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# Chemical Constituents from the Leaf and Twig of *Acer okamotoanum* Nakai and their Cytotoxicity

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**ABSTRACT : As a result of cytotoxic compounds against cancer cell lines from natural sources, senven compounds were isolated from the leaf and twig of *Acer okamotoanum* Nakai. The compounds (1-7) were identified as ethyl gallate (1), methyl gallate (2), gallic acid (3), *trans* resveratrol-3-*O*-$\beta$-D-glucopyranoside (4), acertannin (5), nikoenoside (6), and fraxin (7) by physicochemical and spectroscopic data (including mp, UV, IR, MS, $^1$H-NMR, $^{13}$C-NMR, DEPT, and HMBC) in comparison with those of published papers. All the compounds were tested for their cytotoxic activity against L1210, HL-60, K562, and B16F10 cancer cell lines *in vitro* by MTT assay method. Compounds 1-3 and 5 showed cytotoxic activity against all tested cell lines with $IC_{50}$ values ranged from 12.5 to 72.2 μM. Of the compounds, methyl gallate (2) exhibited the most potent cytotoxic activity against L1210, HL-60, K562, and B16F10 tumor cells with $IC_{50}$ values of 12.5, 48.3, 22.8, and 22.8 μM, respectively. Other compounds did not show any cytotoxic activity against four cancer cell lines.**

***Key words*** : *Acer okamotoanum*, Aceraceae, chemical constituent, MTT, cytotoxicity

## INTRODUCTION

The genus *Acer* comprises 15 species in Korea. These are main maple trees in mountain, the leaf, the branch, and the root of the some species in this genus have been used in folk medicine for the treatment of arthralgia and fracture (Kim *et al.*, 1998a), *Acer okamotoanum* Nakai is an endemic species in Korea and phytochemical studies on this plant have been carried out some flavonol glycosides and phenolic compounds, together with anti-HIV-1 integrase activity previously (Kim *et al.*, 1998b).

In the course of our ongoing research in identifying cytotoxic active compounds from natural plant sources, senven compounds were isolated from the leaf and twig of *A. okamotoanum*. This paper describes the isolation, the structure determination of these compounds, and their cytotoxic activity against L1210, HL-60, K562, and B16F10 cancer cell lines.

## MATERIALS AND METHODS

### Plant material

The leaf and twig of *Acer okamotoanum* Nakai were collected in August 2001 at Ullung-island in Gyungsangbukdo and identified by one of the authors (K. Bae). Voucher specimen (CNU 1289) has been 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 were performed on a Beckman Du-650 UV-VIS recording spectrophotometer. IR spectra were obtained on a JASCO FT/IR-5300 spectrometer with KBr discs. FAB-MS was obtained using a JEOL JMS-DX 300 spectrometer. $^1$H- (300 MHz) and $^{13}$C-NMR (75 MHz) were recorded on a Bruker AM400 FT-NMR spectrometer and TMS was used as an internal standard. HPLC was performed using a Shimazu liquid chromatograph model Class-vp version 6.12, equipped with a SPD-10A UV-VIS detector (Shimadzu). HPLC was performed using a Mightysil column (250 ㎜× 10 ㎜, RP-$C_{18}$, 5 μm, Kanto, Japan) and all solvents for HPLC were filtrated through a 0.45 μm membrane filter (Waters). Column chromatography was performed using silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) and thin layer chromatography (TLC) on pre-coated silica gel 60 $F_{254}$ (0.25 ㎜, Merck).

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## Extraction, fractionation and isolation

The leaf and twig of *A. okamotoanum* (2.7 kg) were extracted with methanol (MeOH) three times under reflux for 24 h and filtrated, concentrated to give MeOH extract (390.0 g). The MeOH extract was suspended in $H_2O$ and extracted with hexane, ethyl acetate (EtOAc), and butanol (BuOH), successively. The resulting fractions were concentrated *in vacuo* to give the hexane-soluble fraction (60.0 g), EtOAc-soluble fraction (65.0 g), and BuOH-soluble fraction (85.0 g), respectively.

