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Cytotoxicity of pregnane glycosides of *Cynanchum otophyllum*

Mi Zhang [a,1], Xiang Li [a,1], Cheng Xiang [a], Yi Qin [a], Jing He [a], Bao-Cai Li [a,*], Peng Li [b,*]

[a] *Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, China*
[b] *State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau 999078, China*

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A B S T R A C T

Fourteen new pregnane glycosides, including nine caudatin glycosides (**1**–**9**), three qinyangshengenin glycosides (**10**–**12**), one kidjoranin glycosides (**13**) and one gagaminin glycosides (**14**), along with twelve known analogs (**15**–**26**) were isolated from roots of *Cynanchum otophyllum* Schneid. Their structures were deduced by detailed analysis of 1D and 2D NMR spectra, as well as HRESIMS. In this study, all pregnane glycosides obtained (**1**–**26**) were evaluated for their cytotoxic activities using three cancer cell lines (HepG2, Hela, U251). As results, except **6** and **10**, other twenty-four pregnane glycosides showed cytotoxicities at different degrees against three cell lines.

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1. Introduction

Pregnane glycosides, also known as C_{21} steroidal glycosides, are the characteristically chemical and bioactive constituents in the genus *Cynanchum* (subfamily Asclepiadoideae within Apocynaceae) [1,2]. Their basic skeletons are the pregnane derivative, whose C-8, C-13, C-15 and C-17 are easy to be substituted by oxygen-containing groups, and C- or D-rings of aglycones are opening occasionally. The sugar moieties in pregnane glycosides consist of 2,6-dideoxysugars mainly, which are usually connected to C-3 of aglycones as a chain [3]. Previous pharmacological experiments have displayed that pregnanes and their glycosides from *Cynanchum* have a variety of bioactivities, such as cytotoxicity, immunoregulation, multidrug-resistance modulation, and antifungus, etc. [4–7].

The perennial herbaceous plant *Cynanchum otophyllum* Schneid., is mainly distributed in southwest of China. Its roots have been used as folk medicine for treatment of rheumatism, lumbago, abdominal pain and distension, etc. [8–12]. In previously chemical studies, our group and other researchers have found this plant is rich in pregnane glycosides [13–23]. As a part of our ongoing research program to isolate novel pregnane glycosides from *Cynanchum* plants, fourteen new pregnane glycosides (**1**–**14**) and twelve known analogs (**15**–**26**) were isolated from roots of *C. otophyllum* (Fig. 1). All steroidal glycosides obtained (**1**–**26**) were evaluated for their cytotoxicities using three cancer cell lines (HepG2, Hela, U251). The results showed all compounds, except **6** and **10**, displayed cytotoxicities against several cell lines at different degrees.

2. Experimental

2.1. General methods

Optical rotations were obtained on a JASCO P-1020 polarimeter. IR spectra were measured on Bruker Tensor 27 spectrometer in KBr pellets. NMR spectra were performed in $CDCl_3$ or C_5D_5N, recorded on Bruker DRX-500 instrument with TMS as internal standard. UV spectra were recorded on Shimadzu UV-2450 spectropolarimeter. HRESIMS were measured on Agilent LC-MSD TOF spectrometer. Silica gel GF_{254} prepared for TLC and silica gel (100–200 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Company, Qingdao, China. Compounds were purified by semi-preparative HPLC (Beijing Tong Heng Innovation Technology Co., Ltd. LC3000), equipped with a column Zorbax SB-C_{18} (9.4 mm × 250 mm, 5 μM).

2.2. Plant material

The roots of *C. otophyllum* were collected from Traditional Chinese Medicine market in Kunming, China, and identified by Prof. Shiming Guo (Yunan Institute of Traditional Chinese Medicine and Material Medica, China). A voucher specimen (No. Co-2012-05) has been deposited in the laboratory of Faculty of Life Science and Technology, Kunming University of Science and Technology.

* Corresponding authors at: 727# Jingming Road, Chenggong, Kunming, Yunnan 650500, China (B.-C. Li).
E-mail addresses: baocaili@hotmail.com (B.-C. Li), pli1978@hotmail.com (P. Li).
[1] Those authors contributed equally to this work.

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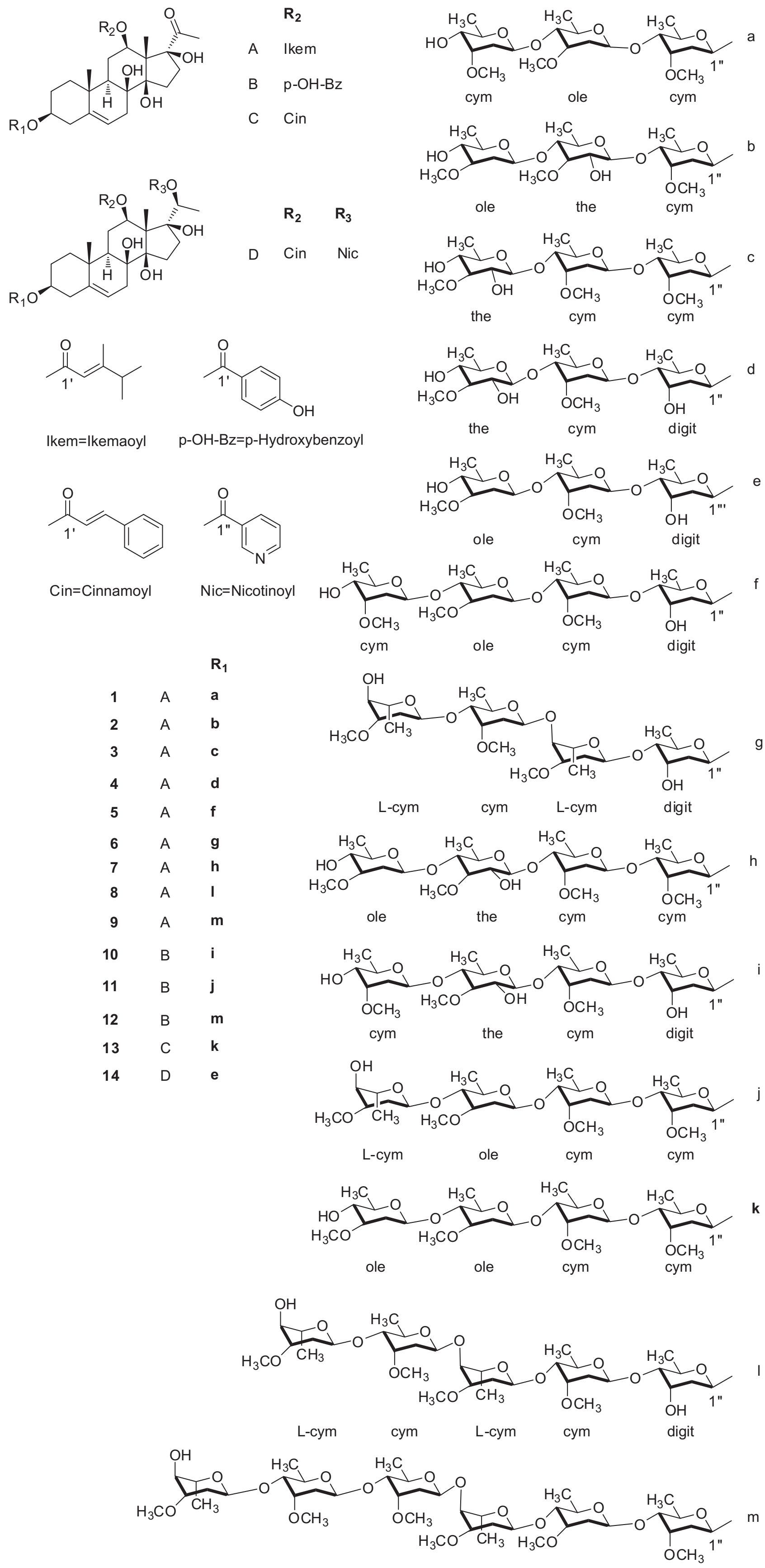

		R_1
1	A	a
2	A	b
3	A	c
4	A	d
5	A	f
6	A	g
7	A	h
8	A	l
9	A	m
10	B	i
11	B	j
12	B	m
13	C	k
14	D	e

Fig. 1. Pregnane glycosides (**1–14**) isolated from the roots of *C. otophyllum*.

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2.3. Extraction and isolation

