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BRIEF COMMUNICATIONS

PREPARATIVE ISOLATION AND PURIFICATION OF FIVE PHENYLETHANOID GLYCOSIDES FROM *Chirita eburnea*

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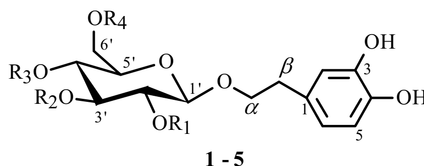
Chirita eburnea Hance, belonging to the family Gesneriaceae, is distributed in Guangxi, Yunnan, Guangdong, and Sichuan Provinces of China [1]. It is a well-known Chinese herbal medicine for the treatment of hemostasis, detoxification, and pulmonary tuberculosis [2]. Naphthoquinones [3], quinine derivatives, and steroids [4] have been isolated from *Chirita eburnea*, and PhGs have been obtained from other genera of the family Gesneriaceae [5–9]. PhGs have many biological functions such as hepatoprotective [10], antioxidant [11], anti-inflammatory, and antinociceptive activity [12]. Gesneriaceae plants are rich in PhGs; 43 PhGs have been isolated from the Gesneriaceae plants [13]. In previous papers the isolation and purification of PhGs usually required the use of multiple chromatographic steps for sample clean-up and isolation [5–9], which usually results in low recovery rates due to irreversible adsorption of PhGs onto the solid support during separation. In contrast, HSCCC have also become an effective alternative to the conventional chromatographic techniques for the separation of some PhGs from plant extracts [14–16]. However, no report has been published on the separation and purification of PhGs from *Chirita eburnea* using the combination of macroporous resin CC and semipreparative HPLC.

In this study, an efficient method to isolate the PhGs from *Chirita eburnea* Hance by the use of a macroporous resin CC and semipreparative HPLC was studied. Five PhGs were obtained from *Chirita eburnea*, and on the basis of spectral data (EI-MS, ^{1}H NMR, ^{13}C NMR), these compounds were identified as plantainoside A (**1**), chiritoside C (**2**), plantainoside B (**3**), plantamajoside (**4**), and desrhamnosylverbascoside (**5**), and their purities analyzed by HPLC were all above 98%. The described method is rapid, efficient, and simple, providing high purification from plant materials for the studies of bioactivities.

An excellent semipreparative HPLC separation was achieved as follows: the mobile phase composed of methanol (A)-water (v/v): 25%A to 26%A 0–5 min; 26% A 5–10 min; 26%A to 27%A 10–14 min; 27%A to 28%A 14–25 min. Flow-rate: 4.0 mL/min. The column temperature and detection wavelength were set at 38°C, 218 nm. Each fraction obtained by semipreparative HPLC, was analyzed by analytical HPLC and the results indicated that compounds **1**–**5** contained only one peak with purities of ca. 98%. These samples were used without further purification for spectroscopic studies.



1: $R_1 = R_3 = R_4$ = H, R_2 = caffeoyl; **2:** $R_1 = R_2 = R_3$ = H, R_4 = Glc-caff
3: R_1 = caffeoyl, $R_2 = R_3 = R_4$ = H; **4:** $R_1 = R_4$ = H, R_2 = Glc, R_3 = caffeoyl
5: $R_1 = R_2 = R_4$ = H, R_3 = caffeoyl

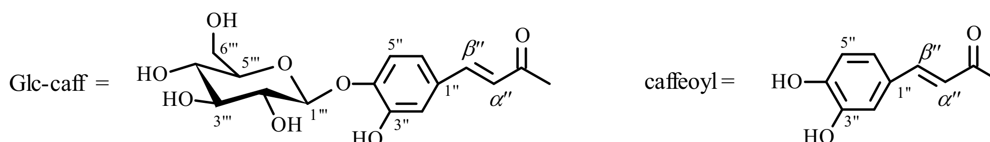


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TABLE 1. ^{13}C NMR Data for Five Phenylethanoid Glycosides in Methanol-d_4

C atom	**1**	**2**	**3**	**4**	**5**
			Aglucone		
1	131.3	131.3	131.7	131.5	131.3
2	116.9	117.0	116.3	117.1	116.3
3	145.9	145.9	144.5	146.1	146.2
4	144.5	144.4	146.0	144.7	144.5
5	116.3	116.3	117.2	116.4	116.9
6	121.1	121.1	121.3	121.3	121.1
α	72.0	72.2	71.8	72.2	72.0
β	36.4	36.6	36.6	36.5	36.3
			Central glucosyl moiety		
1′	104.1	104.3	102.4	104.0	104.2
2′	73.4	74.9	76.3	76.0	75.0
3′	78.9	77.8	75.3	84.2	75.9
4′	69.7	71.7	71.9	70.9	72.3
5′	77.6	75.0	78.1	75.9	75.8
6′	62.3	64.6	62.7	62.3	62.3
			Caffeic acid moiety		
1″	127.7	130.9	127.9	127.7	127.4
2″	115.4	115.7	115.3	115.3	114.5
3″	146.7	148.3	149.5	146.8	146.6
4″	149.3	148.7	146.8	149.7	149.5
5″	116.4	118.1	116.6	116.6	115.0
6″	122.8	122.3	123.1	123.1	122.9
α'	115.1	117.0	115.3	115.2	116.1
β'	146.6	146.2	147.1	147.4	147.4
C=O	169.0	168.3	168.5	168.5	168.4
			Outer glucosyl moiety		
1‴		103.3		105.7	
2‴		74.6		75.9	
3‴		77.4		77.7	
4‴		71.2		71.2	
5‴		78.1		77.9	
6‴		62.3		62.4	

