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Cytotoxic Anthraquinones and Stilbenes from *Reynoutria sachalinensis* (Fr. Schm.) Nakai

WenYi Jin*, MinKyun Na*, GyuYong Song*, Young Mi Lee, and KiHwan Bae*†**

**College of Pharmacy, Chungnam Natl. Univ., Daejeon 305-764, Korea.*
***Dept. of Oriental Pharmacy, College of Pharmacy, Wonkwang Univ., Iksan 540-749, Korea.*

ABSTRACT : Five known anthraquinones, physcion (1), 1-*O*-methylemodin (2), emodin (3), physcion-8-*O*-*β*,-D-glucopyranoside (5), emodin-8-*O*-*β*,-D-glucopyranoside (6) and two known stilbenes, *trans*-resveratrol (4), *trans*-resveratrol-3-*O*-*β*,-D-glucopyranoside (7) were isolated from MeOH extract of *Reynoutria sachalinensis* (Polygonaceae). All structures were unambiguously established by 1D and 2D NMR and MS data and the compounds were evaluated for their cytotoxicity against L1210, HL-60, B16F10 tumor cell lines in MTT assay. Among the compounds, *trans*-resveratrol (4) exhibited significant cytotoxic activity with IC_{50} values of 9.2, 6.7 and 9.8 ㎍/㎖ against the test cell lines respectively, but compounds 1-3 exhibited the moderate cytotoxic activity.

Key words **:** *Reynoutria sachalinensis*, Polygonaceae, anthraquinones, stilbenes, cytotoxicity

INTRODUCTION

Reynoutria sachalinensis (Fr. Schm.) Nakai is a perennial shrub which is distributed in Korea, China, and Japan. The roots of *R. sachalinensis* and *R. japonica* have been used as a source of Reynoutriae Radix-Chinese name of Huzhang (Bae, 1999), for the treatment of arthralgia, jaundice caused by damp-heat, amenorrhea, mass formation in the abdomen, cough with profuse expectoration, scalds, burns, traumatic injuries, carbuncles andscores etc. (Pharmacopia Commission of PRC, 1997).

*R. sachalinensis*has been investigated extensively, resulting in the isolation of anthraquinones (Chi *et al.*, 1983; Umek *et al.*, 1983; Kang *et al.*, 1982b), stilbenes (Chi *et al.*, 1983; 1986), flavonoids (Kang, 1981), flavonoid glycosides (Kang *et al.*, 1982a) and phenolcarboxylic acids (Vechar *et al.*, 1980). Some anthraquinones were also reported to have antidoting activity (Tahara *et al.*, 1993) and stilbenes were reported to have antibacterial and antifungal activities (Chi *et al.*, 1983). As a part of our ongoing study to identify cytotoxic compounds from natural products, the root of *R. sachalinensis*was found to have cytotoxic activity. In this paper, we investigated the cytotoxic constituents of *R. sachalinensis*against murine L1210 leukemia cells, human HL-60 leukemia cells and murine B16F10 melanoma cells.

MATERIALS AND METHODS

Plant material

The root of *R. sachalinensis* was collected in August 2003 at the medicinal plant garden of Chungnam National University and identified by one of the authors (Prof. Bae). Voucher specimen (CNU 482) was deposited at the herbarium in the College of Pharmacy, Chungnam National University.

Instruments and reagents

Melting points were measured by using an Electrothermal melting point apparatus, UV spectra with a Beckman Du-650 UV-VIS recording spectrophotometer and FT-IR spectra with a Jasco Report-100 infrared spectrometer.NMR spectra were measured by using a JEOL 400 or 600 FT-NMR spectrometer (^{1}H, 400, 600 MHz, ^{13}C, 100, 150 MHz) and MS were carried out with a JEOL JMS-HX/HX110A tandem mass spectrometer. Column chromatography was performed by using silica gel (Kieselgel 60, 70~230 mesh and 230~400 mesh, Merck), thin layer chromatography (TLC) on pre-coated silica gel 60 F254 (0.25 ㎜, Merck) and Sephadex LH-20 (Amersham Biosciences).

