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Anti-Human Immunodeficiency Virus-Type 1 Activity of Constituents from *Juglans mandshurica*

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Three naphthalene glycosides (**1-3**), four flavonoids (**4-7**), and two galloyl glycosides (**8-9**) were isolated from the stem-bark of *Juglans mandshurica* (Juglandaceae). Their structures were determined by chemical and spectral means, including to 2D-NMR (COSY, HMQC, and HMBC) experiments. Amongst the isolated compounds, taxifolin (**4**) showed the most potent HIV-induced cytopethic activity against MT-4 cells with complete inhibitory concentration (IC_{100}) value of 25 μg/ml and maximum cytotoxic concentration (CC_{100}) value of above 100 μg/ml. However, naphthalene glycosides (**1-3**), flavonoids (**5-7**)), and galloyl tannins (**8-9**) were inactive against anti-HIV-1 activity.

Key words: Juglans mandshurica, Taxifolin, Anti-HIV activity, MT-4 cells

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) is a pathogenic retrovirus and the etiological agent of acquired immune deficiency syndrome (AIDS) (Goff, 1990; Fauchi, 1988). It is known to be a retrovirus that infects T-cells through binding to CD_4 and $CXCR_4$ protein, receptors present on the surface of the cell, where it eventually replicates inducing immunodeficiency. In the development of the drugs against AIDS, several stages in the replication cycle of HIV have been considered targets for chemotherapeutic intervention. Several useful inhibitors of HIV have been developed which targeted at the reverse transcriptase (RT) and protease (Lim *et al.*, 1997a). The RT inhibitors have been licensed, in the family of nucleoside analogues, such as Zidovudine, Didanoside, Zalcitabine, Stavudine, Lamivudine, and Abacavir (Lim *et al.*, 1997b). On the other hand, the protease inhibitors (Saquinavir, Ritonavir, Indinavir, Nelfinavir, Saquinavir, Amprenavir, and Lopinavir) have been blocked the formation of functional viral enzymes, i.e. reverse transcriptase, integrase, protease, and structural proteins, from fused polyprotein (Kusmoto *et al.*, 1995; Matsuse *et al.*, 1997). However, long-term therapies with these reverse transcriptase and protease inhibitors might be lead to the development of resistant virus and side effects (DeClerq, 1995). Since it is possible that the efficacy of each drug might be enhanced and the toxic effects might be reduced with the combined treatment of multiple drugs that have different antiviral mechanisms, the development of a variety of effective agents has been required (Kawahata *et al.*, 1996).

During the course of our studies on development of anti-HIV agents from natural products, compounds isolated from the stem-bark of *Juglans mandshurica* were evaluated for their anti-HIV activity. *J. mandshurica* has been used as a folk medicine for treatment of cancer in Korea (Son, 1995). Several naphthoquinones, naphthalenyl glucosides, α-tertalonyl glucopyranosides and diarylheptanoyl glucopyranosides have been isolated from this plant, and these compounds have been shown to have cytotoxic activity to human cancer cell lines and inhibitory effects on DNA polymerase and RNase H activities of HIV reverse transcriptase (Son, 1995; Joe *et al.*, 1996; Kim *et al.*, 1998; Min *et al.*, 2000; Lee *et al.*, 2000). In this paper we report anti-HIV-1 activity of compounds isolated from the stem-bark of this plant in MT-4 cells.

