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A new flavonoid glycoside from the leaf of *Cephalotaxus koreana*

KiHwan Bae [a,*], WenYi Jin [a], Phuong Thien Thuong [a], Byung Sun Min [b], MinKyun Na [a], Young Mi Lee [c], Sam Sik Kang [d]

[a] *College of Pharmacy, Chungnam National University, Daejeon 305-764, South Korea*
[b] *College of Pharmacy, Catholic University of Daegu, Gyeongsan 712-702, South Korea*
[c] *Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University, Iksan 540-749, South Korea*
[d] *Natural Products Research Institute and College of Pharmacy, Seoul National University, Seoul 110-640, South Korea*

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Abstract

A new flavone glycoside, apigenin 5-*O*-α−L-rhamnopyranosyl-(1→3)-β-D-glucopyranoside (**1**), along with four known flavonol glycosides (**2**–**5**), were isolated from the leaf of *Cephalotaxus koreana*. The new glycoside **1** showed inhibitory activity in superoxide radical scavenging assay with IC_{50} value of 13.0 μM, while it showed weak activity in 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. Compounds **2**−**5** exhibited antioxidant activity in scavenging DPPH and superoxide radicals with IC_{50} values ranging from 5.7 to 22.3 μM.
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Keywords: *Cephalotaxus koreana*; Apigenin 5-O-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranoside; Antioxidant activity

1. Introduction

Cephalotaxus koreana is endemic in Korea. The leaf has been used as a traditional medicine to treat pierced wounds by insects, and the fruit has been used as a parasiticide [1]. Previous phytochemical investigations on *Cephalotaxus* have resulted in the isolation of flavonoids [2–6], alkaloids [7,8], and sterols [9]. In our continuing study to search for natural antioxidants from plants, a water soluble fraction from the EtOH extract of the leaf of *C. koreana* exhibited strong antioxidant activity in 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (90.7% inhibition at 100 μg/ml), which led us to investigate the antioxidant compounds from the plant. Further fractionation of the water soluble fraction as guided by in vitro DPPH and superoxide radical scavenging assay afforded a new flavone glycoside, apigenin 5-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranoside (**1**), along with four known flavonol glycosides (**2**−**5**), as the active principles (Fig. 1).

* Corresponding author. Tel.: +82 42 821 5925; fax:+82 42 823 6566.
E-mail address: baekh@cnu.ac.kr (K. Bae).

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2. Experimental

2.1. General

Melting points: Electrothermal melting point apparatus.
Optical rotations: JASCO DIP-1000 KUY polarimeter.
UV: Beckman Du-650 spectrophotometer.
IR: JASCO FT/IR-5300 spectrometer with KBr discs.
FAB-MS and HR-FAB-MS: JEOL JMS-DX 300 spectrometer.
^{1}H-(600 and 300 MHz) and ^{13}C-NMR (150 and 75 MHz): Bruker FT-NMR spectrometer.
HPLC: Shimazu LC Class-vp version 6.12, equipped with an RI-8010 detector (Tosoh, Japan) and Shodex OR-2 detector (Showa-Denko, Japan), Mighysil column (250 mm × 10 mm, RP-C_{18}, 5 μm, Kanto, Japan). Column chromatographies were performed using Si–gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck), Diaion HP-20 (Mitsubishi Chemical Corporation, Japan), and Sephadex™ LH-20 (Amersham Biosciences).

2.2. Plant material

Leaf of *C. koreana* Nakai (Taxaceae), collected in October 2002 at Mount Jiri, Korea, was identified by one of the authors, KiHwan Bae. A voucher specimen (CNU 0243) was deposited in the Herbarium of the College of Pharmacy, Chungnam National University.

2.3. Extraction and isolation

The air-dried leaves (6 kg) extracted with 95% EtOH at r.t. for 48 h, filtered and evaporated in vacuo yielding a light yellow residue (960 g). The residue was suspended in water and partitioned with CH_2Cl_2. The water soluble fraction (800 g) showed good activity in DPPH free radical scavenging assay (90.7% inhibition at 100 μg/ml).It was adsorbed on highly porous polymer resin (7.0 kg, Diaion HP-20) and eluted with H_2O–MeOH gradient (H_2O, 20%, 40%, 60%, 80%, and 100% MeOH). The 40% MeOH eluted fraction (86.0 g) was subjected to Si–gel (3.0 kg) CC using CH_2Cl_2–MeOH gradient (from 15:1 to 1:1), to give six fractions (Fr.1–Fr.6). Fractions 2 (1.2 g) and 4 (3.5 g) were over Sephadex LH-20 (0.5 kg) CC with MeOH to afford compound **1** (750 mg) and **2** (14 mg), respectively. By using the

