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Cytotoxic Compounds from the Stem Bark of *Magnolia obovata*

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Abstract – Two sesquiterpenes (**1** - **2**), a tetralone (**3**), and two phenylpropanoids (**4** - **5**) were isolated from the stem bark of *Magnolia obovata* Thunberg (Magnoliaceae) through repeated column chromatography. Their structures were identified as *β*-eudesmol (**1**), cryptomeridiol (**2**), 4*R*-4,8-dihydroxy-*β*-tetralone (**3**), *trans*-*p*-coumaryl aldehyde (**4**), and *p*-coumaric acid (**5**) on the basis of spectroscopic analysis including two dimensional NMR and mass. Compounds **1** - **3** were tested *in vitro* for their cytotoxic activity against the K562, HeLa, A549, and HCT116 cancer cell lines. However, compounds **1** - **3** were inactive in this assay system.
Keywords – *Magnolia obovata*, Magnoliaceae, sesquiterpene, tetralone, cytotoxic activity

Introduction

Magnolia obovata Thunberg (Magnoliaceae) is an endemic to Northeast Asia and is important medicinal plant that has attracted considerable attention on account of their biological and chemical diversity. The stem bark of this plant 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 (Shoji *et al*., 1991). These compounds were shown to display muscle relaxation (Watanabe *et al*., 1975), central depressant effect (Watanabe *et al*., 1983), anti-gastriculcer (Watanabe *et al*., 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). It has also been reported to exhibit a variety of bioactivities of major compounds, magnolol and honokiol, such as anti-inflammation (Wang *et al*., 1995), antimicrobial (Chang *et al*., 1998), and antitumor (Ikeda *et al*., 2003). In the course of our continuing study on cytotoxic compounds from natural sources, the constituents of *M. obovata* have been investigated. This paper deals the isolation and structure elucidation of sesquiterpenes, tetralone and phenylpropanoids from *M. obovata*, as well as cytotoxic activity against K562 (human leukemia), HeLa (cervical epitheloid carcinoma), A549 (human nonsmall lung carcinoma) and HCT116 (human colorectal carcinoma) cancer cell lines.

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Experimental

General experimental procedures – Melting points were measured by using an Electrothermal apparatus and are not corrected. 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, 70 - 230 mesh and 230 - 400 mesh, Merck) was used. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} (0.25 mm, Merck) and RP-18 F_{254} S (0.25 mm, Merck), and spot were detected under an UV light and by spraying with 10% H_2SO_4.

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 H2 was chromatographed on a silica gel column eluting with hexane-EtOAc (100 : 1 to 50 : 1) to give compound **1** (500 mg). The EtOAc-soluble fraction was chromatographed over a silica gel column eluting with $CHCl_3$-MeOH (100 : 0 to 50 : 50) to afford twenty-five fractions (E1 - E25). Fraction E17 was subjected to a silica gel column eluting with $CHCl_3$-MeOH (20 : 1 to 10 : 1) to yeild five subfracitons (E17.1 - E17.5). Subfraction E17.5 was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (20 : 1 to 5 : 1) to afford **2** (200 mg). Fraction E24 was subjected to a silica gel column eluting with hexaneacetone (50 : 1 to 10 : 1) to give six subfracitons (E24.1 - E24.6). Compound **3** (3 mg) was obtained by solvent recrystrallization with MeOH from subfraction E24.1. Fraction E8 was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (20 : 1 to 10 : 1) to give compounds **4** (3 mg) and **5** (30 mg).

***β*-Eudesmol (1)** – white amorphous powder; mp, 73 - 75 °C; $[\alpha]_D^{25}$ –45.1° (*c* 0.9, $CHCl_3$); UV (MeOH) λ_{max} (log ε) nm; 213 (4.17); IR ν_{max} cm^{-1} (KBr): 3600, 1640; EIMS *m/z*: 222 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.22 - 2.29 (8H, m, H-1, 2, 3, 5, 6, 7, 8, 9), 1.20 (6H, s, H-12,13), 4.43 (1H, d, *J* = 1.6 Hz, H-14a), 4.70 (1H, d, *J* = 1.6 Hz, H-14b), 0.70 (3H, s, H-15); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 41.9 (C-1), 23.4 (C-2), 36.8 (C-3), 150.9 (C-4), 49.3 (C-5), 24.9 (C-6), 49.7 (C-7), 22.3 (C-8), 41.1 (C-9), 35.7 (C-10), 72.6 (C-11), 27.0 (C-12), 27.0 (C-13), 105.2 (C-14), 16.2 (C-15).

