Phytochemistry Letters 16 (2016) 38–46

Contents lists available at ScienceDirect

Phytochemistry Letters

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

Short communication

Steroidal glycosides from the roots of *Cynanchum stauntonii* and their effects on the expression of iNOS and COX-2

Chang-Zhi Lai[a,1], Jian-Xin Liu[b,c,1], Shu-Wen Pang[a], Yi Dai[a], Hua Zhou[b], Zhen-Qiang Mu[d], Jun Wu[d], Jin-Shan Tang[a,*], Liang Liu[b], Xin-Sheng Yao[a,*]

[a] Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, People's Republic of China
[b] State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Avenida Wailong, Taipa, Macau, People's Republic of China
[c] College of Pharmacy, Hunan University of Medicine, Huaihua 418000, People's Republic of China
[d] Guangzhou Xiangxue Pharmaceutical Ltd. Co., Guangzhou 510663, People's Republic of China

ARTICLE INFO

Article history:
Received 28 December 2015
Received in revised form 14 February 2016
Accepted 26 February 2016
Available online xxx

Keywords:
Cynanchum stauntonii
C_{21} steroidal glycoside
Chemical constituents
Anti-inflammatory activity

ABSTRACT

Six new steroidal glycosides, named stauntosides O-T (**1**–**6**), along with eight known compounds (**7**–**14**), were obtained from the 95% aqueous ethanol extract of the roots of *Cynanchum stauntonii*. Their chemical structures were elucidated by IR, HR ESI-MS/MS, ^{1}H- and ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HSQC-TOCSY, and HMBC spectroscopic analyses, which showed interesting 13,14:14,15-disecopregnane-type or 14,15-secopregnane-type C_{21} steroidal glycosides. The glycosides' anti-inflammatory effects were investigated by detecting the inhibitory effects of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) on RAW246.7 murine macrophage cells stimulated by lipopolysaccharide (LPS). Our results showed that compounds **1**, **5**, **8**, **9**, **11**, and **13** could significantly inhibit iNOS expression, and compounds **5** and **7** could clearly reduce COX-2 expression in RAW246.7 cells stimulated by LPS compared to cells stimulated with LPS and not treated with other compounds. Thus, compounds **1**, **5**, **7–9**, **11**, and **13** have the potential to mediate anti-inflammatory effects, with compound **5** having a greater anti-inflammatory effect than the other compounds.

1. Introduction

The dried roots of *Cynanchum stauntonii* (Decne.) Schltr.ex Lévl., together with the other species of the same genus, *C. glaucescens* (Decne.) Hand.-Mazz., are recorded in the Chinese Pharmacopoeia (Commission, 2010) as "Bai-Qian" and have been used as antitussive and expectorant in China for a long time. Both of them belong to the family Asclepiadaceae. *C. stauntonii* is widely distributed in the south-central region of China. Chemical investigations revealed that the roots primarily contained C_{21} steroidal glycosides, especially with the aberrant 13,14:14,15-disecopregnane-type or 14,15-secopregnane-type skeleton. It is well known that C_{21} steroidal constituents exhibit considerable bioactivities, including hypolipidemic, antitumor, and anti-inflammatory activities (Day et al., 2001; Zhu et al., 1999). Recent studies *in vitro* also indicated that seco-pregnane steroids from *C. stauntonii* and *C. glaucescens* exhibited inhibitory activity against Na^{+}/K^{+}-ATPase (Shibano et al., 2012) and selective inhibition to alphavirus-like RNA viruses (Li et al., 2007). In our search for anti-inflammatory constituents from the plant of *C. stauntonii*, six new C_{21} steroidal glycosides (stauntosides O-T, **1**–**6**), along with eight known compounds (**7**–**14**), were obtained from the dried roots of *C. stauntonii*. In this study, we describe the isolation and structural elucidation of these new compounds, as well as the anti-inflammatory activity of C_{21} steroidal glycosides.

2. Experimental

2.1. General methods

Optical rotations were obtained on a P-1020 digital polarimeter (Jasco Corporation, Tokyo, Japan). IR spectra were recorded on a Jasco FTIR-480 Plus spectrometer. NMR spectra were measured with Bruker AV 300, 400, 500 and 600 NMR instruments. Chemical shifts were given in ppm (δ) relative to chemical shifts of solvent resonances (C_5D_5N: 8.74 and 150.3 ppm). HR ESI-MS spectra were

* Corresponding authors.
E-mail addresses: gztangjinshan@126.com (J.-S. Tang), tyaoxs@jnu.edu.cn (X.-S. Yao).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.phytol.2016.02.016
1874-3900/

Table 1
^{1}H and ^{13}C NMR data of compounds **1–6** (δ in ppm, J values in Hz).

