Hepatoprotective Triterpenoid Saponins from *Callicarpa nudiflora*

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Four new triterpenoid saponins, 2α,3α,19α,24-tetrahydroxyolean-12-en-28-oic-acid 28-O-β-D-gluco-pyranosyl ester (1), 2α,3α,19α,23-tetrahydroxyolean-12-en-28-oic-acid 28-O-β-D-glycopyranosyl ester (2), 2α,3α,19α-trihydroxyolean-12-en-28-oic-acid 28-O-β-D-glycopyranosyl ester (3), 2α,3α,23,29-tetrahydroxyurs-12,19-dien-28-oic-acid 28-O-β-D-gluco-pyranosyl ester (4), together with three known compounds (5–7), were isolated from the leaves of *Callicarpa nudiflora* Hook. Their structures were established by means of spectroscopic methods and chemical evidence. Hepatoprotective activities of the isolated compounds against D-galactosamine-induced toxicity have been tested. Among them, compounds 1–3 showed pronounced hepatoprotective activities against D-galactosamine-induced toxicity in WB-F344 rat hepatic epithelial stem-like cells.

**Key words** *Callicarpa nudiflora*; triterpenoid saponin; hepatoprotective activity

*Callicarpa nudiflora* Hook. 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. Previous investigation on *C. nudiflora* has led to the isolation of iridoids, flavonoids, triterpenoids, and phenylpropanoid glycosides. Some of them have been shown to exhibit anti-inflammatory, antibacterial, cytotoxic and hemostatic activities. Our previous phytochemical study of *C. nudiflora* yielded two triterpenoid glycosides, an acylated flavone glycoside, and three furan lignans. Further investigation on the 80% EtOH extract of this plant resulted in the isolation of four new compounds, 2α,3α,19α, 24-tetrahydroxyolean-12-en-28-oic-acid 28-O-β-D-glucopyranosyl ester (1), 2α,3α,19α,23-tetrahydroxyolean-12-en-28-oic-acid 28-O-β-D-glycopyranosyl ester (2), 2α,3α,19α-trihydroxyolean-12-en-28-oic-acid 28-O-β-D-glycopyranosyl(-1→2)-β-D-glycopyranosyl ester (3), 2α,3α,23,29-tetrahydroxyurs-12,19-dien-28-oic-acid 28-O-β-D-glycopyranosyl ester (4), along with three known compounds, luteolin (5), 5,4-dihydroxy-3,7,3′-trimethoxyflavone (6), caffeic acid (7). Reported herein are the isolation, structure elucidation and biological activity of these compounds.

**Results and Discussion**

Compound 1 was obtained as a white powder. The molecular formula, C65H86O11, was determined by high resolution-electrospray ionization-mass spectra (HR-ESI-MS) (m/z 665.3878 [M−H]−, Calcd for 665.3901). The 1H-NMR spectrum of 1 in pyridine-d5 showed six methyl singlets (δH 0.99, 1.09, 1.15, 1.19, 1.55, 1.68). Additional proton resonances observed included those ascribed to an olefinic proton at δH 5.52 (1H, brs), two oxymethine protons at δH 4.48 (1H, m) and 4.62 (1H, d, J=1.8 Hz), two oxymethylene protons at δH 3.87 and 4.15 (1H each, d, J=10.8 Hz), and an anomeric proton signal at δH 6.38 (1H d, J=8.4 Hz) correlated in the heteronuclear single quantum coherence (HSQC) spectrum with an anomeric carbons at δC 96.4 in the 13C-NMR spectrum (Table 1). The 13C-NMR spectrum of 1 displayed 36 carbon signals, of which 6 were attributed to the sugar moiety and the remaining 30 to the aglycone. Signals at δC 124.3, 144.8, and 177.7 were assigned to a pair of typical olefinic carbons and a carboxy carbonyl carbon, respectively. These spectroscopic data indicated that 1 is an oleanane-type triterpene with four hydroxyl groups, a trisubstituted double bond. The two oxymethylene proton signals at δH 3.87 and 4.15, which correlated with the carbon resonance at δC 65.7 in the HSQC spectrum, showed heteronuclear multiple bond connectivity (HMBC) correlations with C-3, C-4, and C-24, justifying its assignment to C-23. The assignment of hydroxyl groups at C-2 and C-3 was confirmed from the HMBC correlations from H-2 to C-4 and C-10 and from H-1, H-2, H-23, and H-24 to C-3. Comparison of the NMR spectroscopic data of 1 with sericoside demonstrated that two compounds were almost identical except for the A ring. The splitting pattern of H-3 (1H, d, J=1.8 Hz) suggested that 1 was the C-3 epimer of sericoside. Thus, the C-3 hydroxy group of 1 was α-orientation. From the foregoing evidences it was concluded that 1 was a glycoside of tetrahydroxyolean-12-en-28-oic-acid. Acid hydrolysis of 1 with 2m HCl afforded d-glucose, which was identified by GC analysis of its trimethylsilyl l-cysteine derivatives.

