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Topoisomerase I and II Inhibitory Activities and Cytotoxic Constituents from the Barks of *Tilia amurnesis*

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Abstract – Eight compounds, squalene (**1**), friedelin (**2**), β-sitosterol (**3**), β-sitosterol-3-*O*-glucoside (**4**), α-tocopherol (**5**), betulinic acid (**6**), trilinolein (**7**) and 1-*O*-(9*Z*,12*Z*-Octadecadienoyl)-3-nonadecanoyl glycerol (**8**), were isolated from the barks of *Tilia amurensis*. Their chemical structures were identified by comparing their physicochemical and spectral data with those published in the literature. These isolated compounds were examined for their inhibitory activities against topoisomerase I and II. Compound **7** showed significant inhibition of DNA topoisomerase I and II activities, with percent decreases in activity of 87 and 95%, respectively at a concentration of 100 μM. Compound **6** exhibited cytotoxicity against the human colon adenocarcinoma cell line (HT-29), the human breast adenocarcinoma cell line (MCF-7) and the human liver hepatoblastoma cell line (HepG-2), with IC_{50} values of 20, 59 and 16 μM, respectively.
Keywords – Cytotoxicity, DNA topoisomerases I, DNA topoisomerases II, *Tilia amurensis*

Introduction

Tilia species are large deciduous trees, typically 20 - 40 m tall, belonging to the family Tiliaceae, which are native throughout most of the temperate northern hemisphere, in Asia, Europe and eastern North America (Hickok and Anway, 1972). *Tilia* species are traditional medicinal plants which have been used as sedatives, tranquilizers, diuretics, expectorants and diaphoretics (Ahn, 2003; Park, 2004). Some reports indicate that *Tilia* has additional activities such as anxiolytic (Viola *et al.*, 1994) and anti-stress activities (Aydin *et al.*, 1992). Previous studies on this species have shown the presence of coumarins (Kim *et al.*, 1988; Matsuda *et al.*, 2002; Yu *et al.*, 1990), flavonoids (Matsuda *et al.*, 2002; Pietta, 1993; Toker *et al.*, 2004; Yu *et al.*, 1990), triterpenes (Yu *et al.*, 1990) and hydrocarbons (Yu *et al.*, 1990).

In the present study, eight compounds were isolated from the barks of *Tilia amurnesis* Rupr. (Tiliaceae), and their DNA topoisomerases I and II inhibitory effects and cytotoxicities were investigated.

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Material and Methods

General experimental procedures – Melting point was measured using a capillary melting point apparatus (Electrothermal 9100, Essex, UK) and were uncorrected. Optical rotations were measured using a JASCO DIP-1000 (Tokyo, Japan) automatic digital polarimeter. NMR spectra were recorded on a Bruker 250 MHz (DMX 250) spectrometer using Bruker's standard pulse program. Samples were dissolved in either $CDCl_3$, CD_3OD or pyridine-d_5 and the chemical shifts were reported in ppm downfield from TMS. FAB-MS and EI-MS spectra were measured by a VG TRIO 2A and AUTOSPEC UK mass spectrometer, respectively. The stationary phases used for column chromatography (Silica gel 60, 70 - 230 and 230 - 400 mesh, Lichroprep RP-18 gel, 40 - 63 μm, Merck) and TLC plates (Silica gel 60 F_{254} and RP-18 F_{254s}, 0.25 mm, Merck) were purchased from Merck KGaA (Darmstadt, Germany). Spots were detected under UV radiation and by spraying with 10% H_2SO_4, followed by heating. Camptothecin (CPT) and etoposide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Supercoiled pBR 322 plasmid DNA and calf thymus topoisomerase I was purchased from MBI Fermentas, Inc. (Hanover, MD,