Extraction and isolation

The root of *R. sachalinensis* (4.5 ㎏) was extracted with meth-

† Corresponding author: (Phone) +82-42-821-5925 (E-mail) beakh@cnu.ac.kr
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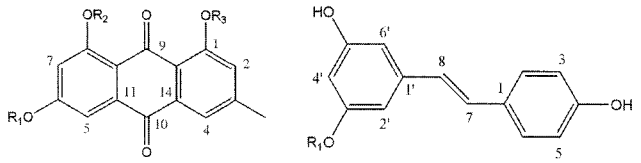

	R_1	R_2	R_3
1	CH_3	H	H
2	H	H	CH_3
3	H	H	H
5	CH_3	D-Glc	H
6	H	D-Glc	H

	R_1
4	H
7	D-Glc

Fig. 1. Structures of compounds **1-7** isolated from R. *sachalinensis*

anol (MeOH) three times under reflux for 15 h, filtered and concentrated to yield MeOH extract (435 g). The MeOH extract was suspended in H_2O and extracted with n-hexane, ethyl acetate (EtOAc) and n-butanol (BuOH) to yield the hexane soluble fraction (27 g), EtOAc soluble fraction (59 g) and BuOH soluble fraction (140 g), respectively. The EtOAc fraction showed moderate cytotoxic activity against L1210 cell line. Therefore, the EtOAc fraction was subjected to column chromatography in silica gel and eluted using a stepwise gradient of methylene dichloride and MeOH to afford eight fractions (Fr. 1~8). Repeated silica gel column chromatography using hexane-EtOAc (15:1) of fraction 1 gave compound 1 (185 mg) and compound 2 (380 mg). Compound 3 (1,097 mg) and 4 (258 mg) were obtained from fractions 2 and 4 by recrystallization in EtOAc. Repeated silica gel column chromatography of fractions 6, 7 and 8 using $CHCl_3$-MeOH (15:1, 10:1, 5:1) and Sephadex LH-20 (100% MeOH) gave compounds 5 (8 mg), 6 (27 mg) and 7 (403 mg), respectively.

Compound 1 – Red brick needle, mp 275~278°C, $FeCl_3$ positive, EI MS *m/z*:284 $[M]^+$, IR $(KBr)_{max}$ cm^{-1}: 3400 (OH), 1622, 1598 (C = O), 1480 (C = C), ^{1}H-NMR (400 MHz, $CDCl_3$): 12.20 (1H, s, OH), 11.99 (1H, s, OH), 7.60 (1H, d, J = 2.4 Hz, H-4), 7.33 (1H, d, J = 2.4 Hz , H-2), 7.06 (1H, d, J = 2.5 Hz, H-5), 6.66 (1H, d, J = 2.5 Hz, H-7), 3.94 (3H, s, OCH_3), 2.43 (3H, s, CH_3), ^{13}C-NMR (100 MHz, $CDCl_3$): 161.8 (s, C-1), 119.5 (d, C-2), 149.2 (s, C-3), 120.8 (d, C-4), 108.0 (d, C-5), 164.8 (s, C-6), 106.5 (d, C-7), 162.1(s, C-8), 192.0 (s, C-9), 181.3 (s, C-10), 137.2 (s, C-11), 113.7 (s, C-12), 115.8 (s, C-13), 133.4 (s, C-14), 22.0 (q, C- CH_3), 56.3 (q, C-OCH_3).

Compound 2 – Orange solid, mp 257~260°C, $FeCl_3$ positive, FAB MS *m/z*: 285 $[M+H]^+$, IR $(KBr)_{max}$ cm^{-1}: 3400 (OH), 1622, 1598 (C = O), 1480 (C = C), ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 12.28 (1H, s, OH-8), 12.11 (1H, s, OH-6), 7.59 (1H, d, J = .4 Hz, H-4), 7.33 (1H, d, J = 2.5 Hz, H-5), 7.06 (1H, d, J = 2.4 Hz, H-2), 6.66 (1H, d, J = 2.5 Hz, H-7), 3.93 (3H, s, OCH_3), 2.44 (3H, s, CH_3), ^{13}C-NMR (100 MHz, $CDCl_3$): 162.3 (s, C-1), 124.4 (d, C-2), 148.3 (s, C-3), 121.1 (d, C-4), 108.2 (d, C-5), 166.4 (s, C-6), 106.7 (d, C-7), 165.0 (s, C-8), 190.6 (s, C-9), 181.8 (s, C-10), 134.9 (s, C-11), 110.2 (s, C-12), 113.6 (s, C-13), 133.1 (s, C-14), 22.2 (q, C-CH_3), 65.1 (q, C- OCH_3).

