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# Isolation and Quantitative Analysis of Peroxynitrite Scavengers from *Artemisia princeps* var. *orientalis*

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Young and mature *Artemisia princeps* var. *orientalis* (APO, Compositae) are used as a health food and a medicinal plant, respectively, in Korea. Here, we identified the *in vitro* potent peroxynitrite ($ONOO^-$)-scavenging effect ($IC_{50}$, 0.26 μg/mL) of the components from the EtOAc fraction. Octadecylsilane column chromatography on the EtOAc fraction yielded two caffeoylquinic acid compounds, 3,5-di-*O*-caffeoyl-muco-quinic acid (**1**) and methyl 4,5-di-*O*-caffeoylquinate (**2**) by NMR spectroscopic data, which have not been reported before from APO. The $IC_{50}$ values of compounds **1** and **2** were 0.18 ± 0.01 μg/mL and 0.12 ± 0.00 μg/mL, respectively, lower than that of the positive control (L-penicillamine). HPLC data indicated that young APO (**1**: 30.3 mg/g dried weight, **2**: 27.7 mg/g) contained considerably higher quantities of the two caffeoylquinic acids than mature APO (**1**: 1.77 mg/g dried weight, **2**: 4.10 mg/g).

**Key words:** *Artemisia princeps* var. *orientalis*, Compositae, Caffeoylquinic acid, Peroxynitrite, HPLC

## INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) formed in neutrophils and help fight infections. The superoxide anion radical ($\cdot O_2^-$) may be dismutated either spontaneously or catalyzed by superoxide dismutase (SOD) to generate ROS (Klotz et al., 2002). Nitric oxide ($\cdot NO$), which is synthesized by nitric oxide synthase (NOS) from L-arginine, is a diffusible molecular mediator in the vascular or nervous system (Marletta et al., 1998). Peroxynitrite ($ONOO^-$) is an RNS formed from the reaction between the superoxide anion radical and nitric oxide, and exerts the peroxidation of lipids and proteins and even results in cytotoxicity and rapid neurotoxicity (Haenen et al., 1997). Peroxynitrite causes diseases such as hypercholesterolemia, atherosclerosis, obesity and diabetes mellitus (Patcher et al., 2005; Drel et al., 2007; Korda et al., 2008).

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Caffeoylquinic acids are a group of natural compounds consisting of one or two caffeic acids and a quinic acid. The usual parent skeleton is quinic acid (Zhao et al., 2006) but can also include stereoisomers, e.g., muco-quinic acid (Kwon et al., 2000; Kim et al., 2007). *Artemisia princeps* var. *orientalis* (APO) belonging to the family Compositae is an edible vegetable or health food at the early stage of growth, but it is almost inedible at the mature stage. The aerial part of young APO is used for atherosclerosis, hypercholesterolemia and thrombosis, with the mature one, Artemisiae Herba, has been used to treat stomachache, fever, chronic hepatitis, digestive disorder, enteritis, bronchitis, asthma, pain, and hemorrhage (Chi and Lee, 1988).

The isolation of the sesquiterpenoids, 3-((S)-2-methylbutyryloxy)-costu-1(10),4(5)-dien-12,6α-olide, 8α-angeloyloxy-3β,4β-epoxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (carlaolide B), and 3β,4β-epoxy-8α-isobutyryloxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (carlaolide A), has been reported from *A. princeps* (Bang et al., 2008). Sesquiterpenes have also been isolated from *A. princeps*: copaene, cyperene, caryophyllene, β-farnesene, α-himachalene, γ-humulene,

and farnesyl acetate (Yana and Nishijima, 1974). In addition, yomogin was isolated from *A. princeps* as a nitric oxide inhibitor (Ryu et al., 1998). *A. princeps* has anti-diabetic effects (Jung et al., 2008) and anti-atherosclerosis effects (Han et al., 2009). Eupatilin isolated from *A. princeps* has antidiabetic, (Kang et al., 2008), anti-inflammatory (Giangaspero et al., 2009) and anti-allergic (Lee et al., 2007) actions.

