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Cynanauriculoside C–E, three new antidepressant pregnane glycosides from *Cynanchum auriculatum*

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ABSTRACT

Based on the bioactive screening results, three new pregnane glycosides named as cynanauriculoside C–E (**1**–**3**), were isolated from the roots of *Cynanchum auriculatum* Royle ex Wight (Asclepiadaceae), together with two known ones, otophylloside L (**4**) and cynauricuoside C (**5**). On the basis of detailed spectroscopic analysis and chemical method, the structures of new compounds were characterized to be qingyangshengenin 3-*O*-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside (**1**), qingyangshengenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4) -α-L-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside (**2**) and caudatin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-digitoxopyranoside (**3**). In the despair mice models, these pregnane glycosides showed significant antidepressant activity at the dosage of 50 mg/kg (i.g.). The most potent one was cyanauriculatoisde D (**2**), which was close to the positive control fluoxetine (20 mg/kg).

1. Introduction

Cynanchum auriculatum Royle ex Wight (Asclepiadaceae) is widely distributed in China. Its root is a famous tonic herbal drug in traditional Chinese medicine, and has been widely used in clinics as a beneficial and tonic agent since ancient times. A recent ethnobotanical revealed that Baishouwu is an important herbal drug for the treatment of gastric disorders (e.g., indigestion and stomach ache) in the ethnomedicine of the Tujia and Miao nationalities in Southwest of China (Shan et al., 2006). According to the current knowledge, *C. auriculatum* contains a variety of bioactive constituents, including pregnane glycosides and baishouwubenzophenone (Chen et al., 1990; Gu et al., 2009; Wang et al., 2002; Zhang et al., 2000a,b, 2007a; Sun et al., 2009). *C. auriculatum* possesses widely useful activities including anti-tumor (Shan et al., 2005; Wang et al., 2003, 2007, 2009; Yao et al., 2009; Zhang et al., 2000a,b), gastroprotective (Shan et al., 2006) and anti-aging (Zhang et al., 2007b). However, there was no report about the antidepressant evaluation of *C. auriculatum*. In our research, the ethanol extract and its fractions of *C. auriculatum* were evaluated for antidepressant activities in mice (Yang, 2007), and here we report the pregnane glycosides isolated from the antidepressant fraction of *C. auriculatum*.

2. Results and discussion

Following the screening results, the active fraction of the $CHCl_3$ extract of the roots of *C. auriculatum was* repeatedly chromatographed and five pregnane glycosides (1–5) were yielded. All of them showed positive effects on Libermann–Buchard (Xu and Chen, 1981) and Keller–Kiliani reactions, indicating the presence of a steroidal skeleton with 2-deoxysugar moiety. Spectroscopic analysis demonstrated that all the glycosides had a pregnane skeleton with an acyl group at C-12 position and a straight sugar chain connected to C-3 group position of the aglycone. In comparison of ^{13}C NMR and ^{1}H-NMR spectra with reported data, compounds **4** and **5** were identified as otophylloside L (**4**) (Ma et al., 2007) and cynauricuoside C (**5**) (Chen et al., 1990) (Fig. 1).

The presence of the monosaccharides in the hydrolysates of each compound was confirmed by co-TLC comparison with authentic sugars. Further GC analysis of the corresponding trimethylsilylated L-cysteine adducts confirmed D-configuration of glucose. For the deoxysugars, since only D-form authentic samples could be obtained, their absolute configurations could not be assigned by GC analysis, but determined to be D-forms by comparison of their ^{13}C NMR spectroscopic data with those reported data. The most significant differences in the ^{13}C NMR data

