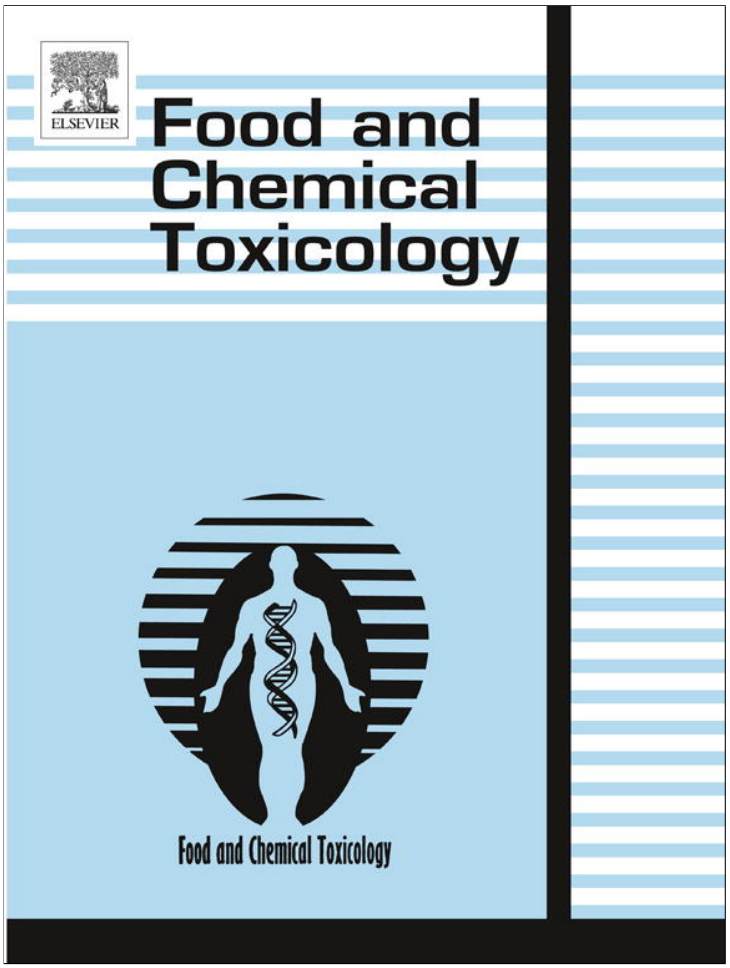

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Lignan constituents of *Tilia amurensis* and their biological evaluation on antitumor and anti-inflammatory activities

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ABSTRACT

In the recent decade, numerous lignan derivatives isolated from plants have been proven to have the potential as an anti-cancer substance. On the search for anti-cancer compounds from Korean medicinal plants, the methanolic extract from the trunk of *Tilia amurensis* Rupr. (Tiliaceae) was found to have significant cytotoxicity against A549 (lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) in our screening test. Hence, a bioassay-guided fractionation and chemical investigation of the methanolic extract resulted in the isolation and identification of 10 lignan derivatives (**1**–**10**) including two new lignan glycosides named tiliamurosides A (**1**) and B (**2**). The structures of these new compounds were determined by spectroscopic methods, namely 1D and 2D nuclear magnetic resonance (NMR) techniques, high resolution mass spectrometry (HRMS), circular dichroism (CD) data, and chemical methods. Tiliamuroside B (**2**) and schizandriside (**3**) showed significant cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with inhibitory concentration (IC_{50}) values of 3.26–8.89 μM. Moreover, (−)-syringaresinol (**8**) and (−)-pinoresinol 4-*O*-β-D-glucopyranoside (**10**) significantly inhibited nitric oxide (NO) production in murine microglia BV-2 with IC_{50} values of 15.05 and 34.35 μM, respectively.

1. Introduction

Cancer, a malignant tumor or a neoplasm, is a generic term for a broad group of diseases that can affect any part of the body via failure of regulation of cell mitosis. The process of cancer development are (1) rapid and abnormal cells division and growth, (2) forming malignant tumors, (3) invasion nearby adjoining parts of the body, and (4) spread to other organs through the lymphatic and/or bloodstream. Cancer is still one of the most threatening disease worldwide affecting human health and quality of life in spite of recent many advances in the knowledge of its molecular biology of induction and progression. Based on the World Health Organization (WHO) 2008 estimates, 7.4 million cancer deaths are estimated to have occurred in 2004, and it is estimated that 83.2 million people more will have died by 2015 without intervention (Olano et al., 2009). The gradual improvement in life expectancy and adoption of cancer-causing behaviors including smoking, physical inactivity, and "westernized" diets are associated with elevated incidence of cancer and mortality from the disease (Jemal et al., 2011). From this fact, we can assume that malignancy will soon be a global problem with its associated burden. Chemotherapy is one of the most frequently used therapeutic modalities for the treatment of cancer, which includes the use of anticancer drugs obtained from natural or synthetic sources. Natural products are the most consistently successful source of drug leads. Approximately 60% of all drugs in clinical trials for the multiplicity of cancer are either natural products or compounds containing pharmacophores derived from natural products, which are composed of various natural product groups including anthracyclines, polyphenols, macrolides, depsipeptides, indolocarbazoles, terpenoids, and lignans (Newman and Cragg, 2007). In the recent decade, renewed interest in investigating natural products has led to the discovery of several important anticancer drugs such as etoposide, etopophos, and teniposide. The three compounds are chemical derivatives of podophyllotoxin, a natural product belonging to the lignan group of natural products that current study concentrates on. Many previous studies have also reported that various

Abbreviations: NMR, nuclear magnetic resonance; HRMS, high resolution mass spectrometry; CD, circular dichroism; IC_{50}, inhibitory concentration; NO, nitric oxide; ESI, electrospray ionization; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser effect spectroscopy.

