Fitoterapia 136 (2019) 104171

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Hepatoprotective steroids from roots of *Cynanchum otophyllum*

Jinrun Dong[a,b,c], Xingrong Peng[a,c], Shuangyang Lu[a,b,c], Lin Zhou[a,c], Minghua Qiu[a,c,*]

[a] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, China
[b] University of the Chinese Academy of Sciences, Beijing, China
[c] Yunnan Key Laboratory of Natural Medicinal Chemistry, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

ARTICLE INFO

Keywords:
Cynanchum otophyllum
Asclepiadaceae
Steriods
Anti-liver fibrosis activity

ABSTRACT

Seven undescribed C21 steroids, namely cynanchin **A–G**, together with thirteen known analogues, were isolated from the roots of *cynanchum otophyllum*. Their structures were elucidated by 1D, 2D NMR and MS spectra, as well as chemical methods. Meanwhile, all of isolates were tested for their anti-hepatic fibrosis activity. Among them, compounds **4–6**, **10–12** and **14–17** showed moderate or significant inhibitory effects for the proliferation of hepatic stellate cells (HSCs) induced by transforming growth factor-β1 (TGF-β1) *in vitro*.

1. Introduction

Cynanchum otophyllum C.K. Schneid (Asclepiadaceae), an endemic species in the People's Republic of China, is distributed primarily in the southwest of China [1], whose dried root and/or rhizome parts have long been used as folk medicine to treat hepatitis, epilepsy, rheumatic pain, phlegm, geriatric diseases, immune deficiency, kidney weakness, and muscle injury [2]. Pharmacodynamic and clinical experiments have established that the chloroform extract and the ethyl acetate extract of the rhizome are particularly effective against epilepsy and chronic hepatitis [3–5]. The main bioactive constituents in this plant were proved to be steroidal glycosides [6–9]. In our continuous efforts to search for novel and interesting bioactive natural products, seven unknown C21 steroids, namely cynanchin **A–G** (**1–7**), together with thirteen known analogues (**8–20**) (Fig. 1), were isolated from the roots of *C. otophyllum*. Herein, we reported the isolation and structural elucidation of these compounds, as well as their anti-hepatic fibrosis effects *in vitro*.

2. Experimental

2.1. General experimental procedures

Column chromatographic materials contain Macroporous resin D-101, Sephadex LH-20 (20–150 μm, Pharmacia), Lichroprep RP-18 (40–63 μm, Merck), and Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc.). Semi-preparative HPLC was performed on an Agilent 1100 or 1260 series instrument (Technologies, Foster City, CA, USA) with ZORBAX SB-C18 column (5 μm, 9.4 × 250 mm). The Bruker AV-400 and AV-600 instruments (Zurich, Switzerland) (internal standard: *tetramethylsilane*, TMS) were used to detect the ^{1}H and ^{13}C NMR spectra. ESIMS and HRTOF-ESIMS data were recorded on an API QSTAR Pulsar spectrometer (Waters, UK) and a Bruker Tensor-27 instrument by using KBr pellets (German) was used for scanning infrared spectra. Chromatogram class methanol and acetonitrile were purchased from Shanghai Youshi Chemical Co., Ltd. (Shanghai, China). Optical rotations were collected on a JASCO P-1020 polarimeter (Tokyo, Japan). A Shimadzu UV2401PC spectrophotometres (Kyoto, Japan) was used to obtain UV spectra.

2.2. Plant material

The roots of *C.otophyllum* were purchased from Traditional Chinese Medicine Market in Kunming, China, and were identified by Prof. Sheng-Ji Pei. A specimen (KUN No0776933) was deposited in the Herbarium of the Department of Taxonomy, Kunming Institute of Botany.

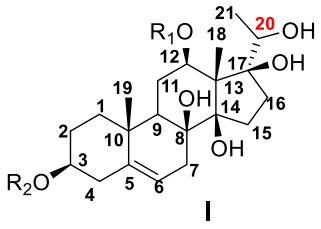
2.3. Extraction and isolation

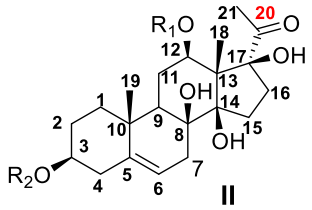
The air-dried roots of *C. otophyllum* (30 kg) were extracted with EtOH at room temperature for three consecutive times. The residue was suspended in water and extracted with $CHCl_3$ to obtain $CHCl_3$ soluble fraction (600 g). The $CHCl_3$ fraction (200 g) was chromatographed on the silica gel column using gradient solvents of $CHCl_3$–MeOH (100:1,

Abbreviation: ole, oleandropyranose; cym, cymaropyranose; digit, digitoxopyranose

* Corresponding author at: State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, China.
E-mail address: mhchiu@mail.kib.ac.cn (M. Qiu).

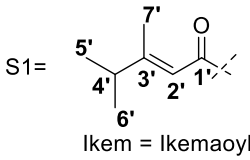
https://doi.org/10.1016/j.fitote.2019.104171
Received 4 April 2019; Received in revised form 7 May 2019; Accepted 10 May 2019
Available online 11 May 2019
0367-326X/



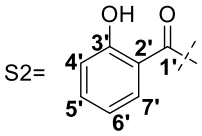


1	R_1= S2	R_2= H
2	R_1= S3	R_2= Ra
6	R_1= S6	R_2= Rh
7	R_1= S6	R_2= Rd
8	R_1= S6	R_2= Rb

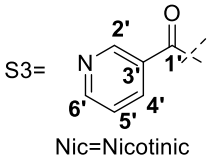
3	R_1= S4	R_2= Rf
4	R_1= S6	R_2= Re
5	R_1= S6	R_2= Rh
9	R_1= S1	R_2= Ra
10	R_1= S1	R_2= Re
11	R_1= S1	R_2= Rc
12	R_1= S1	R_2= Ri
13	R_1= S1	R_2= Rh
14	R_1= S1	R_2= Rg
15	R_1= S5	R_2= Rj
16	R_1= S4	R_2= Ra
17	R_1= S4	R_2= Rd
18	R_1= S4	R_2= Rg
19	R_1= S4	R_2= Rh
20	R_1= S5	R_2= Re