The air-dried roots of *C. otophyllum* (9.0 kg) were extracted with EtOH (75%) for three times (3 h, 10 L ×3). The organic solvent was removed at vacuum, and the residue suspended in water (10 L). Then, it was extracted with $CHCl_3$ (5 L ×4) to afford $CHCl_3$ soluble fraction (240 g). $CHCl_3$ extract (240 g) was subjected to a silica gel (15 cm × 100 cm) column and eluted with $CHCl_3$–MeOH (40:1, 20:1, 10:1, 5:1, v/v) to get six fractions (Frs. 1–6). Purification was focused on Frs. 2–5 by mainly methods of semi-preparative HPLC. Fr. 2 (30.5 g) was separated by MCI CC with MeOH–H_2O (85:15 → 75:25, v/v) to give five fractions (Frs. 2A–2F). Fr. 2A was subjected to ODS CC with MeOH–H_2O (85:15 → 80:20, v/v) to give three fractions (Frs. 2A1–2A3). Frs. 2A3 was performed on preparative HPLC and eluted with MeOH–H_2O (85:15 → 82:18 → 80:20, v/v) to yield **15** (9.4 mg, Rt = 33.5 min), **10** (60.2 mg, Rt = 35.3 min), **2** (7.5 mg, Rt = 38.4 min), **18** (6.5 mg, Rt = 42.0 min), **1** (10.1 mg, Rt = 45.2 min) and **9** (66.3 mg, Rt = 47.8 min). Fr. 2B was separated by ODS CC with MeOH–H_2O (83:17 → 78:22, v/v) to give two fractions (Frs. 2B1–2B2). Frs. 2B1–2B2 were purified by preparative HPLC, eluted with MeOH–H_2O (80:20 → 78:22, v/v) to yield **26** (10.1 mg, Rt = 45.9 min), **3** (110 mg, Rt = 50.4 min), **5** (48.3 mg, Rt = 55.6 min) and **16** (30.2 mg, Rt = 60.5 min). Fr. 2D was purified by preparative HPLC with MeOH–H_2O (77:23, v/v) to yield **25** (5.6 mg, Rt = 36.9 min), **24** (22.3 mg, Rt = 40.3 min), **23** (13.2 mg, Rt = 45 min) and **13** (5.4 mg, Rt = 50.3 min). Fr.3 was subjected to ODS CC with MeOH–H_2O (80:20 → 76:24, v/v) to give three fractions (Frs. 3A1–3A3). Frs. 3A1 was performed on preparative HPLC, eluted with MeOH–H_2O (76:24, v/v) to yield **17** (40.1 mg, Rt = 43.9 min), **7** (23.3 mg, Rt = 46.1 min), **8** (12.5 mg, Rt = 50.9 min) and **19** (20.1 mg, Rt = 55.8 min). Fr. 4 was subjected to ODS CC with MeOH–H_2O (78:22 → 74:26, v/v) to give four fractions (Frs. 4A1–4A4). Fr. 4A2 was performed on preparative HPLC, eluted with MeOH–H_2O (77:23 → 75:25 → 74:26, v/v) to obtain **14** (18.1 mg, Rt = 40.5 min), **6** (13.4 mg, Rt = 45.9 min), **20** (8.5 mg, Rt = 48.9 min) and **12** (40.9 mg, Rt = 57.9 min). Fr. 5 was subjected to ODS CC with MeOH–H_2O (75:25 → 70:30, v/v) to give two fractions (Frs. 5A1–5A2). Fr. 5A1 was performed on preparative HPLC, eluted with MeOH–H_2O (73:27, v/v) to yield **22** (4.1 mg, Rt = 33.9 min), **21** (13.2 mg, Rt = 36.3 min), **4** (60.2 mg, Rt = 42.9 min) and **11** (20.3 mg, Rt = 46.9 min).

2.3.1. Compound **1**

White amorphous powder; $[\alpha]_D^{21}$ −4.7 (*c* 1.06, MeOH); UV (MeOH) λ_{max} (log ε) 226 (3.86) nm; IR (KBr) $\tilde{\nu}_{max}$ 3442, 2965, 2933, 1713, 1641, 1450, 1384, 1368, 1316, 1224, 1166, 1063, 1004, 911, 863; HRESIMS *m/z* 945.5180 ([M+Na]$^+$, $C_{49}H_{78}O_{16}Na$, calcd. 945.5182). ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Tables 1–3.

2.3.2. Compound **2**

White amorphous powder; $[\alpha]_D^{22}$ −5.9 (*c* 3.46, MeOH); UV (MeOH) λ_{max} (log ε) 228 (3.83) nm, 225 (3.81) nm; IR (KBr) $\tilde{\nu}_{max}$ 3443, 2965, 2932, 1713, 1641, 1450, 1384, 1369, 1315, 1224, 1165, 1065, 1005, 913, 862; HRESIMS *m/z* 961.5140 ([M+Na]$^+$, $C_{49}H_{78}O_{17}Na$, calcd. 961.5141). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1–3.

2.3.3. Compound **3**

White amorphous powder; $[\alpha]_D^{22}$ +2.8 (*c* 1.04, MeOH); UV (MeOH) λ_{max} (log ε) 224 (3.87) nm, 226 (3.86) nm; IR (KBr) $\tilde{\nu}_{max}$ 3453, 2968, 2933, 1713, 1642, 1451, 1384, 1368, 1317, 1224, 1167, 1059, 1005, 912, 864; HRESIMS *m/z* 961.5134 ([M+Na]$^+$, $C_{49}H_{78}O_{16}Na$, calcd. 961.5141). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1–3.

2.3.4. Compound **4**

White amorphous powder; $[\alpha]_D^{22}$ +3.2 (*c* 2.01, MeOH); UV (MeOH) λ_{max} (log ε) 220 (3.58) nm; IR (KBr) $\tilde{\nu}_{max}$ 3461, 2969, 2934, 1713, 1643, 1451, 1384, 1369, 1317, 1224, 1166, 1084, 1007, 912, 864; HRESIMS *m/z* 947.4976 ([M+Na]$^+$, $C_{48}H_{76}O_{17}Na$, calcd. 947.4974). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1–3.

2.3.5. Compound **5**

White amorphous powder; $[\alpha]_D^{21}$ +2.6 (*c* 1.16, MeOH); UV (MeOH) λ_{max} (log ε) 224 (3.92) nm; IR (KBr) $\tilde{\nu}_{max}$ 3442, 2968, 2934, 1713, 1641, 1450, 1385, 1370, 1317, 1224, 1165, 1063, 1004, 912, 865; HRESIMS *m/z* 1075.5810 ([M+Na]$^+$, $C_{55}H_{88}O_{19}Na$, calcd. 1075.5812). ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Tables 1–3.

2.3.6. Compound **6**

White amorphous powder; $[\alpha]_D^{22}$ −67.2 (*c* 1.05, MeOH); UV (MeOH) λ_{max} (log ε) 219 (3.74) nm; IR (KBr) $\tilde{\nu}_{max}$ 3445, 2969, 2932, 1714, 1641, 1452, 1384, 1367, 1316, 1223, 1164, 1055, 1005, 913, 865; HRESIMS *m/z* 1075.5815 ([M+Na]$^+$, $C_{55}H_{88}O_{19}Na$, calcd. 1075.5812). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1–3.

2.3.7. Compound **7**

White amorphous powder; $[\alpha]_D^{22}$ +7.8 (*c* 1.60, MeOH); UV (MeOH) λ_{max} (log ε) 219 (3.75) nm; IR (KBr) $\tilde{\nu}_{max}$ 3452, 2969, 2933, 1714, 1643, 1451, 1383, 1368, 1317, 1223, 1164, 1059, 1004, 912, 864; HRESIMS *m/z* 1105.5912 ([M+Na]$^+$, $C_{56}H_{90}O_{20}Na$, calcd. 1105.5917). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1–3.

2.3.8. Compound **8**

White amorphous powder; $[\alpha]_D^{22}$ −2.2 (*c* 1.63, MeOH); UV (MeOH) λ_{max} (log ε) 230 (3.98) nm, 225 3.95)nm; IR (KBr) $\tilde{\nu}_{max}$ 3447, 2968, 2934, 1712, 1641, 1450, 1384, 1369, 1317, 1224, 1166, 1061, 1004, 910, 866; HRESIMS *m/z* 1219.6604 ([M+Na]$^+$, $C_{62}H_{100}O_{22}Na$, calcd. 1219.6598). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1–3.

2.3.9. Compound **9**

White amorphous powder; $[\alpha]_D^{21}$ −9.9 (*c* 1.00, MeOH); UV (MeOH) λ_{max} (log ε) 220 (3.93) nm; IR (KBr) $\tilde{\nu}_{max}$ 3443, 2969, 2932, 1714, 1642, 1452, 1382, 1368, 1317, 1223, 1165, 1065, 1004, 912, 864; HRESIMS *m/z* 1377.7556 ([M+Na]$^+$, $C_{70}H_{114}O_{25}Na$, calcd. 1377.7552). ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Tables 1–3.

2.3.10. Compound **10**

White amorphous powder; $[\alpha]_D^{21}$ +9.9 (*c* 1.08, MeOH); UV (MeOH) λ_{max} (log ε) 259 (3.57) nm; IR (KBr) $\tilde{\nu}_{max}$ 3447, 2972, 2934, 1711, 1610, 1596, 1515, 1451, 1382, 1310, 1276, 1167, 1087, 1059, 911, 856, 772; HRESIMS *m/z* 1101.5242 ([M+Na]$^+$, $C_{55}H_{82}O_{21}Na$, calcd. 1101.5240). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 4–6.

2.3.11. Compound **11**

White amorphous powder; $[\alpha]_D^{22}$ −25.5 (*c* 1.32, MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.00) nm, 259 (3.51) nm; IR (KBr) $\tilde{\nu}_{max}$ 3451, 2972, 2933, 1711, 1611, 1515, 1452, 1383, 1311, 1276, 1164, 1099, 1057, 912, 853, 773; HRESIMS *m/z* 1099.5448 ([M+Na]$^+$, $C_{56}H_{84}O_{20}Na$, calcd. 1099.5446). ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 4–6.

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Table 1
^{13}C NMR data for the aglycone moieties of compounds **1–9** (δ in ppm, 125 MHz).

No.	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]	**8**[b]	**9**[a]
1	38.9	39.0	39.0	39.0	38.7	39.2	39.0	39.0	38.7
2	29.0	29.7	29.9	29.9	28.8	29.8	29.9	29.8	28.8
3	79.0	77.7	77.7	77.7	77.8	77.7	77.7	77.7	77.8
4	38.8	39.3	39.3	39.3	38.7	39.0	39.3	39.3	38.7
5	140.8	139.4	139.5	139.4	140.7	139.4	139.4	139.4	140.7
6	117.6	118.8	118.9	119.1	117.6	119.1	119.9	119.1	117.6
7	34.2	34.7	34.9	34.8	34.2	34.8	34.8	34.8	34.2
8	74.4	74.3	74.4	74.3	74.3	74.3	74.3	74.3	74.8
9	43.9	44.4	44.6	44.6	43.7	44.6	44.6	44.6	43.6
10	37.2	37.4	37.5	37.4	37.1	37.4	37.4	37.4	37.1
11	24.3	25.1	25.1	25.1	24.2	25.0	25.1	25.0	24.2
12	72.5	72.6	72.6	72.6	72.2	73.3	72.6	72.6	72.7
13	58.0	57.9	58.0	58.0	57.9	58.0	58.0	58.0	57.9
14	88.1	89.4	89.5	89.5	87.9	89.4	89.5	89.4	88.0
15	33.9	33.3	33.9	33.8	33.0	33.8	33.8	33.8	33.0
16	32.0	32.5	33.0	33.0	31.8	33.0	33.0	33.0	31.8
17	91.5	92.4	92.5	92.4	91.4	92.4	92.4	92.4	91.5
18	9.4	10.7	10.8	10.7	9.4	10.7	10.7	10.7	9.4
19	18.6	18.2	18.2	18.2	18.6	18.1	18.2	18.1	18.6
20	208.7	209.1	209.4	209.3	208.9	209.3	209.4	209.3	208.9
21	27.1	27.7	27.6	27.5	27.2	27.5	27.5	27.5	27.2
1′	166.7	165.6	166.0	166.0	166.9	165.9	166.0	165.9	166.9
2′	113.1	114.0	114.2	114.2	112.9	114.2	114.2	114.2	112.9
3′	165.9	164.9	165.5	165.3	165.9	165.3	165.4	165.3	165.9
4′	38.2	38.1	38.2	38.1	38.2	38.1	38.2	38.1	38.1
5′	21.0	20.8	20.9	20.8	21.0	20.8	20.9	20.8	20.9
6′	20.9	20.9	21.0	20.9	20.8	20.9	21.0	20.9	20.8
7′	16.5	16.7	16.5	16.5	16.5	16.7	16.5	16.7	16.5

[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.

