Arch Pharm Res Vol 31, No 8, 978-982, 2008
DOI 10.1007/s12272-001-1255-9

Archives of Pharmacal Research
http://apr.psk.or.kr

Cytotoxic Constituents from the Bark of *Salix hulteni*

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(Received March 13, 2008/Revised May 7, 2008/Accepted July 17, 2008)

Eight compounds were isolated from the bark of *Salix hulteni*. Based on spectral data, the isolated compounds were identified as 4-hydroxyacetophenone (**1**), naringenin (**2**), aromadendrin (**3**), catechin (**4**), picein (**5**), sachaliside 1 (**6**), 1-*p*-coumaroyl-β-D-glucoside (**7**), and dihydromyricetin (**8**). Their cytotoxic activities against brine shrimp and a human lung cancer cell line (H1299) were evaluated.

Key words: *Salix hulteni*, Brine shrimp lethality assay, Cytotoxicity, Flavonoids, Acetophenone, Phenylpropanoid

INTRODUCTION

The brine shrimp lethality test is useful in predicting cytotoxicity (Mansoor *et al*., 2007; Zhao *et al*., 1999; Solis *et al*., 1993; Anderson *et al*., 1991) and for performing bioassay-guided isolation (Meyer *et al*., 1982). In the course of searching for cytotoxic compounds from plant sources, we found that the *n*-BuOH soluble fraction of the bark of *Salix hulteni* showed potent cytotoxicity in the brine shrimp lethality assay. The genus *Salix* contains analgesic, antipyretic, and antiinflammatory agents (Fiebich and Chrubasik, 2004; Biegert *et al*., 2004). *Salix purpurea* blocks growth and apoptotic activity in human colon and lung cancer cells (Hostanska *et al*., 2007). The present study reports the isolation of the bioactive constituents of *Salix hulteni* bark and their cytotoxic activities using brine shrimp lethality and *in vitro* cytotoxicity assays.

MATERIALS AND METHODS

General procedures

Melting point was determined on a Fisher Johns melting point apparatus. The UV/Vis spectrum was measured on a V-530 spectrophotometer (JASCO). Optical rotation was measured on a DIP 1000 Digital Polarimeter (JASCO). IR spectrum was measured on a FTIR – 4200 (JASCO). MS spectrum was measured on a micromass M363 (Autospec). NMR spectrum was recorded on Gemini 200 (Varian) and DPX 400 (Bruker). The chemical shifts are represented as part per million (ppm) referenced to the residual solvent signal. Column chromatography was performed using Kieselgel 60, 400-230 mesh (Merck) and YMC gel ODS-A, 150 μm (YMC). TLC was performed on glass-backed Kieselgel 60 F_{254} and RP F_{254s} plates. HPLC analysis was performed using a Spectra system (Thermo Separation Products).

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Plant material

S. hulteni was collected from Mt. Samak (June, 2005) in Korea. A voucher specimen (KNUH-B-0501) was deposited in the herbarium of the College of Pharmacy, Kangwon National University, Korea.

Extraction and isolation

The dried bark of *S. hulteni* (1.2 kg) was extracted with MeOH at room temperature for a week. The MeOH extract (220 g) was suspended in water and then partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH, successively, leaving a residual water-soluble fraction. Each fraction was evaporated *in vacuo* to yield the residues of the *n*-hexane fraction (fr.) (20 g), $CHCl_3$ fr. (12 g), and *n*-BuOH fr. (70 g). Among the solvent fractions, the *n*-BuOH soluble fraction exhibited significant cytotoxic activity, with an LD_{50} value of 30.5 μg/mL in the brine shrimp lethality assay. To isolate active compounds from *n*-BuOH soluble fraction, column chromatography was performed and the purity of isolated compounds was determined by HPLC using a YMC column (A-303, 250×4.6 mm) with acetonitrile : water (30 : 70).

