Fitoterapia 118 (2017) 107–111

Contents lists available at ScienceDirect

Fitoterapia

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

Novel immunosuppressive pregnane glycosides from the leaves of *Epigynum auritum*

Fei Gao [a], Yuan-Cheng Yao [a], Sheng-Bao Cai [a], Tian-Rui Zhao [a], Xiao-Yan Yang [b], Jian Fan [a], Xiao-Nian Li [c], Jian-Xin Cao [a,*], Gui-Guang Cheng [a,*]

[a] *Yunnan Institute of Food Safety, Kunming University of Science and Technology, Kunming 6505000, People's Republic of China*
[b] *Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 6505000, People's Republic of China*
[c] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China*

ARTICLE INFO

Article history:
Received 3 December 2016
Received in revised form 10 February 2017
Accepted 12 February 2017
Available online 12 March 2017

Keywords:
Epigynum auritum
Epigynosides E–G
Pregnane glycosides
Immunosuppressive

ABSTRACT

Phytochemical investigation on the leaves of *Epigynum auritum* led to the isolation of three novel C_{21} pregnane glycosides, epigynosides, E–G (**1**–**3**), together with two known pregnane glycosides, epigynosides A (**4**) and C (**5**). Their structures were elucidated based on extensive spectroscopic data (MS, IR, 1D and 2D NMR) analysis, as well as comparison with the reported data. The immunological activities of the new compounds was evaluated against concanavalin A (Con A)-stimulated proliferation of mice splenocyte in vitro. Compounds **1**–**3** displayed significant immunosuppressive activities, close to the efficacy of the positive control (dexamethasone) at the concentration of 50 μM.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

Immunosuppressive agents are the mainstay treatment for patients that have received organ grafts, and are becoming increasingly important in the treatment of autoimmune diseases [1]. Some clinical drugs (e.g., cyclosporine A [2], lescol [3], and sirolimus [4]), are currently used in immunological therapies but have limitations with undesirable side effects, such as renal injury, diabetes, and cardiovascular disease [5–8]. Searching for new immunosuppressant substances from plants is still challenging. Pregnane glycosides are one of great significance in natural medicine, and are well known for their distinctive structural features and significant diverse bioactivities, such as immunosuppressive [9,10], cytotoxic [11], antidepressant [12], antiepileptic [13], anti-inflammatory [14], antioxidant [15], antibacterial and antifungal [16] activities. The diverse structures combine an aglycone skeleton and a variable number and type of sugar units, typically attached at the C-3 position. Previous phytochemical investigation on *Epigynum auritum* (*Apocynaceae*) reported the isolation of pregnane glycosides, triterpenoid saponins, and phenolic derivatives [17–21]. As part of our continuous studies on biologically active secondary metabolites, three new pregnane glycosides, epigynosides E–G (**1**–**3**), together with two known pregnane glycosides, epigynosides A (**4**) and C (**5**), were isolated from this species (Fig. 1). This paper describes the isolation and structural elucidation of these new pregnane glycosides. Furthermore, the new compounds were tested for their immunosuppressive activities. Compounds **1**–**3** exhibited significantly inhibitory effect on the Con A-stimulated mice splenocyte proliferation in vitro.

2. Experimental

2.1. General methods

Melting points were measured on an X-4 micro melting point apparatus. Optical rotations were measured with a JASCO P-1020 digital polarimeter. UV spectra were obtained using a Shimadzu UV-2401 PC spectrophotometer. IR spectra were recorded on a Bruker Tensor 27 infrared spectrophotometer using KBr pellets. 1D and 2D NMR spectra were recorded on a Bruker AM-400, a DRX-500, an Avance III 600 or AV 600 MHz spectrometer with TMS as internal standard. HRESIMS was recorded on a Thermo high resolution Q Exactive focus mass spectrometer. Column Chromatography was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Rp-18 (40–63 μM, Merck), Sephadex LH-20 (GE Healthcare, Sweden). Fractions were monitored by TLC (GF_{254}, Qingdao Marine Chemical Ltd., Qingdao, China), and by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol.

* Corresponding authors.
E-mail addresses: jxcao@kmust.edu.cn (J.-X. Cao), ggcheng@kmust.edu.cn (G.-G. Cheng).

http://dx.doi.org/10.1016/j.fitote.2017.02.011
0367-326X/© 2017 Elsevier B.V. All rights reserved.

