Isolation of Limonoids and Alkaloids from *Phellodendron amurense* and Their Multidrug Resistance (MDR) Reversal Activity

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Three limonoids and five alkaloids were isolated from the chloroform layer of the MeOH extract of *Phellodendron amurense* (Rutaceae). The structures of the compounds isolated were determined to be obacunone (1), limonin (2), 12α-hydroxylimonin (3), γ-fagarine (4), oxyberberine (5), canthin-6-one (6), 4-methoxy-N-methyl-2-quinolone (7) and oxypalmatine (8) based on the physicochemical and spectroscopic data. Compounds 3, 5, 7, and 8 were first isolated from *Phellodendron amurense*. The isolated compounds were then tested for their cytotoxicity against five human tumor cell lines in *vitro* using the SRB method. Compound 5 showed significant cytotoxicity against the five tumor cell lines with ED₅₀ values ranging from 0.30 to 3.0 μg/mL. The marginal or non-cytotoxic compounds (1, 2, 3, 4, and 7) were examined for their P-gp related MDR reversal activities. Compound 1 showed significant P-gp MDR inhibition activity in MES-SA/DX5 and HCT15 cells with an ED₅₀ value of 0.028 μg/mL and 0.0011 μg/mL, respectively.

**Key words:** *Phellodendron amurense*, Rutaceae, Limonoid, Alkaloid, Multidrug resistance

**INTRODUCTION**

In traditional Chinese Medicine, Phellodendri Cortex (The stem bark of *Phellodendron amurense* Rupr., Rutaceae) has been used to treat dysentery, jaundice, yellow thick foul leukorrhagia, swelling in the knees and feet, urinary tract infections, and infections on the body surfaces (Yan et al., 1999). Isoquinoline alkaloids, phenolic compounds, butenolides and limonoids from the bark of this plant have been reported (Kondo et al., 1985; Wada et al., 1990; Miyaki et al., 1992; Kishi et al., 1992; Ida et al., 1994). Indolopyridoquinazoline alkaloids, furoquinoline alkaloids and isoquinoline alkaloids were also extracted from the callus tissues of bark of this plant (Ikuta et al., 1995, 1998a, 1998b).

As part of an ongoing search for multidrug resistance (MDR) reversal compounds from Korean medicinal plants, the present study examined Phellodendri Cortex because the MeOH extract was found to show P-gp mediated MDR reversal activity in human cancer cells.

The chromatographic separation of the chloroform fraction of the MeOH extract from the bark of *P. amurense* led to the isolation of three limonoids and five alkaloids. Their structures were characterized as obacunone (1), limonin (2), 12α-hydroxylimonin (3), γ-fagarine (4), oxyberberine (5), canthin-6-one (6), 4-methoxy-N-methyl-2-quinolone (7) and oxypalmatine (8) by physicochemical and spectroscopic methods. The compounds were tested for their *in vitro* cytotoxicity against five tumor cell lines using the SRB method. The marginal or non-cytotoxic compounds (1, 2, 3, 4, and 7) were tested for their MDR reversal activity. This paper describes the isolation, structural determination and P-gp expressed MDR reversal activities of the compounds isolated from *P. amurense*.

**MATERIALS AND METHODS**

**General experimental procedures**

The melting points were determined on a Gallenkamp melting point apparatus and were uncorrected. The optical rotations were determined using a Jasco P-1020 polarimeter. The Infrared (IR) spectra were recorded on KBr discs using a Bruker Vector 22 FT-IR spectrometer. The ultraviolet (UV) spectra were obtained using a Shimadzu UV-1601 UV/Visible (Japan) and PDA detector.
(Waters Co.). The nuclear magnetic resonance (NMR) spectra were recorded on Varian VX-500 and JNM-LA400. The El-MS data was obtained using a JMS700 spectrometer (Jeol Co.). The LC-ESI-MS/MS data were obtained using a Quattro micro (Waters Co.). The prep-HPLC was performed using a Prep Nova-Pak HR C18 (6 μm, 19-300 mm) column with a PDA detector (Waters Co., model 2996) and RI detector (Waters Co., model 2414). Silica gel 60 (0.063-0.200 mm, Merck Co.) was used for column chromatography. Kiesel gel 60F254 precoated plates (Merck Co.) and RP-18 F254s precoated plates (Merck Co.) were used for thin layer chromatography (TLC). The packing material used for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.).

The following analytical conditions were used for HPLC: detector = PDA; column = RP18, 5 μm, 4.6 × 150 mm; eluent = gradient 20% MeOH → 90% MeOH (15 min.) → 90% MeOH (10 min.).