The *n*-BuOH soluble fraction (60 g) was separated on a silica gel (800 g, 9×60 cm) using an isocratic elution with $CHCl_3$: MeOH : H_2O (3 : 1 : 0.1), to divide the fraction into four fractions (Fr. 1 – Fr. 4). Among these fractions, Fr. 1 and Fr. 2 showed cytotoxic activity in the brine shrimp lethality test (data not shown). Fr. 1 (1.4 g) was re-separated on an ODS column (150 g, φ 5×50 cm) and Sephadex LH20 column (50 g, φ 3×50 cm) by elution with MeOH : H_2O (40 : 60) and MeOH

(100), to give compound **1** [42 mg, retention time (RT): 7.38 min., 98%], **2** (27 mg, RT: 13.18 min, 99%), and **3** (45 mg, RT: 7.21 min., 93%). Fr. 2 (25 g) was re-separated on a silica gel column (250 g, φ 5×50 cm) by elution with $CHCl_3$: MeOH (6 : 1) to yield five subfractions (Fr. 2-1 – Fr. 2-5). Fr. 2-2 (3 g) was re-separated on an ODS column (70 g, φ 3×50 cm) by elution with MeOH : H_2O (40 : 60 and 20 : 80) to give compound **8** (457 mg, RT: 19.9 min., 95%). Fr. 2-4 (5.6 g) was re-separated on an ODS column (150 g, φ 5×50 cm) by elution with MeOH : H_2O (30 : 70) to yield four subfractions (Fr. 2-2-1 – Fr. 2-2-4). Fr. 2-2-2 (2.3 g) was re-separated on a silica gel (150 g, φ 3×50 cm) by elution with benzene : EtOAc : MeOH (3 : 1 : 1) to give compound **4** (442 mg, RT: 6.13 min., 94%) and **5** (895 mg, RT: 5.27 min., 96%). Fr. 2-2-3 (1.2 g) was re-separated on a Sephdex LH20 column (50 g, φ 3×50 cm) by elution with MeOH : H_2O (20 : 80) to give compound **6** (237 mg, RT: 5.81 min., 93%) and **7** (65 mg, RT: 5.95 min., 97%).

4-Hydroxyacetophenone (1)
mp : 108~109 ºC; (+) EI-MS m/z 136 $[M]^+$; UV (MeOH) λ_{max} 237 nm; IR (KBr) ν_{max} 3306 (OH), 1663 (C=O), 1600, 1570, 1508 (C=C), 1280, 1212, 1163 (C-O); ^{1}H-NMR (DMSO-d_6, 200 MHz) δ : 2.53 (3H, s, COC<u>H</u>$_3$), 6.97 (2H, d, J=7.8 Hz, H-2, H-6), 7.93 (2H, d, J=7.8 Hz, H-3, H-5); ^{13}C-NMR (DMSO-d_6, 50 MHz) δ : 24.65 (CO<u>C</u>H$_3$), 114.44 (C-3, C-5), 128.84 (C-1), 133.05 (C-2, C-6), 161.23 (C-4), 195.23 (<u>C</u>OCH$_3$).

Naringenin (2)
mp : 250~251 ºC; $[\alpha]_D^{21}$ -17.5 (c=0.12, MeOH); (+) EI-MS m/z 272 $[M]^+$; UV (MeOH) λ_{max} 248, 293 nm; IR (KBr) ν_{max} 3112 (OH), 1600 (C=O), 1517, 1493, 1459 (C=C), 1246, 1178, 1154 (C-O); ^{1}H-NMR (DMSO-d_6, 200 MHz) δ : 2.73 (1H, dd, J=2.4, 16.0 Hz, H-3a), 3.19 (1H, dd, J=13.2, 16.0 Hz, H-3b), 5.45 (1H, dd, J=2.4, 13.2Hz, H-2), 5.98 (2H, s, H-6, H-8), 6.91 (2H, d, J=8.0 Hz, H-3', H-5'), 7.40 (2H, d, J=8.0 Hz, H-2', H-6'), 12.20 (1H, s, OH-5); ^{13}C-NMR (DMSO-d_6, 50 MHz) δ : 41.85 (C-3), 78.36 (C-2), 94.31 (C-8), 95.27 (C-6), 101.65 (C-10), 114.66 (C-3', C-5'), 127.52 (C-2', C-6'), 129.25 (C-1'), 157.20 (C-4'), 162.91 (C-9), 163.83 (C-5), 165.84 (C-7), 195.83 (C-4).

