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Gracillosides A–F, six new 8,14-seco-pregnane glycosides from *Adelostemma gracillimum*

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ABSTRACT

Six new 8,14-seco-pregnane glycosides, gracillosides A–F (**1**–**6**), were isolated from the roots of *Adelostemma gracillimum* (Asclepiadaceae). All of them had the same aglycone as gracigenin with a rare 8,14-seco-pregnane type skeleton in nature and possessed an oligosaccharide chain consisting of four to six sugar units at C-3 of the aglycone. Their structures were determined on the basis of detailed spectroscopic analysis and chemical evidence.

1. Introduction

Adelostemma gracillimum Hooker is a lianas distributed in southwest of China. As the folk medicine, its roots have been used as nourishing and roborant drugs and for the treatment of convulsions of children in Shangri-La (Zhongdian) county of Yunnan, China. Previous pharmacological studies showed that the crude glycosides from this species had anti-convulsant and therapeutic actions on chronic epilepsy with good effects [1]. Mu et al. [1] isolated one new aglycone named gracigenin with an unprecedented 8,14-seco-polyoxypregnane ester-type skeleton from the hydrolysate of the crude glycosides of *A. gracillimum*, but the glycosides from this plant have not been reported yet. In this paper, we describe the isolation and characterization of six new 8,14-seco-pregnane glycosides, gracillosides A–F (**1**–**6**) from *A. gracillimum*. All of them had the same aglycone as gracigenin (**7**) and possessed an oligosaccharide chain consisting of four to six sugar units at C-3 of the aglycone. Their structures were determined on the basis of detailed spectroscopic analysis and chemical evidence. To the best of our knowledge, gracillosides A–F (**1**–**6**) are believed to be the third examples to contain an aglycone with a rare 8,14-seco-pregnane type skeleton in nature [2,3]. Furthermore, a structural feature of gracilloside E (**4**) includes both D- and L-cymarose, a pair of optically isomeric sugars in the sugar chain [3–10].

2. Experimental

2.1. General methods

ESI-MS spectra were obtained on a Finnigan LCQ-Advantage mass spectrometer and HRESI-MS spectra were recorded on an API Qstar Pulsar LC/TOF instrument. NMR spectra were measured in C_5D_5N and recorded on a Bruker AM-400 (for ^1H NMR and ^{13}C NMR) and DRX-500 (for 2D NMR) instrument with TMS as internal standard. IR spectra were taken in KBr on a Bio-Rad FTS-135 infrared spectrophotometer. Optical rotations were measured in a JASCO DIP-370 digital polarimeter. UV spectra were measured using a Shimadzu UV-2401PC spectrophotometer. Separation and purification were performed by column chromatography on silica gel (200–300 mesh, Qingdao), RP-18 (Merck), MPLC (Büchi Pump Module C-605, Büchi Pump manager C-615, Büchi Fraction Collector C-660) and on semi-preparative HPLC using an Agilent 1100 instrument (Zorbax reversed phase C_{18} column 9.4 mm × 250 mm, DAD).

2.2. Plant material

The roots of *Adelostemma gracillimum* were collected in October 2005 from Lijiang, Yunnan province, China and identified by

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Prof. Quan-Zhang Mu. A voucher specimen (No. 20051006) was deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air-dried and powdered roots of *A. gracillimum* (10 kg) were extracted with 95% ethanol under reflux (15 L ×3, each 3 h). After removal of the organic solvent *in vacuo*, the residue was suspended in water (2 L) and partitioned with $CHCl_3$ for three times (1.5 L ×3) to give a $CHCl_3$ extract (180 g). Part of the $CHCl_3$ extract (150 g) was subjected to a silica gel CC (12 cm × 150 cm) and eluted with a gradient $CHCl_3$/MeOH (100:0 → 50:50) to afford six fractions (Frs. **1**–**6**). Fr. **3** (30 g) was chromatographed over silica gel with $CHCl_3$/MeOH (100:0 → 90:10) to give three main fractions (Frs. A–C). Fr. C (11 g) was separated by chromatography on a RP-18 column through MPLC using MeOH/H_2O (55:45 → 80:20) to give six fractions (Frs. C_1–C_6). Fr. C_2 (1.2 g) was submitted to CC over RP-18 with MeOH/H_2O (60:40 → 70:30) to give three main fractions (Frs. I–III). Fr. I (145 mg) was subjected to silica gel CC with $CHCl_3$/MeOH (95:5); then semi-preparative HPLC (MeOH/H_2O 68:32) to provide **2** (18 mg). Fr. II (270 mg) was subjected to silica gel CC with $CHCl_3$/MeOH (95:5); RP-18 (MPLC) with acetone/H_2O (50:50) to give four main fractions (Frs. II_1–II_4). Fr. II_1 (28 mg) and II_2 (55 mg) were purified by semi-preparative HPLC to yield **1** (9 mg) from Fr. II_1 with MeOH/H_2O (65:35) and **5** (16 mg) and **6** (7 mg) from Fr. II_2 with CH_3CN/H_2O (40:60), respectively. Fr. II_4 (43 mg) was subjected to silica gel CC with $CHCl_3$/MeOH (95:5) to furnish **3** (18 mg). Fr. III (165 mg) was subjected to silica gel CC with $CHCl_3$/MeOH (95:5); then RP-18 (MPLC) with acetone/H_2O (45:55); then semi-preparative HPLC (CH_3CN/H_2O 40:60) to afford **4** (9 mg).

Another part of the $CHCl_3$ extract (5 g) was dissolved in MeOH (100 mL) and treated with 0.05 M H_2SO_4 (100 mL) at 50 °C for 30 min. Water (100 mL) was added and the whole mixture was concentrated and again warmed at 50 °C for a further 30 min, then the solution was extracted with $CHCl_3$. The $CHCl_3$ phase (1.5 g) was chromatographed repeatedly on silica gel column with petroleum ether (PE)/acetone (50:50); $CHCl_3$/MeOH (98:2) and purified by semi-preparative HPLC (CH_3CN/H_2O 43:57) to afford compound **7** (6 mg).

