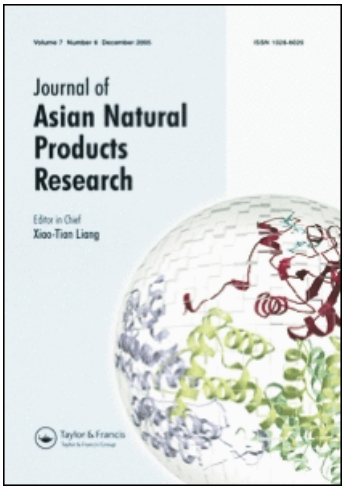


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# Phenylpropanoids from the leaves of *Acanthopanax koreanum* and their antioxidant activity

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By various chromatographic methods, one new phenylpropanoid, acanthopanic acid (**1**), and three known compounds, 1,2-*O*-dicaffeoylcyclopenta-3-ol (**2**), (4*S*)-α-terpineol 8-*O*-β-D-glucopyranoside (**3**), and rutin (**4**), were isolated from the methanol extract of the *Acanthopanax koreanum* leaves. Their structures were elucidated on the basis of spectroscopic analyses, and their antioxidant activities were evaluated by the intracellular reactive oxygen species (ROS) radical scavenging 2′,7′-dichlorofluorescein diacetate assay. Among them, compounds **1**, **2**, and **4** showed significant scavenging capacity with $IC_{50}$ values of 3.8, 2.6, and 2.9 μM, respectively, and compound **3** showed weak scavenging capacity with the inhibition rate of 37% at 40 μM.

**Keywords:** *Acanthopanax koreanum*; Araliaceae; acanthopanic acid; phenylpropanoid; antioxidant; DCF-DA method



## 1. Introduction

Oxidative damage of biological molecules in the human body is involved in degenerative or pathological processes such as aging, coronary heart disease, cancer, arteriosclerosis, and rheumatism [1]. It is well known that natural antioxidants extracted from herbs and spices have high antioxidant activity and are used in many food applications.

*Acanthopanax koreanum* Nakai is a deciduous scrub of the Araliaceae family and an endemic species in Korea. The roots and stems of *A. koreanum* have been used traditionally in Korea as a tonic and in the treatment of rheumatism, hepatitis, and diabetes [2,3]. Previously, lupane-type triterpene glycosides had been isolated and reported from this plant by us [4,5]. As part of continuing the project for antioxidative agents from natural sources, we found that the extract of *A. koreanum* leaves possessed antioxidative effects and we report herein the isolation, structural elucidation, and evaluation of the antioxidant activity of one new phenylpropanoid, acanthopanic acid (**1**), and three known compounds, 1,2-*O*-dicaffeoylcyclopenta-3-ol (**2**), (4*S*)-α-terpineol 8-*O*-β-D-glucopyranoside (**3**), and rutin (**4**) (Figure 1). Their structures were elucidated by physical and spectroscopic methods.

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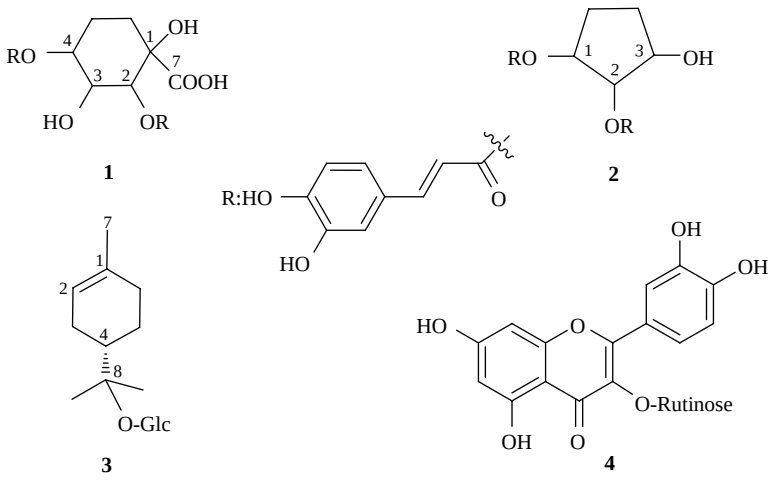


Figure 1. Chemical structures of compounds **1–4**.