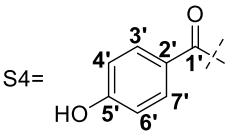
Ikem = Ikemaoyl



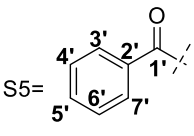
o-OH-Bz = *p*-hydroxybenzoyl



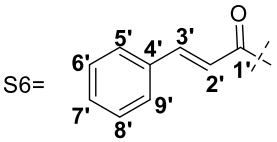
Nic=Nicotinic



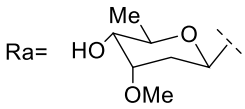
p-OH-Bz = *p*-hydroxybenzoyl



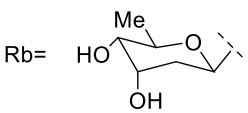
Bz=benzoyl



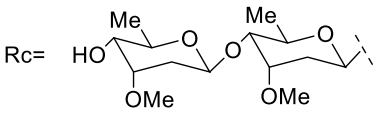
Cin = Cinnamoyl



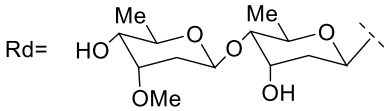
D-cym



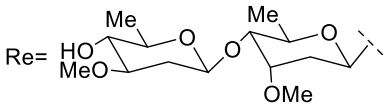
D-digit



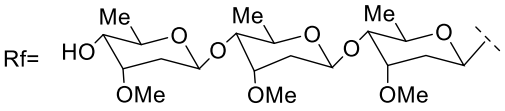
D-cym D-cym



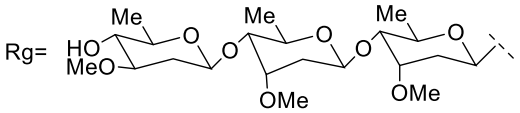
D-cym D-digit



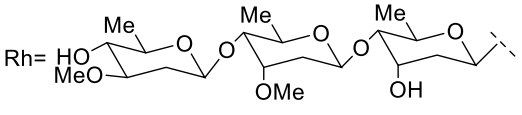
D-ole D-cym



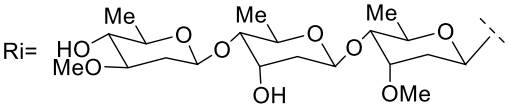
D-cym D-cym D-cym



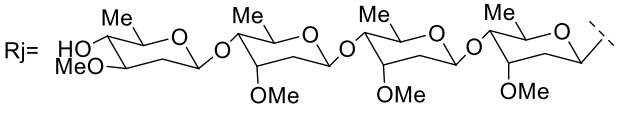
D-ole D-cym D-cym



D-ole D-cym D-digit



D-ole D-digit D-cym



D-ole D-cym D-cym D-cym

Fig. 1. Steroids (**1** − **20**) isolated from the roots of *C. otophyllum*.

80:1,50:1, 20:1, 5:1, 2:1, v/v) to get six fractions (Fr. 1–6). Fr.3 (60 g) was subjected to a silica gel column chromatography (CC) and eluted with CHCl3-MeOH (60:1) to give four fractions (Fr. 3A–3D). Fr. 3A (43.5 g) was submitted to ODS CC, eluted with MeOH–H_2O (40:60 → 100:1, *v*/v) to yield 12 fractions (Fr. 3A1–3A12). Fr. 3A9 was purified by semi-preparative HPLC with CH_3CN–H_2O (68:32, v/v) to give **4** (7 mg), **5** (7 mg), **6** (8 mg), **7** (6 mg), **8** (9 mg), **9** (120 mg), **11** (260 mg) and **20** (8.5 mg). Fr. 3A10 was purified by semi-preparative HPLC with CH_3CN–H_2O (65:35, *v*/v) to give **14** (200 mg). Fr. 3A11 was also treated by semi-preparative HPLC (CH_3CN–H_2O, 66:34, *v*/v) and yield **15** (2.3 mg).Fr. 3C (30 g) was factioned with ODS using MeOH–H_2O (40:60 → 100:1, *v*/v) as eluent andgave 15 fractions (Fr. 3C1−3C15). Subsquently, Fr. 3C8 was treated by semi-preparative HPLC with CH_3CN–H_2O (35:60, v/v) and gave **1** (5 mg) and **2** (7 mg). Compounds **10** (25 mg), **17** (15 mg) and **19** (3 mg) were got from Fr. 3C10, which was purified by semi-preparative HPLC with CH_3CN–H_2O (47:53, *v*/v). Fr. 3C12 was also purified by semi-preparative HPLC (CH_3CN–H_2O, 47:53, *v*/v) to **16** (1 g). Fr. 3C13 and Fr. 3C15 were purified by semi-preparative HPLC with CH_3CN–H_2O (65:35, v/v; 40:60, v/v) to give **3** (16 mg), **12** (35 mg), **13** (6 mg) and **18** (200 mg), respectively.

2.4. Spectroscopic data

2.4.1. Cynanchin A (1)

White amorphous powder; $[\alpha]_D^{26}$ + 35.9 (0.06, MeOH); UV (CH_3OH) λ_{max}(log ε): 206 (4.50), 229 (4.02), 300 (3.48); IR (KBr) ν_{max}: 3430, 2931, 1710, 1613, 1459, 1383, 1224, 1088 and 762 cm $^{-1}$; ^{1}H and ^{13}C NMR data (see Tables 1 and 3); ESIMS *m/z* 525 $[M + Na]^+$, HRESIMS *m/z* 525.2459 $[M + Na]^+$ (calcd for $C_{28}H_{38}NaO_8$, 525.2459).

2.4.2. Cynanchin B (2)

White amorphous powder; $[\alpha]_D^{25.9}$ + 33.8 (0.09, MeOH); UV (CH_3OH) λ_{max} (log ε): 204 (4.15), 260 (3.69); IR (KBr) ν_{max}: 3433, 2925, 1715, 1631, 1383, 1282,1089, and 582 cm $^{-1}$; ^{1}H and ^{13}C NMR data (see Tables 1 and 3); ESIMS *m/z* 632 $[M + H]^+$, HRESIMS *m/z* 632.3407 $[M + H]^+$ (calcd for $C_{34}H_{49}NO_{10}$, 632.3429).

2.4.3. Cynanchin C (3)

White amorphous powder; $[\alpha]_D^{21.7}$ + 15.9 (0.15, MeOH); UV (CH_3OH) λ_{max} (log ε): 202 (4.20) and 259 (4.11); IR (KBr) ν_{max}: 3440, 2930, 1708, 1663, 1399, 1276, 1194, 1058 and 719 cm $^{-1}$; ^{1}H and ^{13}C NMR data (see Tables 1 and 3); ESIMS *m/z* 955 $[M + Na]^+$, HRESIMS *m/z* 955.4662 $[M + Na]^+$ (calcd for $C_{49}H_{72}NaO_{17}$, 955.4662).

2.4.4. Cynanchin D (4)

White amorphous powder; $[\alpha]_D^{19.4}$ + 9.1 (0.17, MeOH); UV (CH_3OH) λ_{max} (log ε): 203 (4.20), 216 (4.14), 278 (4.31) and 385 (1.65); IR (KBr) ν_{max}: 3441, 2927, 1708, 1632, 1384, 1165, 1063 and 771 cm $^{-1}$; ^{1}H and ^{13}C NMR data (see Tables 1 and 3); ESIMS *m/z* 797 $[M –H]^-$, HRESIMS *m/z* 837.3836 $[M + K]^+$ (calcd for $C_{44}H_{62}KO_{13}$, 837.3822).