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lignans have anticancer properties. The lignans isolated from *Schisandra chinensis* showed an antiproliferative and an anti-apoptotic effects in various cancer cell lines (Min et al., 2008; Vandyke et al., 2007; Park et al., 2009; Hwang et al., 2011). Honokiol, a derivative of *Magnolia officinalis* had anti-neoplastic effects on melanoma cells and chondrosarcoma cells (Mannal et al., 2011; Chen et al., 2010). Secoisolariciresinol isolated from *Linum usitatissimum*, and lignans derived from extra-virgin olive oil also exerted anticancer properties in breast carcinoma cells (Theil et al., 2011; Menendez et al., 2008). Moreover, nordihydroguaiaretic acid, a phenolic lignan isolated from *Larrea tridentate* had also anticancer activity *in vitro* and *in vivo* system (Li et al., 2009). Thus, the natural products approach to discovery and development of new lignan derivatives for anticancer drugs is indeed an attractive approach.

Tilia amurensis Rupr. (Tiliaceae) is commonly known as bee tree and widely distributed in countries including Korea, China, and Japan. The flowers of this tree have been applied as a therapeutic agent for alleviating a fever in Korea, and its leaves have also been traditionally used to treat cancer in Korean traditional medicine (Ahn, 2003). A recent pharmacological study revealed the potential of *T. amurensis* as an anticancer agent by evaluating the DNA topoisomerase inhibitory activity of constituents isolated from its MeOH extract (Choi et al., 2008). However, only few constituents associated with the anticancer activity from *T. amurensis* have been reported. In this study, we were interested in lignan derivatives of *T. amurensis* as an anticancer agent. As aforementioned, many reports support the anticancer properties of lignan derivatives. Moreover, lignans not only regulate expression of enzymes, signal transduction pathways, and hormone metabolism, but also induce apoptosis by cell cycle arresting (Huang et al., 2010). Therefore, we assumed that anticancer property of *T. amurensis* in folk medicinal usage can be associated with the presence of bioactive lignan derivatives. In our continuing search for bioactive constituents from Korean medicinal plants, we attempted to investigate the anticancer constituents of *T. amurensis* on the basis of the fact that the MeOH extract from the trunk of *T. amurensis* exhibited significant cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 using a SRB bioassay in our screening test. Using the bioactivity-guided isolation techniques, 10 lignan derivatives including two new lignan glycosides (**1** and **2**) were isolated from the most active EtOAc-soluble fraction. In the present study, we report the isolation and structural elucidation of compounds **1**–**10** and their antitumor and anti-inflammatory activities.

2. Material and methods

2.1. General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter using methanol as a solvent. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer using methanol as a solvent. Circular dichroism (CD) spectra were measured on a Jasco J-715 spectropolarimeter using methanol as a solvent. Ultraviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV–Visible spectrophotometer using methanol as a solvent. High-resolution (HR)-electrospray ionization (ESI) MS and ESIMS spectra were recorded on a Micromass QTOF2-MS. NMR spectra, including ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC), nuclear Overhauser effect spectroscopy (NOESY) experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative high performance liquid chromatography (HPLC) used a Gilson 306 pump with a Shodex refractive index detector. Silica gel 60 (Merck, 230–400 mesh) and RP-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography. Low-pressure liquid chromatography (LPLC) was carried out over a LiChroprep Lobar-A RP-18 column (240 × 10 mm i.d., Merck) with a FMI QSY-0 pump. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co., Ltd.). Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

2.2. Plant material

The trunk of *T. amurensis* was collected at Hwacheon-Myun, Hongcheon city, Gangwon-do, Korea, in March 2010. Samples of plant material were identified by one of the authors (K.R. Lee). A voucher specimen (SKKU 2010–03) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