In the HMBC spectrum of 1, the presence of correlations from Glc-H-1 (δH 6.38) to C-28 (δC 177.7) confirmed that the glucose unit is located at C-28 of the aglycone. The α-orientation of 2-OH, 3-OH, and 19-OH in 1 was deduced by analysis of the nuclear Overhauser effect spectroscopy (NOESY) spectrum which showed nuclear Overhauser effect (NOE) correlations between the following proton pairs: H-2/H-3, H-2/H-24, H-2/H-25, and H-19/H-30. Thus, compound 1 was elucidated as 2α,3α,19α,24-tetrahydroxyolean-12-en-28-oic-acid 28-O-β-D-glucopyranosyl ester.

Compound 2 had the molecular formula C65H86O11, as deduced from the HR-ESI-MS (m/z 821.4290 [M+Na]+, Calcd for 821.4294). The 1H-NMR spectrum of 2 was similar to those of 1 except for the configuration of C-4 and an additional set of β-d-xylose resonances. The proton signal at δH 0.71 assigned to H-24 of 2 was shifted by ~0.97 ppm compared to that of 1, suggesting that the aglycone of 2 was...
| Position | 1\(^\text{a}\) | 2\(^\text{b}\) | 3\(^\text{a}\) | 4\(^\text{b}\) |
|----------|----------------|----------------|----------------|----------------|
|          | \(\delta_H\)  | \(\delta_C\)  | \(\delta_H\)  | \(\delta_C\)  |
| 1        | 1.88 (1H, m)  | 43.5           | 0.89 (1H, d, 12.0) | 47.8           |
|          | 1.97 (1H, m)  | 43.5           | 1.78 (1H, t, 12.0) | 47.8           |
| 2        | 4.48 (1H, m)  | 66.7           | 3.69 (1H, dd, 4.2, 2.4) | 69.7           |
|          | 4.62 (1H, d, 1.8) | 74.8           | 3.35 (1H, d, 2.4) | 78.4           |
| 3        | 4.62 (1H, m)  | 45.6           | 1.60 (1H, m) | 44.1           |
| 4        | 1.88 (1H, m)  | 50.2           | 1.50 (1H, m) | 42.8           |
| 5        | 4.55 (1H, d, 1.8) | 19.7           | 1.60 (1H, m) | 42.8           |
| 6        | 1.51 (1H, m)  | 34.2           | 1.30 (1H, m) | 33.4           |
| 7        | 1.51 (1H, m)  | 1.62 (1H, m)  | 1.63 (1H, m) | 40.5           |
| 8        | 2.15 (1H, m)  | 41.0           | 1.85 (1H, m) | 40.5           |
| 9        | 1.88 (1H, m)  | 40.5           | 1.89 (1H, m) | 39.3           |
| 10       | 2.15 (2H, m)  | 52.0           | 2.0 (2H, m) | 24.9           |
| 11       | 5.52 (1H, brs) | 124.3          | 5.33 (1H, brs) | 124.7          |
| 12       | 144.8         | 144.8          | 144.8          | 144.8          |
| 13       | 42.7          | 42.7           | 42.7           | 42.7           |
| 14       | 1.25 (1H, m)  | 47.2           | 47.2           | 47.2           |
| 15       | 2.37 (1H, m)  | 30.1           | 1.33 (1H, m) | 29.9           |
| 16       | 2.12 (1H, m)  | 28.0           | 2.29 (1H, m) | 28.2           |
| 17       | 2.82 (1H, m)  | 2.81 (1H, m)  | 2.0 (2H, m) | 24.7           |
| 18       | 3.53 (1H, brs) | 45.1           | 4.02 (1H, brs) | 45.2           |
| 19       | 3.57 (1H, d, 3.6) | 81.6           | 3.55 (1H, d, 3.6) | 81.7           |
| 20       | 36.0          | 36.0           | 36.0           | 36.0           |
| 21       | 1.04 (1H, m)  | 29.5           | 2.03 (1H, m) | 29.5           |
| 22       | 2.05 (1H, m)  | 1.69 (1H, m)  | 1.75 (1H, m) | 39.3           |
| 23       | 1.97 (1H, m)  | 33.5           | 1.64 (1H, m) | 33.3           |
| 24       | 2.06 (1H, m)  | 1.76 (1H, m)  | 1.80 (1H, m) | 33.3           |
| 25       | 1.68 (3H, s)  | 66.5           | 3.29 (1H, d, 11.4) | 66.5           |
| 26       | 3.87 (1H, d, 10.8) | 65.7           | 0.71 (3H, s) | 13.8           |
| 27       | 4.15 (1H, d, 10.8) | 65.7           | 0.71 (3H, s) | 13.8           |
| 28       | 1.09 (3H, s)  | 17.5           | 1.04 (3H, s) | 17.5           |
| 29       | 1.19 (3H, s)  | 18.1           | 0.74 (3H, s) | 17.5           |
| 30       | 1.55 (3H, s)  | 25.3           | 1.30 (3H, s) | 17.8           |
| Sugar (C-28) Glc | 72.8 | 72.8 | 72.8 | 72.8 |
| 1        | 6.38 (1H, d, 8.4) | 96.4           | 5.45 (1H, d, 7.8) | 94.0           |
| 2        | 4.38 (1H, m)  | 71.7           | 3.61 (1H, m) | 80.0           |
| 3        | 4.30 (1H, m)  | 79.4           | 3.31 (1H, m) | 78.0           |
| 4        | 4.23 (1H, m)  | 74.6           | 3.41 (1H, m) | 71.2           |
| 5        | 4.04 (1H, m)  | 79.7           | 3.35 (1H, m) | 73.4           |
| 6        | 4.41 (1H, m)  | 62.8           | 3.69 (1H, dd, 10.8, 4.4) | 62.3           |
| 7        | 4.46 (1H, m)  | 3.80 (1H, d, 10.8) | 3.80 (1H, d, 10.8) | 3.80 (1H, d, 10.8) |