2.3.1. Gracilloside A (*1*)

$C_{65}H_{96}O_{25}$; white amorphous powder; $[\alpha]_D^{20}$ −25.0° (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε): 281.8 (4.44), 223.2 (4.23), 218.0 (4.28), 202.6 (4.38) nm; IR (KBr) ν_{max}: 3456, 2935, 1735, 1715, 1636, 1452, 1375, 1252, 1163, 1097, 1073, 1061 cm^{-1}; ESI-MS (positive) *m*/*z* (%): 1299.7 $[M+Na]^+$ (100). HRESI-MS (positive) *m*/*z*: 1299.6110 $[M+Na]^+$ (calcd. for $C_{65}H_{96}O_{25}Na$, 1299.6138); for ^1H NMR and ^{13}C NMR data, see Tables 1 and 2.

2.3.2. Gracilloside B (*2*)

$C_{65}H_{96}O_{25}$; white amorphous powder; $[\alpha]_D^{20}$ −10.0° (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε): 281.8 (4.34), 223.2 (4.15), 218.2 (4.19), 202.6 (4.27) nm; IR (KBr) ν_{max}: 3446, 2934, 1737, 1715, 1636, 1452, 1374, 1253, 1163, 1079 cm^{-1}; ESI-MS (positive) *m*/*z* (%): 1299.7 $[M+Na]^+$ (100). HRESI-MS (positive) *m*/*z*: 1299.6143 $[M+Na]^+$ (calcd. for $C_{65}H_{96}O_{25}Na$, 1299.6138); for ^1H NMR and ^{13}C NMR data, see Tables 1 and 2.

2.3.3. Gracilloside C (*3*)

$C_{58}H_{84}O_{22}$; white amorphous powder; $[\alpha]_D^{20}$ −10.0° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε): 282.2 (4.25), 223.2 (4.02), 218.2 (4.07), 202.0 (4.15) nm; IR (KBr) ν_{max}: 3457, 2935, 1735, 1715, 1636, 1451, 1375, 1252, 1163, 1097 cm^{-1}; ESI-MS (positive) *m*/*z* (%): 1155.7 $[M+Na]^+$ (100). HRESI-MS (positive) *m*/*z*: 1155.5371 $[M+Na]^+$ (calcd.

Table 1
^{13}C NMR spectral data of compounds **1**–**7** (δ in ppm, C_5D_5N).[a].

Position	1	2	3	4	5	6	7
1	37.3	37.3	37.3	37.3	37.3	37.3	37.6
2	29.6	29.6	29.6	29.6	29.6	29.6	31.7
3	76.4	76.4	76.4	76.4	76.4	76.4	70.1
4	38.3	38.3	38.3	38.3	38.3	38.3	42.2
5	141.9	141.9	141.9	141.9	141.9	141.9	142.8
6	118.8	118.7	118.8	118.8	118.8	118.8	118.1
7	41.4	41.4	41.5	41.4	41.4	41.5	41.5
8	209.6	209.6	209.6	209.6	209.6	209.6	209.8
9	55.8	55.8	55.8	55.8	55.8	55.8	56.0
10	43.3	43.3	43.3	43.3	43.3	43.3	43.3
11	26.0	26.0	26.0	26.0	26.0	26.0	26.1
12	71.6	71.8	71.6	71.8	71.6	71.8	71.9
13	62.7	62.7	62.7	62.7	62.7	62.7	62.7
14	217.5	217.5	217.6	217.5	217.6	217.6	217.7
15	34.1	34.0	34.1	34.1	34.1	34.1	34.1
16	30.1	30.1	30.1	30.1	30.1	30.1	30.1
17	82.4	82.4	82.4	82.4	82.4	82.4	82.4
18	12.2	12.2	12.2	12.2	12.2	12.2	12.4
19	18.5	18.7	18.5	18.7	18.6	18.6	18.9
20	74.6	74.6	74.6	74.6	74.6	74.6	74.6
21	14.6	14.6	14.6	14.6	14.6	14.6	14.6
12-*O*-Cin.							
1′	167.8	167.8	167.8	167.8	167.8	167.8	167.8
2′	119.1	119.1	119.1	119.1	119.1	119.1	119.1
3′	145.9	145.7	145.8	145.7	145.8	145.8	145.7
4′	135.0	135.0	135.0	135.0	135.0	135.0	134.9
5′	128.8	128.8	128.9	128.8	128.8	128.9	128.8
6′	129.4	129.4	129.4	129.4	129.4	129.4	129.4
7′	130.9	130.9	130.9	130.8	130.9	130.9	130.9
8′	129.4	129.4	129.4	129.4	129.4	129.4	129.4
9′	128.8	128.8	128.9	128.8	128.8	128.9	128.8
20-*O*-Ac.							
Me	21.3	21.2	21.3	21.2	21.3	21.3	21.3
C=O	169.9	169.9	169.9	169.9	169.9	169.9	169.9
	β-D-digit	β-D-digit	β-D-digit	β-D-digit	β-D-cym	β-D-cym	
1^I	96.5	96.4	96.5	96.5	96.5	96.5	
2^I	39.1	39.0	39.2	39.1	37.1	37.2	
3^I	67.6	67.5	67.6	67.5	77.8	77.8	
4^I	83.6	83.3	83.6	83.6	83.5	83.5	
5^I	68.5	68.5	68.5	68.4	69.0	68.9	
6^I	18.6	18.6	18.7	18.6	18.7	18.5	
OMe					58.8	58.9	
	β-D-ole	β-D-ole	β-D-ole	β-D-ole	β-D-ole	β-D-ole	
1^{II}	101.4	102.0	101.5	101.4	102.0	102.0	
2^{II}	37.4	37.7	37.4	36.9	38.0	37.8	
3^{II}	79.0	78.8	78.9	79.0	79.1	78.8	
4^{II}	82.9	83.2	82.8	81.7	82.9	82.6	
5^{II}	71.8	71.8	71.7	72.1	71.9	71.8	
6^{II}	18.7	18.7	18.7	18.4	18.7	18.7	
OMe	57.5	57.5	57.4	57.2	57.6	57.5	
	β-D-ole	β-D-cym	β-D-ole	α-L-cym	β-D-ole	β-D-cym	
1^{III}	100.2	98.4	100.1	97.5	100.2	98.4	
2^{III}	37.9	36.7	37.6	32.3	37.7	36.7	
3^{III}	79.1	77.7	79.6	73.6	79.0	78.2	
4^{III}	82.9	82.6	83.4	77.9	82.8	83.2	
5^{III}	71.9	69.1	72.2	64.9	71.8	69.7	
6^{III}	18.7	18.5	18.7	18.5	18.7	18.7	
OMe	57.4	58.6	57.4	56.4	57.4	58.6	
	β-D-cym	β-D-cym	β-D-glc	β-D-cym	β-D-cym	β-D-glc	
1^{IV}	98.5	99.8	104.6	95.6	98.5	106.7	
2^{IV}	36.8	36.7	75.8	36.7	36.7	75.5	
3^{IV}	78.2	78.2	78.7	77.7	78.2	78.6	
4^{IV}	83.2	83.2	72.0	82.5	83.2	71.7	
5^{IV}	69.7	69.7	78.3	69.4	69.7	78.4	
6^{IV}	18.7	18.7	63.1	18.5	18.7	63.1	
OMe	58.6	58.9		58.3	58.6		
	β-D-glc	β-D-glc		α-L-cym	β-D-glc		
1^V	106.6	106.6		99.0	106.7		
2^V	75.4	75.4		32.3	75.4		