2.4.5. Cynanchin E (5)

White amorphous powder; $[\alpha]_D^{24.2}$ + 13.8 (0.17, MeOH); UV (CH_3OH) λ_{max} (log ε): 203 (4.43), 213 (4.32) and 278 (4.37); IR (KBr) ν_{max}: 3441, 2929, 1711, 1636, 1451, 1384, 1165, 1060 and 874 cm $^{-1}$; ^{1}H and ^{13}C NMR data (see Tables 2 and 3); ESIMS *m/z*951 $[M+ Na]^+$, HRESIMS *m/z* 951.4738 $[M + Na]^+$ (calcd for $C_{50}H_{72}NaO_{16}$, 951.4713).

2.4.6. Cynanchin F (6)

White amorphous powder; $[\alpha]_D^{19.5}$ + 19.0 (0.1, MeOH); UV (CH_3OH) λ_{max} (log ε): 203 (4.19) , 216 (4.12) and 279 (4.24); IR (KBr) ν_{max}: 3439, 2923, 1708, 1633, 1451, 1383, 1165, 1073 and 581 cm $^{-1}$; ^{1}H and ^{13}C NMR data (see Tables 2 and 3); ESIMS *m/z* 809 $[M+ Na]^+$, HRESIMS *m/z* 809.4089 $[M + Na]^+$(calcd for $C_{43}H_{62}NaO_{13}$, 809.4083).

2.4.7. Cynanchin G (7)

White amorphous powder; $[\alpha]_D^{19.8}$ + 15.3 (0.1, MeOH); UV (CH_3OH) λ_{max} (log ε): 203 (4.29), 216 (4.20), 278 (4.27), 371 (2.07), 481 (1.89) and 591 (1.87); IR (KBr) ν_{max}: 3440, 2921, 2853, 1709, 1633, 1460, 1382, 1164, 1063 and 583 cm $^{-1}$; ^{1}H and ^{13}C NMR data (see Tables 2 and 3); ESIMS *m/z*973 $[M + Na]^+$, HRESIMS *m/z* 953.4856 $[M + Na]^+$(calcd for $C_{50}H_{74}NaO_{16}$, 953.4869).

2.5. Acidic hydrolysis of compounds 2–7

Compounds **2–7** (each 2.0 mg) were dissoved in 2 M HCl (1,4-dioxane/H_2O, 1:1, v/v, 1 mL). The solution was kept at 60 °C for 2 h, and then diluted with H_2O (4 mL). The hydrolyzed mixture was neutralized to pH 7 with saturated $NaHCO_3$ and extracted with $CHCl_3$ (4 mL × 3). The monosaccharides were detected by TLC analysis combined comparison to authentic compounds. The R_f values of cymarose, oleandrose and digitoxose were 0.40, 0.44 and 0.24 with solvent $CHCl_3$–MeOH (10:1); and 0.39, 0.45 and 0.25 with solvent Petroleum ether–acetone (1:1), respectively. As a result, cymaroses were detected in seven new glycosides (**2–7**), oleandroses were detected from **4 to 5** and **7**. Meanwhile, digitoxoses were detected **5–7**. All authentic compounds were purchased from National Institutes for Food and Drug Control, China.

2.6. Determination of the absolute configuration of the monosaccharides

Monosaccharides of compound **2–7** were acquired by above acidic hydrolysis method. And cymarose from compound **2–7**, oleandroses from compound **4–5** and **7**, along with digitoxoses from compound **5–7** were all considered to be of the D-form by comparing their optical rotation (OR) with those reported in literatures (cymarose $[\alpha]_D^{20}$ + 46.2 (*c* = 0.17, H_2O), [11,12]: D-cymarose $[\alpha]_D^{20}$ + 56.0; oleandrose $[\alpha]_D^{20}$–11.0 (*c* = 0.21, H_2O), [11,12,15]: D-oleandrose $[\alpha]_D^{20}$ − 12.0; digitoxose $[\alpha]_D^{20}$ + 40.5 (*c* = 0.20, H_2O), [15]: D-digitoxose $[\alpha]_D^{20}$ + 43.0, respectively.)

2.7. Anti-liver fibrosis activity assay

2.7.1. Cytotoxicity assay of the isolates on HSC-T6 cells

Cells were plated in 96-well plates and treated with chemicals for 12 h. Then viable cells were stained with MTT (0.2 mg/mL, 1 h). The medium was then removed, and formazan crystals produced in the wells were dissolved with the addition of 200 μL of dimethylsulfoxide. Absorbance at 540 nm was measured using a microplate reader (Spectra MAX, Molecular Devices, Sunnyvale, CA). Cell viability was defined relative to untreated controls [i. e., viability (% control) = 100 × (absorbance of treated sample) × (absorbance of control)$^{-1}$].

2.7.2. Effects of the isolates on HSCs proliferation induced by TGF-β1

Hepatic stellate cells (HSCs) activated by TGF-β1 has been long considered to be associated with liver fibrosis, and inhibition for HSC growth has been proposed as a method for treating liver fibrosis [24,25]. The anti-proliferative effects of isolates on HSCs activated by TGF-β1 were determined by an MTT assay [26]. Using the procedures and drug concentrations as described, the experimental groups included the control group, TGF-β1 group, TGF-β1 $^+$ compounds groups. All cell groups except the control group were cultured with DMEM containing 5.0 ng/mL TGF-β1 (without FBS) for 24 h. Inhibitory activity on cell proliferation was calculated as 100 × (absorbance of treated compound – absorbance of background light)/(absorbance of model – absorbance of background light).

Table 1
The ^{1}H NMR (400 MHz) spectroscopic data for compounds **1–4** (δ_H in ppm, *J* in Hz).

