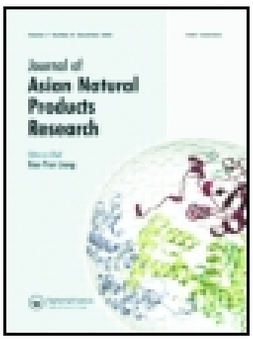

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Hepatoprotective iridoid glucosides from *Callicarpa nudiflora*

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Two new iridoid glucosides, callicoside A (**1**) and callicoside B (**2**), were isolated from the leaves of *Callicarpa nudiflora*. Their structures were elucidated by means of spectroscopic methods and chemical evidences. In an *in vitro* bioassay, compound **1** showed pronounced hepatoprotective activity against D-galactosamine-induced toxicity in WB-F344 rat hepatic epithelial stem-like cells.

Keywords: Verbenacase; *Callicarpa nudiflora*; iridoid glucosides; hepatoprotective activity

1. Introduction

Callicarpa nudiflora Hook, belonging to the family Verbenacase, is distributed widely in Guangdong, Guangxi, and Hainan Provinces in China. The plant is used as traditional Chinese herbal medicines for the treatment of inflammation and bleeding [1]. Previous investigation on *C. nudiflora* has led to the isolation of iridoids, flavonoids, triterpenoids, and phenylpropanoid glycosides [2–5]. Some of them exhibit anti-inflammatory, antibacterial, cytotoxic, and hemostatic activities [6]. In our previous papers, phytochemical studies of *C. nudiflora* yielded six triterpenoid glycosides, three flavones, and three furofuran lignans [6–8]. As part of a continuing search for bioactive constituents from *C. nudiflora*, an 80% EtOH extract of *C. nudiflora* has been investigated and two new compounds (**1** and **2**) obtained. Reported herein are the isolation, structure elucidation, and biological activity of these compounds.

2. Results and discussion

The 80% EtOH extract of *Callicarpa nudiflora* was partitioned with petroleum ether, EtOAc, and *n*-BuOH, successively. The *n*-BuOH-soluble portion was separated by a combination of silica gel, ODS column chromatography, and preparative HPLC and afforded two new compounds (**1** and **2**) (Figure 1). Their structures were elucidated by extensive NMR techniques including 1D NMR (^{1}H and ^{13}C NMR), 2D NMR (COSY, NOESY, HSQC and HMBC), and HRESIMS, as well as chemical evidences.

Compound **1** was obtained as a white amorphous powder. Its molecular formula, $C_{24}H_{30}O_{13}$, was determined from HRESIMS at *m/z* 549.1575 $[M + Na]^+$ and supported by the NMR spectroscopic data. The ^{1}H NMR spectrum of **1** in CD_3OD exhibited two *trans* olefinic protons at δ_H 7.57 (1H, d, $J = 15.6$ Hz) and 6.29 (1H, d, $J = 15.6$ Hz), a set of ABX-type coupled aromatic protons at δ_H 7.05 (1H, d, $J = 1.8$ Hz), 6.96 (1H, dd, $J = 8.4$,1.8 Hz), and 6.78 (1H, d, $J = 8.4$ Hz), and an anomeric proton at δ_H 4.70 (1H, d, $J = 7.8$ Hz) correlated with an anomeric carbon at δ_C 99.4 in the HSQC spectrum (Table 1). In addition, a group of character-

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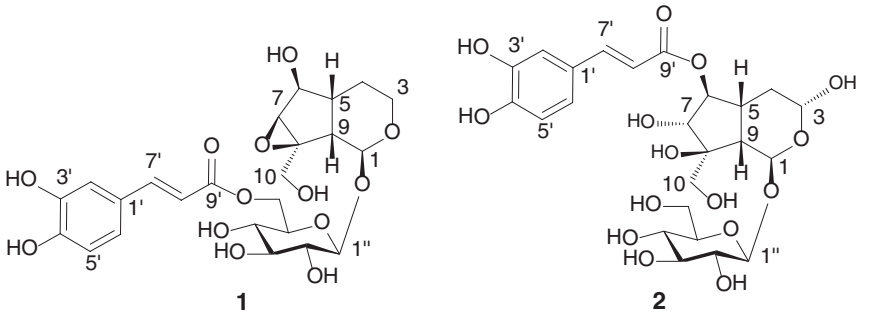