Compound 3 – Orange needle, mp 259~260°C, $FeCl_3$ positive, EI MS *m/z*: 270 $[M]^+$, IR $(KBr)_{max}$ cm^{-1}: 3400 (OH), 1622, 1598 (C = O), 1480 (C = C), ^{1}H-NMR (400 MHz, DMSO-d_6):12.03 (1H, s, OH), 11.95 (1H, s, OH), 7.40 (1H, d, J = 2.4 Hz, H-4), 7.09 (1H, d, J = 2.4 Hz, H-2), 7.05 (1H, d, J = 2.3 Hz, H-5), 6.55 (1H, d, J = 2.3 Hz, H-7), 2.38 (3H, s, CH_3), ^{13}C-NMR (100 MHz, DMSO-d_6): 161.3 (s, C-1), 123.9 (d, C-2), 148.1 (s, C-3), 120.3 (d, C-4), 108.7 (d, C-5), 164.5 (s, C-6), 107.8 (d, C-7), 164.3 (s, C-8), 189.5 (s, C-9), 181.1 (s, C-10), 134.9 (s, C-11),108.7 (s, C-12), 113.1 (s, C-13), 132.6 (s, C-14), 21.4 (q, C-CH_3).

Compound 4 – White needle, mp 258~260°C, $FeCl_3$ positive, EI MS *m/z*: 228 $[M]^+$, IR $(KBr)_{max}$ cm^{-1}: 3350 (OH), 1580, 1513 (aromatic C = C), 965 (*trans* C = C), UV λ_{max} nm: 208, 217, 309, 322, ^{1}H-NMR (400 MHz, CD_3OD): 7.30 (2H, d, J= 8.6 Hz, H-2, 6), 6.90 (1H, d, J = 16.4 Hz, H-7), 6.75 (1H, d, J = 16.4 Hz, H-8), 6.71 (2H, d, J = 8.6 Hz, H-3, 5), 6.40 (2H, d, J = 2.1 Hz, H-2, 6), 6.14 (1H, d, J = 2.1 Hz, H-4), ^{13}C-NMR (100 MHz, CD_3OD): 130.3 (s, C-1), 128.7 (d, C-2, 6), 116.4 (d, C-3, 5), 158.2 (s, C-4), 129.3 (d, C-7), 126.8 (d, C-8), 141.2 (s, C-1), 105.7 (d, C-2, 6), 159.5 (s, C-3, 5), 102.6 (d, C-4).

Compound 5 – Orange needle, mp 230~232°C, $FeCl_3$ positive, FAB MS *m/z*: 492 $[M+Na]^+$, UV λ_{max} nm: 201, 211, 216, 268, IR $(KBr)_{max}$ cm^{-1}: 3400 (OH), 1622, 1598 (C = O), 1088 (glycoside C-O), ^{1}H-NMR (600 MHz, DMSO): 7.48 (1H, s, H-4), 7.36 (1H, d, J = 2.4 Hz, H-5), 7.18 (1H, d, J = 2.4 Hz, H-7), 7.16 (1H, s, H-2), 5.16 (1H, d, J = 7.6 Hz, H-1'), 3.95 (3H, s, OCH_3), 2.40 (3H, s, CH_3), ^{13}C-NMR (150 MHz, DMSO) :161.5 (s, C-1), 124.1 (d, C-2), 147.0 (s, C-3), 119.3 (d, C-4), 107.2 (d, C-5), 164.6 (s, C-6), 106.4 (d, C-7), 160.5 (s, C-8), 186.3 (s, C-9), 181.7 (s, C-10), 136.2 (s, C-11), 114.3 (s, C-12), 114.3 (s, C-13), 131.9 (s, C-14), 21.5 (q, C-15), 56.1 (q, C-16), 100.6 (d, C-1'), 73.2 (d, C-2'), 76.6 (d, C-3'), 69.7 (d, C-4'), 77.4 (d, C-5'), 60.7 (t, C-6').

Compound 6 – Orange needle, mp 192~193°C, $FeCl_3$ positive, FAB MS *m/z*: 455 $[M+Na]^+$, UV λ_{max} nm: 201, 222, 284, IR $(KBr)_{max}$ nm^{-1}: 3400 (OH), 1622, 1598 (C = O), 1088 (glycosidic C-O), ^{1}H-NMR (600 MHz, CD_3OD): 7.44 (1H, d, J =

0.8 Hz, H-4), 7.27 (1H, d, J = 2.4 Hz, H-5), 7.14 (1H, d, J = 0.8 Hz, H-2), 6.98 (1H, d, J = 2.4 Hz, H-7), 5.05 (1H, d, J = 7.6 Hz, H-1'), 2.39 (3H, s, CH_3), ^{13}C-NMR (150 MHz, CD_3OD): 161.6 (s, C-1), 124.1 (d, C-2), 146.8 (s, C-3), 119.2 (d, C-4), 108.2 (d, C-5), 163.9 (s, C-6), 108.2 (d, C-7), 160.9 (s, C-8), 186.3 (s, C-9), 181.9 (s, C-10), 136.4 (s, C-11), 113.2 (s, C-12), 114.3 (s, C-13), 131.9 (s, C-14), 21.5 (q, C-15), 100.6 (d, C-1'), 73.2 (d, C-2'), 76.4 (d, C-3'), 69.4 (d, C-4'), 77.3 (d, C-5'), 60.5 (t, C-6').

