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Antioxidant Caffeic acid Derivatives from Leaves of *Parthenocissus tricuspidata*

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Five caffeic acid derivatives; methyl ester of caffeoylglycolic acid (**1**), dimethyl ester of caffeoyltartaric acid (**2**), dimethyl ester of caffeoyltartronic acid (**3**), monomethyl ester of caffeoyltartronic acid (**4**), methyl ester of caffeic acid (**5**), and some other secondary metabolites including; quercetin, quercetin 3-O-β-D-glucuronide methyl ester, kaempferol, 3,5,7,4'-O-tetramethylkaempferol, β-sitosterol glucoside, 2α-hydroxyursolic acid and 2,24-dihydroxyursolic acid, have been isolated and characterized. All the isolated compounds were characterized with the help of NMR spectroscopy and mass spectrometry. Structure of compound **3** was also confirmed by a single X-ray crystallographic technique. Isolates were evaluated for antioxidant activities and most of the tested compounds were found to be potent in DPPH free radical scavenging (IC_{50} = 4.56-14.17 μg/mL) and superoxide anion scavenging (IC_{50} = 0.58-7.39 μg/mL) assays.

Key words: *Parthenocissus tricuspidata*, Caffeic acid derivatives, Antioxidant activities

INTRODUCTION

Parthenocissus tricuspidata (Sieb. et Zucc.) Planch. is a woody vine that typically grows 30-50'. This is a vigorous tendril climber that needs no support and adheres to flat surfaces (e.g., brick, stone or wood walls) *via* adhesive disks at the tendril ends. It provides excellent covering for walls, trellises, arbors or fences. It may also be grown on the ground to cover old stumps, rock piles or other eyesores for erosion control on slopes.

The leaves have been used as folk medicine in Korea for the treatment of arthritis, jaundice, toothache, neuralgia *etc.* (Hwang *et al.,* 1995). Some phenolic compounds have been reported from this plant (Hwang *et al.,* 1995; Paulino, 1999; Toshiyki *et al.*, 1998, Wang *et al.*, 1982).

Due to the medicinal importance, we decided to search out active constituents from this creeping herb, and from methanolic extract, caffeic acid derivatives, flavonoids, steroids, and triterpenes have been isolated. Most of the compounds have been reported for the first time from this source.

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MATERIALS AND METHODS

General

Optical rotations were measured on Autopol III automatic polarimeter (Rudolph Research Co. Flanders, NJ). ^{1}H-NMR spectra were recorded on Bruker 300 MHz spectrometer using TMS as internal standard. ^{13}C-NMR spectra were recorded on Bruker 75 MHz spectrometer. HMBC and HMQC spectra were obtained with the usual pulse sequences, and data processing was performed with standard Bruker software. Preparative HPLC was performed on a Waters pump model 510 with a photodiode array detector (Waters model 996) using a LichroCART RP-18 (10 mm × 250 mm) column.

Plant material

Leaves were collected from Korea Institute of Science and Technology (KIST) in July 2002 and identified by Prof. Ho Young Choi at Kyung Hee University in Korea. The voucher specimen (944-11A) has been deposited in KIST library.

Extraction and partition

The collected leaves were dried under shade for 2 days (4.8 kg) and then were extracted with methanol (27 × 3 L).

The solvent was evaporated using rotary evaporator. The crude extract (344.2 g) was diluted with water (1000 mL) and extracted successively with methylene chloride, ethyl acetate and butanol (800 mL × 3) each.

Isolation, purification, and characterization

Ethyl acetate fraction (11.29 g) was chromatographed on Sephadex LH-20 eluting with methanol and four fractions were collected on the basis of TLC profiles. Fractions 1 and 2 were mainly containing terpenoids, steroids and esters of tartaric acid. Fraction 3 was containing caffeoyl derivatives and fraction 4 was mainly containing flavonoids.

Fraction 3 was subjected to silica gel column chromatography using a mixture of methylene chloride and methanol (9.7:0.3) as eluting solvent that yielded compounds **2** (5.1 mg, yellowish brown powder) and **4** (4.1 mg, yellowish powder), and a sub-fraction was further purified by HPLC using gradient elution. The gradient elution was carried out by a flow rate of 1.5 mL/min with the increasing percentage of acetonitrile in water (20%-40%) that yielded compounds **3** (119.0 mg, yellowish brown crystals), **1** (45.2 mg, yellowish brown powder), and **5** (9.1 mg, yellow powder).