The EtOAc-soluble fraction (65 g) was chromatographed on a silica gel column eluted with a stepwise gradient of $CHCl_3$-MeOH (40 : 1-1 : 1) to yield five fractions ($E_1$~$E_5$: 0.4 g, 1.2 g, 8.5 g, 10.0 g, 1.2 g). The fraction $E_2$ was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (10 : 1) to obtain three sub-fractions ($E_{21}$~$E_{23}$). The compounds **1** (12.0 mg, $t_R$ 21.0 min), **2** (1209.0 mg, $t_R$ 19.0 min), and **3** (9.8 mg, $t_R$ 18.5 min) were purified from sub-fraction $E_{21}$, $E_{22}$, and $E_{23}$, respectively, by HPLC with 30% MeOH solvent system. By using the similar method, compounds **4** (291.0 mg, $t_R$ 14.0 min) and **5** (49.0 mg, $t_R$ 12.0 min) were isolated from fraction $E_4$ by HPLC in the 35% MeOH solvent system. The BuOH-soluble fraction (85 g) was chromatographed on a silica gel column eluted with a stepwise gradient of $CHCl_3$-MeOH (20 : 1-1 : 1) and to yield five fractions ($B_1$~$B_5$: 1.0 g, 2.5 g, 8.5 g, 9.0 g, 12.0 g). The fraction $B_3$ was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (15 : 1) to obtain three sub-fractions ($B_{31}$~$B_{33}$). The sub-fraction $B_{32}$ was further purified by preparative HPLC on RP-18 using 30% MeOH solvent system to yield compound **6** (39.6 mg, $t_R$ 23.0 min). The fraction $B_4$ was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (10 : 1) to obtain four subfractions ($B_{41}$~$B_{44}$). The subfraction $B_{42}$ was further purified by preparative HPLC on RP-18 using 25% MeOH solvent system to yield compound **7** (15.7 mg, $t_R$ 28.0 min).

**Compound 1** - White needle; mp 160~162℃; UV (MeOH) $\lambda_{max}$ nm: 219, 280; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3350, 1695, 1610, 1535, 1450, 1340, 1250, 1200, 1025; FAB MS *m/z*: 199 $[M+H]^+$; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 7.04 (2H, s, H-2, 6), 4.27 (2H, q, $J$ = 7.1 Hz, H-$OCH_2$), 1.34 (3H, t, $J$ = 7.1 Hz, H-$CH_3$); $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 168.6 (s, C-7), 146.5 (s, C-3, 5), 139.7 (s, C-4), 121.8 (s, C-1), 110.0 (d, C-2, 6), 61.6 (t, C-8), 14.6 (q, C-9).

**Compound 2** - White needle; mp 196~198℃; UV (MeOH) $\lambda_{max}$ nm: 210, 273; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3360, 1690, 1620, 1550, 1445, 1240, 1200, 1040; FAB MS *m/z*: 185 $[M+H]^+$; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 7.04 (2H, s, H-2, 6), 3.81 (3H, s, H-$OCH_3$); $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 169.0 (s, C-7), 146.5 (s, C-3, 5), 139.8 (s, C-4), 121.4 (s, C-1), 110.0 (d, C-2, 6), 52.2 (q, C-8).

**Compound 3** - White needle; mp 270~272℃; UV (MeOH) $\lambda_{max}$ nm: 218, 273; FAB MS *m/z*: 171 $[M+H]^+$; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3320, 1690, 1620, 1520, 1450, 1345, 1260, 1050; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 7.08 (2H, s, H-2, 6); $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 169.5 (s, C-7), 145.4 (d, C-3, 5), 138.6 (s, C-4), 121.0 (s, C-1), 109.4 (d, C-2, 6).

**Compound 4** - White needle; mp 228~229℃; UV (MeOH) $\lambda_{max}$ nm: 215, 318; FAB MS *m/z*: 391 $[M+H]^+$; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3310, 2920, 1620, 1584, 1513, 1088, 965; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 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 (75 MHz, $CD_3OD$) δ: 160.3 (s, C-5'), 159.5 (s, C-3'), 158.3 (s, C-4), 141.3 (s, C-1'), 130.2 (s, C-1), 129.9 (d, C-7), 128.8 (d, C-2, 6), 126.6 (d, C-8), 116.4 (d, C-3, 5), 108.3 (d, C-6'), 106.9 (d, C-2'), 104.0 (d, C-4'), 102.3 (d, C-1"), 78.2 (d, C-5"), 78.0 (d, C-3"), 74.9 (d, C-2"), 71.5 (d, C-4"), 62.6 (t, C-6").