Figure 1. Chemical structures of compounds **1** and **2**.

istic signals for catalpol skeleton [9] were observed at δ_H 4.60 (1H, d, $J = 9.0$ Hz), 4.07 (1H, d, $J = 13.8$ Hz), 4.01 (1H, d, $J = 9.0$ Hz), 3.83~3.85 (1H, m), 3.64 (1H, d, $J = 13.8$ Hz), 3.39~3.41 (1H, m), 2.28 (1H, dd, $J = 9.0$, 7.8 Hz), 2.00 (1H, dd,

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compounds **1** and **2** in CD_3OD (δ in ppm, J in Hz).

	1		**2**	
Position	δ_H	δ_C	δ_H	δ_C
Catalpol				
1	4.60 (1H, d, 9.0)	97.9	5.67 (1H, d, 1.8)	93.1
3a	3.83-3.85 (1H, m)	63.0	5.32 (1H, d, 3.0)	95.9
3b	3.39-3.41 (1H, m)			
4a	1.73-1.75 (1H, m)	23.9	2.44-2.46 (1H, m)	34.4
4b	1.56 (1H, d, 13.8)		2.36-2.38 (1H, m)	
5	2.00 (1H, dd, 13.8, 7.8)	38.3	2.09 (1H, dd, 13.8, 3.0)	34.7
6	4.01 (1H, d, 9.0)	73.3	5.01 (1H, dd, 8.4, 3.0)	87.7
7	3.39-3.41 (1H, m)	62.0	4.44 (1H, d, 8.4)	70.4
8		66.0		79.9
9	2.28 (1H, dd, 9.0, 7.8)	43.3	2.62 (1H, br d, 9.6)	48.3
10a	4.07 (1H, d, 13.8)	61.5	4.04 (1H, d, 13.8, 1.2)	62.1
10b	3.64 (1H, d, 13.8)		3.68 (1H, d, 13.8, 1.2)	
caffeoyl				
1′		127.6		127.6
2′	7.05 (1H, d, 1.8)	115.1	7.07 (1H, d, 1.8)	115.2
3′		146.9		146.8
4′		149.7		149.8
5′	6.78 (1H, d, 8.4)	116.6	6.80 (1H, d, 8.4)	116.5
6′	6.96 (1H, dd, 8.4, 1.8)	123.1	6.97 (1H, dd, 8.4, 1.8)	123.1
7′	7.57 (1H, d, 15.6)	147.2	7.58 (1H, d, 15.6)	147.6
8′	6.29 (1H, d, 15.6)	114.9	6.32 (1H, d, 15.6)	114.5
9′		169.0		168.9
Glc				
1″	4.70 (1H, d, 7.8)	99.4	4.70 (1H, d, 7.8)	98.9
2″	3.25-3.27 (1H, m)	74.8	3.65-3.67 (1H, m)	73.8
3″	3.38-3.40 (1H, m)	77.6	3.29-3.31 (1H, m)	78.1
4″	3.38-3.40 (1H, m)	71.7	3.28-3.30 (1H, m)	71.6
5″	3.50-3.52 (1H, m)	75.8	3.15-3.17 (1H, m)	74.7
6″	4.46 (1H, dd, 11.4, 3.0)	64.1	3.88 (1H, dd, 12.0, 1.2)	62.7
	3.40 (1H, m)		3.67 (1H, dd, 12.0, 1.2)	