Proton	1	2	3	4
1	1.07 (m);1.85(m)	1.08(m);1.83(m)	1.13 (m);1.82(m)	1.11(m);1.81(m)
2	1.28(m);1.75(m)	1.56(m);1.84(m)	1.57(m);1.85(m)	1.57(m);1.85(m)
3	3.42(m)	3.52 m	3.56(m)	3.54(m)
4	2.25 m	2.21(m);2.34(m)	2.21(m);2.36(m)	2.21(m);2.35(m)
6	5.30(s)	5.33(s)	5.35(d,4.9)	5.36(s)
7	2.11(m);2.32(m)	2.13(m);2.33(m)	2.13(m);2.32(m)	2.04(m);2.18(m)
9	1.43(m)	1.44(d,13.1)	1.57(m)	1.55(m)
11	1.79(m)	1.58(m);1.84(m)	1.78(m);1.97(m)	1.76(m);1.94(m)
12	5.47(d,6.4)	4.83(d,2.0)	4.76 m	4.65(dd,11.7,4.1)
15	1.90(m)	1.77(m);1.89(m)	1.92(m);2.05(m)	1.91(m);2.08(m)
16	1.87(m);2.77(m)	1.82(m);2.84(m)	1.72(m);2.86(m)	1.72(m);2.86(m)
18	1.36(s)	1.36(s)	1.63(s)	1.60(s)
19	1.14(s)	1.12(s)	1.12(s)	1.16(s)
20	3.45(m)	3.46 m		
21	1.36(d, 6.2)	1.36(d, 6.3)	2.05(s)	2.22(s)
		Nic	p –OH-Bz	Cin
2′		9.22(d, 2.0)		6.40(d, 16.0)
3′			7.79(d,8.8)	7.62(d, 16.1)
4′	6.74(m)	8.49 m	6.81(d,8.8)	
5′/9′	7.43(m)	7.55(dd,8.0,5.0)		7.34(d,5.6)
6′/8′	6.90(t)	8.7(dd,5.2,1.5)	6.81(d,8.8)	7.53(d, 5.3)
7′	8.04(m)		7.79(d,8.8)	7.41(m)
		D-cym	D-cym	D-cym
1″		4.83(d, 9.6)	4.90 m	4.89 m
2″		1.51(m);2.13(m)	1.57(m);2.13(m)	1.51(m);2.05(m)
3″		3.58 m	3.84 m	3.85 m
4″		3.25 m	3.22 m	3.25 m
5″		3.46 m	3.79 m	3.81 m
6″		1.21(d,6.2)	1.22(d,6.2)	1.28(d,6.1)
OMe		3.42 s	3.42(d,2.0)	3.43(d,6.2)
			D-cym	D-ole
1‴			4.77 m	4.60(d,9.5)
2‴			1.57(m);2.13(m)	1.35(m);2.32(m)
3‴			3.84 m	3.19 m
4‴			3.59 m	2.96(d,9.0)
5‴			3.81 m	3.26 m
6‴			1.20(d,6.3)	1.21(d,6.2)
OMe			3.42(d,2.0)	3.43(d,6.2)
			D-cym	
1⁗			4.77 m	
2⁗			1.31(m);2.32(m)	
3⁗			3.54 m	
4⁗			3.85 m	
5⁗			3.81 m	
6⁗			1.18(d,6.3)	
OMe			3.30(m)	

Measured in CD_3OD. The assignments were based on COSY, HSQC, and HMBC experiments.

2.7.3. Statistical analysis

Each experiment repeated three times and data were expressed as means ± standard deviation (SD). Value with $p < .05$ was considered to be statistically significant.

3. Results and discussion

3.1. Isolation and structure elucidation of compounds 1–7

Roots of *C. otophyllum* were extracted using 95% MeOH and partitioned between H_2O and chloroform.The chloroform extract was separated and purified by various column chromatography and semi-preparative HPLC to give seven previously undescribed steroidal derivatives.

The molecular formula of compound **1** was determined to be $C_{28}H_{38}O_8$ based on the HRESIMS ($[M + Na]^+$, *m/z* 525.2459; calcd 525.2459). The ^{1}H NMR spectrum of **1** revealed the presence of two singlet methyl protons (δ_H 1.36, Me-18; δ_H 1.14, Me-19) and one doublet methyl protons (δ_H 1.36, Me-21), one olefinic proton (δ_H 5.30, H-6) and four aromatic protons of a *o*rthosubstituted benzene ring [δ_H 6.74 (m, H-4′), δ_H 7.43 (m, H-5′), δ_H 6.90 (t, H-6′) and δ_H 8.04 (m, H-7′)]. The ^{13}C NMR spectrum of **1** displayed 28 carbon resonances, belonging to three methyls, seven methylenes, eight methines (including five olefinic/aromatic and four oxygenated), and nine quateranry carbons (including one ketone carbonyl, three olefinic/aromatic, and five oxygenated). Aforementioned data indicated that **1** was a typical C21 steroid and was very similar with 12-*O*-benzoylsareostin [10], except for a *o*-hydroxybenzoyl at C-12 in **1** instead of a *p*-hydroxybenzoyl in the latter. And the HMBC correlations from δ_H 6.74 (m, H-4′) to δ_C 118.1 (C-2′) and δ_C 162.9 (C-3′), from δ_H 7.43 (m, H-5′) to δ_C 162.9 (C-3′), from δ_H 6.90 (t, H-6′) to δ_C 118.1 (C-2′), from δ_H 8.04 (m, H-7′) to δ_C 170.6 (C-1′), δ_C 118.1 (C-2′) and δ_C 162.9 (C-3′) confirmed it. Hence, **1** was determined to be 12-*O*-3′-hydroxy-benzoylsarcostin and named cynanchin **A** (**1**).

Compound **2** had a molecular formula of $C_{34}H_{49}NO_{10}$ based on HRESIMS *m/z* 632.3407 $[M + H]^+$ (calcd. 632.3429). IR spectrum showed the absorption bands for hydroxyl (3433 cm^{-1}), carbonyl (1631 cm^{-1}) groups.1D NMR data (Tables 1,3) of **2** showed that it had 34 carbon resonances, including four methyls, eight methylenes, thirteen methines, and eight quaternary carbons. This implied that the aglycone fraction of **2** was like compound **1**, with the notable differences being the nicotinic group at C-12 in **2** instead of a *o*-

Table 2
The ^{1}H NMR (400 MHz) spectroscopic data for compounds **5–7** (δ_H in ppm, *J* in Hz).

Proton	5[a]	6[b]	7[b]
1	1.11(m);1.88(m)	1.12(m);1.85(m)	1.11(m);1.85(m)
2	1.62(m);1.90(m)	1.56(m);1.84(m)	1.56(m);1.83(m)
3	3.57 m	3.56 m	3.54 m
4	2.29(m);2.40(m)	2.21(m);2.38(m)	2.21(m);2.37(m)
6	5.37(d,3.8)	5.34 m	5.34(d,4.5)
7	2.20(m);2.32(m)	2.11(m);2.32(m)	2.12(m);2.32(m)
9	1.56(m)	1.54(m)	1.54(m)
11	1.78(m);1.88(m)	1.74(m);2.07(m)	1.74(m);2.06(m)
12	4.69(dd,10.4,5.5)	4.78 m	4.76(dd,12.0,4.6)
15	1.98(m)	1.76(m);1.94(m)	1.76(m);1.94(m)
16	1.90(m);2.87(m)	1.78(m);2.87(m)	1.78(m);2.84(m)
18	1.47(s)	1.58(s)	1.58(s)
19	1.13(s)	1.11(s)	1.07(s)
20		3.55(q,6.4)	3.54 m
21	2.20(s)	1.27(d, 6.1)	1.27(d, 6.2)
	Cin	Cin	Cin
2′	6.40(d, 16.0)	6.63(d, 16.0)	6.63(d, 16.0)
3′	7.62(d, 16.0)	7.78(d, 16.0)	7.78(d, 16.0)
5′/9′	7.52(dd,6.6,2.9)	7.62(d, 4.0)	7.62(d, 3.9)
6′/8′	7.39(m)	7.40(m)	7.40(m)
7′	7.39(m)	7.40(m)	7.56(d,8.0)
	D-digit	D-digit	D-digit
1‴	4.93(dd,9.6,2.1)	4.92 m	4.95(d,9.9)
2‴	1.73(m);2.11(m)	1.65(m);1.94(m)	1.64(m);1.94(m)
3‴	4.24(d,3.4)	4.22(d,2.9)	4.21 m
4‴	3.22(dq,5.9,2.8)	3.21 m	3.26 m
5‴	3.80 m	3.74 m	3.80 m
6‴	1.32(d,6.2)	1.16(s)	1.16(s)
	D-cym	D-cym	D-cym
1⁗	4.82(dd,9.8,2.1)	4.80 m	4.81 m
2⁗	1.61(m);1.89(m)	1.59(m);2.21(m)	1.54(m);1.98(m)
3⁗	3.82 m	3.58 m	3.85 m
4⁗	3.22(dq,5.9,2.8)	3.54 m	3.26 m
5⁗	3.92 m	3.18 m	3.80 m
6⁗	1.25(s)	1.21 s	1.20s
OMe	3.46 s	3.40 m	3.42(d,8.3)
	D-ole		D-ole
1⁗′	4.50(dd,9.7 , 2.0)		4.59(dd,9.8 , 2.0)
2⁗′	1.49(m);2.32(m)		1.36(m);2.32(m)
3⁗′	3.22 m		3.20 m
4⁗′	3.13 m		2.96 m
5⁗′	3.30 m		3.27 m
6⁗′	1.13(s)		1.21(d,6.0)
OMe	3.39 s		3.42 (d,8.3)