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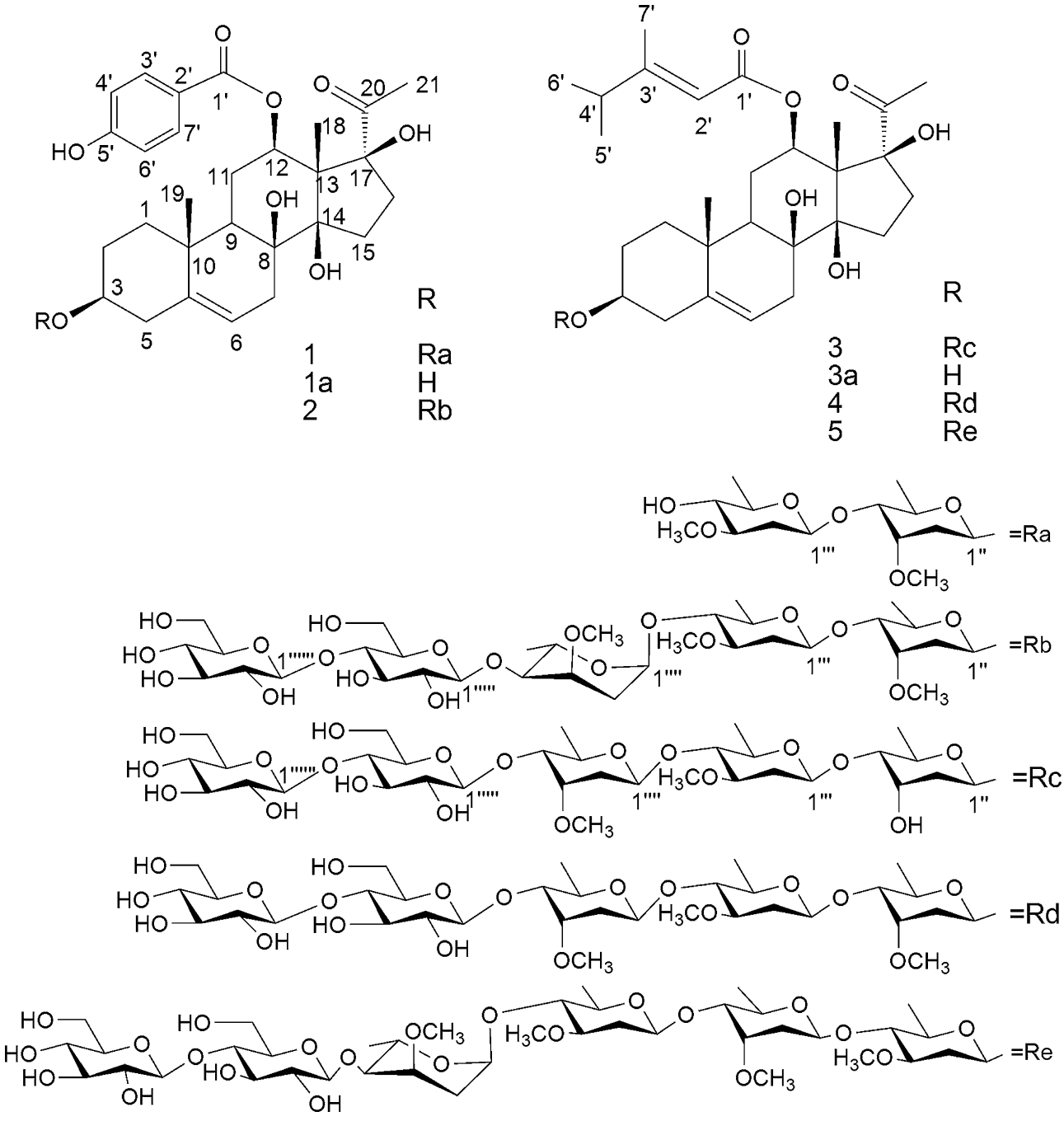

Fig. 1. Pregnane glycosides from *Cynanchum auriculatum*.

between D- and L-configuration cymaropyranose involve the resonances of C-2. The chemical shift of C-2 in the L-cymaropyranose is less than 35.0 ppm, but that of C-2 in the D-cymaropyranose appears above 36.0 ppm (Hamed et al., 2004; Li et al., 2006; Wang et al., 2002; Zhu et al., 1999). As to digitoxopyranosyl and oleandropyranosyl, the configuration was supposed as D-configuration (Ma et al., 2007), and also due to their similar ^{13}C NMR chemical shifts with those reported in literatures (Bai et al., 2005; Mu et al., 1986; Yoshikawa et al., 1996, 1999; Zhang and Zhou, 1983).