Table 1 (*Continued*)

Position	1	2	3	4	5	6	7
3^V	78.5	78.5		73.4	78.4		
4^V	71.9	71.9		78.9	71.8		
5^V	78.4	78.4		64.9	78.4		
6^V	63.1	63.1		18.7	63.1		
OMe				56.9			
				β-D-glc			
1^{VI}				102.3			
2^{VI}				75.3			
3^{VI}				78.5			
4^{VI}				71.9			
5^{VI}				78.7			
6^{VI}				63.0			

[a] Measured at 125 MHz. Assignments were confirmed by 2D NMR spectra.

for $C_{58}H_{84}O_{22}Na$, 1155.5351); for ^1H NMR and ^{13}C NMR data, see Tables 1 and 2.

*2.3.4. Gracilloside D (**4**)*

$C_{72}H_{108}O_{28}$; white amorphous powder; $[\alpha]_D^{20}$ −87.1° (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε): 282.0 (4.44), 223.2 (4.17), 218.0 (4.22), 203.0 (4.30) nm; IR (KBr) ν_{max}: 3443, 2935, 1734, 1715, 1636, 1553, 1452, 1374, 1252, 1163, 1078, 1057 cm^{-1}; ESI-MS (positive) *m/z* (%): 1443.9 $[M+Na]^+$ (100). HRESI-MS (positive) *m/z*: 1443.6928 $[M+Na]^+$ (calcd. for $C_{72}H_{108}O_{28}Na$, 1143.6924); for ^1H NMR and ^{13}C NMR data, see Tables 1 and 2.

*2.3.5. Gracilloside E (**5**)*

$C_{66}H_{98}O_{25}$; white amorphous powder; $[\alpha]_D^{20}$ −29.4° (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε): 281.8 (4.36), 223.0 (4.18), 218.2 (4.22), 202.4 (4.27) nm; IR (KBr) ν_{max}: 3458, 2935, 1734, 1715, 1637, 1552, 1452, 1375, 1253, 1163, 1101, 1060 cm^{-1}; ESI-MS (positive) *m/z* (%): 1313.8 $[M+Na]^+$ (100). HRESI-MS (positive) *m/z*: 1313.6298 $[M+Na]^+$ (calcd. for $C_{66}H_{98}O_{25}Na$, 1313.6294); for ^1H NMR and ^{13}C NMR data, see Tables 1 and 2.

*2.3.6. Gracilloside F (**6**)*

$C_{59}H_{86}O_{22}$; white amorphous powder; $[\alpha]_D^{20}$ −17.7° (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε): 282.0 (4.32), 223.2 (4.10), 218.2 (4.15), 202.8 (4.20) nm; IR (KBr) ν_{max}: 3437, 2935, 1732, 1715, 1635, 1452, 1374, 1252, 1163, 1098, 1060 cm^{-1}; ESI-MS (positive) *m/z* (%): 1169.6 $[M+Na]^+$ (100). HRESI-MS (positive) *m/z*: 1169.5514 $[M+Na]^+$ (calcd. for $C_{59}H_{86}O_{22}Na$, 1169.5508); for ^1H NMR and ^{13}C NMR data, see Tables 1 and 2.

*2.3.7. Gracigenine (**7**)*

$C_{32}H_{40}O_8$; white amorphous powder; ESI-MS (positive) *m/z* (%): 575.4 $[M+Na]^+$ (100); ^1H NMR (C_5D_5N, 500 MHz): δ 0.81 (3H, s, H-19), 1.48 (3H, d, *J* = 6.0 Hz, H-21), 1.91 (3H, s, H-18), 1.96 (3H, s, H-2 of Ac), 3.70 (1H, m, H-3), 5.22 (1H, brs, H-6), 5.30 (1H, q, *J* = 6.0 Hz, H-20), 5.85 (1H, d, *J* = 10.5 Hz, H-12), 6.91 (1H, d, *J* = 16.0 Hz, H-2′), 7.35 (3H, m, H-6′/7′/8′), 7.68 (2H, m, H-5′/H-9′), 8.01 (1H, d, *J* = 16.0 Hz, H-3′); for ^{13}C NMR data, see Table 1.

*2.4. Acid hydrolysis of **1**–**6***

A solution of **1**–**6** (each 5 mg) in 3 mL 50% dioxane and 3 mL 0.05 M H_2SO_4 was heated at 95 °C for 2 h. After dioxane was removed *in vacuo*, the solution was extracted with $CHCl_3$. The $CHCl_3$ layer of each compound was compared with authentic sample of gracigenin (**7**) by TLC analysis which revealed to be the same aglycones of compounds **1**–**6**, respectively. The H_2O layer of each compound was neutralized with sat. aq. $Ba(OH)_2$ soln. and the precipitation was filtered off. The filtrate was evaporated and identified by TLC comparison with authentic samples. Digitoxose was detected from **1** to **4**; cymarose was detected from **1** to **2**, **4** to **6**; oleandrose and glucose were detected from **1** to **6**.