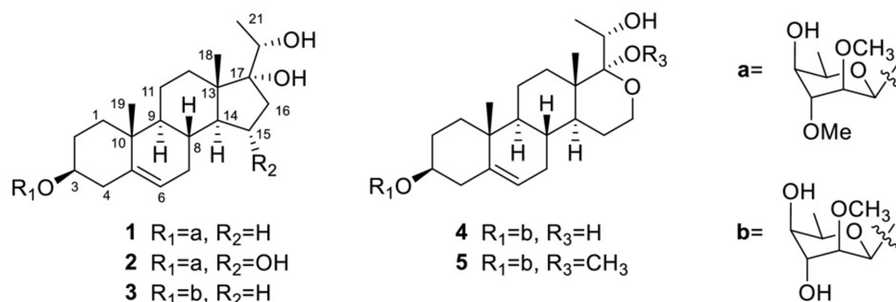

Fig. 1. Chemical structures of compounds **1–5**.

2.2. Plant material

The leaves of *E. auritum* were collected from Xishuangbanna Autonomous Prefecture, Yunnan Province, People's Republic of China, and identified by Wenqiang Xiao of Xishuangbanna Botanic Garden. A voucher specimen (No. Cao20150608) has been deposited at the Yunnan Institute of Food Safety, Kunming University of Science and Technology.

2.3. Extraction and isolation

The air dried and crushed leaves of *E. auritum* (4.5 Kg) were extracted with MeOH (40 L, 48 h) three times at room temperature. After filtration, all the solvent was evaporated under reduced pressure at 50 °C to yield the extract. This crude extract was exhaustively partitioned between water and EtOAc to obtain the EtOAc fraction (57 g). The EtOAc fraction (57 g) was chromatographed over macroporous resin D101, eluted with MeOH-H_2O (20:80, 40:60, 60:40, 80:20, and 100:0, v/v) to give five fractions (Fr. A–E). Fr. B (4.6 g) was purified on a MPLC column with a gradient of MeOH-H_2O (30:70–70:30) to yield six subfractions B1–B6. Subfraction B2 (2.5 g) was further subjected to column chromatography over silica gel (200–300 mesh), eluting with a gradient of chloroform-acetone (10:1–3:1) to yield four subfractions B2a–B2d. Fractions B2b (298 mg) and B2c (200 mg) was subjected to Sephadex LH-20 ($CHCl_3$–CH_3OH, 1:1) respectively to yield compounds **2** (5.5 mg) and **5** (8.0 mg). Fraction D (6.4 g) was chromatographed over Sephadex LH-20 (MeOH-H_2O, 1:1) to yield four subfractions D1–D4. Fraction D2 (1.3 g) was subjected to MPLC (MeOH-H_2O, 40:60 → 80:20) and further purified by a semi-preparative HPLC (MeCN–H_2O, 50:50) to obtain compounds **1** (23.2 mg). Fraction D3 (110 mg) was subjected to column chromatography over silica gel (200–300 mesh) and eluted with chloroform-acetone (4:1) and further by Sephadex LH-20 ($CHCl_3$–CH_3OH, 1:1) to yield compound **3** (5.7 mg). Fraction E (7.2 g) was subjected to Sephadex LH-20 (MeOH–H_2O, 1:1) to yield three subfractions E1–E3. Subfraction E2 (960 mg) was chromatographed over RP-C_{18} gel (MeOH-H_2O, 50:50 → 70:30) and further purified by a semi-preparative HPLC (MeCN-H_2O, 55:45) to give compound **4** (6.3 mg).

*2.3.1. Epigynoside E (**1**)*

Colorless crystals (CH_3OH–H_2O, 9:1); mp 227–229 °C; $[\alpha]_D^{26} - 98.5$ (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε): 203 (3.5) nm; IR (KBr) ν_{max} 3473, 2902, 2831, 1733, 1717, 1458, 1375, 1329, 1047, 962 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 531.3298 $[M + Na]^+$ (calcd. for $C_{29}H_{48}O_7Na^+$, 531. 3298).

*2.3.2. Epigynoside F (**2**)*

White amorphous powder, mp 189–190 °C; $[\alpha]_D^{26} - 205.9$ (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε): 203 (3.8) nm; IR (KBr) ν_{max} 3429, 2933, 1632, 1517, 1379, 1179, 1097, 1036 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; positive-ion HRESIMS *m/z* 547.3245 $[M + Na]^+$ (calcd. for $C_{29}H_{48}O_8Na^+$, 547.3247).