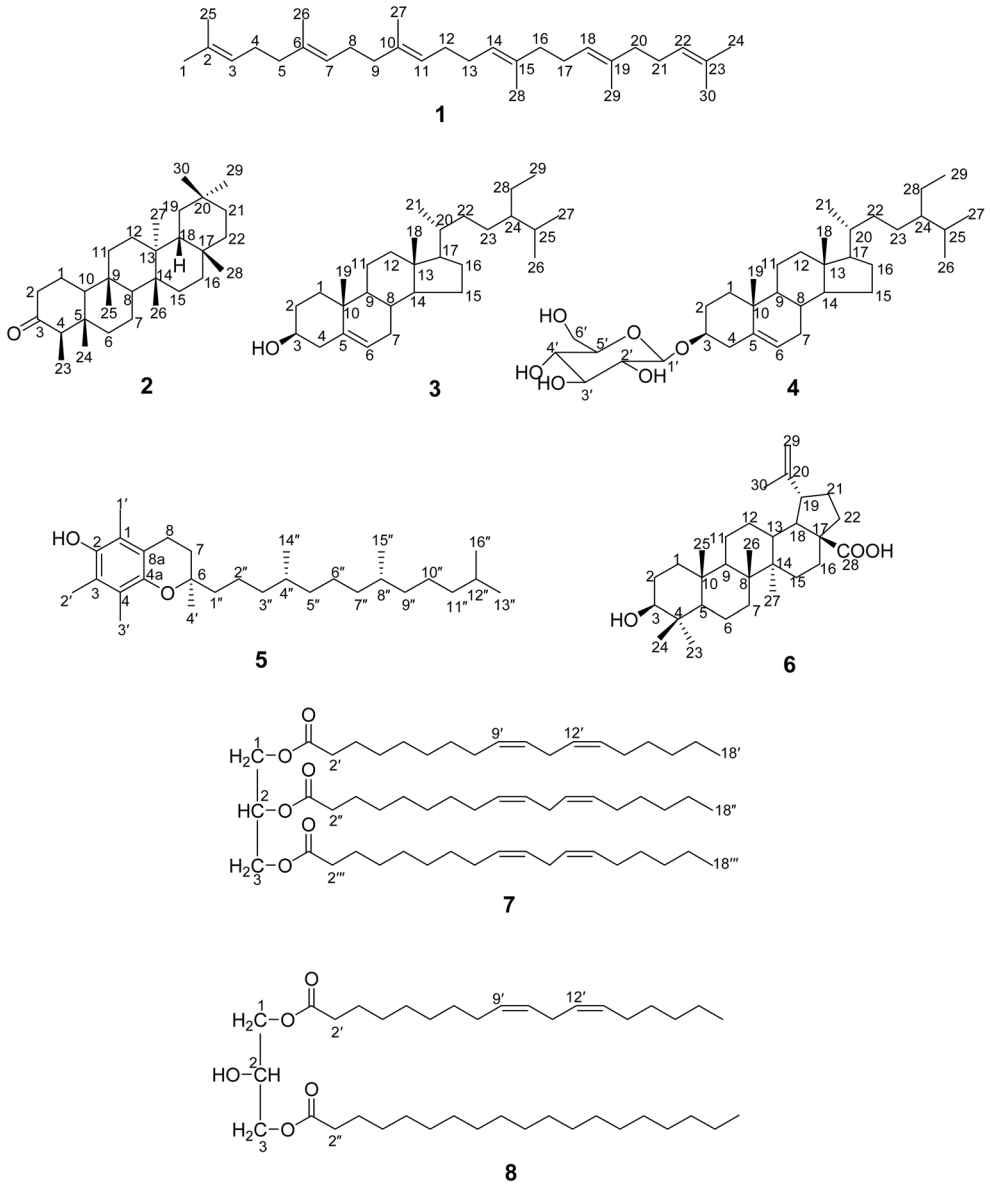


Fig. 1. The structures of compounds isolated from the bark of *Tilia amurensis*.

USA). Human topoisomerase II was purchased from TopoGEN, Inc. (Columbus, OH, USA). One unit of topoisomerase I or II completely relaxes 1 μg of pBR322 DNA in 30 min at 37 °C. All other chemicals and solvents were analytical grade and used without further purification.

Plant materials – The barks of *Tilia amurensis* was purchased in May 2002 from a folk medicine market, "Yak-ryong-si" in Deagu, Republic of Korea. This material was taxonomically confirmed by Professor Ki Hwan Bae, Chungnam National University, Daejeon, Republic of Korea. A voucher specimen (YNTA-2004) has been preserved at the College of Pharmacy, Yeungnam University.

