMBC) were recorded on a Bruker Avance 500 spectrometer. For column chromatography, silica gel (Kieselgel 60, 70230 mesh and 230 - 400 mesh, Merck) was used. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F254 (0.25 mm, Merck).

Plant material – The dried stem bark of *M. obovata* was purchased from Uchida Co., Ltd., Tokyo, Japan on March 2005. The crude drug was identified by one of the authors, K. Bae. The voucher specimen (CNU-594) was deposited at the herbarium of the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and Isolation – The dried stem bark of *M. ovobata* (20 kg) was extracted with methanol (MeOH) three times under reflux for 4 h. The MeOH solutions were combined, filtered, and concentrated to yield a dry MeOH extract (3 kg). The MeOH extract (3 kg) was suspended in distilled water and fractionated with hexane, EtOAc, and BuOH to give hexane (600 g), EtOAc (1000 g), and BuOH-soluble fractions (780 g), successively. The hexane-soluble fraction was chromatographed over a silica gel column eluting with hexane / EtOAc (100 : 0 to 50 : 50) to afford nine fractions (H1-H9). Fraction H4 was chromatographed on a silica gel column eluting with hexane / EtOAc (100 : 1 to 20 : 1) to give compound **6** (5 g). Fraction H9 was subjected to a silica gel column eluting with hexane / EtOAc (50 : 1 to 10 : 1) to give three subfracitons (H9.1H9.3). Subfraction H9.1 was chromatographed on a silica gel column eluting with hexane / EtOAc (50 : 1 to 10 : 1) to give **1** (100 mg), **2** (50 mg), and **3** (50 mg). Subfraction H9.2 was subjected to a silica gel column eluting with hexane / EtOAc (50 : 1 to 10 : 1) to give **4** (80 g) and **5** (50 g).

Eudeshonokiol B (1) – Colorless oil; $[1]_D^{25}$ −70.5° (*c* 1.05, $CHCl_3$); UV λ_{max} (ε): 208 (56000), 256 (20000): FABMS *m/z*: 511 $[M+Na]^+$; IR ν_{max} cm^{-1} (KBr): 3500, 1640, 1600, 1450; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.84 (3H, s, H-11), 1.02 (3H, s, H-12), 1.32 (3H, s, H-13), 1.24 (3H, s, H-14), 1.63 (1H, d, *J* = 10.8 Hz, H-6β), 2.17 (1H, d, *J* = 10.8 Hz, H-6α), 3.38 (2H, d, *J* = 6.0 Hz, H-7"), 3.48 (2H, d, *J* = 6.0 Hz, H-7'), 5.02 (4H, m, H-9", 9'), 5.92 (2H, m, H-8", 8'), 6.94 (1H, dd, *J* = 9.0, 2.4 Hz, H-4"), 6.99 (1H, d, *J* = 9.0 Hz, H-3"), 7.09 (1H, d, *J* = 6.9 Hz, H-3'), 7.11 (1H, d, *J* = 2.4 Hz, H-6"), 7.14 (1H, dd, *J* = 6.9, 2.1 Hz, H-6'), 7.16 (1H, d, *J* = 2.1 Hz, H-2'), ^{13}C-NMR (75 MHz, $CDCl_3$): δ 152.1 (C-4'), 148.9 (C-2"), 138.3 (C-8'), 137.4 (C-8"), 136.6 (C-1"), 134.9 (C-5"), 132.7 (C-2'), 132.5 (C-1'), 131.3 (C-6"), 129.4 (C-6'), 128.8 (C-4"), 128.5 (C-3'), 124.7 (C-3"), 118.5 (C-9"), 116.1 (C-9'), 115.5 (C-5'), 88.3 (C-4), 73.0 (C-13), 51.1 (C-5), 50.0 (C-7), 44.9 (C-9), 40.5 (C-1), 39.7 (C-7"), 39.5 (C-7'), 37.7 (C-3), 35.1 (C-10), 31.0 (C-14), 27.2 (C-15), 22.7 (C-8), 22.1 (C-6), 21.0 (C-12), 20.0 (C-2), 19.2 (C-11).