*2.3.3. Epigynoside G (**3**)*

White amorphous powder; 181–183 °C; $[\alpha]_D^{26.2} - 177.7$ (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 203 (3.6) nm; IR (KBr) ν_{max} 3419, 2931, 1713, 1633, 1457, 1376, 1255, 1104, 1041, 884 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; positive-ion HRESIMS *m/z* 517.3143 $[M + Na]^+$ (calcd. for $C_{28}H_{46}O_7Na^+$, 517.3141).

Table 1
^1H NMR and ^{13}C NMR spectroscopic data for compounds **1–3**[a] (δ in ppm).

Position	1		2		3	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1a	1.84 m	37.2	1.88 m	38.3	1.86 m	37.2
1b	1.06 td (13.7, 3.5)		1.07 td (13.4, 3.2)		1.08 td (13.7, 3.5)	
2a	1.96 m	29.4	1.93 m	30.6	1.98 m	29.4
2b	1.60 ov[b]		1.59 m		1.62 ov	
3	3.56 ov	78.3	3.51 m	79.6	3.60 br, s	78.2
4a	2.33 m	38.7	2.34 m	39.8	2.36 m	38.7
4b	2.24 m		2.20 m		2.26 m	
5		140.6		141.7		140.6
6	5.35 m	121.6	5.37 m	121.7	5.36 m	121.6
7a	1.99 m	31.9	1.95 m	33.0	2.01 m	31.8
7b	1.60 ov		1.56 ov		1.63 m	
8	1.48 m	31.7	1.46 m	32.6	1.51 m	31.9
9	0.96 dd (11.7, 4.0)	49.6	0.96 td (11.8, 4.6)	51.3	0.97 dd (11.7, 4.0)	49.6
10		36.7		37.8		36.7
11a	1.58 m	20.4	1.56 ov	21.1	1.60 m	20.4
11b	1.43 dd (12.8, 3.9)		1.49 dd (7.8, 2.2)		1.45 dd (13.0, 3.7)	
12a	1.99 m	31.0	1.96 m	32.7	2.01 m	31.0
12b	1.60 ov		1.56 ov		1.62 ov	
13		45.6		47.0		45.6
14	1.70 ov	51.3	1.85 m	50.5	1.73 ov	51.3
15a	1.72 m	23.5	4.34 dd(9.0, 2.0)	77.4	1.75 m	23.5
15b	1.16 m				1.18 m	
16a	2.03 m	37.6	1.72dd (13.0, 4.1)	35.3	2.05 m	37.6
16b	1.70 ov		1.47 m		1.73 ov	
17		85.7		84.0		85.7
18	0.73 s	13.9	0.77 s	14.9	0.74 s	13.9
19	0.99 s	19.3	1.02 s	19.7	1.01 s	19.3
20	3.83 ov	72.2	3.83 m	72.8	3.85 m	72.3
21	1.18 d (6.3)	18.5	1.18 d (6.3)	18.5	1.19 d (6.3)	18.5
1′	4.75 s	97.4	4.82 s	98.7	4.90 br, s	96.9
2′	3.32 d (3.1)	77.8	3.34 m	79.1	3.28 m	79.8
3′	3.65 d (2.5)	78.0	3.63 t (3.6)	79.1	4.22 d (2.9)	69.1
4′	3.35 br, s	68.6	3.37 m	69.8	3.27 m	71.6
5′	3.83 ov	70.6	3.87 dd (6.6, 1.7)	72.0	3.97 q (6.6)	70.3
6′	1.27 d (6.6)	16.3	1.24 d (6.6)	16.7	1.31 d (6.6)	16.3
2′–CH_3	3.56 ov	60.3	3.53 s	60.3	3.57 s	60.2
3′–CH_3	3.40 s	57.9	3.44 s	58.4		

[a] Compounds **1** and **2** were measured in $CDCl_3$, **3** in methanol-d_4.
[b] ov: overlap.

2.4. X–ray crystallographic analysis of **1**

The intensity data for epigynoside E (**1**) were collected at 100 K on a Bruker APEX DUO diffractometer equipped with an APEX II CCD, using Cu Kα radiation. Cell refinement and data reduction were performed with Bruker SAINT. Structure was resolved by direct methods, expanded by Fourier techniques, and refined by the program and full-matrix least-squares calculations [22]. All non-hydrogen atoms have been refined anisotropically, and all hydrogen atoms were fixed at calculated positions. Crystallographic data for epigynoside E (**1**) has been placed in Cambridge Crystallographic Data Center as supplementary publications (number: CCDC 1519960). Copies of the data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, U.K.; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).