Aromadendrin (3)
mp : 247~248 ºC; $[\alpha]_D^{22}$ + 38.6 (c=0.145, MeOH); (+) EI-MS m/z 288 $[M]^+$; UV (MeOH) λ_{max} 251, 295 nm; IR (KBr) ν_{max} 3171 (OH), 1634 (C=O), 1597, 1517, 1469 (C=C), 1231, 1163, 1130 (C-O); ^{1}H-NMR (Acetone-d_6, 200 MHz) δ : 4.67 (1H, d, J=11.4 Hz, H-3), 5.11 (1H, d, J=11.4 Hz, H-2), 5.97 (1H, s, H-6), 6.01 (1H, s, H-8), 6.91 (2H, d, J=8.4 Hz, H-3, H-5'), 7.44 (2H, d, J=8.4 Hz, H-2', H-6'); ^{13}C-NMR (Acetone-d_6, 50 MHz) δ : 71.39 (C-3), 82.66 (C-2), 94.36 (C-8), 95.38 (C-6), 114.24 (C-3', C-5'), 127.47 (C-1'), 128.67 (C-2', C-6'), 157.13 (C-4'), 162.57 (C-9, C-5), 166.20 (C-7), 196.71 (C-4).

(+)-Catechin (4)
mp : 175~177 ºC; $[\alpha]_D^{26}$ -18.8 (c=0.5, MeOH); EI-MS *m/z* : 290 $[M]^+$; UV (MeOH) λ_{max} 281, 372 nm; IR (KBr) ν_{max} 3321 (OH), 1455 (C=C), 1257, 1080 (C-O); ^{1}H-NMR (MeOH-d_4, 200 MHz) δ : 2.50 (1H, dd, J=8.2, 16.2 Hz, H-4b), 2.85 (1H, dd, J=5.2, 16.2 Hz, H-4a), 3.98 (1H, m, H-3), 4.56 (1H, d, J=7.4 Hz, H-2), 5.85 (1H, d, J=2.4 Hz, H-6), 5.93 (1H, d, J=2.4 Hz, H-8), 6.75 (3H, m, H-2', H-5', H-6'); ^{13}C-NMR (MeOH-d_4, 50 MHz) δ : 28.90 (C-4), 69.22 (C-3), 83.26 (C-2), 95.51 (C-8), 96.69 (C-6), 101.21 (C-10), 115.67 (C-2'), 116.50 (C-5'), 120.47 (C-6'), 132.64 (C-1'), 146.69 (C-3', C-4'), 157.36 (C-7), 158.04 (C-5), 158.29 (C-9).

Picein (5)
mp : 198~199 ºC; (+)FAB-MS m/z : 299 $[M+H]^+$; UV (MeOH) λ_{max} 224 nm; IR (KBr) ν_{max} 3350 (OH), 1658 (C=O), 1600, 1575, 1508 (C=C), 1246, 1183, 1081 (C-O) cm^{-1}; ^{1}H-NMR (DMSO-d_6, 200 MHz) δ : 2.53 (3H, s, COC<u>H</u>$_3$), 5.02 (1H, d, J=7.6 Hz, anomeric H), 7.13 (2H, d, J=8.4 Hz, H-3, H-5), 7.92 (2H, d, J=8.4 Hz, H-2, H-6); ^{13}C-NMR (DMSO-d_6, 50 MHz) δ : 27.12 (CO<u>C</u>H$_3$), 61.20 (C-6'), 70.24 (C-4'), 73.80 (C-2'), 77.16 (C-5'), 77.74 (C-3'), 100.42 (C-1'), 116.55 (C-3, C-5), 131.04 (C-2, C-6), 131.49 (C-1), 161.79 (C-4), 197.24 (<u>C</u>OCH$_3$).