Compound 1 was obtained as a white amorphous powder. Its molecular formula was assigned to be $C_{42}H_{60}O_{14}$ on the basis of negative HRESI-MS *m/z* (787.3978 [M ($C_{42}H_{60}O_{14}$)−H]$^-$, calculated: 787.3849) and ^{13}C NMR (DEPT) data (Table 1). IR spectrum showed the absorption bands for hydroxyl (3443 cm^{-1}), carbonyl (1710 cm^{-1}) groups and benzene ring (1612 and 1591 cm^{-1}). The ^1H NMR spectrum of 1 revealed the presence of three singlet methyl groups δ_H 1.14, 1.64, 2.05 (each 3H, s, Me-19, -18, -21)] one olefinic proton [δ_H 5.36 (br. s, H-6)] and four aromatic protons on a *para*-substituted benzene ring [δ_H 6.81 (2H, d, J = 8.8 Hz, H-4′,6′) and δ_H 7.80 (2H, d, J = 8.8 Hz, H-3′,7′)] (Tables 1 and 2) in its aglycone moiety. The above observation suggested qingyangshengenin seemed to be the aglycone of compound 1. Proton signals were also assigned to two secondary methyl groups [δ_H 1.20 (d, J = 6.4 Hz) and 1.27 (d, J = 6.4 Hz)], two methoxyl groups [δ_H 3.45 and 3.42] and two anomeric protons [δ_H 4.84 (dd, J = 1.6, 9.6 Hz), 4.61 (d, J = 8.4 Hz)] whose multiplicities suggested the presence of three 2,6-dideoxy-sugar in a disaccharides chain and the β-configuration of the two hexose units. Acid hydrolysis of 1 gave qingyangshengenin (**1a**) as the aglycone, and oleandrose as well as

Table 1
^{13}C NMR spectroscopic data of compounds **1–3** (C_5D_5N, δ in ppm).

