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Triterpenoids of *Chrysosplenium carnosum*

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ABSTRACT

A phytochemical study of the ethanolic extract of *Chrysosplenium carnosum* Hook. f. et Thoms. led to the isolation of two new oleanane-type triterpenoids, 6β-hydroxy-3-oxoolean-12-en-27-oic acid (**1**) and 3β, 21α-dihydroxyolean-12-en-27-oic acid (**2**), along with fourteen known compounds (**3**–**16**), all of which were isolated from this plant for the first time. The structures of these compounds were established on the basis of spectroscopic methods. Compounds **1**–**4** were evaluated for their *in vitro* anti-tumor activities on B16F10, SP2/0 and Hep-G2 cells lines. Compounds **1**, **3** and **4** exhibited strong inhibitory activity against B16F10 and SP2/0 cells' growth, compared with moderate cytotoxic activity against Hep-G2 cells. However, compound **2** showed to be inactive against these tumor cells.

1. Introduction

The genus *Chrysosplenium* (Saxifragaceae) consists of 65 species of herbaceous plants mainly distributed in Asia temperate zone, and 35 of which were discovered in China [1]. Referring to the domestic reports, many plants of this genus were widely applied in folk as medicinal plants, such as: *Chrysosplenium forrestii*, *Chrysosplenium nudicaule*, *Chrysosplenium absconditicapsulum*, *Chrysosplenium griffithii*, *Chrysosplenium alternifolium*, *Chrysosplenium delavayi*, *Chrysosplenium axillare*, *Chrysosplenium macrophyllum*, *Chrysosplenium sinicum*, *Chrysosplenium hydrocotylifolium*, *Chrysosplenium grayanum*, *Chrysosplenium uniflorum* and *Chrysosplenium nepalense* and so on. Most of them were described to possess antimicrobial [2], anti-tumor [3,4] as well as anti-virus activities [5], and consequently were extensively used to relieve heat and toxic activities and for the treatment of hepatopathy.

According to the previous literature on the *Chrysosplenium* plants, highly O-methylated flavonoids proved to be the characteristic constituents [6], which were found responsible for the biological activities of the plants. Moreover, other chemical components such as terpenoids, sterides, volatile oils and monosaccharide [6] were discovered. Two C-27 highly oxygenated olean triterpenoids isolated from *C. grayanum* [7] and *C. griffithii* [8], plants of the genus *Chrysosplenium*, were also tested to possess strong inhibitory activities against tumor cells' growth. While, C-27 highly oxygenated pentacyclic triterpenoids which were found rare in nature, seemly widely distributed in Saxifragaceae plants [9–11].

Chrysosplenium carnosum Hook. f. et Thoms., one of the species of the genus *Chrysosplenium* (Saxifragaceae), is a perennial herb distributed abundantly in Western Sichuan and east of Tibet Autonomous Region of China [12], and commonly used for relieving heat and pain in Tibetan medicine. However, there are few reports on phytochemical investigations of this plant up to now. In order to find out the chemical composition and medicinal value, the ethanol extract of *C. carnosum* was investigated. Sixteen compounds were isolated and identified as follows: 6β-hydroxy-3-oxoolean-12-en-27-oic acid (**1**), 3β, 21α-dihydroxyolean-12-en-27-oic acid (**2**), 3β, 6β-dihydroxy-olean-12-en-27-oic acid (**3**) [13], 3β, 24β-dihydroxyolean-12-en-27-oic acid (**4**) [14], 3-oxoolean-12-en-27-oic acid (**5**) [15], 3β-hydroxy-olean-12-en-27-oic acid (**6**) [7], chrysosplenol B (**7**) [16], chrysosplenol C (**8**) [17], chrysosplenol D (**9**) [16], penduletin (**10**) [18], oxyayanin B (**11**) [8], chrysosplenoside B (**12**) [16], 5, 6, 4′-trihydroxy-3, 7, 3′-trimethoxy-flavone-6-O-β-D-glucopyranoside (**13**) [19], β-sitosterol (**14**), β-daucosterol (**15**), and adenosine (**16**) [8] (Fig. 1). Among the isolates, terpenoids 1 and 2 were

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discovered as new natural compounds. The structures were determined mainly on the basis of various spectroscopic evidence including 1D and 2D NMR, HRESIMS and IR data. The anti-tumor activities of compounds **1**–**4** were evaluated *in vitro*, compounds **1**, **3** and **4** of them showed strong inhibitory activities against B16F10 and SP2/0 cells' growth with an IC_{50} ranging from 15.7 to 18.3 μM and from 13.1 to 31.5 μM, respectively, and moderate activities on Hep-G2 cells with an IC_{50} value ranging from 47.3 to 61.0 μM. However, compound **2** showed to be inactive against these tumor cells. Herein, the isolation and structural elucidation of the two new triterpenoids will be described.