Sachaliside 1 (6)
mp : 176~177 ºC; (+)FAB-MS m/z : 313 $[M + H]^+$; UV (MeOH) λ_{max} 223, 257(sh) nm; IR (KBr) ν_{max} 3253 (OH), 1610, 1590, 1440 (C=C), 1163, 1081, 1032 (C-O) cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ : 4.15 (1H, dd, J=6.5, 13.5 Hz, H-9a), 4.20 (1H, d, J=7.7 Hz, anomeric H), 4.38 (1H, dd, J=5.7, 15.9 Hz, H-9b), 6.12 (1H, dt, J=6.5, 15.9 Hz, H-8), 6.53 (1H, d, J=15.9, H-7), 6.72 (2H, d, J=8.5 Hz, H-2, H-6), 7.26 (2H, d, J=8.5 Hz, H-3, H-5); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ : 61.46 (C-6'), 69.22 (C-9), 70.45 (C-4'), 73.85 (C-2'), 77.10 (C-5'), 77.22 (C-3'), 102.30 (C-1'), 115.77 (C-3, C-5), 122.97 (C-8), 127.86 (C-1), 128.03 (C-2, C-6), 132.03 (C-7), 157.51 (C-4).

1-*p*-Coumaroyl-β-D-glucoside (7)
mp : 225~227 ºC; (+)FAB-MS m/z : 327 $[M + H]^+$; UV (MeOH) λ_{max} 275 nm; IR (KBr) ν_{max} 3313 (OH), 1707 (C=O), 1606, 1582, 1514 (C=C), 1289, 1163, 1073 (C-O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ : 5.46 (1H, d, J=7.9 Hz, anomeric H), 6.39 (1H, d, J=15.9 Hz, H-8), 6.80 (2H, d, J=8.6 Hz, H-3, H-5), 7.57 (2H, d, J=8.6 Hz, H-2, H-6), 7.64 (1H, d, J=15.9 Hz, H-7); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ : 60.93 (C-6'), 69.84 (C-4'), 72.85 (C-2'), 76.81 (C-5'), 78.61 (C-3'), 94.57 (C-1'), 113.86 (C-8), 116.23 (C-3, C-5), 125.26 (C-1), 130.92 (C-2, C-6), 146.42 (C-7), 160.56 (C-4), 165.79 (C-9).

Dihydromyricetin (8)
mp : 245~246 ºC; $[\alpha]_D^{22}$ + 28.2 (c=0.125, MeOH); EI-MS m/z : 320 $[M]^+$; UV (MeOH) λ_{max} 225 (sh), 291 nm; IR (KBr) ν_{max} 3258 (OH), 1629 (C=O), 1605, 1546, 1474 (C=C), 1266, 1163, 1077 (C-O); ^{1}H-NMR (DMSO-d_6, 200 MHz) δ : 4.45 (1H, d, J=11.0 Hz, H-3), 4.92 (1H, d, J=11.0 Hz, H-2), 5.88 (1H, d,

J=1.2 Hz, H-8), 5.92 (1H, d, J=1.2 Hz, H-6), 6.42 (2H, s, H-2', H-6'), 11.90 (1H, s, OH-5); ^{13}C-NMR (DMSO-d_6, 50 MHz) δ : 72.34 (C-3), 83.97 (C-2), 95.72 (C-6), 96.73 (C-8), 101.22 (C-10), 107.71 (C-2', C-6'), 127.91 (C-1'), 134.24 (C-4'), 146.50 (C-3', C-5'), 163.34 (C-5), 164.16 (C-9), 167.62 (C-7), 198.53 (C-4).

Acid hydrolysis of compounds 5-7

Each compound (5 mg) was refluxed with 5% H_2SO_4 (5 mL) in water for 1 h. The reaction mixture was diluted with water and fractionated by EtOAc. Each EtOAc-soluble fraction was concentrated and examined by TLC with authentic samples. Each remaining aqueous layer was adjusted to pH 7 with $NaHCO_3$ and filtered. The filtrate was concentrated and examined by TLC with authentic sugars.