Planta material

The bark of P. amurense was purchased at the Kyungdong herbal market, in March, 2003, Seoul, Korea and a voucher specimen was deposited in the College of Pharmacy at Sungkyunkwan University.

Extraction and isolation

The dried, chopped bark (2.5 kg) was extracted three times with 80% MeOH (6 L × 3) at room temperature. The resulting extracts (150 g) were suspended with distilled water (3 L), followed by fractionation with n-hexane and chloroform. The chloroform layer (40 g) was subjected to column chromatography with a Sephadex LH-20 column (n-hexane : ethylacetate : methanol = 10 : 10 : 0.3) to afford compounds 6 (10 mg), 7 (10 mg) and 8 (5 mg).

Obacunone (1)

Colorless crystal; mp 228–235°C (CH2Cl2/MeOH); [α]D20 46.4° (c 0.24, CHCl3); IR (neat) νmax cm−1: 2988, 1745, 1703, 1630, 1572, 1503, 1451, 1393, 1282; El-MS m/z (rel. int.): 454 (M+*, 0.4), 439 (4), 397 (3), 363 (15), 347 (2), 331 (100), 313 (7); 1H-NMR (500 MHz, CDC13): δ 7.42 (1H, m, H-21), 7.40 (1H, t, J = 1.8 Hz, H-23), 6.51 (1H, d, J = 11.7 Hz, H-1), 6.36 (1H, dd, J = 1.8, 0.9 Hz, H-22), 5.96 (1H, d, J = 11.7 Hz, H-2), 5.46 (1H, s, H-17), 3.65 (1H, s, H-15), 2.98 (1H, t, J = 14.1 Hz, H-6δ)), 2.60 (1H, dd, J = 14.1, 5.0 Hz, H-5), 2.28 (1H, dd, J = 14.1, 5.0 Hz, H-6δ), 2.14 (1H, br.d, J = 8.5, 3.5 Hz, H-9), 1.50 (6H, s, H-29 and H-30), 1.45 (3H, s, H-28), 1.24 (3H, s, H-19), 1.12 (3H, s, H-18); 13C-NMR (100 MHz, CDC13): δ 208.10 (C-7), 167.59 (C-3)*, 167.36 (C-16)*, 157.45 (C-1), 143.88 (C-23), 141.70 (C-21), 123.67 (C-2), 120.78 (C-20), 110.44 (C-22), 84.66 (C-4), 78.67 (C-17), 65.74 (C-14), 58.03 (C-5), 54.02 (C-15), 53.65 (C-8), 49.92 (C-9), 43.83 (C-10), 40.60 (C-6), 38.14 (C-13), 33.47 (C-12), 32.73 (CH3), 27.51 (CH3), 21.83 (CH3), 20.16 (CH3), 17.69 (C-11), 17.14 (CH3).

Limonin (2)

Colorless crystal; mp 287–293°C (CH2Cl2/MeOH); [α]D20 127.7° (c=0.2, CHCl3); IR (neat) νmax cm−1: 2997, 1747, 1714, 1503, 1460, 1285, 1025; El-MS m/z (rel. int.): 470 (M+, 0.6), 454 (4), 412 (13), 347 (100), 329 (15), 135 (22); 1H-NMR (500 MHz, CDC13): δ 7.41 (1H, m, H-21), 7.40 (1H, t, J = 1.8 Hz, H-23), 6.51 (1H, dd, J = 1.8, 0.9 Hz, H-22), 5.96 (1H, d, J = 11.7 Hz, H-2), 5.46 (1H, s, H-17), 3.65 (1H, s, H-15), 2.98 (1H, t, J = 14.1 Hz, H-6δ)), 2.60 (1H, dd, J = 14.1, 5.0 Hz, H-5), 2.28 (1H, dd, J = 14.1, 5.0 Hz, H-6δ), 2.14 (1H, br.d, J = 8.5, 3.5 Hz, H-9), 1.50 (6H, s, H-29 and H-30), 1.45 (3H, s, H-28), 1.24 (3H, s, H-19), 1.12 (3H, s, H-18); 13C-NMR (100 MHz, CDC13): δ 208.10 (C-7), 167.59 (C-3)*, 167.36 (C-16)*, 157.45 (C-1), 143.88 (C-23), 141.70 (C-21), 123.67 (C-2), 120.78 (C-20), 110.44 (C-22), 84.66 (C-4), 78.67 (C-17), 65.74 (C-14), 58.03 (C-5), 54.02 (C-15), 53.65 (C-8), 49.92 (C-9), 43.83 (C-10), 40.60 (C-6), 38.14 (C-13), 33.47 (C-12), 32.73 (CH3), 27.51 (CH3), 21.83 (CH3), 20.16 (CH3), 17.69 (C-11), 17.14 (CH3).