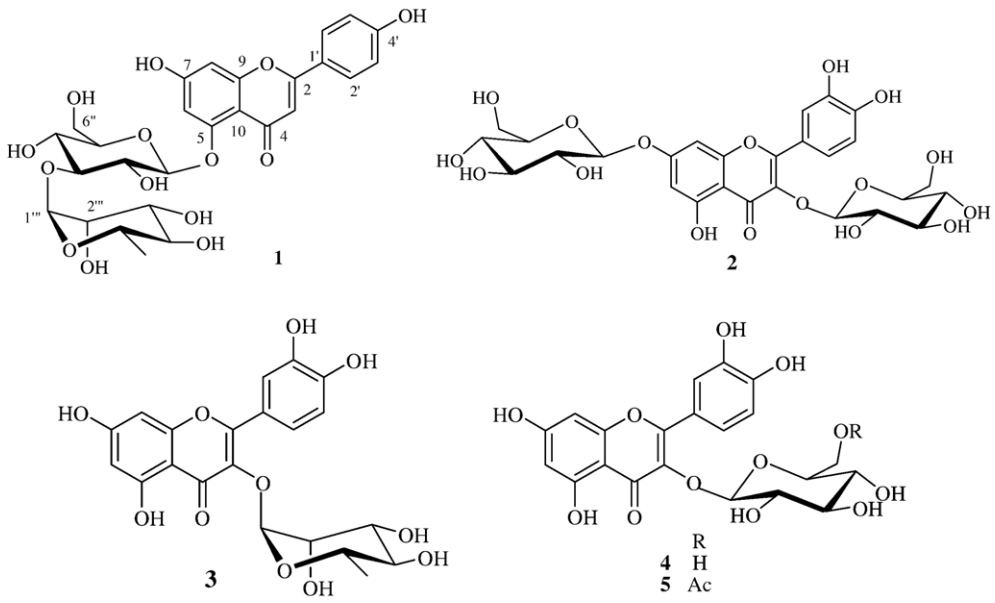

Fig. 1. Structures of compound **1**–**5**.

same method, compounds **3** (25 mg) and **4** (299 mg) were isolated from 60% MeOH eluted fraction (35 g). Compound **5** (350 mg) was purified from 80% MeOH eluted fraction (46 g). These compounds showed a satisfactory purity, >95%, as evidenced by NMR and HPLC analysis.

Compound 1. Yellow amorphous powder; mp 183–184 °C; $[\alpha]_D^{25}-22.5°$ (c 0.4, MeOH); UVmax (MeOH): 263, 328 nm; IR bands (KBr): 3400, 2930, 1720, 1650, 1610, 1510, 1455, 1360, 1260, 1180, 1080, 835 cm^{-1}; HR-FAB-MS m/z: 601.1536 [M+Na]+. Calc. for C27H30O14Na: 601.1533; 1H-NMR (600 MHz, CDCl3): δ: 7.85 (2 H, d, J 8.7 Hz, H-2', 6'), 6.91 (2 H, d, J 8.7 Hz, H-3', 5'), 6.59 (1 H, d, J 2.1 Hz, H-8), 6.52 (1 H, d, J 2.1 Hz, H-6), 6.50 (1 H, s, H-3), 5.28 (1 H, d, J 6.6 Hz, H-1"), 5.21 (1 H, d, J 0.6 Hz, H-1"'), 3.65–3.68 (2 H, m, H-6"), 3.66 (2 H, m, H-3", 3"'), 3.64 (1 H, m, H-5"'), 3.46 (1 H, m, H-5"), 3.41 (1 H, m, H-2"), 3.37 (1 H, m, H-2"'), 3.33 (1 H, m, H-4"), 3.10 (1 H, m, H-4"'), 1.02 (3 H, d, J 6.0 Hz, H-6"'); 13C-NMR (150 MHz, CDCl3): 176.3 (C-4), 162.9 (C-7), 161.3 (C-2), 160.9 (C-4'), 159.5 (C-9), 158.4 (C-5), 128.7 (C-2', 6'), 122.3±C-1'), 116.8 (C-3', 5'), 108.3 (C-10), 106.8 (C-3), 100.5 (C-1"'), 99.7 (C-6), 98.5 (C-1"), 97.1 (C-8), 77.8 (C-5"), 77.6 (C-2"), 77.5 (C-3"), 73.1 (C-4"'), 71.4 (C-3"'), 71.2 (C-2"'), 70.6 (C-4"), 68.3 (C-5"'), 61.5 (C-6"), 18.7 (C-6"').
Compound 2. ^1H and ^{13}C-NMR spectral data were in accordance with published data [10,11].
Compound 3. ^1H and ^{13}C-NMR spectral data were in accordance published data [12,13].
Compound 4. ^1H- and ^{13}C-NMR spectral data were in accordance with published data [14,15].
Compound 5. ^1H- and ^{13}C-NMR spectral data were in accordance with published data [16].

