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A Cytotoxic Monoterpene-Neolignan from the Stem Bark of *Magnolia officinalis*†

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Abstract – A monoterpene-neolignan, piperitylmagnolol (**1**), was isolated from the stem bark of *Magnolia officinalis*, together with syringaresinol (**2**), caffeic acid (**3**), and sinapaldehyde (**4**). The isolated compounds were established on the basis of spectroscopic and physicochemical analyses including 1D- and 2D- NMR techniques, as well as on comparing the spectral data with those in the literature and of authentic samples. Compounds **1** - **4** were tested for their cytotoxic activity against the HeLa, K562, A549, and HCT116 cancer cell lines *in vitro*. Of the isolates, piperitylmagnolol (**1**) exhibited cytotoxic activity against the tested cancer cell lines with IC_{50} values of 7.7 - 9.5 μg/mL.
Keywords – *Magnolia officinalis*, Magnoliaceae, Monoterpene-neolignan, Cytotoxic activity

Introduction

The stem bark of *Magnolia officinalis* Rehd. et Wils. (Magnoliaceae) has been used as a 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 as constituents of this plant. Regarding bioactive constituents in stem bark, neolignans were shown to have muscle relaxant (Watanabe *et al*., 1975), central depressant (Watanabe *et al*., 1983), anti-gastric ulcer (Watanabe, 1986), vasorelaxant (Teng *et al*., 1990), antiallergic (Shin *et al*., 2001), antibacterial (Namba *et al*., 1982; Bae *et al*., 1998), and neurotrophic effects (Fukuyama *et al*., 1992). They were also cytotoxic against cancer cell lines (OVCAR-3, HepG2 and HeLa) (Syu *et al*., 2004). During the course of our continuing study on cytotoxic compounds, four known compounds **1** - **4** were isolated from the stem bark of *M. officinalis*. In this paper, the isolation and structure elucidation of compounds **1** - **4**, as well as the evaluation of their cytotoxic activity against HeLa (cervical epitheloid carcinoma), K562 (human lymphocytic leukemia), A549 (human nonsmall lung), and HCT116 (human colorectal carcinoma) cancer cell lines are described.

Experimental

General experimental procedures – Melting points were measured using an electrothermal apparatus. UV and IR spectra were recorded on a U-3000 spectrophotometer (Hitachi, Japan) and an FTS 135 FT-IR spectrometer (Bio-Rad, CA), respectively. 1D and 2D NMR experiments were performed on a UNITY INOVA 400 MHz FT-NMR instrument (Varian, CA) with tetramethylsilane (TMS) as internal standard. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (0.25 mm, Merck). Silica gel (230 - 400 mesh, Merck, Germany) and RP-18 (YMC-pack ODS-A, 12 nm, S-150 μm) were used for column chromatography.

Plant material – The dried stem bark of *M. officinalis* was purchased from a local market in Daejeon, Korea in June 2005, and was identified by one of the authors (K. B.). A voucher specimen (CNU 594) was deposited at the herbarium of the College of Pharmacy, Chungnam National

†Dedicated to professor KiHwan Bae for his leading works on bioactive natural products.
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Extraction and isolation – The dried stem bark of *M. officinalis* (5 kg) was extracted with methanol (MeOH) three times under reflux for 4 h. The MeOH solutions were combined, filtered, and concentrated to yield a dried residue (640 g). The residue (640 g) was suspended in distilled water and fractionated with n-hexane, EtOAc and n-BuOH to give n-hexane (180 g), EtOAc (270 g) and n-BuOH soluble fractions (80 g), respectively. The EtOAc-soluble fraction was chromatographed over a silica gel column eluting with $CHCl_3$-MeOH (100 : 1 to 5 : 1) to afford twelve fractions (E1-E12). Fraction E5 was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (50 : 1 to 5 : 1) to afford five subfractions (E5.1-E5.5) according to their TLC profiles. Subfraction E5.3 was subjected to HPLC [YMC-pack ODS-A, MeOH-H_2O (60 : 40)] to yield compounds **1** (6 mg, t_R 70 min) and **2** (3 mg, t_R 80 min), respectively. Fraction E7 was chromatographed on a silica gel column using $CHCl_3$-MeOH (50 : 1 to 10 : 1) to afford six subfractions (E7.1-E7.6). Subfraction E7.5 was subjected to silica gel chromatography, using $CHCl_3$-MeOH (30 : 1 to 5 : 1), to give **3** (8 mg) and **4** (5 mg), respectively.