Position	1	2	3	Position	1	2	3
1	39.8 (CH_2)	39.7 (CH_2)	39.8 (CH_2)	1″	β-D-ole 97.3 (CH)	β-D-ole 97.2 (CH)	β-D-digit 97.2 (CH)
2	30.2 (CH_2)	30.2 (CH_2)	30.2 (CH_2)	2″	36.7 (CH_2)	36.7 (CH_2)	39.0 (CH_2)
3	79.3 (CH)	79.3 (CH)	79.3 (CH)	3″	78.6 (CH)	78.5 (CH)	67.7 (CH)
4	39.8 (CH_2)	39.7 (CH_2)	39.8 (CH_2)	4″	83.9 (CH)	83.8 (CH)	83.9 (CH)
5	140.2 (C)	140.2 (C)	140.2 (C)	5″	70 .0 (CH)	69.9 (CH)	70.7 (CH)
6	119.7 (CH)	119.7 (CH)	119.7 (CH)	6″	18.5 (CH_3)	18.6 (CH_3)	18.3 (CH_3)
7	35.2 (CH_2)	35.2 (CH_2)	35.2 (CH_2)	OMe	58.5 (CH_3)	58.5 (CH_3)	
8	75.0 (C)	75.0 (C)	75.0 (C)	1‴	β-D-cym 102.8 (CH)	β-D-cym 102.7 (CH)	β-D-ole 101.6 (CH)
9	45.1 (CH)	45.0 (CH)	45.2 (CH)	2‴	37.4 (CH_2)	37.3 (CH_2)	36.7 (CH_2)
10	38.1 (C)	38.1 (C)	38.1 (C)	3‴	81.6 (CH)	80.1 (CH)	80.9 (CH)
11	25.5 (CH_2)	25.5 (CH_2)	25.4 (CH_2)	4‴	77.0 (CH)	83.2 (CH)	83.6 (CH)
12	74.1 (CH)	74.1 (CH)	73.3 (CH)	5‴	73.3 (CH)	72.8 (CH)	71.7 (CH)
13	59.1 (C)	59.1 (C)	58.8 (C)	6‴	18.4 (CH_3)	18.7 (CH_3)	18.6 (CH_3)
14	90.0 (C)	90.0 (C)	89.9 (C)	OMe	57.4 (CH_3)	57.0 (CH_3)	58.6 (CH_3)
15	34.3 (CH_2)	34.3 (CH_2)	34.2 (CH_2)	1⁗		α-L-cym 98.5 (CH)	β-D-cym 100.6 (CH)
16	33.5 (CH_2)	33.5 (CH_2)	33.3 (CH_2)	2⁗		32.4 (CH_2)	36.3 (CH_2)
17	93.1 (C)	93.1 (C)	93.0 (C)	3⁗		74.2 (CH)	80.9 (CH)
18	10.6 (CH_3)	10.6 (CH_3)	10.4 (CH_3)	4⁗		79.3 (CH)	83.3
19	18.5 (CH_3)	18.6 (CH_3)	18.2 (CH_3)	5⁗		65.1 (CH)	72.5 (CH)
20	212.1 (C)	212.1 (C)	211.6 (C)	6⁗		18.1 (CH_3)	18.5 (CH_3)
21	27.9 (CH_3)	27.8 (CH_3)	27.6 (CH_3)	OMe		56.9 (CH_3)	57.7 (CH_3)
1′	166.8 (C)	166.7 (C)	167.5 (C)	1⁗′		β-D-glc 101.5 (CH)	β-D-glc 104.6 (CH)
2′	120.4 (C)	122.3 (C)	114.3 (CH)	2⁗′		75.0 (CH)	75.3 (CH)
3′	132.8 (CH)	132.8 (CH)	167.4 (C)	3⁗′		76.6 (CH)	70.73 (CH)
4′	116.2 (CH)	116.1 (CH)	39.4 (CH)	4⁗′		80.9 (CH)	81.5 (CH)
5′	163.6 (C)	163.5 (C)	21.4 (CH_3)	5⁗′		76.2 (CH)	77.1 (CH)
6′	116.2 (CH)	116.1 (CH)	21.3 (CH_3)	6⁗′		62.0 (CH_2)	62.8 (CH_2)
7′	132.8 (CH)	132.8 (CH)	16.7 (CH_3)	1⁗″		β-D-glc 104.6 (CH)	β-D-glc 106.1 (CH)
				2⁗″		74.9 (CH)	74.9 (CH)
				3⁗″		78.1 (CH)	78.4 (CH)
				4⁗″		71.3 (CH)	71.4 (CH)
				5⁗″		77.8 (CH)	77.6 (CH)
				6⁗″		62.4 CH_2)	62.5 (CH_2)

cymarose as sugar residues. The ^{13}C NMR shifts of each sugar unit were assigned unambiguously by HSQC, HMBC and HSQC-TOCSY analyses (Table 1). The existence of one D-oleandropyranosyl and one D-cymaropyranosyl units were confirmed by their comparison with the spectroscopic data in the literatures (Mu et al., 1986; Yoshikawa et al., 1996, 1999). Compared with spectral data of qingyangshengenin (Ma et al., 2007), the glycosylation shifts effects of C-2 (−0.2 ppm), C-3 (+7.9 ppm), and C-4 (−1.7 ppm) showed the linkage position of the sugar moiety was at the C-3 hydroxyl group of the aglycone, confirmed by the HMBC correlation from δ_H 4.84 (H-1″ of terminal β-D-cymaropyranosyl) to δ_C 79.3 (C-3). The sequence of the two sugar units was demonstrated by HMBC spectrum, in which distinct correlations from δ_H 4.61 (H-1‴ of terminal β-D-oleandropyranosyl) to δ_C 83.9 (C-4″ of inner β-D-cymaropyranosyl) and correlation from H δ_H 3.28 (H-4″ of inner β-D-cymaropyranosyl) to δ_C 102.8 (C-1‴ of β-D-oleandropyranosyl) were observed, respectively. Thus, the structure of compound **1** was established as qingyangshengenin-3-*O*-β-D-oleandropyranosyl- (1 → 4)-β-D-cymaropyranoside, and named cynanauriculoside C.

