Studies on Constituents with Cytotoxic Activity from the Stem Bark of *Syringa velutina*

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Received February 1, 1999; accepted April 24, 1999

Cytotoxic compounds, oleuropein (1) and a phenylethanoid glycoside (2) were isolated from the stem bark of *Syringa velutina* KOM. along with coniferylaldehyde 4-*O*-glucoside, syringin, ligstroside, (+)-syringaresinol 4-*O*-glucoside, (+)-medioresinol 4″-*O*-glucoside and (−)-olivil 4″-*O*-glucoside. Phenylethanoid glycoside (2) was identified to be 3,4-dihydroxyphenylethyl alcohol 8-*O*-β-D-glucopyranoside. This compound showed the most potent cytotoxic effect on several tumor cell lines (P-388, L-1210, SNU-5 and HL-60) among eight compounds isolated in the present study. We suggest that the 3,4-dihydroxyphenylethoxy moiety of this compound contributes to cytotoxicity.

Key words *Syringa velutina*; phenylethanoid glycoside; oleuropein; 3,4-dihydroxyphenylethyl alcohol 8-*O*-β-D-glucopyranoside; tumor cell; cytotoxicity

In Korea, the stem bark of *Syringa velutina* (Oleaceae) has been used for the treatment of tooth pain, intestinal disorders and diarrhea.[1)] The wild plants of the Syringa species in Korea include *Syringa reticulata* (Bl.) HARA var. *mandshurica* (MAXIM.) HARA, *S. wolfi* SCHNEID., *S. dilatata* NAKAI and *S. velutina* KOM.[2)] *S. vulgaris* L., one of the Western Syringa species, is commonly refferred to as "lilac". Earlier investigations of this species led to the isolation of syringopicroside, syringoxide and syringenone from the leaves, acteoside and neoacteoside from the flowers, and syringin from the leaves, green fruits and bark. In addition, syringalactone A and B, which are secoiridoid glycosides, have been isolated from this species.[3)]

In our continuous search for natural antitumor agents, we tried to isolate constituents from the stem bark of *S. velutina*, and performed an *in vitro* cytotoxicity test of isolated compounds against several tumor cell lines. In this paper, the relationship between the chemical structures of these products and their cytotoxic activities is discussed.

Experimental

Plant Material The stem bark of *Syringa velutina* was collected in May 1997 on Kariwang Mountain, Kangwon Province, Korea. It was identified by Prof. G. T. Kim (Dept. of Forestry, Sangji University, Wonju, Korea), and a voucher specimen is deposited in the Herbarium of Life Science and Natural Resources, Sangji University, Wonju, Korea.

General Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. IR and UV spectra were recorded on Hitachi 260-01 (in KBr disks) and Shimadzu UV-2200 UV-VIS spectrophotometers. EI-MS (ionization voltage, 70 eV) and FAB-MS were measured with a JEOL JMS DX-300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with TMS as an internal standard.

Cells P-388 cells (mouse lymphoma), L-1210 (mouse lymphocytic leukemia), SNU-5 cells (human stomach cancer), and HL-60 cells (human promyelocytic leukemia) were obtained from the Korean Cell Line Bank.

Chemicals RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and adriamycin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Extraction, Fractionation and Isolation The air-dried stem bark (7.2 kg) of *S. velutina* was finely cut and extracted three times with hot MeOH under reflux. The MeOH extract was then filtered and evaporated on a rotary evaporator under reduced pressure to obtain a viscous mass (350 g) of MeOH extract. This material was suspended with H_2O and partitioned with $CHCl_3$, EtOAc and *n*-BuOH to give a $CHCl_3$-soluble fraction (115 g), an EtOAc-soluble fraction (95 g) and an *n*-BuOH-soluble fraction (75 g). A part of the EtOAc-soluble fraction (20 g) was subjected to column chromatography on silica gel (Merck, Art. No. 7734, Germany). The column was eluted with $CHCl_3$–MeOH–H_2O (7 : 3 : 1), and five fractions were collected. Repeated preparative TLCs on fractions 1 and 2 afforded (+)-syringaresinol 4-*O*-glucoside and (+)-medioresinol 4″-*O*-glucoside, respectively. Fraction 3 was subjected to Sephadex LH-20 chromatography to give coniferylaldehyde 4-*O*-glucoside. Recrystallization of fractions 4 and 5 with acetone yielded ligstroside and oleuropein (**1**). Recrystallization of fraction 6 with MeOH yielded syringin. The *n*-BuOH-soluble fraction was also subjected to column chromatography to afford fractions 7 and 8. Recrystallization of fractions 7 and 8 yielded (−)-olivil 4″-*O*-glucoside and a phenylethanoid glycoside (**2**), respectively.

