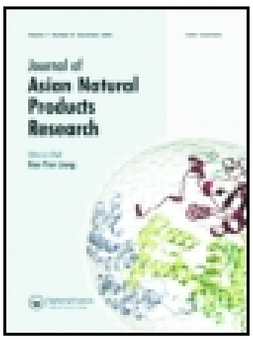

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# Two new iridoid glycosides from the leaves of *Callicarpa nudiflora*

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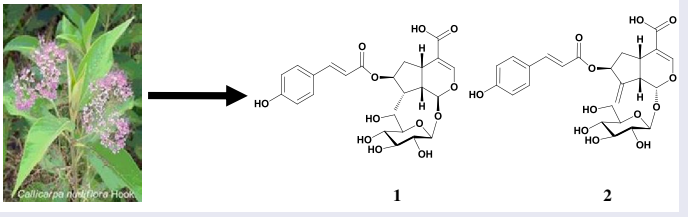
**ABSTRACT**

Two new iridoid glycosides, callicoside C (**1**) and callicoside D (**2**), together with three known compounds (**3**–**5**), were isolated from the leaves of *Callicarpa nudiflora*. Their structures were established by 1D and 2D NMR spectroscopy and mass spectrometry. 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.

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Verbenacase; *Callicarpa nudiflora*; iridoid glycosides; hepatoprotective activity

## 1. Introduction

*Callicarpa nudiflora* Hook belongs to the family Verbenacase and has been used as traditional Chinese herbal medicines to treat inflammation and bleeding [1]. Previous investigation on *C. nudiflora* has led to the isolation of iridoids, flavonoids, triterpenoides, and phenylpropanoid glycosides [2–5]. Some of them have been shown to exhibit anti-inflammatory, antibacterial, cytotoxic and hemostatic activies [6]. *C. nudiflora* has been intensively studied in our previous work and was found containing diverse constituents including triterpenoid glycosides, flavonoids, furofuran lignans, and iridoid glucosides, among which two iridoid glucosides, callicoside A and callicoside B, showed pronounced hepatoprotective activity

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against D-galactosamine-induced toxicity in WB-F344 rat hepatic epithelial stem-like cells [6–9]. In continuation of our investigation on the iridoid composition of *C. nudiflora*, two further new iridoid glycosides, callicoside C (**1**), callicoside D (**2**) and three known compounds 6-*O*-caffeoyl ajugo (**3**), nudifloside (**4**), minecoside (**5**) were isolated from the 80% EtOH extract of *C. nudiflora*. 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) together with three known compounds 6-*O*-caffeoylajugo (**3**) [10], nudifloside (**4**) [2], minecoside (**5**) [11]. 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 evidence.

Compound **1** was obtained as a white amorphous powder. Its molecular formula, $C_{25}H_{30}O_{12}$, was determined from HRESIMS at *m/z* 521.1656 $[M - H]^-$ and supported by the NMR spectroscopic data. The $^1$H NMR spectrum of **1** in $CD_3OD$ exhibited two *trans* olefinic protons at $\delta_H$ 7.61 (1H, d, $J$ = 15.6 Hz) and 6.32 (1H, d, $J$ = 15.6 Hz), one disubstituted olefinic protons at $\delta_H$ 7.45 (1H, s), a set of AA′BB′-type coupled aromatic protons at $\delta_H$ 7.46 (2H, d, $J$ = 7.8 Hz) and 6.80 (2H, d, $J$ = 7.8 Hz), two hemiacetal protons at $\delta_H$ 5.55 (1H, d, $J$ = 4.2 Hz) and 4.69 (1H, d, $J$ = 7.8 Hz), one secondary methyl at $\delta_H$ 1.12 (3H, d, $J$ = 7.2 Hz) (Table 1). The $^{13}$C NMR spectrum of **1** displayed 25 carbon signals including two carboxy carbonyl carbons at $\delta_C$ 170.6 and 169.0. From the foregoing evidence it was concluded that **1** was likely to be a carboxylated iridoid glucoside. Comparison of the NMR spectroscopic data of **1** (Table 1) with those of linearoside [12–14] demonstrated that two compounds were almost identical. The only difference between **1** and linearoside is the configurations at C-7 and C-8. Acid hydrolysis of **1** with 2 M HCl afforded D-glucose, which was identified by GC analysis of their trimethylsilyl L-cysteine derivatives [15,16]. In the HMBC spectrum of **1**, the HMBC correlation from H-7 ($\delta_H$ 4.94) to C-9′ ($\delta_C$ 169.0) confirmed that the *p*-coumaroyl ester group is attached to C-7 of the aglycone (Figure 2). In addition, a HMBC correlation from Glc-H-1″ ($\delta_H$ 4.69) to C-1 ($\delta_C$ 96.0) indicated the *β*-D-glucopyranosyl unit is located