J = 13.8, 7.8 Hz), 1.73~1.75 (1H, m), and 1.56 (1H, d, J = 13.8 Hz) (Table 1). The ^{13}C NMR spectrum of **1** in combination with the HSQC spectrum displayed 25 carbon signals, including those for a carbonyl carbon at δ_C 169.0, a (*E*)—C═C bond at δ_C 147.2 and 114.9, for an aromatic ring at δ_C 149.7, 146.9, 127.6, 123.1, 116.6, 115.1, and for a sugar moiety at δ_C 99.4, 77.6, 75.8, 74.8, 71.7, and 64.1. These data suggested the presence of a caffeoyl moiety and a sugar unit in the structure of **1**. Comparison of the NMR spectroscopic data of **1** (Table 1) with those of piscroside B [10] demonstrated that two compounds were almost identical except that the *p*-coumaroyl residue in piscroside B was replaced by a caffeoyl group. Acid hydrolysis of **1** with 2 M HCl afforded D-glucose, which was identified by GC analysis of their trimethylsilyl L-cysteine derivatives [11–12]. In the HMBC spectrum of **1**, the HMBC correlation from H-6″ (δ_H 4.46) to C-9′ (δ_C 169.0) confirmed that the caffeoyl ester group is attached to C-6″ of the glucose (Figure 2). In addition, the HMBC correlation from Glc-H-1″ (δ_H 4.70) to C-1 (δ_C 97.9) indicated the β-D-glucopyranosyl unit is located at C-1. The relative configuration of **1** was established by a combination of the ^{1}H NMR spectral data and the NOESY experiment, as well as a comparison with the spectroscopic data of those reported ones [13–15]. The large coupling of H-1 (J = 9.0 Hz) with H-9 (δ_H 2.28) demonstrated the dihedral angle to be 180° between vicinally coupled protons (H-1 and H-9) [14]. H-9 gave a double doublet (δ_H 4.60, J = 9.0, 7.8 Hz) due to coupling with H-1 (δ_H 4.60) and H-5 (δ_H 2.00). Appreciable coupling with H-5 indicated that the fused ring was *cis* [14]. In the NOESY spectrum of **1**, the NOESY cross peaks of H-4α/H-6, H-7/H-10, and H-1/H-10 indicated that the hydroxy group at C-6 and the oxo-bridge from C-7 to C-8 should be β-oriented. Full assignments of the proton and carbon resonances of **1** could be achieved by comprehensive analysis of ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY spectra (Table 1). Thus, compound **1** was determined as 6′-*O*-caffeoyl-dihydrocatalpol, named callicoside A.

Compound **2** was obtained as a white amorphous powder. The HRTOFMS of **1** showed a quasi-molecular ion peak at *m*/*z* 559.1656 [M−H]$^-$, indicating a molecular formula of $C_{24}H_{32}O_{15}$. Acid hydrolysis of **2** with 2 M HCl afforded D-glucose, which was identified by GC analysis of their trimethylsilyl L-cysteine derivatives [10, 11]. On the basis of the coupling constant of anomeric proton and the chemical shift of the anomeric carbon, the anomeric configuration of the glucose moiety was determined as β [16]. Comparison of the NMR data of **2** with those of **1** indicated **2** consisted of the following structural fragments: a catalpol skeleton, a caffeoyl group, and a glucose moiety. The differences between **1** and **2** were in the catalpol moiety and the connection position of

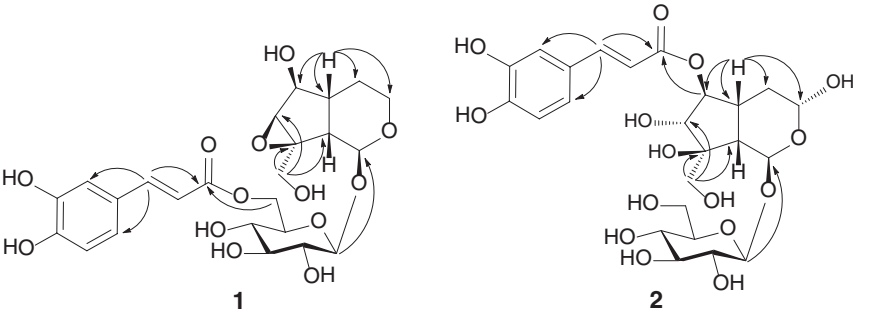

Figure 2. Key HMBC (H → C) correlations of compounds **1** and **2**.