Compound **1**: Amorphous powder, $[\alpha]_D$ −128.4° (c=0.61 in EtOH). IR ν_{max} (KBr) cm^{-1}: 3400 (broad, OH), 1700 (C=O), 1620 (C=C), 1590, 1510, (aromatic C=C). ^{1}H-NMR (300 MHz, CD_3OD) δ: 5.91 (1H, s, H-1), 7.52 (1H, s, H-3), 3.96 (1H, dd, J=9.2, 4.5 Hz, H-5), 2.43 (1H, dd, J=14.2, 9.2 Hz, Ha-6), 2.71 (1H, dd, J=14.2, 4.5 Hz, Hb-6), 6.08 (1H, q, J=6.9 Hz, H-8), 1.65 (3H, d, J=6.9 Hz, H-10), 3.72 (3H, s, OMe), 4.85 (1H, d, J=7.8 Hz, anomeric proton of D-glucose), 6.70 (1H, d, J=1.5 Hz, H-2″), 6.73 (1H, d, J=8.0 Hz, H-5″), 6.57 (1H, d, J=8.0, 1.5 Hz, H-6″), 4.11 (1H, dt, J=10.7, 7.0 Hz, Ha-α), 4.22 (1H, dt, J=10.7, 7.0 Hz, Hb-α), 2.77 (2H, t, J=7.0 Hz, H-β). ^{13}C-NMR (75.5 MHz, CD_3OD) δ: 95.6 (C-1), 155.6 (C-3), 109.6 (C-4), 31.2 (C-5), 41.7 (C-6), 173.7 (C-7), 125.4 (C-8), 130.6 (C-9), 14.0 (C-10), 169.1 (C-11), 52.7 ($COO\underline{C}H_3$), 101.2 (C-1′), 75.0 (C-2′), 78.1 (C-3′), 71.7 (C-4′), 78.6 (C-5′), 63.0 (C-6′), 131.2 (C-1″), 117.5 (C-2″), 146.5 (C-3″), 145.6 (C-4″), 116.9 (C-5″), 121.8 (C-6″), 35.7 (C-α), 67.3 (C-β).

Compound **2**: Pale yellowish powder (MeOH), $[\alpha]_D$ −23.8° (c =1.00 in MeOH). IR ν_{max} (KBr): 3400 (broad, OH), 1598, 1517 (aromatic C=C), 1274 (C–O), 1092 (glycoside). ^{1}H-NMR (300 MHz, CD_3OD) δ: 2.81 (1H, dd, J=7.3, 14.7 Hz, Hb-7), 3.71 (1H, dd, J=7.3, 14.7 Hz, Ha-7), 4.07 (2H, t, J=7.3 Hz, H-8), 4.33 (1H, d, J=7.7 Hz, H-1 of D-glucose), 6.72 (1H, d, J=8.4 Hz, H-5), 6.64 (1H, dd, J=2.1, 8.4 Hz, H-6), 6.73 (1H, d, J=2.1 Hz, H-2). ^{13}C-NMR (300 MHz, CD_3OD): 37.0 (C-7), 63.2 (C-6′), 72.0 (C-8), 72.1 (C-4′), 75.6 (C-2′), 78.3 (C-3′), 78.5 (C-5′), 104.8 (C-1′), 116.8 (C-2), 117.6 (C-5), 121.8 (C-6), 132.0 (C-1), 145.0 (C-4), 146.5 (C-3). FAB-MS m/z: 317 $[M+H]^+$.

Alkaline hydrolysis of **1**: Compound **1** (120 mg) was hydrolyzed in 2% KOH in MeOH–H_2O (1 : 1) under reflux for 1 h. The pH of the reaction mixture was adjusted to pH 11 with NaOH solution and further partitioned with EtOAc. The EtOAc fraction was washed with H_2O and dried *in vacuo*. The

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residue was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (7 : 3 : 1) to give **1a** and **1b**.