3. Results and discussion

The $CHCl_3$ soluble fraction of the ethanolic extract of the roots of *A. gracillimum* was subjected to repeated column chromatography on silica gel and reversed phase silica gel, followed by semi-preparative HPLC to provide six new compounds (**1**–**6**). All of them showed positive Liebermann–Burchard and Keller–Kiliani reactions, indicating that they were all steroidal glycosides containing 2-deoxy sugar moieties. Each of the isolates was subjected to detailed spectroscopic analysis to establish their chemical structures. Acidic hydrolysis of the $CHCl_3$ extract obtained an aglycone which was identified to be gracigenin [7] by comparison of its spectral data with those in the literature [1].

The presence of sugar units in the glycosides was determined by comparison of its ^{13}C NMR spectroscopic data with those reported data and confirmed by TLC analysis. The configuration of glucose was determined to be D-form as tentative from biogenetic consideration. As regards the deoxysugar, its configuration was determined to be D- or/and L-form based on the literature, in which a survey of closely related glycosides from the Asclepiadaceae family reveals that all the β-linked 2,6-dideoxy sugars have the D-configuration, whereas the α-linked sugars are mostly L-sugars [3]. Further, chemical shift values for C-2 of the 2-deoxy sugars (cymarose, oleandrose and digitoxose) can be used as argument to determine its configuration. For example, the chemical shift values for C-2 of sugar moieties in compound **4** showed that the two α-linked cymaroses (both at δ_C 32.3) had the L-configuration and the β-linked cymarose (δ_C 36.7), oleandrose (δ_C 36.9) as well as digitoxose (δ_C 39.1) all the D-configuration [3,10]. It is noteworthy that the most significant differences in the ^{13}C NMR data between D- and L-cymarose sugars involve the resonances of C-2 [10] (Fig. 1).

Gracilloside A (**1**) was obtained as a white amorphous power, its molecular formula was determined as $C_{65}H_{96}O_{25}$ by positive HRESI-MS (*m/z* 1299.6110 $[M+Na]^+$, calcd. 1299.6138). The IR spectrum showed the absorption bands for hydroxyl (3456 cm^{-1}), carbonyl (1735 and 1715 cm^{-1}) and olefinic (1636 cm^{-1}) groups. The compound displayed 65 carbon signals in its ^{13}C NMR spectrum, of which 32 were assigned to the aglycone part and 33 to the sugar moiety (Table 1). Two tertiary methyl group at δ_H 1.90 (s) and 0.74 (s), and one olefinic proton at δ_H 5.18 (brs) observed in the ^1H NMR spectrum (Table 2), coupled with the information from the ^{13}C NMR spectrum (two methyl carbons at δ_C 12.2 and 18.5, two olefinic carbons at δ_C 141.9 and 118.8, and two ketone carbonyl carbons at δ_C 209.6 and 217.5), indicated that the aglycone possessed a 8,14-seco-pregnane type skeleton [1]. Two acyl substituentes were assigned to one cinnamoyl group and one acetyl group based on the ^1H NMR and ^{13}C NMR data shown in Tables 1 and 2. In the HMBC spectrum, the carbonyl signal of the cinnamoyl group at δ_C 167.8 was correlated with the signal of methine proton (H-12) at δ_H 5.84 (d, *J* = 10.5 Hz) on an oxygen-bearing carbon (C-12) at δ_C 71.6, and that of the acetyl group at δ_C 169.9 was correlated with the signal of methine proton (H-20) at δ_H 5.30 (q, *J* = 6.0 Hz) on an oxygen-bearing carbon (C-20) at δ_C 74.6, establishing that in **1,** the cinnamoyl group is located at C-12 and the acetyl group at C-20. Thus, aglycone was identified as gracigenin (**7**), which was confirmed by comparison of its spectroscopic data to those in the literature [1]. On mild acid hydrolysis, **1** afforded gracigenin (**7**) as aglycone and digitoxose, oleandrose, cymarose as well as glucose as sugar residues. The NMR spectral data of **1** revealed that it contained five anomeric carbon signals at δ_C 96.5, 98.5, 100.2, 101.4, and 106.6, correlating with anomeric proton signals at δ_H 5.36 (brd, *J* = 9.5 Hz), 5.25 (brd, *J* = 10.0 Hz), 4.86 (brd, *J* = 10.0 Hz), 4.72 (brd,

Table 2
^{1}H NMR spectral data of compounds **1**–**6** (δ in ppm, J in Hz, C_5D_5N).[a,b].