caffeoyl residue. Meanwhile, detailed NMR analysis showed an additional hydroxy group in **2**. In the ^{13}C NMR spectrum of **2**, C-3 (δ_C 95.9) was deshielded by $\Delta\delta_C$ 32.9 as compared to **1**, indicating that the hydroxyl group is located at C-3 in **2**. In the HMBC spectrum of **2**, HMBC correlations from Glc-H-1″ (δ_H 4.70) to C-1 (δ_C 93.1) and H-6 (δ_H 5.01) to C-9′ (δ_C 168.9) indicated β-D-glucopyranosyl unit and the caffeoyl ester group are attached to C-1 and C-6 of aglycone, respectively. The relative configuration of **2** was established by a combination of the NOESY experiment and the biogenetic ground. The small coupling of H-1 ($J = 1.8$ Hz) with H-9 (δ_H 2.62) confirmed the β-orientation of H-9, since, naturally occurring iridoids display an α-orientation for H-1 [17–18]. The large coupling of H-9 ($J = 9.6$ Hz) with H-5 (δ_H 2.09) confirmed the β-orientation of H-5, demonstrating that the stereochemistry of the catalpol ring fusion was *cis*. In the NOESY spectrum of **2**, the presence of NOE interactions of H-3/H-5 and H-7/H-9 indicated that the hydroxy groups at C-3 and C-7 are in α-positions. Meanwhile, NOE interactions were observed among H-1, H-6, and H-10, which indicated the hydroxy groups at C-6 and C-8 should be β-oriented. Thus, compound **2** was elucidated as 7-*O*-caffeoyl-3, 6, 8-trihydroxy-8-(hydroxymethyl)cyclopenta[*c*]pyran-1-*O*-β-D-glucopyranoside, named callicoside B.

In an *in vitro* bioassay, compound **1** at 10^{-5} M showed pronounced hepatoprotective activities against D-galactosamine-induced toxicity in WB-F344 rat hepatic epithelial stem-like cells (Table 2).

Table 2. Hepatoprotective effects of compounds **1**–**2** against D-galactosamine-induced toxicity in WB-F344 cells.[a]

Compound	$\overline{X} \pm s$	Cell survival rate (% of normal)
Normal	1.026 ± 0.037	100
Control	0.325 ± 0.076	28[##]
Bicyclol[b]	0.537 ± 0.049	50*
1	0.558 ± 0.085	50*
2	0.323 ± 0.058	26

[a] Results are expressed as means ± SD ($n = 3$); [##] $p < 0.001$, significantly different from control by Student's *t*-test; * $p < 0.05$, significantly different from normal by Student's *t*-test.
[b] Positive control compound.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on an Autopol IV-T/V (Rudolph Research Analytical, New Jersey, USA). Melting points were determined on an XT digital melting-point apparatus with a microscope and are uncorrected (Shanghai Eastern Analytical Instrument Co., Ltd., Shanghai, China). UV spectra were recorded in MeOH on a Jasco V650 spectrophotometer (JASCO, Inc., Easton, MD, USA). The ^1H (600 MHz), ^{13}C (150 MHz), and 2D NMR spectra were recorded on a Bruker AVANCE III 600 instrument using TMS (Tetramethylsilane) as an internal reference (Bruker Company, Boston, MA, USA). HRTOFMS data were obtained on an Agilent 7890-7000A mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Preparative HPLC (high performance liquid chromatography) was conducted with an Agilent Technologies 1200 series instrument with a MWD (multiple wavelength detector) using a YMC-pack ODS (Octadecylsilyl)-A column (5 μm, 250 mm × 20 mm). Column chromatography was performed with silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Develosil ODS (50 μm, Nomura Chemical Co. Ltd., Osaka, Japan), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). TLC (thin layer chromatography) was carried out with glass precoated with silica gel GF_{254}. Spots were visualized under UV light or by spraying with 10% sulfuric acid in EtOH followed by heating.