2.4.1. Crystallographic data for epigynoside E (**1**)

$C_{29}H_{48}O_7 \cdot H_2O$, $M = 526.69$, $a = 7.3861(4)$ Å, $b = 9.9803(5)$ Å, $c = 19.0795(10)$ Å, $\alpha = 84.8750(10)°$, $\beta = 88.381(2)°$, $\gamma = 89.622(2)°$, $V = 1400.26(13)$ Å^3, $T = 100(2)$ K, space group $P1$, $Z = 2$, μ (CuKα) = 0.724 mm^{-1}, 12,734 reflections measured, 6127 independent reflections ($R_{int} = 0.0429$). The final R_1 values were 0.0915 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.2429 ($I > 2\sigma(I)$). The final R_1 values were 0.0920 (all data). The final $wR(F^2)$ values were 0.2446 (all data). The goodness of fit on F^2 was 1.228. Flack parameter = 0.21(12).

2.5. Acid hydrolysis of **1–4**

Compounds **1**–**4** (each 3 mg) were dissolved with 2 M HCl (1, 4-dioxane/H_2O 1:1, 2 mL) and hydrolyzed on water bath at 90 °C for 2 h. After cooling, the reaction products were partitioned with $CHCl_3$ for three times (3 × 5 mL). The aqueous layer was neutralized with 2 M NaOH. Compounds **3** and **4** had the same monosaccharide, which was identified as 2-*O*-methyl-6-deoxy-β-D-idopyranose by co-TLC with authentic sugar, while compounds **1** and **2** had the same sugar unit, eluting with chloroform/methanol/water/acetic acid 40:10:1.0:0.5 (*RF* = 0.56 for 2,3-dimethoxy-6-deoxy-β-D-idopyranose, 0.45 for 2-*O*-methyl-6-deoxy-β-D-idopyranose). After evaporated to dryness the aqueous layer, the residue was dissolved in 1 mL anhydrous pyridine and treated with L-cysteine methyl ester hydrochloride (1.5 mg) and stirred at 60 °C for 1 h. Trimethylsilylimidazole (1.5 mL) was added to the reaction mixtures, and they were kept at 60 °C for 30 min [23]. The mixture was subjected to GC analysis, run on a Shimadzu GCMS-QP2010 equipped with a 30 m × 0.32 mm i.d. 30QC2/AC-5 quartz capillary column and an H_2 flame ionization detector with the following conditions: column temperature, 180–280 °C; programmed increase, 3 °C/min; carrier gas, N_2 (1 mL/min); injector and detector temperature, 250 °C; injection volume, 4 μL; and split ratio 1/50. The configuration of the sugar moiety was determined by comparing the retention time. The retention time of the sugar moiety for **1** and **2** was 22.5 min, and for **3** and **4** was 17.5 min. So the glycosides of compound **1**–**4** were determined to be all D configurations.

2.6. Splenocyte proliferation assay

Splenocytes were isolated from Male BALB/c mice as described previously [24]. Cells were seeded into 96-well flat-bottom microtiter plates (Nunc) at the density of 1×10^6 cell/mL, and exposed to the test compound at various concentrations in the presence of concanavalin A (Con A, 10 μg/mL), using the Con A-treated splenocytes as the experimental control, dexamethasone (DXM) as a positive control, and splenocytes without Con A-treated as the negative control. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO_2, 10 μL of CCK-8 was added and incubated for another 4 h. The tests were conducted for three independent replicates, and the data were calculated as the mean of the three individual experiments. The viability of cells was evaluated using the CCK-8 assay by detecting absorbance at 450 nm on a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures / the absorbance value for non-stimulated cultures.

2.7. Cytotoxicity assay

The cytotoxicity of **1**–**5** was evaluated towards five human cancer cell lines, MCF-7 (breast cancer), SMMC-7721 (hepatocellular carcinoma), HL-60 (myeloid leukemia), A-549 (lung cancer), and SW480 (colon cancer), using the MTT method in 96-well microplates [25]. Each tumor cell line was exposed to each test compound at various concentrations in triplicate for 48 h, with cisplatin (Sigma, USA) as a positive control. IC_{50} values were calculated following Reed and Muench's method [26].

3. Results and discussion

The MeOH extracts of *E. auritum* was partitioned between EtOAc and water. The EtOAc extract was fractionated by macroporous resin D101, and purified by repeated MPLC, Sephadex LH-20, and semi–preparative HPLC to yield three new pregnane glycosides, epigynosides E–G (**1**–**3**), and two known compounds. The aglycone of compounds **1**–**5** was identified with the same skeleton of (3β,17α,20*S*) pregn-5-ene-3,17,20-triol by detailed analysis of their NMR spectra data in the literature information. The structures of the new compounds were established mainly by spectroscopic methods including 1D and 2D NMR experiments (HSQC, COSY, HMBC, NOESY, and TOCSY) and mass spectrometry, whereas the known compounds were identified as epigynosides A (**4**) and C (**5**) by comparison with the reported data [17].