APO activity shows seasonality, with plants collected in April showing more potent antimicrobial and antioxidant effects, and particularly inhibiting xanthine oxidase than those collected in August (Yun et al., 2008). The polyphenol of *A. princeps* inhibits a bovine albumin fragmentation, but the polyphenol compounds are unidentified (Toda, 2004). We performed phytochemical isolation, a peroxynitrite assay and HPLC analysis to identify the peroxynitrite scavengers and quantitative levels in APO.

## MATERIALS AND METHODS

### Instruments and reagents

HPLC chromatograms were measured on a Varian HPLC system consisting of a Prostar 210 solvent delivery module, Prostar UV-Vis detector and 20 μL sample loop (Rheodyne, Rohert Park) with Shiseido Capcell Pak C18 column (5 μL, 250 mm×4.6 mm I.D.) purchased from Hamilton. HPLC grade solvents were used as the mobile phase, purchased from J.T. Baker.

Optical rotation was measured on a Perkin Elmer Model 341 polarimeter at 20°C. The $^{1}$H-NMR spectra (δ ppm, *J* in Hz) was recorded in DMSO-$d_6$ or $CD_3OD$ on a Bruker AM-500 spectrometer (500 MHz) with tetramethylsilane (TMS) as an internal standard, while $^{13}$C-NMR spectra were recorded in the same solvents on a Bruker AM-500 spectrometer at 125 MHz. Dihydrorhodamine 123 (DHR 123) and peroxynitrite were used for the peroxynitrite assay (Molecular Probes and Cayman Chemical Co., respectively).

### Plant materials

*Artemisia princeps* var. *orientalis* (APO, Compositae) was collected in April and August in Wonju, Korea. Young APO is designated as the aerial part of APO less than 20 cm high; mature APO means more than 50 cm high. These were dried at a cool site, cut, and used for extraction. These plant materials were identified by Dr. Won-Bae Kim (Highland Agriculture Research Center, Rural Development Administration, Pyeongchang 232-950, Korea). Voucher specimens (young APO, Natchem #42; mature APO, Natchem #43) were deposited at the laboratory of Natural product Chemistry, Sangji Univeristy.

### Extraction and fractionation

Young APO (390 g) was extracted under reflux for 6 h three times. The MeOH solution was filtered and evaporated *in vacuo* on a rotatory evaporator to give a viscous MeOH extract. This was further subjected to freeze-drying to give a solid MeOH extract (72.1 g) which was further partitioned between distilled water (1.00 L) and ether (800 mL) for fractionation in a separating funnel. The ether-soluble portion was concentrated to dryness to give an ether fraction. A residual water layer was successively fractionated in the same way into EtOAc-soluble and BuOH-soluble portions. The three fractions were freeze-dried to produce an ether fraction (11.2 g, % yield 19.6%), an EtOAc fraction (4.90 g, 8.60%), and a BuOH fraction (14.2 g, 24.9%). Similarly, the mature APO (400 g) was extracted and fractionated into a MeOH extract (78.0 g), an ether fraction (13.6 g), an EtOAc fraction (5.20 g) and a BuOH fraction (16.8 g). Those extracts and fractions used to measure the peroxynitrite scavenging effect.

### Isolation

Since the EtOAc fraction of young APO exhibited the highest biological activity, it was used to isolate the bioactive components. The EtOAc fraction was chromatographed on an Octadecylsilane (ODS) column (Ö 50 mm × 34 cm) using MeOH-$H_2O$ (1:1) as a mobile phase, and the aliquots were collected in 30 mL. After checking on TLC, the aliquots were combined into the three groups {Fr.1 (#1-2), Fr.2 (#3-11) and Fr.3 (#12-17)}. Fr.2 concentrated was again subjected to ODS column (Ö 30 mm × 38 cm) using MeOH-$H_2O$ (1:1) and collected in 30 mL. One group of the aliquots (#1-5) and the other group (#13-22) were concentrated, dissolved in MeOH and allowed to stand one night to yield compounds **1** (210 mg) and **2** (280 mg), respectively.