Position	**1**	**2**	**3**	**4**	**5**	**6**
1	1.30 (m) 1.85 (m)	1.27 (m) 1.81 (m)	1.29 (m) 1.84 (m)	1.30 (m) 1.84 (m)	1.30 (m) 1.84 (m)	1.29 (m) 1.83 (m)
2	1.58 (m) 2.06 (m)	1.56 (m) 2.04 (m)	1.58 (m) 2.06 (m)	1.58 (m) 2.07 (m)	1.57 (m) 2.06 (m)	1.56 (m) 2.06 (m)
3	3.68 (m)	3.67 (m)	3.68 (m)	3.68 (m)	3.66 (m)	3.67 (m)
4	2.14 (m) 2.46 (m)	2.11 (m) 2.44 (m)	2.13 (m) 2.47 (m)	2.11 (m) 2.47 (m)	2.14 (m) 2.45 (m)	2.14 (m) 2.46 (m)
6	5.18 (brs)	5.18 (brs)	5.19 (brs)	5.18 (brs)	5.20 (brs)	5.19 (brs)
7	2.72 (m) 3.23 (dt, 19.5, 3.0)	2.71 (m) 3.22 (dt, 19.5, 3.0)	2.72 (m) 3.23 (dt, 19.5, 3.0)	2.70 (m) 3.22 (dt, 19.5, 3.0)	2.71 (m) 3.24 (dt, 19.5, 3.0)	2.72 (m) 3.23 (dt, 19.5, 3.0)
9	2.69 (m)	2.69 (m)	2.68 (m)	2.69 (m)	2.69 (m)	2.69 (m)
11	1.85 (m) 2.33 (m)	1.82 (m) 2.32 (m)	1.83 (m) 2.33 (m)	1.83 (m) 2.31 (m)	1.84 (m) 2.32 (m)	1.85 (m) 2.33 (m)
12	5.84 (d, 10.5)	5.83 (d, 10.5)	5.82 (d, 10.5)	5.85 (d, 10.5)	5.83 (d, 10.5)	5.84 (d, 10.5)
15	2.71 (m) 3.00 (ddd, 19.0, 11.0, 1.5)	2.70 (m) 3.00 (ddd, 19.0, 11.0, 1.5)	2.71 (m) 3.00 (ddd, 19.0, 11.0, 1.5)	2.70 (m) 3.00 (ddd, 19.0, 11.0, 1.5)	2.69 (m) 2.99 (ddd, 19.0, 11.0, 1.5)	2.69 (m) 3.00 (ddd, 19.0, 11.0, 1.5)
16	2.14 (m) 2.38 (m)	2.12 (m) 2.38 (m)	2.14 (m) 2.38 (m)	2.11 (m) 2.38 (m)	2.12 (m) 2.37 (m)	2.12 (m) 2.39 (m)
18	1.90 (s)	1.90 (s)	1.89 (s)	1.90 (s)	1.90 (s)	1.90 (s)
19	0.74 (s)	0.74 (s)	0.75 (s)	0.74 (s)	0.74 (s)	0.73 (s)
20	5.30 (q, 6.0)	5.28 (q, 6.0)	5.29 (q, 6.0)	5.30 (q, 6.0)	5.29 (q, 6.0)	5.30 (q, 6.0)
21	1.48 (d, 6.0)	1.47 (d, 6.0)	1.48 (d, 6.0)	1.47 (d, 6.0)	1.48 (d, 6.0)	1.46 (d, 6.0)
12-*O*-Cin.						
2′	6.94 (d, 16.0)	6.93 (d, 16.0)	6.91 (d, 16.0)	6.94 (d, 16.0)	6.93 (d, 16.0)	6.94 (d, 16.0)
3′	8.04 (d, 16.0)	8.04 (d, 16.0)	8.03 (d, 16.0)	8.05 (d, 16.0)	8.04 (d, 16.0)	8.03 (d, 16.0)
5′	7.72 (m)	7.71 (m)	7.71 (m)	7.71 (m)	7.70 (m)	7.70 (m)
6′	7.36 (m)	7.34 (m)	7.35 (m)	7.35 (m)	7.34 (m)	7.34 (m)
7′	7.36 (m)	7.34 (m)	7.35 (m)	7.35 (m)	7.34 (m)	7.34 (m)
8′	7.36 (m)	7.34 (m)	7.35 (m)	7.35 (m)	7.34 (m)	7.34 (m)
9′	7.72 (m)	7.71 (m)	7.71 (m)	7.71 (m)	7.70 (m)	7.70 (m)
20-*O*-Ac.						
Me	1.97 (s)	1.96 (s)	1.97 (s)	1.96 (s)	1.96 (s)	1.97 (s)
C = O						
	β-D-digit	β-D-digit	β-D-digit	β-D-digit	β-D-cym	β-D-cym
1 ^{I}	5.36 (brd, 9.5)	5.34 (brd, 9.5)	5.35 (brd, 9.5)	5.35 (brd, 9.5)	5.14 (dd, 9.5, 1.5)	5.14 (dd, 9.5, 1.5)
2 ^{I}	2.02 (m) 2.38 (m)	1.99 (m) 2.36 (m)	1.99 (m) 2.36 (m)	1.98 (m) 2.35 (m)	1.83 (m) 2.27 (m)	1.81 (m) 2.24 (m)
3 ^{I}	4.62 (brs)	4.60 (brs)	4.62 (brs)	4.60 (brs)	4.02 (m)	4.01 (m)
4 ^{I}	3.50 (m)	3.46 (m)	3.47 (m)	3.46 (m)	3.45 (m)	3.45 (m)
5 ^{I}	4.26 (m)	4.21 (m)	4.26 (m)	4.24 (m)	4.15 (m)	4.14 (m)
6 ^{I}	1.40 (d, 6.0)	1.38 (d, 6.0)	1.38 (d, 6.0)	1.38 (d, 6.0)	1.34 (d, 6.0)	1.32 (d, 6.0)
OMe					3.55 (s)	3.54 (s)
	β-D-ole	β-D-ole	β-D-ole	β-D-ole	β-D-ole	β-D-ole
1 ^{II}	4.72 (brd, 9.5)	4.66 (brd, 9.5)	4.72 (brd, 9.5)	4.67 (brd, 9.5)	4.67 (dd, 9.5, 1.5)	4.65 (dd, 10.0, 1.5)
2 ^{II}	1.65 (m) 2.44 (m)	1.68 (m) 2.44 (m)	1.64 (m) 2.43 (m)	1.53 (m) 2.44 (m)	1.71 (m) 2.44 (m)	1.71 (m) 2.42 (m)
3 ^{II}	3.51 (m)	3.51 (m)	3.51 (m)	3.38 (m)	3.50 (m)	3.49 (m)
4 ^{II}	3.41 (m)	3.40 (m)	3.41 (m)	3.29 (m)	3.48 (m)	3.47 (m)
5 ^{II}	3.49 (m)	3.47 (m)	3.49 (m)	3.42 (m)	3.48 (m)	3.47 (m)
6 ^{II}	1.35 (d, 6.0)	1.33 (d, 6.0)	1.34 (d, 6.0)	1.28 (d, 6.0)	1.40 (d, 6.0)	1.38 (d, 6.0)
OMe	3.52 (s)	3.50 (s)	3.52 (s)	3.40 (s)	3.52 (s)	3.49 (s)
	β-D-ole	β-D-cym	β-D-ole	α-L-cym	β-D-ole	β-D-cym
1 ^{III}	4.86 (brd, 10.0)	5.25 (brd, 10.0)	4.86 (brd, 10.0)	5.04 (brd, 2.5)	4.86 (dd, 10.0, 1.5)	5.25 (brd, 10.0)
2 ^{III}	1.73 (m) 2.45 (m)	1.75 (m) 2.25 (m)	1.73 (m) 2.48 (m)	1.80 (m) 2.33 (m)	1.71 (m) 2.44 (m)	1.75 (m) 2.24 (m)
3 ^{III}	3.51 (m)	4.02 (m)	3.61 (m)	3.76 (m)	3.50 (m)	4.13 (m)
4 ^{III}	3.49 (m)	3.44 (m)	3.73 (t, 9.0)	3.86 (m)	3.48 (m)	3.67 (m)
5 ^{III}	3.49 (m)	4.12 (m)	3.61 (m)	4.70 (m)	3.48 (m)	4.26 (m)
6 ^{III}	1.39 (d, 6.0)	1.30 (d, 6.0)	1.71 (d, 6.0)	1.53 (d, 6.0)	1.40 (d, 6.0)	1.61 (d, 6.0)
OMe	3.52 (s)	3.51 (s)	3.52 (s)	3.27 (s)	3.48 (s)	3.50 (s)