No.	1		2		3		4		5		6	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	37.0 (t)	1.84,m/0.97,m	37.0	1.85,m/0.97, m	36.9	1.87,m/0.98, m	37.0	1.84,m/0.96,m	37.0	1.86,m/0.97, m	38.0	1.78,m/1.11,m
2	30.6 (t)	2.11,m/1.71,m	30.6	2.11,m/1.71, m	30.5	2.12,m/1.71, m	30.5	2.12,m/1.71,m	30.5	2.13,m/1.72, m	30.7	1.73,m/1.39,m
3	78.0 (d)	3.79,m	78.1	3.77,m	78.7	3.85,m	78.7	3.83,m	78.5	3.82,m	78.7	3.84,m
4	39.5 (t)	2.58,m/2.32, m	39.5	2.56,m/2.29, m	39.4	2.65,m/2.34, m	39.5	2.65,m/2.33,m	39.5	2.63,m/2.32, m	39.7	2.64,m/2.44,m
5	141.1 (s)		141.1		141.1		141.1		141.1		140.7	
6	121.0 (d)	5.43, d(5.4)	121.0	5.42,d(4.9)	120.8	5.35,d(5.2)	120.9	5.35,d(5.2)	120.9	5.36,d(4.8)	122.2	5.36,overlapped
7	29.0 (t)	2.66,m/2.17,m	28.9	2.66,m/2.16, m	28.9	2.62,m/2.12, m	28.9	2.62,m/2.12,m	28.9	2.63,m/2.10, m	26.0	2.50,m/2.22,m
8	41.2 (d)	2.52,m	41.2	2.52,m	41.1	2.48,m	41.2	2.47,m	41.2	2.47,m	31.8	1.96,m
9	53.7 (d)	1.25,t(10.4)	53.7	1.25,m	53.7	1.24,t(10.4)	53.7	1.23,t(10.3)	53.7	1.24,t(10.5)	46.3	1.65,m
10	39.2 (s)		39.2		39.1		39.2		39.2		37.4	
11	24.5 (t)	2.64,m/1.40, m	24.4	2.62,m/1.39, m	24.4	2.62,m/1.39, m	24.4	2.62,m/1.38,m	24.4	2.62,m/1.38, m	20.5	1.48,m/1.41,m
12	30.5 (t)	2.06,m/1.41,m	30.5	2.04,m/1.39, m	30.4	2.06,m/1.41, m	30.5	2.04,m/1.39,m	30.5	2.06,m/1.38, m	30.8	2.11,dd(12.2,4.3)/ 1.60,m
13	114.9 (s)		114.9		114.8		114.9		114.9		49.2	
14	176.0 (s)		176.0		175.9		176.0		176.0		109.5	
15	68.3 (t)	4.28,m/3.98, m	68.3	4.28,m/3.96, m	68.2	4.25,m/3.97, m	68.3	4.28,m/3.96,m	68.3	4.26,m/3.94, m	71.8	4.05,d(9.3)/3.83,m
16	76.1 (d)	5.48,m	76.0	5.47,m	76.0	5.46,m	76.1	5.46,m	76.0	5.46,q(7.9)	77.6	4.47,m
17	56.7 (d)	3.41,m	56.7	3.58,m	56.7	3.57,m	56.7	3.57,d(8.4)	56.7	3.57,m	58.5	2.32,d(2.6)
18	144.4 (d)	6.52,s	144.4	6.51,s	144.3	6.49,s	144.4	6.51,s	144.4	6.50,s	16.0	1.10,s
19	18.4 (q)	0.86,s	18.4	0.87,s	18.3	0.81,s	18.3	0.80,s	18.3	0.81,s	19.7	0.93,s
20	119.0 (s)		119.0		119.0		119.0		119.0		114.1	
21	25.3 (q)	1.57,s	25.3	1.57,s	25.2	1.56,s	25.3	1.57,s	25.3	1.57,s	24.4	1.74,s
1′	β-D-ole 98.6 (d)	4.84,dd(9.7, 1.8)	β-D-can 98.7	4.90,d(9.9)	β-D-the 102.8	4.83,d(7.6)	β-D-the 102.8	4.84,d(7.8)	β-D-the 102.8	4.83,d(7.7)	β-D-the 102.7	4.83,d(7.7)
2′	38.5(t)	2.45,m/1.83, m	40.6	2.54,m/1.99, m	75.1	3.94,m	75.1	3.95,m	75.1	3.92,m	75.1	3.93,m
3′	79.6 (d)	3.63,m	70.6	3.98,m	86.2	3.71,m	86.3	3.72,m	86.3	3.68,m	86.4	3.69,m
4′	83.7 (d)	3.61,m	89.0	3.34,t(8.8)	83.1	3.71,m	83.4	3.73,m	83.2	3.69,m	83.3	3.69,m
5′	72.3 (d)	3.60,m	71.4	3.56,m	72.0	3.71,m	72.1	3.70,m	72.1	3.70,m	72.0	3.70,m
6′	19.4 (q)	1.50,d(5.6)	18.6	1.39,d(6.0)	18.8	1.49,d(4.0)	19.2	1.48,d(6.0)	19.2	1.48,d(3.0)	19.2	1.47,d(3.5)
3′-OCH_3	58.0 (q)	3.59,s			60.9	3.95,s	61.0	3.95,s	61.0	3.94,s	61.0	3.95,s
1″	β-D-digt 99.4 (d)	5.58,dd(9.7, 1.8)	β-D-digt 100.3	5.29,d(9.5)	β-D-cym 99.2	5.33,d(7.1)	β-D-digt 99.4	5.53,dd(9.6,1.2)	β-D-cym 100.4	5.31,d(9.5)	β-D-cym 99.3	5.32,dd(9.8,1.5)
2″	40.3 (t)	2.47,m/2.02, m	38.7	2.46,m/2.00, m	37.5	2.36,m/1.87, m	39.6	2.45,m/2.02,m	37.5	2.35,m/1.86, m	37.5	2.37,m/1.87,m
3″	69.3 (d)	4.47,m	68.0	4.50,d(2.7)	78.5	4.09,m	68.2	4.65,d(2.6)	78.7	4.22,m	78.5	4.10,m
4″	74.6 (d)	3.63,m	81.3	3.43,m	83.7	3.49,m	83.6	3.50,m	83.7	3.49,m	83.7	3.50,m
5″	71.1 (d)	4.31,m	69.8	4.20,m	69.7	4.24,m	69.3	4.31,m	69.7	4.19,m	69.7	4.23,m
6″	19.6 (q)	1.61,d(6.2)	18.5	1.33,d(6.1)	19.0	1.38,d(5.8)	19.0	1.43,d(6.0)	18.9	1.37,d(6.2)	19.1	1.40,d(6.2)
3″-OCH_3					59.3	3.69,s			59.4	3.63,s	59.3	3.63,s
1‴			α-L-cym 98.9	5.04,br s	β-D-cym 100.6	5.10,d(9.5)	β-D-cym 100.1	5.14,dd(9.6,1.2)	β-D-cym 100.7	5.08,d(9.6)	β-D-cym 100.7	5.10,dd(9.7,1.6)
2‴			32.8	2.33,m/1.81, m	35.6	2.43,m/1.75, m	35.3	2.42,m/1.69,m	35.5	2.41,m/1.72, m	35.6	2.44,m/1.75,m
3‴			74.0	3.94,m	77.9	3.97,m	77.9	3.90,d(2.4)	78.0	3.92,m	78.0	3.96,m
4‴			78.5	3.99,m	82.6	3.47,m	82.8	3.40,dd (9.5,2.4)	82.8	3.45,m	82.7	3.47,m
5‴			66.8	4.62,m	69.7	4.24,m	69.8	4.22,m	69.7	4.19,m	69.7	4.23,m
6‴			18.6	1.37,d(6.6)	19.1	1.40,d(6.1)	19.0	1.31,d(6.0)	19.1	1.39,d(6.4)	18.9	1.39,d(6.2)
3″-OCH_3			57.4	3.45,s	57.7	3.55,s	57.8	3.54,s	57.8	3.54,s	57.8	3.55,s
1⁗			β-D-glu 103.0	5.02,d(7.3)	α-L-dign 101.5	5.24,d(3.1)	α-L-dign 101.6	5.20,d(3.2)	α-L-dign 101.6	5.22,d(2.4)	α-L-dign 101.6	5.24,d(3.0)

obtained with a Micromass Q-TOF mass spectrometer. HPLC was performed with a Sunfire C_{18} column (4.6 × 250 mm, 5 μm) and a HPLC system equipped with a Waters e2695 system, a Waters 2998 PDA detector and an Alltech 3300 ELSD detector. Semi-preparative HPLC was performed on a HPLC system equipped with a Waters 1515 pump and a Waters 2489 detector, using a Sunfire C_{18} column (10.0 × 250 mm, 5 μm). Open column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Haiyang Chemical Group Corp., Qingdao), ODS column (50 μm, YMC Co., Ltd., Kyoto, Japan), and Sephadex LH-20 (Pharmacia Corp., United States). TLC analysis was performed on pre-coated silica gel GF254 plates (Qingdao Haiyang Chemical Group Corp., Qingdao). Lipopolysaccharide (LPS) used for stimulation of RAW246.7 cells and the drug dexamethasone, used as a positive control or standard, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant material

The Chinese Medicine "Bai-Qian," the dried roots of *C. stauntonii*, was obtained from Guangzhou Xiangxue Pharmaceutical Co., Ltd., in November, 2012. It was identified by Associate Prof. Ying Zhang, College of Pharmacy, Jinan University. A voucher specimen (CS201211-1) was deposited into the Institute of Traditional Chinese Medicine and Natural Products, Jinan University.

2.3. Extraction and isolation

The dried and chopped roots (9.5 kg) of *C. stauntonii* were refluxed three times in 95% (v/v) aqueous EtOH (90 L × 3). The combined ethanol extract was evaporated under vacuum to yield a dark-brown residue (ca. 1100 g). The residue was directly chromatographed over an open macroporous Diaion HP 20 columns (φ 12.0 × 63.0 cm) and eluted with a gradient of EtOH:H_2O (0, 30, 50, and 95% of EtOH) to yield 4 fractions (A-D). Fraction D (93.0 g, 95% ethanol) was applied to a silica gel column for chromatography (φ 9.5 × 68.0 cm) using a gradient of mixtures of petroleum ether:ethyl acetate (EtOAc) (100:0, 98:2, 95:5, 9:1, 7:3, 0:100) and EtOAc:methanol (CH_3OH) (95:5, 9:1, 7:3, 0:100) to yield 13 subfractions (D1-D13) according to their TLC profiles and HPLC behavior. Fraction D10 (20.8 g) was subjected to open ODS CC (φ 4.0 × 30.0 cm) using a CH_3OH:H_2O gradient to afford 13 subfractions (D10-1∼D10-13). Subfraction D10-6 was subjected to ODS CC using a gradient of CH_3OH:H_2O as the eluent to yield 8 subfractions (D10-6-1∼D10-6-8). Subfraction D10-6-4 was subjected to Sephadex LH-20CC (CH_3OH) and then applied to a semi-preparative RP-HPLC system (CH_3O H-H_2O, 70: 30) to afford compound **11** (10.0 mg). Subfraction D10-7 was separated by ODS CC using gradient solvents of CH_3OH:H_2O to yield 7 subfractions (D10-7-1∼D10-7-7). Subfraction D10-7-6 was subjected to Sephadex LH-20CC (CH_3OH) and then applied to a semi-preparative RP-HPLC system (CH_3OH:H_2O, 70:30) to yield compounds **6** (34.0 mg), **8** (12.0 mg), and **12** (11.0 mg). Subfraction D10-7-7 was subjected to Sephadex LH-20CC (CH_3OH) and then semi-preparative RP-HPLC (CH_3O H-H_2O, 70: 30) to yield compounds **1** (23.0 mg) and **9** (13.0 mg). Subfraction D10-8 was chromatographed over silica gel CC using petroleum ether:EtOAc (7:3, 1:1, 0:100, v/v) as the eluent to give 10 subfrations (D10-8-1∼D10-8-10). Subfraction D10-8-6 was applied to a semi-preparative RP-HPLC system (CH_3OH:H_2O, 70:30) to yield compound **10** (58.0 mg). Subfraction D10-8-7 was applied to semipreparative RP-HPLC system (CH_3OH:H_2O, 70:30) to yield compound **14** (86.0 mg). Subfraction D10-8-8 was applied to a semi-preparative RP-HPLC system (CH_3OH:H_2O, 70:30) to yield compounds **3** (84.0 mg) and **13** (8.0 mg). Subfraction D12 (39.0 g) was subjected to ODS CC (φ 4.0 × 30.0 cm) using a gradient of CH_3OH:H_2O to afford 13 subfractions (D12-1∼D12-13). Subfraction D12-5 was applied to a semi-preparative RP-HPLC system (CH_3OH:H_2O, 70:30) to obtain compound **4** (50.0 mg). Subfraction D12-8 was subjected to Sephadex LH-20CC (CH_3OH) and then applied to a semi-preparative RP-HPLC system (CH_3OH:H_2O, 70:30) to yield compounds **2** (25.0 mg), **5** (86.0 mg), and **7** (25.0 mg).

