SHORT REPORT

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Chemical Constituents of *Gymnosporia stylosa* and Their Anti-inflammatory Activities

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Abstract: A new flavanone glycoside, gymnosporioside (**1**), and three known compounds (**2**–**4**) were isolated from the leaves of *Gymnosporia stylosa*. The structure of gymnosporioside (**1**) was determined by nuclear magnetic resonance (NMR), time-of-flight mass spectrometry (TOFMS), and circular dichroism (CD) spectral data. The known compounds were identified as prunin (**2**), acteoside (**3**), and isoacteoside (**4**), by comparing the NMR data from this study with those reported in the literature. These known compounds, **2**–**4**, have not been previously isolated from *G. stylosa*. Their anti-inflammatory activities were evaluated against lipopolysaccharides (LPS)-induced activation of nitric oxide (NO) production in RAW264.7 cells, *in vitro*. Compounds **3** and **4** showed significant inhibitory activities against LPS-induced NO production in RAW264.7 cells, with IC_{50} values of 17.8 ± 0.4 and 19.3 ± 0.3 μM, respectively.

Keywords: *Gymnosporia stylosa*; Celastraceae; flavanone; anti-inflammatory.

1. Plant Source

The leaves of *Gymnosporia stylosa* was collected in Thua Thien Hue province, Vietnam, and authenticated by Dr. Nguyen Quoc Binh, Vietnam National Museum of Nature, VAST. A voucher specimen (VN1844) has been stored at Institute of Natural Products Chemistry, VAST.

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2. Previous Studies

G. stylosa is a member of the *Gymnosporia* genus, which is one of the largest genera in the Celastraceae family. This family consists of approximately 100 genera and 1,300 species, which are primarily distributed in tropical regions [1]. *Gymnosporia* species are rich in triterpenoid, sesquiterpenoid, alkaloid, flavonoid, and phenolic compounds [2]. *Gymnosporia* species have also been found to possess biological activities, including antioxidative [3], hepatoprotective [4], cytotoxic [5], antimicrobial [6], and anti-inflammatory activities [7]. *G. stylosa* has been recognized as an oriental medicinal plant, with cytotoxic activity [2]. Previous studies examining the chemical constituents in *G. stylosa* have identified the presence of triterpenoid and phenolic compounds [2].

3. Present Study

Dried, powdered *G. stylosa* leaves (2 kg) were extracted using MeOH (3 × 8 L), by refluxing, to produce 165 g crude extract. The crude extract was suspended in water (800 mL) and successively partitioned, using $CHCl_3$ (4 × 1 L) and EtOAc (4 × 1 L). The EtOAc extract (65.0 g) was applied to an open silica gel column chromatography (CC) and eluted with $CHCl_3$–MeOH (10:1 to 0:1), yielding eighteen fractions (E1–E18). Fraction E11 (5.2 g) was further subjected to silica gel CC and eluted with $CHCl_3$–EtOAc–MeOH (5:1:0 to 0:1:1), yielding ten sub-fractions (E11.1–E11.10). Further purification of the sub-fraction E11.5 (510 mg), using an Agilent 1260 HPLC, Optima Pak RP-C18 column (10 x 250 mm ID, 5 μM particle size) and MeOH-H_2O + 0.1% formic acid (25:75 → 35:65) as the eluent, resulted in the isolation of compounds **1** (12.5 mg) and **2** (16.7 mg). Fraction E17 (1.8 g) was also subjected to silica gel CC, eluted with $CHCl_3$–MeOH (5:1 to 0:1), yielding five sub-fractions (E17.1 – E17.5). Further purification of sub-fraction E17.3 (325 mg), using an Agilent 1260 HPLC, Optima Pak RP-C18 column (10 x 250 mm ID, 5 μM particle size) and MeOH-H_2O + 0.1% formic acid (15:85 → 30:70) as the eluent, resulted in the isolation of compounds **3** (20.8 mg) and **4** (15.5 mg).

Determination of NO Production. The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatants as described previously [8-10].