Table 2 (*Continued*)

Position	1	2	3	4	5	6
	β-D-cym	β-D-cym	β-D-glc	β-D-cym	β-D-cym	β-D-glc
1 IV	5.25 (brd, 10.0)	5.14 (brd, 10.0)	5.10 (d, 8.0)	5.25 (brd, 10.0)	5.25 (dd, 9.5, 1.5)	4.94 (d, 8.0)
2 IV	1.75 (m) 2.24 (m)	1.75 (m) 2.25 (m)	4.00 (m)	1.80 (m) 2.31 (m)	1.73 (m) 2.22 (m)	4.00 (m)
3 IV	4.14 (m)	4.11 (m)	4.24 (m)	3.88 (m)	4.13 (m)	4.21 (m)
4 IV	3.68 (m)	3.68 (m)	4.16 (m)	3.44 (m)	3.66 (m)	4.19 (m)
5 IV	4.27 (m)	4.26 (m)	3.99 (m)	4.18 (m)	4.26 (m)	3.99 (m)
6 IV	1.62 (d, 6.0)	1.62 (d, 6.0)	4.54 (dd, 12.0, 3.0) 4.38 (dd 12.0, 5.0)	1.35 (d, 6.0)	1.62 (d, 6.0)	4.59 (dd, 12.0, 3.0) 4.40 (m)
OMe	3.51 (s)	3.55 (s)		3.54 (s)	3.50 (s)	
	β-D-glc	β-D-glc		α-L-cym	β-D-glc	
1 V	4.94 (d, 8.0)	4.94 (d, 8.0)		4.93 (brd, 2.5)	4.94 (d, 8.0)	
2 V	4.01 (m)	4.00 (m)		1.80 (m) 2.33 (m)	4.00 (m)	
3 V	4.25 (m)	4.24 (m)		3.93 (m)	4.23 (m)	
4 V	4.18 (m)	4.18 (m)		3.96 (m)	4.18 (m)	
5 V	3.99 (m)	3.98 (m)		4.70 (m)	3.98 (m)	
6 V	4.40 (dd, 12.0, 5.0) 4.56 (m)	4.40 (dd, 12.0, 5.0) 4.56 (m)		1.46 (d, 6.0)	4.40 (dd, 12.0, 5.0) 4.58 (dd, 12.0, 3.0)	
OMe				3.43 (s)		
				β-D-glc		
1 VI				4.99 (d, 8.0)		
2 VI				3.98 (m)		
3 VI				4.25 (m)		
4 VI				4.20 (m)		
5 VI				3.96 (m)		
6 VI				4.38 (dd, 12.0, 5.0) 4.56 (dd, 12.0, 3.0)		

Digit: digitoxopyranosyl; ole: oleandropyranosyl; cym: cymaropyranosyl; glc: glucopyranosyl.

[a] Measured at 500 MHz.

[b] Assignments were confirmed by 2D NMR spectra.

J = 9.5 Hz), and 4.94 (d, J = 8.0 Hz), respectively, which indicated that there were five sugars with β-linkages in **1** [11]. Complete ^{1}H and ^{13}C NMR resonance assignments for the saccharide units were carried out unambiguously on the basis of ^{1}H–^{1}H COSY, HSQC, HMBC, TOCSY, HSQC–TOCSY and ROESY experiments. The existence of one D-digitoxopyranosyl, two D-oleandropyranosyl, one D-cymaropyranosyl and one D-glucopyranosyl units was confirmed by comparison of its spectroscopic data with those in the literatures [8,12–19]. Comparing with **7**, the glycosidation shifts were observed at C-2 (−2.1 ppm), C-3 (+6.3 ppm), and C-4 (−3.9 ppm) in the aglycone moiety. Therefore, the sugar moiety linked to the C-3 hydroxyl group of the aglycone. The sugar sequence of **1** was confirmed by the HMBC spectrum, in which distinct correlations from (i) H-1^{V} of the β-D-glucopyranosyl (δ_H 4.94) to C-4^{IV} of the β-D-cymaropyranosyl (δ_c 83.2); (ii) H-1^{IV} of the β-D-cymaropyranosyl (δ_H 5.25) to C-4 ^{III} of the outer β-D-oleandropyranosyl (δ_c 82.9); (iii) H-1^{III} of the outer β-D-oleandropyranosyl (δ_H 4.86) to C-4^{II} of the inner β-D-oleandropyranosyl (δ_c 82.9); (iv) H-1^{II} of the inner β-D-oleandropyranosyl (δ_H 4.72) to C-4^{I} of the β-D-digitoxopyranosyl (δ_c 83.6); and (v) H-1^{I} of the β-D-digitoxopyranosyl (δ_H 5.36) to C-3 (δ_c 76.4) were observed, respectively. Thus, the structure of gracilloside A (**1**) was established as gracigenin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-digitoxopyranoside.