2.3.1. Stauntoside O (*1*)

White amorphous powder; $[\alpha]_D^{28}$ −42.5 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3443, 2934, 1735, 1516 and 1068 cm^{-1}; ESI–MS (positive mode) *m/z* 657.9 $[M+Na]^+$. HR ESI–MS (positive mode) *m/z* 657.3260 $[M+Na]^+$ (calcd for $C_{34}H_{50}O_{11}Na$, 657.3251); ^1H (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N) data see Table 1.

2.3.2. Stauntoside P (*2*)

White amorphous powder; $[\alpha]_D^{28}$ −32.9 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3447, 2938, 1739, 1515 and 1077 cm^{-1}; ESI-MS (positive

Table 1 (*Continued*)

No.	1		2		3		4		5		6	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2‴			75.8	4.01,m	31.4	2.40,m/2.09, m	32.7	2.36,m/2.05,m	32.7	2.35,m/2.06, m	31.4	2.41,m/2.11,m
3‴			79.2	3.99,m	76.2	3.88,m	75.6	3.87,m	75.6	4.01,m	76.3	3.89,m
4‴			72.2	4.22,m	68.1	4.09,m	74.8	4.28,m	74.8	4.26,m	68.1	4.10,m
5‴			78.9	4.25,m	68.2	4.34,m	68.6	4.28,m	68.5	4.29,m	68.2	4.34,m
6‴			63.4	4.58,m/4.38, m	18.1	1.59,d(6.5)	18.4	1.71,d(6.4)	18.4	1.71,d(6.3)	18.3	1.59,d(6.5)
3″-OCH_3					55.4	3.34,s	56.2	3.54,s	56.2	3.53,s	55.5	3.35,s
1⁗							β-D-glu 105.9	4.99,d(7.8)	β-D-glu 105.9	4.97,d(7.7)		
2⁗							75.7	4.04,t(7.5)	75.7	3.88,m		
3⁗							79.1	4.24,d(8.9)	79.1	4.19,m		
4⁗							72.3	4.19,m	72.2	4.17,m		
5⁗							78.8	3.96,m	78.8	4.20,m		
6⁗							63.5	4.55,dd (11.4,2.3)/ 4.36,dd (11.4,5.4)	63.5	4.52,m/4.34, m		

Multiplicities by DEPT experiments in parentheses; s:quanternary, d: CH, t:CH2, and q: CH3C-atoms.

mode) *m/z* 949.9 $[M+Na]^+$. HR ESI-MS (positive mode) *m/z* 949.4405 $[M+Na]^+$ (calcd for $C_{46}H_{70}O_{19}Na$, 949.4409); ^1H (400 MHz, C_5D_5N) and ^{13}C NMR (75 MHz, C_5D_5N) data see Table 1.

2.3.3. Stauntoside Q (**3**)

White amorphous powder; $[\alpha]_D^{28}$ −45.3 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3495, 2935, 1734, 1450 and 1072 cm^{-1}; ESI-MS (positive mode) *m/z* 976.1 $[M+Na]^+$. HR ESI-MS (positive mode) *m/z* 975.4924 $[M+Na]^+$ (calcd for $C_{49}H_{76}O_{18}Na$, 975.4929); ^1H (400 MHz, C_5D_5N) and ^{13}C NMR (100 MHz, C_5D_5N) data see Table 1.

2.3.4. Stauntoside R (**4**)

White amorphous powder; $[\alpha]_D^{28}$ −31.8 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3450, 2943, 1733, 1522 and 1076 cm^{-1}; ESI-MS (positive mode) *m/z* 1123.9 $[M+Na]^+$. HR ESI-MS (positive mode) *m/z* 1123.5300 $[M+Na]^+$ (calcd for $C_{54}H_{84}O_{23}Na$, 1123.5301); ^1H (600 MHz, C_5D_5N) and ^{13}C NMR (75 MHz, C_5D_5N) data see Table 1.

2.3.5. Stauntoside S (**5**)

White amorphous powder; [α]28 D −24.5 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3462, 2976, 1732, 1453 and 1076 cm^{-1}; ESI–MS (positive mode) *m/z* 1138.1 $[M+Na]^+$. HR ESI–MS (positive mode) *m/z* 1137.5468 $[M+Na]^+$ (calcd for $C_{55}H_{86}O_{23}Na$, 1137.5458); ^1H (400 MHz, C_5D_5N) and ^{13}C NMR (75 MHz, C_5D_5N) data see Table 1.

2.3.6. Stauntoside T (**6**)

White amorphous powder; $[\alpha]_D^{28}$ −48.7 (*c* 0.5, CH_3OH); IR (KBr) ν_{max}: 3507, 2938, 1540, 1455 and 1061 cm^{-1}; ESI-MS (positive mode) *m/z* 961.6 $[M+Na]^+$. HR ESI-MS (positive mode) *m/z* 961.5137 $[M+Na]^+$ (calcd for $C_{49}H_{78}O_{17}Na$, 961.5137); ^1H (300 MHz, C_5D_5N) and ^{13}C NMR (75 MHz, C_5D_5N) data see Table 1.

2.4. Determination of absolute configuration of sugars

2.4.1. Acid hydrolysis of compounds **2**, **4**, and **5** and determination of the absolute configuration of sugars

Each solution of compounds **2**, **4**, and **5** (20 mg) in CH_3OH (2 mL) was refluxed with 10% hydrochloric acid (HCl) (3 mL) at 75 °C for 2.5 h. After cooling, the reaction mixture was extracted thoroughly with chloroform ($CHCl_3$) (3 mL × 2), and the aqueous layer was neutralized with barium hydroxide [$Ba(OH)_2$] saturated with H_2O. The precipitates were filtered out, and the solution was dried to yield a crude sugar fraction. The crude sugar fraction was subjected to silica gel CC, successively eluting with $CHCl_3$:CH_3OH at the ratios indicated to yield monosugars. For compound **2**, cymarose (97:3, v/v), digitoxose (93:7, v/v), canarose (9:1, v/v), and glucose (0:100, v/v) were obtained from the crude sugar fraction; for compound **4**, diginose (98:2, v/v), cymarose (97:3, v/v), digitoxose (93:7, v/v), thevetose (85:15, v/v), and glucose (0:100, v/v) were obtained from the crude sugar fraction; and for compound **5**, diginose (98:2, v/v), cymarose (97:3, v/v), thevetose (85:15, v/v), and glucose (0:100, v/v) were obtained from the crude sugar fraction.

According to the procedure described previously (Tanaka et al., 2007), the absolute configurations of glucoses were defined using HPLC by comparing the retention times of derivatives of glucoses obtained from hydrolyzing the compounds with those of standards. The monosugars obtained by acid hydrolysis as described above were dissolved in pyridine and reacted with L-cysteine methyl ester hydrochloride at 60 °C for 1 h. Then, arylisothiocyanates were added to the reaction mixtures, which were then stirred at 60 °C for another 1 h. The reaction mixtures were analyzed using HPLC with a UV detector at 250 nm. Sugar standards were derivatized in the same manner. The retention times (min) of the derivatized standards were as follows: 19.26 min (D-glucose) and 17.94 min (L-glucose). By comparing their retention times with those of the standards, the glucoses in compounds **2**, **4**, and **5** were determined to be d-configuration.