Compound 7 – White needle, mp 228~229°C, $FeCl_3$ positive, FAB MS *m/z*: 391 $[M+H]^+$, IR $(KBr)_{max}$ cm^{-1}: 3310 (OH), 1584, 1513 (aromatic C = C), 1088 (glycoside C-O), 965 (trans C=C), UV λ_{max} nm: 215, 318, ^{1}H-NMR (400 MHz, CD_3OD): 7.32 (2H, d, J = 8.4 Hz, H-2, 6), 6.97 (1H, d, J = 16.4 Hz, H-7), 6.80 (1H, d, J = 16.4 Hz, H-8), 6.73 (2H, d, J = 8.4 Hz, H-3, 5), 6.56 (2H, d, J = 2.1 Hz, H-2, 6), 6.40 (1H, d, J = 2.1 Hz, H-4), 4.84 (1H, J = 7.2 Hz, H-1"), 3.67 (1H, dd, J = 5.6, 12.0 Hz, H-2"), 3.44 (3H, m, H-3", 4", 5"), ^{13}C-NMR (100 MHz, CD_3OD): 130.2 (s, C-1), 128.8 (d, C-2, 6), 116.4 (d, C-3, 5), 158.3 (s, C-4), 129.9 (d, C-7), 126.6 (d, C-8), 141.3 (s, C-1), 106.9 (d, C-2), 159.5 (s, C-3), 104.0 (d, C-4), 160.3 (s, C-5), 108.3 (d, C-6), 102.3 (d, C-1"), 74.9 (d, C-2"), 78.0 (d, C-3"), 71.5 (d, C-4"), 78.2 (d, C-5"), 62.6 (t, C-6").

Cytotoxicity assay – Cells were maintained in RPMI 1640 including L-glutamine (JBI) with 10% FBS (JBI) and 2% penicillin-streptomycin (GIBCO). Trypsin-EDTA was used to separate cell from the culture flask. All cell lines were cultured at 37℃ in an atmosphere of 5% CO_2 incubater.

Cytotoxicity was measured by a modification of the Microculture Tetrazolium (MTT) assay (Mosmann, 1983). Viable cells were seeded in the growth medium (180 ㎛) into 96 well microtiter plate (1×10^4 cells per each well) and allowed to attach in 37℃, 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to the final sample concentrations ranging from 30 ㎍/㎖ to 1.875 ㎍/㎖ by diluting with the growth medium. Each sample was prepared to triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing 2 h, 20 ㎕ of test samples were added to each wells in same concentration of DMSO and were added in the control group. After 48 h test sample addition, 20 ㎕ MTT (final concentration, 5 ㎍/㎖) was added to the each wells. Two hours later, the plate was centrifuged for 5 minutes in 1,500 rpm, the medium was removed and formed formazan crystals were dissolved with 150 ㎕ DMSO. The optical density (O.D.) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow). The IC_{50} value was defined as the concentration of sample to reduce a 50% of absorbance relative to the vehicle-treated control.

RESULTS AND DISCUSSTION

Compound 1 was obtained as a red brick needle. Its EI-MS spectrum gave a molecular ion peak at *m/z* 284 $[M]^+$, with a molecular formula $C_{16}H_{12}O_5$, which was supported by the ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum of 1 showed two aromatic protons appeared at δ 7.60 (1H, d) and 7.33 (1H, d), which could be assigned as *meta*-coupled protons of an anthraquinone moiety. Two additional *meta*-coupled protons were observed at δ 7.06 (1H, d, J = 2.5 Hz) and 6.66 (1H, d, J = 2.5 Hz). The signals at δ 3.94 and 2.43 showed the typical resonance of methoxyl and methyl group, respectively, attached to a benzene ring. Two hydrogen-bonded hydroxyl protons appeared at δ 12.20 and 11.99. In the ^{13}C-NMR spectrum, two ketone signals (δ 181.3 and 192.0), twelve aromatic carbons, one methoxyl and one methyl signal were detected. The structure of compound 1 was finally identified as physcion (emodin 3-methyl ether) by comparing its physicochemical and spectroscopic data with these reported reference (Ko *et al.*, 1995). The ^{1}H- and ^{13}C-NMR spectra data of compounds 2, 3, 5 and 6 were similar to those of 1. Compound 2 was identified as 1-*O*-methylemodin (William & Latchezar, 1997; Peter & Neil, 1995), 3 as emodin (Francis & Holt, 1998). Compounds 5 and 6 were found to have a sugar moiety, which identified as glucose on TLC by a comparison with an authentic compound after acid hydrolysis. Furthermore, the sugar was determined to be D-glucose by the GLC of its trimethylsilylated L-cysteine methyl ester derivative (Melek *et al.*, 2003).