Compound **1**: FAB-MS (negative-ion mode): *m/z* 251 (for $C_{12}H_{12}O_6$); ^{1}H-NMR (Acetone-d_6, 300 MHz): δ 7.68 (1H, d, J = 15.7 Hz, H-7), 7.24 (1H, d, J = 1.9 Hz, H-2), 7.11 (1H, dd, J = 8.1 & 1.9 Hz, H-6), 6.92 (1H, d, J = 8.1 Hz, H-5), 6.42 (1H, d, J = 15.7 Hz, H-8), 4.77 (2H, s, H-1') and 3.67 (3H, s, OMe); ^{13}C-NMR (Acetone-d_6, 75 MHz): δ 127.8 (C-1), 114.8 (C-2), 147.5 (C-3), 149.5 (C-4), 115.7 (C-5), 123.3 (C-6), 146.8 (C-7), 116.8 (C-8), 167.3 (C-9), 61.4 (C-1'), 169.6 (C-2') and 52.7 (OMe).

Compound **2**: $[\alpha]_D^{25}$ +7.05° (*c* 0.0085, MeOH); FAB-MS (negative-ion mode): *m/z* 339 (for $C_{15}H_{16}O_9$); ^{1}H-NMR (CD$_3$OD, 300 MHz): δ 7.58 (1H, d, J = 15.9 Hz, H-7), 6.96 (1H, d, J = 1.9 Hz, H-2), 6.88 (1H, dd, J = 8.1 & 1.9 Hz, H-6), 6.70 (1H, d, J = 8.1 Hz, H-5), 6.22 (1H, d, J = 15.9 Hz, H-8), 5.46 (1H, d, J = 2.6 Hz, H-2'), 4.78 (1H, d, J = 2.6 Hz, H-3'), 3.69 (3H, s, OMe) and 3.65 (3H, s, OMe); ^{13}C-NMR (CD$_3$OD, 75 MHz): δ 128.0 (C-1), 114.0 (C-2), 149.0 (C-3), 150.4 (C-4), 115.6 (C-5), 123.7 (C-6), 147.3 (C-7), 116.9 (C-8), 168.1 (C-9), 169.6 (C-1'), 75.1 (C-2'), 72.3 (C-3'), 172.2 (C-4'), 53.5 (OMe) and 53.1 (OMe).

Compound **3**: FAB-MS (negative-ion mode): *m/z* 309 (for $C_{14}H_{14}O_8$); ^{1}H-NMR (Acetone-d_6, 300 MHz): δ 7.69 (1H, d, J = 15.8 Hz, H-7), 7.23 (1H, d, J = 2.0 Hz, H-2), 7.12 (1H, dd, J = 8.1 & 2.0 Hz, H-6), 6.90 (1H, d, J = 8.1 Hz, H-5), 6.42 (1H, d, J = 15.8 Hz, H-8), 5.66 (1H, s, H-2') and 3.80 (6H, s, 2 × OMe); ^{13}C-NMR (Acetone-d_6, 75 MHz): δ 126.7 (C-1), 112.9 (C-2), 145.9 (C-3), 148.9 (C-4), 115.0 (C-5), 122.7 (C-6), 147.8 (C-7), 116.0 (C-8), 165.6 (C-9), 165.4 (C-1'), 71.9 (C-2'), 165.4 (C-3') and 52.9 (2 × OMe).

Compound **4**: $[\alpha]_D^{25}$ -7.78° (*c* 0.0018, MeOH); FAB-MS (negative-ion mode): *m/z* 295 (for $C_{13}H_{12}O_8$); ^{1}H-NMR (CD$_3$OD, 300 MHz): δ 7.59 (1H, d, J = 15.8 Hz, H-7), 6.97 (1H, br s, H-2), 6.85 (1H, br d, J = 7.8 Hz, H-6), 6.66 (1H, d, J = 8.1 Hz, H-5), 6.28 (1H, d, J = 15.8 Hz, H-8), 5.28 (1H, s, H-2') and 3.67 (3H, s, OMe); ^{13}C-NMR (CD$_3$OD, 75 MHz): d 125.6 (C-1), 112.3 (C-2), 146.6 (C-3), 151.0 (C-4), 113.6 (C-5), 122.5 (C-6), 147.5 (C-7), 115.7 (C-8), 167.5 (C-9), 169.2 (C-1'), 74.8 (C-2'), 169.0 (C-3') and 51.9 (OMe).