1a: Amorphous power (MeOH), IR ν_{max} (KBr): 3397 (broad, OH), 1726 (ester), 1025 (glycoside). ^{1}H-NMR (300 MHz, CD_3OD) δ: 1.77 (3H, d, J=7.0 Hz, H-10), 2.30 (1H, dd, J=9.4, 13.5 Hz, Ha-6), 2.66 (1H, dd J= 4.2, 13.5 Hz, Hb-6), 3.72 (3H, s, $COOCH_3$), 4.80 (1H, d, J=7.7 Hz, anomeric proton of D-glc), 4.02 (1H, dd, J=4.2, 9.4 Hz, H-5), 5.96 (1H, s, H-1), 6.08 (1H, q, J=7.0 Hz, H-8), 7.49 (1H, s, H-3). ^{13}C-NMR (75.5 MHz, CD_3OD) δ: 14.2 (C-10), 32.9 (C-5), 43.6 (C-6), 52.7 ($COO\underline{C}H_3$), 63.2 (C-6′), 72.0 (C-4′), 75.3 (C-2′), 78.4 (C-3′), 78.9 (C-5′), 96.0 (C-1) 101.4 (C-1′), 110.8 (C-4), 124.8 (C-9), 131.4 (C-8), 155.3 (C-3), 169.4 (C-11), 177.5 (C-7). FAB-MS m/z: 404.3 $[C_{17}H_{24}O_{11}]^+$.

1b: Syrupy (MeOH), IR ν_{max} (KBr): 3388 (broad, OH), 1703 (COOH). ^{1}H-NMR (200 MHz, CD_3OD) δ: 2.05 (3H, d, J=7.0 Hz, H-10), 2.55—2.62 (2H, m, H-6), 4.26 (1H, dd, J=4.3, 7.2 Hz, H-5), 6.78 (1H, q, J=7.0 Hz, H-9), 9.30 (1H, s, H-3). ^{13}C-NMR (50 MHz, CD_3OD) δ: 15.3 (C-10), 31.3 (C-5), 39.1 (C-6), 91.9 (C-1), 98.3 (C-4), 125.5 (C-9), 145.8 (C-8), 155.4 (C-3), 177.5 (C-7), 197.3 (C-11). MS m/z: 228.3 $[C_{10}H_{12}O_6]^+$.

Cytotoxicity Assay The *in vitro* tests against P-388, L-1210, SNU-5 and HL-60 cells were performed essentially according to the method described previously.[4] Cells (1×10^4) were seeded in each well containing 100 μl of RPMI medium supplemented with 10% FBS in a 96-well microtiter plate and incubated overnight. All the test samples were dissolved in dimethylsulfoxide (DMSO) and were added in serial dilution (the final DMSO concentrations in all assays did not exceed 0.01%). Twenty-four hours after seeding, 100 μl new media or test compounds were added, and the plates were incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue which formed in the cells was replaced by adding 50 μl DMSO. Optical density was measured at 540 nm. Adriamycin was used as a positive control.