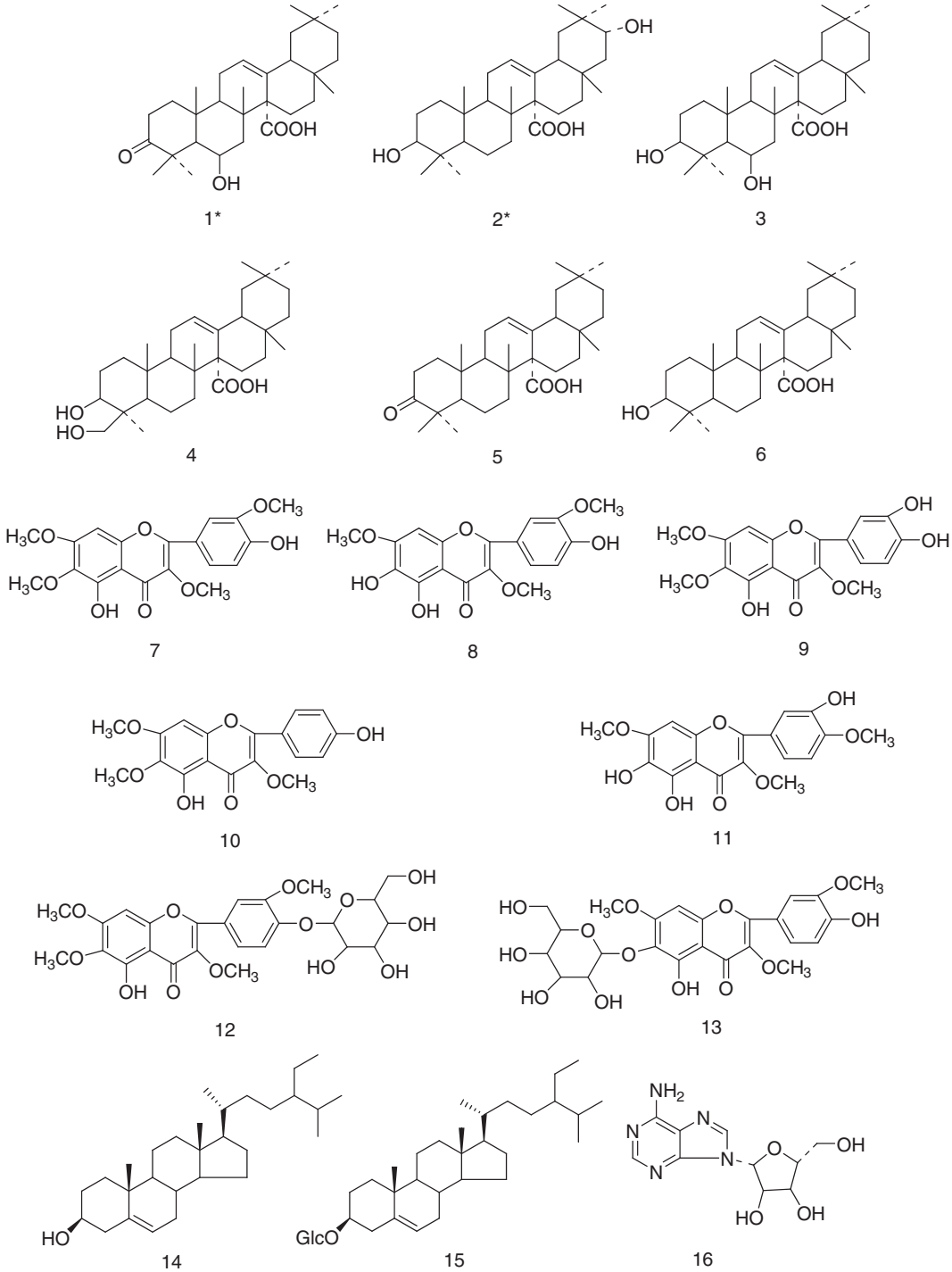

Fig. 1. Chemical structures of compounds **1**–**16**.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a WZZ-2B spectropolarimeter. IR spectra were recorded on a NICOLET IR200 FT-IR spectrophotometer. NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). HRESIMS was carried out on an Agilent Technologies 6224 TOF LC–MS apparatus. Column chromatography (CC) was performed with silica gel (200–300 mesh, Qingdao Marine Chemical, Inc.). MCI GEL CHP20p (75–150 μ, Mitsubishi Kasei Corporation.), Sephdex LH-20 (20-100 μ, Pharmacia) and Macroporous resin AB~8 (Anhui Sanxing Resin Technology Co., Ltd) were also used for CC. TLC was carried out on silica gel GF_{254} plates (10–40 μm; Qingdao Marine Chemical, Inc.). Petroleum ether, ethyl acetate and other reagents were purchased from Nanjing Wanqing reagent, Inc. Spots were observed by UV light as well as by spraying with 10% H_2SO_4–EtOH followed by heating. Tumor cells were incubated in a HF-212UV CO_2 incubator and observed on an OLYMPUS CKX41 inverted microscope. Optical density (OD) values were read on a BIO-RAD Model 680 microplate reader.

2.2. Plant material

The dried whole plant of *C. carnosum* was purchased in July 2010 from Tibetan medicine market in Lhasa, Tibet Autonomous Region of China. It was identified by Prof. Xue-feng Lu of Northweast Institute of Plateau Biology, Chinese Academy of Sciences, Xining, China. A voucher specimen (no. 10-01-06) was deposited at the laboratory of Zhixin Liao, Southeast University, Nanjing, China.

2.3. Extraction and Isolation

The dried whole plant (5.00 kg) of *C. carnosum* was percolated with 90% ethanol (4×7 days) at room temperature. The supernatants were combined and concentrated in vacuum to give a concrete (763 g). The crude extract was then suspended in H_2O (1.50 L), extracted successively with ethyl acetate (5×800 mL), and n-BuOH (4×800 mL). After the removal of the solvents, two residues were obtained respectively.