*2.3.12. Compound **12***

White amorphous powder; $[\alpha]_D^{22}$ −52.8 (*c* 0.75, MeOH); UV (MeOH) λ_{max} (log ε) 260 (3.89) nm; IR (KBr) $\tilde{\upsilon}_{max}$ 3446, 2972, 2933, 1713, 1611, 1610, 1516, 1452, 1381, 1313, 1274, 1164, 1100, 1059, 913, 853, 772; HRESIMS *m*/*z* 1387.7018 ([M+Na]$^+$, $C_{70}H_{108}O_{26}Na$, calcd. 1387.7021). ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 4–6.

*2.3.13. Compound **13***

White amorphous powder; $[\alpha]_D^{21}$ +15.8 (*c* 1.90, MeOH); UV (MeOH) λ_{max} (log ε) 216 (4.30) nm, 278 (3.80) nm; IR (KBr) $\tilde{\upsilon}_{max}$ 3442, 2992, 1713, 1636, 1496, 1450, 1384, 1368, 1311, 1165, 1088, 911, 864, 769; HRESIMS *m*/*z* 1109.5649 ([M+Na]$^+$, $C_{58}H_{86}O_{19}Na$, calcd. 1109.5655). ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Tables 4–6.

*2.3.14. Compound **14***

White amorphous powder; $[\alpha]_D^{22}$ +111.3 (*c* 1.49, MeOH); UV (MeOH) λ_{max} (log ε) 217 (3.89) nm, 282 (3.81) nm; IR (KBr) $\tilde{\upsilon}_{max}$ 3447, 2972, 2934, 2309, 1717, 1637, 1593, 1450, 1379, 1286, 1165, 1074, 989, 952, 911, 866, 743; HRESIMS *m*/*z* 1036.5267 ([M+H]$^+$, $C_{56}H_{78}NO_{17}$, calcd. 1036.5264). ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 4–6.

2.4. Acidic hydrolysis

A solution of **1–14** (each 2.0 mg) in MeOH (2 mL) was added 0.025 M H_2SO_4 (1 mL). The solution was kept at 60 °C for 2 h, and diluted with H_2O (5 mL). The hydrolyzed mixture was neutralized with saturated aqueous $Ba(OH)_2$ solution. The precipitate was filtered and the filtrate evaporated to dryness. The residue was isolated to CC (Toyopearl-HW-40C gel, MeOH) to afford sugars and aglycone parts successively. The total yields of digitoxoses, cymaroses, oleandroses and thevetoses from the hydrolysates of **1–14** were 0.5 mg, 1.1 mg, 0.6 mg and 0.3 mg, and each monosaccharide was confirmed by TLC, comparing with its R_f value of authentic sugar, respectively. As results, cymaroses were detected from **1–14**, digitoxoses were detected from **3–6**, **8** and **10**, oleandroses were detected from **1**, **2**, **5**, **6**, **8** and **11–14**, and thevetoses were detected from **3–4**, **7** and **10**. The R_f values of thevetose, digitoxose, cymarose, and oleandrose were 0.10, 0.18, 0.38, and 0.40 with solvent $CHCl_3$–MeOH (10:1); 0.05, 0.09, 0.15, and 0.17 with solvent petroleum ether–Me_2CO (7:3), respectively. All authentic compounds were purchased from National Institutes for Food and Drug Control, China.

2.5. Determination of the absolute configuration of the sugars

Compound **10** (20.0 mg) was hydrolyzed by the above method to get digitoxose, cymarose, thevetose, respectively, which absolute configurations were confirmed by comparison of their optical rotations with those reported in literatures (digitoxose $[\alpha]_D^{21}$ +42.5 (*c* 0.16, H_2O), [15]: D-digitoxose $[\alpha]_D^{20}$ +43.0; cymarose $[\alpha]_D^{21}$ +53.5 (*c* 0.20, H_2O), [24]: D-cymarose $[\alpha]_D^{27}$ +56.0; thevetose $[\alpha]_D^{21}$ +36.5 (*c* 0.10, H_2O), [24]: D-thevetose $[\alpha]_D^{27}$ +35.0). By the same method, D-oleandrose and L-cymarose were detected from **6** (5.0 mg), and their optical rotations were determined and compared with reported data (oleandrose $[\alpha]_D^{21}$ −7.5 (*c* 0.10, H_2O), [24]: D-oleandrose $[\alpha]_D^{27}$ −12.0; cymarose $[\alpha]_D^{21}$ −44.9 (*c* 0.10, H_2O); [23]: L-cymarose $[\alpha]_D^{20}$ −47.3).

2.6. Cytotoxic bioassay

The protocol of the cytotoxic bioassay was provided in a previously published paper [25]. Briefly, each cell line (HepG2, HeLa, U251 human cancer cell lines) was cultured in DMEM medium in 5% CO_2 at 37 °C. Then 100 µL of the cell suspension was seeded onto 96-well cell culture plates at an initial density of 1×10^4 cells/mL and allowed to adhere for 12 h. Each tumor cell line was exposed to each test compound at various concentrations for 48 h, with 5-FU and cisplatin (Sigma, St. Louis, MO, USA) used as positive

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Table 2
^{13}C NMR data for the sugar moieties of compounds **1–9** (δ in ppm, 125 MHz).

No.	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]	**8**[b]	**9**[a]
	D-cym	D-cym	D-cym	D-digit	D-digit	D-digit	D-cym	D-digit	D-cym
1′′	96.1	96.3	96.5	96.4	95.9	96.4	96.4	95.6	94.6
2′′	35.8	37.2	37.3	39.0	37.1	38.8	37.4	39.0	35.1
3′′	77.0	77.9	78.1	67.5	66.7	67.8	78.1	67.5	77.1
4′′	82.3	83.1	83.2	83.5	82.5	82.3	81.6	83.4	82.4
5′′	68.5	68.9	69.1	68.6	68.7	68.8	69.0	68.7	68.8
6′′	18.2	18.5	18.6	18.7	18.3	18.6	18.7	18.6	18.2
OMe	58.3	58.8	58.9				58.9		57.7
	D-ole	D-the	D-cym	D-cym	D-cym	L-cym	D-cym	D-cym	D-ole
1′′′	101.4	104.1	100.5	99.7	98.4	99.0	100.5	99.7	101.3
2′′′	36.4	75.2	37.1	36.7	35.3	32.5	37.3	36.7	35.9
3′′′	79.0	88.2	78.2	78.1	76.9	73.6	78.0	77.7	78.5
4′′′	82.7	83.4	83.4	83.2	82.4	77.7	82.4	82.3	81.8
5′′′	71.2	72.0	69.4	69.5	68.2	66.3	69.3	69.3	71.5
6′′′	18.3	18.8	18.6	18.5	18.2	18.2	18.6	18.6	17.7
OMe	56.6	60.9	58.9	58.8	58.3	57.1	58.9	58.5	56.8
	D-cym	D-ole	D-the	D-the	D-ole	D-cym	D-the	L-cym	L-cym
1′′′′	98.2	101.6	106.3	106.3	101.4	96.0	106.0	99.0	98.4
2′′′′	33.9	36.8	75.1	75.1	36.4	36.7	74.8	32.2	31.6
3′′′′	77.6	79.2	87.9	87.9	78.9	77.3	85.6	73.2	72.0
4′′′′	71.7	76.2	75.9	75.9	82.2	81.1	83.2	77.7	76.9
5′′′′	71.1	72.9	72.8	72.8	71.1	69.4	71.6	65.3	63.8
6′′′′	18.4	18.5	18.6	18.5	18.2	18.6	18.6	18.5	18.1
OMe	57.1	57.3	61.0	60.9	56.7	58.3	60.4	56.6	56.3
					D-cym	L-cym	D-ole	D-cym	D-cym
1′′′′′					98.2	98.4	100.4	96.4	96.6
2′′′′′					33.7	32.2	37.0	36.8	35.4
3′′′′′					77.5	76.4	83.4	77.7	76.9
4′′′′′					71.5	73.3	76.3	82.1	82.4
5′′′′′					71.2	66.6	73.1	69.3	68.5
6′′′′′					18.3	18.5	18.5	18.5	18.2
OMe					57.1	56.6	57.1	58.2	57.9
								L-cym	D-cym
1′′′′′′								98.9	98.3
2′′′′′′								32.3	35.5
3′′′′′′								76.4	76.9
4′′′′′′								73.3	81.9
5′′′′′′								66.4	68.3
6′′′′′′								18.4	18.2
OMe								56.9	58.3
									L-cym
1′′′′′′′									97.4
2′′′′′′′									31.8
3′′′′′′′									74.5
4′′′′′′′									71.6
5′′′′′′′									65.5
6′′′′′′′									18.2
OMe									56.2

D-digit: β-D-digitoxopyranosyl. D-cym: β-D-cymaropyranosyl. L-cym: α-L-cymaropyranosyl. D-ole: β-D-oleandropyranosyl. D-the: β-D-thevetopyranosyl.
[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.

controls. At the end of the treatment period, 20 μL (5 mg/mL) of the MTT was added to each well and the plates were incubated for 4 h at 37 °C. The medium was removed and MTT reduction product (formazan crystals) was dissolved in dimethylsulfoxide (DMSO) and measured with an ELISA reader (Bio-Rad, USA) at a wavelength of 492 nm. IC_{50} value of each test compound was calculated, given in Table 7. Each assay was done in triplicate.