2.3. Extraction, fractionation, and purification methods

The air-dried and pulverized trunk of *T. amurensis* (6 kg) was extracted twice with 80% aqueous MeOH (2 × 4 h) under reflux, and then filtered. The filtrate was evaporated under vacuum to obtain a MeOH extract (384 g), which was suspended in distilled water and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 12.5, 40.4, 11.0, and 69.3 g residues, respectively. Each fraction was evaluated for its cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines using a SRB bioassay. Among the four fractions, the EtOAc-soluble fraction showed the most significant cytotoxicity against the tested tumor cell lines. The EtOAc-soluble fraction (11.0 g) was subjected to column chromatography on a silica gel column using a gradient of increasing MeOH in $CHCl_3$ from 10% to 50% to give nine fractions (E1–E9). Fraction E2 (530 mg) was applied to column chromatography on a RP-C_{18} silica gel column with a gradient of increasing MeOH in H_2O from 80% to 100% to give three fractions (E21–E23). Fraction E22 (80 mg) was applied to C_{18} Waters Sep-Pak Vac 6 cc with 100% MeOH and purified by preparative C_{18} RP HPLC using a 250 × 10 mm i.d., 10 μm, Econosil RP-18 column (Alltech Co., Ltd.) with a solvent system of MeOH-H_2O (4:1, flow rate; 2 mL/min) to afford compound **8** (3 mg). Fraction E5 (1.4 g) was subjected to column chromatography on Sephadex LH-20 with 100% MeOH to give seven fractions (E51–E57). Fraction E52 (130 mg) was applied to C_{18} Waters Sep-Pak Vac 6 cc with 40% MeOH and purified by preparative C_{18} RP HPLC using 38% MeOH to yield compounds **4** (27 mg) and **5** (23 mg). Fraction E53 (350 mg) was subjected to column chromatography on LiChroprep Lobar-A RP-18 column with 40% MeOH to give five fractions (E531–E535). Fraction E533 (84 mg) was purified by preparative C_{18} RP HPLC using 40% MeOH to obtain compounds **1** (5 mg), **2** (8 mg), **3** (20 mg), **6** (4 mg), and **10** (3 mg). Compound **9** (7 mg) was isolated from fraction E56 (120 mg) through preparative C_{18} RP HPLC purification using 70% MeOH. Fraction E7 (1.1 g) was subjected to column chromatography on Sephadex LH-20 with 100% MeOH to give eight fractions (E71–E78). Compound **7** (6 mg) was obtained from fraction E73 (76 mg) through repeated column chromatography on C_{18} Waters Sep-Pak Vac 6 cc with 45% MeOH and preparative C_{18} RP HPLC with 45% MeOH.

2.3.1. Tiliamuroside A (1)

Colorless gum; $[\alpha]_D^{25}$+24.8 (*c* 0.18, MeOH); IR (KBr) ν_{max} 3400, 2935, 1513, 1460, 1355, 1113, 1029 cm^{-1}; UV (MeOH) λ_{max} (log ε) 228 (4.2), 284 (3.3) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 220 (−13.7), 244 (+14.6), 276 (+5.2) nm; ^{1}H and ^{13}C NMR spectra, see Table 1; HR-ESIMS (positive-ion mode) *m/z* 545.1995 [M+Na]$^+$ (calcd for $C_{26}H_{34}NaO_{11}$, 545.1999).

2.3.2. Tiliamuroside B (*2*)

Colorless gum; $[\alpha]_D^{25}$−75.3 (*c* 0.35, MeOH); IR (KBr) ν_{max} 3405, 2937, 1514, 1460, 1358, 1114, 1029 cm^{-1}; UV (MeOH) λ_{max} (log ε) 223 (4.0), 283 (3.5) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 213 (−16.2), 240 (−13.4), 276 (−4.1) nm; ^{1}H and ^{13}C NMR spectra, see Table 1; HR–ESIMS (positive-ion mode) *m/z* 515.1888 [M+Na]$^+$ (calcd for $C_{25}H_{32}NaO_{10}$, 515.1893).

2.4. Enzymatic hydrolysis of *1* and *2*

A solution of compounds **1** and **2** (each 3 mg) in H_2O (3 mL) was individually hydrolyzed with cellulase (from *Aspergillus niger*, Nagase Biochemical Co., 6.5 mg) at 37 °C for 96 h. Each reaction mixture was extracted with an equal amount of EtOAc (3×), and the individual EtOAc extract was evaporated under reduced pressure. The combined EtOAc layers from **1** and **2** were purified by a silica gel Waters Sep-Pak Vac 6 cc ($CHCl_3$–MeOH, 15:1) to give each aglycone, (+)-8-methoxyisolariciresinol [1.2 mg, $[\alpha]_D^{25}$+58.1 (*c* 0.06, MeOH); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 241 (+10.3), 278 (+6.2) nm] from **1** and burselignan [1.0 mg, $[\alpha]_D^{25}$−48.9 (*c* 0.05, MeOH); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 242 (−14.1), 275 (−7.8) nm] from **2**. The aglycones were identified by the comparison of their ^{1}H NMR, MS, specific rotation, and CD data with those reported in the literature (Raju and Pillai, 1989, 1990; Jutiviboonsuk et al., 2005). The aqueous phase of the hydrolysates of **1** and **2** were subjected separately to column chromatography on a silica gel column eluting with MeCN–H_2O (8:1) to yield D-xylose with positive specific rotation ($[\alpha]_D^{25}$+20.5 in H_2O for **1**; $[\alpha]_D^{25}$+18.5 in H_2O for **2**). The D-xylose was analyzed by silica gel co-TLC by comparison with authentic sample [solvent system ($CHCl_3$/MeOH/H_2O, 8:5:1); TLC (R_f of xylose 0.56)].

2.5. Cell cutures

All tumor cell cultures were maintained using RPMI1640 cell growth medium (Gibco, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. Human tumor cell lines such as

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of compounds **1**–**2** (δ in ppm, J values in parentheses)[a].