The ethyl acetate fraction (105 g) was subjected to silica gel column chromatography (with 1.00 kg silica gel dissolved in 2700 mL petroleum ether) eluted with a gradient system of petroleum ether–ethyl acetate (100:0 to 0:100) to yield 4 fractions (Fr.1–Fr.4), which were combined according to TLC detection. Fr.1 (13.0 g, petroleum ether–ethyl acetate 100:1–10:1) was abandoned without further separation because it is mainly comprised of volatile oil components of low polarity. The extractums of Fr.2 (24.1 g), Fr.3 (34.8 g) and Fr.4 (6.20 g) were firstly purified over MCI GEL eluted with 80% ethanol to remove the pigments. The remaining materials were evaporated respectively for further isolation. Fr.2 (petroleum ether–ethyl acetate 9:1–4:1) was subjected to separation repeated by silica gel column chromatography (with 500 g silica gel dissolved in 1200 mL petroleum ether) eluted with petroleum ether–ethyl acetate (9:1–8:1) to yield **14** (881 mg) and **5** (540 mg), followed by **3** (180 mg) and **6** (12.3 g) when eluted with petroleum ether–ethyl acetate (5:1). Fr.3 (petroleum ether–ethyl acetate 3:1–1:1) was subjected to CC over silica gel (with 750 g silica gel dissolved in 1800 mL petroleum ether) eluted with petroleum ether–ethyl acetate (2:1–1.5:1) to give **7** (12.4 g) and **8** (6.97 g). A repeated separation of the residue over silica gel column leads to the isolation of **1** (684 mg), followed by **10** (5.00 mg), **2** (22.0 mg), **4** (544 mg) and **9** (898 mg), flavonoids of which were finally purified over Sephadex LH-20 eluted with 70% ethanol. Fr.4 (petroleum ether–ethyl acetate 1:1.5–1:4) was chromatographed over silica gel (with 280 g silica gel dissolved in 600 mL petroleum ether) with petroleum ether–ethyl acetate (1:2–1:3) to afford **15** (163 mg).

The n-BuOH extract fraction (102 g) was purified over Macroporous resin AB~8 to remove the water-soluble substances such as sugars, proteins and so on. The portion collected was condensed to dryness. The residue (36.0 g) was fractionated by CC over silica gel (with 700 g silica gel dissolved in 1800 mL chloroform) with the gradient system of increasing polarity (chloroform–methanol 50:1–1:1). Two fractions (Fr.1–Fr.2) were obtained. Compound **11** (3.00 mg) was isolated from Fr.1 (8.15 g) when eluted with chloroform–methanol (12:1) by CC over silica gel. Compounds **12** (1.50 g), **16** (322 mg) and **13** (7.21 g) were precipitated successively from Fr.2 (20.3 g) with increasing polarity of chloroform–methanol from 8:1 to 5:1.