[a] Measured in $CDCl_3$.
[b] Measured in CD_3OD. The assignments were based on COSY, HSQC, and HMBC experiments.

hydroxybenzoyl in **1**, and the presence of a sugar moiety in **2**. The identification of nicotinoyl group was deduced from the proton signals at δ_H 9.22 (1H, d, *J* = 2.0 Hz, H-2′), δ_H 8.49 (m, 1H, H-4′), δ_H 7.55 (1H, dd, *J* = 5.0, 8.0 Hz, H-5′), δ_H 8.7 (1H, dd, *J* = 1.5, 5.2 Hz, H-6′), and the corresponding ^{13}C NMR signals at δ_C 165.0 (C-1′), δ_C 151.5 (C-2′), δ_C 129.0 (C-3′), δ_C 139.2 (C-4′), δ_C 125.1 (C-5′), δ_C 153.5 (C-6′). The position of the nicotinoyl group was identified as at C-12 based on a long-range HMBC (see Fig. 2) correlations of δ_H 4.82 (1H, d, *J* = 2.0 Hz, H-12) with C-1′ (δ_C 165.0). For the sugar moiety, the characteristic anomeric proton and carbon signals at δ_H 4.83 (d, *J* = 9.6, Hz) and δ_C 97.2 and a series of ^{1}H—^{1}H COSY correlations of H-1″/H-2″/H-3″/H-4″/H-5″/H$_3$–6″, together with the HMBC correlation of the methoxyl with C-3″, indicated that the sugar moiety was a cymaropyranose. Meanwhile, the critical HMBC correlation of H-1″ with C-3 confirmed that this cymaropyranose was located at C-3. Furthermore, The sugar was identified as *β*-D-cymaropyranose by comparison co-TLC and OR data of monosaccharides in the hydrolysates with authentic compounds and published spectroscopic data [11,12], respectively. Hence, **2** was determined to be 3-*O*-*β*-D-cymaropyranosyl-12-*O*-Nicotinoylsarcostin and named cynanchin **B** (**2**).

Table 3
The ^{13}C NMR (150 MHz) data for compounds **1–7** (δ_C in ppm, *J* in Hz).

Carbon	1[a]	2[a]	3[a]	4[a]	5[b]	6[a]	7[a]
1	39.8	39.8	39.8	39.8	39.8	39.8	39.8
2	31.7	31.2	30.2	30.2	30.1	30.2	30.2
3	71.3	79.3	79.3	79.3	79.3	79.3	79.3
4	42.9	39.8	39.8	39.8	39.8	39.8	39.8
5	140.8	140.3	140.2	140.2	140.2	140.1	140.1
6	119.4	119.9	119.7	119.7	119.7	119.9	119.9
7	34.4	35.3	35.2	35.2	35.2	35.5	35.2
8	75.1	75.1	75.0	75.0	75.0	75.0	75.0
9	45.4	45.3	45.1	45.1	45.1	44.9	44.9
10	37.8	38.0	38.2	38.2	38.2	38.1	38.1
11	25.5	29.8	25.5	25.4	25.4	26.0	26.0
12	77.1	77.4	74.5	74.3	74.3	75.5	75.5
13	59.3	59.3	59.1	58.8	58.6	57.6	57.6
14	88.8	88.9	90.0	90.0	90.0	89.1	89.1
15	34.4	34.4	34.3	34.2	34.2	34.3	34.3
16	34.1	34.1	33.5	33.3	33.4	33.6	33.6
17	89.5	89.4	93.1	93.1	93.1	89.4	89.4
18	9.7	9.8	10.6	10.4	10.4	11.2	11.2
19	18.6	18.7	18.7	18.4	18.4	18.4	18.4
20	72.7	71.4	212.1	212.2	212.1	71.8	71.8
21	15.2	15.1	27.8	27.8	27.8	18.8	18.8
	o-OH-B	Nic	*p*-OH-Bz	Cin	Cin	Cin	Cin
1′	170.6	165.0	166.8	167.3	167.3	168.4	168.4
2′	118.1	151.5	122.2	118.9	118.9	119.3	119.3
3′	162.9	129.0	132.8	146.4	146.4	146.8	146.8
4′	114.7	139.2	116.2	135.7	135.7	135.9	135.9
5′/9′	136.3	125.1	163.8	129.3	129.3	129.4	129.4
6′/8′	120.1	153.5	116.2	130.1	130.1	130.0	130.0
7′	131.7		132.8	131.6	131.6	131.4	131.5
		D-cym	D-cym	D-cym	D-digit	D-digit	D-digit
1″		97.2	97.2	97.2	97.0	97.0	97.0
2″		35.9	36.6	36.7	38.9	38.9	38.9
3″		79.2	78.5	78.5	76.9	68.6	75.5
4″		74.5	83.8	83.9	83.8	83.6	83.8
5″		71.3	70.0	69.9	68.6	69.2	68.6
6″		18.6	18.6	18.6	18.6	18.7	18.8
OMe		58.1	58.4	58.5			58.1
			D-cym	D-ole	D-cym	D-cym	D-cym
1‴			101.2	102.8	100.6	100.6	100.6
2‴			36.3	37.4	36.3	35.2	36.3
3‴			79.2	81.6	78.5	79.1	78.5
4‴			71.3	77.0	83.6	71.4	83.6
5‴			69.9	73.3	69.5	74.4	69.4
6‴			18.5	18.5	18.4	18.5	18.5
OMe			58.4	57.4	58.6	58.1	58.5
			D-cym		D-ole		D-ole
1⁗			101.3		102.8		102.8
2⁗			35.6		37.4		37.4
3⁗			79.2		81.6		81.6
4⁗			78.6		76.9		76.9
5⁗			69.9		70.0		70.0
6⁗			18.5		18.4		18.5
OMe			58.1		57.4		57.4

[a] Measured in CD_3OD.
[b] Measured in $CDCl_3$. The assignments were based on COSY, HSQC, and HMBC experiments.