**Compound 5** - White crystal; mp 162~165℃; UV (MeOH) $\lambda_{max}$ nm: 216, 278; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3420, 1710, 1610, 1540, 1525, 1450, 1355; FAB MS *m/z*: 469 $[M+H]^+$; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 7.26, 7.25 (4H, H-phenoic), 5.06 (2H, dt, $J$ = 10.0, 5.5 Hz, H-2), 4.71, 4.55 (2H, d, $J$ = 12.0 Hz, H-6), 4.26 (1H, dd, $J$ = 11.0, 5.5 Hz, H-$1_{eq}$), 3.87 (1H, t, $J$ = 9.0 Hz, H-3), 3.68 (2H, m, H-4, H-5), 3.51 (1H, t, $J$ = 10.5 Hz, H-$1_{ax}$); $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 168.2, 167.6 (s, C-COO), 146.4 (4C, s, *m*-C), 139.8 (2C, s, *p*-C ), 121.3, 121.0 (2C, s, arom.-C-1), 110.2 (4C, d, *o*-C), 80.1 (d, C-5), 76.9 (d, C-6), 73.1 (d, C-2), 71.9 (d, C-4), 67.9 (t, C-1), 64.9 (t, C-6).

**Compound 6** - White powder; mp 146~148℃; UV (MeOH) $\lambda_{max}$ nm: 271; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3453, 2936, 1595, 1508, 1466, 1327, 1032; FABMS *m/z*: 383 $[M+Na]^+$; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 6.78 (2H, brs, 2,6-H), 4.86, 4.67 (1H each, both d, $J$ = 13.2 Hz, 7-$H_2$), 4.34 (1H, d, $J$ = 7.4 Hz, 1'-H), 3.85 (6H, s, 3,5-$OCH_3$), 3.73 (3H, s, 4-$OCH_3$); $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 154.1 (s, C-3, 5), 137.4 (s, C-4), 134.1 (s, C-1), 105.3 (d, C-2, 6), 101.9 (d, C-1'), 77.1 (d, C-3', 5'), 74.1 (d, C-2'), 70.8 (d, C-4'), 70.5 (t, C-7), 61.9 (t, C-6').

**Compound 7** - Colourless needle; mp 205~206℃; UV (MeOH) $\lambda_{max}$ nm: 210, 231, 293, 344; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3420, 2920, 1660, 1510, 1460, 1070; FABMS *m/z*: 371 $[M+H]^+$; $^1$H-NMR (300 MHz, $CD_3OD$) δ: 7.85 (1H, d, $J$ = 9.5 Hz, H-4), 6.93 (1H, s, H-4), 6.20 (1H, d, $J$ = 9.5 Hz, H-3), 4.96 (1H, d, $J$ = 7.6 Hz, H-1'), 3.87 (3H, s, 6-$OCH_3$); $^{13}$C-NMR (75 MHz, $CD_3OD$) δ: 163.2 (s, C-2), 147.4 (s, C-4), 146.4 (d, C-9), 145.7 (s, C-6), 144.2 (s, C-7), 133.2 (s, C-8), 113.1 (d, C-3), 112.2 (s, C-10), 106.0 (d, C-5), 105.1 (d, C-1'),

78.6 (d, C-3'), 77.7 (d, C-5'), 75.5 (d, C-2'), 70.7 (d, C-4'), 62.2 (d, C-6'), 57.0 (q, C-$OCH_3$).

### Cytotoxicity assay

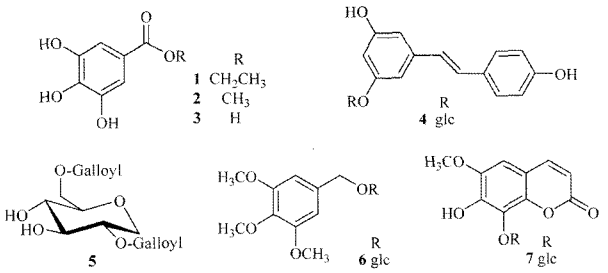
All cancer cell lines (L1210, HL-60, K562, B16F10) were maintained in RPMI 1640 which included L-glutamine (JBI) with 10% FBS (JBI) and 2% penicillin-streptomycin (GIBCO). Trypsin-EDTA was used to separate cells (B16F10) from the culture flask. Cells were cultured at 37 ℃ in a 5% $CO_2$ incubator.