6β-hydroxy-3-oxoolean-12-en-27-oic acid (**1**): white powder, mp: 224–225 °C, $[\alpha]^{20}_D = +14.7$ (c = 0.00061, MeOH), IR (KBr): 3465, 3330, 2947, 2926, 1692 cm^{-1}. ^{1}H and ^{13}C NMR (DMSO) (see Table 1). HR-ESI-MS: 471.3470 ($[M+H]^+$, $C_{30}H_{47}O_4^+$; calc. 471.3474).

3β, 21α-dihydroxyolean-12-en-27-oic acid (2): white amorphous powder, mp: 241–242 °C, $[\alpha]^{20}_D = +10.3$ (c = 0.00025, MeOH), IR (KBr): 3523, 3468, 3432, 2966, 2946, 1683 cm^{-1}. ^{1}H and ^{13}C NMR (DMSO) (see Table 2). HR-ESI-MS: 471.3479 ($[M-H]^-$, $C_{30}H_{47}O_4^-$; calc. 471.3474).

2.4. Anti-tumor activity experiments

The anti-tumor activities of compounds **1**–**4** were evaluated *in vitro* against the cancer cell lines B16F10, SP2/0 and Hep-G2 by MTT assay method. The cell suspension was distributed into 96-well cell culture plate and cultured at 36–37 °C, with 5% CO_2 in incubator for 24 h. Each sample was dissolved with limited DMSO and diluted to five different concentrations with culture medium, then added to the corresponding well. The blank controls were added with the same amount of DMSO and incubated under the same conditions. After 48 h cultivation, MTT was added to each well for another 4 h cultivation. Finally, the supernatant was discarded and limited DMSO was added to each well to dissolve the blue-violet crystals completely, then optical density (OD) values were read on the microplate reader. Origin was used to figure out the median inhibitory rate IC_{50}, and the results are presented in Table 3.

3. Results and discussion

The extract from whole plant of *C. carnosum* was subjected to repeated column chromatography for a systematical phytochemical investigation and compounds **1**–**16** were obtained. The two new compounds were identified by means

Table 1
^{1}H NMR, ^{13}C NMR and HSQC spectral data of 1.

Position	^{1}H NMR	^{13}C NMR	HSQC
1a	1.15 (1H, m)	41.3	1.15 (41.3)
1b	1.85 (1H, m)		1.85 (41.3)
2a	2.12 (1H, m)	33.8	2.12 (33.8)
2b	2.76 (1H, m)		2.76 (33.8)
3		215.3	
4		48.0	
5	0.98 (1H, m)	55.7	0.98 (55.7)
6a	4.23 (1H, s)	66.4	4.23 (66.4)
6-OH	4.40 (OH, d, 3.45 Hz)		
7a	1.23 (1H, m)	42.9	1.23 (42.9)
7b	1.77 (1H, m)		1.77 (42.9)
8		38.4	
9	2.26 (1H, m,)	46.0	2.26 (46.0)
10		36.0	
11a	1.94 (1H, m)	22.5	1.94 (22.5)
11b	2.01 (1H, m)		2.01 (22.5)
12	5.56 (1H, t, 2.25 Hz)	124.7	5.56 (124.7)
13		136.9	
14		55.5	
15a	1.66 (1H, m)	21.7	1.66 (21.7)
15b	1.88 (1H, m)		1.88 (21.7)
16a	0.76 (1H, m)	27.3	0.76 (27.3)
16b	1.96 (1H, m)		1.96 (27.3)
17		32.5	
18	1.94 (1H, m)	48.6	1.94 (48.6)
19a	0.93 (1H, m)	43.4	0.93 (43.4)
19b	1.31 (1H, m)		1.31 (43.4)
20		30.6	
21a	1.06 (1H, m)	34.0	1.06 (34.0)
21b	1.22 (1H, m)		1.22 (34.0)
22a	1.16 (1H, m)	36.1	1.16 (36.1)
22b	1.37 (1H, m)		1.37 (36.1)
23	1.02 (3H, s)	25.5	1.02 (25.5)
24	1.31 (3H, s)	23.1	1.31 (23.1)
25	1.44 (3H, s)	16.2	1.44 (16.2)
26	1.23 (3H, s)	19.5	1.23 (19.5)
27		176.6	
28	0.83 (3H, s)	28.0	0.83 (28.0)
29	0.80 (3H, s)	33.2	0.80 (33.2)
30	0.83 (3H, s)	23.4	0.83 (23.4)

^{1}H, ^{13}C NMR Data and HSQC of 1 (at 500 and 125 MHz, in DMSO at 30 °C; δ in ppm).