12α-Hydroxylimonin (3)

White powder; [α]D20 141.7° (c 0.02, CHCl3); IR (neat) νmax cm−1: 3526, 2969, 1742, 1505, 1459, 1281, 1022; El-MS m/z (rel. int.): 486 (M+, 3), 471 (3), 440 (19), 429 (14), 363 (100), 345 (11); 1H-NMR (500 MHz, CDC13): δ 7.52 (1H,
The human ovarian cancer cell line, SK-OV-3; the human colon carcinoma cell line, COLO-205; the human small cell lung adenocarcinoma, SK-LC-1; the human melanoma, SK-MEL-2; (skin melanoma); XF498 (CNS) and HCT15 (colon).

Cytotoxicity test in vitro
The sulfonfuranad B assay (SRB) was used for the cytotoxicity test. The activity of the compounds was tested at several concentrations against five cultured human tumor cell lines in vitro (Skehan et al., 1990); A549 (non small cell lung adenocarcinoma). SK-OV-3 (ovarian), SK-MEL-2 (skin melanoma), XF498 (CNS) and HCT15 (colon).

MDR reversal activity
The human ovarian cancer cell line, SK-OV-3; the human colorectal cancer cell line, HCT15 cells; the human uterine sarcoma cell line, MES-SA; the human MDR uterine sarcoma cell line, MES-SA/DX5; were provided by the...
National Cancer Institute (NCI), and were maintained in the Korea Research Institute of Chemical Technology (KRICT). The SK-OV-3 cells did not express P-gp and were not multidrug resistant cancer cells. Meanwhile, HCT15 cells and MES-SA/DX5 cells showed high level P-gp expression. The cell cultures were conducted with RPMI 1640 medium supplied with 5% FBS previously reported (Choi et al., 1996). The cells were incubated with serial dilutions of paclitaxel in the presence or absence of each isolated compound (10 μM) or verapamil (10 μM) for 72 h. The procedure for calculating the survival fractions was identical to that of the cytotoxicity assay. In this assay, the controls contained each isolated compound or verapamil without paclitaxel.

RESULTS AND DISCUSSION

Obacunone (1) (Sugimoto et al., 1988a), limonin (2) (Sugimoto et al., 1988a, 1988b), γ-fagarine (4) (Robertson et al., 1963; Narasimhan et al., 1974) and canthin-6-one (6) (Ohmoto et al., 1976; Koike et al., 1985) were identified by a comparison of their spectral data (UV, IR, MS, 1H-NMR, 13C-NMR) with those reported in the literature.

Compound 3 was obtained as colorless powder. The IR spectrum showed the presence of OH group at 3526 cm⁻¹ and C=O group at 1742 cm⁻¹, respectively. The molecular ion peak of compound 3 in the EI-MS spectrum was m/z 486. The 1H- and 13C-NMR spectra of compound 3 were almost same as those of compound 2. However, the major differences were the H-12 proton chemical shift at δ 3.93, and the C-12 carbon chemical shift at δ 68.56 in compound 3, indicating the presence of an OH group at C-12. Based on the above data and the literature survey, the structure of compound 3 was determined to be 12α-hydroxylimonin. The NMR and physical data of compound 3 was in good agreement with those in reported literature (Sugimoto et al., 1988a).

Compound 5 was obtained as a yellow powder and tested positive on the dragendorff reagent. The IR spectrum showed amide carbonyl group at 1647 cm⁻¹. The molecular ion peak of compound 5 in the EI-MS spectrum was observed at m/z 351 as the base peak. The 1H- 13C-NMR and IR spectral data suggested compound 5 to be an 8-oxo-protoberberine derivative (Patra et al., 1987). The 1H-NMR spectrum showed H-11 and H-12 signals of the protoberberine D-ring at δ 7.32 and 7.27 (d, J = 8.8 Hz), H-13 signal of the C-ring at δ 7.21 (s), H-1 and H-4 signals of the A-ring at δ 6.70 (s) and 6.71 (s) and H-5 and H-6 signals of the isoquinoline B-ring at δ 2.89 and 4.29 (2H, t, J = 6.2 Hz), respectively. The 1H-NMR spectrum also showed two methoxyl groups (δ 3.95 and 4.01), an amide carbonyl (C-8, δ 160.10) and a dioxymethylene group (-OCH2O-, δ 101.40). Based on the above spectral data, the structure of compound 5 was determined to be oxyberberine. The NMR data and physical data of compound 5 were in good agreement with those reported in the