Gracilloside B (**2**) was isolated as a white amorphous power with a molecular formula of $C_{65}H_{96}O_{25}$, which was deduced from the positive HRESI-MS (m/z 1299.6143 [M+Na]$^+$, calcd. 1299.6138). Mild acid hydrolysis of **2** afforded **7** as the aglycone and the same sugar compositions as compound **1**. In the ^{13}C NMR spectra of **1** and **2** (Tables 1 and 2), the structure of **2** corresponded to **1** except that the outer β-D-oleandrose was replaced with β-D-cymarose by the result of correlation from H-1^{III} of the inner β-D-cymaropyranosy (δ_H 5.25) to C-4^{II} of the β-D-oleandropyranosyl (δ_c 83.2) in HMBC experiment. Therefore, the structure of gracilloside B (**2**) was deduced to be gracigenin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyanosyl-(1 → 4)-β-D-digitoxopyranoside.

Gracilloside C (**3**) was isolated as a white amorphous power. Based on the positive HRESI-MS data, the molecular formula of **3** was determined to be $C_{58}H_{84}O_{22}$ (m/z 1155.5371 [M+Na]$^+$, calcd. 1155.5351). Mild acid hydrolysis of **3** afforded **7** as the aglycone, and digitoxose, oleandrose as well as glucose as sugar residues. The ^{1}H and ^{13}C NMR spectra of **3** were very similar to those of **1** except that signals due to one set of D-cymaropyranosyl unit vanished in **3**. This conclusion and the sugar moieties linkage were further confirmed by the 2D-spectra. Hence, compound **3** was deduced to be gracigenin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-digitoxopyranoside.

Gracilloside D (**4**) was suggested to have the molecular formula $C_{72}H_{108}O_{28}$, based on the positive HRESI-MS (m/z 1443.6928 [M+Na]$^+$, calcd. 1443.6924). Mild acid hydrolysis of **4** afforded **7** as the aglycone and the same sugar compositions as compounds **1** and **2**. The NMR spectral data of **4** showed that it contained six anomeric proton signals at δ_H 4.67 (brd, J = 9.5 Hz), 4.93 (brd, J = 2.5 Hz), 4.99 (d, J = 8.0 Hz), 5.04 (brd, J = 2.5 Hz), 5.25 (brd, J = 10.0 Hz), and 5.35 (brd, J = 9.5 Hz), correlating with

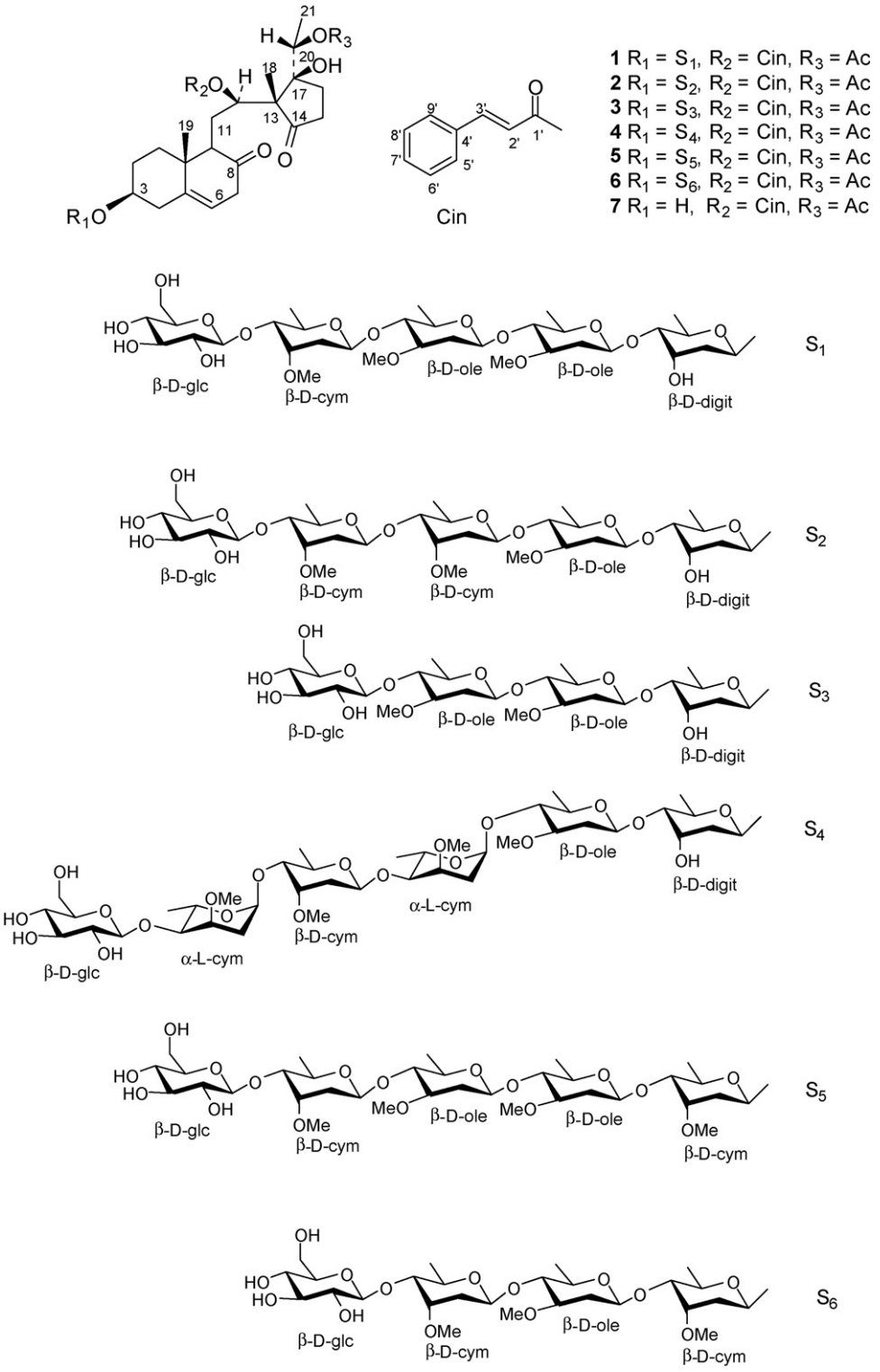

Fig. 1. The chemical structures of compounds **1**–**6**.