Eudesobovatol B (2) – Colorless oil; $[\alpha]_D^{25}$ −20.5° (*c* 1.15, $CHCl_3$); UV λ_{max} (ε): 205 (50000), 275 (4800): FABMS *m/z*: 503 $[M-H]^-$, 282; IR ν_{max} cm^{-1} (KBr): 3550, 1640, 1610; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.85 (3H, s, H-11), 1.00 (3H, s, H-14), 1.02 (3H, s, H-15), 1.32 (3H, s, H-12), 1.38 (1H, d, *J* = 12.0 Hz, H-6β), 2.01 (1H, d, *J* = 12.0 Hz, H-6α), 3.19 (2H, d, *J* = 6.8 Hz, H-7"), 3.31 (2H, d, *J* = 6.8 Hz, H-7'), 4.98 (4H, m, H-9", 9'), 5.82 (2H, m, H-8", 8'), 6.27 (1H, d, *J* = 2.0 Hz, H-3'), 6.57 (1H, d, *J* = 2.0 Hz, H-5'), 6.79 (2H, d, *J* = 8.4 Hz, H-2", 6"), 7.06 (2H, d, *J* = 8.4 Hz, H-3", 5"), ^{13}C-NMR (100 MHz, $CDCl_3$): δ 156.0 (C-1"), 152.4 (C-2'), 150.5 (C-6'), 137.7 (C-8"), 137.1 (C-8'), 136.8 (C-4'), 134.3 (C-4"), 132.6 (C-1'), 129.8 (C-3", 5"), 117.5 (C-2", 6"), 116.2 (C-9'), 115.9 (C-9"), 112.6 (C-5'), 111.2 (C-3'), 87.7 (C-4), 72.9 (C-13), 53.1 (C-5), 49.7 (C-7), 45.1 (C-9), 40.7 (C-1), 39.9 (C-7"), 39.6 (C-7'), 38.6 (C-3), 35.2 (C-10), 27.3 (C-14), 26.6 (C-15), 22.6 (C-8), 21.9 (C-6), 21.2 (C-12), 20.4 (C-2), 19.3 (C-11).

Clovanemagnolol (3) – Colorless oil; $[\alpha]_D^{25}$ +20.0° (*c* 1.50, $CHCl_3$); UV λ_{max} (ε): 205 (46000), 285 (56000): EIMS *m/z*: 486 $[M]^+$; IR ν_{max} cm^{-1} (KBr): 3550, 1640; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.83 (3H, s, H-13), 0.91 (3H, s, H-14), 0.94 (3H, s, H-15), 3.25 (1H, brs, H-9β), 3.36 (2H, d, *J* = 6.0 Hz, H-7'), 3.40 (2H, d, *J* = 6.0 Hz, H-7"), 4.14 (1H, dd, *J* = 9.3, 5.7 Hz, H-2α), 5.04 (4H, m, H-9", 9'), 5.95 (2H, m, H-8", 8'), 6.93 (1H, d, *J* = 8.1 Hz, H-3'), 6.98 (1H, d, *J* = 8.1 Hz, H-3"), 7.05 (1H, d, *J* = 2.1 Hz, H-6'), 7.08 (1H, dd, *J* = 8.1, 2.1 Hz, H-4"), 7.13 (1H, d, *J* = 2.1 Hz, H-6"), 7.14 (1H, dd, *J* = 8.1, 2.1 Hz, H-4'), ^{13}C-NMR (75 MHz, $CDCl_3$): δ 154.6 (C-2'), 152.1 (C-2"), 138.2 (C-8"), 137.6 (C-8'), 134.4 (C-5'), 132.5 (C-6'), 132.4 (C-5"), 131.3 (C-6"), 129.4 (C-4"), 129.3 (C-1'), 129.0 (C-4'), 127.1 (C-1"), 117.4 (C-3"), 116.6 (C-3'), 116.0 (C-9'), 115.5 (C-9"), 90.2 (C-2), 75.1 (C-9), 50.2 (C-5), 45.0 (C-1), 44.6 (C-3), 39.6 (C-7"), 39.6 (C-7'), 37.8 (C-4), 35.7 (C-12), 34.9 (C-8), 33.2 (C-7), 31.4 (C-14), 28.5 (C-15), 26.7 (C-11), 26.4 (C-10), 25.6 (C-13), 20.8 (C-6).