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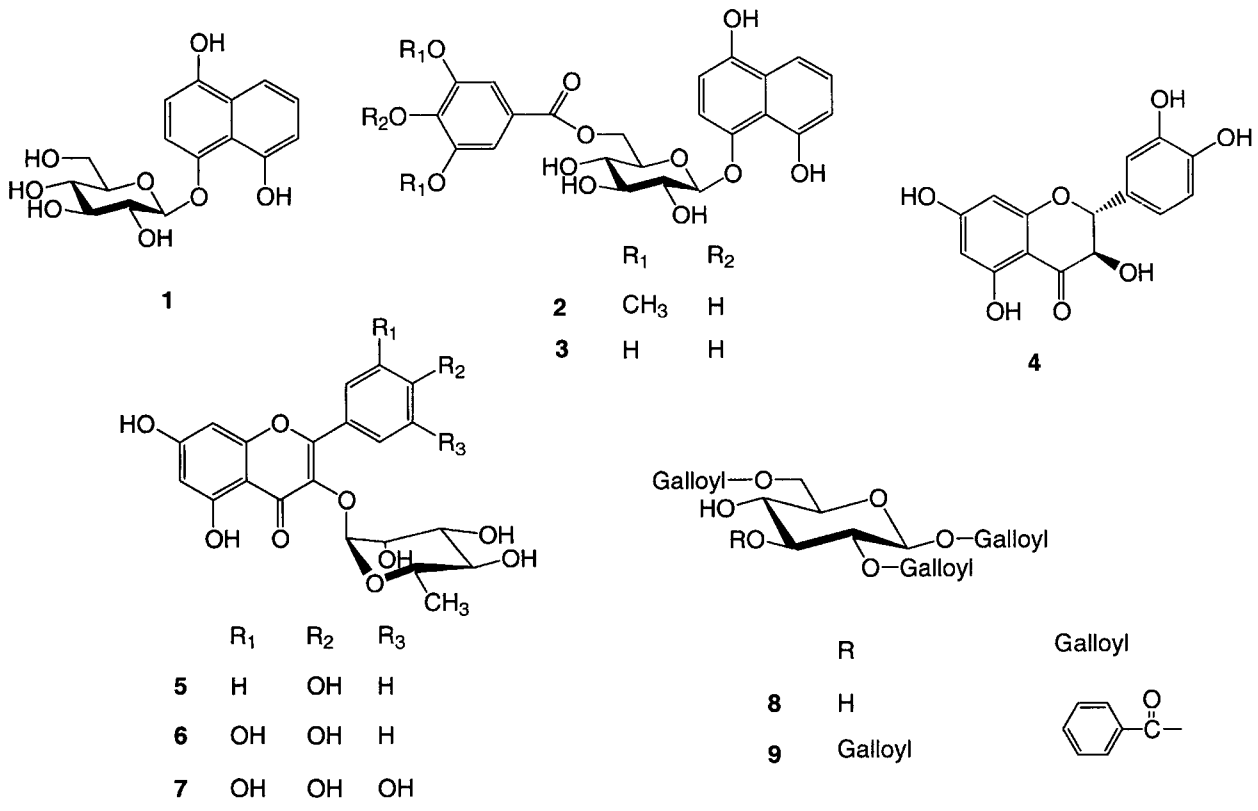

Fig. 1. Structures of isolated of compounds

MATERIALS AND METHODS

General

Optical rotations were measured with a DIP-360 automatic polarimeter (JASCO). UV spectra were measured with a UV-2200 UV-VIS recording spectrophotometer (Shimadzu). IR spectra were measured with a FT/IR-230 infrared spectrometer (JASCO). ^{1}H- and ^{13}C-NMR spectra were measured with JNA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; JEOL) spectrophotometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. HR-EIMS were measured with a JMX-AX 505 HAD mass spectrophotometer (JEOL) using glycerol as a matrix. Column chromatography was carried out on silica-gel (Kieselgel 60, 70-230 mesh, Merck), Sephadex LH-20 (Pharmacia), and ODS (Chromatorex, 100-200 mesh, Fuji Silysia). MPLC was carried out on LiChroprep RP-18 (size A, Merck). Thin layer chromatography (TLC) was carried out on pre-coated Silica-gel 60 F_{254} plates (0.25 mm, Merck) and RP-18 F_{254S} (0.25 mm, Merck), and spots were detected under a UV light and by spraying 10% H_2SO_4 followed by heating.

Plant Material

The stem-bark of *J. mandshurica* was collected during September 1998 at a mountain area of Kimchun, Kyungbook, Korea, and dried at room temperature for 3 weeks. A voucher specimen is deposited at the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Isolation Procedure

Stem-bark of *J. mandshurica* (3.0 kg) was extracted with MeOH at room temperature for 24h to give a dark-brown extract (390 g). The MeOH extract (300 g) was suspended in H_2O (2500 ml) and extracted with hexane (2500 × 3) to give a hexane-soluble fraction (48 g). The resulting H_2O layer was extracted with CH_2Cl_2 (3000 ml × 3), EtOAc (3000 ml × 3), and BuOH (3000 ml × 3), successively. The EtOAc-soluble fraction (90 g) was chromatographed on a column of silica gel (1 kg). The column was eluted using a gradient of $CHCl_3$, MeOH, and H_2O to give 6 fractions (Fr. A-F: 2.7 g, 15.9 g, 23.9 g, 7.5 g, 5.4 g, and 10.2 g, respectively). Repeated column chromatography of Fr. B on silica gel ($CHCl_3$-MeOH, 9 : 1), Sephadex LH-20 ($CHCl_3$-MeOH, 1 : 9), and ODS column (50% aqueous MeOH), followed by MPLC on RP-18 (50% aq. MeOH and 70% aq. CH_3CN) afforded compounds **2**, **4**, and **5**. Repeated column chromatography of Fr. C on Sephadex LH-20 (MeOH and $CHCl_3$-MeOH, 1 : 9), silica gel ($CHCl_3$-MeOH, 8 : 2), and ODS column (40% aq. MeOH),

followed by MPLC on RP-18 (40% aq. MeOH and 80% aq. CH_3CN) furnished compounds **1**, **3**, **6**, **7**, **8**, and **9**.