3. Results and discussion

This study aims to systematically elucidate the structure and cytotoxicities of C_{21} steroids in *C. otophyllum*. TLC was used to preliminarily determine the existence of pregnane glycosides. The TLC spots, showing characteristically green color (Liebermann–Burchard Reaction) of C_{21} steroids, were rich in $CHCl_3$ fraction from 75% ethanol extract of its roots. Thus, the $CHCl_3$ fraction were successively and repeatedly subjected to silica gel and ODS column chromatography, and purified by semi-preparative HPLC to afford 26 pregnane glycosides.

Compound **1** was obtained as a white powder. Its molecular formula $C_{49}H_{78}O_{16}$ was defined by HRESIMS. The ^{13}C NMR spectrum (Tables 1 and 2) showed 49 carbon signals, seven of which at δ_C 166.7, 113.1, 165.9, 38.2, 21.0, 20.9 and 16.5 were assigned to an ikemaoyl group by study of HSQC and HMBC spectra. The aglycone part of **1** was identified as caudatin considering the NMR data [21].

Table 3

^{1}H NMR spectral data of compounds **1–9** (δ in ppm, J in Hz, 500 MHz).

No.	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]	**8**[b]	**9**[a]
1	1.08 m; 1.87 m	1.11 m; 1.81 m	1.08 m; 1.78 m	1.10 m; 1.80 m	1.08 m; 1.87 m	1.08 m; 1.79 m	1.09 m; 1.78 m	1.08 m; 1.79 m	1.08 m; 1.85 m
2	1.60 m; 1.91 m	1.80 m; 2.08 m	1.77 m; 2.04 m	1.78 m; 2.08 m	1.61 m; 1.89 m	1.78 m; 2.06 m	1.78 m; 2.05 m	1.78 m; 2.07 m	1.59 m; 1.90 m
3	3.57 m	3.84 m	3.84 m	3.86 m	3.56 m	3.84 m	3.84 m	3.84 m	3.55 m
4	2.29 m; 2.36 m	2.40 m; 2.55 m	2.39 m; 2.55 m	2.40 m; 2.51 m	2.28 m; 2.36 m	2.39 m; 2.50 m	2.41 m; 2.52 m	2.39 m; 2.50 m	2.28 m; 2.36 m
6	5.36 s	5.28[c]	5.27[c]	5.25 s	5.35 s	5.28 s	5.27[c]	5.26[c]	5.36 s
7	2.18 m	2.47 m	2.44 m	2.45 m	2.18 m	2.44 m	2.44 m	2.43 m	2.17 m
9	1.51 m	1.73 m	1.70 m	1.72 m	1.51 m	1.70 m	1.72 m	1.70 m	1.49 m
11	1.84 m	2.16 m; 2.26 m	2.13 m; 2.23 m	2.15 m; 2.25 m	1.81 m	2.13 m; 2.24 m	2.14 m; 2.24 m	2.14 m; 2.23 m	1.80 m
12	4.56 t (7.5)	5.04[d]	5.02 dd (4.0, 11.5)	5.02[d]	4.55 t (7.5)	5.05[d]	5.0 5[d]	5.04[d]	4.54 t (7.5)
15	1.95 m	2.11 m	2.08 m	2.10 m	1.93 m	2.10 m	2.09 m	2.09 m	1.93 m
16	1.84 m; 2.84 m	2.02 m; 3.27 m	2.01 m; 3.27 m	2.02 m; 3.27 m	1.83 m; 2.84 m	2.02 m; 3.26 m	2.03 m; 3.26 m	2.01 m; 3.26 m	1.84 m; 2.84 m
18	1.40 s	1.98 s	1.97 s	1.98 s	1.40 s	1.99 s	1.98 s	1.99 s	1.39 s
19	1.12 s	1.32 s	1.31 s	1.30 s	1.12 s	1.31 s	1.31 s	1.31 s	1.11 s
21	2.12 s	2.51 s	2.50 s	2.50 s	2.16 s	2.50 s	2.50 s	2.50 s	2.14 s
2′	5.51 s	5.86 s	5.85 s	5.86 s	5.51 s	5.86 s	5.86 s	5.86 s	5.50 s
4′	2.35 m	2.25 m	2.26 m	2.25 m	2.36 m	2.25 m	2.25 m	2.25 m	2.34 m
5′	1.05 d (7.0)	0.92 d (7.5)	0.92 d (7.5)	0.92 d (6.5)	1.05 d (7.0)	0.92 d (7.5)	0.92 d (6.5)	0.92 d (7.5)	1.04 d (7.0)
6′	1.06 d (7.0)	0.94 d (7.5)	0.99 d (7.5)	0.94 d (6.5)	1.06 d (7.0)	0.94 d (7.5)	0.93 d (6.5)	0.94 d (7.5)	1.05 d (7.0)
7′	2.16 s	2.27 s	2.26 s	2.25 s	2.12 s	2.27 s	2.26 s	2.27 s	2.13 s
	D-cym	D-cym	D-cym	D-digit	D-digit	D-digit	D-cym	D-digit	D-cym
1″	4.82 dd (9.5, 1.5)	5.26 d (9.5)	5.25 d (10.0)	5.46 dd (10.0, 1.0)	4.91 dd (9.5, 1.5)	5.41 dd (9.0, 1.0)	5.26[c]	5.47 dd (9.5, 1.0)	4.84[c]
2″	1.58 m; 2.10 m	1.88 m; 2.33 m	1.85 m; 2.29 m	2.06 m; 2.38 m	1.68 m; 2.10 m	2.03 m; 2.38 m	1.85 m; 2.33 m	2.07 m; 2.39 m	1.61 m; 2.09 m
3″	3.81 m	4.05 m	4.05 m	4.62 m	4.23 m	4.48 m	4.06 m	4.62 m	3.73 m
4″	3.17 m	3.49 m	3.48 m	3.48 m	3.18 m	3.48 m	3.49 m	3.45 m	3.22 m
5″	3.86 m	4.21 m	4.20 m	4.30 m	3.76 m	4.19 m	4.21 m	4.28 m	3.81 m
6″	1.29 d (5.5)	1.41 d (6.0)	1.37 d (6.0)	1.42 d (6.0)	1.28 d (6.0)	1.40 d (6.5)	1.36 d (6.0)	1.42 d (6.0)	1.19 d (6.0)
OMe	3.41 s	3.56 s	3.60 s				3.60 s		3.42 s
	D-ole	D-the	D-cym	D-cym	D-cym	L-cym	D-cym	D-cym	D-ole
1‴	4.44 dd (9.5, 1.5)	4.94 d (8.0)	5.10 d (9.5)	5.16 dd (9.5, 1.0)	4.87 dd (9.5, 1.5)	4.97 d (3.0)	5.11[d]	5.12 dd (9.5, 1.0)	4.44 d (9.5)
2‴	1.52 m; 2.32 m	3.92 m	1.81 m; 2.31 m	1.78 m; 2.32 m	1.61 m; 2.33 m	1.69 m; 2.26 m	1.81 m; 2.30 m	1.78 m; 2.33 m	1.53 m; 2.32 m
3‴	3.55 m	3.60 m	4.04 m	4.05 m	3.80 m	3.73 m	4.03 m	3.85 m	3.24 m
4‴	3.23 m	3.67 m	3.58 m	3.52 m	3.21 m	3.89 m	3.55 m	3.45 m	3.19 m
5‴	3.58 m	3.57 m	4.19 m	4.21 m	3.90 m	4.55 m	4.22 m	4.18 m	3.26 m
6‴	1.22 d (6.5)	1.70 d (6.0)	1.57 d (6.0)	1.53 d (6.0)	1.22 d (6.0)	1.40 d (6.0)	1.47 d (5.5)	1.36 d (6.0)	1.17 d (6.0)
OMe	3.40 s	3.88 s	3.55 s	3.55 s	3.42 s	3.36 s	3.61 s	3.52 s	3.36 s
	D-cym	D-ole	D-the	D-the	D-ole	D-cym	D-the	L-cym	L-cym
1⁗	4.84 dd (9.5, 1.5)	4.69 d (9.5)	4.75 d (7.5)	4.75 d (6.0)	4.44 dd (9.5, 1.5)	5.23 dd (9.5, 1.5)	4.71 d (8.0)	4.96 d (3.5)	4.83[c]
2⁗	1.94 m; 2.20 m	1.78 m; 2.47 m	3.91 m	3.90 m	1.71 m; 2.10 m	1.77 m; 2.30 m	3.88 m	1.82 m; 2.32 m	1.83 m; 2.24 m
3⁗	3.62 m	3.58 m	3.61 m	3.61 m	3.21 m	3.60 m	3.69 m	3.60 m	3.56 m
4⁗	3.29 m	3.62 m	3.60 m	3.60 m	3.16 m	3.45 m	3.68 m	3.85 m	3.88 m
5⁗	3.64 m	3.73 m	3.72 m	3.70 m	3.29 m	4.17 m	3.67 m	4.62 m	4.27 m
6⁗	1.29 d (5.5)	1.58 d (6.0)	1.58 d (7.0)	1.56 d (6.0)	1.30 d (6.0)	1.35 d (6.0)	1.58 d (6.0)	1.50 d (6.5)	1.24 d (6.0)
OMe	3.45 s	3.52 s	3.89 s	3.89 s	3.40 s	3.52 s	3.88 s	3.35 s	3.33 s
					D-cym	L-cym	D-ole	D-cym	D-cym
1‴″					4.80 dd (9.5, 1.5)	5.05 s	4.99 d (9.5)	4.54 dd (9.5, 1.0)	4.82[c]
2‴″					1.56 m; 2.19 m	1.78 m; 2.27 m	1.74 m; 2.47 m	1.78 m; 2.33 m	1.61 m; 2.09 m
3‴″					3.60 m	3.73 m	3.48 m	3.85 m	3.78 m
4‴″					3.21 m	3.60 m	3.50 m	3.45 m	3.24 m
5‴″					3.59 m	4.56 m	3.61 m	4.18 m	3.83 m
6‴″					1.22 d (6.0)	1.51 d (6.0)	1.58 d (6.0)	1.36 d (6.0)	1.19 d (6.0)
OMe					3.45 s	3.37 s	3.48 s	3.52 s	3.45 s
								L-cym	D-cym
1‴‴								4.93 d (2.5)	4.73 d (9.5)
2‴‴								1.80 m; 2.35 m	1.53 m; 2.11 m