Based on the HRESI-MS data (*m*/*z* 1255.5938 [M ($C_{61}H_{92}O_{27}$)−H]$^-$, calculated: 1255.5640) and ^{13}C NMR (DEPT) data (Table 1), compound **2** was shown to have a molecular formula $C_{61}H_{92}O_{27}$. Acidic hydrolysis 2 afforded **1a** as the aglycone and glucose, cymarose as well as oleandropyranose as sugar residues. The ^{13}C NMR shifts of each sugar unit were assigned unambiguously by HSQC, HMBC and HSQC-TOCSY analyses (Table 1). The existence of two D-glucopyranosyl, one L-cymaropyranosyl, one D-oleandropyranosyl and one D-cymaropyranosyl units were confirmed by their comparison with the spectroscopic data in the literatures (Ma et al., 2007; Gu et al., 2009; Xiang et al., 2009). In the HMBC experiment, the linkage of the sugar moiety to C-3 of the aglycone was proved by the correlation observed from δ_H 4.89 (H-1″ of terminal β-D-cymaropyranosyl) to δ_C 79.3 (C-3), and the sequence of the five sugar units was elucidated by significant correlations observed from δ_H 4.61 (H-1‴ of terminal β-D-oleandropyranosyl) to δ_C 83.8 (C-4″ of inner β-D-cymaropyranosyl), from δ_H 3.23 (H-4‴ of inner β-D-oleandropyranosyl) to δ_C 98.5 (C-1⁗ of α-L-cymaropyranosyl), from δ_H 4.88 (H-1⁗ of terminal α-L-cymaropyranosyl) to δ_C 83.2 (C-4‴ of inner β-D-oleandropyranosyl), from δ_H 4.43 (H-1⁗′ of

Table 2
Effect of compounds **1–5** on the forced swimming test and tail suspension test in mice (means ± S.E.M.).

	n	Forced swimming test		Tail suspension test	
		Immobility duration (s)	Reduction (%)	Immobility duration (s)	Reduction (%)
Control	8	89.7 ± 35.5		112.7 ± 21.5	
Fluoxetine	8	31.3 ± 11.5**	65.1	46.1 ± 18.5**	59.1
1	8	52.5 ± 13.5**	41.5	73.2 ± 28.1**	35.0
2	8	42.7 ± 15.2**	52.4	55.5 ± 21.9**	50.8
3	8	55.3 ± 12.6**	38.4	91.3 ± 27.6*	19.0
4	8	62.5 ± 16.5*	30.3	86.1 ± 25.7*	23.6
5	8	69.3 ± 15.1*	22.7	75.3 ± 24.5**	33.2

* $p < 0.05$
** $p < 0.01$.

terminal β-D-glucopyranosyl) to δ_C 79.3 (C-4‴″ of inner α-L-cymaropyranosyl), and from δ_H 4.41 (H-1‴″″ of terminal β-D-glucopyranosyl) to δ_C 80.9 (C-4‴″″ of inner β-D-glucopyranosyl). Thus, the structure of 2 was established as qingyangshengenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside, and named cynanauriculoside D.