## 2. Results and discussion

Compound **1** was obtained as a yellowish powder and its molecular formula, $C_{25}H_{24}O_{12}$, was determined on the basis of ESI-MS at *m/z* 539.1 $[M + Na]^+$ (positive), 517.0 $[M + H]^+$ (positive), and HR-EI-MS at *m/z* 517.1360 $[M + H]^+$. The $^1$H NMR spectrum of **1** (methanol-$d_4$) showed signals due to four olefinic protons [$\delta_H$ 6.31, 6.41, 7.58, 7.62 (each, d, $J = 16.0$ Hz)] and two 1,3,4-trisubstituted aromatic rings with ABX coupling patterns [$\delta_H$ 6.78 (d, $J = 8.0$ Hz), 6.94 (dd, $J = 1.5$, 8.0 Hz), 7.06 (d, $J = 1.5$ Hz), 6.78 (d, $J = 8.0$ Hz), 6.97 (dd, $J = 1.5$, 8.0 Hz), 7.08 (d, $J = 1.5$ Hz)]. From these observations, along with the analysis of the $^{13}$C NMR spectroscopic data (Table 1), two caffeoyl groups were inferred to be present in the molecule of **1**. The $^1$H and $^{13}$C NMR spectra of **1** were similar with those of 3,4-di-*O*-caffeoyl quinic acid [6], except for the position of hydroxyl groups. The presence of a 1,2,3,4-tetrahydroxycyclohexane-1-carboxylic acid moiety was suggested by the characteristic $^{13}$C NMR signals due to three oxymethines ($\delta_C$ 72.6, 73.4, and 74.6), two sp$^3$ methylenes ($\delta_C$ 37.8 and 41.1), one oxygenated quaternary carbon ($\delta_C$ 76.5), and one carboxyl carbon ($\delta_C$ 181.7). Moreover, the long-range H–C correlations between H-2 ($\delta_H$ 5.39) and C-1 ($\delta_C$ 76.5), C-3 ($\delta_C$ 73.4), C-4 ($\delta_C$ 72.6), C-9′ ($\delta_C$ 169.1), and C-7 (181.7) and between H-4 ($\delta_H$ 5.54) and C-3 ($\delta_C$ 73.4), C-5 ($\delta_C$ 41.1), and C-9″ ($\delta_C$ 169.6) were observed in the HMBC spectrum (Figure 2), confirming one hydroxyl group at C-3, one carboxyl group at C-1, and two caffeoyl groups at C-2 and C-4, respectively. These assignments were further supported by the analysis of the $^1$H–$^1$H COSY correlations (H-2/H-3, H-3/H-4, and H-4/H-5). The spin–spin coupling constants ($J_{2,3} = 3.5$ Hz, $J_{3,4} = 10.0$ Hz) in cyclohexane ring confirmed one equatorial proton (H-2) and two axial protons (H-3 and H-4). From all the above evidence, the structure of **1** was characterized as 2,4-*O*-dicaffeoyl-1,3-dihydroxycyclohexane-1-carboxylic acid, a new compound named as acanthopanic acid.

Compounds **2–4** were identified as 1,2-*O*-dicaffeoylcyclopenta-3-ol [7], (4*S*)-α-terpineol 8-*O*-β-D-glucopyranoside [8], and rutin [9], respectively, by comparison of the NMR and mass spectral data with those of literature values. All these compounds were initially isolated from *A. koreanum.*

The antioxidant activities of all compounds were evaluated by the intracellular

Table 1. The NMR spectral data of compound **1**.

| | **1** | |
|---|---|---|
| Pos. | $\delta_C$[a,b] | $\delta_H$[a,c] mult. |
| 1 | 76.5 | – |
| 2 | 74.6 | 5.39 (d, $J = 3.5$ Hz) |
| 3 | 73.4 | 3.91 (dd, $J = 3.5$, 10.0 Hz) |
| 4 | 72.6 | 5.54 (dt, $J = 5.5$, 10.0 Hz) |
| 5 | 41.1 | 2.08[d] |
| 6 | 37.8 | 2.28 (dd, $J = 2.0$, 15.5 Hz)<br>2.07[d] |
| 7 | 181.7 | – |
| *2-Caffeoyl* | | |
| 1′ | 128.0 | – |
| 2′ | 115.3 | 7.06 (d, $J = 1.5$ Hz) |
| 3′ | 146.9 | – |
| 4′ | 149.5 | – |
| 5′ | 116.6 | 6.78 (d, $J = 8.0$ Hz) |
| 6′ | 123.1 | 6.94 (dd, $J = 1.5$, 8.0 Hz) |
| 7′ | 147.0 | 7.58 (d, $J = 16.0$ Hz) |
| 8′ | 115.7 | 6.31 (d, $J = 16.0$ Hz) |
| 9′ | 169.1 | – |
| *4-Caffeoyl* | | |
| 1″ | 128.2 | – |
| 2″ | 115.3 | 7.08 (d, $J = 1.5$ Hz) |
| 3″ | 146.9 | – |
| 4″ | 149.6 | |
| 5″ | 116.6 | 6.78 (d, $J = 8.0$ Hz) |
| 6″ | 123.1 | 6.97 (dd, $J = 1.5$, 8.0 Hz) |
| 7″ | 146.9 | 7.62 (d, $J = 16.0$ Hz) |
| 8″ | 116.2 | 6.41 (d, $J = 16.0$ Hz) |
| 9″ | 169.6 | – |

Notes: [a] Measured in $CD_3OD$.
[b] 125 MHz.
[c] 500 MHz.
[d] Overlapped signals, assignments were done by HMQC, HMBC, and COSY.