Table 3 (*continued*)

No.	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[a]	**6**[b]	**7**[b]	**8**[b]	**9**[a]
3″″″								3.70 m	3.80 m
4″″″								3.73 m	3.06 m
5″″″								4.53 m	3.84 m
6″″″								1.50 d (6.0)	1.19 d (6.0)
OMe								3.51 s	3.46 s
									L-cym
1″″″′									4.79 d (3.5)
2″″″′									1.83 m; 2.21 m
3″″″′									3.68 m
4″″″′									3.27 m
5″″″′									4.04 m
6″″″′									1.24 d (6.0)
OMe									3.33 s

D-digit: β-D-digitoxopyranosyl. D-cym: β-D-cymaropyranosyl. L-cym: α-L-cymaropyranosyl. D-ole: β-D-oleandropyranosyl. D-the: β-D-thevetopyranosyl.
[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.
[c] Partial overlapped with other signals.
[d] Overlapped with H_2O signals.

Table 4
^{13}C NMR data for the aglycone moieties of compounds **10–14** (δ in ppm, 125 MHz).

No.	**10**[b]	**11**[b]	**12**[b]	**13**[a]	**14**[b]
1	39.0	39.3	39.0	38.7	39.3
2	29.9	29.9	29.8	29.1	29.9
3	77.7	77.7	77.7	77.8	77.7
4	39.3	38.9	39.2	38.7	39.1
5	139.4	139.5	139.4	140.7	139.4
6	119.1	119.1	119.1	117.5	119.3
7	34.8	34.8	34.8	34.5	34.9
8	74.3	74.4	74.3	75.4	74.3
9	44.6	44.5	44.5	43.6	44.1
10	37.4	37.4	37.4	37.1	37.3
11	25.1	25.2	25.2	24.5	25.7
12	72.6	73.4	73.4	72.7	74.6
13	58.0	58.4	58.4	58.7	57.2
14	89.5	89.5	89.5	87.7	89.0
15	33.8	33.9	33.9	33.1	34.1
16	33.0	33.1	33.2	31.9	33.7
17	92.4	92.5	92.5	91.4	87.5
18	10.7	10.8	10.8	9.4	11.5
19	18.2	18.2	18.1	18.6	18.0
20	209.3	209.7	209.8	209.1	76.4
21	27.5	27.8	27.9	27.6	15.4
1′	166.0	165.4	165.3	165.7	166.8
2′	114.2	122.0	122.0	117.7	120.3
3′	165.3	132.4	132.4	145.4	144.0
4′	38.1	116.2	116.2	134.3	134.9
5′	20.8	163.6	163.6	128.1	128.5
6′	20.9	116.2	116.2	128.9	129.3
7′	16.5	132.4	132.4	130.4	128.5
8′				128.9	129.3
9′				128.1	128.5
1″					164.7
2″					153.8
3″					127.0
4″					137.3
5″					123.0
6″					151.4

[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.

The anomeric carbons at δ_C 96.1 (cym C-1), 101.4 (ole C-1), and 98.2 (cym C-1), and the corresponding proton signals at δ_H 4.82 (dd, J = 9.5, 1.5 Hz), 4.44 (dd, J = 9.5, 1.5 Hz), and 4.86 (dd, J = 9.5, 1.5 Hz), respectively, showed correlations in the HSQC experiment. Analysis of the coupling constants of three secondary methyl groups [δ_H 1.29 (d, J = 5.5 Hz), 1.22 (d, J = 6.5 Hz) and 1.29 (d, J = 5.5 Hz)] in ^{1}H NMR (Table 3) and of the anomeric protons, as well as other signals in 1D NMR spectra (Tables 1–3) indicated a β-linkage for the sugar chain, which was formed by two D-cymarose and one D-oleandrose units. This fact was confirmed by comparison of the TLC and optical rotation (OR) analysis of acid hydrolysate of **1** with those of authentic samples. The sequence of these three sugar units linked C-3 of the caudatin was illustrated by distinct HMBC correlations from δ_H 4.82 (β-D-cymaropyranosyl H-1″) to δ_C 78.0 (C-3), from δ_H 4.44 (inner β-D-oleandropyranosyl H-1‴) to δ_C 82.3 (β-D-cymaropyranosyl C-4″), from δ_H 4.86 (terminal β-D-cymaropyranosly H-1″″) to δ_C 82.7 (β-D-oleandropyranosyl C-4‴). Thus, the structure of **1** was determined to be caudatin-3-*O*-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Comparison of 1D NMR data (Tables 1 and 3) and the key correlations in 2D NMR with those reported in the literatures [21,22] suggested that aglycone parts of **2**–**9** were caudatin-3-O-multiosides, as the same as **1**. The differences observed among them were orders of linkage and types of sugar units, which were identified by NMR analysis, HRESIMS, and TLC and optical rotation comparison with authentic samples, respectively.

Compounds **2** and **3** had the same molecular formula $C_{49}H_{78}O_{17}$ based on HRESIMS. The ^{13}C NMR data of sugar parts in **2** (Tables 1 and 2) exhibited three anomeric carbon signals at δ_C 96.3, 104.1, 101.6, correlating with anomeric protons at δ_H 5.26 (brd, 9.5 Hz), 4.94 (brd, J = 8.0 Hz), and 4.69 (brd, J = 9.5 Hz) confirmed by HSQC experiment, respectively, along with three groups of well recognized methoxyl proton [δ_H 3.60, 3.55, 3.98 (each 3H, s)] and three methyl signals [δ_H 1.41 (d, J = 6.0 Hz), 1.70 (d, J = 6.0 Hz), 1.58 (d, J = 6.0 Hz)] in ^{1}H NMR (Table 3), suggesting that the sugar chain for **2** consisted of one β-D-cymarose, one β-D-oleandrose and one β-D-thevetose [22]. The sequence of these sugar units of **2** was inferred by the correlations in HMBC analysis between δ_H 4.69 (β-D-oleandropyranosyl H-1″″) and δ_C 83.4 (middle β-D-thevetopyranosyl C-4‴), and δ_H 4.94 (middle β-D-thevetopyranosyl H-1‴) and δ_C 83.1 (β-D-cymaropyranosyl C-4″). Similarly, three anomeric carbons at δ_C 96.5, 100.5, 106.3, and the corresponding protons at δ_H 5.25 (d, J = 10.0 Hz), 5.10 (d, J = 9.5 Hz) and 4.75 (d, J = 7.5 Hz), were shown in HSQC experiment of **3**. The coupling constants of three anomeric protons and of three secondary methyl groups [δ_H 1.37 (d, J = 6.0 Hz), 1.57 (d, J = 6.0 Hz) and 1.58 (d, J = 7.0 Hz)] in ^{1}H NMR (Table 3), as well as other signals in 1D NMR spectra (Tables 1–3) indicated that the sugar chain in **3** was formed by two β-D-cymaropyranosides and one β-D-thevetopyranoside. The sequence of sugar units of **3** was elucidated by HMBC experiment in which the correlations between δ_H 4.75 (β-D-thevetopyranosyl H-1⁗) and δ_C 83.4 (middle β-D-cymaropyranosyl C-4‴), and δ_H 5.10 (middle β-D-cymaropyranosyl H-1‴) and δ_C 83.2 (β-D-cymaropyranosyl C-4″) were observed. Comparing with reported spectral data, sugar moiety of **3** bore a resemblance to that of stemucronatoside J [5]. Further, all the monosaccharides in **2** and **3** were also confirmed by comparison of the TLC and OR analysis of acid hydrolysates of **2** and **3** with those of authentic samples. Hence, the structures of **2** and **3** was determined to be caudatin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-thevetopyranosyl(1 → 4)-β-D-cymaropyranoside and caudatin-3-O-β-D-thevetopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside, respectively.

Compound **4** had a molecular formula $C_{48}H_{76}O_{17}$. Its ^{1}H NMR spectral data of sugar part existed three anomeric protons at δ_H 5.46 (dd, J = 10.0, 1.5 Hz), 5.16 (dd, J = 9.5, 1.0 Hz), and 4.75 (brd, J = 7.5 Hz), whose coupling constants revealed the existence of two 2, 6-dideoxy sugars and one 6-dideoxy sugar with all β-linkages. According to 2D NMR analysis and comparison of the spectral data, TLC and OR data of acid hydrolysate of **4** with those of analogs [22], it indicated the existence of one β-D-digitoxopyranosyl, one β-D-cymaropyranosyl and one β-D-thevetopyranosyl in **4**. The sequence of those sugar units of **4** was inferred by the long-range correlations in HMBC analysis from δ_H 4.75 (β-D-thevetopyranosyl H-1⁗) to δ_C 83.2 (β-D-cymaropyranosyl C-4‴), and from δ_H 5.15 (β-D-cymaropyranosyl H-1‴) to δ_C 83.5 (β-D-digitoxopyranosyl C-4″). Consequently, **4** was established to be caudatin-3-O-β-D-thevetopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside.