The negative HRESI-MS of 3 showed the quasi-molecular ion peak at *m/z* 1231.6278 ([M−H]$^-$, calculated: 1231.6008), in agreement with the molecular formula $C_{60}H_{96}O_{26}$, which was supported by the ^{13}C NMR and DEPT experiments (Table 1). IR spectrum showed the absorption bands for hydroxyl (3445 cm^{-1}) and carbonyl (1713 cm^{-1}) groups as well as C=C band (1644 cm^{-1}). Acidic hydrolysis of 3 afforded a sugar mixture of cymarose, oleandrose, digitoxose and glucose, and the aglycone, which was identical to caudatin (**3a**), by co-TLC comparison with authentic samples. Inspection of the NMR spectral data of compound **3** (Table 1) showed that besides the signals arising from the aglycone (**1a**), it contained five anomeric carbons at δ_C 97.2 (digit C-1), 100.6 (cym C-1), 101.6 (ole C-1),104.6 (glu C-1), and 106.1 (glu C-1), corresponding to five anomeric proton signals at δ_H 4.88 (d, *J* = 9.6, 1.6 Hz), 4.79 (d, *J* = 8.4 Hz), 4.62 (dd, *J* = 9.6, 1.6 Hz), 4.43 (d, *J* = 8.8 Hz), and 4.39 (d, *J* = 7.6 Hz), respectively. The ^{13}C signals of each sugar unit (Table 1) were assigned by HSQC and HMBC analyses, suggesting the existence of one D-digitoxose, one D-cymaropyranosyl, one D-oleandropyranosyl, and two D-glucopyranosyl units compared with the spectroscopic data in the literatures (Li et al., 2006; Bai et al., 2005, 2008; Warashina and Noro, 1997; Xiang et al., 2009). Compared to the ^{13}C chemical shifts of 3a (Ma et al., 2007), glycosylation shifts were observed at the C-3 (+8.0 ppm), and C-4 (−1.7 ppm) positions, thus proving the attachment of the sugar chain at the C-3 hydroxyl group of the aglycone in 3. The sugar sequence of 3 was demonstrated by HMBC correlations from δ_H 4.39 (H-1‴″″of terminal β-D-glucopyranosyl) to δ_C 81.5 (C-4‴″ of inner β-D-glucopyranosyl); from δ_H 4.43 (H-1‴″ of outer β-D-glucopyranosyl) to δ_C 83.3 (C-4‴″ of innerβ-D-cymaropyranosyl); from δ_H 4.79 (H-1‴″ of outer β-D-cymaropyranosyl) to δ_C 83.6 (C-4‴ of inner β-D-oleandropyranosyl); from δ_H 4.62 (H-1‴ of β-D-oleandropyranosyl) to δ_C 83.9 (C-4″ of inner β-D-digitoxoyranosyl); from δ_H 4.88 (H-1″ of inner β-D-digitoxopyranosyl) to δ_C 79.3 (C-3). Consequently, the structure of 3 was deduced to be caudatin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-digitoxopyranoside, and named cynanauriculoside E.

Compounds (**1–5**) were evaluated for their antidepressant activities on a forced swimming test and a tail suspension test in mice (Porsolt et al., 1977; Steru et al., 1985). Treating compounds (**1–5**) in mice by gavage for five consecutive days respectively, the immobility duration of forced swimming test and tail suspension test was significantly reduced (Table 2), while the open field test showed that compounds (**1–5**) did not affect the spontaneous activity of mice (Supplementary Material III). These results confirmed the antidepressant activity of the compounds (**1–5**). The most potent one was compound **2**, which show a reduction degree close to the positive control fluoxetine.