Cytotoxic Assay – Cells were maintained in RPMI 1640 including L-glutamine (JBI), 10% FBS (JBI), and 2% penicillin-streptomycin (GIBCO). Trypsin-EDTA was used to separate cell from culture flask. All cell lines were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxic activity was measured by a modified Microculture Tetrazolium (MTT) assay (Mosmann *et al.*, 1983). Viable cells were seeded in the growth medium (180 μg/mL) into 96 well microtiter plates (1×10^4 cells per each well) and incubated at 37 °C, 5% CO_2. A test sample was dissolved in DMSO and adjusted to the final sample concentrations ranging from 1.875 μg/mL to 30 μg/mL by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing for 2 h, 20 μL of the test sample was added to each well. The same volume of DMSO was added to the control group well. Forty-eight hours after the test sample was added, 20 μL MTT was added to each well (final concentration of 5 μg/mL). Two hours later, the plate was centrifuged for 5 minutes at 1500 rpm, then

the medium was removed and the resulting formazan crystals were dissolved in 150 μL DMSO. The optical density (O.D.) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow). The IC_{50} value was defined as the needed concentration of sample to reduce 50% of absorbance relative to the vehicle-treated control.

Results and Discussion

Repeated chromatography of the hexane-soluble fraction of the MeOH extract from the stem bark of *M. obovata* on silica gel, YMC-pack RP-C_{18} columns led to the isolation of seven compounds (**1** - **6**). The isolated compounds were identified as eudeshonokiol B (**1**) (Fukuyama *et al.*, 1992), eudesobovatol B (**2**) (Fukuyama *et al.*, 1992), clovanemagnolol (**3**) (Fukuyama *et al.*, 1992), magnolol (**4**) (Shoji *et al.*, 1991), honokiol (**5**) (Shoji *et al.*, 1991), and obovatol (**6**) (Kazuo *et al.*, 1982).

Compound **1** was obtained as colorless oil and it showed a molecular ion peak at *m/z* 511 $[M + Na]^+$ in the FABMS. The IR spectrum showed the presence of hydroxyl group at 3500 cm^{-1} and aromatic ring at 1600 cm^{-1}. The ^{1}H-NMR spectrum of **1** revealed the presence of two 1,2,4-trisubstituted benzene rings at δ 6.94 (1H, dd, J = 9.0, 2.4 Hz), 6.99 (1H, d, J = 9.0 Hz), 7.11 (1H, d, J = 2.4 Hz), 7.09 (1H, d, J = 6.9 Hz), 7.14 (1H, dd, J = 6.9, 2.1 Hz) and 7.16 (1H, d, J = 2.1 Hz), and two allyl groups at δ 3.38 (2H, d, J = 6.0 Hz), 5.02 (2H, m), 5.92 (1H, m), 3.48 (2H, d, J = 6.0 Hz), 5.02 (2H, m) and 5.92 (1H, m). The ^{13}C-NMR and DEPT spectra of **1** showed the twelve aromatics and two allyl group carbons, which were assignable to a *neo*-lignan moiety, as compared with those of honokiol (**5**). Moreover, the remaining fifteen carbon signals indicated the occurrence of four quaternary methyls at δ_C 19.2, 21.0, 27.2 and 31.0, six methylenes at δ_C 20.0, 22.1, 22.7, 37.7, 40.5 and 44.9, two methins at δ_C 50.0 and 51.1, a quaternary carbon at δ_C 35.1, and two oxygenate quaternary carbons at δ_C 88.3 and 73.0. In addition, the ^{1}H-NMR spectrum showed signals for four methyl groups at δ_H 0.84, 1.02, 1.32, and 1.24. These signals were almost the same as those of a eudesman-type sesquiterpene isolated from *Laggera pterodonta* (Zhao *et al.*, 1997). Long-range correlations between δ_H 1.02 (H-12) and δ_C 88.3 (C-4"), 6.94 (H-4") and 148.9 (C-2") confirmed that the hydroxyl group at C-2" on the honokiol ring was linked to the C-4 position in eudesmol moiety associated with the ether bond. On the basis of the above evidence, compound **1** was identified as eudeshonokiol B by comparison of several physical