Cytotoxic activity was measured by a modified Microculture Tetrazolium (MTT) assay (Mosmann, 1983). Viable cells were seeded in the growth medium (180 ㎕) into 96 well microtiter plates ($1\times10^4$ cells per each well) and incubated at 37℃ in 5% $CO_2$ incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 6.25 μM to 100 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing for 2 h, 20 ㎕ of the test sample was added to each well. The same volume of DMSO was added to the control group well. Forty-eight hours after the test sample was added, 20 ㎕ MTT was also added to the each well (final concentration, 5 ㎍/㎖). Two hours later, the plate was centrifuged for 5 minutes at 1500 rpm, the medium was then removed and the resulting formazan crystals were dissolved with 150 ㎕ DMSO. The optical density (O.D.) was measured at 570 ㎚ 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 DISCUSSION

In our continuing search for cytotoxic active compounds from natural plant sources, three simple phenolic compounds (**1-3**), a stilbene glycoside (**4**), a galloyl glucose (**5**), an aromatic glycoside (**6**), and a coumarin glycoside (**7**) were isolated from the MeOH extract of leaf and twig of *A. okamotoanum* by column chromatography and HPLC method.

Compounds **1-3** were obtained as white needle, the UV maxima at 273 or 380 ㎚ was typical of phenolic compounds. The IR absorption at 1690 $cm^{-1}$ showed the presence of carbonyl group. It is easily understand that the R group in the **Fig. 1** was different from $^1$H- and $^{13}$C-NMR spectra data. Therefore, compounds **1-3** were identified as ethyl gallate (**1**), methyl gallate (**2**), and gallic acid (**3**) by comparison with those of published papers (Redwane *et al.*, 2002; Charles, 1993). Compound **4** was obtained as a white needle, a colorizing reaction with $FeCl_3$ showed dark blue, and the UV spectrum of **4** exhibited




**1**: ethyl gallate, **2**: methyl gallate, **3**: gallic acid, **4**: *trans* resveratrol-3-*O*-*β*-D-glucopyranoside, **5**: acertannin, **6**: nikoenoside, **7**: fraxin

**Fig. 1.** Structures of compounds **1-7** from *A. okamotoanum* Nakai.

bands at 215 and 318 ㎚ to suggest the presence of a stilbene skeleton (Hata *et al.*, 1975). FAB-MS gave a quasimolecular ion peak at *m/z* 391 $[M+H]^+$ providing the formula $C_{20}H_{22}O_8$. The configuration of the glycosidic linkage was determined as *β* on the basis of the $J_{1,2}$ value of the anomeric proton of 7.2 Hz (δ 4.84). The attachment position of the sugar to the aglycon was deduced from HMBC correlation, which were correlated between $\delta_H$ 4.84 (H-1") and $\delta_C$ 159.5 (C-3'). Finally, the compound (**4**) was identified as *trans* resveratrol-3-*O*-*β*-D-glucopyranoside (**4**) by comparison with those of reported values (Yaki *et al.*, 1971; Pierre *et al.*, 1998). Compound **5** was obtained as white crystal, UV showed phenolic bands at 216 and 278 ㎚. The IR spectrum of **5** showed the characteristic bands for hydroxyl group at 3420 $cm^{-1}$, carbonyl group at 1710 $cm^{-1}$, aromatic rings at 1610 and 1540 $cm^{-1}$. FAB-MS gave a quasimolecular ion peak at *m/z* 469 $[M+H]^+$ providing the formula $C_{20}H_{20}O_{13}$. Accroding to the $^1$H- and $^{13}$C-NMR spectrum data and described data, compound **5** was identified as acertannin (**5**) by comparison with those of reported data (Klaus *et al.*, 1980). Compound **6** was obtained as a white powder, showed an absorption maximum at 271 ㎚ in UV spectrum. The IR spectum of **6** showed absorption bands at 3453, 1595, 1508, and 1032 $cm^{-1}$, suggestive of glycosidic and aromatic functions. FAB-MS gave a quasimolecular ion peak at *m/z* 383 $[M+Na]^+$ providing the formula $C_{16}H_{24}O_9$. The $^1$H- and $^{13}$C-NMR spectra of **6** indicated the presence of a 3,4,5-trimethoxylbenzyl moiety [δ 3.73 (3H, 4-$OCH_3$), 3.85 (6H, s, 3,5-$OCH_3$), 4.67, 4.86 (1H, each, both d, $J$= 13.2 Hz, 7-$H_2$), 6.78 (2H, brs, 2,6-H)] and *β*-D-glucopyranosyl part [4.34 (1H, d, $J$= 7.4 Hz, 1'-H)]. On the basis of these findings, compound **6** was identified as nikoenoside (**6**) (Morikawa *et al.*, 2003). Compound **7** was obtained as colourless needle and the UV maxima at 231, 293, and 344 ㎚ were typical bands of coumarins. The IR spectum of **7** showed absorption bands at 3420, 1660, 1510, and 1070 $cm^{-1}$, suggestive of glycosidic and aromatic functions. FAB-MS gave a quasimolecular ion peak