2.4.2. Mild acid hydrolysis of compounds **1**, **3**, and **6** and determination of the absolute configuration of sugars

Each solution of compounds **1**, **3**, and **6** (20 mg) in CH_3OH (2 mL) was mixed with 0.05 N HCl (3 mL), stirred for 15 min at 70 °C and then evaporated in vacuum to remove CH_3OH. The aqueous solution was extracted with $CHCl_3$ (3 mL × 3). The aqueous layer was neutralized with $Ba(OH)_2$ saturated with H_2O. The precipitates were filtered out and the solution dried to yield a crude sugar fraction. The crude sugar fraction was subjected to silica gel CC, successive eluting with $CHCl_3$:MeOH at the ratios indicated to yield monosugars. For compound **1**, oleandrose (97:3, v/v) and digitoxose (93:7, v/v) were obtained from the aqueous layer; for compound **3**, diginose (98:2, v/v), cymarose (97:3, v/v), and thevetose (85:15, v/v) were obtained from the aqueous layer; and for compound **6**, diginose (98:2, v/v), cymarose (97:3, v/v), and thevetose (85:15, v/v) were obtained from the aqueous layer.

D-digitoxose was obtained as a colorless gum with the following analytical properties: $[\alpha]_D^{27}$ +11.7 (*c* 0.1, H_2O) [Ref. (Wang et al., 2010) $[\alpha]_D^{25}$ +48.0, H_2O]. ^1H NMR (β-anomer, major, D_2O) δ_H: 1.05 (3H, d, *J* = 6.4 Hz), 1.52 (1H, m), 1.87 (1H, m), 3.13 (1H, dd, *J* = 9.6, 3.0 Hz), 3.65 (1H, m), 3.92 (1H, d, *J* = 3.5 Hz), 4.91 (1H, dd, *J* = 10.3, 2.0 Hz). ^{13}C NMR (β-anomer, D_2O) δ_C: 18.0, 39.0, 68.1, 70.1, 73.0, 92.1. The data were consistent with those of D-digitoxose (Hidekazu et al., 2010).

D-thevetose was obtained as a colorless gum with the following analytical properties: $[\alpha]_D^{27}$ +19.9 (*c* 0.3, H_2O) [Ref. (Liu et al., 2007) $[\alpha]_D^{25}$ +3.5, H_2O]. ^1H NMR (β-anomer, D_2O, 600 MHz) δ_H: 1.25 (3H, d, *J* = 6.3 Hz), 3.02 (1H, m), 3.03 (1H, m), 3.17 (1H, t, *J* = 8.6 Hz), 3.31 (1H, m), 3.62 (3H, s), 4.45 (1H, d, *J* = 7.9 Hz); ^1H NMR (α-anomer, D_2O, 600 MHz) δ_H: 1.20 (3H, d, *J* = 6.3 Hz), 2.99 (1H, m), 3.32 (1H, overlap), 3.40 (1H, d, *J* = 3.8 Hz), 3.85 (1H, m), 3.63 (3H, s), 5.01 (1H, d, *J* = 3.8 Hz). ^{13}C NMR (β-anomer, D_2O, 150 MHz) δ_C: 18.2, 61.3, 73.3, 76.4, 76.8, 87.7, 98.1; ^{13}C NMR (α-anomer, D_2O, 150 MHz) δ_C: 18.3, 61.1, 68.4, 74.0, 77.3, 84.8, 94.0. The data were consistent with those of D-thevetose (Li et al., 2015).

D-canarose was obtained as a colorless gum with the following analytical properties: $[\alpha]_D^{27}$ +58.5 (*c* 0.5, H_2O) [Ref. (Toshiyuki et al., 1997) $[\alpha]_D^{20}$ +45.7, H_2O]. ^1H NMR (β-anomer, CD_3OD, 400 MHz) δ_H: 1.27 (3H, d, *J* = 6.3 Hz), 1.47 (1H, m), 2.13 (1H, dd, *J* = 12.5, 5.3 Hz), 2.90 (1H, t, *J* = 9.0 Hz), 3.26 (1H, m), 3.50 (1H, m), 4.75 (1H, dd, *J* = 9.6, 1.8 Hz); ^1H NMR (α-anomer, CD_3OD, 400 MHz) δ_H: 1.22 (3H, d, *J* = 6.3 Hz), 1.58 (1H, td, *J* = 12.2, 3.6 Hz), 2.03 (1H, dd, *J* = 12.8, 5.2 Hz), 2.92 (1H, t, *J* = 9.6 Hz), 3.81 (1H, m), 3.84 (1H, m), 5.19 (1H, bd, *J* = 3.5 Hz). ^{13}C NMR (β-anomer, CD_3OD, 100 MHz) δ_C: 18.2, 42.0, 72.2, 73.3, 78.4, 94.8; ^{13}C NMR (α-anomer, CD_3OD, 100 MHz) δ_C: 18.3, 39.8, 68.7, 69.4, 79.2, 92.6. The data were consistent with those of D-canarose (Toshiyuki et al., 1997).

L-cymarose was obtained as a colorless gum with the following analytical properties: $[\alpha]_D^{27}$ −10.1 (*c* 0.1, H_2O) [Ref. (Brasholz and Reißig, 2009) $[\alpha]_D^{22}$ −49.8, H_2O]. ^1H NMR (β-pyranose, CD_3OD, 400 MHz,) δ_H: 1.23 (3H, d, *J* = 6.7 Hz), 3.43 (3H, s), 4.95 (1H, dd, *J* = 9.7, 2.5 Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ_C: 18.8, 36.8, 58.1, 71.6, 74.6, 79.4, 93.1. ^1H NMR (β-furanose, CD_3OD, 400 MHz) δ_H: 1.22 (3H, d, *J* = 6.3 Hz), 3.30 (3H, s), 5.49 (1H, dd *J* = 5.4, 2.0 Hz); ^{13}C NMR (CD_3OD, 100 MHz,) δ_C: 19.5, 41.2, 57.1, 69.3, 82.6, 89.6, 99.9. The data were consistent with those of L-cymarose (Brasholz and Reißig, 2009).

D-cymarose was obtained as a colorless gum with the following analytical properties: $[\alpha]_D^{27}$ +31.3 (*c* 0.1, H_2O) [Ref. (Liu et al., 2007) $[\alpha]_D^{25}$ + 47.6, H_2O]. ^{13}C NMR (β-pyranose, CD_3OD, 100 MHz) δ_C: 18.8,

36.8, 58.1, 71.6, 74.6, 79.4, 93.1; ^{13}C NMR (β-furanose, CD_3OD, 100 MHz) δ_C: 19.5, 41.2, 57.1, 69.3, 82.6, 89.6, 99.9. The data were consistent with those of D-cymarose (Liu et al., 2007; Brasholz and Reißig, 2009).

L-diginose was obtained as a colorless gum with the following analytical properties: $[\alpha]_D^{27}$ −24.8 (*c* 0.1, H_2O) [Ref. (Brasholz and Reißig, 2009) $[\alpha]_D^{25}$ −66.0, H_2O]. ^{13}C NMR (β-anomer, CD_3OD, 100 MHz) δ_H: 16.2, 32.6, 54.9, 66.0, 70.7, 77.3, 93.8; ^{13}C NMR (α-anomer, CD_3OD, 100 MHz) δ_H: 16.3, 29.7, 55.2, 66.7, 67.1, 74.2, 91.5. The data were consistent with those of L-diginose (Brasholz and Reißig, 2009).

D-oleandrose was obtained as a colorless gum with the following analytical properties: $[\alpha]_D^{27}$ +36.4 (*c* 0.1, H_2O) [Ref. (Shibano et al., 2012) $[\alpha]_D$ +20.8, H_2O]. ^{13}C NMR (β-anomer, CD_3OD, 100 MHz) δ_H: 18.5, 38.8, 57.5, 73.5, 79.4, 81.9, 95.1. ^{13}C NMR (α-anomer, CD_3OD, 100 MHz) δ_H: 18.5, 36.6, 57.5, 69.0, 77.9, 79.1, 92.8. The data were consistent with those of D-oleandrose (Brasholz and Reißig, 2009).