3.2. Plant material

The leaves of *Callicarpa nudiflora* Hook were collected from Wuzhishan, Hainan, China, in August 2011 and identified by Prof. Guiping Yuan at Jiangxi Provincial Institute for Drug and Food Control, China. A voucher specimen (No. 20110817) has been deposited in the Herbarium of Jiangxi Provincial Institute for Drug and Food Control.

3.3. Extraction and isolation

The powdered dried leaves of *Callicarpa nudiflora* (9.6 kg) were extracted three times with 80% EtOH under reflux (2 h each). The extracting solution was evaporated under reduced pressure to yield a dark brown residue (1.8 kg). The residue was suspended in water (15 L) and then successively partitioned with petroleum ether (3 L × 15 L), EtOAc (3 L × 15 L), and *n*-BuOH (3 L × 15 L). After removing the solvent, the *n*-BuOH extract (545 g) was passed through a XAD-7 macroporous resin column eluted with H_2O and H_2O-EtOH (5:95, v/v), respectively. The H_2O-EtOH (5:95, v/v) fraction (236 g) was separated by silica gel column chromatography (CC) using $CHCl_3$-MeOH gradient mixtures (99:1 − 60:40, v/v) to give 13 fractions (E1–E13). Fraction E6 (23.9 g) was subjected to silica gel CC and eluted with $CHCl_3$-MeOH (85:15 − 75:25, v/v) to afford five fractions (E6-1 − E6-5). Fraction E6-4 (7.18 g) was again subjected to silica gel CC and eluted with $CHCl_3$-MeOH (95:5 − 80:20, v/v) to afford 12 fractions (E6-4-1 − E6-4-12). Fraction E6-4-11 (1.53 g) was separated by ODS CC (10 − 70%, MeOH − H_2O) to give 21 subfractions. Subfraction 4 (145 mg) was further separated by preparative HPLC (YMC-ODS-A 5 μm, 250 mm × 20 mm, detection at 210 nm) using 15% CH_3CN-H_2O (5 ml/min) containing 0.01% TFA (trifluoroacetic acid) as mobile phase to yield compounds **1** (25.0 mg, t_R 87 min) and **2** (2.1 mg, t_R 105 min).

3.3.1. Callicoside A (*1*)

White amorphous powder; mp 179–180°C; $[\alpha]_D^{20}$ −58.8 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε): 205 (4.72) and 329 (4.38) nm; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectral data see Table 1; HRESIMS: *m/z* 549.1575 $[M + Na]^+$ (calculated for $C_{24}H_{30}O_{13}Na$, 549.1579).

3.3.2. Callicoside B (*2*)

Yellow amorphous powder; mp 117–119°C; $[\alpha]_D^{20}$ −10 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.46), 244 (3.65), and 330 (4.23) nm; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectral data see Table 1; HRTOFMS: *m/z* 559.1656 $[M - H]^-$ (calculated for $C_{24}H_{31}O_{15}$, 559.1663).

3.4. Determination of absolute configurations of the sugar moieties in 1–2

The determination of the absolute configuration of the sugars in compounds **1**–**2** was conducted as described previously [7].

3.5. Protective effect on cytotoxicity induced by D-galactosamine in WB-F344 cells

The hepatoprotective effects of compounds **1**–**2** was conducted as described previously [8].

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Disclosure statement

No potential conflict of interest was reported by the authors.

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