### Compound 1

Yellowish gum, $[\alpha]_D$ -153.8° (c = 0.51, MeOH), HR-FAB-MS *m/z* 517.1349 $[M + H]^+$, $^{1}$H-NMR (500 MHz, DMSO-$d_6$) δ 1.98 (1H, m, $H_{eq}$-2), 2.13 (1H, m, $H_{ax}$-2), 5.13 (1H, brs, H-3), 3.80 (1H, brs, H-4), 5.19 (1H, brs, H-5), 1.98 (1H, m, $H_{eq}$-6), 2.15 (1H, m, $H_{ax}$-6), 5.30 (1H, brs, 4-OH), 3-caffeoyl 7.00 (1H, d, *J* = 2.0 Hz, H-2'), 6.79 (1H, d, *J* = 8.0 Hz, H-5'), 7.01 (1H, brd, *J* = 8.0 Hz, H-6'), 7.46 (1H, d, *J* = 16.0 Hz, H-7'), 6.23 (1H, d, *J* = 16.0 Hz, H-8'), 5-caffeoyl 7.00 (1H, d, *J* = 2.0 Hz, H-2'), 6.79 (1H, d, *J* = 8.0 Hz, H-5'), 7.06 (1H, brd, *J* = 8.0 Hz, H-6'), 7.43 (1H, d, *J* = 16.0 Hz, H-7'), 6.22 (1H, d, *J* = 16.0 Hz, H-8'), $^{13}$C-NMR (125 MHz, DMSO-$d_6$) δ quinic acid 73.0 (C-1), 34.8 (C-2), 72.8 (C-3), 68.3 (C-4),

71.7 (C-5), 36.4 (C-6), 176.0 (C-7), 3-caffeoyl 126.2 (C-1'), 115.3 (C-2'), 146.1 (C-3'), 148.9 (C-4'), 115.3 (C-5'), 121.5 (C-6'), 145.0 (C-7'), 116.4 (C-8'), 166.5 (C-9'), 5-caffeoyl 126.1 (C-1''), 115.3 (C-2''), 146.1 (C-3''), 148.7 (C-4''), 115.2 (C-5''), 121.7 (C-6''), 145.3 (C-7''), 116.3 (C-8''), 166.8 (C-9'').

**Compound 2**

Light yellow powder, $[\alpha]_D$ -210.7 (c = 0.13, MeOH), HR-FAB-MS *m/z* 529.1349 $[M-H]^+$, $^1$H-NMR (500 MHz, $CD_3OD$) δ Quinic acid methyl ester 2.10 (1H, m, $H_{eq}$-2), 2.29 (1H, m, $H_{ax}$-2), 4.35 (1H, dt, *J* = 3.0, 2.5, H-3), 5.11 (1H, dd, *J* = 9.0, 3.0 Hz, H-4), 5.62 (1H, m, H-5), 2.23 (2H, m, H-6), 3.87 ($COOCH_3$), 4-caffeoyl 7.01 (1H, d, *J* = 2.0 Hz, H-2'), 6.91 (1H, d, *J* = 8.0 Hz, H-5'), 6.74 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 7.60 (1H, d, *J* = 16.0 Hz, H-7'), 6.27 (1H, d, *J* = 16.0 Hz, H-8'), 5-caffeoyl 6.99 (1H, d, *J* = 2.0 Hz, H-2''), 6.89 (1H, d, *J* = 8.0 Hz, H-5''), 6.74 (1H, dd, *J* = 8.0, 2.0 Hz, H-6''), 7.51 (1H, d, *J* = 16.0 Hz, H-7''), 6.18 (1H, d, *J* = 16.0 Hz, H-8''); $^{13}$C-NMR (125 MHz, $CD_3OD$) δ 76.6 (C-1), 38.9 (C-2), 69.4 (C-3), 75.2 (C-4), 69.5 (C-5), 39.8 (C-6), 56.8 ($COO\underline{C}H_3$), 177.3 ($\underline{C}OOCH_3$), 4-caffeoyl 123.6 (C-1), 115.8 (C-2), 147.6 (C-3), 148.2 (C-4), 117.0 (C-5), 123.6 (C-6), 150.1 (C-7), 115.3 (C-8), 169.1 (C-9), 5-caffeoyl 123.6 (C-1), 115.8 (C-2), 147.2 (C-3), 148.1 (C-4), 117.0 (C-5), 123.6 (C-6), 150.1 (C-7), 115.2 (C-8), 168.7 (C-9).