a) Recorded in pyridine-\(d_5\). b) Recorded in CD\(_3\)OD.
the C-4 epimer. The hydroxymethyl at C-4 was α-oriented, as deduced from the NOESY correlation between the following proton pairs: H-2/H-24, H-2/H-25, and H-3/H-24. The linkage position of the sugar units with the aglycone was established from the following HMBC correlations: Glc-H-1 ($\delta_{H}$ 5.45)/aglycone-C-28 ($\delta_{C}$ 178.7) and Xyl-H-1 ($\delta_{H}$ 4.63)/Glc-C-2 ($\delta_{C}$ 80.0). Thus, compound 2 was determined to be 2α,3α,19α,23-tetrahydroxyolean-12-en-28-oic-acid 28-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl ester.

Compound 3 was isolated as a white powder. The HR-ESI-MS peak at $m/z$ 781.4370 [M−H]− indicated the molecular formula of 3 to be C_{44}H_{56}O_{16} with one oxygen atom less than that of 2 (821.4290 [M+Na]+). The structure of the sugar chain was determined to be the same as that of 2 by comparison of their 1H- and 13C-NMR spectroscopic data (Table 1). Comparing the NMR spectra of 3 with those of 2 indicated that signals due to a hydroxymethyl group [δ_{H} (3.29, 3.50) (each, d, δ_{C} 71.3, 17.7)] at C-4 in 2 was replaced by a signal due to the α-methyl group [δ_{H} 1.20 (3H, s), δ_{C} 29.9] in 3, which was further confirmed by HMBC and NOESY experiments on 3. Thus, compound 3 was concluded to be 2α,3α,19α-trihydroxyolean-12-en-28-oic-acid 28-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl ester.