X-ray structure determination of 3

Suitable yellowish crystals of **3** were obtained by recrystallization from MeOH. The X-ray data was collected on an Enraf-Nonius CAD4 automated diffractometer equipped with a Mo X-ray tube (λ = 0.71073 Å) and a graphite crystal monochromator. The crystal (0.24 × 0.35 × 0.35 mm) belongs to the triclinic system, space group *P*1, with a = 7.0027 (17) Å, b = 7.934 (2) Å, c = 13.349 (3) Å, V = 688.9 (3) Å^3, $Z = D_{calcd}$ = 2, 1.496 Mg/cm^3. The orientation matrix and unit cell dimensions were determined from 25 machine centered reflections in the 2θ range of from 1.5 to 25°. The variation of intensities was monitored by a repeated check of intensities of three reflections every 1 h during the data collection period. A direct method was employed to find all the atoms. Subsequent cycles of Fourier map and least squares refinements were followed (Sheldrick, 1997). All non-hydrogen atoms were refined anisotropically for **3**. Hydrogen atoms were included in the structure factor calculation using a riding model. All the calculations were carried out using VAX and PC computers. The refinement converged to a final $R1$ = 0.0413, $wR2$ = 0.1068, where $R1 = \Sigma||F_o| - |F_c||/\Sigma|F_o$, $wR2 = \{\Sigma w(F_o^2 - F_c^2)^2/\Sigma wF_o^4\}^{1/2}$, for 1860 observed reflections [$I > 2\sigma(I)$] and 201 variable parameters.

Protocol for antioxidant activities

Scavenging effects on DPPH radical

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was determined by using a reaction mixture containing test samples (dissolved in EtOH) and 100 μM DPPH ethanolic solution in 96-well micro titer plates. The reaction mixture was incubated at 37°C for 30 minutes and absorbance was measured at 515 nm. Percentage inhibition was determined by comparison with an ethanol treated control group.

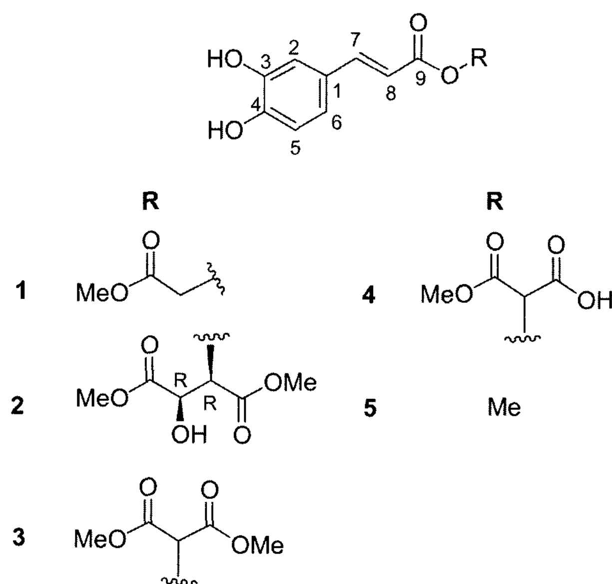
IC_{50} values denote the concentration of samples required to scavenge 50% DPPH free radicals (Gamez *et al.,* 1998).

Scavenging effects on superoxide anion

The superoxide anion radical scavenging activities were

measured following the method reported in literature (Shisuo *et al.,* 1991). The standard reaction mixture containing 1.0 mM xanthine and 1.0 mM EDTA, bovine serum albumin (50 μg of protein/mL), 40 mM sodium carbonate (pH 10.2), 25 μM nitrobluetetrazolium, sample solution and 7×10^{-3} U/mL xanthine oxidase, with a final volume of 3 mL was incubated at 25°C for 20 minutes. The reaction was terminated after incubation by the addition of 6.6 μL of 6 mM $CuCl_2$ solution. The absorbance of the formazan produced was determined at 560 nm; 0.1 mM formazan was produced in the absence of the sample solution. Inhibitory effects of the samples on the generation of superoxide anion were estimated using the following equation:

$$\text{Inhibitory ratio} = \frac{(\text{Absorbance without sample} - \text{Absorbance with sample})}{\text{Absorbance without sample}} \times 100$$