Piperitylmagnolol (1) – Colorless oil; $[\alpha]_D^{25}$ −135.5 (*c* 1.05, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 208 (3.85), 258 (3.80) nm; FABMS *m/z*: 403 $[M+H]^+$; IR ν_{max} cm^{-1} (KBr): 3400, 1650, 1610, 1455; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.82 (3H, d, *J* = 6.4 Hz, H-9"), 0.90 (3H, d, *J* = 6.4 Hz, H-10"), 1.75 (3H, s, H-7"), 3.38 (4H, d, *J* = 6.8 Hz, H-7 and H-7'), 3.50 (1H, m, H-3"), 5.02 (4H, m, H-9 & 9'), 5.43 (1H, br s, H-2"), 5.90-6.45 (2H, m, H-8 & 8'), 6.75 (1H, d, *J* = 2.0 Hz, H-4), 6.98 (1H, d, *J* = 2.0 Hz, H-2), 7.10 (1H, d, *J* = 2.0 Hz, H-2'), 6.80 (1H, dd, *J* = 8.4, 2.0 Hz, H-4'), 6.58 (1H, d, *J* = 8.4 Hz, H-5'); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 132.1 (C-1), 129.5 (C-2), 125.3 (C-3), 128.9 (C-4), 133.5 (C-5), 149.0 (C-6), 40.0 (C-7), 137.5 (C-8), 115.5 (C-9), 132.4 (C-1'), 132.5 (C-2'), 126.6 (C-3'), 130.1 (C-4'), 118.5 (C-5'), 151.7 (C-6'), 40.0 (C-7'), 137.9 (C-8'), 114.8 (C-9'), 136.1 (C-1"), 123.5 (C-2"), 44.9 (C-3"), 40.5 (C-4"), 22.7 (C-5"), 30.5 (C-6"), 23.7 (C-7"), 28.1 (C-8"), 17.0 (C-9"), 22.2 (C-10").

Syringaresinol (2) – White powder; $[\alpha]^{25}$ +5.0 (*c* 1.50, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 240: (3.85), 275 (3.80) nm; EIMS *m/z*: 418 $[M]^+$; IR ν_{max} cm^{-1} (KBr): 3430, 1614; ^{1}H-NMR (400 MHz, CD_3OD): δ 2.92-3.06 (2H, m, H-1 & H-5), 3.60-4.25 (4H, m, H-4 & H-8), 4.62 (4H, d, *J* = 4.0, H-2 & H-6), 6.60 (4H, s, H-2', H-6', H-2", H-6"), 3.87 (12H, s, OCH_3); ^{13}C-NMR (100 MHz, CD_3OD): δ 54.5 (C-1 & C-5), 66.5 (C-2 & C-6), 85.7 (C-4 & C-8), 132.1 (C-1' & C-1"), 104.5 (C-2', C-6', C-2", C-6"), 148.0 (C-3', C-5', C-3", C-5"), 133.5 (C-4' & C-4"), 57.2 (OCH_3).

Caffeic acid (3) – White powder; UV (MeOH) λ_{max} (log ε): 285 (4.30) nm; EIMS *m/z*: 180 $[M]^+$; IR ν_{max} cm^{-1} (KBr): 3440, 1616; ^{1}H-NMR (400 MHz, CD_3OD): δ 6.20 (1H, d, *J* = 15.8, H-2), 6.52 (1H, d, *J* = 8.0, H-5'), 6.80 (1H, dd, *J* = 8.0, 2.0, H-6'), 7.10 (1H, d, *J* = 2.0, H-2'), 7.50 (1H, d, *J* = 15.8, H-3); ^{13}C-NMR (100 MHz, CD_3OD): δ 171.5 (C-1), 115.7 (C-2), 147.1 (C-3), 128.2 (C-1'), 114.8 (C-2'), 146.4 (C-3'), 149.5 (C-4'), 116.4 (C-5'), 121.3 (C-6').