**Sample preparation for HPLC**

Plant materials (20 g, young and mature APO) was sonicated in 400 mL at 40°C for 6 h. The extracted solution was filtered and evaporated under reduced pressure. For HPLC analysis, the viscous MeOH extract was freeze-dried to produce a solid MeOH extract. The two MeOH extracts were dissolved in 80% MeOH and filtered using 0.50 μg syringe filter prior to injection.

**HPLC analysis**

The two MeOH extracts, and fractionated extracts (ether-, EtOAc- and BuOH fractions) and compounds **1** and **2** were dissolved in 80% MeOH and filtered using 0.50 μg syringe filter prior to injection. UV detection was performed at a fixed wavelength of 246 nm. The mobile phase consisted of 0.05% phosphoric acid as the A solvent and MeOH as the B solvent, and was eluted with a flow rate 1.00 mL/min: (0-10 min, 60% A : 40% B; 10-20 min, 50% A : 50% B; 20-30 min, 40% A : 60% B; 30-35 min, 60% A : 40% B). Test samples of the three concentrations (50, 100 and 200 μg/mL) of the two standard compounds **1** and **2** were run. Using peak areas obtained, regression equations were established: y = 384.13x - 362.5, $t_R$ 9.7 min (compound **1**) and y = 142.01x - 161.0, $t_R$ 17.0 min (compound **2**), where y is peak area (μV), x is (concentration, μg/mL). Both $R^2$ values were more than 0.998. The concentration levels in the plant materials, the extract or fractions were expressed as mg/g unit or percentage ratio of the extract, calculated from the regression equation using the peak area on HPLC chromatograms.

**Assay for peroxynitrite scavenging activity**

Peroxynitrite-scavenging activity was determined by a modified Kooy's method (Kooy et al., 1994). This assay monitors highly fluorescent rhodamine 123 rapidly formed from non-fluorescent DHR 123. Rhodamine buffer (pH 7.4) is consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final concentration of DHR 123 was 5 μM. Buffer solution was prepared before use and placed on ice. The plant materials were dissolved in 10% DMSO (f.c. 5 μg/mL) as test samples. The background and fluorescent intensity of oxidized DHR 123 was measured after treatment with or without the addition of authentic $ONOO^-$ (10 μM) dissolved in 0.3 N sodium hydroxide. The fluorescent intensity of oxidized DHR 123 was evaluated using microplate fluorescent reader FL 500 (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 480 and 530 nm, respectively. The level of peroxynitrite scavenging effect was calculated by subtracting background fluorescence from the final fluorescence intensity via the detection of DHR oxidation. L-penicillamine was used as a positive control. Data of peroxynitrite assay was shown as mean ± S.E.M.

## RESULTS AND DISCUSSION

The MeOH extracts from young and mature APOs were fractionated into ether-, EtOAc- and BuOH fractions. As shown in Table I, the peroxynitrite scavenging effects were strong throughout all the extracts and fractions, though young APO had generally more potent activity than the mature one. The $IC_{50}$ of the MeOH extract from young APO was 0.26 μg/mL and the EtOAc fraction $IC_{50}$ was 0.26 μg/mL. These suggest that potent peroxynitrite scavengers will be primarily distributed in the EtOAc fraction.