Brine shrimp lethality assay

The assay was performed as described previously (Meyer *et al*., 1982) using brine shrimp nauplii. The eggs (San Francisco Bay Brand, U.S.A.) were placed in brine and hatched within 48 h. Extracts and compounds (3 mg) were dissolved in 3 μL DMSO to prepare a stock solution of 1 mg/mL, with transfer of 300, 30, and 3 mL to vials corresponding to 100, 10, and 1 μg/mL, respectively.

***In vitro* cytotoxicity assay**

Cell culture

Human lung carcinoma cells (H1299) obtained from the American Type Culture Collection (Rockville, U.S.A.) were cultured in DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 20 mM glutamine, 10 U/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37 °C in a humidified atmosphere with 5% CO_2. All experiments were performed on sub-confluent cultures.

Determination of cell viability (MTT)

The MTT assay was performed as previously prescribed (Yoo *et al*., 2002). In brief, H1299 human lung cancer cells were cultured in a 24-well plate (Corning Inc., Corning, NY) at 5×10^4 cells per well. The cells were then treated with 10 μM or 100 μM of each compound or 10 nM of taxol as a positive control. After 24 h incubation with each compound, cells were washed and treated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which yields a blue formazan product in living cells, but not in dead cells or their lytic debris, and then incubated further at 37°C for 2 h. The resultant formazan product was extracted for 4 h with extraction solution (20% SDS, 50% DMF and 2% acetic acid) and detected by a UV-VIS spectrometer (Perkin Elmer Co.) at 570 nm.

Statistics

All values are expressed as mean ± SE from three independent experiments. Data were analyzed using Student's t-test and considered significantly different when the two-tailed *p* value was <0.05.

RESULTS AND DISCUSSION

Repeated silica gel, ODS, and Sephadex LH 20 column chromatography led to the isolation of compounds **1**-**8**. Compounds **1**-**5**, **7**, and **8** were identified by comparison with spectral data from the literature as 4-Hydroxyacetophenone (**1**)

Table I. Brine shrimp lethality of extracts and isolates from *S. hulteni*

	LD_{50} (μg/mg)[a]
MeOH extract	36.8
n-Hexane extract	67.2
$CHCl_3$ extract	46.4
n-BuOH extract	30.5
4-Hydroxyacetophenone (**1**)	73.7
Narigenin (**2**)	52.3
Aromadendrin (**3**)	55.3
(+)-Catechin (**4**)	76.8
Picein (**5**)	>100
Sachialisie 1 (**6**)	>100
1-*p*-Coumaroyl-β-D-glucoside (**7**)	>100
Dihydromyricetin (**8**)	14.1
Camptothecin[b]	2.1

[a] LD_{50} values were calculated by the Finny program using three different concentrations in triplicate experiments.
[b] Used as a positive control.

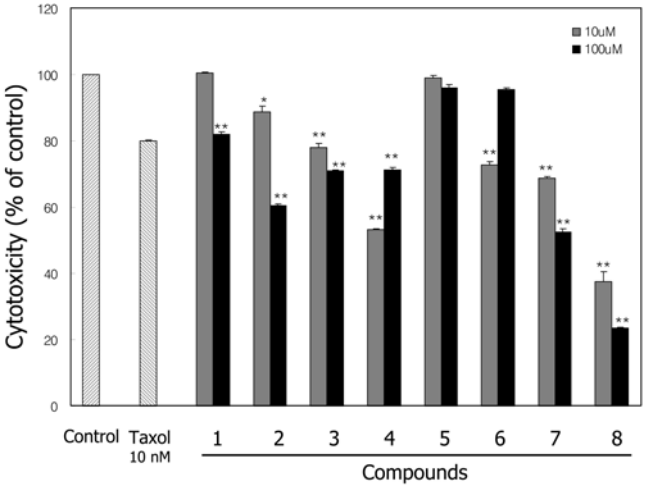

Fig. 1. Cytotoxic effects of compounds in H1299 human lung carcinoma cells. Cell viability was determined by MTT assay. Gray and black bars represent 10 μM and 100 μM of each compound, respectively. Experimental values are calculated as mean ± SE and expressed as percent of control from three independent experiments. *p<0.05, **p<0.01 indicate statistically significant differences from control.