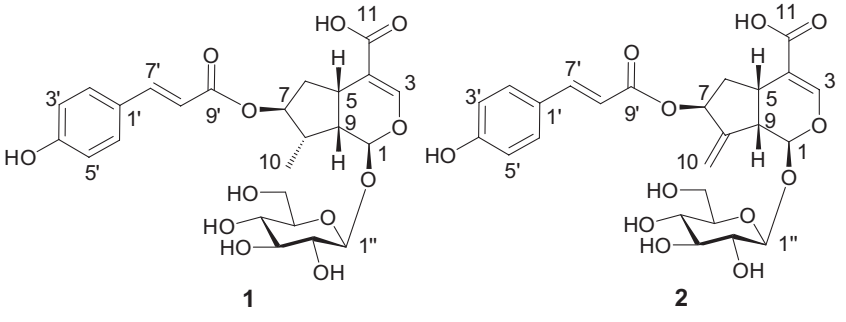


**Figure 1.** Chemical structures of compounds **1** and **2**.

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

| | **1**[a] | | **2**[b] | |
|---|---|---|---|---|
| Position | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| iridoid | | | | |
| 1 | 5.55 (1H, d, 4.2) | 96.0 | 5.84 (1H, d, 5.4) | 96.4 |
| 3 | 7.45 (1H, s) | 152.7 | 7.97 (1H, s) | 152.8 |
| 4 | | 113.7 | | 111.9 |
| 5 | 3.09–3.11 (1H, m) | 31.8 | 3.65 (1H, d, 7.2) | 33.3 |
| 6 | 2.24–2.26 (1H, m) | 39.1 | 2.58–2.60 (1H, m) | 38.9 |
| | 1.98–2.00 (1H, m) | | 2.43–2.45 (1H, m) | |
| 7 | 4.94 (1H, overlap) | 82.6 | 5.93 (1H, br t) | 75.9 |
| 8 | 2.46–2.48 (1H, m) | 43.1 | | 149.0 |
| 9 | 2.60–2.62 (1H, m) | 43.3 | 3.27 (1H, s) | 44.8 |
| 10 | 1.12 (3H, d, 7.2) | 14.3 | 5.67 (1H, s) | 116.4 |
| | | | 5.55 (1H, s) | |
| 11 | | 170.6 | | 169.7 |
| *p*-coumaroyl | | | | |
| 1′ | | 127.2 | | 126.6 |
| 2′ | 7.46 (1H, d, 7.8) | 131.1 | 7.61(1H, d, 8.4) | 131.2 |
| 3′ | 6.80 (1H, d, 7.8) | 116.9 | 7.18 (1H, d, 8.4) | 117.3 |
| 4′ | | 161.2 | | 162.0 |
| 5′ | 6.80 (1H, d, 7.8) | 116.9 | 7.18 (1H, d, 8.4) | 117.3 |
| 6′ | 7.46 (1H, d, 7.8) | 131.1 | 7.61 (1H, d, 8.4) | 131.2 |
| 7′ | 7.61 (1H, d, 15.6) | 146.5 | 7.96 (1H, d, 15.6) | 145.8 |
| 8′ | 6.32 (1H, d, 15.6) | 111.5 | 6.59 (1H, d, 15.6) | 115.8 |
| 9′ | | 169.0 | | 167.6 |
| Glc | | | | |
| 1″ | 4.69 (1H, d, 7.8) | 99.9 | 5.50 (1H, d, 8.4) | 101.2 |
| 2″ | 3.19–3.21 (1H, m) | 74.8 | 4.16 (1H, t, 8.4) | 75.4 |
| 3″ | 3.36–3.38 (1H, m) | 78.1 | 4.32–4.34 (1H, m) | 79.0 |
| 4″ | 3.23–3.25 (1H, m) | 71.8 | 4.30–4.32 (1H, m) | 72.1 |
| 5″ | 3.31–3.33 (1H, m) | 78.4 | 4.05–4.07 (1H, m) | 79.4 |
| 6″ | 3.91 (1H, d, 11.4) | 63.0 | 4.59 (1H, dd, 12.0, 2.4) | 63.3 |
| | 3.64–3.66 (1H, m) | | 4.41 (1H, dd, 12.0, 5.4) | |

[a]Recorded in $CD_3OD$.
[b]Recorded in pyridine-$d_5$.