Compound 4 was isolated as a white powder, with the molecular formula C_{36}H_{56}O_{11}, as deduced from the [M+Na]+ peak at $m/z$ 687.3729 by HR-ESI-MS. The $^1$H-NMR spectrum of 4 in CD_{3}OD showed five methyl singlets (δ_{H} 0.78, 0.89, 1.02, 1.06, 1.67). Additional proton resonances observed included those ascribed to an olefinic proton at δ_{H} 5.53 (1H, t, J=3.61Hz), two oxymethine protons at δ_{H} 3.61 (1H, d, J=2.44Hz) and 3.90 (1H, m), two oxymethylene protons at δ_{H} 3.95 and 4.17 (1H each, δ_{C} 23.6, 23.9), and an anomic proton signal at δ_{H} 5.40 (1H d, J=8.41Hz) correlated in the HSQC spectrum with an anomeric carbons at δ_{C} 95.9 in the $^{13}$C-NMR spectrum (Table 1). The $^{13}$C-NMR spectrum of 4 displayed 36 carbon signals including five methyl (δ_{C} 16.9, 17.7, 17.9, 18.4, 22.6), four olefinic (δ_{C} 129.1, 129.2, 133.7, 138.3), of which δ_{C} 129.2 and 138.3 are replaced by the double bond at C-12(13) of an ursane-type triterpene, and a carboxyl signal (δ_{C} 177.7) (Table 1). Comparing the NMR spectra of 4 with those of rubuside (4a) showed that 4 had two more OH groups. After several NMR experiments, including HMBC, heteronuclear multiple quantum coherence (HMOC), and NOESY, it was apparent that the signals due to α-methyl group at C-4 and methyl group at C-29 in 4 were replaced by signals due to two hydroxymethyl groups [δ_{H} 3.39, 3.53 (each, d, J=10.2Hz), and δ_{C} 71.3] and [δ_{H} 3.95, 4.17 (each, d, J=11.4Hz), and δ_{C} 63.7]. Acid hydrolysis of 4 with 2M HCl afforded o-glucose, which was identified by GC analysis of its trimethylsilyl t-cysteine derivatives. In the HMBC spectrum of 4, a long-range correlation from Glc-H-1 (δ_{H} 5.40) to C-28 (δ_{C} 177.7) confirmed that the β-D-glucopyranosyl unit is attached to C-28. In the NOESY spectrum, NOE correlations were observed between the following proton pairs: H-2/H-24, H-2/H-25, and C-2 and C-23 were all oriented to α position. Thus, compound 4 was characterized as 2α,3α,19α-trihydroxyolean-12,19-dien-28-oic-acid 28-O-β-D-glucopyranosyl ester.

The structures of known compounds 5–7 were determined as luteolin (5), 5,4′-dihydroxy-3,7,3′-trimethoxyflavone (6), and caffeic acid (7), respectively, by detailed spectroscopic analysis and comparison of their spectral data with reported values in the literatures.

Hepatoprotective activities against α-galactosamine-induced toxicity of the four new compounds (1–4) and three known compounds (5–7) were examined in WB-F344 cells. Compounds 1–3 were found to show hepatoprotective activity at 10^{-5}M in vitro. The hepatoprotective activity of 1 was more than that of bicyclol (10^{-5}M), a drug showing potent hepatoprotective activity. This is the first report of hepatoprotective activity from a Callicarpa L.

**Experimental**

**General Experimental Procedures** Optical rotations were measured on a Perkin-Elmer 341 polarimeter. The UV spectra were recorded in MeOH on a Perkin-Elmer Lambda...
25 UV-Vis spectrophotometer. The $^1$H- (600 MHz), $^{13}$C- (150 MHz), and 2D-NMR spectra were recorded on a Bruker AVANCE III 600 instrument using tetramethylsilane (TMS) as an internal reference. ESI-MS data were collected on a MDS SCIEX API 2000 LC/MS/MS instrument. HR-ESI-MS data were obtained on an Acquity UPLC Xevo G2Q-Tof mass spectrometer. Preparative HPLC (high performance liquid chromatography) was conducted with an Agilent Technologies 1200 series instrument with a MWD detector using a YMC-pack ODS (Octadecylsil)-A column (5 µm, 250 × 20 mm) and a Shimadzu LC-6A instrument with a RID-10A refractive index detector using a X Terra prep MS C$_{18}$ column (10 µm, 300 × 19 mm). Column chromatography was performed with silica gel 60 (100–200 mesh, Qingdao Marine Chemical Ltd., Qingdao, China) and Devosil ODS (75 µm, Nomura Chemical Co., Ltd., Japan). 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.

**Plant Material** The leaves of *Callicarpa nudiflora* were collected from Wuzhishan, Hainan, China, in Aug. 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.

**Extraction and Isolation** The powdered dried leaves of *Callicarpa nudiflora* (9.6 kg) were extracted three times with 80% EtOH under reflux (2h 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 × 15 L), EtOAc (3 × 15 L), and n-BuOH (3 × 15 L). After removing the solvent, the n-BuOH extract (545 g) was passed through a XAD-7 macroporous resin column eluted with H$_2$O for 1 h. Then, the cultured cells were exposed to 40 mM D-glucose for 24 h. Cytotoxic effects of test samples were measured simultaneously in the absence of D-galactosamine. The Student’s t-test for unpaired observations between normal or control and tested samples was carried out to identify statistically significant differences; p values less than 0.05 were considered as significantly different.

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