In this study, acetonitrile–water and methanol–water were used as the mobile phase in the preparation. The results show that the effects of the mobile phase were very similar, hence the methanol–water system was used as the mobile phase in the preparation. It is also important to carefully compare the time of the elution procedure. The maximum flow rate that can be achieved on a separation column is limited by the pressure drop. Therefore, the effect of preparation on a flow rate of 2 mL/min, 3 mL/min, and 4 mL/min was studied. Although flow rates of 2 mL/min and 3 mL/min gave a relatively good performance in separating the PhGs of *Chirita eburnea*, they had a long settling time. When the flow rate was 4 mL/min, it required 26 min not only to complete an elution unit but also to maintain good separation efficiency.

The structural identification of compounds **1**–**5** was performed with ESI-MS and NMR. Data for each compound are as follows.

Compound **1**: ESI-MS *m/z* 479 $[M + H]^+$. ^1H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.59 (1H, d, J = 16, H-β''), 7.07 (1H, d, J = 2, H-2″), 6.96 (1H, dd, J = 2 and 8, H-6″), 6.79 (1H, d, J = 8, H-5″), 6.69 (1H, d, J = 2, H-2), 6.67 (1H, d, J = 8, H-5), 6.56 (1H, dd, J = 2 and 8, H-6), 6.34 (1H, d, J = 16, H-α''), 5.06 (1H, t, J = 9, H-3′), 4.42 (1H, d, J = 8, H-1′), 4.05 (1H, m, H-α), 3.89 (1H, dd, J = 2 and 12, H-6′), 3.73 (2H, m, H-α and H-6′), 3.55 (1H, t, J = 9, H-4′), 3.41 (2H, m, H-2′ and H-5′), 2.78 (2H, m, H_2-β). ^{13}C NMR: see Table 1. Comparison the above data with [17] shows that compound **1** is 3,4-dihydroxy-phenethylalcohol-3-*O*-caffeoyl-β-D-glucopyranoside (plantainoside A).

Compound **2**: ESI-MS *m/z* 641 $[M + H]^+$. ^1H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.56 (1H, d, J = 16, H-β''), 7.17 (1H, d, J = 8.5, H-5″), 7.09 (1H, d, J = 2, H-2″), 6.90 (1H, dd, J = 2 and 8.5, H-6″), 6.68 (1H, d, J = 2, H-2), 6.65 (1H, d, J = 8, H-5), 6.53 (1H, dd, J = 2 and 8, H-6), 6.36 (1H, d, J = 16, H-α''), 4.89 (1H, d, J = 7, H-1′), 4.52 (1H, dd, J = 2 and 12, H-6′), 4.34 (1H, d, J = 8, H-1′), 4.32 (1H, dd, J = 6 and 12, H-6′), 2.79 (1H, t, J = 7.5, H_2-β). ^{13}C NMR: see Table 1. The results are very similar to those in [6]; compound **2** corresponds to 3,4-dihydroxyphenethylalcohol-6-*O*-glucopyranosylcaffeoyl-β-D-glucopyranoside (chiritoside C).

Compound **3**: ESI-MS *m/z* 479 $[M + H]^+$. ^1H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.58 (1H, d, J = 16, H-β''), 7.09 (1H, d, J = 2, H-2″), 6.98 (1H, dd, J = 2 and 8, H-6″), 6.81 (1H, d, J = 8.5, H-5″), 6.63 (1H, d, J = 2, H-2), 6.60 (1H, d, J = 8, H-5), 6.49 (1H, dd, J = 2 and 8, H-6), 6.30 (1H, d, J = 16, H-α''), 4.81 (1H, dd, J = 9 and 8, H-2′), 4.52 (1H, d, J = 8, H-1′), 3.99 (1H, m, H-α), 3.89 (1H, dd, J = 2 and 12, H-6′), 3.72 (1H, dd, J = 6 and 12, H-6′), 3.64 (1H, m, H-α), 3.59 (1H, t, J = 9, H-3′), 3.42 (1H, t, J = 9, H-4′), 3.32 (1H, m, H-5′), 2.67 (2H, m, H_2-β). ^{13}C NMR: see Table 1. Compared with the data given in [17], compound **3** corresponds to 3,4-dihydroxyphenethylalcohol-2-*O*-caffeoyl-β-D-glucopyranoside (plantainoside B).