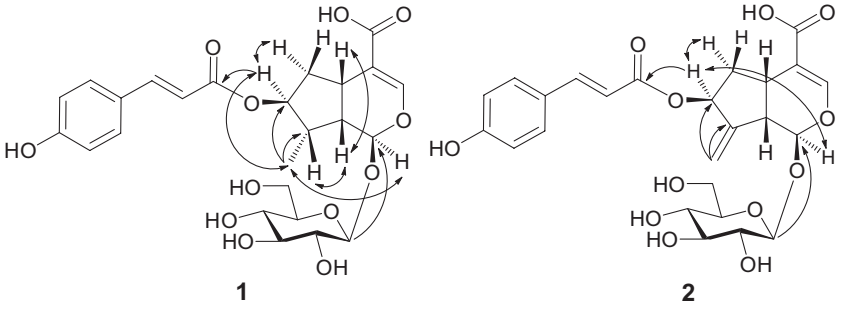


**Figure 2.** Key HMBC (H→C) and NOE (H↔H) correlations of compounds **1** and **2**.

at C-1. In the NOESY spectrum of **1**, NOESY correlations were observed between the following proton pairs: H-5/H-6$\beta$, H-5/H-9, and H-9/H-8, indicating that methyl group at C-8 should be $\alpha$-oriented. However, NOE interactions were observed among H-1, H-7, and H-10, which indicated the hydroxyl group at C-7 should be $\beta$-oriented. Further structure analysis suggested the configurations at C-7 and C-8 in compound **1** was 7S, 8S, while the

configurations at C-7 and C-8 in linearoside was 7S, 8R. 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. Thus, compound **1** was determined as 7-*O*-*E*-*p*-coumaroyl-8-*epi*-loganic acid, named callicoside C.

Compound **2** was obtained as a white amorphous powder. The HRESIMS of **2** showed a quasi-molecular ion peak at *m/z* 543.1479 $[M + Na]^+$, indicating a molecular formula of $C_{25}H_{28}O_{12}$. The $^{1}$H NMR spectrum (Table 1) of **2** was similar to that of **1**, including a *p*-coumaroyl group and a glucose unit, as well as the resonance at $\delta_H$ 7.97 (1H, s, H-3) and $\delta_H$ 5.84 (1H, d, $J$ = 5.4 Hz, H-1). The major differences were in the iridoid moiety. Thus, additional resonances [$\delta_H$ 5.67 (1H, s) and 5.55 (1H, s)] were present, while that from a methyl group was missing. In the $^{13}$C NMR spectrum of **2**, the expected 25 carbon signals were observed. When compared with the spectrum of **1**, only the resonances for the iridoid aglycone differed. The two resonances at $\delta_C$ 149.0 and 116.4 suggested an iridoid with a 8, 10-double bond, which was further confirmed by HMBC correlations from $H_{2-10}$ to C-7, C-8, and C-9. However, comparison of the NMR data of **2** with those of gardoside [17] indicated that **2** is a derivative of gardoside. HMBC correlations from Glc-H-1″ ($\delta_H$ 5.50) to C-1 ($\delta_C$ 96.4) and H-7 ($\delta_H$ 5.93) to C-9′ ($\delta_C$ 167.6) indicated *β*-D-glucopyranosyl unit and *p*-coumaroyl group are attached to C-1 and C-7 of aglycone, respectively. In the NOESY spectrum of **2**, NOE interactions were observed among H-1, H-7, and H-6*α*, which indicated the hydroxyl groups at C-1 and C-7 should be *β*-oriented. Thus, compound **2** was elucidated as 7-*O*-*E*-*p*-coumaroyl-gardoside, named callicoside D.

In an *in vitro* bioassay, compound **1** at 10 μM showed pronounced hepatoprotective activities against D-galactosamine-induced toxicity in WB-F344 rat hepatic epithelial stem-like cells (Table 2). Structure analysis indicated that compound **1** was very similar to compound **2** except that the methyl group of C-8 in **1** was replaced by the olefinic bond of C-8 in **2**. However, there were significant differences in the activity of the two compounds. It may be that the methyl group of C-8 is an active group.

## 3. Experimental

### 3.1. General experimental procedures

Optical rotations were measured on an Autopol IV-T/V (Rudolph Research Analytical, New Jersey, USA). UV spectra were recorded in MeOH on a Jasco V650 spectrophotometer (JASCO, Inc., Easton, Maryland, USA). The $^{1}$H (600 MHz), $^{13}$C (150 MHz), and 2D NMR spectra were recorded on a Bruker AVANCE III 600 instrument using TMS

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

| Compound | $x \pm s$ | 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** | 0.572 ± 0.016 | $52^{**\triangle}$ |
| **2** | 0.311 ± 0.067 | 27 |

[a]Results are expressed as means ± SD ($n$ = 3).
[b]Positive control compound.
$^{\#\#}p < 0.001$, significantly different from control by Student's $t$-test; $^{*}p < 0.05$; $^{**}p < 0.01$, significantly different from normal by Student's $t$-test; $^{\triangle}p < 0.05$, significantly different from positive control by Student's $t$-test.