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1		128.6		127.2
2		125.1		132.0
3		146.1	6.33 s	115.8
4		137.4		144.4
5		147.1		146.6
6	6.56 s	106.3	6.70 s	111.0
7	2.60 dd (15.0, 11.5) 2.71 dd (15.0, 4.5)	32.5	2.69 dd (16.5, 10.5) 2.91 dd (16.5, 5.5)	32.0
8	1.70 m	39.1	1.98 m	33.8
9	3.53 dd (11.0, 6.5) 3.66 dd (11.0, 4.5)	64.6	3.52 dd (11.0, 6.5) 3.57 dd (11.0, 4.5)	63.9
1′		138.7		134.4
2′	6.76 d (2.0)	112.1	6.81 d (2.5)	114.3
3′		147.2		146.8
4′		143.9		144.3
5′	6.66 d (8.0)	114.3	6.61 d (8.0)	114.0
6′	6.50 dd (8.0, 2.0)	120.2	6.32 dd (8.0, 2.5)	122.9
7′	4.35 d (6.5)	41.2	4.25 br d (4.5)	45.4
8′	2.06 m	45.4	2.23 m	41.2
9′	3.42 dd (9.5, 4.5) 3.47 dd (9.5, 5.0)	69.6	3.48 dd (9.5, 4.5) 3.68 dd (9.5, 5.0)	70.1
1″	4.21 d (7.5)	104.0	4.21 d (7.5)	104.1
2″	3.22 dd (8.5, 7.5)	73.5	3.26 dd (8.5, 7.5)	74.1
3″	3.32 m	76.5	3.34 m	76.7
4″	3.84 m	69.8	3.84 m	70.1
5″	3.17 dd (11.0, 10.5) 3.82 m	65.5	3.20 dd (11.0, 10.5) 3.80 m	65.7
3-OCH_3	3.32 s	58.5		
5-OCH_3	3.85 s	55.1	3.83 s	55.1
3′-OCH_3	3.77 s	55.0	3.79 s	55.0

[a] ^{1}H and ^{13}C NMR data of **1** and **2** in CD_3OD; The assignments were based on ^{1}H,^{1}H-COSY, HMQC, and HMBC experiments.

A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) were provided by the National Cancer Institute (NCI). BV2 (microglia from murine) was generously provided by Dr. E. Choi from Korea University (Seoul, Korea). It was maintained in Dulbecco's modified Eagle (DMEM) medium supplemented with 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

2.6. Cytotoxicity assessment

The cytotoxicity of the compounds against cultured human tumor cell lines was evaluated by the sulforhodamine B (SRB) method (Skehan et al., 1990). Each tumor cell line was inoculated over standard 96-well flat-bottom microplates and then incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were then incubated with the serially diluted lignan samples. After continuous exposure to the compounds for 48 h, the culture medium was removed from each well and the cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again and then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader.

2.7. Measurement of nitric oxide production and cell viability

BV-2 cells were plated into a 96-well plate (3×10^4 cells/well.). After 24 h, cells were pretreated with compounds **1**–**10** for 30 min, and then stimulated with 100 ng/ml of LPS for another 24 h. Nitrite, a soluble oxidation product of nitric oxide (NO), were measured in the culture media using the Griess reaction. The supernatant (50 μl) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 570 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate the NO_2^- concentration. Cell viability was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay. N^G-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, USA), a well-known NOS inhibitor, was tested as a positive control (Reif and McCreedy, 1995).

3. Results and discussion

3.1. Isolation of compounds *1*–*10* from the trunk of T. amurensis

The 80% MeOH extract from the trunk of *T. amurensis* (6 kg) was suspended in water and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. Each fraction was evaluated for its cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines using a SRB bioassay. The EtOAc-soluble fraction showed the most significant cytotoxicity against the tested tumor cell lines. Thus, the EtOAc-soluble fraction was further subjected to repeated column chromatography, which led to the isolation of 10 compounds including two new lignan glycosides (**1** and **2**), together with eight known lignan derivatives (Fig. 1). Among them, the known compounds were identified as schizandriside (**3**) (Kwon et al., 2010), lyoniside (**4**) (Šmite et al., 1995; Lee et al., 2001), nudiposide (**5**) (Šmite et al., 1995; Lee et al., 2001), lariciresinol 4-*O*-*β*-D-glucopyranoside (**6**) (Baderschneider and Winterhalter, 2001), urolignoside (**7**) (Matsuda et al., 1996), (−)-syringaresinol (**8**) (Ito et al., 1994), (−)-sesamin (**9**) (Ina et al., 1987), and (−)-pinoresinol 4-*O*-β-D-glucopyranoside (**10**) (Sugiyama and Kikuchi, 1991) respectively, by the comparison of their spectroscopic and physical data with those previously reported. To the best of our knowledge, above known compounds except for the two isolates (**4** and **5**) are reported here for the first time from this plant.

3.2. Tiliamuroside A (*1*)

Tiliamuroside A (**1**) was obtained as a colorless gum, $[\alpha]_D^{25}$+24.8 (*c* 0.18, MeOH). The molecular formula was established as $C_{26}H_{34}O_{11}$ by the molecular ion peak $[M+Na]^+$ at *m/z* 545.1995 (calcd for $C_{26}H_{34}NaO_{11}$, 545.1999) in the positive-ion HR–ESIMS. The IR absorptions of **1** implied the presence of phenyl (2935 and 1460 cm^{-1}) and hydroxy (3400 cm^{-1}) groups and its UV maximum absorption at 284 nm suggested the presence of phenolic units in **1**. The ^{1}H and ^{13}C NMR spectra (Table 1) were very similar to those of lyoniside (**4**) (Šmite et al., 1995; Lee et al., 2001), indicating that both **1** and **4** shared the same carbon framework. A major difference between them lies on the substitution pattern of one of the two aromatic rings in **1**, which was identified as 1,3,4-substitued aromatic ring, instead of 1,3,4,5-substitued one in **4** by a typical aromatic ABX coupling system [δ_H 6.50 (1H, dd, J = 2.0, 8.0 Hz), 6.66 (1H, d, J = 8.0 Hz), and 6.76 (1H, d, J = 2.0 Hz)] in the ^{1}H