Extraction and isolation – The barks of *Tilia amurensis* (9.8 kg) were extracted with 70% MeOH three times by refluxing for 24 h and the 70% MeOH solution was evaporated to dryness (1.75 kg). The MeOH extract was suspended in H_2O (1 L) then partitioned successively with hexane, CH_2Cl_2, EtOAc and BuOH (each 1 L × 3). The hexane extract (110 g) was loaded on a silica-gel column (10 × 120 cm, silica-gel 230 - 400 mesh), and eluted with gradient of hexane-EtOAc (from hexane 100% to EtOAc 100%; 100:0, 10 L; 95:5, 10 L; 90:10, 10 L, 70:30, 10 L; 50:50, 10 L; 30:70, 15 L; 10:90, 15 L; 0:100, 20 L) and then EtOAc-MeOH (from EtOAc 100% to MeOH 100%; 100:0, 15 L; 97:3, 15 L; 95:5, 15 L; 90:10, 15 L, 70:30,

20 L; 50:50, 20 L; 30:70, 20 L; 0:100, 20 L). The eluents (500 mL in each flask) were combined into 58 fractions (TAH1-58) on the basis of silica gel TLC. Fractions TAH2 (2 g) and TAH22 (1 g) were recrystallized from 100% $CHCl_3$ and left in a refrigerator for 24 h, which yielded squalene [**1**, 1.78 g, oil] and *β*-sitosterol [**3**, 47.9 mg, white crystals, mp 274 - 275 °C], respectively. Fraction TAH11 (180 mg) was recrystallized in 100% MeOH and left in a refrigerator for 24 h, which yielded α-tocopherol [**5**, 47.9 mg, yellow oil, $[\alpha]^{25}_D$ –60.2° (*c* 0.2, MeOH)]. The fractions TAH9 (1 g) and TAH16 (1 g) were subjected to chromatography on a silica-gel column (3 × 50 cm, silica-gel 230 - 400 mesh) with a gradient elution of hexane-EtOAc (100:0, 3 L; 99:1, 5 L; 95:5, 3 L; 90:10, 5 L; 70:30, 2 L; 50:50, 2 L; 100% EtOAc, 3 L) to afford friedelin [**2**, 22.3 mg, amorphous powder, mp 261 - 262 °C, $[\alpha]^{25}_D$ –22.2° (*c* 0.2, $CHCl_3$)] and trilinolein [**7**, 13.6 mg, oil]), respectively. Fractions TAH37 (800 mg) and TAH49 (1 g) were subjected to chromatography on a silica-gel column (4 × 60 cm, silica-gel 230 - 400 mesh) with a CH_2Cl_2-MeOH gradient elution (100:0, 5 L; 99:1, 5 L; 97:3, 3 L; 95:5, 3 L; 90:10, 3 L; 70:30, 2 L; 50:50, 2 L; 30:70, 2 L; 100% MeOH, 3 L) to afford 1-*O*-(9*Z*,12*Z*-Octadecadienoyl)-3-nonadecanoyl glycerol [8, 40.1 mg, yellow oil] and betulinic acid [**6**, 13.5 mg, amorphous powder, mp 277 - 281 °C, $[\alpha]^{25}_D$ +6.8° (*c* 0.1, $CHCl_3$)], respectively. Fraction TAH47 (700 mg) was subjected to chromatography on a silica-gel column (4 × 60 cm, silica-gel 230 - 400 mesh) with an EtOAc-MeOH gradient elution (100% EtOAc, 3 L; 99:1, 5 L; 97:3, 3 L; 95:5, 3 L; 90:10, 5 L; 70:30, 3 L; 50:50, 2 L; 30:70, 2 L; 100% MeOH, 3 L) to afford *β*-Sitosterol-3-*O*-glucoside [**4**, 26.2 mg, oil].

Assay for DNA topoisomerase I inhibition *in vitro* – Activity of DNA topoisomerase I was determined by measuring the relaxation of supercoiled DNA pBR322. The reaction mixture was comprised of 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 0.01% bovine serum albumin, 200 ng pBR322, 0.3 U calf thymus DNA topoisomerase I (Amersham), and topoisomerase I inhibitors (prepared compounds) in a final volume of 10 μL. The reaction mixture was incubated at 37 °C for 30 min. The reactions were terminated by adding 2.5 μL of solution comprising 10% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol and 30% glycerol. The mixture was applied to a 1% agarose gel and electrophoresed for 10 h with a running buffer of Tris-borate-EDTA. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and supercoiled DNA was quantitated by an image analyzer and LabWork 4.5 software (UVP).