*Compound **1***: Yellow amorphous powder; $[\alpha]_D^{25}$ − 112.4° (*c* 0.2, MeOH); UV λ_{max} (MeOH): 282, 332 nm; IR (KBr) ν_{max} 3400 (OH), 2858, 1710, 1628 (C = O), 1588, 1518 cm^{-1}; TOFMS *m*/*z* 647.1717 $[M + Na]^+$ (Calcd for $C_{32}H_{32}O_{13}Na$, 647.1735); CD (*c* 0.01, MeOH): $\Delta\varepsilon_{277}$ – 16.55, $\Delta\varepsilon_{303}$ + 8.75; ^{1}H NMR (400 MHz, Methanol-d_4) δ_H (ppm): 5.44 (1H, dd, *J* = 2.8, 13.2 Hz, H-2), 2.74 (1H, dd, *J* = 13.2, 16.8 Hz, H-3$_{ax}$), 2.88 (1H, dd, *J* = 2.8, 16.8 Hz, H-3$_{eq}$), 5.90 (1H, s, H-6), 7.28 (1H, d, *J* = 8.8 Hz, H-3′), 6.75 (1H, dd, *J* = 2.8, 8.8, H-4′), 7.07 (1H, d, *J* = 2.8 Hz, H-6′), 4.93 (1H, d, *J* = 8.0 Hz, H-1″), 5.12 (1H, dd, *J* = 8.0, 9.6 Hz, H-2″), 3.68 (1H, m, H-3″), 3.49 (1H, m, H-4″), 3.51 (1H, m, H-5″), 4.01 (1H, br d, *J* = 12.0 Hz, H-6″a), 3.79 (1H, dd, *J* = 5.2, 12.0 Hz, H-6″b), 7.36 (2H, m, H-2‴/H-6‴), 7.34 (2H, m, H-3‴/H-5‴), 7.39 (1H, m, H-4‴), 7.42 (1H, d, *J* = 16.0 Hz, H-7‴), 6.37 (1H, d, *J* = 16.0 Hz, H-8‴), 3.70 (3H, s, 7-OC$\underline{H}_3$), 3.47 (3H, s, 8-OC$\underline{H}_3$); ^{13}C NMR (100 MHz, Methanol-d_4) δ_C (ppm): 75.8 (C-2), 43.5 (C-3), 198.0 (C-4), 103.8 (C-4a), 160.8 (C-5), 94.2 (C-6), 162.4 (C-7), 130.6 (C-8), 154.8 (C-8a), 131.2 (C-1′), 148.3 (C-2′), 119.1 (C-3′), 116.5 (C-4′), 154.6 (C-5′), 113.2 (C-6′), 102.9 (C-1″), 75.1 (C-2″), 75.7 (C-3″), 71.6 (C-4″), 78.4 (C-5″), 62.5 (C-6″), 135.2 (C-1‴), 129.8 (C-2‴/C-6‴), 129.4 (C-3‴/C-5‴), 131.6 (C-4‴), 147.4 (C-7‴), 118.0 (C-8‴), 167.7 (C-9‴), 56.5 (7-O$\underline{C}H_3$), 61.3 (8-O$\underline{C}H_3$).

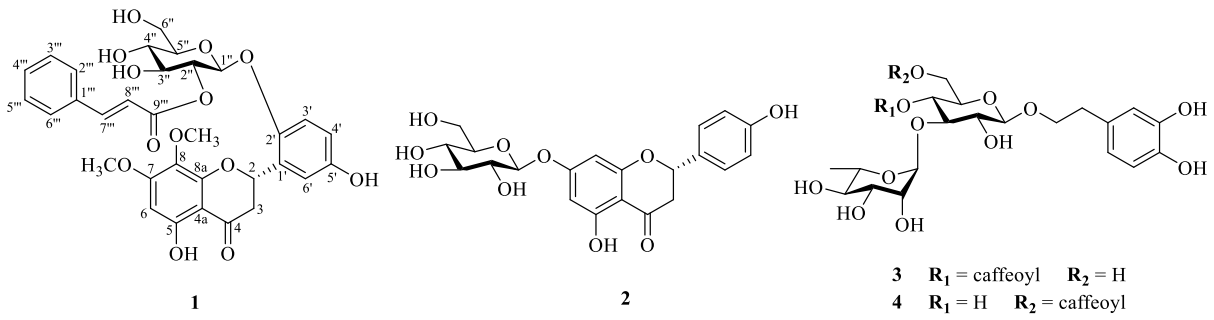

Figure 1. Chemical structure of the compounds (**1–4**) isolated from *G. stylosa*