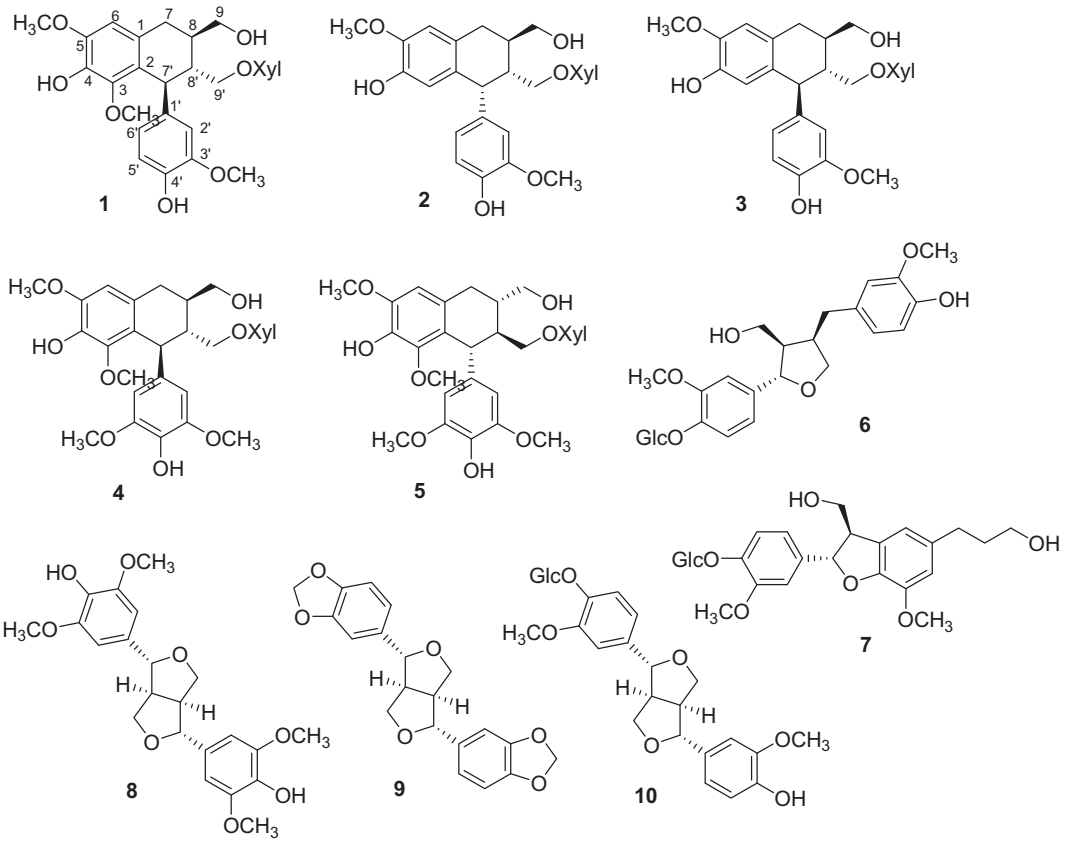

Fig. 1. Chemical structures of compounds **1**–**10**.

NMR spectrum of **1**. This structure was confirmed by analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra (Fig. 2). Moreover, the presence of a β-D-xylose unit was suggested by its ^{1}H and ^{13}C NMR data (Šmite et al., 1995; Lee et al., 2001), and enzymatic hydrolysis of **1** with cellulase liberating D-xylose ($[\alpha]_D^{25}$+20.5 in H_2O). The HMBC correlation from H-1″ (δ_H 4.21) to C-9′ (δ_C 69.6) indicated that the xylose unit was linked to the oxygen at C-9′ (Fig. 2). Furthermore, the positions of three methoxy groups were confirmed to be at C-3, C-5, and C-3′, respectively by the HMBC cross-peaks of 3-OCH_3/C-3, 5-OCH_3/C-5, and 3′-OCH_3/C-3′, respectively. The absolute configuration of **1** was established on the basis of the examination of CD spectrum of **1** in combination with the NOESY experiment. The observed NOESY correlations of H-8/H-9′, H-9/H-2′, H-7′/H-9′, and H-8′/H-9 indicated the relative configuration as 7′*S**,8*R**,8′*R** (Fig. 3). The CD spectrum of **1** showed Cotton effects at 244 ($\Delta\varepsilon$ + 14.6) and 276 ($\Delta\varepsilon$ + 5.2) nm (Fig. 3) consistent with those of the reported compound, (+)-lyoniresinol 3α-*O*-β-D-glucopyranoside (Ohashi et al., 1994). Consequently, the absolute configuration of **1** was determined to be 7′*S*,8*R*,8′*R*, which was also supported by enzymatic hydrolysis of **1** affording the aglycone, (+)-8-methoxyisolariciresinol. The identity of the aglycone of **1** was performed by the comparison of its ^{1}H NMR, MS, specific rotation, and CD data with those reported in the literature (Raju and Pillai, 1989, 1990). On the basis of above data, compound **1** was assigned as shown in Fig. 1.