2.5. Cell culture

The RAW264.7 murine macrophage cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in complete DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) (Gibco BRL Co, Grand Island, NY) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.6. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression bioassay

Cells were cultured in six-well plates at a density of 4×10^5 cells/well and incubated for 24 h at 37 °C. Next, cells were pretreated with 33 μM of the compounds to be tested (**1–14**) for 1 h and were stimulated with LPS (100 ng/mL) for another 18 h. The cell culture medium was removed, and cells were washed twice with cold PBS. Cells were subsequently incubated in RIPA

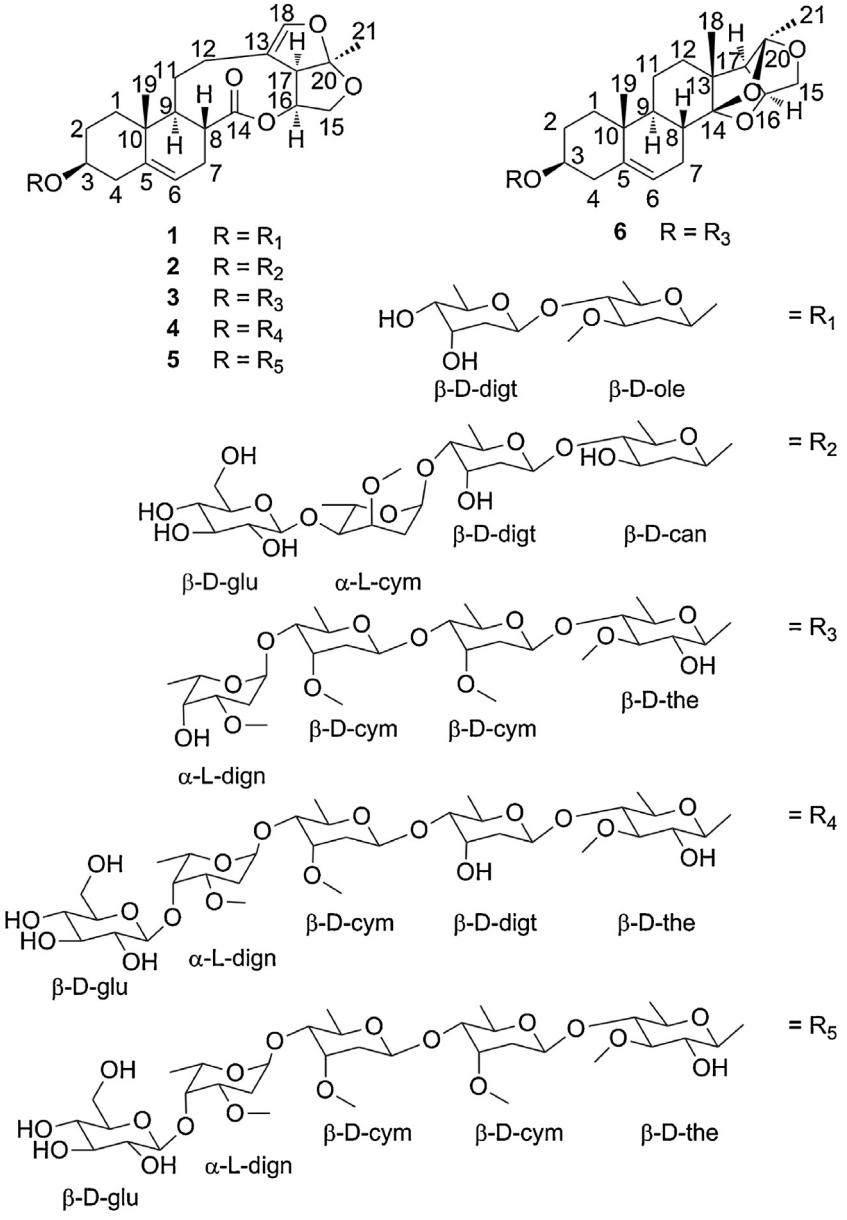


Fig. 1. Structures of compounds **1–6**.

lysis buffer (Cell Signaling Technology, Boston, MA) containing 1 × protease inhibitor mix (Roche Applied Science) on ice for 30 min. The resulting cell solution was centrifuged at 10,000*g* for 20 min, and the supernatant was immediately collected and transferred. The protein concentration of each cell lysate was determined with a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Forty micrograms of protein from each sample was separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein transfer to a nitrocellulose membrane (GE Healthcare Life Sciences, Buckinghamshire). The membrane was blocked in 5% skim milk for 1 h at room temperature and then incubated with the corresponding primary antibody. Antibodies for COX-2 and iNOS were obtained from Cell Signaling Technology, Inc. (Boston, MA). Antibody for β-actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antigen-antibody complexes were subsequently probed with IRDye 800CW-conjugated goat anti-mouse IgG (H + L) or IRDye 800CW-conjugated goat anti-rabbit IgG (H + L) secondary antibodies (Li-COR, Lincoln, NE).

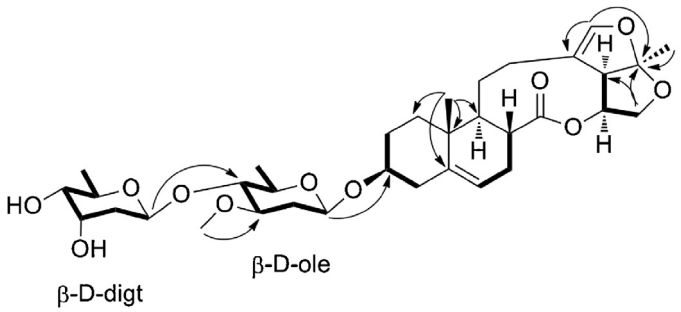
3. Results and discussion

3.1. Chemistry

Compounds **1**–**6** were obtained as white amorphous powders and showed positive reactions to the Liebermann-Burchard and Keller-Kiliani tests, suggesting that they were steroidal glycosides with 2-deoxysugar units in their structures (Fig. 1).

Stauntoside O (**1**) was determined to possess the molecular formula $C_{34}H_{50}O_{11}$ by its pseudomolecular ion at *m/z* 657.3260 $[M+Na]^+$ in the positive HR ESI-MS experiment. The IR spectrum showed the absorption bands for hydroxyl (3443 cm^{-1}), carbonyl (1735 cm^{-1}) and olefinic (1516 cm^{-1}) groups. The ^1H NMR spectrum of **1** revealed the presence of two tertiary methyl protons [δ_H 0.86 (3H, s, H-19) and 1.57 (3H, s, H-21)], two olefinic protons [δ_H 5.43 (1H, d, J = 5.4 Hz, H-6) and 6.52 (1H, s, H-18)], two oxygen-substituted methine protons [δ_H 3.79 (1H, m, H-3) and 5.48 (1H, m, H-16)] and methylene protons [δ_H 3.98 (m, H-15β) and 4.28 (m, H-15α)]. Comparison of the ^1H- and ^{13}C NMR data with those of cynatratoside B (Sugama et al., 1986) revealed that **1** contained the aglycone of glaucogenin C, which was confirmed by analyses of the ^1H-^1H COSY and HMBC spectra (Fig. 2). The ^1H NMR spectrum showed two anomeric protons [δ_H 4.84 (1H, dd, J = 9.7, 1.8 Hz, H-1′) and 5.58 (1H, dd, J = 9.7, 1.8 Hz, H-1″)] and two secondary methyl groups [δ_H 1.50 (3H, d, J = 5.6 Hz, H-6′) and 1.61 (3H, d, J = 6.2 Hz, H-6″)], indicating that it contained two 6-deoxysugars in the structure. The sugars were determined to be oleandrose and digitoxose by comparing their ^1H- and ^{13}C NMR data with those of corresponding deoxysugar units of glaucoside D and stauntoside K (Nakagawa et al., 1983a,b,c; Yu et al., 2013a,b). The specific rotations of digitoxose ($[\alpha]_D^{27}$ +11.7) and oleandrose ($[\alpha]_D^{27}$ +36.4) were obtained from an aqueous solution after 24 h of equilibration. The d-configurations of digitoxose and oleandrose were verified by comparison of the experimental and reported specific rotations (Shibano et al., 2012; Wang et al., 2010). The splitting patterns of anomeric proton signals indicated that **1** has two sugar units with β-linkages (Lin et al., 1995). The HMBC correlation from δ_H 5.58 (H-1″) to δ_C 83.7 (C-4′) suggested that digitoxopyranose was linked to the C-4′ position of oleandropyranose. The HMBC correlation between δ_H 4.84 (1H, dd, J = 9.7, 1.8 Hz, H-1′) and δ_C 78.0 (C-3) revealed that the sugar chain was attached at C-3 of the aglycone, which was confirmed by glycosylation shifts of the aglycone at C-2 (−2.0), C-3 (+6.6), and C-4 (−3.8) compared with glaucogenin C (Nakagawa et al., 1983a,b,c). The ^1H- and ^{13}C NMR data of **1** were assigned by the ^1H- and ^{13}C NMR, ^1H-^1H COSY, HSQC, and HMBC spectra (Table 1). Thus, the structure of **1** was established as glaucogenin C 3-*O*-β-d-digitoxopyranosoyl-(1 → 4)-β-d-oleandropyranoside, named "stauntoside O".