Compound **1** was obtained as a yellow, amorphous powder, with an optical rotation of $[\alpha]_D^{25}$ −112.4°. The molecular formula of **1** was determined to be $C_{32}H_{32}O_{13}$, based on the molecular ion peak at *m/z* 647.1717 for the $[M + Na]^+$ ion (calcd. for $C_{32}H_{32}O_{13}Na$, 647.1735) in the TOFMS analysis. The ultraviolet (UV) spectrum displayed maximum absorption bands at 282 and 332 nm. The infrared (IR) spectrum of compound **1** indicated the presence of a hydroxyl group (3400 cm^{-1}), ketone (1710 cm^{-1}), carbonyl carbon (1628 cm^{-1}), and aromatic rings (1588 and 1518 cm^{-1}). The presence of an oxymethine group [δ_H 5.44 (1H, dd, *J* = 2.8, 13.2 Hz, H-2)/δ_C 75.8 (C-2)], a methylene group [δ_H 2.74 (1H, dd, *J* = 13.2, 16.8 Hz, H-3$_{ax}$) and 2.88 (1H, dd, *J* = 2.8, 16.8 Hz, H-3$_{eq}$)/δ_C 43.5 (C-3)], and a ketone at δ_C 198.0 (C-4), in the ^{1}H and ^{13}C NMR spectra, indicated that compound **1** was a flavanone (Figure 1 and Supporting information) [11,12]. The ^{1}H NMR spectrum of compound **1** further revealed the presence of an aromatic proton, at δ_H 5.90 (1H, s, H-6), two methoxy protons [δ_H 3.70 (3H, s, 7-OCH$_3$) and 3.47 (3H, s, 8-OCH$_3$)], a sugar moiety [δ_H 4.93 (1H, d, *J* = 8.0 Hz, H-1′′), 5.12 (1H, dd, *J* = 8.0, 9.6 Hz, H-2′′), 3.68 (1H, m, H-3′′), 3.49 (1H, m, H-4′′), 3.51 (1H, m, H-5′′), 4.01 (1H, br d, *J* = 12.0 Hz, H-6′′a), and 3.79 (1H, dd, *J* = 5.2, 12.0 Hz, H-6′′b)], and three other aromatic protons [δ_H 7.28 (1H, d, *J* = 8.8 Hz, H-3′), 6.75 (1H, dd, *J* = 2.8, 8.8, H-4′), and 7.07 (1H, d, *J* = 2.8 Hz, H-6′)], which were characterized as a 1,2,5-trisubstituted benzene ring (Figure 1). The signals of two *trans*-olefinic protons [δ_H 7.42 (1H, d, *J* = 16.0 Hz, H-7′′′), 6.37 (1H, d, *J* = 16.0 Hz, H-8′′′)] and five aromatic protons [δ_H 7.36 (2H, m, H-2′′′/H-6′′′), 7.34 (2H, m, H-3′′′/H-5′′′), and 7.39 (1H, m, H-4′′′)] indicated the presence of a cinnamoyl group (Figure 1 and Supporting information). The ^{13}C NMR and heteronuclear single quantum correlation (HSQC) spectra exhibited thirty-two signals, due to two methoxy carbons, at δ_C 56.5 and 61.3 ppm, and twelve *sp*^{2} carbons, at δ_C 94.2–162.4 ppm, indicating the presence of two aglycone benzene rings. In addition, the six-carbon signals of the sugar moiety, at δ_C 102.9 (C-1′′) and δ_C 62.5–78.4 (C-2′′–C-6′′), and nine carbon signals of the cinnamoyl group [δ_C 135.2 (C-1′′′), 129.8 (C-2′′′/C-6′′′), 129.4 (C-3′′′/C-5′′′), 131.6 (C-4′′′), 147.4 (C-7′′′), 118.0 (C-8′′′), and 167.7 (C-9′′′)] were also detected in the ^{13}C NMR spectrum (Figure 1 and Supporting information). Acid hydrolysis of compound **1** yielded D-glucose, which was confirmed by gas chromatography (GC) analysis, according to the previously published method [13]. The glucose linkage was determined as *β*, based on the $J_{1,2}$ value (8.0 Hz) for the anomeric proton [14-16]. Cross-peaks between H-2 and H-3, H-3′and H-4′, H-2′′ and H1′′/H-3′′, and H-7′′′ and H-8′′′ were observed in the correlation spectroscopy (COSY) spectrum (Figure 2 and Supporting information). In the heteronuclear multiple bond correlation (HMBC) spectrum, the oxygenated methine proton at δ_H 5.44 (H-2) demonstrated long-range correlations with carbon signals at δ_C 198.0 (C-4), 154.8 (C-8a), 131.2 (C-1′), 148.3 (C-2′), and 113.2 (C-6′); the aromatic proton at δ_H 5.90 (H-6) was correlated with carbons at δ_C 160.8 (C-5), 162.4 (C-7), and 130.6 (C-8), as well as two methoxy proton signals (δ_H 3.70 and 3.47), which showed correlations with carbon resonances, at δ_C 162.4 (C-7) and 130.6 (C-8), respectively, indicating the presence of two methoxy groups at C-7 and C-8 (Figure 2 and Supporting information). The anomeric proton at δ_H 4.93 (H-1′′) showed long-range correlation with the carbon signal at δ_C 148.3 (C-2′), in the HMBC spectrum, suggested that the sugar moiety was located at C-2′

(Figure 2). Interestingly, the HMBC correlations of the protons at δ_H 7.42 (H-7‴), δ_H 6.37 (H-8‴), and δ_H 5.12 (H-2″) with the carbon signal at δ_C 167.7 (C-9‴) suggested that the cinnamoyl group was located at C-2″ of the sugar moiety (Figure 2 and Supporting information).