3. Experimental

3.1. General experimental procedures

Melting points were measured on an XRC-I micromelting point apparatus and were uncorrected. Optical rotations were obtained on a SEPA-300 automatic digital polarimeter. IR spectra were determined on a Bruker Tensor 27 spectrometer in KBr pellets. NMR spectra were performed in CD_3OD unless otherwise noted and recorded on Bruker DRX-400 at 400 MHz (^{1}H) and 100 MHz (^{13}C), with TMS as internal standard. HRESI-MS data were detected on a Bruker Daltonics micrO-TOF-II MS system. Silica gel HF_{254} prepared for TLC and silica gel (200–300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Company, Qingdao, China. Reversed phase silica gel Rp-18 and Rp-8 for CC were purchased from Merck & Co. Inc. L-Glucose, L-glucose, L-cysteine methyl ester hydrochloride, and 1-(trimethylsilyl)imidazole were purchased from Sigma (USA), Supelco (USA), Aldrich (USA), and Fluka (Switzerland), respectively.

3.2. Plant material

C. auriculatum was collected in Duyun, Guizhou province, China, in September 2007, and identified by Qing-Fu Chen (School of Life Sciences, Guizhou Normal University, Guiyang, China). A voucher specimen (No. 0709-08) is deposited at Herbarium of School of Life Sciences, Guizhou Normal University, Guiyang, China.

3.3. Extraction and isolation

The air-dried roots of *C. auriculatum* (4.5 kg) were extracted with 90% EtOH at room temperature for three times (72, 72, and 48 h, 10 L × 3). After removal of the organic solvent *in vacuo*, the residue was suspended in water (2 L) and partitioned with $CHCl_3$ for three times (1.5 L × 3) to give a $CHCl_3$ extract (125.0 g), part of which (80.0 g) was subjected to a silica gel CC (11 cm × 120 cm) and eluted with $CHCl_3$-MeOH (10:1.5) to give five fractions (Frs. 1-5). (The Fr. 5 was found to be the most active fraction in our previous screening for antidepressant activity). Fr. 5 (6.2 g) was repeatedly subjected to silica gel [$CHCl_3$–MeOH (10:1) and EtOAc–EtOH–H_2O (10:1:0.5)], and gave two fractions (Frs. 5–1 to 5–3). Fr. 5–1 (200 mg) was subjected to repeatedly Rp-18 (MeOH–H_2O, 60% → 100%) to afford 1 (80 mg). Fr 5–2 (2.1 g) was subjected repeatedly to silica gel ($CHCl_3$–MeOH, 9:1) and Rp-18 (MeOH–H_2O, 60% → 100%) CC to yield 3 (120 mg) and 4 (70 mg), Fr 5–3 (1.8 g) was subjected repeatedly to silica gel ($CHCl_3$–MeOH, 7:1) and Rp-18 (MeOH–H_2O, 60% → 90%) CC to yield 2 (150 mg) and 5 (100 mg).

3.4. Cynanauriculoside C (1)

White amorphous powder, mp 215–218 °C, $[\alpha]_D^{26}$ + 2.8° (*c* = 0.60, MeOH), IR (KBr) ν_{max} 3443, 2938, 2934, 1710, 1612, 1591, 1512, 1383, 1317, 1254, 1066, 911, 860, 781 cm^{-1}. HRESI-MS: *m/z* 787.3978 [M ($C_{42}H_{60}O_{14}$)−H]$^-$ (calculated: 787.3849). ^{1}H and ^{13}C NMR: see Table 1 and Supplementary Material II.

3.5. Cynanauriculoside D (2)

White amorphous powder, mp 203–206 °C, $[\alpha]_D^{26}$ + 5.6° (*c* = 0.72, MeOH), IR (KBr) ν_{max} 3441, 2936, 1710, 1621, 1503, 1385, 1312, 1162, 1069, 910, 856, 772 cm^{-1}. HRESI-MS: *m/z* 1255.5938 [M ($C_{61}H_{92}O_{27}$)−H]$^-$ (calculated: 1255.5640). ^{1}H and ^{13}C NMR: see Table 1 and Supplementary Material II.