Assay for DNA topoisomerase II inhibition *in vitro* – DNA topoisomerase II inhibition was measured by assessing relaxation of supercoiled pBR322 plasmid DNA. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin, 0.2 μg pBR322 plasmid DNA, 0.3 U human DNA topoisomerase IIα (TopoGEN), and topoisomerase II inhibitors (prepared compounds) in a final volume of 20 μL. The reactions were incubated for 30 min at 37 °C and terminated by the addition of 3 μL of solution containing 0.77% sodium dodecyl sulfate in 77 mM EDTA. Samples were mixed with 2 μL of solution containing 30% sucrose, 0.5% bromophenol blue and 0.5% xylene cyanol, and subjected to electrophoresis on a 1% agarose gel at 1.5 V/cm for 10 h with a running buffer of Tris-borate-EDTA. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and supercoiled DNA was quantitated by an image analyzer and LabWork 4.5 software (UVP).

Cytotoxicity bioassays – A tetrazolium-based colorimetric assay (MTT assay) was used to determine cytotoxicities towards human colon adenocarcinoma (HT-29), human breast adenocarcinoma (MCF-7) and human liver hepatoblastoma (HepG-2) cell lines (Rubinstein *et al.*, 1990).

Results and Discussion

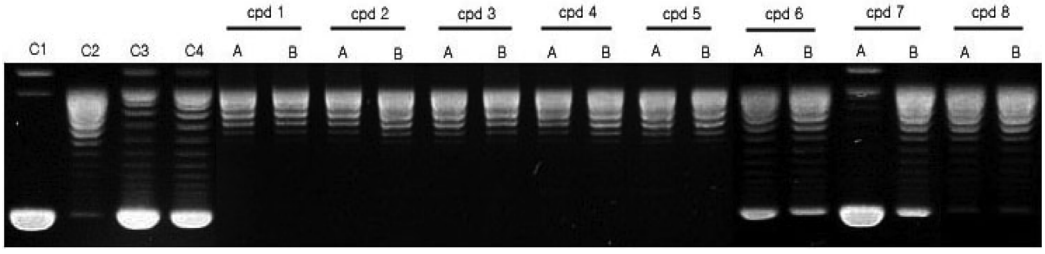
The MeOH extract of the barks of *Tilia amuenesis* was partitioned with hexane, CH_2Cl_2, EtOAc, BuOH and H_2O sequentially. The hexane extract was subjected to repeated normal-phase silica-gel column chromatography to afford eight compounds (**1** - **8**). By comparing their optical rotation values, ^{1}H- and ^{13}C-NMR and MS data with published values, compounds **1** - **8** were characterized as squalene (**1**) (Ngnokam *et al.*, 1993; Nishiyama *et al.*, 1996), friedelin (**2**) (Ali *et al.*, 1999; Klass *et al.*, 1992), *β*-sitosterol (**3**) (Kim *et al.*, 2005), *β*-sitosterol-3-*O*-glucoside (**4**) (Kim *et al.*, 2005), α-tocopherol (**5**) (Kitajima *et al.*, 1998; Nozawa *et al.*, 2000; Sakamoto *et al.*, 1991), betulinic acid (**6**) (Chatterjee *et al.*, 1999; Nick *et al.*, 1995), trilinolein (**7**) (Mannina *et al.*, 1999), and 1-*O*-(9*Z*,12*Z*-Octadecadienoyl)-3-nonadecanoyl glycerol (**8**) (Ma *et al.*, 2002).