The molecular formulas of **5** and **6** were both determined as $C_{55}H_{88}O_{19}$ by their HRESIMS. TLC analysis of the acidic hydrolysate displayed there were cymarose, oleandrose and digitoxose in **5**, and cymarose and digitoxose in **6**, respectively. The ^{1}H NMR spectrum of **5** showed four secondary methyl signals [δ_H 1.28 (d, J = 6.0 Hz), 1.22 (d, J = 6.0 Hz), 1.30 (d, J = 6.0 Hz), 1.22 (d, J = 6.0 Hz)], three methoxyl signals [δ_H 3.42, 3.40, 3.45 (each 3H, s) and four anomeric proton signals [δ_H 4.91 (1H, dd, J = 9.5, 1.5 Hz), 4.80 (1H, dd, J = 9.5, 1.5 Hz), 4.44 (1H, dd, J = 9.5, 1.5 Hz), 4.87 (1H, dd, J = 9.5, 1.5 Hz)] (Table 3). Those information proved that four 2,6-dideoxysugars were composed as sugar residue with a β-linkage in **5**. The carbon shifts of each sugar unit were assigned by HMBC and HSQC experiments (Table 2). Compared with the spectral data of analogs [26–29], it suggested the sugar chain of **5** was formed from two D-cymaropyranosyl, one D-digitoxopyranosyl and one D-oleandropyranosyl units. The sequence of those four sugar units of **5** was elaborated by HMBC long correlations from δ_H 4.87 (terminal β-D-cymaropyranosyl H-1″‴) to δ_C 82.2 (inner β-D-oleandropyranosyl C-4⁗), from δ_H 4.44 (β-D-oleandropyranosyl H-1⁗) to δ_C 82.4 (inner β-D-cymaropyranosyl C-4‴), and from δ_H 4.81 (β-D-cymaropyranosyl H-1‴) to δ_C 82.5 (inner β-D-digitoxopyranosyl C-4″). Similarly, based on the reporting data [21] that the chemical shift of C-2 is <δ_C 35.0 ppm in an L-cymaropyranosyl and >δ_C 36.0 ppm in a D-cymaropyranosyl, respectively, as well as the ^{13}C NMR signals of the five sugar unit assigning unambiguously by HSQC and HMBC analysis (Table 2), and the resonance of C-2 at δ_C 32.5, 36.7 and 32.2 in cymaropyranosyl, respectively, it suggested the existence of two α-L-cymaropyranosyl, one β-D-cymaropyranosyl and one β-D-digitoxopyranosyl in sugar moieties in **6**. The sequence of sugar units was determined by the analysis of HMBC correlations from δ_H 5.05 (outer α-L-cymaropyranosyl H-1″‴) to δ_C 81.1 (β-D-cymaropyranosyl C-4⁗), from δ_H 5.23 (β-D-cymaropyranosyl H-1⁗) to δ_C 77.3 (β-L-cymaropyranosyl C-4‴), and from δ_H 4.97 (α-L-cymaropyranosyl H-1‴) to δ_C 82.3 (β-D-digitoxopyranosyl C-4″). Therefore, the structure of **5** and **6** were established as caudatin-3-*O*-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside and caudatin-3-O-α-L-cymaropyranosyl-(1 → 4)-$\hat{a}$-D-cymaropyranosyl-(1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside, respectively.

Compound **7** was suggested to have a molecular formula $C_{56}H_{90}O_{20}$ on the basis of the HRESIMS. The analysis of acid hydrolysate of **7** suggested that the sugar units consisted of cymarose, thevetose and oleandrose. Based on the 1D NMR data of sugar part, it suggested two β-D-cymaropyranosyl, one β-D-oleandropyranosyl and one β-D-thevetopyranosyl existed in **7**. These four sugar units were formed as one chain connected to C-3 of the aglycone, which was confirmed by long-range correlations in HMBC analysis from δ_H 4.99 (outer β-D-oleandropyranosyl H-1″‴) to δ_C 83.3 (β-D-thevetopyranosyl C-4⁗), from δ_H 4.71 (β-D-thevetopyranosyl C-1⁗) to δ_C 82.4 (β-D-cymaropyranosyl C-4‴), from δ_H 4.98 (β-D-cymaropyranosyl H-1‴) to δ_C 81.6 (β-D-cymaropyranosyl C-4″), and from δ_H 5.26 (β-D-cymaropyranosyl H-1″) to δ_C 77.7 (C-3). Thus, **7** was determined to be caudatin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **8** was isolated as colorless powder. According to the HRESIMS, its molecular formula was determined to be $C_{62}H_{100}O_{22}$. Its ^{1}H NMR showed five anomeric proton signals [δ_H 5.47 (H, dd, J = 9.5, 1.0 Hz), 5.12 (H, dd, J = 9.5, 1.0 Hz), 4.96 (H, d, J = 3.5 Hz), 5.24 (H, dd, J = 9.5, 1.0 Hz) and 4.93 (H, d, J = 2.5 Hz)], whose coupling constants observed suggested that **8** had three sugar units with β-linkages and two sugar units with α-linkages (Table 3), respectively. Acid hydrolysis of **8** gave digitoxose and cymarose as residues. The NMR data of sugar part of **8** bore a resemblance to that of **6**, except for the presence of an additional β-D-cymaropyranosyl in **8**. The sequence of the sugar part was assigned by HMBC spectrum in which distinct correlations from δ_H 4.93 (outer α-L-cymaropyranosyl H-1″⁗) to δ_C 82.1 (β-D-cymaropyranosyl C-4″‴), from δ_H 5.24 (β-D-cymaropyranosyl H-1″‴) to δ_C 77.7 (α-L-cymaropyranosyl C-4⁗), from δ_H 4.96 (α-L-cymaropyranosyl H-1⁗) to δ_C 82.3 (β-D-cymaropyranosyl C-4‴), and from δ_H 5.15 (β-D-cymaropyranosyl H-1‴) to δ_C 83.4 (β-D-digitoxopyranosyl C-4″) were observed. Hence, **8** was deduced to be caudatin-3-O-α-L-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside.

The molecular formula of **9** was inferred to be $C_{70}H_{114}O_{25}$ according to HRESIMS. In ^{1}H NMR spectrum, six methoxyl groups and six secondary methyl groups were shown, suggesting that the sugar chain consisted of six 2,6-deoxysugar units. The coupling constants of six anomeric proton signals, which were shown at δ_H 4.84 (brd, partially overlapped), 4.44 (brd, J = 9.5 Hz), 4.82 (d, partially overlapped), 4.83 (brd, partially overlapped), 4.74 (brd, J = 9.5 Hz), 4.79 (d, J = 3.5 Hz) in ^{1}H NMR spectrum, indicated **9** possessed six sugar units with two α-linkages and four β-linkages (Table 3), respectively. Each sugar unit signal was assigned to three D-cymaropyranosyl units, two L-cymaropyranosyl units and one D-oleandropyranosyl unit by 1D and 2D NMR analysis (Tables 2 and 3) and comparison of their NMR information with those of analogs [26–29]. The sequence of four sugar units in **9** was elaborated by HMBC correlations from δ_H 4.79 (terminal α-L-cymaropyranosyl H-1″″‴) to δ_C 81.9 (inner β-D-cymaropyranosyl C-4″″″), from δ_H 4.73 (β-D-cymaropyranosyl H-1″″″) to δ_C 82.4 (inner β-D-cymaropyranosyl C-4″⁗), from δ_H 4.83 (β-D-cymaropyranosly H-1″⁗) to δ_C 76.9 (α-L-cymaropyranosyl C-4″‴), from $\ddot{a}_H$ 4.82 (α-L-cymaropyranosyl H-1″‴) to δ_C 81.8 (β-D-oleandropyranosyl C-4‴), and from $\ddot{a}_H$ 4.44 (β-D-oleandropyranosyl H-1‴) to δ_C 82.4 (β-D-cymaropyra-

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nosyl C-4″). Thus, the structure of **9** was established to be caudatin-3-O-α-L-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **10**, isolated as white powder, had a molecular formula $C_{55}H_{82}O_{21}$. For aglycone moiety, the ^{1}H NMR signals of **10** suggested the presence of four aromatic protons attributed to a benzene ring [δ_H 8.30 (2H, m, H-3′, 7′), 7.26 (2H, m, H-4′, 6′)], three singlet methyl groups [δ_H 1.29, 2.08, 2.41 (each 3H, s, H-19, 18, 20)], and an olefinic proton (δ_H 5.27, partially overlapped, H-6), which features were in good agreement with qingyangshengenin by comparing with spectral data (Table 6) of analogs in the literatures [21,22]. Furthermore, four anomeric protons [δ_H 5.47 (H, brd, J = 9.5 Hz), 5.15 (H, brd, partially overlapped), 4.69 (H, brd, J = 8.0 Hz), 5.26 (H, brd, partially overlapped)], four secondary methyl groups [δ_H 1.44 (H, d, J = 6.5 Hz), 1.42 (H, d, J = 6.0 Hz), 1.54 (H, d, J = 6.0 Hz), 1.53 (H, d, J = 5.5 Hz)], and three methoxyl groups [δ_H 3.56, 3.87, 3.48, s, each 3H] were exhibited in ^{1}H NMR, indicating that three 2,6-dideoxy sugar units and one 6-deoxy sugar unit with β-linkage formed the sugar moiety in **10**. The carbon signals of each sugar unit were assigned unambiguously by HSQC and HMBC analysis (Tables 5 and 6), suggesting the presence of one digitoxopyranosyl, one thevetopyranosyl, and two cymaropyranosyl in **10**. Comparing spectral data of **10** with those of analogs [21,22], the absolute configurations of sugar units were all elucidated as D-series. According to HMBC spectrum, the sequence of the deoxysugars located at C-3 of the aglycone as a chain was confirmed by the HMBC long range correlations observed between δ_H 4.69 (outer β-D-cymaropyranosyl H-1⁗′) and δ_C 82.4 (β-D-thevetopyranosyl C-4⁗), from δ_H 4.69 (β-D-thevetopyranosyl H-1⁗) and δ_C 83.2 (β-D-cymaropyranosyl C-4‴), from δ_H 5.15 (β-D-cymaropyranosyl H-1‴) and δ_C 83.5 (β-D-digitoxopyranosyl C-4″), from δ_H 5.47 (β-D-digitoxopyranosyl H-1″) and δ_C 77.7 (C-3). Thus, **10** was determined to be qingyangshengenin-3-O-β-D-cymaropyranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside.