3.6. Cynanauriculoside E (3)

White amorphous powder, mp 187–190 °C, $[\alpha]_D^{26}$ + 2.8° (*c* = 0.55, MeOH), IR (KBr) ν_{max} 3445, 2971, 2933, 1713, 1644, 1451, 1382, 1369, 1317, 1224, 1162, 1066, 1003, 914, 865 cm^{-1}. HRESI-MS: *m/z* 1277.6353 [M ($C_{60}H_{96}O_{26}$) + HCOOH−H]$^-$, 1231.6278 [M ($C_{60}H_{96}O_{26}$)−H]$^-$ (calculated: 1231.6008); 917.4585 [M ($C_{60}H_{96}O_{26}$)–Glc–Glc−H]$^-$. ^{1}H and ^{13}C NMR: see Table 1 and Appendix B Supplementary Material II.

3.7. Acid hydrolysis of 1–3

A solution of 1 and 2 (each 5 mg) in MeOH (5 mL) was treated separately with 5% HCl (5 mL) at 50 °C for 15 min. After added H_2O (5 mL), the reaction mixture was evaporated to 10 mL under vacuum for removing of MeOH, and then kept in 60 °C for another 15 min. The hydrolyzed mixture was neutralized to pH 7 with NaOH (4 mol/L) and condensed to dryness under reduced pressure. The residue was dissolved in MeOH, and compared by TLC analysis with authentic samples of qingyanshengenin (**1a**) and caudatin (**3a**) which thus revealed to be the aglycones of compounds **1**–**3**, respectively. The presence of the monosaccharides in the hydrolysates of each compound was confirmed by TLC comparison with authentic sugars, TLC Solvent system A: $CHCl_3$–MeOH (10:1) and Solvent system B: *n*-hexane–Me_2CO (1:1) were used for qingyangshengenin, caudatin, digitoxose, oleandrose, and cymarose, while solvent system C: $CHCl_3$–MeOH–H_2O (7:3:0.5) was used for glucose (Ma et al., 2007). Cymarose was detected from 1–3, digitoxose was detected from **3**; oleandrose was detected from **1**–**3**; glucose was detected from **2** and **3**.

3.8. Determination of absolute configuration of glucose moieties

The procedure was conducted as described (Ma et al., 2007), and the absolute configurations of the glucoses were confirmed to be D-series by comparison of the retention times of glucose derivatives with those of standard samples.

3.9. Animals

Male ICR mice (18–22 g) were used in the TST, FST and OFT, supplied by Guiyang Medical University (Guiyang, China). Mice (10 mice per group) were treated with compounds **1**–**5** (intragastric administration, i.g.), twice a day for 5 consecutive days, respectively. Animals were housed free access to food and water in a room with 12:12 h light–dark cycle, lights on at 7:00 am, temperature (24–26 °C) and humidity (50–60%), for a week before the experiment. All animals used in this study were treated humanely according to the 'Principles of Laboratory Animal Care' and the 'Guide for the Care and Use of Laboratory Animals of Guizhou Normal University'. The experiments were carried out under the approval of the Committee of Experimental Animal Administration of the University.

3.10. Drugs administration

All tested samples were freshly prepared in 0.5% carboxymethylcellulose sodium (CMC-Na) each day before testing. Fluoxetine (Shanghai Fudan Fuhua Pharma Ltd., Shanghai, China) was used as positive control, and suspended in 0.5% CMC-Na and tested at a dose of 20 mg/kg, 0.5% CMC-Na was applied as vehicle control, test samples and controls were administrated by gavage once a day for 5 consecutive days, respectively.

3.11. Forced swimming test (FST), tail suspension test (TST) and open-field test (OFT)

These tests were performed according to the method described (Porsolt et al., 1977; Steru et al., 1985; Cunha et al., 2008) with slight modifications. The details are described in Supplementary Material.

3.12. Data analysis

Data were reported as the means ± S.E.M. Overall differences according to the treatment were confirmed using the one-way analysis of variance (ANOVA). Analysis of variance and least significant difference tests were conducted to identify differences among means.

Conflict of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytol.2011.02.009.

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