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Chemical Constituents from the Bark of *Phellodendron amurense* and Their Cytotoxic Effects on HL-60 Human Leukemia Cells

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Abstract – Phellodendri Cortex, phellodendron bark, has been used as a stomachic for intestinal function control and as an antimicro and anti-inflammatory agent. In this phytochemical study, eight compounds, berberine (**1**), palmatine (**2**), syringin (**3**), (+)-syringaresinol di-*O*-*β*-D-glucopyranoside (**4**), salvadoraside (**5**), citrusin B (**6**), osmanthuside H (7), and kelampayoside A (**8**), were isolated from the bark of *Phellodendron amurense*. Their structures were elucidated by comparing spectroscopic data with reported values. Compounds **1** - **8** were evaluated for cytotoxic activity against HL-60 human promyelocytic leukemia cells *in vitro*. Among them, compounds **1** and **2** reduced the viability of HL-60 cells significantly, with IC_{50} values of 26.0 and 18.5 μM, respectively.
Keywords – *Phellodendron amurense*, Rutaceae, Cytotoxicity, HL-60 leukemia cells

Introduction

Plants of the genus *Phellodendron* are rich sources of berberine and aphorphine alkaloids, flavonoids, various coumarins, lignans, and limonoids (Gray *et al*., 1988, Wu *et al*., 2003, Chiu *et al*., 2005, Chiang *et al*., 2006). Phellodendri Cortex, the bark of *Phellodendron amurense* Rupr. (amur cork tree, Rutaceae), has been used in traditional Chinese medicine to treat meningitis, bacillary dysentery, pneumonia, tuberculosis, and liver cirrhosis (Li *et al*., 2009). In recent study, *P. amurense* bark extract prevented the development of prostate cancer in a murine model of prostate cancer and inhibited prostate cancer cell proliferation (Garcia *et al*., 2006, Kumar *et al*., 2007).

Leukemia is cancer of the blood or bone marrow characterized by the abnormal proliferation of blood cells. Human leukemia results from multiple mutations that lead to abnormalities in either the expression or function of gene products that affect the delicate balance among proliferation, differentiation and apoptosis (Ikeda *et al.*, 2006; Cuong *et al.*, 2010). In our study, a methanol extract of *P. amurense* was isolated by serial column chromatography, and the isolates were evaluated for antileukemic properties. Specifically, their cytotoxic activity against HL-60 human promyelocytic leukemia cells was examined using the 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

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Experimental

General – Melting points were obtained with an Electrothermal 9100 melting point apparatus (Electrothermal Ltd.). Optical rotations were determined using a Jasco DIP-370 automatic polarimeter. The FT-IR spectra were measured using a Jasco Report-100 infrared spectrometer, and the ESI-MS using an Aglient 1200 LC-MSD Trap spectrometer. Column chromatography was performed using a silica gel (Kieselgel 60, 70 - 230, and 230 - 400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica gel 60 F_{254} and RP-18 $F_{254}S$ plates (both 0.25 mm, Merck, Darmstadt, Germany).

Plant Material – Dried bark of *P. amurense* was purchased from Naemome Dah, Ulsan, Korea in November 2010 and identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 10108) was deposited at the Herbarium of College of Pharmacy, Chungnam National University, Korea.

Extraction and Isolation – Dried bark of *P. amurense* (2.0 kg) was extracted with MeOH under reflux for 10h (7 L × 3 times). The extract was suspended in water and partitioned with ethyl acetate and *n*-BuOH. (each 1.5 L ×