Table 2
^{1}H NMR, ^{13}C NMR and HSQC spectral data of 2.

Position	^{1}H NMR	^{13}C NMR	HSQC
1a	0.83 (1H, m)	38.3	0.83 (38.3)
1b	1.57 (1H, m)		1.57 (38.3)
2a	1.45 (1H, m)	26.9	1.45 (26.9)
2b	1.46 (1H, m)		1.46 (26.9)
3	2.95 (1H, m)	76.9	2.95 (76.9)
4		38.4	
5	0.56 (1H, m)	55.0	0.56 (55.0)
6a	1.25 (1H, m)	17.9	1.25 (17.9)
6b	1.44 (1H, m)		1.44 (17.9)
7a	1.13 (1H, m)	36.0	1.13 (36.0)
7b	1.54 (1H, m)		1.54 (36.0)
8		36.5	
9	2.08 (1H, m)	46.4	2.08 (46.4)
10		38.9	
11a	1.81 (1H, m)	22.4	1.81 (22.4)
11b	1.82 (1H, m)		1.82 (22.4)
12	5.52 (1H, t, 3.65 Hz)	125.0	5.52 (125.0)
13		136.6	
14		55.1	
15a	1.59 (1H, m)	21.8	1.59 (21.8)
15b	1.86 (1H, m)		1.86 (21.8)
16a	0.91 (1H, m)	28.8	0.91 (28.8)
16b	1.90 (1H, m)		1.90 (28.8)
17		34.6	
18	1.93 (1H, m)	48.1	1.93 (48.1)
19a	0.99 (1H, m)	44.0	0.99 (44.0)
19b	1.35 (1H, m)		1.35 (44.0)
20		35.9	
21	3.21 (1H, m)	71.3	3.21 (71.3)
22a	1.22 (1H, m)	44.9	1.22 (44.9)
22b	1.29 (1H, m)		1.29 (44.9)
23	0.86 (3H, s)	28.2	0.86 (28.2)
24	0.66 (3H, s)	16.0	0.66 (16.0)
25	0.88 (3H, s)	16.0	0.88 (16.0)
26	0.93 (3H, s)	17.8	0.93 (17.8)
27		176.4	
28	0.81 (3H, s)	28.0	0.81 (28.0)
29	0.81 (3H, s)	29.5	0.81 (29.5)
30	0.73 (3H, s)	17.2	0.73 (17.2)

^{1}H, ^{13}C NMR Data and HSQC of 2 (at 500 and 125 MHz, in DMSO at 30 °C; δ in ppm).

of IR, HRESIMS and NMR spectroscopy (^{1}H NMR, ^{13}C NMR, HSQC, HMBC and ROESY).

Compound **1**, obtained as a white powder, mp: 224–225 °C, $[\alpha]^{20}_{D} = +14.7$ (c = 0.00061, MeOH). The molecular formula was deduced to be $C_{30}H_{46}O_4$ based on the ^{1}H, ^{13}C NMR data and the molecular ion peak at m/z 471.3470 $[M+H]^{+}$ (calc. 471.3474) in the HRESIMS. The assignment was confirmed with the help of 2D NMR (HSQC, HMBC and ROESY) spectrum.

There are seven methyl groups (δ 1.44, 1.31, 1.23, 1.02, 0.83×2 and 0.80) in the ^{1}H NMR spectrum and 30 carbon signals in ^{13}C NMR spectrum, which suggested that compound **1** was a triterpenoid. An olefinic proton at δ 5.56 (d, J = 2.25 Hz, 1H) with a pair of olefinic carbon signals at δ 124.7 and 136.9 indicated a double bond in the structure. The proton signal at δ 12.20 (s, 1H) together with the carbon signal at δ176.6 revealed the presence of a carboxylic group. These data revealed that it was a triterpenoid with a skeleton of olean-12-en-27-oic acid [20]. Moreover, an oxygen-bearing methine proton at δ 4.23 (s, 1H) and hydroxyl proton at δ 4.40 (d, J = 3.45 Hz 1H), correlated with the signal at δ 66.4 confirmed that a hydroxyl was attached to the structure. Another carbon signal at δ 215.3 testified the existence of a carbonyl group. The proton and carbon signals in the HSQC spectrum of **1** were shown in Table 1.