Compound **11** was obtained as colorless powder and its molecular formula was established as $C_{56}H_{84}O_{20}$. The NMR characteristics implied that **11** had the same aglycone of qingyangshengenin as **10**. Observation of the NMR spectral data showed that sugar moiety of **11** contained four anomeric carbons at δ_C 96.4, 100.5, 101.9 and 97.3, and four corresponding anomeric proton signals at δ_H 5.28 (dd, partially overlapped), 5.10 (dd, J = 9.5, 1.5 Hz), 4.66 (dd, J = 9.5, 2.0 Hz), and 5.08 (dd, J = 3.5, 1.0 Hz), confirmed by HSQC experiment. On the basis of further analysis of HSQC and HMBC spectra and comparison of the spectral data with those of analogs [22] (Tables 5 and 6), the signals of the sugar units were exactly assigned, implying the existence of two β-D-cymaropyranosyl, one β-D-oleandropyranosyl, and one α-L-cymaropyranosyl in **11**. The sugar chain was attached at the C-3 of aglycone by HMBC analysis in which correlations from δ_H 5.08 (outer α-L-cymaropyranosyl H-1⁗′) to δ_C 81.5 (β-D-oleandropyranosyl C-4⁗), from δ_H 4.66 (β-D-oleandropyranosyl C-1⁗) to δ_C 83.2 (β-D-cymaropyranosyl C-4‴), from δ_H 5.10 (β-D-cymaropyranosyl H-1‴) to δ_C 83.5 (β-D-cymaropyranosyl C-4″), and from δ_H 5.28 (β-D-cymaropyranosyl H-1″) to δ_C 77.7 (C-3) were observed. Therefore, **11** was established to be qingyangshengenin-3-O-α-L-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **12** had a molecular formula $C_{70}H_{108}O_{26}$. The NMR features of aglycone demonstrated that **12** was a qingyangshengenin-type steroid (Table 4). Its sugar moiety consisted of three β-D-cymaropyranosyl, two α-L-cymaropyranosyl and one β-D-oleandropyranosyl based on analysis of 1D and 2D NMR data of **12** and comparison of its NMR information with reported data of analogs [22] (Tables 5 and 6). Meantime, the similarity of the 1D NMR spectroscopic data of sugar part of **12** (Tables 2, 3, 5 and 6) to those of **9** indicated that **12** possesses the same substituted sugar moiety as **9**, which was confirmed by analysis of the key HMBC correlations in **12**. Thus, **12** was confirmed to be qingyangshengenin-3-O-α-L-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **13**, obtained as a colorless powder, had a molecular formula $C_{58}H_{86}O_{19}$, established by HRESIMS and ^{13}C NMR spectrum. Its ^{1}H NMR spectrum (Table 6) showed a series of characteristic proton signals at δ_H 6.30 (d, H-2′, J = 16.0 Hz), 7.62 (d, H-3′, J = 16.0 Hz), 7.51 (m, H-5′ and H-9′), 7.38 (m, H-6′ and H-8′), and 7.39 (m, H-7′), corresponding to the carbon signals at δ_C 165.7 (C-1′), 117.6 (C-2′), 145.4 (C-3′), 134.3 (C-4′), 128.1 (C-5′, C-9′),

Table 5
^{13}C NMR data for the sugar moieties of compounds **10–14** (δ in ppm, 125 MHz).

No.	**10**[b]	**11**[b]	**12**[b]	**13**[a]	**14**[b]
	D-digit	D-cym	D-cym	D-cym	
1″	96.4	96.4	95.6	96.1	
2″	39.0	37.2	36.7	35.7	
3″	67.6	78.1	77.6	77.0	
4″	83.5	83.5	83.1	82.4	
5″	69.5	69.1	68.9	68.3	
6″	18.6	18.7	18.6	18.2	
OMe		59.0	58.9	58.5	
	D-cym	D-cym	D-ole	D-cym	D-digit
1‴	99.8	100.5	101.9	99.7	96.4
2‴	36.8	37.1	37.2	35.7	38.8
3‴	78.1	77.8	79.1	78.0	67.5
4‴	83.2	83.2	81.6	82.2	83.4
5‴	68.6	69.0	72.1	68.5	68.6
6‴	18.7	18.6	18.5	18.4	18.7
OMe	58.9	58.8	56.4	58.3	
	D-the	D-cym	L-cym	D-ole	D-cym
1⁗	106.1	101.9	97.4	101.3	99.7
2⁗	74.7	37.3	32.3	36.6	36.7
3⁗	85.5	79.1	73.6	79.1	77.8
4⁗	82.4	81.5	78.0	82.5	83.2
5⁗	71.7	72.2	64.9	71.6	69.1
6⁗	18.6	18.5	18.3	18.2	18.7
OMe	60.7	56.9	57.2	56.9	58.9
	D-cym	L-cym	D-cym	D-ole	D-ole
1⁗′	98.9	97.3	96.4	100.2	102.2
2⁗′	36.0	32.2	37.2	35.8	37.2
3⁗′	79.0	76.7	77.9	80.7	81.4
4⁗′	74.2	73.4	83.1	75.4	76.2
5⁗′	71.3	66.1	69.1	70.9	73.0
6⁗′	19.1	18.5	18.6	17.9	18.5
OMe	58.1	56.4	58.8	56.6	57.1
			D-cym		
1⁗″			100.5		
2⁗″			37.0		
3⁗″			77.7		
4⁗″			82.3		
5⁗″			69.4		
6⁗″			18.6		
OMe			58.3		
			L-cym		
1⁗‴			99.0		
2⁗‴			32.3		
3⁗‴			76.4		
4⁗‴			73.3		
5⁗‴			66.4		
6⁗‴			18.5		
OMe			56.9		

[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.

Table 6
^{1}H NMR spectral data of compounds **10–14** (δ in ppm, J in Hz, 500 MHz).

No.	**10**[b]	**11**[b]	**12**[b]	**13**[a]	**14**[b]
1	1.09 m; 1.78 m	1.07 m; 1.77 m	1.08 m; 1.78 m	1.09 m; 1.87 m	1.03 m; 1.71 m
2	1.77 m; 2.07 m	1.77 m; 2.05 m	1.77 m; 2.05 m	1.63 m; 1.92 m	1.77 m; 2.06 m
3	3.86 m	3.85 m	3.86 m	3.56 m	3.86 m
4	2.40 m; 2.51 m	2.40 m; 2.52 m	2.41 m; 2.53 m	2.30 m; 2.34 m	2.33 m; 2.47 m
6	5.27[c]	5.28[c]	5.28[c]	5.37 s	5.29[c]
7	2.46 m	2.45 m	2.45 m	2.21 m	2.47 m
9	1.75 m	1.75 m	1.75 m	1.56 m	1.73 m
11	2.18 m; 2.31 m	2.18 m; 2.31 m	2.18 m; 2.31 m	1.88 m	2.01 m; 2.33 m
12	5.32 dd (11.5, 4.0)	5.29 dd (11.5, 4.0)	5.32 dd (11.5, 4.0)	4.74 t (7.5)	5.32[d]
15	2.13 m	2.11 m	2.12 m	1.99 m	2.07 m
16	2.05 m; 3.27 m	2.02 m; 3.27 m	2.03 m; 3.26 m	1.90 m; 2.86 m	2.07 m; 2.12 m
18	2.08 s	2.09 s	2.09 s	1.46 s	2.10 s
19	1.29 s	1.30 s	1.30 s	1.12 s	1.28 s
20					5.04[d]
21	2.51 s	2.41 s	2.41 s	2.20 s	1.55[c]
2′				6.30 d (16.0)	6.54 d (16.0)
3′	8.29 m	8.30 m	8.30 m	7.62 d (16.0)	7.85 d (16.0)
4′	7.26 m	7.23 m	7.22 m		
5′				7.51 m	7.42 m
6′	7.26 m	7.23 m	7.22 m	7.37 m	7.33 m
7′	8.29 m	8.30 m	8.30 m	7.38 m	7.33 m
8′				7.37 m	7.33 m
9′				7.51 m	7.42 m
	D-digit	D-cym	D-cym	D-cym	
1″	5.47 d (9.5)	5.28[c]	5.24[c]	4.84 dd (9.5, 1.5)	
2″	2.03 m; 2.39 m	1.90 m; 2.35 m	1.79 m; 2.30 m	1.42 m; 2.07 m	9.52 s
3″	4.61 m	4.08 m	3.99 m	3.80 m	
4″	3.48 m	3.52 m	3.49 m	3.16 m	8.29 d (8.0)
5″	4.21 m	4.22 m	4.16 m	3.85 m	7.17[c]
6″	1.44 d (6.5)	1.38[c]	1.36[c]	1.22 d (6.5)	8.80 d (5.0)
OMe		3.60 s	3.54 s	3.44 s	
	D-cym	D-cym	D-ole	D-cym	D-digit
1‴	5.15[d]	5.10[d]	4.64 d (9.5)	4.74 dd (9.5, 1.5)	5.48 dd (9.5, 1.0)
2‴	1.74 m; 2.30 m	1.83 m; 2.32 m	1.62 m; 2.49 m	1.54 m; 2.10 m	2.01 m; 2.43 m
3‴	4.02 m	4.00 m	3.40 m	3.78 m	4.65 m
4‴	3.50 m	3.48 m	3.36 m	3.16 m	3.51 m
5‴	4.29 m	4.16 m	3.44 m	3.86 m	4.30 m
6‴	1.42 d (6.5)	1.38[c]	1.37[c]	1.22 d (6.0)	1.43 d (6.0)
OMe	3.56 s	3.55 s	3.29 s	3.44 s	
	D-the	D-ole	L-cym	D-ole	D-cym
1⁗	4.69 d (8.0)	4.66 dd (9.5, 1.5)	5.06 d (3.0)	4.44 dd (9.5, 1.5)	5.17 dd (9.5, 1.0)
2⁗	3.86 m	1.78 m; 2.46 m	1.86 m; 2.32 m	1.54 m; 2.30 m	1.78 m; 2.32 m
3⁗	3.65 m	3.41 m	3.77 m	3.37 m	4.05 m
4⁗	3.64 m	3.40 m	3.88 m	3.22 m	3.46 m
5⁗	3.63 m	3.47 m	4.71 m	3.31 m	4.20 m
6⁗	1.54 d (6.0)	1.38 d (6.0)	1.54 d (6.0)	1.30 d (6.0)	1.35 d (6.0)
OMe	3.87 s	3.43 s	3.40 s	3.39 s	3.56 s
	D-cym	L-cym	D-cym	D-ole	D-ole
1⁗′	5.26[c]	5.08 dd (3.5, 1.0)	5.27[c]	4.70 dd (9.5, 1.5)	4.75 dd (9.5, 1.0)
2⁗′	1.77 m; 2.37 m	1.86 m; 2.30 m	1.82 m; 2.30 m	1.55 m; 2.32 m	1.74 m; 2.47 m
3⁗′	3.76 m	3.73 m	4.08 m	3.16 m	3.57 m
4⁗′	3.54 m	3.62 m	3.50 m	3.14 m	3.48 m
5⁗′	4.13 m	4.65 m	4.20 m	3.57 m	3.59 m
6⁗′	1.53 d (5.5)	1.54 d (6.0)	1.36[c]	1.34 d (6.0)	1.56 d (6.0)
OMe	3.48 s	3.30 s	3.60 s	3.39 s	3.45 s
			D-cym		
1⁗″			5.13 dd (9.5, 1.0)		
2⁗″			1.78 m; 2.30 m		
3⁗″			3.88 m		
4⁗″			3.44 m		
5⁗″			4.20 m		
6⁗″			1.38[c]		
OMe			3.52 s		
			L-cym		
1⁗‴			4.96 d (3.0)		
2⁗‴			1.81 m; 2.34 m		
3⁗‴			3.70 m		