![Fig. 1. The Structures of compounds 1–8 from the bark of Phellodendron amurense](image-url)
Compound 7 was obtained as a colorless powder and tested positive to the dragendorff reagent. The molecular ion peak of compound 7 in EI-MS spectrum showed m/z 189 as the base peak. The 1H-NMR spectrum showed a H-3 olefinic proton at δ 6.06 (1H, s, H-3), and aromatic ring system protons at δ 7.35 (1H, dd, J = 5.7, 0.6 Hz, H-7), 7.30 (1H, dd, J = 5.7, 0.6 Hz, H-7), 7.60 (1H, dd, J = 5.7, 4.8, 0.6 Hz, H-6), and 7.99 (1H, dd, J = 5.3, 1.0 Hz, H-8). The 13C-NMR spectrum showed quinolone ring carbons at δ 163.82 (C-2), 162.64 (C-4), 139.75 (C-9), 131.18 (C-5), 123.34 (C-7), 121.61 (C-6), 116.50 (C-10), 114.01 (C-8), and 96.49 (C-3). From the above spectral data and literature survey, the structure of compound 7 was determined to be 4-methoxy-N-methyl-2-quinolone. The NMR and physical data of compound 7 were in good agreement with the reported literature (Nayar et al., 1971).

Compound 8 was obtained as a yellow powder and tested positive to the dragendorff reagent. The molecular ion peak of compound 8 in EI-MS spectrum was observed as the base peak at m/z 367. The 1H-, 13C-NMR spectra of compound 8 were similar to those of compound 5. The differences were the presence of four methoxyl groups at δ 3.94, 3.96, 3.99 and 4.02, and the absence of a dioxy-methylene group in the 1H-NMR spectrum of compound 8. The result of the spectral data suggested compound 8 to be an 8-oxo-protoberberine derivative (Patra et al., 1987). The structure of compound 8 was determined to be oxypalmatine. The NMR data and physical data of compound 8 were in good agreement with the reported literature (Pinho et al., 1992).

Compounds 3, 5, 7, and 8 have not yet reported from this plant.

Compounds 1, 2, 3, 4, and 7 showed little cytotoxicity against the human cancer cell lines, A549, SK-OV-3, SK-MEL-2, XF498 and HCT15. However, compound 5 showed significant cytotoxicity against the five tumor cell lines with an ED50 value ranging from 0.3 to 3 μg/mL (Table I). The noncytotoxic compounds were tested for their MDR reversal activity in the P-gp nonexpressed (non-MDR) cell line, SK-OV-3 cell, and the P-gp expressed MDR cell line, MES-SA/DX5 cell and HCT15 cell. Compounds 1, 2, 3, 4 and 7 showed P-gp MDR inhibition and very weak cytotoxicity to the non-MDR cell line (SK-OV-3). In particular, the P-gp MDR inhibition activity of compound 1 was similar to verapamil, with an ED50 value of 0.028 μg/mL and 0.0011 μg/mL in the MES-SA/DX5 and HCT15 cells, respectively (Table II).

**Table I.** Cytotoxic activities of compounds (1-8) isolated from *Phellodendron amurense*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ED50(μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td>1</td>
<td>25.46</td>
</tr>
<tr>
<td>2</td>
<td>&gt;30.0</td>
</tr>
<tr>
<td>3</td>
<td>&gt;30.0</td>
</tr>
<tr>
<td>4</td>
<td>22.41</td>
</tr>
<tr>
<td>5</td>
<td>1.82</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>23.19</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* - not tested

**Table II.** MDR reversal activities of compounds 1-4 and 7 isolated from *Phellodendron amurense*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>SK-OV-3</th>
<th>HCT15</th>
<th>MES-SA/DX5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pacitaxel (P)</td>
<td>0.0004</td>
<td>0.1130</td>
<td>0.817</td>
</tr>
<tr>
<td>P + 1</td>
<td>0.0003</td>
<td>0.0011</td>
<td>0.028</td>
</tr>
<tr>
<td>P + 2</td>
<td>0.0003</td>
<td>0.0210</td>
<td>0.392</td>
</tr>
<tr>
<td>P + 3</td>
<td>0.0007</td>
<td>0.0180</td>
<td>0.449</td>
</tr>
<tr>
<td>P + 4</td>
<td>0.0004</td>
<td>0.0100</td>
<td>0.264</td>
</tr>
<tr>
<td>P + 7</td>
<td>0.0003</td>
<td>0.0170</td>
<td>0.335</td>
</tr>
<tr>
<td>P + verapamil</td>
<td>0.0007</td>
<td>0.0003</td>
<td>0.022</td>
</tr>
</tbody>
</table>

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**REFERENCES**


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