Compound **3** was obtained as white amorphous powders, had a molecular formula $C_{49}H_{72}O_{17}$ as determined by HRESIMS *m/z* 955.4662 ($[M + Na]^+$, calcd. 955.4662). IR spectrum showed the absorption bands for hydroxyl (3440 cm^{-1}), carbonyl (1708 m^{-1}) and olefinic (1609 cm^{-1}) groups. The ^{1}H NMR spectrum of **3** revealed the presence of three singlet methyl protons (δ_H 1.63, s; δ_H 1.12, s; δ_H 2.05, s), three doublet methyl protons (δ_H 1.22, d, *J* = 6.2 Hz; δ_H 1.20, d, *J* = 6.3 Hz; δ_H 1.18, d, *J* = 6.3 Hz), one olefinic proton [δ_H 5.35 d, *J* = 4.9 Hz, H-6)] and four aromatic protons of a parasubstituted benzene ring [δ_H 6.81 (2H, d, *J* = 8.8 Hz, H-4′,6′) and 7.79 (2H, d, *J* = 8.8 Hz, H-3′,7′)]. The ^{13}C NMR spectrum of **3** displayed 49 carbon resonances, belonging to six methyls, ten methylenes, twenty methines (including five olefinic/aromatic, fourteen oxygenated), and nine quateranry carbons (including three olefinic/aromatic, two ester carbonyl,

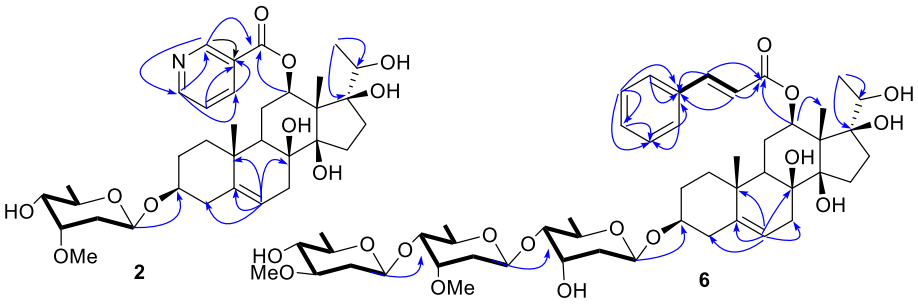


Fig. 2. Selected HMBC (H → C), and $^{1}H-^{1}H$ COSY correlations of compounds **2** and **6**.

three oxygenated). Aforementioned data indicated that **3** was a typical C21 steroidal glycoside with a *p*-hydroxybenzoyl at C-12 and three sugar moieties. By comparing with the reported literature, **3** had the same aglycone fraction as qingyangshengenin [13]. As for sugar moiety, in the NMR spectrums of compound **3** (Tables 1-3), three anomeric carbon resonances at δ_C 97.2, δ_C 101.2, and δ_C 101.3 correlating with anomeric protons at δ_H 4.90 (m), δ_H 4.77 (m) and δ_H 4.77 (m) respectively, were observed revealed the presence of three sugar residues, and the sugar was identified as *β*-D-cymaropyranose by comparison co-TLC and OR data of monosaccharides in the hydrolysates with authentic compounds and published literature [11,12] respectively. Furthermore, the HMBC correlations of H-1″ with C-3; of H-1‴ with C-4″; of H-1⁗ with C-4″ confirmed the sugar chain fragment was 3-*O*-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-cymaropyranosyl. Finally, the structure of **3** was determined as 3-*O*-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-cymaropyranosyl- qingyangshengenin and named Cynanchin **C** (**3**).

The molecular formula of compound **4** was determined to be $C_{44}H_{62}O_{13}$ on the basis of its HRESIMS *m/z* 837.3836 $[M + Na]^+$ (cald 837.3822). IR spectrum showed the absorption bands for hydroxyl (3441 cm $^{-1}$), carbonyl (1708 cm $^{-1}$), and olefinic (1632 cm $^{-1}$) groups. The ^{13}C NMR spectrum of **4** showed 44 carbon signals, consisting of four olefinic carbons, six aromatic carbons, one ketone carbonyl and one ester group, as well as five methyls, two methoxyls, nine methylenes, nineteen methines, and nine quaternary carbons. The detailed analysis of the NMR and MS data and comparison with references indicated that its aglycone is kidjoranin [14]. In addition, two deoxysuger units in **4** were characterized by NMR signals at δ_H 4.89 (m) and δ_H 4.60 (d, J = 9.5 Hz); δ_C 97.2 and δ_C 102.8, indicating that **4** possessed a *β*-oleandropyranose and *β*-cymaropyranosyl sugar sequence, in accordance with the results of acid hydrolysis of **4**. Furthermore, the HMBC correlation from H-1″ to C-3 and from H-1″ to C-4′ indicated that the sugar moiety was *β*-oleandropyranose-(1 → 4)-*β*-cymaropyranosyl and linked to C-3. Meanwhile, the D configurations of the monosaccharide moiety were identified according to their OR values by comparing with those literatures [11,12]. Thus, **4** was identified as 3-*O*-*β*-D-oleanderopyranose-(1 → 4)-*β*-D-cymaropyranoside-kidjoranin and named cynanchin **D** (**4**).

The molecular formula of **5** was determined to be $C_{50}H_{72}O_{16}$ by analyzing the HRESIMS *m/z* 951.4738 $[M + Na]^+$ (calcd. 951.4713) and ^{13}C NMR spectrum. Comparison 1D NMR spectroscopic data (Tables 1 and 2) of **5** and **4** showed that they had the same aglycone, and the significant difference was the presence of an additional sugar moiety in **5**. As well, combinned the characteristic 1D NMR data and coupling constants of three sugars and hydrolysis experiment, as well as TLC analysis, three sugars were assigned unambiguously to be *β*-digitoxopyranosyl, *β*-oleandropyranosyl and *β*-cymaropyranosyl units. Furthermore, the absolute configuration of the deoxysugars was D-series by comparing with the OR value reported in the literature [11,12]. The sequence of the three sugar units located at C-3 of the aglycone was elucidated by HMBC spectrum, in which distinct correlations from δ_H 4.93 (dd, J = 2.1, 9.6 Hz, H-1″) to δ_C 79.3 (C-3); from δ_H 4.82 (dd, J = 2.1, 9.8 Hz, H-1‴) to δ_C 83.6 (C-4″); from δ_H 4.50(dd, J = 2.0, 9.7 Hz, H-1⁗) to δ_C 76.9 (C-4‴) were observed. Therefore, **5** was deduced to be 3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-cymaropyranoside-(1 → 4)-*β*-D-digitoxopyranosyl-deacetylmetaplexigenin and named Cynanchin **E** (**5**).