3), successively. The water layer was subjected to a Diaion HP-20 column eluted with a gradient of MeOH in H_2O (0, 30, 60, and 100% MeOH) to give four fractions (1a - d). The *n*-BuOH extract (52.0 g) was soaked with 2 - 3% HCl solution for three days. The acidic aqueous phase was basified to pH 9 - 10 with NH_4OH and extracted with CH_2Cl_2. The aqueous layer was further basified with saturated NaOH solution to pH 12 and extracted with *n*-BuOH to afford alkaloid extract (5.0 g). The alkaloid extract was further chromatographed on a silica gel column using $CHCl_3$-MeOH (10 : 1 v/v) to give compound **1** (2.1 g) and **2** (805.0 mg). Fraction 1c was chromatographed on a silica gel column using a gradient of $CHCl_3$-MeOH-H_2O (10 : 1 : 0 - 0 : 1 : 0 v/v/v) to afforded four subfractions (2a–d). Fraction 2a was further chromatographed on a RP chromatography column with MeOH-H_2O (0.35 : 1 v/v) to give compound **3** (130.0 mg) and **8** (15.0 mg). Fraction 2b was chromatographed on a silica gel column using a gradient of $CHCl_3$-MeOH-H_2O (2.5 : 1 : 0.1 - 0 : 1 : 0 v/v/v) to afford three subfractions (3a - c). Fraction 3a was purified using a RP column chromatography with MeOH-H_2O (0.5 : 1 v/v) to yield compound **6** (33.0 mg). Fraction 3c was purified using a RP column chromatography with acetone-H_2O (0.15 : 1 v/v) to yield compound **4** (5.0 mg). Fraction 2c was chromatographed on a silica gel column using a gradient of $CHCl_3$-MeOH-H_2O (4 : 1 : 0.15 - 0 : 1 : 0 v/v/v) to afford three subfractions (4a - c). Fraction 4c was purified using a RP column chromatography with acetone-MeOH-H_2O (0.15 : 0.15 : 1 v/v/v) to yield compounds **5** (35.0 mg) and **7** (90.0 mg).

Berberine (1): Yellow amorphous powder; UV (MeOH): λ_{max} 222, 265, 349, 429 nm; ESI-MS: *m/z* 337 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Kim *et al*., 2000).

Palmatine (2): Yellow amorphous powder; mp 195 - 198 ºC; UV (MeOH): λ_{max} 228, 268, 344, 430 nm; ESI-MS: *m/z* 353 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Wafo *et al*., 1999).

Syringin (3): White amorphous powder; mp 109 - 111 ºC; $[\alpha]_D^{28} = -32.0$ (*c* 0.5, MeOH); UV (MeOH): λ_{max} (log ε) 222 (4.23), 265 (3.99) nm; IR (KBr): ν_{max} 3411, 1743, 1643, 1587, 1507 cm^{-1}; ESI-MS: *m/z* 373 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Calis *et al*., 1993).

(+)-Syringaresinol di-*O*-*β*-D-glucopyranoside (4): Brown amorphous powder; $[\alpha]_D^{28} = +28.0$ (*c* 0.4, MeOH); ESI-MS: *m/z* 741 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Houghton and Lian, 1986).

Salvadoraside (5): White amorphous powder; mp 260 - 262 ºC; ESI-MS: *m/z* 743 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Kamel *et al*., 1992).

Citrusin B (6): White amorphous powder; $[\alpha]_D^{20} = -43.6$ (*c* 1.72, MeOH); FAB-MS *m/z* 575.2 $[M + Li]^+$, 591.2 $[M + Na]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Deyama *et al*., 1987, Yuan *et al*., 2007).

Osmanthuside H (7): Brownish oil; $[\alpha]_D^{20} = -78.9$ (*c* 0.3, MeOH); ESI-MS: *m/z* 743 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Sugiyama and Kikuchi, 1993).

Kelampayoside A (8): White amorphous solid; $[\alpha]_D^{21} = -81.7$ (*c* 0.9, MeOH); UV (MeOH): λ_{max} (log ε) 223 (3.73), 275 (2.80) nm; IR (KBr): ν_{max} 3394, 2938, 1601, 1506, 1464, 1128, 1060 cm^{-1}; FAB-MS: *m/z* 501 $[M + Na]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Kitagawa *et al*., 1996, Jiang *et al*., 2005).