Cryptomeridiol (2) – white amorphous powder; mp, 133 - 135 °C; $[\alpha]_D^{25}$ –36.6° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) nm; 273 (3.40); IR ν_{max} cm^{-1} (KBr): 3500, 1380; EIMS *m/z*: 240 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.02 - 1.96 (8H, m, H-1, 2, 3, 5, 6, 7, 8, 9), 1.21 (6H, s, H-12,13), 0.87 (3H, s, H-14), 1.12 (3H, s, H-15); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 41.2 (C-1), 20.3 (C-2), 43.6 (C-3), 72.5 (C-4), 55.0 (C-5), 21.7 (C-6), 50.1 (C-7), 22.7 (C-8), 44.8 (C-9), 34.7 (C-10), 73.1 (C-11), 27.2 (C-12), 27.5 (C-13), 18.8 (C-14), 22.8 (C-15).

4*R*-4,8-Dihydroxy-*β*-tetralone (3) – white crystal; mp, 75 - 76 °C; $[\alpha]_D^{25}$ +14.5° (*c* 0.3, $CHCl_3$); UV (MeOH) λ_{max} (log ε) nm; 259 (3.50), 343 (3.10); IR ν_{max} cm^{-1} (KBr): 3500, 1590, 1460; EIMS *m/z*: 178 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.90 (1H, m, H-2α), 2.66 (1H, m, H-2β), 2.30 (1H, m, H-3α), 2.10 (1H, m, H-3β), 7.07 (1H, dd, *J* = 8.3, 0.9 Hz, H-5), 7.50 (1H, dd, *J* = 8.3, 7.6 Hz, H-6), 6.84 (1H, dd, *J* = 7.6, 0.9 Hz, H-7); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 206.3 (C-1), 36.1 (C-2), 32.6 (C-3), 68.3 (C-4), 148.6 (C-4a), 118.8 (C-5), 137.9 (C-6), 117.6 (C-7), 163.7 (C-8), 116.5 (C-8a).

***trans-p*-Coumaryl aldehyde (4)** – white amorphous powder; mp, 138 - 140 °C; IR ν_{max} cm^{-1} (KBr): 3150, 1650, 1600; EIMS *m/z*: 148 $[M]^+$; ^{1}H-NMR (300 MHz, CD_3OD): δ 7.50 (1H, d, *J* = 8.5 Hz, H-2), 6.90 (1H, d, *J* = 8.5 Hz, H-3), 6.90 (1H, d, *J* = 8.5 Hz, H-5), 7.50 (1H, d, *J* = 8.5 Hz, H-6), 7.42 (1H, d, *J* = 15.9 Hz, H-7), 6.62 (1H, dd, *J* = 15.9, 7.7 Hz, H-8), 9.56 (1H, d, *J* = 7.7 Hz, H-9); ^{13}C-NMR (75 MHz, CD_3OD): δ 127.2 (C-1), 132.1 (C-2), 117.2 (C-3), 162.5 (C-4), 117.2 (C-5), 132.1 (C-6), 156.1 (C-7), 126.5 (C-8), 196.3 (C-9).

***p*-Coumaric acid (5)** – white amorphous powder; mp, 145 - 147 °C; UV (MeOH) λ_{max} (log ε) nm; 236 (3.40), 320 (3.30); IR ν_{max} cm^{-1} (KBr): 3250, 1605; EIMS *m/z*: 164 $[M]^+$; ^{1}H-NMR (300 MHz, CD_3OD): δ 7.42 (1H, d, *J* = 8.4 Hz, H-2), 6.79 (1H, d, *J* = 8.4 Hz, H-3), 6.79 (1H, d, *J* = 8.4 Hz, H-5), 7.42 (1H, d, *J* = 8.4 Hz, H-6), 7.57 (1H, d, *J* = 16.2 Hz, H-7), 6.25 (1H, d, *J* = 16.2 Hz, H-8); ^{13}C-NMR (75 MHz, CD_3OD): δ 127.3 (C-1), 131.2 (C-2), 116.9 (C-3), 161.2 (C-4), 116.9 (C-5), 131.2 (C-6), 146.8 (C-7), 115.7 (C-8), 171.1 (C-9).