Compound **1** was obtained as colorless crystals. Its molecular formula was assigned as $C_{29}H_{48}O_7$ based on the sodium adduct (+)-HRESIMS ion at *m/z* 531.3298 $[M + Na]^+$ (calcd 531.3298) and ^{13}C NMR data. The IR spectrum featured a strong absorption at 3473 cm^{-1} due to the presence of hydroxy groups. The ^{1}H NMR spectrum of **1** showed two singlet methyl proton signals at δ_H 0.73 (3H, s, CH_3-18), 0.99 (3H, s, CH_3-19), two doublet methyl proton signals at 1.18 (3H, d, J = 6.3 Hz, CH_3-21) and 1.27 (3H, J = 6.6 Hz, CH_3-6′), two methoxy proton signals at δ_H 3.56 (3H, s, OCH_3-2′) and 3.40 (3H, s, OCH_3-3′) and one olefinic proton

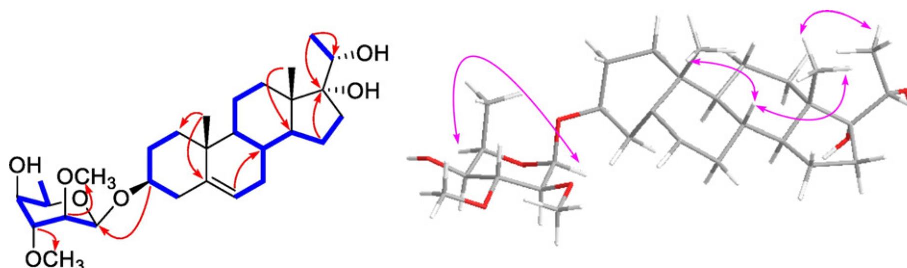

Fig. 2. Key HMBC ( ), ^{1}H—^{1}H COSY ( ) and ROESY ( ) correlations for **1**.

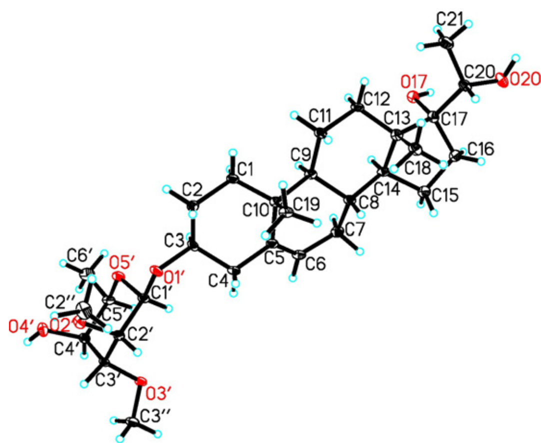

Fig. 3. X-ray ORTEP drawing of **1** with ellipsoids drawn at the 30% probability level.

at δ_H 5.35 (1H, m, H-6). In the ^{13}C NMR and DEPT spectrum of **1**, twenty nine carbon signals were observed. Among them, twenty one of which could be assigned to the C_{21}-steroidal aglycone moiety including three methyls (δ_C 13.9, 18.5, and 19.3), eight methylenes (δ_C 20.4, 23.5, 29.4, 31.0, 31.9, 37.2, 37.6, and 38.7), five methines (δ_C 31.7, 49.6, 51.3, 72.2, and 78.3), three quaternary carbons (δ_C 36.7, 45.6, and 85.7), as well as two olefinic carbons (δ_C 121.6 and 140.6). Detailed interpretation of the 2D NMR (^{1}H–^{1}H COSY, HSQC and HMBC) spectra of **1** allowed the assignment of all proton and carbon resonances (Table 1). The aglycone of **1** was identified as (3β,17α,20*S)* pregn-5-ene-3,17,20-triol by comparison of the NMR data of **1** with those published in the literature [27]. Besides, the downfield shift of C-1′ (δ_C 97.4), five oxymethines carbon resonances (δ_C 97.4, 77.8, 78.0, 68.6, and 70.6), an upfield methyl doublet (CH_3-6′, δ_C 16.3), and an HMBC correlation of H-1′ (δ_H 4.75) with C-5′ (δ_C 70.6) suggested the presence of a 6-dehydro monosaccharide [21]. Interpretation of ^{1}H–^{1}HCOSY correlations delineated a spin system from H-1′ to CH_3-6′ (Fig. 2). The HMBC correlations of δ_H 3.32 (H-2′) with δ_C 60.3 (q, OCH_3-2′), and of δ_H 3.65 (H-3′) with δ_C 57.9 (q, OCH_3-3′) indicated methylation of the C-2′ and C-3′ hydroxy groups. Chair conformations of the monosaccharide were deduced by the ROESY and coupling constants. The HMBC correlation from H-3 to C-1′ placed the monosaccharide at C-3 of the aglycone. The ROESY correlations of CH_3-19 with H-8 and H-4b, and of CH_3-18 with H-20 and CH_3-21 showed that these groups were β-oriented, while correlations of H-4a with H-3 established the α-orientation of H-3 (Fig. 2). A single-crystal X-ray diffraction confirmed the absolute configuration the structure of **1**, which were analyzed by X-ray diffraction with Cu Kα radiation (Fig. 3). Therefore, the structure of **1** was unequivocally assigned as (3β,17α,20*S*) pregn-5-ene-3,17,20-triol 2,3-dimethoxy-6-deoxy-β-D-idopyranose, and named as epigynoside E.