Results and Discussion

In the present study, we isolated a phenylethanoid glycoside (**2**) along with three lignan glycosides, two secoiridoid glycosides and two phenylpropanoid glycosides from *S. velutina*. Among these constituents, syringin,[5,6] coniferylaldehyde 4-*O*-glucoside,[7] ligstroside,[8] (+)-syringaresinol 4-*O*-glucoside,[9] (+)-medioresinol 4″-*O*-glucoside[10] and (−)-olivil 4″-*O*-glucoside[11] were identified by the interpretation of 2D-NMR spectral data and by comparison of the data in literature. The structure of another secoiridoid glycoside (**1**) was intensively studied in ^{1}H–^{1}H correlation spectrometry (COSY)-, ^{1}H–^{13}C COSY-, heteronuclear multiple quantum coherence (HMBC) and nuclear overhauser effect and exchange spectroscopy (NOESY) NMR, spectra as shown in Fig. 1, and the NMR assignment was in good agreement with oleuropein reported by previous researchers.[12,13] When this structure was compared with that of lilacoside isolated from *S. vulgaris*, the δ-lactone ring of lilacoside was opened in **1**. Furthermore, oleuropein also differs from lilacoside with respect to the linkage position of the 3,4-dihydroxyphenylethoxy moiety. Syringalactone B is the strong bitter principle of *S. vulgaris*, while oleuropein showed no bitterness in this study. This fact suggests that the δ-lactone ring of secoiridoid glycosides is an important part of that molecule's bitterness. In addition, since this compound showed moderate to significant cytotoxic activity (Table 1), **1** was hydrolyzed in an alkaline solution in order to examine which part of the molecule is responsible for cytotoxic effect. Compounds **1a** and **1b** were obtained by repeated chromatography of all the hydrolysates shown on TLC. The interpretation of ^{1}H- and ^{13}C-NMR spectra revealed that **1a** was produced as a result of hydrolysis of the 3,4-dihydroxyphenylethyl moiety of **1**. In contrast, **1b** was a remaining product of **1** in which the methyl group and D-glucose of **1**, as well as the aromatic moiety, were hydrolyzed away (Fig. 2). Compared

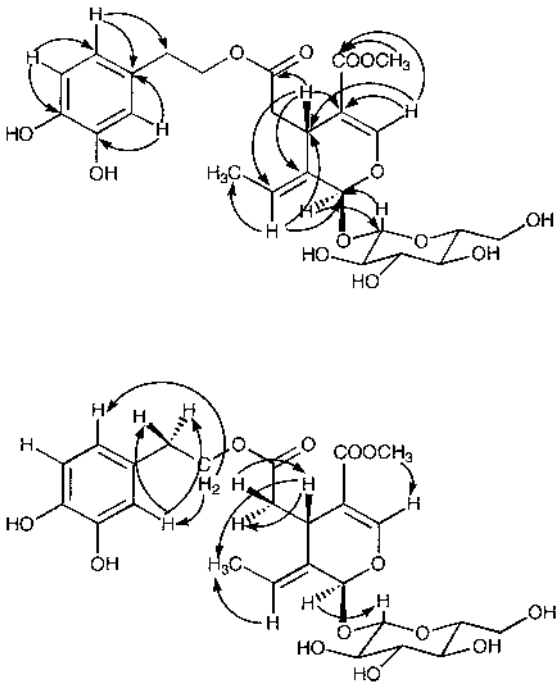

Fig. 1. HMBC (Above) and NOESY (Below) Correlations of Oleuropein (**1**)

Table 1. Cytotoxic Activities of Compounds from the Stem Bark of *Syringa velutina* on Tumor Cell Growth

Compound	IC_{50}[a] (μg/ml)			
	P-388	L-1210	SNU-5	HL-60
Oleuropein (**1**)	75.5	46.5	13.4	22.5
1a	>1000	>1000	>1000	>1000
3,4-Dihydroxyphenylethyl alcohol 8-β-D-glucopyranoside (**2**)	2.7	2.7	47.9	8.0
Ligstroside	>1000	>1000	>1000	>1000
(+)-Syringaresinol 4-glc	>1000	>1000	>1000	>1000
(+)-Medioresinol 4″-glc	>1000	>1000	>1000	>1000
(−)-Olivil 4″-glc	>1000	>1000	>1000	>1000
Coniferylaldehyde 4-glc	>1000	>1000	>1000	>1000
Syringin	>1000	>1000	>1000	>1000
Adriamycin	4.2×10^{-2}	7.1×10^{-2}	2.3×10^{-2}	9.1×10^{-2}

a) IC_{50} is defined as the concentration which resulted in a 50% decrease in cell number. The values represent the mean of three independent experiments.