3.3. Tiliamuroside B (**2**)

Tiliamuroside B (**2**) was isolated as a colorless gum, $[\alpha]_D^{25}$-75.3 (*c* 0.35, MeOH) with the molecular formula of $C_{25}H_{32}O_{10}$ based on the positive-ion HR-ESIMS data (*m/z* 515.1888 $[M+Na]^+$, calcd for $C_{25}H_{32}NaO_{10}$, 515.1893). The ^{1}H NMR spectrum (Table 1) showed a 1,2,4,5-tetrasubstituted aromatic ring [δ_H 6.33 (1H, s, H-3) and 6.70 (1H, s, H-6)] and a 1′,3′,4′-trisubstituted aromatic ring system [δ_H 6.32 (1H, dd, *J* = 2.5, 8.0 Hz, H-6′), 6.61 (1H, d, *J* = 8.0 Hz, H-5′), and 6.81 (1H, d, *J* = 2.5 Hz, H-2′)]. The ^{1}H and ^{13}C NMR spectra (Table 1) of **2** were similar to those of schizandriside (3) (Kwon et al., 2010) with apparent differences in their chemical shifts at C-2, C-8, C-1′, C-7′, and C-8′, indicating that compound **2** was the same aryltetralin type lignan as 3 but different in terms of the configuration at C-8, C-7′, and C-8′. The structural framework of **2** was confirmed by the analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra (Fig. 2). The HMBC correlation of H-1″ (δ_H 4.21)/C-9′ (δ_C 70.1) revealed the presence of a β-D-xylose unit at C-9′, which was confirmed by its ^{1}H and ^{13}C NMR data, and enzymatic hydrolysis of **2** with cellulase affording D-xylose ($[\alpha]_D^{25}$+18.5 in H_2O). The absolute configuration of **2** was assigned on the basis of the examination of CD spectrum of **2** in combination with the NOESY experiment. The small coupling constant (*J* = 4.5 Hz) of H-7′ and H-8′ observed in the ^{1}H NMR spectrum of **2**, as opposed to the large coupling constant (*J* = 7.5 Hz) between H-7′ and H-8′ in **3**, established that H-7′ and H-8′ are in the same orientation (Jutiviboonsuk et al., 2005; Ohashi et al., 1994). Moreover, the NOESY correlations of H-8/H-2′, H-9/H-7′, H-7′/H-8′, H-8′/H-9, and H-9′/H-2′ indicated the relative configuration as 7′*R**,8*R**,8′*R** (Fig. 3). The CD spectrum of **2** displayed Cotton effects at 240 ($\Delta\varepsilon$ −13.4) and 276 ($\Delta\varepsilon$ −4.1) nm (Fig. 3), which were in good agreement with those of the reported compound, (−)-4-epi-lyoniresinol 3α-*O*-β-D-glucopyranoside (Ohashi et al., 1994). Furthermore, compound **2** showed high similarity of ^{1}H and ^{13}C NMR data at C-2, C-8, C-1′, C-7′, and C-8′ to those of (−)-4-epi-lyoniresinol 3α-*O*-β-D-glucopyranoside (Ohashi et al., 1994). Thus, the absolute configuration of **2** was established to be 7′*R*,8*R*,8′*R*, which was also supported by enzymatic hydrolysis of **2** affording the aglycone, burselignan

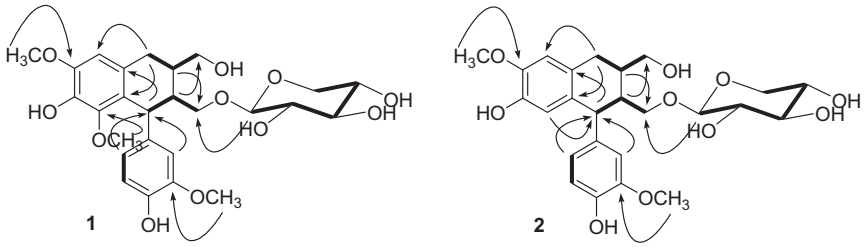

Fig. 2. ^{1}H–^{1}H COSY (bold lines) correlations and key HMBC (arrows) of **1** and **2**.