Stauntoside P (**2**) had the molecular formula of $C_{46}H_{70}O_{19}$, determined by the pseudomolecular ion at *m/z* 949.4405 $[M+Na]^+$ in the positive HR ESI-MS experiment. The IR spectrum showed the absorption bands for hydroxyl (3447 cm^{-1}) and carbonyl (1739 cm^{-1}) groups. The ^1H- and ^{13}C NMR data were assigned by analyses of ^1H-^1H COSY, HSQC, HMBC and HSQC-TOCSY spectra (Table 1). The aglycone of **2** was the same as that of **1** by comparison of their ^1H- and ^{13}C NMR data. The ^1H NMR spectrum of **2** showed four anomeric proton signals at δ_H 4.90 (1H, d, J = 9.9 Hz, H-1′), 5.02 (1H, d, J = 7.3 Hz, H-1‴), 5.04 (1H, br s, H-1‴), and 5.29 (1H, d, J = 9.5 Hz, H-1″), suggesting that it contained four sugar units in its structure. The presence of three secondary methyl proton signals at δ_H 1.33 (3H, d, J = 6.1 Hz, H-6″), 1.37 (3H, d, J = 6.6 Hz, H-6‴), and 1.39 (3H, d, J = 6.0 Hz, H-6′) revealed that three of them were 6-deoxysugars. By comparing its ^1H- and ^{13}C NMR data with those of Komaroside G and glaucogenin C 3-*O*-α-L-cymaropyranosyl-(1 → 4)-β- D-digitoxopyranosyl-β-D-canaropyranoside (**14**) (Wang et al., 2004; Fu et al., 2007), the sugars in **2** were determined to be canarose, digitoxose, cymarose, and glucose. The monosugars were obtained by acid hydrolysis of **2** and subsequent silica gel column chromatography purification. The d-configuration of glucose was determined by acid hydrolysis and appropriate derivatization of the resulting sugar (Tanaka et al., 2007). The absolute configurations of D-canarose, D-digitoxose, and L-cymarose were identified according to their specific rotation values (Wang et al., 2010; Toshiyuki et al., 1997; Brasholz and Reißig, 2009). The splitting patterns of anomeric proton signals indicated that the l-cymarose was in an α-linkage, and the other three sugars were in β-linkages (Lin et al., 1995). The 1 → 4 linkage of the sugars was determined by the HMBC correlations from δ_H 5.02 (H-1‴) to δ_C 78.5 (C-4‴), from δ_H 3.43 (H-4″) to δ_C 98.9 (C-1‴), and from δ_H 5.29 (H-1″) to δ_C 89.0 (C-4′). The HMBC



1

Fig. 2. Key ^1H-^1H COSY and HMBC correlations of **1**.

correlation from δ_H 4.90 (H-1′) to δ_C 78.1 (C-3) revealed that the sugar chain was linked to C-3 of the aglycone. Thus, the structure of **2** was determined to be glaucogenin C 3-*O*-β-D-glucopyranosoyl-(1→4)-α-L-cymaropyranosoyl-(1→4)-β-D-digitoxopyranosoyl-(1→4)-β-D-canaropyranoside, named "stauntoside P".

Stauntoside Q (**3**) was determined to possess the molecular formula $C_{49}H_{76}O_{18}$ by its pseudomolecular ion at *m/z* 975.4924 $[M+Na]^+$ in the positive HR ESI-MS experiment. The ^{1}H- and ^{13}C NMR data were assigned by analyses of ^{1}H-^{1}H COSY, HSQC, HMBC, and HSQC-TOCSY spectra (Table 1). Comparison of its ^{1}H- and ^{13}C NMR data with those of **1** revealed that they had the same aglycone and that the difference lay in the sugar chain. Comparison of its ^{1}H- and ^{13}C NMR data with those of stauntoside G (Yu et al., 2013a,b) suggested that they had the same sugar chain, which was confirmed by analyses of ^{1}H-^{1}H COSY, HSQC, HMBC and HSQC-TOCSY experiments. The L configuration of diginose and D configurations of cymarose and thevetose were determined according to their specific rotation values after mild acid hydrolysis of **3** and subsequent silica gel CC purification (Liu et al., 2007; Brasholz and Reißig, 2009). The sequence of the sugars was confirmed by the HMBC correlations from δ_H 5.24 (H-1‴ of L-diginopyranose) to δ_C 82.6 (C-4‴), from δ_H 5.10 (H-1‴ of d-cymaropyranose) to δ_C 83.7 (C-4″), and from δ_H 5.33 (H-1″ of d-cymaropyranose) to δ_C 83.1 (C-4′). The HMBC correlation from δ_H 4.83 (H-1′ of d-thevetopyranose) to δ_C 78.7 (C-3) revealed that the sugar chain was attached at the C-3 of the aglycone. The splitting patterns of anomeric proton signals indicated that the l-diginopyranose was in an α-linkage and that the other three sugars were in β-linkages (Lin et al., 1995). Thus, **3** was determined to be glaucogenin C 3-*O*-α-L-diginopyranosoyl-(1→4)-β-D-cymaropyranosoyl-(1→4)-β-D-cymaropyranosoyl-(1→4)-β-D-thevetopyranoside, named "stauntoside Q".

The positive HR ESI-MS of stauntoside R (**4**) showed a pseudomolecular ion at *m/z* 1123.5300 $[M+Na]^+$, corresponding to the molecular formula $C_{54}H_{84}O_{23}$. The ^{1}H- and ^{13}C NMR data were assigned by analyses of ^{1}H-^{1}H COSY, HSQC, HMBC and HSQC-TOCSY spectra (Table 1). Comparison of its ^{1}H- and ^{13}C NMR data with those of **1** revealed that they had the same aglycone and differed in the sugar chain. The ^{1}H NMR spectrum of **4** showed five anomeric proton signals at δ_H 4.84 (1H, d, *J* = 7.8 Hz, H-1′), 4.99 (1H, d, *J* = 7.8 Hz, H-1⁗), 5.14 (1H, dd, *J* = 9.6, 1.2 Hz, H-1‴), 5.20 (1H, d, *J* = 3.2 Hz, H-1⁗), and 5.53(1H, dd, *J* = 9.6, 1.2 Hz, H-1″), suggesting that it contained five sugar units in the structure. Four secondary methyl proton signals at δ_H 1.31 (3H, d, *J* = 6.0 Hz, H-6‴), 1.43 (3H, d, *J* = 6.0 Hz, H-6″), 1.48 (3H, d, *J* = 6.0 Hz, H-6′), and 1.71 (3H, d, *J* = 6.4 Hz, H-6⁗) revealed the presence of four 6-deoxysugars. The sugar units were identified as thevetose, digitoxose, cymarose, diginose, and glucose by comparing their ^{1}H and ^{13}C NMR data with those found in the literature (Yu et al., 2013a,b; Nakagawa et al., 1983a,b,c). The l configuration of diginose and d configurations of digitoxose, cymarose and thevetose were determined according to their specific rotations after acid hydrolysis of **4** and subsequent silica gel CC purification (Wang et al., 2010; Liu et al., 2007; Brasholz and Reißig, 2009). The absolute configuration of glucose was determined to be d by acid hydrolysis and appropriate derivatization of the resulting sugar (Tanaka et al., 2007). The 1→4 linkage of sugar units was determined by the HMBC correlations from δ_H 4.99 (H-1⁗) to δ_C 74.8 (C-4⁗), from δ_H 5.20 (H-1‴) to δ_C 82.8 (C-4‴), from δ_H 5.14 (H-1‴) to δ_C 83.6 (C-4″), and from δ_H 5.53 (H-1″) to δ_C 83.4 (C-4′). The HMBC correlation from δ_H 4.84 (H-1′) to δ_C 78.7 (C-3) revealed that the sugar chain was attached at C-3 of the aglycone. The splitting patterns of anomeric proton signals indicated that only l-diginopyranose was in an α-linkage and that the other four sugars were in β-linkages in **4** (Lin et al., 1995). Hence, **4** was identified as glaucogenin C 3-*O*-β-D-glucopyranosoyl-(1→4)-α-L-diginopyranosoyl-(1→4)-β-D-cymaropyranosoyl-(1→4)-β-D-digitoxopyranosoyl-(1→4)-β-D-thevetopyranoside, named "stauntoside R".