Cell Culture – HL-60 cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO Inc., Grand Island, NY, U.S.A.) and 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO Inc., Grand Island, NY, U.S.A.) at 37 ºC in a humidified 5% CO_2. Briefly, HL-60 cells were seeded into 96-well plates at a density of 3×10^5 cells/well. The exponentially growing cells were used throughout the experiments.

Cytotoxicity Assay – HL-60 cells were seeded at 3×10^5 cells/mL in 96-well microplate. After 4h, the cells were treated with the samples at concentrations 10, 50 and 100 μM. Mitoxantrone (MX) (Sigma-Aldrich, MO, U.S.A.) was used as the positive control. After 72 h, 50 μL MTT (2 mg/mL, Sigma Chemical Co., MO, U.S.A.) was added to each well. Plates were incubated at 37 ºC for 4 h, the media was carefully aspirated. 150 μL Dimethylsulfoxide (DMSO, Amresco, OH, U.S.A.) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., NY, U.S.A.). All the experiments were performed at least three times in triplicate and the mean absorbance values were calculated. A dose-response curve was generated and the inhibitory concentration of 50% (IC_{50}) was determined for each compound as well as each cell line.

Results and Discussion

From our phytochemical analyses, two alkaloids (**1** and

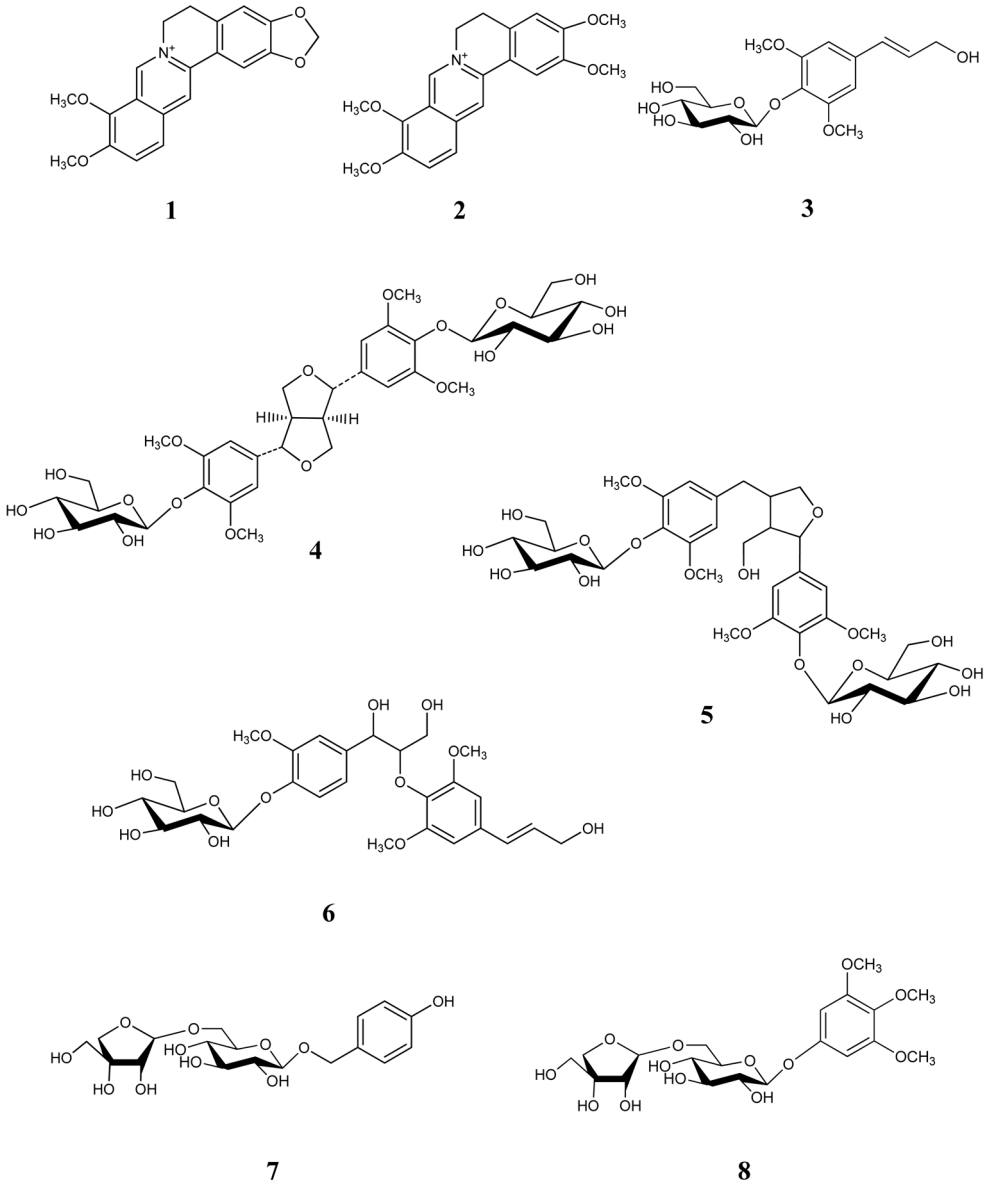

Fig. 1. Structures of compounds **1** - **8** from the bark of *P. amurense.*

2), three lignans (**4** - **6**) and three phenolic glucosides (**3**, **7**, and **8**) were isolated from the bark of *P. amurense* using a combination of various column chromatographies. Their structures were elucidated by 1D-, 2D-NMR, and MS data, and comparing spectroscopic data to published values. The isolates were characterized as berberine (**1**), palmatine (**2**), syringin (**3**), (+)-syringaresinol-di-*O*-*β*-D-glucopyranoside (**4**), salvadoraside (**5**), citrusin B (**6**), osmanthuside H (**7**), and kelampayoside A (**8**) (Fig. 1). All of the compounds were purified previously, but this is the first time that compounds **4** - **6** and **8** have been isolated from this plant.