(Tetramethylsilane) as an internal reference (Bruker Company, Massachusetts, USA). HRESIMS data were obtained on an Agilent 7890–7000A mass spectrometer (Agilent Technologies, Santa Clara, USA). Preparative HPLC (high-performance liquid chromatography) was conducted with an Angilent Technologies 1200 series instrument with a MWD detector using a YMC-pack ODS (Octadecylsilyl)-A column (5 μm, 250 × 20 mm). Column chromatography was performed with silica gel (200–300 mesh, Qingdao Haiyang 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. WB-F344 cells come from the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing.

### 3.2. Plant material

The leaves of *Callicarpa nudiflora* Hook were collected from Wuzhishan, Hainan, China 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 with 80% EtOH at reflux for 3 × 2 h, and the extract was evaporated under reduced pressure to yield a dark brown residue (1.8 kg), which was suspended in water (15 L) and then partitioned with petroleum ether (3 × 15 L), EtOAc (3 × 15 L), and *n*-BuOH (3 × 15 L), successively. 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), eluting with $CHCl_3$-MeOH gradient mixtures (99:1–60:40, v/v), to afford 13 fractions, E1–E13. Fraction E6 (23.9 g) was fractionated via silica gel CC, eluting with $CHCl_3$-MeOH (85:15–75:25, v/v) to give 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 E6–4-10 (4.31 g) was separated by ODS CC (30–100%, MeOH–$H_2O$) to give 17 subfractions. Subfraction 9 (457 mg) was further separated by preparative HPLC (YMC-ODS-A 5 μm, 250 mm × 20 mm, detection at 210 nm) using 19% $CH_3CN$-$H_2O$ (5 ml/min) containing 0.01%TFA as mobile phase to yield compounds **1** (14.0 mg, $t_R$ 84 min) and **2** (47.4 mg, $t_R$ 93 min). Subfraction 12 (457 mg) was purified further by preparative HPLC (YMC-ODS-A 5 μm, 250 mm × 20 mm, detection at 210 nm) using 16% $CH_3CN$-$H_2O$ (7 ml/min) as mobile phase to yield compounds **3** (7.0 mg, $t_R$ 121 min), **4** (12.6 mg, $t_R$ 133 min) and **5** (6.7 mg, $t_R$ 148 min).

#### 3.3.1. Callicoside C (1)

White amorphous powder; $[\alpha]_D^{20} - 58.8$ (*c* 0.08, MeOH); UV (MeOH) $\lambda_{max}$ (log$\varepsilon$): 228 (4.72) and 312 (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* 521.1656 $[M - H]^-$ (calcd for $C_{25}H_{29}O_{12}$, 521.1659).

### 3.3.2. Callicoside D (2)

White amorphous powder; $[\alpha]_D^{20}$ − 25.3 (*c* 0.07, MeOH); UV (MeOH) $\lambda_{max}$ (log$\varepsilon$): 228 (4.42) and 312 (4.26) nm; $^1$H NMR (600 MHz, $C_5D_5N$) and $^{13}$C NMR (150 MHz, $C_5D_5$ N) spectral data see Table 1; HRESIMS: *m/z* 543.1479 $[M + Na]^+$ (calcd for $C_{25}H_{28}O_{12}Na$, 543.1478).

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

The determination of the absolute configuration of the sugars in compounds **1** and **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** and **5** were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay in WB-F344 cells, with some modification [8]. Each cell suspension of $1 \times 10^4$ cells in 200 µl of Dulbecco's modified Eagle's medium containing fetal calf serum (3%), penicillin (100 units/ml), and streptomycin (100 µg/ml) was planted in a 96-well microplate and precultured for 24 h at 37 °C under a 5% $CO_2$ atmosphere. Fresh medium (200 µl) containing bicyclol and test samples was added, and the cells were cultured for 1 h. Then, the cultured cells were exposed to 40 mM D-galactosamine for 24 h. Cytotoxic effects of test samples were measured simultaneously in the absence of D-galactosamine. The medium was changed into a fresh one containing 0.5 mg/ml MTT. After 3.5 h incubation, the medium was removed and 150 µl of dimethyl sulfoxide was added to dissolve formazan crystals. The optical density (OD) of the formazan solution was measured on a microplate reader at 492 nm.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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