The molecular formula of stauntoside S (**5**) was determined to be $C_{55}H_{86}O_{23}$ by its pseudomolecular ion at *m/z* 1137.5468 $[M+Na]^+$. The ^{1}H- and ^{13}C NMR data were assigned by analyses of ^{1}H-^{1}H COSY, HSQC, HMBC and HSQC-TOCSY spectra (Table 1). Comparison of its ^{1}H- and ^{13}C NMR data with those of **1** revealed that they had the same aglycone. The presence of five anomeric proton signals at δ_H 4.83 (1H, d, *J* = 7.7 Hz, H-1′), 4.97 (1H, d, *J* = 7.7 Hz, H-1⁗), 5.08 (1H, d, *J* = 9.6 Hz, H-1‴), 5.22 (1H, d, *J* = 2.4 Hz, H-1⁗), and 5.31 (1H, d, *J* = 9.5 Hz, H-1″) in the ^{1}H NMR spectrum indicated that it contained five sugar units. The presence of four secondary methyl proton signals at δ_H 1.37 (3H, d, *J* = 6.2 Hz, H-6″), 1.39 (3H, d, *J* = 6.4 Hz, H-6‴), 1.48 (3H, d, *J* = 3.0 Hz, H-6′), and 1.71 (3H, d, *J* = 6.3 Hz, H-6⁗) suggested that four of them were 6-deoxysugars. The ^{1}H- and ^{13}C NMR data of **5** were almost identical with those of **4** except that the digitoxose was replaced by cymarose. The deoxysugars of d-cymarose, d-thevetose, and l-diginose were determined by their specific rotation values after mild acidic hydrolysis of **5** and subsequent silica gel CC purification (Liu et al., 2007; Brasholz and Reißig, 2009). The d-glucose in **5** was determined by acid hydrolysis and appropriate derivatization of the resulting sugar (Tanaka et al., 2007). The splitting patterns of anomeric proton signals indicated that only l-diginopyranose was in an α-linkage and that the other four sugars in **5** were in β-linkages (Lin et al., 1995). The HMBC correlation from δ_H 4.83 (H-1′) to δ_C 78.5 (C-3) revealed that the sugar chain was attached to C-3 of aglycone. Thus, **5** was identified to be glaucogenin C 3-*O*-β-D-glucopyranosoyl-(1→4)-α-L-diginopyranosoyl-(1→4)-β-D-cymaropyranosoyl-(1→4)-β-D-cymaropyranosoyl-(1→4)-β-D-thevetopyranoside, named "stauntoside S".

The molecular formula of stauntoside T (**6**) was determined to be $C_{49}H_{78}O_{17}$ by its pseudomolecular ion at *m/z* 961.5137 $[M+Na]^+$. The IR spectrum showed the absorption bands for hydroxyl (3507 cm^{-1}) and olefinic (1540 cm^{-1}) groups. The ^{1}H-and ^{13}C NMR data were assigned by analyses of ^{1}H-^{1}H COSY, HSQC, HMBC and HSQC-TOCSY experiments (Table 1). The ^{1}H NMR spectrum of **6** showed the presence of three tertiary methyl protons at δ_H 0.93 (3H, s, H-19), 1.10 (3H, s, H-18), and 1.74 (3H, s, H-21). Comparison

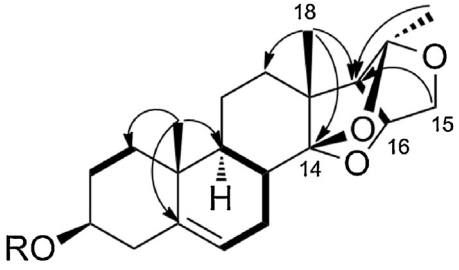


Fig. 3. Key ^{1}H-^{1}H COSY and HMBC correlations of the aglycone of **6**.

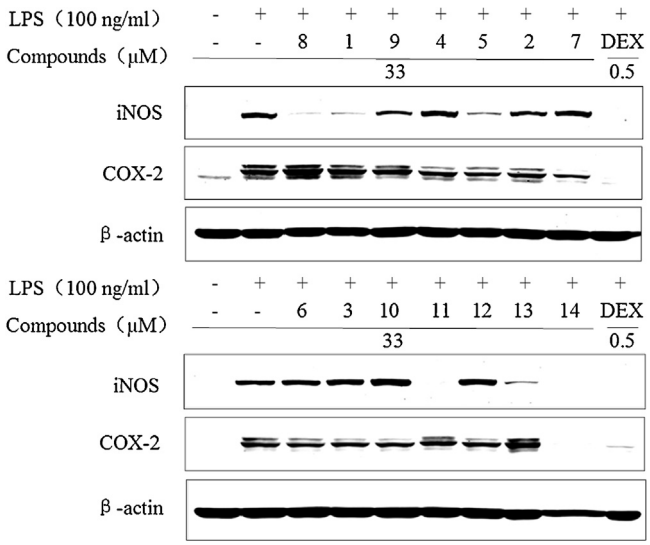


Fig. 4. Inhibitory effects of compounds **1**–**14** on LPS-induced iNOS and COX-2 expression in RAW264.7 macrophages.

of its ^{1}H and ^{13}C NMR data with those of argeloside C revealed that they had the same aglycone and that the difference lay in the sugar chain (Plaza et al., 2005), which was confirmed by analyses of the ^{1}H-^{1}H COSY and HMBC spectra (Fig. 3). In the ROESY spectrum, H-8 (δ_H 1.96, 1H, m) correlated with H-18 (δ_H 1.10, 3H, s) and H-19 (δ_H 0.93, 3H, s), while H-1α (δ_H 1.11, 1H, m) correlated with H-3 (δ_H 3.84, 1H, m) and H-9 (δ_H 1.65, 1H, m); this suggested a *trans* BC ring junction, while 18- and 19-CH_3 groups had axial orientation. The small *J* value of H-17 (2.6 Hz) revealed the *cis* DE ring junction. Thus, the relative configuration of the aglycone for **6** was determined to be the same as previously reported (Plaza et al., 2005; Perrone et al., 2006). Based on the generally accepted β-orientation of CH_3-19 in 14,15-secopregnane-type skeletons, the aglycone was determined to be (14*S*,16*S*,20*R*)-14,16–14,20–15,20-triepoxy-14,15-secopregn-5-en-3-ol. Comparison of the ^{1}H- and ^{13}C NMR data with those of **3** suggested that they had the same sugar chain in the structure, which was confirmed by analyses of ^{1}H-^{1}H COSY, HSQC, HMBC and HSQC-TOCSY spectra. The absolute configurations of d-cymarose, d-thevetose, and L-diginose were determined by their specific rotations after mild acidic hydrolysis of **6** and subsequent silica gel CC purification (Liu et al., 2007; Brasholz and Reißig, 2009). The 1→4 linkage of the sugars was confirmed by the HMBC correlations from δ_H 5.24 (H-1‴ of l-diginopyranose) to δ_C 82.7 (C-4‴), from δ_H 5.10 (H-1‴ of d-cymaropyranose) to δ_C 83.7 (C-4″), from δ_H 5.32 (H-1″ of d-cymaropyranose) to δ_C 83.3 (C-4′), and from δ_H 4.83 (H-1′ of D-thevetopyranose) to δ_C 78.7 (C-3). The splitting patterns of anomeric proton signals indicated that the l-diginopyranose was α-linkage, and the other three sugars were β-linkages (Lin et al., 1995). Thus, **6** was determined to be (14*S*,16*S*,20*R*)-14,16–14,20–15,20-triepoxy-14,15-secopregn-5-en-3-ol-3-*O*-α-L-diginopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-thevetopyranoside, named "stauntoside T".

The eight known pregnane glycosides were cynatratoside D (**7**) (Wang et al., 2007), stauntoside L (**8**) (Yu et al., 2013a,b), stauntoside M (**9**) (Yu et al., 2013a,b), cynatratoside B (**10**) (Sugama et al., 1986), glaucogenin C mono-d-thevetoside (**11**) (Nakagawa et al., 1983a,b,c), glaucoside G (**12**) (Nakagawa et al., 1983a,b,c), stauntoside B (**13**) (Yu et al., 2013a,b) and glaucogenin C 3-*O*-α-L-cymaropyranosyl-(1→4)-β-D-digitoxopyranosyl-β-D-canaropyranoside (**14**) (Fu et al., 2007). The identification of these substances was established by comparing their physical and spectroscopic data with those reported previously.