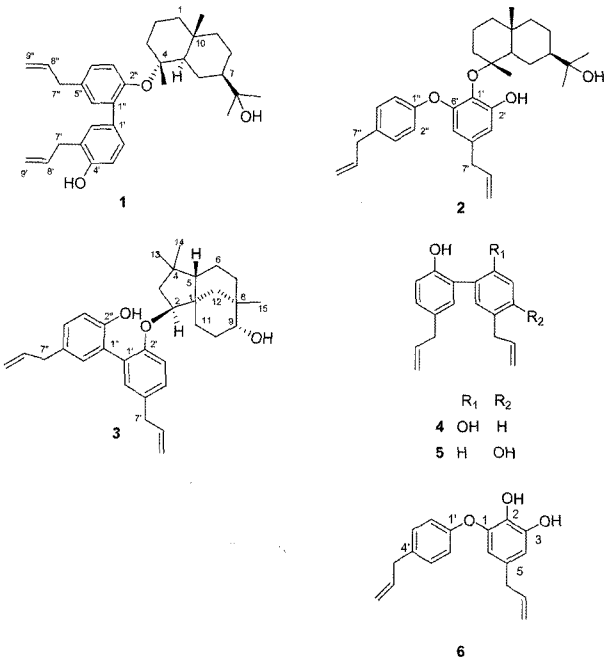
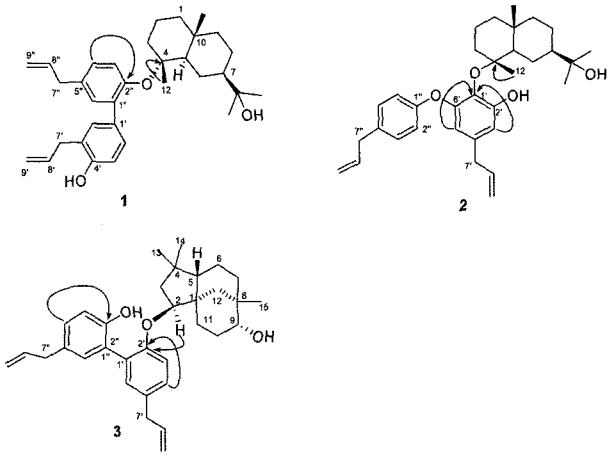


Fig. 1. Chemical structures of compounds **1** - **6** from stem bark of *M. obovata*.

and spectral data with those reported in the literature (Fukuyama *et al.*, 1992).

Compound **2** was obtained as colorless oil and it showed a molecular ion peak at *m/z* 504 $[M - H]^+$ in the FABMS. The IR spectrum indicated the presence of hydroxyl group at 3550 cm^{-1} and aromatic ring at 1600 cm^{-1}. The ^{1}H-NMR spectrum of **2** showed the presence of two AB type aromatic protons at δ 6.79 (2H, d, J = 8.4 Hz) and 7.06 (2H, d, J = 8.4 Hz), *meta*-coupled aromatic protons at δ 6.27 (1H, d, J = 2.0 Hz) and 6.57 (1H, d, J = 2.0 Hz), and two allyl groups at δ 3.19 (2H, d, J = 6.8 Hz), 4.98 (2H, m), and 5.82 (1H, m) and δ 3.31 (2H, d, J = 6.8 Hz), 4.98 (2H, m), and 5.82 (1H, m), which were assignable to be an obovatol moiety (**6**). In addition, ^{1}H-NMR spectrum of **2** showed four methyl groups at δ 0.85, 1.00, 1.02, and 1.32, and indicated that the presence of eudesmol moiety, as compared with that of **1**. The ^{13}C-NMR spectrum of **2** also indicated the presence of an obovatol moiety and a sesquiterpene moiety, the latter of which consisted of four quarternary methyls (δ_C 19.3, 21.2, 26.6 and 27.3), six methylenes (δ_C 20.4, 22.9, 22.6, 38.6, 40.7 and 45.1), two methins (δ_C 49.7 and 53.1), a quarternary carbon (δ_C 35.2), and two oxygenate quarternary carbons (δ_C 87.7 and 72.9). Furthermore, the long-range correlations between δ_H 1.32 (H-12) and δ_C 87.7 (C-4), δ_H 6.27 (H-3')/6.57 (H-5') and δ_C 132.6 (C-1'), estimated an ether linkage through the –OH group at C-1 in obovatol and C-4 in eudesmol. Therefore, compound **2**



HMBC : H → C

Fig. 2. Key HMBC correlations of **1**, **2**, and **3**.