RESULTS AND DISCUSSION

The medicinal importance of this herb motivated us to search for biologically active secondary metabolites. Therefore, its methanolic extract was partitioned in various solvents and different fractions were screened for antioxidant activities. Among the tested fractions, ethyl acetate fraction showed some potential DPPH radical scavenging effect (IC_{50} = 7.87 ± 0.44 μg/mL) and superoxide anion radical anion scavenging effect on X/XO system (IC_{50} = 4.47 ± 0.33 μg/mL).

Column chromatography of ethyl acetate fraction was carried out using Sephadex LH-20 as stationary phase and was eluted with methanol to give four fractions. All the fractions were chromatographed individually, and caffeic acid derivatives, flavonoids, steroids, and triterpenes were isolated.

Compound **1** was obtained as a yellowish white powder and its molecular formula ($C_{12}H_{12}H_6$) was established by negative-ion mode FABMS as the molecular ion peak was observed at *m/z* 251. In the ^{1}H-NMR spectrum, the signals at δ 7.24 (d, *J* = 1.9 Hz), 7.11 (dd, *J* = 8.1 & 1.9 Hz) and δ 6.92 (d, *J*= 8.1 Hz) corresponding to three aromatic protons of ABX system and the signals at δ 7.68 (d, *J* = 15.7 Hz) and 6.42 (d, *J* = 15.7 Hz) corresponding to the *trans* olefinic protons indicated the presence of a *trans*-caffeoyl group in **1**. The signals at δ 4.77 (s, H-1') and 3.67 were attributed to oxygenated methylene of glycolic acid moiety and methoxyl group of methyl ester, respectively. In the ^{13}C-NMR spectrum of **1**, the carbonyl carbons at δ 167.3 (C-9) of caffeoyl moiety and δ 169.6 (C-2') of glycolic acid moiety also supported the above assignments. Furthermore, the signals at δ 6.42 (H-8) and 4.77 (H-1') were correlated with the signal at δ 167.3 (C-9) and the signal at δ 3.67 of methoxy group was correlated with the signal at δ 169.6 (C-2') in HMBC spectrum. On the basis of these results, the structure of **1** was characterized as caffeoylglycolic acid methyl ester, which has never been reported from any natural source. However, compound **1** could be the artifact of caffeoylglycolic acid (Budzianowski, 1997) and reported as a fragment during mass spectrometric analysis of caffeoyltartronic acid (Snook *et al.,* 1993; Kolodynska *et al.,* 1967).

Compound **2** was obtained as a yellowish white powder. The ^{1}H-NMR spectrum showed altogether nine signals five of them were attributed to caffeoyl moiety. The ^{1}H-NMR spectrum afforded two doublets at δ 5.46 (*J*= 2.6 Hz, H-2') and 4.78 (*J*= 2.6 Hz, H-3') due to two oxygenated methines and singlets at δ 3.69 and 3.65 for two methoxyl units. In the ^{13}C-NMR, six signals at δ 53.3 and 53.1 (OMe), 75.1 and 72.3 (CH), and 172.2 and 169.6 (C=O) were identified as tartaric acid dimethyl ester in addition to the signals of caffeic acid. The downfield chemical shift of the methine proton at δ 5.46 (H-2) revealed its connectivity to the carboxylic function of caffeic acid, which was correlated with the signal at δ 168.1 (C-9) in HBMC spectrum and thus the compound was characterized as caffeoyltartaric acid dimethyl ester (**2**). Absolute and relative stereochemistry were assigned as 2'*R* and 3'*R* by analyzing coupling constant (2.6 Hz) between H-2' and H-3' in H-NMR spectra, and positive value of optical rotation ($[\alpha]_D^{25}$ +7.05°), which was compared with the given value for L-tartaric acid ($[\alpha]_D$+12.1°). As a result, compound **2** was characterized as caffeoyltartaric acid dimethyl ester, which has been reported as artifactual of caffeoyltartaric acid (Budzianowski, 1997; Baldi *et al.,* 1993; Luo *et al.,* 2003; Sumaryono *et al.,* 1991; Veit *et al.,* 1995).