On the basis of HRESIMS *m/z* 809.4089 [M+ Na] +, (calcd. 809.4083), the molecular formula of **6** was assigned as $C_{43}H_{62}O_{13}$. The 1D NMR spectra of **6** indicated that its aglycone is also 12-*O*-Cinnamoylsarcostin like **8**, except for differences in sugar moiety. In the ^{13}C NMR spectrum of compound **6**, two anomeric carbon resonances at δ_C 97.0 and δ_C 100.6 revealed the presence of two sugar residues, and they were assigned to be one cymarose and one digitoxopyranosyl by comparing the 1D NMR spectroscopic data with compound **17**. Furthermore, the HMBC correlations of H-1″ with C-3; of H-1‴ with C-4″ along with comparing the co-TLC and OR data of monosaccharides in the hydrolysates with authentic compounds and published spectroscopic data [11,12,15] respectively, confirmed the sugar chain fragment was-β-D-cymaropyranosyl (1 → 4) -β-D-digitoxopyranosyl. Finally, the structure of **6** was determined as 3-*O*-*β*-D-cymaropyranoside-(1 → 4)-*β*-D-digitoxopyranosyl-12-*O*-cinnamoylsarcostin and named Cynanchin **F** (**6**).

The molecular formula of compound **7** was determined to be $C_{50}H_{74}O_{16}$ based on the HRESIMS ([M + Na] +, *m/z* 953.4856; calcd 953.4869). Analysis of the 1D NMR data (Tables 1 and 2) of **7** revealed that its structure resembles that of 9, except for the presence of an additional sugar moiety in **7**. in the ^{13}C NMR spectrum of compound **7**, three anomeric carbon resonances at δ_C 97.0, δ_C 100.6, and δ_C 102.8 and three corresponding anomeric proton signals at δ_H 4.95(d, J = 9.9 Hz), δ_H 4.81 (m), δ_H 4.59(dd, J = 2.0, 9.8 Hz) respectively revealed the presence of three sugar residues the sugar chain fragments were completely same with that of **5** by attentively comparing the NMR spectroscopic data. Therefore, the structure of **7** was determined as 3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-cymaropyranoside-(1 → 4)-*β*-D-digitoxopyranosyl-12-*O*-Cinnamoylsarcostin and named Cynanchin **G** (**7**).

The thirteen known C21 steroidal glycosides were identified by comparison of experimental and literature spectroscopic data as cynsaccatol L (8) [16], caudatin 3-*O*-*β*-D-cymaropyranoside (**9**) [17], caudatin-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-cymaropyranoside (**10**) [18], Caudatin 3-*O*-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-cymaropyranoside (**11**) [19], caudatin-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D–digitoxopyranosyl-(1 → 4)-*β*-D-cymaropyranoside (**12**) [20], Otophylloside F (**13**) [22], otophylloside B (**14**) [22], caudatin-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-cymar-opyranoside(**15**) [21], cynotophylloside C (**16**) [21], qingyangshengenin3-*O*-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-digitoxopyranoside (**17**) [20], otophylloside A (**18**) [21], qinyangshengenin-3-*O*-*β*-D-oleandropyranosyl-(1 → 4)-*β*-D-cymaropyranosyl-(1 → 4)-*β*-D-digitoxopyranoside (**19**) [18], Cynanotin D (**20**) [23].

3.2. Biological evaluation

All the isolates were evaluated for their inhibitory effects against the

Table 4
Inhibitory effects of compounds **4–6**, **10–12** and **14–17** on HSC-T6 cell proliferation induced by TGF-β1[a].

Groups	Concentration (μM)	OD values	Cells survival rate	Inhibition rate of cell proliferation (%)
Control	–	1.116 ± 0.030	100.00	–
TGF-β1 model	–	1.305 ± 0.078[b]	116.97	–
4	10	0.769 ± 0.093[d]	101.12	10.10
5	10	0.714 ± 0.125[c]	94.00	16.43
6	10	0.739 ± 0.081[c]	97.22	13.56
10	10	2.087 ± 0.284[b]	118.85	17.67
11	10	2.258 ± 0.070[c]	128.61	11.91
12	10	0.713 ± 0.152[c]	95.87	16.54
14	10	2.074 ± 0.355[b]	118.13	18.17
15	5	0.756 ± 0.124[d]	99.47	11.57
16	10	2.266 ± 0.173[c]	129.04	10.62
17	10	2.345 ± 0.238[d]	133.52	7.51

[a] n = 3, mean ± SD. Control: a set of cells maintained in culture medium with DMSO. Model: a set of cells maintained in culture medium with DMSO and treated only with TGF-β1.
[b] $p < 0.001$, compared to control group.
[c] $p < 0.01$, compared to model group.
[d] $p < 0.05$, compared to model group.

proliferation of HSC-T6 cells induced by TGF-β1. Cytotoxicity assay of the isolates on HSC-T6 cells showed that their maximum non-toxic concentration was 10 μM (Table S1). At the concentration of 10 μM, compound **4–6**, **10–12** and **14–17** showed moderate or marked inhibitory activities against the proliferation of HSC-T6 induced by TGF-β1 with the inhibition rate of 10.1%, 16.0%, 13.6%, 17.7%, 10.9%, 16.5%, 18.2%, 11.6%, 10.6%, and 7.5%, respectively (see Table 4). Analysis of the structure-activity relationship shows that the keto-carbonyl group at C-20 was a key bioactive functionality. When the hydroxyl group was at C-20, compounds **1–2** and **7–8** were inactive. In addition, different substitution group at C-12 gave rise to disparate activity. The inhibitory rate of the ikemaoyl group at C-12 was stronger than that of the cinnamoyl benzene ring group, while the benzoyl or *p*-hydroxybenzoyl group at C-12 had weak activity. Meanwhile, *o*-hydroxybenzoyl or Nicotinic groups lead to inactive compounds, like compounds **1**, **2**. With respect to the number of sugars on C-3, diglycosylation and triglycosylation especially the latter would intensify their activity, whereas mono- and tetraglycosylation showed weaker or no activity (triglycosylation > diglycosylation > tetraglycosylation > monoglycosylation).

4. Conclusions

In conclusion, seven undescribed steroids, namely cynanchin A–**G**(**1–7**), together with thirteen known analogues, were identified from the roots of *C. otophyllum*. Among them, compound **4–6**, **10–12** and **14–17** showed moderate or significant inhibitory effects for the proliferation of hepatic stellate cells (HSCs) induced by transforming growth factor-β1 (TGF-β1) *in vitro*, indicating that they displayed anti-liver fibrosis activities. Our findings not only enrich the structural diversity of *Cynanchum otophyllum*, but also indicate that it play an important role in liver-protection.