anomeric carbon signals at δ_C 101.4, 99.0, 102.3, 97.5, 95.6, and 96.5, respectively, which indicated that there were six sugar units with two α-linkages and four β-linkages in **4**. The signals of each sugar unit were assigned exactly by 2D-NMR analysis and indicated the existence of one β-D-digitoxopyranosyl, one β-D-oleandropyranosyl, one β-D-glucopyranosyl, one β-D-cymaropyranosyl and two α-L-cymaropyranosyl units [3–10]. The sequence of sugars in **4** was determined by the important correlations from (i) H-1^{VI} of the β-D-glucopyranosyl (δ_H 4.99) to C-4^V of the outer α-L-cymaropyranosyl (δ_c 78.9); (ii) H-1^V of the outer α-L-cymaropyranosyl (δ_H 4.93) to C-4^{IV} of the β-D-cymaropyranosyl (δ_c 82.5); (iii) H-1^{IV} of the β-D-cymaropyranosyl (δ_H 5.25) to C-4^{III} of the inner α-L-cymaropyranosyl (δ_c 77.9); (iv) H-1^{III} of the inner α-L-cymaropyranosyl (δ_H 5.04) to C-4^{II} of the β-D-oleandropyranosyl (δ_c 81.7); (v) H-1^{II} of the β-D-oleandropyranosy (δ_H 4.67) to C-4^I of the β-D-digitoxopyranosyl

(δ_C 83.6); and (vi) H-1^I of the β-D-digitoxopyranosyl (δ_H 5.35) to C-3 (δ_C 76.4) observed in the HMBC spectrum, respectively. In addition, the position of each sugar residue was confirmed by ROESY experiment that showed a cross peak between the signal at δ_H 5.35 (H-1^I of the β-D-digitoxopyranosyl) and the signal at δ_H 3.68 (H-3), and other key correlation peaks between the signal at δ_H 4.67 (H-1^{II} of the β-D-oleandropyranosyl) and δ_H 3.46 (H-4^I of the β-D-digitoxopyranosyl), δ_H 5.04 (H-1^{III} of the inner α-L-cymaropyranosyl) and 3.29 (H-4^{II} of the β-D-oleandropyranosyl), δ_H 5.25 (H-1^{IV} of the β-D-cymaropyranosyl) and 3.86 (H-4^{III} of the inner α-L-cymaropyranosyl), δ_H 4.93 (H-1^V of the outer α-L-cymaropyranosyl) and 3.44 (H-4^{IV} of the β-D-cymaropyranosyl), and δ_H 4.99 (H-1^{VI} of the β-D-glucopyranosyl) and 3.96 (H-4^V of the outer α-L-cymaropyranosyl). Thus, the structure of gracilloside D (**4**) was deduced to be gracigenin-3-*O*-β-D-glucopyranosyl-(1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-digitoxopyranoside.

Gracilloside E (**5**) was obtained as a white amorphous power. Analysis of positive HRESI-MS (*m*/*z* 1313.6298 $[M+Na]^+$, calcd. 1313.6294) led to the molecular formula of $C_{66}H_{98}O_{25}$. Mild acid hydrolysis of **5** yielded a sugar mixture of cymarose, oleandrose, as well as glucose and aglycone, which was identical to gracigenin (**7**), by co-TLC comparison with authentic samples. Five anomeric carbon signals at δ_C 96.5, 98.5, 100.2, 102.0, and 106.7, corresponding to five anomeric proton signals at δ_H 5.14 (dd, *J* = 9.5, 1.5 Hz), 5.25 (dd, *J* = 9.5, 1.5 Hz), 4.86 (dd, *J* = 10.0, 1.5 Hz), 4.67 (dd, *J* = 9.5, 1.5 Hz), and 4.94 (d, *J* = 8.0 Hz) were observed in the NMR spectra. Based on the ^1H–^1H COSY, HSQC, HMBC, TOCSY, HSQC-TOCSY and ROESY spectra, the data of these five sugar were assigned to be two D-oleandropyranosyl, two D-cymaropyranosyl and one D-glucopyranosyl units compared with the spectroscopic data in the literature [12–19]. The glycosidation shifts of the carbon signals of **5** were observed at C-2, C-3, and C-4, by −2.1, +6.3, and −3.9 ppm, respectively, compared to **7**, indicating that the sugar chain linked to the C-3 hydroxyl group of the aglycone. The sugar sequence of compound **5** was confirmed by the HMBC spectrum, in which distinct correlations from (i) H-1^V of the β-D-glucopyranosyl (δ_H 4.94) to C-4^{IV} of the outer β-D-cymaropyranosyl (δ_C 83.2); (ii) H-1^{IV} of the outer β-D-cymaropyranosyl (δ_H 5.25) to C-4^{III} of the outer β-D-oleandropyranosyl (δ_C 82.8); (iii) H-1^{III} of the outer β-D-oleandropyranosyl (δ_H 4.86) to C-4^{II} of the inner β-D-oleandropyranosyl (δ_C 82.9); (iv) H-1^{II} of the inner β-D-oleandropyranosyl (δ_H 4.67) to C-4^I of the inner β-D-cymaropyranosyl (δ_C 83.5); and (v) H-1^I of the inner β-D-cymaropyranosyl (δ_H 5.14) to C-3 (δ_C 76.4) were observed, respectively. Consequently, the structure of gracilloside E (**5**) was established as gracigenin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Gracilloside F (**6**) possessed a molecular formula $C_{59}H_{86}O_{22}$ on the basis of the HRESI-MS spectrum (*m*/*z* 1169.5514 $[M+Na]^+$, calcd. 1169.5508). In the acid hydrolysis experiment, the same aglycone and sugar components as those of **5** were obtained by TLC comparison with authentic samples. Inspection of the NMR data of compound **6** and the comparison with those of **5** showed that the signals for one oleandropyranosyl unit was absent in **6**, which was further confirmed by ^1H–^1H COSY, HSQC, HMBC, TOCSY, HSQC-TOCSY and ROESY experiments. Based on the above evidence, compound **6** was proved to be gracigenin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-oleandropyranosyl (1 → 4)-β-D-cymaropyranoside.

Present phytochemical investigation has resulted in isolation and characterization of six new steroidal glycosides which have not been reported in the literature. Due to the small available for each isolated glycoside, the antiepileptic activity of these compounds to animals have not been tested, so it is unclear whether these compounds are effective ingredients of the crude glycosides from this plant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2009.03.001.

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