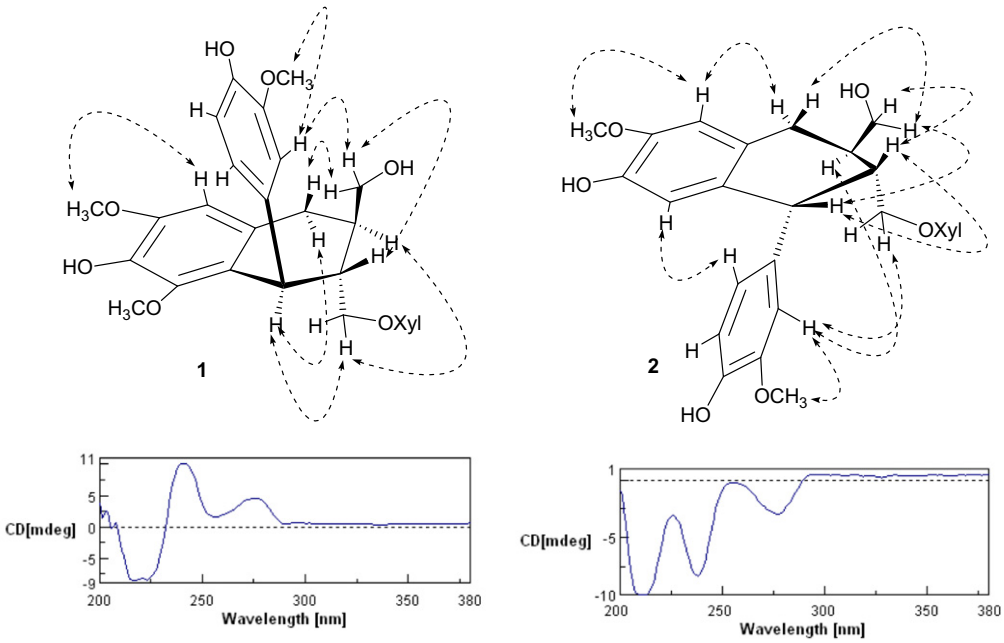

Fig. 3. Key NOSEY correlations (dashed arrows) and CD spectra of **1** and **2**.

(Jutiviboonsuk et al., 2005). On the basis of above data, compound **2** was determined as shown in Fig. 1.

3.4. Biological activities studies

Lignans are a class of secondary metabolites produced by oxidative dimerization of two phenylpropanoid units. Although their molecular backbone only consists of two phenylpropane (C_6–C_3) units, lignans show an enormous structural diversity. There is a growing interest in lignans and their synthetic derivatives due to its applications in cancer chemotherapy and other various pharmacological effects, particularly anticancer and anti-inflammatory activities (Hirano et al., 1994; Bao-Ning et al., 2004).

In this investigation, the isolated compounds **1**–**10** from the MeOH extract of *T. amurensis* were evaluated for their antiproliferative activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cell lines using the SRB bioassay (Skehan et al., 1990). The results (Table 2) showed that most of the tested isolates (**1**–**10**) had cytotoxicity against above tested cell lines. In compounds **1**–**5**, the same aryl-tetralin type lignans, they showed significant cytotoxicity against all of the cell lines tested with IC_{50} values of 3.26–29.70 μM. In particular, compounds **2** and **3** exhibited the strongest cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines (IC_{50} (**2**): 7.32, 8.89, 7.84, and 6.18 μM, and IC_{50} (**3**): 6.90, 5.88, 3.26, and 6.65 μM, respectively). These results indicated that the absence of a methoxy group at C-3 in the aryl-tetralin type lignan seems to increase the cytotoxic activity against the tested cell lines in consideration of above obtained data. Based on this evidence, the pharmacological activity of the well-known antitumor agent, podophyllotoxin, which is also the pharmacological precursor for the important anticancer drug etoposide, can be explained by the absence of the methoxy group at C-3 (Canel et al.,

Table 2
Cytotoxic activities of compounds (**1**–**10**) isolated from *T. amurensis*.

Compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	20.24 ± 0.83[b]	22.73 ± 2.43	19.39 ± 1.87	29.70 ± 4.53
2	7.32 ± 0.92	8.89 ± 0.27	7.84 ± 0.80	6.18 ± 1.18
3	6.90 ± 0.31	5.88 ± 0.13	3.26 ± 0.68	6.65 ± 0.90
4	21.09 ± 1.84	14.47 ± 1.28	14.10 ± 1.21	20.08 ± 1.71
5	24.33 ± 2.93	23.19 ± 3.23	13.91 ± 4.29	19.03 ± 2.66
6	>30.0	>30.0	13.46 ± 1.53	>30.0
7	15.29 ± 2.11	>30.0	12.29 ± 2.21	28.14 ± 5.02
8	22.08 ± 0.23	18.78 ± 2.19	6.15 ± 0.77	29.30 ± 3.44
9	>30.0	>30.0	12.57 ± 0.38	>30.0
10	23.11 ± 1.25	25.35 ± 4.04	15.21 ± 2.61	20.06 ± 1.74
Doxorubicin[c]	0.001 ± 0.001	0.003 ± 0.002	0.002 ± 0.004	0.081 ± 0.017

[a] IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.
[b] Data are expressed as mean ± SEM of three independent experiments.
[c] Doxorubicin as a positive control.

Table 3
Inhibitory effect on NO production of compounds **1**–**10** in LPS-activated BV-2 cells.

Compound	IC_{50} (μM)[a]	Cell viability (%)[b]	Compound	IC_{50} (μM)[a]	Cell viability (%)[b]
1	83.65	98.1 ± 3.4	**7**	73.80	98.2 ± 2.5
2	80.85	96.7 ± 2.8	**8**	15.05	103.5 ± 2.2
3	156.02	97.0 ± 2.1	**9**	>500	88.1 ± 3.7*
4	57.01	97.2 ± 1.9	**10**	34.35	98.1 ± 3.7
5	133.74	96.7 ± 3.3	NMMA[c]	16.44	96.7 ± 5.2
6	53.31	97.6 ± 3.0			

[a] IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] Cell viability after treatment with 20 μM of each compound was expressed as a percentage (%) of the LPS only treatment group. The results are averages of three independent experiments, and the data are expressed as mean ± SD. Statistical comparisons were performed using an one-way ANOVA test with Student's *t*-test.
[c] NMMA as a positive control.
* Only *p*-value < 0.05 was indicated as statistically significant.