Compound **2** displayed a molecular ion at *m/z* 547.3245 $[M + Na]^+$ in the HRESIMS, indicating a molecular formula, $C_{29}H_{48}O_8$, differing from **1** by the addition of 16 mass units. In addition, the UV and IR spectra of these two compounds were also similar. The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) showed a close correspondence with those of **1**, except for the presence of an additional hydroxymethine (δ_C 77.4) in **2** instead of a methylene in **1**. The key HMBC correlations from H-15 (δ_H 4.34, dd, $J = 9.0, 2.0$ Hz) to C-13 (δ_C 47.0, s), C-16 (δ_C 35.3, t), and C-17 (δ_C 84.0, s) showed that **2** was the presence of hydroxyl of **1** at C-15. Biosynthetically, the methyl groups at C-18 and C-19 in pregnane were typical β-configuration [28]. The ROESY correlations of CH_3-18 (δ_H 0.77) with H-8 (δ_H 1.46), and H-15 (δ_H 4.34) placed these protons at the same side (β-orientation). Hence, the structure of **2** was established, and named epigynoside F.

The HRESIMS of compound **3** gave a molecular formula of $C_{28}H_{46}O_7$ by the molecular ion peak at *m/z* 517.3143 $[M + Na]^+$, indicating one CH_2 less than compound **1**. Comparison of the NMR spectroscopic data of **1** with those of **3** (Table 1) showed that they were the structural analogue, except for the presence of a 2-*O*-methyl-6-deoxy-β-D-idopyranose sugar unit in **3**. The observations of an anomeric proton signal at δ_H 4.90 (1H, br. s, H-1′), one doublet methyl proton signals at δ_H 1.31 (3H, d, $J = 6.6$ Hz, CH_3-6′) and one methoxy proton signals at δ_H 3.57 (3H, s, OCH_3-2′) in the ^{1}H NMR spectrum, and the characteristic carbon signals at δ_C 96.9, 79.8, 69.1, 71.6, 70.3, 16.3, and 60.2, confirmed the deduction in combination with TLC evidence of the acidic hydrolyzates of **3** and epigynoside A [17]. The location of the sugar moiety was constructed by the HMBC correlation from the anomeric proton at δ_H 4.90 to δ_C 78.2 (d, C-3). Thus, the structure **3** was established as shown, and given the trivial name epigynoside G.

The immunosuppressive activity of compounds **1**–**3** was evaluated on mitogen-stimulated mice splenocyte proliferation with dexamethasone as a positive control (Fig. 4). Compounds **1**–**3** significantly inhibited Con A-stimulated mice splenocyte proliferation in a dose-dependent manner, which was close to the efficacy of the dexamethasone at the concentration of 50 μM. However, there were no significant differences of compounds **2** and **3** in inhibitory effect on mitogen-stimulated splenocyte proliferation, at the concentration of 12.5 μM. In addition, all the isolated compounds were tested for their in vitro cytotoxicity against MCF-7 (breast cancer), SMMC-7721 (hepatocellular carcinoma), HL-60 (myeloid leukemia), A-549 (lung cancer), and SW480 (colon cancer) human cancer cell lines. Unfortunately, none of them showed cytotoxic activity ($IC_{50} > 10$ μM).