Compound **4**: ESI-MS *m/z* 641 $[M + H]^+$. ^1H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.54 (1H, d, J = 16, H-β''), 7.03 (1H, d, J = 2, H-2″), 6.94 (1H, dd, J = 2 and 8, H-6″), 6.75 (1H, d, J = 8, H-5″), 6.70 (1H, d, J = 2, H-2), 6.66 (1H, d, J = 8, H-5), 6.52 (1H, dd, J = 2 and 8, H-6), 4.90 (1H, t, J = 9, H-4′), 3.90 (1H, t, J = 9, H-3′), 2.75 (2H, m, H_2-β). ^{13}C NMR: see Table 1. Compared with the reported data given in Ref. [8], compound **4** corresponds to 3,4-dihydroxyphenethylalcohol-β-D-glucopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside (plantamajoside).

Compound **5**: ESI-MS *m/z* 479 $[M + H]^+$. ^1H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.56 (1H, d, J = 16, H-β''), 7.04 (1H, d, J = 2, H-2″), 6.96 (1H, dd, J = 2 and 8, H-6″), 6.79 (1H, d, J = 8, H-5″), 6.69 (1H, d, J = 2, H-2), 6.68 (1H, d, J = 8, H-5), 6.56 (1H, dd, J = 2 and 8, H-6), 6.30 (1H, d, J = 16, H-α''), 4.84 (1H, t, J = 9, H-4′), 4.37 (1H, d, J = 8, H-1′), 4.05 (1H, m, H-α), 3.71 (1H, m, H-α), 2.76 (2H, m, H_2-β). ^{13}C NMR: see Table 1. The data are very similar to those in [18], and compound **5** corresponds to 3,4-dihydroxyphenethylalcohol-4-*O*-caffeoyl-β-D-glucopyranoside (desrhamnosylverbascoside).

The IC_{50} of these five PhGs on Fe^{3+}-ADP/NADPH lipid peroxidation in rat liver microsome is as follows: **1** – 0.53 μM, **2** – 0.78 μM, **3** – 0.48 μM, **4** – 0.64 μM, **5** – 0.52 μM. All PhGs were very effective.

HPLC Analysis. Agilent series 1100 HPLC (Agilent, USA) including an Eclipse XDB-C18 (4.6 × 150 mm, 5 μm) (Agilent, USA), a G1311A Quat Pump, a G1315B DAD, and a 2000ES ELSD (Alltech Associates, USA), a G1328B Manual injector (equipped with a 7725i six-way valve (Rheodyne, USA)), a G1379A degasser, Agilent HPLC workstation, and Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus, Hangzhou, China).

Semipreparative HPLC. Agilent series 1200 HPLC (Agilent, USA) including an Eclipse XDB-C18 (9.4 × 250 mm, 5 μm) (Aglient, USA), a G1311A Quat Pump, a G1314B VWD detector, a G1329A Auto-sampler injector, a G1322A degasser, and Agilent HPLC workstation.

Mass spectra were recorded on an Agilent series 1100 electrospray ionization (ESI) mass spectrometer.

The nuclear magnetic resonance (NMR) spectrometer used was a Bruker AMX-500 instrument (Bruker, Germany).

All organic solvents used for preparation of crude extract were of analytical grade (Nanjing Reagent Factory, Nanjing, China). The methanol used for HPLC was of chromatographic grade (Fisher, USA), and the water used was distilled water. Methanol-d_4 was used as the solvent for NMR determination. MCI gel CHP-20P (Mitsubishi Chemicals, Japan) was used for enrichment of PhGs. TLC analysis was performed on polyamide plates (TZSHSL, China), developed with water–*n*-butanol–acetone–acetic acid (16:2:2:6, v/v), and colorized with 5% $FeCl_3$ ethanol.

The roots of *Chirita eburnea* were obtained from Yanshan, Guilin, Guangxi province, China, in June 2008 and identified by Prof. Fanan Wei (Guangxi Institute of Botany). A voucher specimen (CE08061202) of the plant is deposited at the Herbarium of Guangxi Institute of Botany, China.

Extraction. The dried roots of *Chirita eburnea* were ground to powder (about 30 mesh) by a disintegrator. The powder (5 kg) was extracted three times with 50% ethanol (10 L). The extract was combined and evaporated to dryness under reduced pressure, which yielded 700 g of dry powder. Then, the dry powder was dissolved in 0.5 L water. After filtration, the aqueous solution was extracted three times with 0.5 L of water-saturated light petroleum, ethyl acetate, and *n*-butanol successively, which yielded 80 g of light petroleum extract, 120 g of ethyl acetate extract, and 200 g of *n*-butanol extract after being combined and evaporated to dryness under reduced pressure. In order to enrich the target components and remove impurities, the residue of *n*-butanol was loaded on an MCI gel CHP-20P column and eluted with distilled water and 30, 40, 50, 60, 70, and 100% methanol successively. As a result, five major PhGs in considerable amounts were enriched from the total ethanol extract on TLC analysis. The 40% methanol effluent was collected and evaporated at 65°C under reduced pressure, and about 680 mg of residue was obtained. All the residues were stored in a refrigerator (3°C) for the subsequent semi-HPLC separation.

The influence of PhGs on microsomal lipid peroxidation induced by Fe^{3+}-ADP/NADPH was examined using the procedure described in Ref. [19].

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