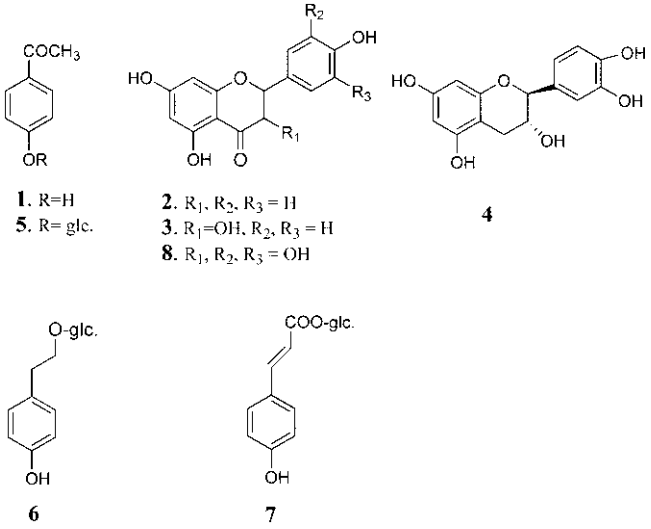

Fig. 2. Structures of **1-8**

(Pouchert and Behnke, 1993; Miyajima *et al*., 1971), naringenin (**2**) (Duddeck *et al*., 1978), aromadendrin (**3**) (Chang *et al*., 2004), (+)-catechin (**4**) (Morimoto *et al.*, 1985), picein (**5**) (Whang *et al*., 1995; Ushiyama and Furuya, 1989), 1-*p*-coumaroyl-β-D-glucoside (**7**) (Kashiwada *et al*., 1984; Zhang *et al*., 2008), and dihydromyricetin (**8**) (Miller and Bohm, 1979). Compound **6** was *trans*-*p*-coumaryl glycoside according to the ^{1}H- and ^{13}C-NMR data. The FAB-MS spectrum of compound **6** showed a quasi-molecular ion at *m/z* 335 $[M+Na]^{+}$. In the ^{1}H-NMR spectrum of compound **6**, signals at δ 4.15 (1H, dd, J=6.5, 13.5 Hz, H-9a), 4.38 (1H, dd, J=5.7, 15.9 Hz, H-9b), 6.12 (1H, dt, J=6.5, 15.9 Hz, H-8), 6.53 (1H, d, J=15.9, H-7), and 6.72/7.26 (each 2H, d, J=8.5 Hz, H-2, H-6/H-3, H-5) suggested the presence of a *trans*-*p*-coumaroyl moiety. The ^{1}H- and ^{13}C-NMR spectrum also showed a sugar anomeric signals at δ_H 4.20 (1H, d, J=7.7 Hz, H-1')/δ_C 102.30 (C-1'). The ^{1}H-^{1}H COSY, DEPT, and HMQC spectral data revealed that the sugar residue of 6 consisted of β-glucopyranose. Acid hydrolysis with 5% H_2SO_4 afforded D-glucose in compound **6**. The HMBC correlation of compound 6 from H-9a/b to C-1' and from H-1' to C-9 confirmed the attachment of a glucose unit to aglycone. On the basis of the above spectral data and literature values (Naidoo *et al*., 1992; Tolonen *et al*., 2003), the structure of compound **6** was identified as sachaliside 1.

Dihydromyricetin (**8**) showed significant brine shrimp lethality, with an LD_{50} value of 14.1 μg/mL (Table I). The cytotoxic activities of compounds **1-8** were evaluated *in vitro* using human lung carcinoma cells (H1299). Taxol (10 nM), an antitumor agent, reduced cell viability by 20% (Fig. 1). Compounds **2**, **3**, **4**, and **7** showed mild cytotoxic activity, with compound **8** showing the greatest inhibition, 62.34% and 76.36%, respectively. Compounds **1**, **5**, and **6** showed no significant cytotoxic activity. These results suggest that flavonoid compounds are responsible for the cytotoxic activity of *S. hulteni*.

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