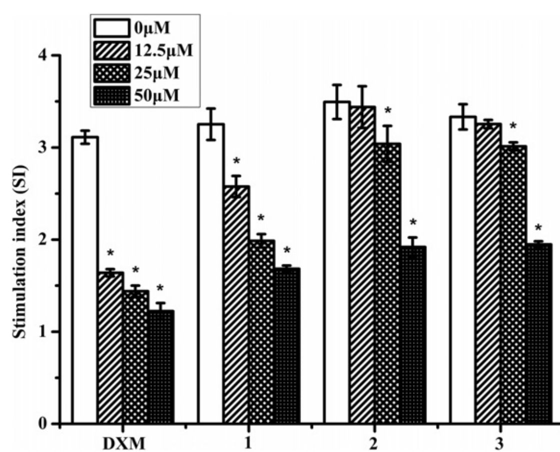

Fig. 4. Effect of compounds **1**–**3** on Con A-stimulated splenocyte proliferation in vitro. DXM was used as the positive control. The values were presented as mean ± SD of triplicate replicates. Statically significant difference was determined by ANOVA and Tukey test (*p < 0.05, compared with the control group).

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [grant numbers 31460083 and 31600274] and the Natural Science Foundation of Yunnan Province (KKSY201562017).

Appendix A. Supplementary data

1D and 2D NMR, IR, UV, HRESIMS spectra of **1**–**3**, and X-ray crystallographic data for **1** are available. This material is available free of charge via the Internet at http://dx.doi.org/10.1016/j.fitote.2017.02.011.

References

[1] X. Li, H. Sun, Y. Ye, F. Chen, Y. Pan, C-21 steroidal glycosides from the roots of *Cynanchum chekiangense* and their immunosuppressive activities, Steroids 71 (2006) 61–66.

[2] J.L. Ader, L. Rostaing, Cyclosporin nephrotoxicity: pathophysiology and comparison with FK-506, Curr. Opin. Nephrol. Hypertens. 7 (1998) 539–545.

[3] A.G. Jardine, B. Fellstrom, J.O. Logan, E. Cole, G. Nyberg, C. Gronhagen-Riska, S. Madsen, H.-H. Neumayer, B. Maes, P. Ambuhl, A.G. Olsson, T. Pedersen, H. Holdaas, Cardiovascular risk and renal transplantation: post hoc analyses of the Assessment of Lescol in Renal Transplantation (ALERT) Study, Am. J. Kidney Dis. 46 (2005) 529–536.

[4] J.W. Moses, M.B. Leon, J.J. Popma, P.J. Fitzgerald, D.R. Holmes, C. O'Shaughnessy, R.P. Caputo, D.J. Kereiakes, D.O. Williams, P.S. Teirstein, Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery, New Engl. J. Med. 349 (2003) 1315–1323.

[5] A. Mika, P. Stepnowski, Current methods of the analysis of immunosuppressive agents in clinical materials: a review, J. Pharm. Biomed. Anal. 127 (2016) 207–231.

[6] J.D. Ellis, D.A.H. Neil, N.G. Inston, E. Jenkinson, M.T. Drayson, P. Hampson, S.J. Shuttleworth, A.R. Ready, M. Cobbold, Inhibition of histone deacetylase 6 reveals a potent immunosuppressant effect in models of transplantation, Transplantation 100 (2016) 1667–1674.

[7] A.O. Ojo, A. Leichtman, R. Merion, Chronic renal failure after transplantation of a nonrenal organ, N. Engl. J. Med. 349 (2003) 2564–2565.

[8] P.F. Halloran, Immunosuppressive drugs for kidney transplantation, N. Engl. J. Med. 351 (2004) 2715–2729.

[9] Z.J. Zhang, M.L. Ding, L.J. Tao, M. Zhang, X.H. Xu, C.F. Zhang, Immunosuppressive C_{21} steroidal glycosides from the root of *Cynanchum atratum*, Fitoterapia 105 (2015) 194–201.

[10] J.L. Li, J. Zhou, Z.H. Chen, S.Y. Guo, C.Q. Li, W.M. Zhao, Bioactive C_{21} steroidal glycosides from the roots of *Cynanchum otophyllum* that suppress the seizure-like locomotor activity of zebrafish caused by pentylenetetrazole, J. Nat. Prod. 78 (2015) 1548–1555.

[11] J.Y. Zheng, Q. Wang, Z.X. Liu, C.X. Liu, Z.Y. Guo, H.Q. Zhang, H.B. He, X. Tu, K. Zou, Two new steroidal glycosides with unique structural feature of 14α-hydroxy-5β-steroids from *Reineckia carnea*, Fitoterapia 115 (2016) 19–23.