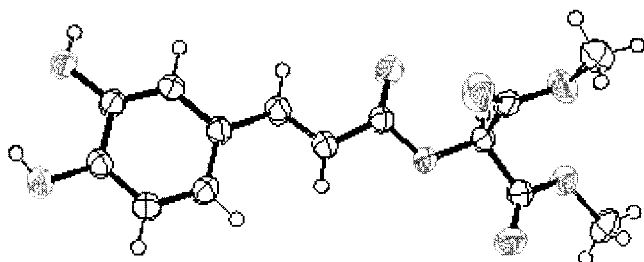


Fig. 1. X-ray drawing of **3**

The ^{1}H- and ^{13}C-NMR spectra of **3** indicated structural feature similar to those of compound **2**. In the ^{1}H-NMR spectrum, a singlet signal at δ 5.66 (H-2') corresponding to the methine proton indicated C-2' to be the connection site of tartronyl moiety to caffeoyl group. One downfield signal (δ 165.4) for two carbonyl units and two methoxyl units at δ 52.9 were resonated at the same position due to symmetry in the tartronyl methyl ester moiety. The structure of the compound **3** was confirmed as a caffeoyltartronic acid dimethyl ester with the help of the most sophisticated a single X-ray crystallographic technique. This is a new secondary metabolite from any plant source but could be the artifact due to using methanol during extraction protocol, and has been reported for the first time from our nvestigated source.

The whole NMR data of compound **4** was very similar to that of compound **3** except that in ^{1}H-NMR spectrum, only one methoxyl unit was found to resonate at δ 3.67. In addition, two carbonyl signals appeared at different positions (δ 169.2 and 169.0) due to asymmetry in tartronyl moiety. Therefore, with the help of this spectroscopic information the compound **4** was characterized as caffeoyltartronic acid monomethyl ester, which was further confirmed by negative-ion mode FAB mass spectrometry.

In addition to these compounds caffeic acid, methyl ester of caffeic acid, dimethyl & monomethyl esters of tartaric acid, β-sitosterol glucoside, 2α-hydroxyursolic acid, 2, 24-dihydroxyursolic acid, quercetin, quercetin 3-*O*-β-D-glucuronide methyl ester, kaempferol, and 3,5,7,4'-*O*-tetramethylkaempferol have also been isolated from this source. Among these, β-sitosterol glucoside, 2α-hydroxyursolic acid, 2,24-dihydroxyursolic acid, quercetin 3-*O*-β-D-glucuronide methyl ester, and 3,5,7,4'-*O*-tetramethyl-kaempferol have been reported for the first time from this source.

The antioxidant activities of all the isolated caffeic acid derivatives were investigated in DPPH free radical and superoxide anion scavenging assays. The activity data of vitamin C, vitamin E and quercetin were included for comparison. The results are shown in Table I. Among all the tested compounds, the methyl ester of caffeic acid (**5**) showed the most potent DPPH radical scavenging and superoxide anion scavenging activities IC_{50} = 4.56 and 0.58 μg/mL, respectively, consistent with previous biological results (Motoyuki and Yasuyuki, 1998).

Table I. Antioxidant activities of compounds isolated from leaves of *P. tricuspidata*

Compound	DPPH radical scavenging activity (IC_{50}, μg/mL)	Superoxide anion scavenging activity (IC_{50}, μg/mL)
1	9.67 ± 1.97	1.29 ± 0.10
2	10.71 ± 1.63	2.74 ± 0.27
3	14.17 ± 4.15	2.44 ± 0.27
4	13.13 ± 0.96	7.39 ± 3.37
5	4.56 ± 0.17	0.58 ± 0.02
Vitamin C	6.49 ± 1.07	>50
Vitamin E	12.64 ± 0.42	>50
Quercetin	4.75 ± 0.57	19.57 ± 0.44

IC_{50} values with standard deviation are at least from three independent experiments.

Other tested compounds also showed excellent inhibitory activities in superoxide anion scavenging assay than vitamin C, vitamin E and quercetin, whereas, moderate activity in DPPH radical scavenging assay (Table I) was observed.

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