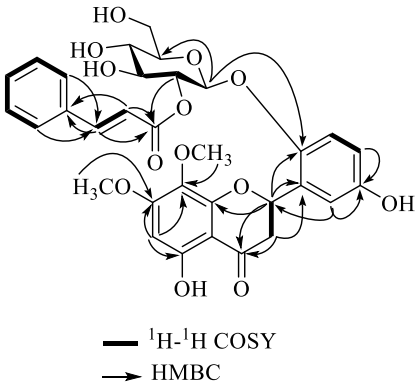

Figure 2. Important ^{1}H-^{1}H-COSY and HMBC correlations for gymnosporioside (**1**)

Flavanones that have a 2*S*-configuration can exhibit positive Cotton effects, due to an n–π^* transition (approximately 330 nm), and negative Cotton effects, due to a π–π^* transition (270–290 nm), in the circular dichroism (CD) spectra [17, 18]. In our experiment, the CD curve for compound **1** (Figure 3) exhibited positive Cotton effects at $\Delta\varepsilon_{303}$ +8.75 and negative Cotton effects at $\Delta\varepsilon_{77}$ −16.55, which established the 2*S*-configuration [17, 18]. Based on these findings, compound **1** was determined to be (2*S*)-5,5′-dihydroxy-7,8-dimethoxyflavanone-2′-*O*-*β*-D-(2″-*O*-cinnamoyl)glucopyranoside, named gymnosporioside.

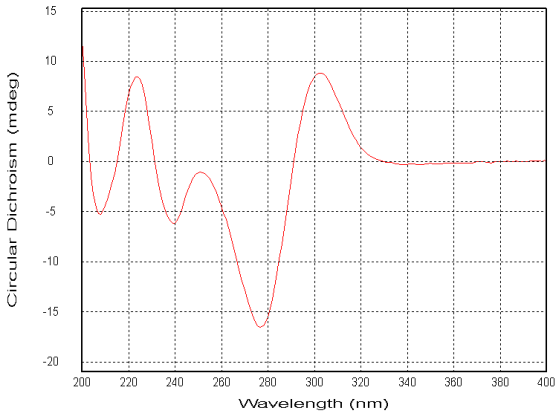

Figure 3. Experimental ECD spectrum of compound **1** in MeOH

The known compounds were identified as prunin (**2**) [19], acteoside (**3**) [20], and isoacteoside (**4**) [21], by comparing their NMR data (Supporting information) from this study with those reported in the literatures.

To test the cytotoxic effects of compounds **1**–**4** in RAW 264.7 cells, we evaluated their cytotoxicity, in the presence and/or absence of lipopolysaccharides (LPS), by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. These compounds showed no significant cytotoxic effects on cell viability, even at doses as high as 50 μM, after 24 h incubations, regardless of the presence or absence of LPS (data not shown). To examine the nitric oxide (NO) production inhibitory activity of these compounds, RAW 264.7 cells were treated with isolated compounds, at several concentrations (1, 3, 10 and 30 μM), and the level of NO production was determined by assessing nitrite quantities in the cell culture supernatants. The results demonstrated that compounds **3** and **4** effectively inhibited NO production, with IC_{50} values of 17.8 ± 0.4 and 19.3 ± 0.3 μM, respectively (Table 1). However, compounds **1** and **2** were inactive (IC_{50} values > 30 μM). In

this assay, celastrol, a natural secondary metabolite, was used as a positive inhibitor. Celastrol expressively inhibited LPS-induced NO production with an IC_{50} value of 1.0 ± 0.1 μM [9, 10, 22].

Table 1. NO production inhibitory activity of isolated compounds **1**–**4**

Compound	IC_{50} value (μM)[a]
1	> 30
2	> 30
3	17.8 ± 0.4
4	19.3 ± 0.3
Celastrol[b]	1.0 ± 0.1

[a] The inhibitory effects are represented as the molar concentration (μM) giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments (mean ± S.D).
[b] Positive control.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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