[12] X. Li, Y. Luo, G.P. Li, Q.X. Yang, Pregnane glycosides from the antidepressant active fraction of cultivated *Cynanchum otophyllum*, Fitoterapia 110 (2016) 96–102.

[13] J.L. Li, Z.B. Gao, W.M. Zhao, Identification and evaluation of antiepileptic activity of C_{21} steroidal glycosides from the roots of *Cynanchum wilfordii*, J. Nat. Prod. 79 (2016) 89–97.

[14] C.Z. Lai, H.B. Liu, J.X. Liu, Q. Ouyang, S.W. Pang, H. Zhou, H.Y. Tian, L. Liu, X.S. Yao, J.S. Tang, Hirundigenin type C_{21} steroidal glycosides from *Cynanchum stauntonii* and their anti-inflammatory activity, RSC Adv. 6 (2016) 59257–59268.

[15] K. Ounaissia, D. Pertuit, A.C. Mitaine-Offer, T. Miyamoto, C. Tanaka, S. Delemasure, P. Dutartre, D. Smati, M.A. Lacaille-Dubois, New pregnane and phenolic glycosides from *Solenostemma argel*, Fitoterapia 114 (2016) 98–104.

[16] D. Zhao, B. Feng, S. Chen, G. Chen, Z. Li, X. Lu, X. Sang, X. An, H. Wang, Y. Pei, C_{21} steroidal glycosides from the roots of *Cynanchum paniculatum*, Fitoterapia 113 (2016) 51–57.

[17] J.X. Cao, Y.J. Pan, Y. Lu, C. Wang, Q.T. Zheng, S.D. Luo, Three novel pregnane glycosides from *Epigynum auritum*, Tetrahedron 61 (2005) 6630–6633.

[18] J.X. Cao, G.F. Lai, Y.F. Wang, L.B. Yang, S.D. Luo, A new triterpenoid saponin and a new glycoside from *Epigynum auritum*, Chin. J. Chem. 21 (2003) 1665–1668.

[19] Q. Jin, Q. Mu, Constituents from *Epigynum auritum*, Nat. Prod. Res. Dev. 15-18 (1990).

[20] Q.D. Jin, Q.Z. Mu, Glycosidal constituents from *Epigynum auritum*, Acta Pharm. Sin. 26 (1991) 841–845.

[21] F. Gao, Y.C. Yao, Z. Wan, S.B. Cai, Jian Fan, T.R. Zhao, J.X. Cao, G.G. Cheng, Secopregnane glycosides from the stems of *Epigynum auritum*, Nat. Prod. Res. 31 (2016) 1102–1105.

[22] G.G. Cheng, D. Li, B. Hou, X.N. Li, L. Liu, Y.Y. Chen, P.K. Lunga, A. Khan, Y.P. Liu, Z.L. Zuo, Melokhanines A–J, bioactive monoterpenoid indole alkaloids with diverse skeletons from *Melodinus khasianus*, J. Nat. Prod. 79 (2016) 2158–2166.

[23] G.G. Cheng, H.Y. Zhao, L. Liu, Y.L. Zhao, C.W. Song, J. Gu, W.B. Sun, Y.P. Liu, X.D. Luo, Non-alkaloid constituents of *Vinca major*, Chin. J. Nat. Med. 14 (2016) 56–60.

[24] X. Li, B. Xuan, Q. Shou, Z. Shen, New flavonoids from *Campylotropis hirtella* with immunosuppressive activity, Fitoterapia 95 (2014) 220–228.

[25] G.G. Cheng, Y.L. Zhao, Y. Zhang, P.K. Lunga, D.B. Hu, Y. Li, J. Gu, C.W. Song, W.B. Sun, Y.P. Liu, X.D. Luo, Indole alkaloids from cultivated *Vinca major*, Tetrahedron 70 (2014) 8723–8729.

[26] M.H. Reed L, A simple method of estimating fifty percent endpoint, Am. J. Hyg. 27 (1938) 493–497.

[27] H. Itokawa, J. Xu, K. Takeya, Studies on chemical constituents of the antitumor fraction from *Periploca sepium*. IV. Structures of new pregnane glycosides, periplocosides D, E, L, and M, Chem. Pharm. Bull. 36 (1988) 2084–2089.

[28] C.W. Song, P.K. Lunga, X.J. Qin, G.G. Cheng, J.L. Gu, Y.P. Liu, X.D. Luo, New antimicrobial pregnane glycosides from the stem of *Ecdysanthera rosea*, Fitoterapia 99 (2014) 267–275.