2000). It was also reported that a lignan glycoside, aviculin, which has the same carbon framework as **2** and **3** with the only difference being its glycosidation of rhamnose, exhibited a potent inhibitory effect on cancer cell invasion through a rat mesothelium monolayer using an MM1 cell line in an *in vitro* system (Ohashi et al., 2003). The lariciresinol-type lignan, compound **6** showed the antiproliferative activity against only the SK-MEL-2 cell line with IC_{50} value of 13.46 μM. The neolignan, compound **7** showed cytotoxicity against the tested cell lines except for SK-OV-3 cell with IC_{50} values of 12.29–28.14 μM. In compounds **8**–**10** in the furofuran lignan group, compounds **8** and **10** exhibited relatively moderate cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with IC_{50} values of 6.15–29.30 μM. However, compound **9** with the methylene dioxy group in the benzene ring exhibited the cytotoxic activity against only the SK-MEL-2 cell line with IC_{50} value of 12.57 μM. It seems that the presence of the methylene dioxy group decreased the activity against the A549, SK-OV-3, and HCT-15 cell lines in consideration of above obtained data even though this cannot apply to other type of lignans such as podophyllotoxin with the methylene dioxy group. Interestingly, the furofuran-type lignans, compounds **8**–**10** exhibited selective cytotoxicity against the SK-MEL-2 cell line (IC_{50} (**8**): 6.15 μM, IC_{50} (**9**): 12.57 and IC_{50} (**10**): 15.21 μM).

It has been known that progression of cancer is associated with inflammatory responses. The expression of inflammatory genes is often negatively correlated with cancer stage and prognosis (Chang et al., 2004; Wang et al., 2006; Galon et al., 2006). Moreover, nonsteroidal anti-inflammatory drugs show preventive effects against cancer (Ulrich et al., 2006). Therefore, cancer and inflammation are related by epidemiology, histopathology, and inflammatory profiles (Rakoff-Nahoum, 2006). On the basis of the expanded understanding that inflammation plays a crucial role in tumor progression, we evaluated the anti-inflammatory activities of the isolate compounds (**1**–**10**) in the murine microglia BV-2 cell line. Many investigations about the anti-inflammatory effects of lignans have been announced (Baumgartner et al., 2011; Kou et al., 2011; Guo et al., 2011; Pellegrini et al., 2010). In this study, compounds **8** and **10** significantly reduced the production of LPS-induced pro-inflammatory factor, NO, in BV-2 cells without cell toxicity (Table 3). Compounds **8** and **10** exhibited the inhibitory activity with an IC_{50} of 15.05 and 34.35 μM. In particular, the activity of **8** was more potent than that of positive control, L-NMMA, in inhibiting NO production with an IC_{50} of 15.05 μM. Previously, several studies showed that NO produced in inflamed tissues could contribute to the carcinogenesis process (Ohshima and Bartsch, 1994; Tamir and Tannenbaum, 1996; Liu and Hotchkiss, 1995). Toxicity induced by overproduction of NO, when it becomes chronic, can lead to enhanced cell replication which is a risk factor for many cancers (Ames and Gold, 1990). Moreover, it is known that oxidative damage to DNA by NO may be associated with the development of cancer (Feig et al., 1994; Tamir and Tannenbaum, 1996). Continued exposure to NO lead to accumulation of populations of cells with activated oncogenes or impaired tumor suppressor genes, which induce cell death and mutagenesis. In this study, although compounds **8** and **10** inhibited NO production induced by LPS in BV-2 cells strongly, they did not effectively exert cytotoxicity against cancer cell lines. Moreover, compounds **2** and **3**, which had strong cytotoxic activities against cancer cell lines, did not exert significantly the effects on inhibition of NO production. This result demonstrates that the inhibitory activities on NO production may be a different status with cytotoxic effects against cancer cell lines. Therefore, we suggest that the inhibitory activities on NO production of lignans isolated from *T. amurensis* may be exerted independently from their cytotoxic properties. However, it is known that various bioactivities of lignans are responsible for their properties on cancer prevention. According to this consideration, we suggest that cytotoxicity of lignans from *T. amurensis* against cancer cell lines can be also associated with other bioactivities of lignans (e.g. antioxidant, anti-angiogenic, and anti-apoptotic properties). Further studies will be needed to investigate the precise mechanisms of isolated lignans from *T. amurensis* on cytotoxic activities against cancer cell lines and inhibitory activities on NO production.

4. Conclusion

This study deals with the isolation and biological study of 10 lignan derivatives from the most active EtOAc-soluble fraction from MeOH extract of *T. amurensis*. This tree *T. amurensis* has been used in Korean traditional medicine as an anticancer agent. However, the constituents of this tree responsible for anticancer activity and its underlying mechanism are still unclear. In this study, we isolated and identified 10 lignan derivatives (**1**–**10**) including two new lignan glycosides (tiliamurosides A and B) from the EtOAc-soluble fraction through the bioactivity-guided isolation techniques. This study indicates that lignan derivatives are the main components of the trunk of *T. amurensis*. With regard to bioactivity, we could confirm the anticancer effects of the lignan derivatives through their bioactive evaluation on antitumor and anti-inflammatory activities.

The present study thus suggests that the lignan derivatives isolated from *T. amurensis* may be valuable antitumor agents as contributors to the anticancer activity of this tree and expected to possess beneficial therapeutic potential against various cancers and inflammation-related diseases. This study proves that the use of *T. amurensis* as Korean traditional medicine to treat cancer is reasonable because of the anticancer effects found from the isolated lignan derivatives.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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