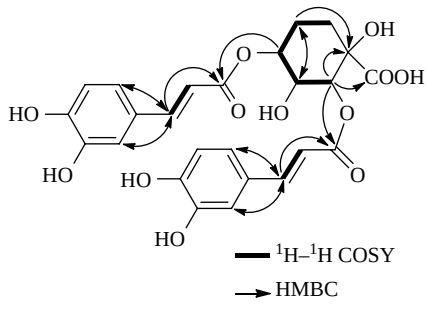


Figure 2. Significant $^1$H–$^1$H COSY and HMBC correlations of compound **1**.

reactive oxygen species (ROS) radical scavenging 2′,7′-dichlorofluorescein diacetate (DCF-DA) assay (Figure 3). The results showed that compounds **1**, **2**, and **4** exhibit significant antioxidant activity with $IC_{50}$ values of 3.8, 2.6, and 2.9 μM, respectively. Compound **3** was considered void of antioxidant activity with inhibition rate of 37% at 40 μM. Furthermore, by considering the antioxidant activity of the two phenylpropanoids, **2** showed stronger effects than **1**. Compound **4** was isolated as the main component from this plant and showed significant antioxidant activity.

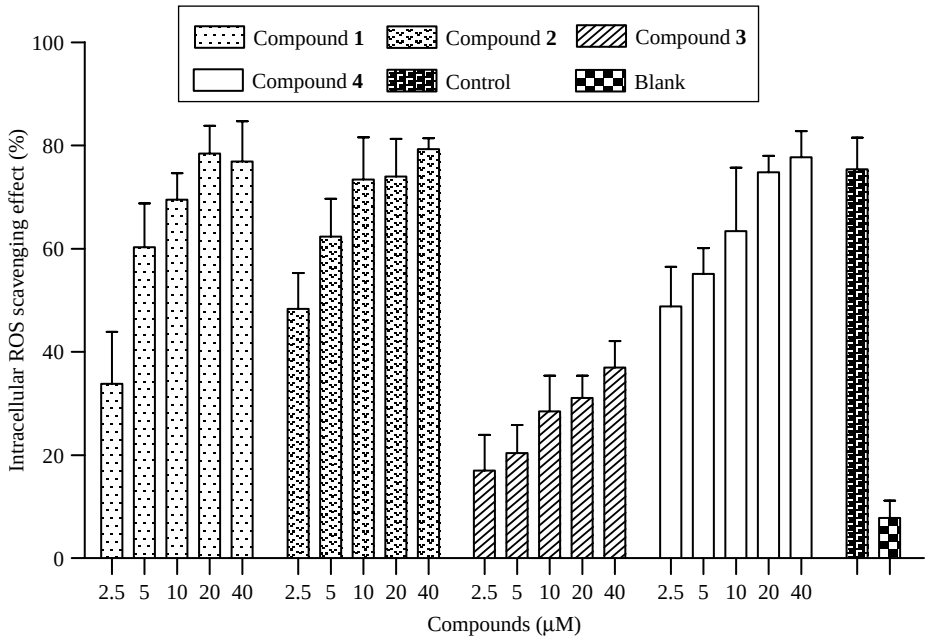


Figure 3. The scavenging effect of **1**–**4** on intracellular ROS. The cells were treated with the samples at 40, 20, 10, 5.0, and 2.5 μM. After 30 min, 1 mM of $H_2O_2$ was added to the plate. After an additional 30 min, DCF-DA was added and the intracellular ROS generated were detected by spectrofluorometry. Significantly different from control cells ($p < 0.05$) as examined by Tukey test. NAC was used as positive control at concentration of 1 mM.



## 3. Experimental

### 3.1 General experimental procedures

Melting points were determined using an Electrothermal IA-9200. The IR spectra were obtained from a Hitachi 270-30 type spectrometer with KBr disks. Optical rotations were determined on a Jasco DIP-1000 KUY polarimeter. The ESI mass spectra were obtained using an Agilent 1200 LC-MSD Trap spectrometer. The HR-EI-MS spectra were obtained using a JEOL JMS-AX505 HR-5890 series spectrometer. The $^1$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer and tetramethylsilane (TMS) was used as an internal standard. Column chromatography was performed on silica gel (Kieselgel 60, 70–230 mesh, and 230–400 mesh, Merck, Whitehouse Station, NJ, USA) and YMC RP-18 resins (30–50 μm, Fujisilisa Chemical Ltd., Kasugai, Japan). Thin layer chromatography was performed on DC-Alufolien 60 silica gel $F_{254}$ (Merck 1.05554.0001) or DC Platten $RP_{18}$ $F_{254s}$ (Merck 1.15685.0001) plates. Spots were visualized by spraying 10% $H_2SO_4$ aqueous and heating for 5 min.