2.4. Enzymatic hydrolysis of 1

Naringinase (200 mg, from *Penicillium decumbens*) was added to a suspension of **1** (10.0 mg) in 50 mM acetate buffer (pH 5.5) and the mixture was stirred at 37 °C for 5 h. The reaction mixture was extracted with EtOAc (3×1000 ml), and the organic layer was evaporated to dryness. The residue was Si–gel CC using a gradient of $CHCl_3$-MeOH (20:1 to 10:1) to give apigenin (4.8 mg) as a yellow amorphous powder. The water layer was passed through a Sep–Pak C_{18} cartridge (Waters, Milford, MA), which was then analyzed by HPLC under the following condition: solvent MeCN–H_2O (3:1); flow rate 0.5 ml/min; detection RI and OR. The identification of D-glucose and L-rhamnose present in the water layer were carried out by the comparison of their retention times and polarities with those of authentic samples: t_R 17.5 min (d-glucose, positive polarity) and t_R 11.7 min (l-rhamnose, negative polarity).

2.5. Bioassys

Free radical (DPPH) scavenging activity was examined as previously reported [17]. In brief, 20 μl of each sample in DMSO was seeded to 980 μl of 150 μM methanolic DPPH and incubated at r.t. After 30 min, the absorbance of the mixture was measure at 517 nm on a Shimazu UV-1240 spectrometer.

Superoxide radical scavenging activity was assayed by NBT reduction method [18]. The 495 μl assay mixture consisted of 50 mM sodium carbonate buffer (pH 10.2), 0.1 mM xanthine and 25 μM nitro blue tetrazolium (NBT). The reaction was initiated by addition of 5 μl 20 nM xanthine oxidase in the presence or absence of each compound. The increase in absorbance at 560 nm was read after 5 min on a spectrophotometer Shimadzu UV-1240. Superoxide radical scavenging activity was expressed by the degree of NBT reduction decrease of test group in comparison with that of the control group [19].

3. Results and discussion

Repeated column chromatography (including normal silica gel, Diaion HP-20, and Sephadex LH-20) of the water soluble fraction from EtOH extract of leaf of *C. koreana* led to the isolation of a new flavone glycoside (**1**) together with four known flavonol glycosides (**2**–**5**). Compounds **2**–**5** were identified as quercetin 3,7-di-*O*-glucoside (**2**) [10,11], quercetin 3-*O*-α-L-rhamnopyranoside (**3**) [12,13], quercetin-3-*O*-β-D-glucopyranoside (**4**) [14,15], and quercetin 3-*O*-(6"-*O*-acetyl)-β-d-glucoside (**5**) [16] (Fig. 1).

Compound **1** was obtained as light yellowish amorphous powder with $[\alpha]_D^{25}-22.5°$ (c 0.4, MeOH). It showed greenish brown color in $FeCl_3$ test and red color in HCl–Mg reaction. Bands were observed at 263 and 328 nm in UV spectrum. The IR spectrum of **1** showed the bands for hydroxyl group at 3400 cm^{-1}, carbonyl group at 1720 cm^{-1},

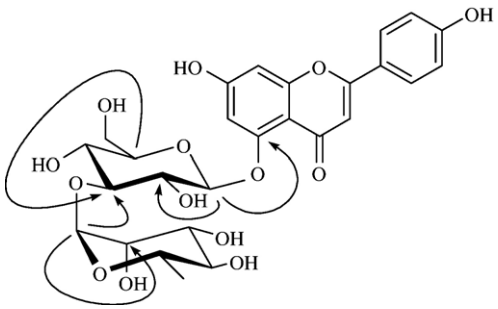

Fig. 2. The HMBC key correlations of compound **1**.