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 200 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- and EtOAc-soluble fractions of the MeOH extract from the stem bark of *M. obovata* on silica gel columns led to the isolation of two sesquiterpenes (**1 - 2**), one tetralone (**3**), and two phenylpropanoids (**4 - 5**).

Compound **1** was obtained as white amorphous powder and it showed a negative optical rotation $[\alpha]_D^{25}$ –45.1° (*c* 0.9, $CHCl_3$) with a molecular ion peak at *m/z* 222 $[M]^+$ in the EIMS. The IR spectrum showed the presence of hydroxyl group at 3600 cm^{-1} and olefinic double bond at 1640 cm^{-1}. The ^{1}H-NMR spectrum of **1** revealed the presence of three methyl group signals at δ 0.70 and 1.20 (6H), and *exo*-methylene protons at δ 4.43 (1H, d, J = 1.6 Hz), 4.70 (1H, d, J = 1.6 Hz). The ^{13}C-NMR and DEPT spectra of **1** showed the fifteen carbons with three methyl signals at δ 16.2 (C-15), 27.0 (C-12) and 27.0 (C-13), an oxygenated carbon at δ 72.6 (C-11), and two olefinic carbons at δ 105.2 (C-14) and 150.9 (C-4). These signals were almost the same as those of a eudesman-type sesquiterpene isolated from *Laggera pterodonta* (Zhao *et al*., 1997). On the basis of the above evidence, compound **1** was identified as β-Eudesmol by comparison of several physical and spectral data with those reported in the literature (Fukuyama *et al*., 1992).

Compound **2** was obtained as white amorphous powder and it showed a molecular ion peak at *m/z* 240 $[M]^+$ in the EIMS. The IR spectrum indicated the presence of hydroxyl group at 3500 cm^{-1} and geminal dimethyl group at 1380 cm^{-1}. The ^{1}H-NMR spectrum of **2** showed the presence of four methyl groups at δ 0.87, 1.12 and 1.21 (× 2), 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 a sesquiterpene moiety, which consisted of four quarternary methyls (δ 18.8, 22.8, 27.2 and 27.5), six methylenes (δ 20.3, 21.7, 22.7, 41.2, 43.6 and 44.8), two methins (δ 50.1 and 55.0), a quarternary carbon (δ 34.7), and two oxygenate quarternary carbons (δ 73.1 and 72.5). Therefore, compound **2** was identified as cryptomeridiol, which had previously been synthesized from β-Eudesmol (Viqar *et al*., 1992).

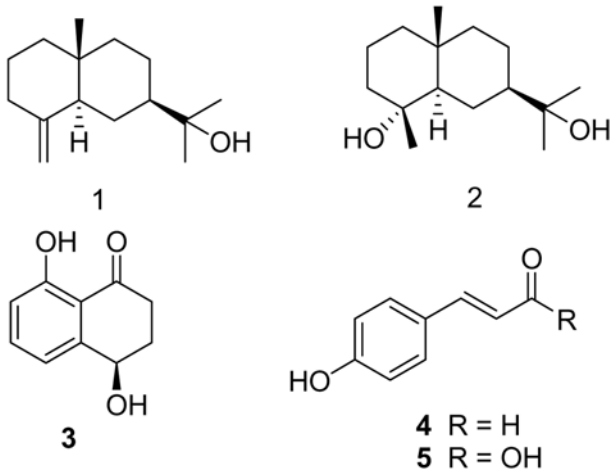

Fig. 1. Structures of compounds **1 - 5**.

Compound **3** was obtained as white amorphous powder, mp 75 - 76 °C. It showed a molecular ion peak at *m/z* 178 $[M]^+$ in the EIMS. The IR spectrum of **3** showed the characteristic bands for hydroxyl group at 3500 cm^{-1} and aromatic group at 1590 cm^{-1}. The ^{1}H-NMR spectrum indicated the presence of three aromatic protons at δ 7.50 (1H, dd, J = 8.3, 7.6 Hz), 7.07 (1H, dd, J = 8.3, 0.9 Hz) and 6.84 (1H, dd, J = 7.6, 0.9 Hz), and an oxygenated methin proton at δ 4.84 (1H, dd, J = 8.0, 3.8 Hz). The ^{13}C-NMR and DEPT spectra of **3** displayed the presence of ten carbons and consisted of a carbonyl carbon at δ 206.3, six aromatic carbons with an oxygenated at δ 68.3, and two methylene carbons at δ 36.1 and 32.6. This evidences indicated that the structure of **3** is a tetrarone derivative, compared with that of α-tetralonyl glucopyranoside isolated from *Juglans mandshurica* (Min *et al*., 2000). Therefore, compound **3** was confirmed as isosclerone 4*R*-4,8-dihydroxy-β-tetralone by comparing with spectral data and optical rotation (lit. $[\alpha]_D$ +24°), with reported in the literature (Kokubun *et al*., 2003).