ODS column chromatography of the fraction produced compounds **1** and **2**. Compound **1** was identified as 3,5-di-*O*-caffeoyl-muco-quinic acid (Kwon et al., 2000; Kim et al., 2007) and compound **2** was identified as methyl 3,4-di-*O*-caffeoylquinic acid based (Basnet et al., 1996) (Fig. 1). In the peroxynitrite scavenging assay, the $IC_{50}$ values of **1** and **2** were 0.18 μg/mL and

**Table I.** $IC_{50}$ value (μg/mL) on peroxynitrite scavenging activity of MeOH extracts, its fractions and the isolated compounds of young and mature APOs

| Treatment | Young APO | Mature APO | Compound |
|---|---|---|---|
| MeOH extract | 0.26 ± 0.01 | 0.93 ± 0.03 | - |
| Ether fraction | 0.30 ± 0.01 | 4.04 ± 0.29 | - |
| EtOAc fraction | 0.26 ± 0.00 | 0.68 ± 0.04 | - |
| BuOH fraction | 0.28 ± 0.00 | 0.89 ± 0.04 | - |
| **1** | - | - | 0.18 ± 0.01 |
| **2** | - | - | 0.12 ± 0.00 |
| L-Penicillamine* | - | - | 0.28 ± 0.03 |

$ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123 as described in materials and methods. Data are mean ± S.E.M. of triplicate experiments. *used as a positive control.

0.12 μg/mL, respectively, lower than the positive control (L-penicillamine).

Using **1** and **2** as standard compounds, HPLC analysis was performed to compare the levels in young and mature APOs (Fig. 2). HPLC chromatograms of the MeOH extract and EtOAc fraction of young APO were shown in Fig. 2 as typical chromatograms. The percentage ratio of the MeOH extract and EtOAc fraction were determined to assess viability for practical use. Young APO had much higher levels (**1**: 30.3 mg/g and **2**: 27.7 mg/g), than mature APO (**1**: 1.77 mg/g and **2**: 4.10 mg/g) (Table II). The proportion of those compounds in the young APO was 50.5% and 35.7% of the EtOAc fraction for compounds **1** and **2**, respectively, indicating that these are primary components of the EtOAc fraction.

A variety of peroxynitrite scavengers occur in natural resources: hesperetin ($IC_{50}$ 4.67 μg/mL) obtainable

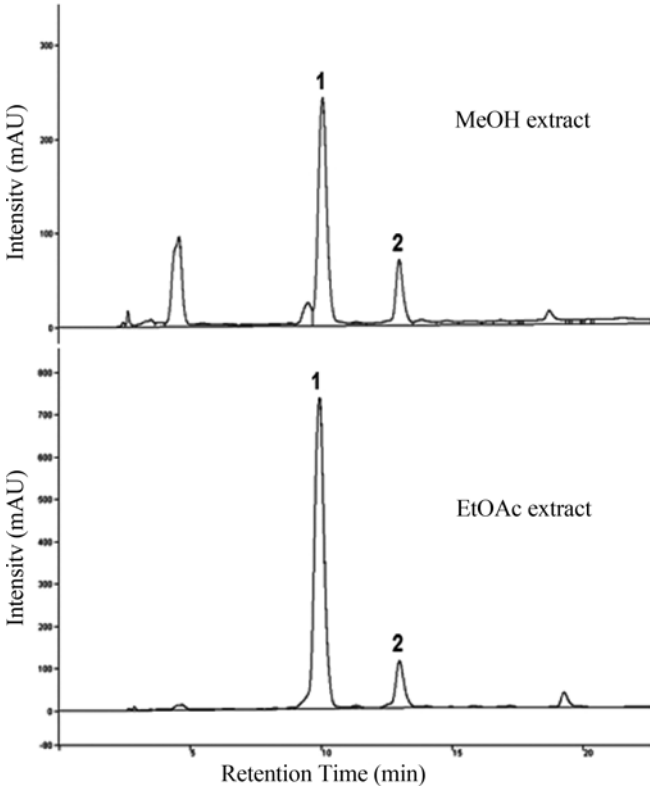


**Fig. 2.** HPLC chromatograms MeOH extract and EtOAc extract of *Artemicia princeps* var. *orientalis*

from Citrus species, (Kim et al., 2004) prenylated flavonoids ($IC_{50}$ 0.62-7.30 μg/mL) such as kushenol C from *Sophora flavescens* (Jung et al., 2008) and aurantioobtusin (an anthraquinone, $IC_{50}$ 1.43 μg/mL) from *Cassia tora* (Hyun et al., 2009).