**Table 1.** The cytotoxicity of compounds 1-7 from *Acer okamotoanum* Nakai.

| Compounds | $IC_{50}$ (μM) [a] | | | |
|---|---|---|---|---|
| | L1210 | HL-60 | K562 | B16F10 |
| 1 | 32.8 ± 2.6 | 65.6 ± 2.4 | 72.2 ± 2.1 | 46.9 ± 2.2 |
| 2 | 12.5 ± 1.9 | 48.3 ± 2.1 | 22.8 ± 1.5 | 22.9 ± 1.2 |
| 3 | 49.4 ± 1.8 | 55.9 ± 2.4 | 50.0 ± 2.5 | 50.1 ± 1.8 |
| 4 | > 100 | > 100 | > 100 | > 100 |
| 5 | 20.9 ± 1.5 | 38.7 ± 1.7 | 29.3 ± 2.1 | 43.3 ± 2.2 |
| 6 | > 100 | > 100 | > 100 | > 100 |
| 7 | > 100 | > 100 | > 100 | > 100 |
| AM [b] | 1.5 ± 0.3 | 5.2 ± 0.9 | 2.6 ± 0.8 | 1.7 ± 1.1 |

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

at *m/z* 371 $[M + H]^+$ providing the formula $C_{16}H_{18}O_{10}$. The $^1$H- and $^{13}$C-NMR spectra also reveal the coumarin skeleton. Two tipical olefinic protons of coumarins were observed at δ 7.85 (1H, d, $J$ = 9.5 Hz, H-4) and δ 6.20 (1H, d, $J$ = 9.5 Hz, H-3) in the $^1$H-NMR and two olefinic carbons at δ 147.4 (C-4) and δ 113.1 (C-3) in the $^{13}$C-NMR. One singlet proton was observed at 6.93 (1H, s, H-4), indicating the ring of B of coumarin skeleton was trisubstituted. Accordingly, compound **7** was identified as fraxin (**7**) by comparison with those of published paper (Liu *et al.*, 2005). Except compound **2** (Kim *et al.*, 1998b), all other compounds were isolated for the first time from this plant.

All the compounds were tested for their cytotoxic activity against L1210, HL-60, K562, and B16F10 cancer cell lines *in vitro* by MTT assay method. The results ($IC_{50}$ values) are shown in **Table 1**. Of these, three simple phenolic compounds (**1-3**) and galloyl glucose (**5**) showed cytotoxic activity against tested cancer cell lines with $IC_{50}$ values ranged from 12.5 to 72.2 μM. The ethyl gallate (**1**) and gallic acid (**3**) have been isolated from *Canarium bengalense* by Du *et al.* as cell cycle inhibitors (Du *et al.*, 2003) and showed almost the same cytotoxic activity against rat hepatocytes (Nakagawa and Tayama, 1995). Methyl gallate (**2**) and acertannin (**5**) have been isolated from *Acer ginnala* by Choi *et al.* as the main cytotoxic compounds with $IC_{50}$ values of 6.7-69.8 μM against cancer cell lines (Choi *et al.*, 2005). Our result of **2** and **5** were in accordance with reported data (Choi *et al.*, 2005). On the other hand, *trans* resveratrol-3-*O*-β-D-glucopyranoside (**4**), nikoenoside (**6**), and fraxin (**7**) did not show any cytotoxic activity against all tested cell lines. All these result suggested that the importance of galloyl group to maximize cytotoxic activity against cancer cell lines and the important cytotoxic components were to be simple phenolic compounds and galloyl glucose in the *A. okamotoanum*.

## ACKNOWLEDGEMENT

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