1,4,8-Trihydroxynaphthalene 1-*O*-β-D-glucopyranoside (1)

Brown amorphous powder, $[\alpha]_D$ -85° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1615, 1520, 1405, 1375, 1260, 1070. UV λ_{max} nm (log ε): 224 (4.6), 306 (4.2), 326 (4.1), 342 (4.1). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

1,4,8-Trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(4″-hydroxy-3″,5″-dimethyoxybenzoyl)]glucopyranoside (2)

Light brown amorphous powder, $[\alpha]_D$ -49° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1680, 1610, 1520, 1460, 1420, 1340, 1215, 1120. UV λ_{max} nm (log ε): 224 (4.8), 288 (4.1), 326 (4.1), 342 (4.1). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

1,4,8-Trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(3″,4″,5″-trihydroxybenzoyl)] glucopyranoside (3)

Brown needles (MeOH-H_2O), $[\alpha]_D$ -53° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1690, 1610, 1520, 1445, 1350. UV λ_{max} nm (log ε): 224 (4.8), 282 (4.1), 326 (4.1), 342 (3.9). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

Taxifoline (5,7,3′,4′-tetrahyrdoxyflavanol, 4)

White amorphous powder, $[\alpha]_D$ +20° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3420, 1620, 1520, 1470, 1360, 1265, 1165. UV λ_{max} nm (log *e*): 288 (4.2), 328 (sh). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

Afzelin (kaempferol 3-*O*-α-L-rhamnopyranoside, 5)

Yellow amorphous powder, $[\alpha]_D$ -184° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3280, 1655, 1615, 1500, 1450, 1365. UV λ_{max} nm (log ε): 264 (4.3), 342 (4.1). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

Quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside, 6)

Yellow amorphous powder, $[\alpha]_D$ -178° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3320, 1660, 1610, 1500, 1450, 1360. UV λ_{max} nm (log *e*): 254 (4.2), 314 (sh), 350 (4.1). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

Myricitrin (myricetin 3-*O*-α-L-rhamnopyranoside, 7)

Yellow amorphous powder, $[\alpha]_D$ -141° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3270, 1655, 1610, 1500, 1455, 1340. UV λ_{max} nm (log *e*): 254 (4.2), 312 (sh), 354 (4.1). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

1,2,6-Trigalloylglucopyranose (8)

White amorphous powder, $[\alpha]_D$ -94° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3420, 1710, 1610, 1540, 1525, 1450, 1355. UV λ_{max} nm (log ε): 216 (4.6), 278 (4.4). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

1,2,3,6-Tetragalloylglucopyranose (9)

White amorphous powder, $[\alpha]_D$ +39° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1700, 1610, 1540, 1455, 1355. UV λ_{max} nm (log ε): 216 (4.9), 278 (4.5). ^1H- and ^{13}C-NMR data: see reference (Min *et al.*, 2000).

Cells

The HTLV-1-carrying cell line MT-4 cells were used. They were maintained at 37°C under 5% CO_2 in RPMI-1640 medium (Flow Laboratories), supplemented with 10% fetal calf serum (FCS, Flow Laboratories), 100 μg/ml of streptomycin (Meiji Seika) and 100 U/ml of penicillin G (Banyu Pharmaceutical).

Virus

HIV-1 (strain HTLV-III^B) was obtained from the supernatant of MOLT-4/HTLV-III^B cells that had been persistently infected with LAV-1.

Assay of cytopathic effect (CPE) of HIV-1 on MT-4 cells

MT-4 cells were infected for 1h with HIV-1 (HTLV-IIIB) at 50%-tissue culture infective dose ($TCID_{50}$) of 0.001/cell. Then, the cells were resuspended at 1×10^5 cells/ml in RPMI-1640 medium and 200 μl/well of the cell suspension was cultured for 5 days in a 96-well culture plate containing various concentrations (12 doses, maximum 1860-850 μg/ml and minimum 0.89-0.42 μg/ml) of the tested compounds. Control assays were performed in the absence of test compound with HIV-1 infected and uninfected cultures. On day 5, the inhibitory concentration (IC) of the test sample required to prevent HIV-1-induced cytopathic effect completely was examined through an optical microscope and the cell growth was visualized to give a cytotoxic concentration (CC) that reduces the viability of MT-4 cells (Harada *et al.*, 1985).