3.2. Bioactivity

Immune cells, including macrophages and monocytes, are involved in various inflammatory diseases, which are pathophysiologically complex (Mcinnes and Schett, 2011; Galkina and Ley, 2009; Vergani and Mieli, 2008). Lipopolysaccharide (LPS) can activate macrophages to produce proinflammatory mediators, including nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Chao et al., 2008). Both NO and PGE_2 are among the most prominent inflammatory mediators. NO is induced by NO synthase (iNOS), and prostaglandins are regulated by cyclooxygenase-2 (COX-2; prostaglandin H2 synthase). Given the key role of macrophages in inflammation, in our current study, the anti-inflammatory effects of compounds **1**–**14** were investigated by measuring their ability to inhibit protein expression of iNOS and COX-2 in RAW246.7 cells stimulated by LPS (Fig. 4). Dexamethasone (DEX) was used as a positive control at a working concentration of 0.5 μM. Pharmacological results revealed that compounds **1**, **5**, **8**, **9**, **11**, and **13** could clearly inhibit protein expression of iNOS, while compounds **5** and **7** could, to a certain extent, decrease COX-2 protein expression in LPS-stimulated RAW246.7 cells compared to LPS-stimulated cells that received no other treatment. Based on these results, we speculate that these compounds affected the release of NO and/or PGE_2, respectively. Compound **14** could dramatically inhibit iNOS and COX-2 protein expression compared to LPS stimulation alone; however, it also showed strong cytotoxicity at a concentration of 33 μM. Therefore, we considered this inhibitory effect on iNOS and COX-2 to be related to the cytotoxic effects. Our results indicate that compounds **1**, **5**, **7**–**9**, **11**, and **13** have the potential to medicate anti-inflammatory effect, with compound **5** having a stronger anti-inflammatory effect than other compounds.

Acknowledgments

We are grateful to the State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology for the anti-inflammatory activity testing. Associate Professor Ying Zhang's help in plant authentication is also appreciated. We are also grateful to Mr. T. Shi, Mr. J. Geng, and Dr. Y. Yu for the HR ESI-MS and NMR measurements. The major chemical work was accomplished in Guangzhou Xiangxue Pharmaceutical Ltd.

References

Brasholz, M., Reißig, H.U., 2009. Alkoxyallene-based de novo synthesis of rare deoxy sugars: new routes to L-cymarose, L-sarmentose, L-diginose and L-oleandrose. Eur. J. Org. Chem. 21, 3595–3604.

Chao, C.H., Wen, Z.H., Su, J.H., Chen, I., Huang, H.C., Dai, C.F., Sheu, J.H., 2008. Further study on anti-inflammatory oxygenated steroids from the octocoral *Dendronephthya griffini*. Steroids 73, 1353–1358.

Commission, C.P., 2010. Pharmacopoeia of the People's Republic of China. China Medical Science Press, Beijing, pp. 101.

Day, S.H., Wang, J.P., Won, S.J., Lin, C.N., 2001. Bioactive constituents of the roots of *Cynanchum atratum*. J. Nat. Prod. 64, 608–611.

Fu, M.H., Wang, Z.J., Yang, H.J., Maimai, M., Fang, J., Tang, L.Y., Yang, L., 2007. A new C21-steroidal glycoside from *Cynanchum stauntonii*. Chin. Chem. Lett. 18, 415–417.

Galkina, E., Ley, K., 2009. Immune and inflammatory mechanisms of atherosclerosis. Annu. Rev. Immunol. 27, 165.

Hidekazu, H., Hideaki, A., Keisuke, K., Hiroyuki, A., 2010. Nucleophilic addition to 2,3-disubstituted butanal derivatives and their application to natural product synthesis. Chem. Pharm. Bull. 58, 1411–1418.

Li, Y.M., Wang, L.H., Li, S.L., Chen, X.Y., Shen, Y.M., Zhang, Z.K., He, H.P., Xu, W.B., Shu, Y.L., Liang, G.D., Fang, R.X., Hao, X.J., 2007. Seco-pregnane steroids target the subgenomic RNA of alphavirus-like RNA viruses. Proc. Natl. Acad. Sci. U. S. A 104, 8083–8088.

Li, J.L., Zhou, J., Chen, Z.H., Guo, S.Y., Li, C.Q., Zhao, W.M., 2015. Bioactive C21-steroidal glycosides from the roots of *Cynanchum otophyllum* that suppress the seizure-like locomotor activity of zebrafish caused by pentylenetetrazole. J. Nat. Prod. 78, 1548–1555.

Lin, Y.L., Lin, T.C., Kuo, Y.H., 1995. Five new pregnane glycosides from *Cynanchum taiwanianum*. J. Nat. Prod. 58, 1167–1173.

Liu, Y.B., Tang, W.Z., Yu, S.S., Qu, J., Liu, J., Liu, Y., 2007. Eight new C-21 steroidal glycosides from *Dregea sinensis* var corrugata. Steroids 72, 514–523.

Mcinnes, I.B., Schett, G., 2011. The pathogenesis of rheumatoid arthritis. N. Engl. J. Med. 365, 2205–2219.

Nakagawa, T., Hayashi, K., Wada, K., Mitsuhashi, H., 1983a. Studies on the constituents of Asclepiadaceae plants—LII. The structures of five glycosides glaucoside A, B, C, D, and E from Chinese drug Pai-ch'ien *Cyanchum glaucescens* Hand-Mazz. Tetrahedron 39, 607–612.

Nakagawa, T., Hayashi, K., Mitsuhashi, H., 1983b. Studies on the constituents of Asclepiadaceae plants. LIII. The structures of glaucogenin-A, -B, and -C mono-D-thevetoside from the Chinese drug Pai-ch'ien *Cynanchum glaucescens* Hand-Mazz. Chem. Pharm. Bull. 31, 870–878.

Nakagawa, T., Hayashi, K., Mitsuhashi, H., 1983c. Studies on the constituents of Asclepiadaceae plants. LIV. The structures of glaucoside-F and -G from the Chinese drug Pai-ch'ien: *Cynanchum glaucescens* Hand-Mazz. Chem. Pharm. Bull. 31, 879–882.

Perrone, A., Plaza, A., Ercolino, S.F., Hamed, A.I., Parente, L., Pizza, C., Piacente, S., 2006. 14,15-Secopregnane derivatives from the leaves of *Solenostemma argel*. J. Nat. Prod. 69, 50–54.

Plaza, A., Perrone, A., Balestrieri, C., Balestrieri, M.L., Bifulco, G., Carbone, V., Hamed, A., Pizza, C., Piacente, S., 2005. New antiproliferative 14,15-secopregnane glycosides from *Solenostemma argel*. Tetrahedron 61, 7470–7480.

Shibano, M., Misaka, A., Sugiyama, K., Taniguchi, M., Baba, K., 2012. Two secopregnane-type steroidal glycosides from *Cynanchum stauntonii* (Decne.) Schltr.ex Levl. Phytochem. Lett. 5, 304–308.

Sugama, K., Hayashi, K., Mitsuhashi, H., Kaneko, K., 1986. Studies on the constituents of Asclepiadaceae plants. LXVI. The structures of three new glycosides, cynapanosides A, B, and C, from the Chinese drug Xu-Chang-Qing, *Cynanchum paniculatum* Kitagawa. Chem. Pharm. Bull. 34, 4500–4507.

Tanaka, T., Nakashima, T., Ueda, T., Tomii, K., Kouno, I., 2007. Facile discrimination of aldose enantiomers by reversed-phase HPLC. Chem. Pharm. Bull. 55, 899–901.

Toshiyuki, N., Tsutomu, F., Naoko, O., Wataru, H., Junko, N., Tadatake, O., 1997. Synthesis of highly deoxidized monosaccharides from methyl 3-O-acetyl-6-O-(p-tolylsulfonyl)-β-D-glucopyranoside. J. Appl. Glycosci. 44, 175–181.

Vergani, D., Mieli, V.G., 2008. Aetiopathogenesis of autoimmune hepatitis. World J. Gastroenterol. 14, 3306–3312.

Wang, L.Q., Shen, Y.M., Xing, X., Wei, Y.Q., Zhou, J., 2004. Five new C21 steroidal glycosides from *Cynanchum komarovii* Al. Iljinski. Steroids 69, 319–324.

Wang, L.Q., Lu, Y., Zhao, Y.X., Zhou, J., Zheng, Q.T., 2007. Three new C21-steroidal glycosides from the roots of *Cynanchum inamoenum*. J. Asian Nat. Prod. Res. 9, 771–779.

Wang, L., Yin, Z.Q., Wang, Y., Zhang, X.Q., Li, Y.L., Ye, W.C., 2010. Perisesaccharides A–E: new oligosaccharides from the root barks of *Periploca sepium*. Planta Med. 76, 909–915.

Yu, J.Q., Deng, A.J., Qin, H.L., 2013a. Nine new steroidal glycosides from the roots of *Cynanchum stauntonii*. Steroids 78, 79–90.

Yu, J.Q., Zhang, Z.H., Deng, A.J., Qin, H.L., 2013b. Three new steroidal glycosides from the roots of *Cynanchum stauntonii*. Bio. Med. Res. Int. 816145.

Zhu, N.Q., Wang, M.F., Kikuzaki, H., Nakatani, N., Ho, C.T., 1999. Two C21-steroidal glycosides isolated from *Cynanchum stauntoi*. Phytochemistry 52, 1351–1355.