Compound **4** was isolated as white crystal, mp 138 - 140 °C and showed a molecular ion peak at *m/z* 148 in the EIMS spectrum. The IR spectrum showed the characteristic bands for hydroxyl group at 3150 cm^{-1} and aromatic group at 1650 cm^{-1}. The ^{1}H-NMR spectrum of **4** exhibited an aldehyde proton at δ 9.56 (d, J = 7.7 Hz), two olefinic protons at δ 7.42 (d, J = 15.9 Hz) and 6.62 (dd, J = 7.7, 15.9 Hz), and four aromatic protons at δ 7.50 (2H, d, J = 8.5 Hz) and 6.90 (2H, d, J = 8.5 Hz). The ^{13}C-NMR and DEPT spectra revealed seven carbons, including an aldehyde carbon at δ 196.3 and two olefinic carbons at δ 156.1 and 126.5, indicated that the presence of an α,β-unsaturated aldehyde group in the moiety of **4**. In addition, the ^{13}C-NMR spectrum showed an oxygenated

aromatic at δ 162.5, a quarterary carbon at δ 127.2, and four aromatic methin carbons at δ 117.2 (C-3,5) and 132.1 (C-2,6). Therefore, compound **4** was identified as *trans*-*p*-coumaryl aldehyde, compared with that of literature (Stange *et al*., 1999).

Compound **5** was isolated as white crystal, mp 145 - 147 °C and showed a molecular ion peak at *m/z* 164 in the EIMS spectrum. The IR spectrum showed the characteristic bands for hydroxyl group at 3250 cm^{-1} and aromatic group at 1605 cm^{-1}. The ^{1}H-NMR spectrum of **5** showed two olefinic protons at δ 7.57 (d, J = 16.2 Hz) and 6.25 (d, J = 16.2 Hz), and four aromatic protons at δ 7.42 (2H, d, J = 8.4 Hz) and 6.79 (2H, d, J = 8.4 Hz), and indicated that the presence of coumaric moiety, as compared with that of **4**. The ^{13}C-NMR spectrum also indicated the presence of a α,β-unsaturated carbonyl group, which consisted of a carbonyl carbon at δ 171.1, two olefinic carbons at δ 146.2 and 115.7. In addition, the ^{13}C-NMR spectrum showed an oxygenated aromatic at δ 161.2, a quarterary carbon at δ 127.3, and four aromatic methin carbons at δ 116.9 (C-3,5) and 131.2 (C-2,6). Therefore, compound **5** was identified as *p*-coumaric acid, which was synthesized from *p*-hydroxybenzaldehyde (Iiyama *et al*., 1990).

Compounds **1** - **3** were tested *in vitro* for their cytotoxic activity against K563, HeLa, A549, and HCT116 cancer cell lines (Table 1). β-Eudesmol (**1**), cryptomeridiol (**2**) and 4*R*-4,8-dihydroxy-β-tetralone (**3**) were completely inactive against tested cancer cell lines. *trans*-*p*-Coumaryl aldehyde (**4**) and *p*-coumaric acid (**5**) were not tested their cytotoxic activity in this test. However, some sesquiterpene-neolignan, eudeshonokiol B, eudesobovatol and clovane-magnolol isolated from *M. obovata*, 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 (Youn *et al*., 2008). Referring to the previous report (Kim *et al*., 1999), the cytotoxicity of magnolol and honokiol were comparable to sesquiterpene-neolignans against various cancer cell lines. This result indicated that eudesmane-type sesquiterpene did not enhance the cytotoxic activity against cancer cell lines.

Table 1. Cytotoxicity of compounds against cultured K562, HeLa, A549, and HCT116 cancer cell lines

Compounds	IC_{50} (μg/ml)[a]			
	K562	HeLa	A549	HCT116
1	> 100[b]	> 100	> 100	> 100
2	> 100	> 100	> 100	> 100
3	> 100	> 100	> 100	> 100
Adriamycin[c]	2.5 ± 0.1	1.4 ± 0.1	2.2 ± 0.1	1.2 ± 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.

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