Caffeoylquinic acids also show the inhibition of NO formation (Olmos et al., 2008), neuroprotective (Hur

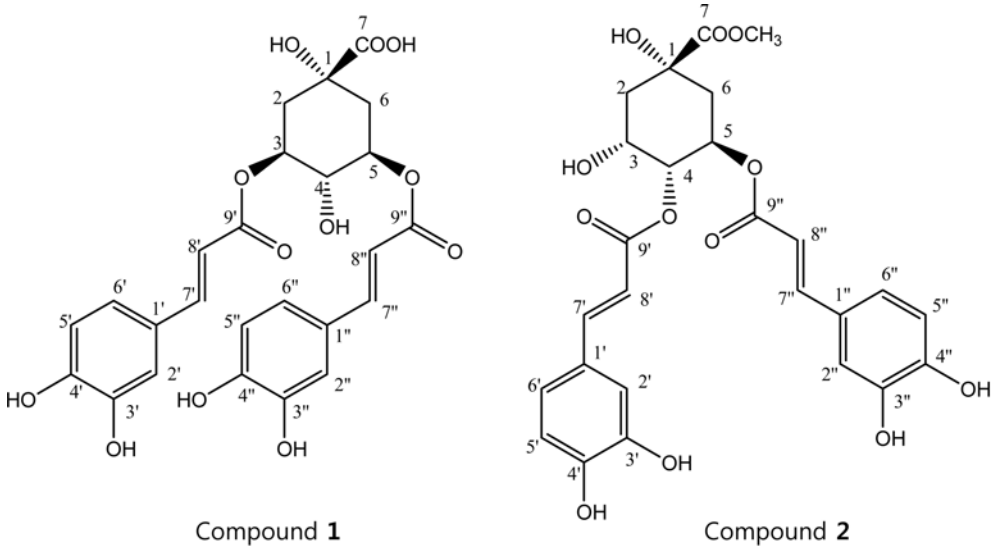


**Fig. 1.** Structure of compounds **1** and **2** isolated from APO

**Table II.** Contents of compounds **1** and **2** in the plant materials and its MeOH extracts and its EtOAc fraction of young and mature APOs

| | Young APO | | | Mature APO | | |
|---|---|---|---|---|---|---|
| | mg/g dw | % of MeOH ext. | % of EtOAc fr. | mg/g dw | % of MeOH ext. | % of EtOAc fr. |
| **1** | 30.3 ± 0.62 | 17.0 ± 0.35 | 50.5 ± 0.51 | 1.77 ± 0.04 | 2.01 ± 0.05 | 9.61 ± 0.29 |
| **2** | 27.7 ± 2.65 | 15.6 ± 1.49 | 35.7 ± 1.62 | 4.10 ± 4.10 | 4.66 ± 0.16 | 20.0 ± 1.14 |

et al., 2001) and antiviral effects (Kwon et al., 2000). Overproduction of peroxynitrite causes hypercholesterolemia, atherosclerosis, obesity and diabetes mellitus (Patcher et al., 2005; Drel et al., 2007; Korda et al., 2008).

In conclusion, young APO had higher levels of the two peroxynitrite scavengers of 3,5-di-*O*-caffeoyl-mucoquinic acid and methyl 4,5-di-*O*-caffeoylquinic acid than mature APO. Caffeoylquinic acids contribute to the pharmacological action of APO, and potentially its antidiabetic, (Jung et al., 2008) antiallergic (Lee et al., 2007) and antiatherosclerotic activities (Han et al., 2009). The EtOAc fraction of young APO has high levels of potent peroxynitrite scavengers. This is the first report of the isolation of **1** and **2** from APO.

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