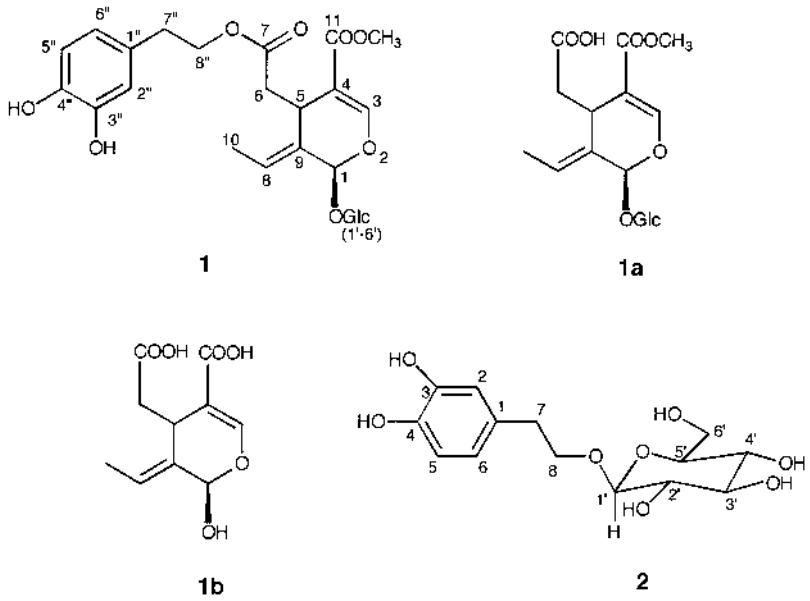

Fig. 2. Structures of Oleuropein (**1**), Its Alkaline Hydrolysates (**1a** and **1b**) and 3,4-Dihydroxyphenylethyl Alcohol 8-*O*-*β*-D-Glucopyranoside (**2**)

with the signals of **1a**, the signal of H-3 (δ_H 9.30) and C-11 (δ_C 197.3) of **1b** in NMR spectra were shown to be extremely deshielded, probably due to the partial structure of α,β-unsaturated carboxylic acid.

From overall consideration of the NMR spectra of **2**, the structure was found to be 3,4-dihydroxyphenylethyl alcohol 8-*O*-*β*-D-glucopyranoside (Fig. 2), which had been reported by Shimomura *et al.*[14] Very simple phenylethanoid glycosides such as 2-hydroxyphenylethyl alcohol 8-*O*-*β*-D-glucopyranoside,[15] 4-methoxyphenylethyl alcohol 8-*O*-*β*-D-glucopyranoside[16] and 3,4-dihydroxyphenylethyl alcohol 4-*O*-*β*-D-glucopyranoside[17] have been reported. It has been known that phenylethanoid glycosides show antioxidative activities, and their effect is related to the phenolic functional ligand.[18,19]

Next, we tested the cytotoxicities of all isolated compounds, together with **1a** on several tumor cell lines such as P-388, L-1210, SNU-5 and HL-60 (Table 1). Oleuropein (**1**) and 3,4-dihydroxyphenylethyl alcohol 8-*O*-*β*-D-glucopyranoside (**2**) exhibited significant activities, and **2** was more potent than **1**. However, the product (**1a**) in which only 3,4-dihydroxyphenylethyl alcohol was hydrolyzed away from **1** was shown to be inactive. Therefore, these observations suggest that the cytotoxic effect of **1** is caused by the 3,4-dihydroxyphenylethoxy moiety. This finding can be supported by the fact that **2** containing the same aromatic moiety showed potent cytotoxic activity. Verbascoside, a phenylethanoid glycoside, has been reported to show an inhibitory effect on the protein kinase C (PKC) of tumor cell lines,[20] as well as a cell differentiation effect on human leukemia cell lines.[21] Moreover, Herbert *et al.*[20] have demonstrated by pharmacokinetic study that this molecule interacts with the catalytic domain of PKC, and that it is a competitive inhibitor for ATP (K_i= 22 μM) and a non-competitive inhibitor for the phosphate acceptor (histone III). Since the molecule of **2** consists of verbascoside as a partial structure, the cytotoxic effects can be attributed to the 3,4-dihydroxyphenylethoxy moiety which may act as a competitive inhibitor to the catalytic domain of PKC. Therefore, we suggest that **2** is an essential parent skeleton for most cytotoxic phenylethanoid glycosides. The 4-hydroxyphenylethoxy moiety may serve as a parent skeleton for cytotoxic activity because ligstroside exhibited no cytotoxic activity. Thus, it is possible that natural compounds containing a 3,4-dihydroxyphenylethoxy moiety should exhibit this effect by a similar biological mechanism, although structural differences in other parts of these compounds may cause considerable variation in cytotoxic effect.

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