Conflict of interests

The authors declare that there is no conflict of interest.

Acknowledgements

This project was financially supported by Foundational Project of Yunnan Key Laboratory of Tobacco Chemistry, R&D Center of China Tobacco Yunnan Industrial Co., Ltd. (KCFZ-2017-1096) and Autonomous Deployment Project (KIB2017010) of Kunming Institute of Botany, CAS. The authors are grateful to the Analytical and Testing Centre at Kunming Institute of Botany for NMR data collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2019.104171.

References

[1] P.T. Li, G.G. Michael, W.D. Stevens, Z.Y. Wu, H.R. Peter, Flora of China, Science Press, Beijing, 1995, pp. 205–223.
[2] X.X. Ma, F.T. Jiang, Q.X. Yang, X.H. Liu, Y.J. Zhang, C.R. Yang, New pregnane glycosides from the roots of *Cynanchum otophyllum*, Steroids. 72 (2007) 778–786.
[3] Q.Z. Mu, Y.M. Shen, Q.L. Zhou, X.J. Hao, The Pharmaceutical Application of Otophyllosides A and B. CN 1064235C, Kunming Institute of Botany, Chinese Academy of Sciences, 2001.
[4] Q.Z. Mu, Y.M. Shen, Q.L. Zhou, X.J. Hao, The Preparation And Application of Otophyllosides C–G. CN 1064048C, Kunming Institute of Botany, Chinese Academy of Sciences, 2001.
[5] Y. Ni, Y.P. Ye, Distribution of C21 steroidal glycosides in plants of Asclepiadaceae and their pharmacological activities, Chin. Trad. Herbal.Drugs. 41 (2010) 162–165.
[6] Y.N. Sun, J.L. Yan, The Clinical Use of the National Medicine Qingyangginseng, 40 Chin. national folk med Magazine, 1999, p. 309.
[7] G. Chen, N. Xu, Z.F. Li, Q.H. Zhan, H.H. Wu, Y.H. Pei, Steroidal glycosides withanti-tumor activity from the roots of *Cynanchum wallichii Wight*, J. Asian Nat. Prod. Res. 12 (2010) 453–457.
[8] Z.J. Wu, L.S. Ding, S.X. Zhao, Recent advance in the chemical and biological studies of *Cynanchum* plants, World.Phytomed. Plant.Med. 6 (1991) 147–154.
[9] W. Liu, Z. Zhang, L. Wu, Y. Dai, Q. Wu, Advance on chemical constituents and pharmacological activities of *Cynanchum* plants, Zhong Yao Cai. 26 (2006) 216–217.
[10] S.X. Qiu, Z.X. Zhang, J. Zhou, Studies on the constituents from *marsdenia globifera*, J. Integr. Plant Biol. 32 (1990) 936–942.
[11] Abe Fumiko, H. Okabe, T. Yamauchi, K. Honda, N. Hayashi, Pregnane glycosides from *Marsdenia tomentosa*, Chem. Pharm. Bull. 47 (1999) 869–875.
[12] Y. Liu, W. Tang, S. Yu, J. Qu, J. Liu, Y. Liu, Eight new C-21 steroidal glycosides from *Dregea sinensis var. corrugate*, Steroids 72 (2007) 514–523.
[13] L.F. Ma, W.G. Shan, Z.J. Zhan, Polyhydroxypregnane glycosides from the roots of *Cynanchum otophyllum*, Helv. Chim. Acta. 94 (2011) 2272–2282.
[14] H.K. Tamihiko, M. Hiroshi, Structure of kidjolanin and the position of the ester linkage of penupogenin, Chem.Pharm.Bull. 20 (1972) 628–629.
[15] G. Wang, X. Liu, L.F. Ma, Z.J. Zhan, Pregnane Glycosides from *Marsdenia tomentosa*, J. Chem. Res. 36 (2012) 38–40.
[16] X. Qian, B. Li, P. Li, et al., C21 steroidal glycosides from *Cynanchum auriculatum* and their neuroprotective effects against H_2O_2 -induced damage in PC12 cells[J], Phytochemistry. 140 (2017) 1–15.
[17] Y. Li, J. Zhang, X. Gu, Y. Peng, W. Huang, S. Qian, Two new cytotoxic preganne glycosides from *Cynanchum auriculatum*, Planta Med. 74 (2008) 551–554.
[18] G. Chen, N. Xu, Y.H. Pei, C_{21} steroidal glycosides from *Cynanchum wallichii Wight*, J. Asian Nat. Prod. Res. 11 (2009) 177–182.
[19] J.L. Li, J. Zhou, Z.H. Chen, S.Y. Guo, C.Q. Li, W.M. Zhao, Bioactive C21 steroidal glycosides from the roots of *Cynanchum otophyllum* that suppress the seizure-like locomotor activity of zebra fish caused by Pentylenetetrazole, J. Nat. Prod. 78 (2015) 1548–1555.
[20] X. Li, M. Zhang, C. Xiang, Y. Qin, J. He, B.C. Li, P. Li, C21 steroids from the roots of *Cynanchum otophyllum*, China J. Chin. Materia Med. 39 (2014) 1450–1456.
[21] X. Li, M. Zhang, C. Xiang, B.C. Li, P. Li, Antiepileptic C21 steroids from the roots of *Cynanchum otophyllum*, J. Asian.Nat.Prod.Res. 17 (2015) 724–732.
[22] Q.Z. Mu, J.R. Lu, Q.L. Zhou, Two new antiepilepsy compounds–otophyllosides A and B, Sci. Sin. B. 29 (1986) 295–301.
[23] J.R. Dong, X.R. Peng, L. Li, S.Y. Lu, L. Zhou, M.H. Qiu, C21 steroidal glycosides with cytotoxic activities from *Cynanchum otophyllum*, Bioorg.Med.Chem.Lett. 28 (2018) 1520–1524.
[24] S. Gelinas, M.G. Martinoli, Neuroprotective effect of estradiol and phytoestrogens on MPP^+-induced cytotoxicity in neuronal PC12 cells, J. Neurosci. Res. 70 (2002) 90–96.
[25] J. Bartalis, F.T. Halaweish, In vitro and QSAR studies of cucurbitacins on HepG2 and HSC-T6 liver cell lines, Bioorg. Med. Chem. Lett. 19 (2011) 2757–2766.
[26] Y.Q. Liu, Z. Wang, S.Q. Kwong, E.L.H. Liu, F.R. Friedman, F.R. Li, R.W.C. Lam, G.C. Zhang, H. Zhang, T. Ye, Inhibition of PDGF, TGF-β, and Abl signaling and reduction of liver fibrosis by the small molecule Bcr-Abl tyrosine kinase antagonist Nilotinib, J. Hepatol. 55 (2011) 612–625.