To evaluate the potential of the isolates for leukemia treatment, their cytotoxic activity was tested against HL-60 cells, using the MTT assays. Among the compounds **1** and **2**, which are isoquinoline alkaloids, significantly reduced HL-60 cell viability; their IC_{50} values were 26.0 and 18.5 μM, respectively. However, the lignans (**4** - **6**) and phenolic glucosides (**3**, **7**, and **8**) lacked cytotoxic activity; their IC_{50} values were > 90 μM in all cases (Table 1). Previously, berberine (**1**) was shown to exert cytotoxic effects against 8505C and TPC1 cells, with an IC_{50} value of 10.0 μM in both cases (Park *et al*., 2012). In addition, the cytotoxic activity of berberine (**1**) and palmatine (**2**) chloride analogs has been demonstrated against seven human cancer cell lines (7701QGY, SMMC7721, HepG2,

Table 1. IC_{50} values of compounds **1** - **8** on the growth of HL-60 human leukemia cells

Compounds	IC_{50} values (μM)[a]
1	26.05 ± 1.01
2	18.51 ± 2.18
3	94.42 ± 4.39
4	94.63 ± 7.47
5	>100
6	>100
7	>100
8	>100
Mitoxantrone[b]	6.80 ± 0.90

[a]Results are the means ± SD of three independent experiments in triplicates, [b]Positive control.

CEM, CEM/VCR, KIII, Lewis), yielding IC_{50} values of 0.02 - 13.5 μM (Zhang *et al*., 2012). The results of the present study suggest that isoquinoline alkaloids (e.g., compounds **1** and **2** from *P. amurense*) a source of active metabolites with cytotoxic activity against HL-60 cells; moreover, these compounds may be valuable for the treatment of human leukemia.

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