### 3.2 Plant material

The leaves of *A. koreanum* were collected in Susin Ogapi (Cheonan, Korea) and identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 070614) has been deposited at the Herbarium of College of Pharmacy, Chungnam National University, Korea.

### 3.3 Extraction and isolation

The dried leaves of *A. koreanum* (4.0 kg) were extracted with hot MeOH (three times, 50°C, 5 liters each) to yield the methanol extract (80 g), which was then suspended in water (2 liters) and extracted,

in turn, with ethyl acetate (3 × 2 liters) to give the ethyl acetate (AK1, 30 g) and water (AK2, 50 g) extracts. The AK2 extract (50 g) was chromatographed on a Diaion HP-20P column eluting with water containing increasing concentrations of MeOH (0, 25, 50, 75, and 100% MeOH, 0.5 liters each) to give four corresponding fractions, AK2A–AK2D. Fraction AK2D (6 g) was chromatographed on an YMC RP-18 column (40 g, 3 × 50 cm) eluting with acetone/water (2:1, v:v, 2.0 liters, fractions of 10 ml were collected) to yield four sub-fractions, AK2D1–AK2D4. Sub-fraction AK2D1 (300–500 ml, 1.5 g) was further chromatographed on a silica gel column (40 g, 2 × 40 cm) eluting with chloroform/methanol/water (30:10:1, v:v:v, 1500 ml, fractions of 15 ml were collected) to obtain compounds **2** (yellowish powder, 5.0 mg) and **1** (yellowish powder, 4.5 mg). Sub-fraction AK2D2 (700–850 ml, 1.7 g) was further chromatographed on a silica gel column (45 g, 2 × 45 cm) eluting with dichloromethane/methanol/water (25:10:1.4, v:v:v, 1500 ml, fractions of 10 ml were collected) to obtain compounds **3** (700–850 ml, white powder, 6.4 mg) and **4** (1100–1200 ml, yellow powder, 200 mg).

#### 3.3.1 Compound *1*

A yellowish powder; $[\alpha]_D^{25}$: −147.2° ($c = 0.5$, MeOH); UV $\lambda_{max}$(log ε, MeOH): 328 (4.5), 299 (4.3), 230 (4.0), 220 (4.4) nm; IR (KBr) $\nu_{max}$ 3420, 1750, 1702, 1260, 1035, and 988 $cm^{-1}$; $^1H$ NMR and $^{13}C$ NMR spectral data: see Table 1; positive EI-MS *m/z*: 539 $[M + Na]^+$, and HR-EI-MS found *m/z*: 517.1360 $[M + H]^+$ (calcd $C_{25}H_{25}O_{12}$ for 517.1346).

### 3.4 Antioxidant assay

#### 3.4.1 Chemicals and sample preparation

DCF-DA, *N*-acetylcysteine (NAC), and glucose were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and reagents were of analytical grade. The compounds (**1**–**4**), glucose, and NAC were dissolved in DMSO.

#### 3.4.2 Cell culture

V79-4 Chinese hamster lung fibroblast cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained at 37°C and 5% $CO_2$ in a humidified incubator, as well as in Dulbecco's modified Eagle's medium, which contained 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 unit/ml).

#### 3.4.3 Intracellular ROS measurement

The DCF-DA method was used to detect intracellular ROS. DCF-DA diffuses into cells, where it is further hydrolyzed by intracellular esterase to polar 2′,7′-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog is trapped in cells and can be oxidized to the highly fluorescent 2′,7′-dichlorofluorescein by intracellular oxidants [10]. Thus, the dichlorofluorescein is directly proportional to the amount of intracellular radical generation. Cells were seeded at a density of $1 \times 10^5$ cells/ml in a 96-well plate. After plating for 16 h, cells were treated with the samples, followed by the addition of 1 mM $H_2O_2$ to the plate 30 min later. The final DMSO concentration was kept at 0.1% in order not to affect cell viability. Next, cells were incubated for an additional 30 min at 37°C. The fluorescence of 2′,7′-dichlorofluorescein was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Perkin-Elmer LS-5B spectrofluorometer. The ROS scavenging activity was expressed as the percent inhibition, which was calculated using the following formula:

$$\text{ROS scavenging activity } (\%) = [(OD_{control} - OD_{sample})/\ OD_{control}] \times 100.$$

### 3.5 Statistical analysis

The data were presented as mean ± standard error of three different experiments

in triplicate. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. Values of $p < 0.05$ were considered significantly.

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