Sinapaldehyde (4) – White powder; UV (MeOH) λ_{max} (log ε): 285 (3.80) nm; IR ν_{max} cm^{-1} (KBr): 3350, 1625; EIMS *m/z*: 208 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ 6.63 (1H, dd, *J* = 15.8, 7.8, H-2), 6.82 (2H, s, H-2' & H-6'), 7.40 (1H, d, *J* = 15.8, H-3), 9.70 (1H, d, *J* = 7.8, H-1), 3.95 (6H, s, OCH_3); ^{13}C-NMR (100 MHz, CD_3OD): δ 192.5 (C-1), 125.7 (C-2), 150.1 (C-3), 130.0 (C-1'), 110.3 (C-2' & C-6'), 149.6 (C-3' & C-5'), 135.8 (C-4'), 57.3 (OCH_3).

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 cells from the culture flask. All cell lines were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxic activity was measured by the MTT [3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983). Viable cells were seeded in the growth medium (180 μg/ml) into 96 well microtiter plates (1×10^4 cells per well) and incubated at 37 °C, and 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 of MTT was added to each well (final concentration of 5 g/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 of 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 concentration of sample needed to reduce absorbance to 50% of the vehicle-treated control.

Results and Discussion

The MeOH extract of the stem bark of *M. officinalis*

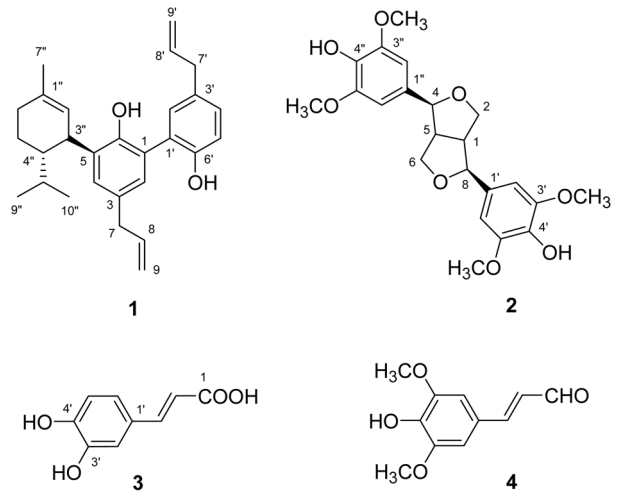

Fig. 1. Chemical structures of compounds **1 - 4** from the stem bark of *M. officinalis*.

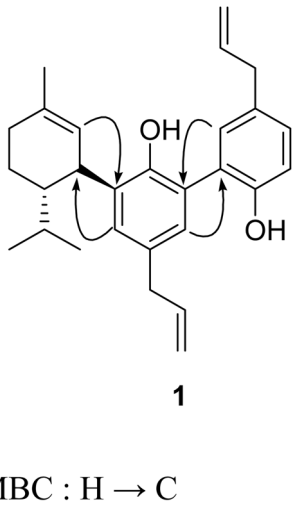

Fig. 2. Selected HMBC correlations of compound **1**.

was suspended in H_2O and successively partitioned with n-hexane, EtOAc, and n-BuOH. The EtOAc-soluble fraction was separated by silica gel and prep-HPLC columns, which led to the isolation of four compounds (**1** - **4**).