Table 1. Cytotoxicity of compounds against cultured HeLa, A549, and HCT116 cancer cell lines

Compounds	IC_{50} (mg/mL) [a]		
	HeLa	A549	HCT116
1	7.8 ± 0.8	8.8 ± 1.6	8.5 ± 1.4
2	7.1 ± 0.5	10.1 ± 1.6	8.7 ± 0.9
3	9.1 ± 1.4	9.2 ± 0.8	10.5 ± 2.0
4	8.6 ± 1.4	7.7 ± 1.2	12.2 ± 1.5
5	11.1 ± 1.2	11.2 ± 0.7	11.4 ± 0.7
6	12.4 ± 1.0	14.1 ± 0.9	14.4 ± 0.6
Adriamycin [c]	0.8 ± 0.1	1.2 ± 0.1	0.7 ± 0.1

[a]IC_{50} is defined as the concentration that resulted in a 50% decrease in cell number and the results are means ± standard deviation of three independent replicates.
[b]The IC_{50} greater than 30 μg/mL was considered to be no cytotoxicity.
[c]Positive control substance.

was identified as eudesobovatol B, which had previously been isolated from *M. obovata* (Fukuyama *et al.*, 1992).

Compound **3** was obtained as colorless oil and it showed a molecular ion peak at *m/z* 486 $[M]^+$ in the EIMS. The ^{1}H-NMR spectrum indicated the presence of an aromatic and sesquiterpene moieties. The ^{1}H-NMR spectrum of **1** was readily assigned as magnolol (**4**) such as two allyl groups (δ 3.36, 5.04, 5.95, 3.40, 5.04 and 5.95) and two 1,2,4-trisubstituted aromatic rings (δ 6.93, 7.05, 7.14, 6.98, 7.08 and 7.13). However, the spectral data of the sesquiterpene moiety were totally different from those of eudesmol-type structure. The ^{13}C-NMR spectrum of **3** displayed the presence of fifteen carbons and consisted of three methyls (δ_C 25.6, 28.5 and 31.4), six methylenes (δ_C 20.8, 26.4, 26.7, 33.2, 35.7 and 44.6), one methine (δ_C 50.2), two oxygen bearing methins (δ_C 75.1 and 90.2), and three quaternary carbons (δ_C 34.9, 37.8 and 45.0). These spectral features were similar to those of a known sesquiterpene compound, clovandiol (Fukuyama *et al.*, 1992). The connectivity of a lignan moiety and the sesquiterpene moiety was further supported by the HMBC spectrum (Fig. 2), which showed the correlation between δ_H 4.14 (H-2) and δ_C 154.6 (C-2'). Therefore, compound **3** was confirmed as clovanemagnolol (Fukuyama *et al.*, 1992).

Among isolates, compounds **1** - **3** were belonged to a unique class of natural products made up of a sesquiterpene and biphenyl-type neolignan *via* an ether bond.

Compounds **1** - **6** were tested *in vitro* for their cytotoxic activity against HeLa, A549, and HCT116 cancer cell lines (Table 1). Eudeshonokiol B (**1**), eudesobovatol B (**2**), clovanemagnolol (**3**), magnolol (**4**), honokiol (**5**), and obovatol (**6**) showed cytotoxic activity against the HeLa, A549, and HCT116 cancer cell lines, with IC_{50} values ranging from 7.1 to 14.4 μg/mL. Referring to the previous report (Kim *et al.*, 1999), the cytotoxicity of honokiol was comparable to magnolol against various cancer cell lines. The cytotoxic activities of honokiol and magnolol were also close to previous data (Kim *et al.*, 1999) against tested cell lines in the present study. Also, the sesquiterpene-neolignans **1** - **3** were isolated from *M. obovata* as neurotrophic activity on a neuronal cell culture system derived from fetal rat hemispheres (Fukuyama *et al.*, 1992) and nitric oxide production in lipopolysaccharide-activated macrophages (Matsuda *et al.*, 2001). However, to the best of our knowledge, the cytotoxicity of sesquiterpene-neolignans against cancer cell lines is being reported for the first time in this study. Our results indicate that the cytotoxic activities of this unique type of sesquiterpene-neolignan warrant further investigation and optimization.

Acknowledgments

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