Table 1. Cytotoxic activity of compounds **1-7** isolated from *R. sachalinensis*

Compounds	IC_{50} (㎍/㎖)[a]		
	L1210	HL-60	B16F10
1	15.5	25.1	17.2
2	17.3	22.2	20.1
3	12.2	10.2	15.3
4	9.2	6.7	9.8
5	>30	>30	>30
6	>30	>30	>30
7	>30	>30	>30
AM[b]	0.8	1.9	0.9

[a]IC_{50} values mean the 50% inhibition concentration and were calculated from regression lines using five different concentrations in triplicate experiments.
[b]Adriamycin (AM) was used as a positive control.

Therefore, compound 5 was identified as physcion-8-*O*-β-D-glucopyranoside (Takeshi & Yutake, 1987; Maksut *et al.*, 1990) and 6 as emodin-8-β-D-glucopyranoside (Takeshi & Yutake, 1987; Maksut *et al.*, 1990). Compound 4 showed different pattern from compounds 1, 2, 3, 5 and 6. In 1H-NMR spectrum, *ortho*-coupling protons at δ 7.30 and 6.71 (J = 8.6 Hz), *meta*-coupling two protons at δ 6.40 (J = 2.1 Hz) and one proton showing meta coupling as doublet at δ 6.14 (J = 2.1 Hz) were observed, which indicative of 1,4-disubstituted and 1,3,5-trisubstituted benzene moieties. Trans olefinic protons were observed at δ 6.90 and 6.75 (J = 16.4 Hz), suggesting the presence of a stilbene moiety. In combination with ^{13}C-NMR, 4 was supposed to be *trans*-resveratrol, which finally identified by comparison with those of reported data (Ko *et al.*, 1998). The NMR spectra of compound 7 were similar to those of 4 except the additional sugar moiety. The sugar was identified as D-glucose with the same method of compound 5 and 6. Therefore, compound 7 was identified as *trans*-resveratrol-3-β-D-glucopyranoside, which was accorded with these reported data (Pierre *et al.*, 1998; Yaki *et al.*, 1971).

The isolated compounds 1-7 were evaluated for their cytotoxicity against murine L1210 leukemia cells, human HL-60 leukemia cells and murine B16F10 melanoma cells using a MTT assay. As shown in Table 1, compounds 1-3 exhibited the moderate cytotoxic activity with IC_{50} values of 10.2~22.2 $\mu g/m\ell$, whereas, anthraquinone glycosides, compound 5 and 6, were found to have no cytotoxic activity, suggesting that hydroxyphenyl moiety is important for the cytotoxicity. These results are in accordance with reported data that is anthraquinone aglycones possess strong cytotoxic acitivity but glycosides are inactive (Yeh *et al.*, 1988). Compound 4, resveratrol, exhibited the strongest cytotoxic activity against L1210, HL-60 and B16F10 tumor cell lines with IC50 values of 9.2, 6.7 and 9.8 $\mu g/m\ell$, respectively. Resveratrol was came into the spotlight as potential therapeuticagents for several pathological diseases (Bianchini & Vainio, 2003; Bhat *et al.*, 2001). Moreover, The cytotoxic activity of resveratrol has been reported to have potent cytotoxic activity on various cancer cells such as leukemia, colon, breast and prostate cancer cells and has been suggested as one of the cancer chemopreventive agents (Schneider *et al.*, 2000; Gautam *et al.*, 2000; Dorrie *et al.*, 2001; Michell *et al.*, 1999; Damianaki *et al.*, 2000). For this reason, extensive searches for novel naturally occurring stilbene derivatives have been undertaken (Huang *et al.*, 2000; Ito *et al.*, 2003). Interestingly, in our observations, compound 7, a glycoside of 4, was found to be completely devoid of cytotoxicity against all tumor cell lines. This result suggested that 3,5-dihydroxyphenyl moiety of stilbenes may be important for cytotoxicity. Therefore, it will be in much interest to synthesize reseveratrol analogues and evaluate their cytotoxicity. Further studies will be undertaken to elucidate the antitumor activity of the compounds 1-4.

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