The conversion of supercoiled pBR322 plasmid DNA to relaxed DNA using calf thymus topoisomerase I and II

Table 1. Inhibitory effects of compounds **1 - 8** on DNA topoisomerases I and II (% inhibition ratio of relaxation) and their IC_{50} values against HT-29, MCF-7 and HepG-2 cell lines

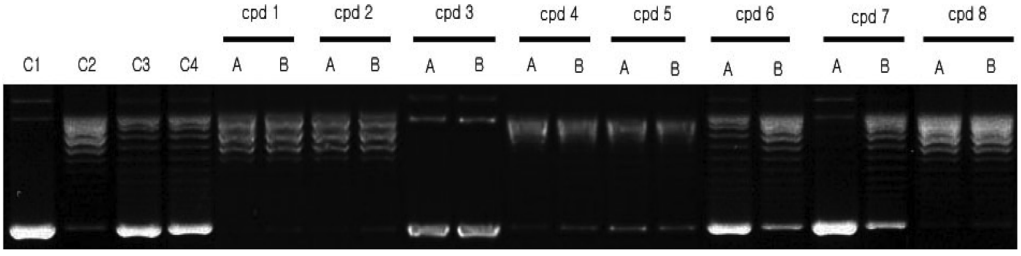
Compounds	Inhibition ratio for Topoisomerase I (%)		Inhibition ratio for Topoisomerase II (%)		Cytotoxicity IC_{50} (mM)		
	100 μM	20 μM	100 μM	20 μM	HT-29[a]	MCF-7[b]	HepG2[c]
1	0	0	0	3	> 100	> 100	> 100
2	1	2	0	16	83	46	> 100
3	25	4	100	82	59	68	> 100
4	2	0	0	0	59	47	71
5	1	1	0	0	> 100	> 100	> 100
6	25	11	55	15	20	59	16
7	87	16	95	25	> 100	> 100	> 100
8	0	0	0	0	> 100	> 100	> 100
CPT[d]	70	62	N/A[e]	N/A	0.1	10.2	1.06
Etoposide[f]	N/A	N/A	91	65	N/A	N/A	N/A

[a]HT-29: human colon carcinoma; [b]MCF-7: human breast carcinoma; [c]HepG-2: human hepatoblastoma. [d]Camptothecin (CPT): positive control for topoisomerase I; [e]NA: not applicable; [f]etoposide: positive control for topoisomerase II.



Lane C1: supercoiled DNA alone, Lane C2: supercoiled DNA + topoisomerase I (calf thymus), Lane C3: supercoiled DNA + topoisomerase I (calf thymus) + camptothecin (100 μM) -Positive control, Lane C4: supercoiled DNA + topoisomerase I (calf thymus) + camptothecin (20 μM) -Positive control, Lane A: compounds **1 - 8** (100 μM), Lane B: compounds **1 - 8** (20 μM).

Fig. 2. DNA topoisomerase I inhibitory activity of compounds **1 - 8**.



Lane C1: supercoiled DNA alone, Lane C2: supercoiled DNA + topoisomerase II (human), Lane C3: supercoiled DNA + topoisomerase II (human) + etoposide (100 μM), Positive control, Lane C4: supercolied DNA + topoisomerase II (human) + etoposide (20 μM) Positive control, Lane A: compounds **1 - 8** (100 μM). Lane B: compounds **1 - 8** (20 μM).

Fig. 3. DNA topoisomerase II inhibitory activity of compounds **1 - 8**.

were examined in the presence of compounds **1 - 8** (Table 1, Fig. 2, 3). At a concentration of 100 μM, compound **7** showed strong inhibitory activities toward both DNA topoisomerases I and II. Also, compound **3** showed strong inhibitory activity toward DNA topoisomerase II at concentrations of 20 and 100 μM.

All isolates (**1 - 8**) were evaluated for cytotoxicity against human colon adenocarcinoma (HT-29), human breast adenocarcinoma (MCF-7) and human liver hepatoblastoma (HepG-2) cell lines (Table 1). Compounds **2 - 4** and **6** exhibited weak cytotoxicity on the HT-29 cell line with IC_{50} values of 83, 59, 59 and 20 μM, respectively

(IC_{50} value of positive control camptothecin, 0.1 μM). Compounds **2 - 4** and **6** exhibited a weak cytotoxicity against the MCF-7 cell line with IC_{50} values of 46, 68, 47 and 59 μM, respectively (IC_{50} values of positive control camptothecin, 10.2 μM). In the HepG-2 cell line, compound **4** and **6** showed weak cytotoxic activities with IC_{50} values of 71 and 16 μM, respectively (IC_{50} values of positive control camptothecin, 1.06 μM).

Acknowledgement

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