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Table 6 (*continued*)

No.	**10**[b]	**11**[b]	**12**[b]	**13**[a]	**14**[b]
4″″″′			3.60 m		
5″″″′			4.54 m		
6″″″′			1.51 d (6.0)		
OMe			3.36 s		

D-digit: β-D-digitoxopyranosyl. D-cym: β-D-cymaropyranosyl. L-cym: α-L-cymaropyranosyl.
D-ole: β-D-oleandropyranosyl. D-the: β-D-thevetopyranosyl.
[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.
[c] Partial overlapped with other signals.
[d] Overlapped with H_2O signal.

Table 7
Cytotoxicities of compounds **1**–**26**, 5-FU and Cisplatin against three cancer cell lines.

Compound	IC_{50} (μM)		
	HepG2	Hela	U251
1	41.82 ± 2.91	38.69 ± 3.85	46.16 ± 4.80
2	86.28 ± 2.90	86.27 ± 3.97	>100
3	37.87 ± 3.08	57.69 ± 5.20	22.52 ± 4.74
4	34.27 ± 4.35	72.35 ± 4.45	>100
5	30.71 ± 4.02	27.44 ± 4.88	40.37 ± 8.92
6	>100	>100	>100
7	44.44 ± 2.03	46.92 ± 8.73	53.94 ± 4.86
8	25.99 ± 2.66	44.85 ± 7.82	34.70 ± 6.28
9	30.47 ± 5.40	20.89 ± 6.03	45.94 ± 4.49
10	>100	>100	>100
11	61.91 ± 3.95	79.47 ± 6.79	50.39 ± 2.43
12	72.50 ± 2.28	96.83 ± 3.87	83.28 ± 3.87
13	41.76 ± 1.83	35.09 ± 5.14	52.02 ± 7.21
14	98.19 ± 1.01	>100	97.18 ± 4.87
15	96.21 ± 1.85	>100	69.28 ± 2.51
16	38.91 ± 3.21	31.24 ± 4.63	35.81 ± 4.91
17	51.84 ± 4.33	45.37 ± 2.05	35.37 ± 3.60
18	16.75 ± 1.42	25.33 ± 3.06	23.96 ± 6.06
19	66.9 ± 1.00	47.48 ± 2.53	>100
20	74.75 ± 4.42	89.13 ± 2.48	54.64 ± 3.33
21	95.26 ± 2.89	92.62 ± 3.65	77.98 ± 8.33
22	49.93 ± 1.81	42.71 ± 10.33	64.85 ± 3.95
23	70.69 ± 6.89	>100	65.98 ± 5.52
24	43.72 ± 5.99	>100	51.71 ± 15.26
25	28.48 ± 3.28	51.08 ± 4.46	>100
26	64.15 ± 4.78	56.88 ± 4.23	69.81 ± 2.85
5-FU	20.09 ± 0.79	7.80 ± 3.85	47.45 ± 0.71
Cisplatin	4.85 ± 0.97	10.61 ± 1.27	5.38 ± 0.93

Data are present as the mean ± SD of three experiments performed in triplicate.

128.9 (C-6′, C-8′), and 130.4 (C-7′), respectively, confirmed by HSQC experiment, indicating that a cinnamoyl group was present in **13** and it was linked in C-12 of aglycone based on the long-range correlation from δ_C 165.7 (C-1′) to δ_H 4.74 (m, H-12) in HMBC spectrum. Compared to features of NMR data of known aglycones in Asclepiadaceae subfamily, the aglycone of **13** was determined to be kidjoranin [26,27]. For the sugar units, detailed analysis of 1D and 2D NMR of **13** suggested that the sugar sequence and linkage position to the aglycone were exactly similar to that of gagaminin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside [28]. Thus, **13** was elucidated to be kidjoranin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **14** had a molecular formula $C_{56}H_{77}NO_{17}$. Detailed analysis of 1D NMR signals assigned to aglycone indicated that a cinnamoyl group [δ_H 6.54 (d, H-2′, J = 16.0 Hz), 7.85 (d, H-3′, J = 16.0 Hz), 7.42 (m, H-5′ and H-9′), 7.33 (m, H-6′ and H-8′), 7.33 (m, H-7′); δ_C 166.8 (C-1′), 120.3 (C-2′), 144.0 (C-3′), 134.9 (C-4′), 128.5 (C-5′, C-9′), 128.5 (C-6′, C-8′), 130.5 (C-7′)] and a nicotinoyl group [δ_H 9.52 (s, H-2″), 8.27 (d, H-4″, J = 8.0 Hz), 7.17 (dd, H-5″, J = 8.0, 5.0 Hz), 8.80 (d, H-6″, J = 5.0 Hz); δ_C 164.7 (C-1″), 153.8 (C-2″), 127.0 (C-3″), 137.3 (C-4″), 123.0 (C-5″), 151.4 (C-6″)] were present in **14** (Tables 4–6), which were linked in C-12 and C-20 of aglycone, confirmed by the HMBC correlations from δ_C 166.8 (C-1′) to δ_H 5.32 (H-12), and from δ_C 164.7 (C-1′) to δ_H 5.04 (H-20) in HMBC, respectively. Comparison of remaining spectral data of **14** with those of analogs [24,30], along with above two characteristically substituted groups, indicated that aglycone of **14** was gagaminin. For sugar moiety, the signals of the sugar units were unambiguously assigned on the basis of 1D and 2D NMR analysis (Tables 5 and 6), which suggested the existence of one β-D-cymaropyranosyl, one β-D-oleandropyranosyl, one β-D-digitoxopyranosyl. The linkage position and sequence of three sugar units were determined by the distinct correlations in HMBC spectrum from δ_C 102.2 (β-D-oleandropyranosyl C-1″″) to δ_H 3.46 (β-D-cymaropyranosyl H-4″″), from δ_C 99.7 (β-D-cymaropyranosyl C-1″″) to δ_H 3.51 (β-D-digitoxopyranosyl H-4‴), and from δ_C 96.4 (β-D-digitoxopyranosyl H-1‴) to δ_H 3.86 (H-3). Hence, **14** was confirmed as gagaminin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside.

Twelve known steroids were otophylloside T (**15**) [14], caudatin-3-O-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside (**16**) [26], caudatin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-digitoxopyranosyl-(1 → 4)-β-D-cymaropyranoside (**17**) [12], caudatin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside (**18**) [12], Otophylloside O (**19**) [23], otophylloside N (**20**) [23], qinyangshengenin-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranoside (**21**) [5], wallicoside J (**22**) [31], mucronatoside C (**23**) [32], gagamine-3-O-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside (**24**) [28], stephanoside H (**25**) [5], sinomarinoside B (**26**) [33].

Compounds **1**–**26** were evaluated for their cytotoxicity using HepG2, Hela and U251 human cancer cell lines. All of them, except **6** and **10** (IC_{50} > 100 μM), displayed the cytotoxic activities (IC_{50} < 100 μM) at different degrees against those cell lines, compared with the reference compounds 5-FU and cisplatin against HepG2, Hela and U251 with IC_{50} values of 20.09, 7.80 and 47.45 μM, and 4.85, 10.61 and 5.38 μM, respectively (Table 7). Notably, those steroidal glycosides, whose aglycones were caudatin, such as **1**, **3**, **5**, **7**–**9**, **13**, **16**–**18**, showed better cytotoxicities (IC_{50} < 50 μM) than another analogs against cancer cell lines. It suggested that the aglycone of caudatin may play an important role in the cytotoxic activity.

4. Conclusion

Systematic research on the pregnane glycosides from *C. otophyllum* was carried out in this study. As a result, fourteen new pregnane glycosides (**1**–**14**) were isolated, along with twelve known analogs (**15**–**26**). Their structures possessed five types of aglycones, including caudatin, qinyangshengenin, gagaminin, kidjoranin and

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penupogenin, and four types of deoxysugars, including digitoxose, oleandrose, cymarose, and thevetose, which were the mainly characteristic structures of the pregnane glycosides in the genus *Cynanchum* plants (subfamily Asclepiadoideae). In cytotoxic bioassay, all steroidal glycosides obtained (**1**–**26**), except **6** and **10**, showed the cytotoxicities against HepG2, Hela, U251 cancer cell lines at different degrees. And **18** displayed obvious activities against HepG2 with IC_{50} at 16.75 ± 1.42 μM (IC_{50} < 20 μM).

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

HRESIMS, 1D and 2D NMR spectra of compounds **1**–**14** are available in the online version, at http://dx.doi.org/10.1016/j.steroids.2015.08.010.

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