RESULTS AND DISCUSSION

We have evaluated anti-HIV effects of natural sources from China, Indonesia, Panama, and Sri Lanka to research HIV-1 agents. Some plants such as *Swietenia*

Table I. Inhibition of HIV-1-induced CPE by compounds 1-9

Compound	Anti-HIV-1 (μg/ml)	
	IC_{100}	CC_{100}
1,4,8-Trihydroxynaphthalene 1-O-β-D-glucopyranoside (**1**)	>100	25
1,4,8-Trihydroxynaphthalene 1-O-β-D-[6′-O-(4″-hydroxy-3″,5″-dimethoxybenzoyl)]glucopyranoside (**2**)	>100	>100
1,4,8-Trihydroxynaphthalene 1-O-β-D-[6′-O-(3″,4″,5″-trihydroxybenzoyl)]glucopyranoside (**3**)	>100	25
Taxifolin (**4**)	25	>100
Afzelin (**5**)	>100	>100
Quercitrin (**6**)	>100	>100
Myricitrin (**7**)	>100	>100
1,2,6-Trigalloylglucopyranose (**8**)	>100	>100
1,2,3,6-Tetragalloylglucopyranose (**9**)	>100	25
DS 8000[a)]	3.9	>1000

[a]Dextran sulfate (prepared from average M_r 8000; Moriya *et al.*, 1993)

mahagoni L. and *Croton tiglium* L. inhibited the replication of HIV-1. Of these, 12-*O*-acetylphorbol-13-decanoate and 12-*O*-decanoylphorbol-13-(2-methylbutyrate) isolated form *C. tiglium* showed potent cytopathic effect of HIV-1 with IC_{100} values of 7.6 ng/ml and 7.81 μg/ml, respectively, without activation of protein kinase C (PKC) associated with tumorpromoting action at concentrations of 10 and 100 ng/ml (El-Mekkawy *et al.*, 2000).

In previous study, we isolated 1,4,8-trihydroxynaphthalene 1-*O*-β-D-glucopyranoside (**1**), 1,4,8-trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(4″-hydroxy-3″,5″-dimethoxybenzoyl)] glucopyranoside (**2**), 1,4,8-trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(3″,4″,5″-trihydroxybenzoyl)]glucopyranoside (**3**), taxifolin (**4**), afzelin (**5**), quercitrin (**6**), myricitirin (**7**), 1,2,6-trigalloylglucopyranose (**8**), and 1,2,3,6-tetragalloylglucopyranose (**9**), from the stem-bark of *J. mandshurica* (Min *et al.*, 2000). Three naphthalene glycosides (**1-3**), four flavonoids (**4-7**), and two galloyl glucosides (**8-9**) tested for their ability to inhibit the replication of HIV-1 on MT-4 cells, a human CD_4-posotive cell line caring HTLV-1, and the results presented in Table 1. Of compounds tested, taxifolin (**4**) had the most potent complete inhibition of CPE at concentration (IC_{100} value) of 25 μg/ml, with maximum cytotoxic concentration (CC_{100}) of 100 μg/ml. While compounds (**1-3**, **5-9**) showed no anti-HIV-1 activity against MT-4 cells. Compounds 1, 3, and 9 exhibited cytotoxic activity with CC_{100} value of 25 μg/ml, respectively.

In previous study, 1,2,6-trigalloylglucopyranose (**8**) and 1,2,3,6-tetragalloylglucopyranose (**9**) showed the inhibitory activity of RNA-dependent DNA polymerase activity of HIV-1 reverse transcriptase at 50% inhibitory concentration (IC_{50}) of 67 and 40 nM, together with the inhibition of RNase H activity, which is catalyzed hydrolysis of the RNA component of the hydride leaving small RNA primers for the subsequent synthesis of complementary plus DNA strand by the DNA-dependent DNA polymerase (Loya & Hizi, 1993), with IC_{50} values of 310 and 39 μM, respectively (Min *et al.*, 2000). Four flavonoids, compounds **4-7**, were not observed any inhibition of RNA-dependent DNA polymerase and RNase H activities of HIV-1 reverse transcriptase with IC_{50} values of above 500 μM, respectively. From these results, compounds **8-9** had potent inhibitory effect on DNA polymerase and RNase H activities of HIV-1 reverse transcriptase without anti-HIV activity. Taxifolin (**4**) was demonstrated the inhibitory activity of HIV-1 replication, while no active for HIV-1 essential enzyme. The action mechanism of taxifolin in HIV-induced cytopathic activity will be of particular interest to be investigated in future.

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