Compound **1** was obtained as a colorless oil and it showed a molecular ion peak at *m/z* 403 $[M + H]^+$ in the FABMS. The IR spectrum showed the presence of a hydroxyl group at 3400 cm^{-1} and an aromatic ring at 1610 cm^{-1}. The NMR spectra of **1** showed three methyls at [δ_H 0.82 (3H, d, J = 6.4 Hz)/δ_C 17.0 (C-9"), 0.90 (3H, d, J = 6.4 Hz)/δ_C 22.2 (C-10"), and 1.75 (3H, s)/δ_C 23.7 (C-7")], three methines at δ_H 1.20-1.80 (2H, m)/δ_C 40.5 (C-4) & 28.1 (C-8) and 3.50 (1H, m)/δ_C 44.9 (C-3"), two methylenes at δ_H 1.20 - 1.80 (2H, m)/δ_C 22.7 (C-5") and 2.06 (2H, m)/30.5 (C-6"), an olefinic group at δ_H 5.43 (1H, s)/123.5 (C-2"), and an olefinic quaternary carbon at δ_C 136.1 (C-1"), which were the same as those of a *p*-menth-1-ene (piperityl) type monoterpene isolated from *Platipus quercivorus* (Kashiwagi *et al*., 2006). In addition, the ^{1}H-NMR spectrum of **1** revealed the presence of two *meta* coupled aromatic protons at δ 6.75 (1H, d, J = 2.0 Hz, H-4) and 6.98 (1H, d, J = 2.0 Hz, H-2), 1,2,4-trisubstituted aromatic protons at [δ 7.10 (1H, d, J = 2.0 Hz, H-2'), 6.80 (1H, dd, J = 8.4, 2.0 Hz, H-4'), and 6.58 (1H, d, J = 8.4 Hz, H-5')], and two allyl group protons at [δ 3.38 (4H, d, J = 6.8 Hz, H-7 and H-7'), 5.90 - 6.45 (2H, m, H-8 and 8'), and 5.02 (4H, m, H-9 and 9')]. Furthermore, the ^{13}C-NMR and DEPT spectra of **1** showed twelve aromatic and two allyl group carbons, which were assignable to a *neo*-lignan moiety, as compared with those of magnolol, except for the substitution of the peperityl group. The connectivity of the lignan and monoterpene moiety was further supported by the HMBC spectrum (Fig. 2), which showed correlations of δ_H 6.75 (H-4) with δ_C 44.9 (C-3") and δ_H 5.43 (H-2") with δ_C 133.5 (C-5), respectively. The configurations at C-3" and C-4" were determined to be 3 (*S*) and 4 (*S*), respectively, on the basis of optical rotation ($[\alpha]_D^{20}$ –135.0°) in comparison with published data ($[\alpha]^{20}$ –146.0°) (Shoji *et al*., 1991). On the basis of the above evidence, compound **1** was identified as piperitylmagnolol by comparison of several physical and spectral data with those reported in the literature (Shoji *et al*., 1991).

Compound **2** was obtained as a white powder. It showed a molecular ion peak at *m/z* 418 $[M]^+$ in the EIMS. The IR spectrum indicated the presence of a hydroxyl group at 3430 cm^{-1} and an aromatic ring at 1614 cm^{-1}. The NMR spectra of **2** showed the presence of an aromatic signal at δ_H 6.60 (1H, s)/δ_C 104.5 (C-2' & C-6'), an oxygenated methine group at δ_H 3.60 - 4.25 (1H, m)/δ_C 85.7 (C-4 & C-8), an oxygenated methylene group at δ_H 4.62 (2H, d, J = 4.0 Hz)/δ_C 66.5 (C-2 & 6), a methine signal at δ_H 2.92 - 3.06 (1H, m)/δ_C 54.5 (C-1 & C-5), and a methoxy group at δ_H 3.87 (3H, s)/δ_C 57.2 (OCH_3), which were assignable to a symmetrical furofuran type lignan (Park *et al*., 2003). These observations were further supported by the molecular ion peak at *m/z* 418 $[M]^+$ ($C_{22}H_{26}O_8$). The configurations at C-4 and C-8 were determined to be *R* and *R*, respectively, on the basis of optical rotation ($[\alpha]_D^{20}$ +5.0°) in comparison with published data ($[\alpha]_D^{20}$ +2.4°) (Park *et al*., 2003). Therefore, compound **2** was identified as syringaresinol, which had previously been isolated from *Sophra subprostrata* (Park *et al*., 2003).