aromatic rings at 1650 and 1510 cm^{-1}, and glycosidic C–O at 1080 cm^{-1}. HR–FAB–MS gave a quasimolecular ion peak at *m/z* 601.1536 $[M+Na]^+$ providing the formula $C_{27}H_{30}O_{14}$ (calcd for $C_{27}H_{30}O_{14}Na$: 601.1533). The ^{1}H-NMR spectrum of *1* showed signals assignable to four *ortho* coupled aromatic protons at δ 7.85 and 6.91, two *meta* coupled aromatic protons at δ 6.59 and 6.52, an siglet proton at δ 6.50, two anomeric protons at δ 5.28 (1H, *d*, *J* 6.6 Hz) and 5.21 (1H, *d*, *J* 0.6 Hz), two methylene protons at δ 3.65−3.68, eight methine protons at δ 3.10–3.66, and a doublet methyl proton at δ 1.02. The ^{13}C-NMR and DEPT spectra revealed 27 carbon signals, including a carbonyl carbon at δ 176.3, twenty aromatic carbons at δ 97.1–162.9, two anomeric carbons at δ 98.5 and 100.5, eight oxygenated methine carbons at δ 68.3–77.8, a methylene carbons at δ 61.5, and a methyl carbon at δ 18.7. From these data, compound **1** was considered as a flavone with two sugar residues. The aglycone (**1a**), obtained from an enzymatic hydrolysis of **1**, was identified as apigenin [20]. The enzymatic hydrolysis of **1** afforded two monosaccharide units, which were identified as D-glucose and l-rhamnose by HPLC. The configurations of the glycosidic linkage of the glucopyranoside and rhamnopyranoside were determined to be β and α, respectively, on the basis of the $J_{H-1''}$ value (6.6 Hz) and small $J_{H-1'''}$ value (0.6 Hz, anti-diequatorial) of the anomeric protons [11]. The attachment position of the sugar to the aglycone was deduced from the ^{13}C-NMR spectroscopic data and was verified by the result of HMBC study. By comparison of the carbon signals in the ^{13}C-NMR spectrum of **1** with those of **1a**, glycosylation shift was observed at C-5 of **1**, where the chemical shift of **1** was observed in higher field (δ 158.4) than δ 161.4 of **1a**. The C-3" of glucose moiety was shifted to lower field (δ 77.5) compared to **1a** [12]. These observation were further supported by the HMBC correlation between δ_H 5.28 (H-1") and δ_C 158.4 (C-5), δ_H 5.21 (H-1"') and δ_C 77.5 (C-3") (Fig. 2). Therefore, based on the above data, the structure of compound **1** was established as apigenin 5-*O*-α−L-rhamnopyranosyl-(1→3)-β-D-glucopyranoside (Fig. 1).

Compounds **1**−**5** were tested for their antioxidant activity against DPPH [17] and superoxide radical [18]. The results are presented in Table 1. In the DPPH radical assay, the flavonol glycosides **2**–**5** which have a quercetin moiety as the aglycone exhibited stronger scavenging activity than compound 1. This indicates that the presence of *ortho*-dihydroxyl group in B ring contributes to the strong radical scavenging activity of the compounds, which is in accordance with those previously reported. To further characterize the isolates as antioxidants, their ability to scavenge

Table 1
Antioxidant activities of compounds **1**–**5** isolated from *C. koreana*

Compounds	DPPH radical scavenging, IC_{50} (μM)[a]	Superoxide radical scavenging, IC_{50} (μM)[a]
1	>100	13.0±1.7
Apigenin	>100	2.7±0.8
2	17.2±2.1	12.8±2.3
3	22.3±1.5	12.6±1.6
4	21.1±1.9	5.7±1.1
5	18.1±2.5	5.8±0.9
Quercetin[b]	9.2±1.2	2.1±0.8
α-Tocopherol[b]	24.6±1.8	–[c]

[a] The values presented mean±SD from triplicate experiments.
[b] Positive control.
[c] Inactive.

superoxide radical was examined. As shown in Table 1, all the isolated compounds scavenged superoxide radical with IC_{50} values ranged from 5.7 to 13.0 μM. Both the aglycones, apigenin (**1a**) and quercetin, showed stronger scavenging activity than their glycosides, suggesting that substitution of sugars is responsible for a loss of antioxidant activity.

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