Table 1. Cytotoxic activity of compounds against HeLa, K562, A549, and HCT116 cancer cell lines

	IC_{50} (μg/ml) [a]			
	HeLa	K562	A549	HCT116
1	7.7 ± 0.5	9.2 ± 1.2	8.8 ± 0.6	9.5 ± 1.2
2	> 30 [b]	> 30	> 30	> 30
3	> 30	> 30	> 30	> 30
4	> 30	> 30	> 30	> 30
Adriamycin[c]	0.8 ± 0.1	2.5± 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. The results are means ± standard deviation of three independent replicates. [b]An IC_{50} greater than 30 μg/mL was considered to represent no cytotoxic activity. [c]Positive control substance.

Compound **3** was obtained as a white powder. It showed a molecular ion peak at *m/z* 180 $[M]^+$ in the EIMS. The IR spectrum indicated the presence of a hydroxyl group at 3440 cm^{-1} and an aromatic ring at 1616 cm^{-1}. The NMR spectra of **3** showed the presence of an ABX type aromatic group at [δ_H 6.52 (1H, d, J = 8.0 Hz)/δ_C 116.4 (C-5'), δ_H 6.80 (1H, dd, J = 8.0, 2.0 Hz)/δ_C 121.3 (C-6'), and δ_H 7.10 (1H, d, J = 2.0 Hz)/δ_C 114.8 (C-2')], an α,β-unsaturated olefinic group at [δ_H 6.20 (1H, d, J = 15.8 Hz)/δ_C 115.7 (C-2) and at δ_H 7.50 (1H, d, J = 15.8 Hz)/δ_C 147.1 (C-3)], two oxygenated aromatic carbons at δ_C 146.4 (C-3') & 149.5 (C-4'), and a carbonyl carbon at δ_C 171.5 (C-1), which were indicative of the presence of a phenyl propanoic acid. Therefore, compound **3** was confirmed as caffeic acid by comparison of several physical and spectral data with those reported in the literature (Sakakibara *et al*., 2007).

Compound **4** was obtained as a white powder. It showed a molecular ion peak at *m/z* 208 $[M]^+$ in the EIMS. The NMR spectra of **4** showed the presence of an aromatic signal at δ_H 6.82 (1H, s)/δ_C 110.3 (C-2' & C-6'), an α,β-unsaturated aldehyde group at [δ_H 6.63 (1H, dd, J = 15.8, 7.8 Hz)/δ_C 125.7 (C-2), δ_H 7.40 (1H, d, J = 15.8 Hz)/δ_C 150.1 (C-3), and δ_H 9.70 (1H, d, J = 7.8 Hz)/δ_C 192.5 (C-1)], two oxygenated aromatic carbons at δ_C 149.6 (C-3') & 135.8 (C-4'), and a methoxy group at δ_H 3.95 (3H, s)/δ_C 57.3 (OCH_3), which were assignable to symmetrical phenyl propane aldehyde. These observations were further supported by the molecular ion peak at *m/z* 208 $[M]^+$ ($C_{11}H_{12}O_4$). Accordingly, compound **4** was confirmed as sinapaldehyde by comparison of physical and spectral data with those reported in the literature (Shoji *et al*., 1991).

Compounds **1** - **4** were tested *in vitro* for their cytotoxic activity against HeLa, K562, A549, and HCT116 cancer cell lines using MTT assays. Syu *et al*. previously reported that compound **1** had cytotoxic activity against OVCAR-3, HepG2, and HeLa cancer cell lines (Syu *et al*., 2004). To facilitate our understanding of the cytotoxicity of the compounds we isolated from *M. officinalis*, we examined their cytotoxic activity against others cancer cell lines. As the results in Table 1 indicate, piperitylmagnolol (**1**) showed cytotoxic activity against the HeLa, K562, A549, and HCT116 cells, with IC_{50} values of 7.7, 9.2, 8.8, and 9.5 μg/mL, respectively. On the other hand, all of the remaining compounds (**2** - **4**) were inactive (IC_{50} > 30 μg/mL) against four cancer cell lines. Among isolates, compound **3** was isolated for the first time from this plant source.

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