In the HMBC spectrum (Fig. 2), correlations of C-12 with H-9 and H-18, C-13 with H-11, H-15 and H-19 proved a double bond across C-12/C-13. The correlations between C-27 and H-15 revealed a carboxylic group located at C-14. In addition, the position of the carbonyl group was confirmed to be C-3 by observation of HMBC correlations between C-3 and

Table 3
The median inhibitory rate (IC_{50}) of compounds **1**, **3** and **4**.

Sample	Median inhibitory rate(IC_{50}) (μM)		
	B16F10	SP2/0	Hep-G2
1	15.9	17.0	47.3
3	15.7	31.5	61.0
4	18.3	13.1	59.8

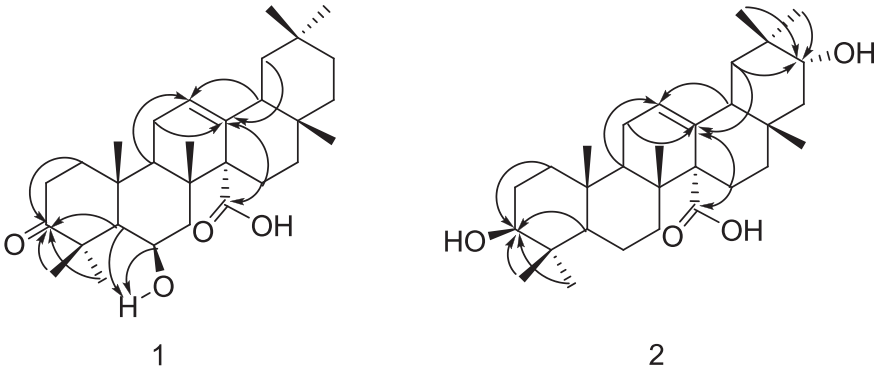

Fig. 2. Key HMBC (H→C) correlations of compounds **1**–**2**.

H-1, H-5, H-23 along with H-24. The hydroxyl function was located at C-6 according to the HMBC cross-peaks of the hydroxyl proton (δ 4.40) with C-5 and C-6.

The relative configuration of **1** was determined by a ROESY experiment. In the ROESY spectrum (Fig. 3), H-6 was coupled with H-5 and H-23, which suggested that H-6 was α-axial [9], consequently, the hydroxyl group at C-6 was β-equatorial. Therefore, compound **1** was identified as 6β-hydroxy-3-oxoolean-12-en-27-oic acid.

Compound **2**, obtained as a white amorphous powder, mp: 241–242 °C, $[\alpha]^{20}_{D} = +10.3$ (c = 0.00025, MeOH). The molecular formula was determined as $C_{30}H_{48}O_4$ based on the ^1H, ^{13}C NMR data and the molecular ion peak at m/z 471.3479 $[M-H]^-$ (calc. 471.3474) in the HRESIMS.

Similar to compound **1**, compound **2** was also a triterpenoid with a skeleton of olean-12-en-27-oic acid based on the signals as follows: 30 carbon signals with seven methyl groups (δ 0.93, 0.88, 0.86, 0.81 × 2, 0.73, 0.66); a pair of olefinic carbon signals (δ 125.0 and 136.6) with the corresponding olefinic proton signal at δ 5.52, moreover, a proton signal at δ 12.18 together with the carbon signal at δ 176.4, all of which helped to testify the structure. In addition to the characteristic functional group, the structure of compound **2** was found to possess two hydroxyl groups, which was based on the following data. There were two oxygen bearing methine protons at δ 2.95 and δ 3.21 in the ^1H NMR spectrum, which was correlated with the carbon signals at δ 76.9 and δ 71.3, respectively. The proton and carbon signals in the HSQC spectrum of **2** were shown in Table 2.

In the HMBC spectrum (Fig. 2), the correlations of C-12 with H-9 and H-18, C-13 with H-11, H-15 and H-19, C-27 with H-15 demonstrated the skeleton. The correlations between C-3 (δ 76.9) and H-1, H-5, H-23, along with H-24 confirmed that this hydroxyl group was attached to C-3. Another hydroxyl function was located at C-21 according to the correlations of C-21 (δ 71.3) with H-19, H-29 and H-30.

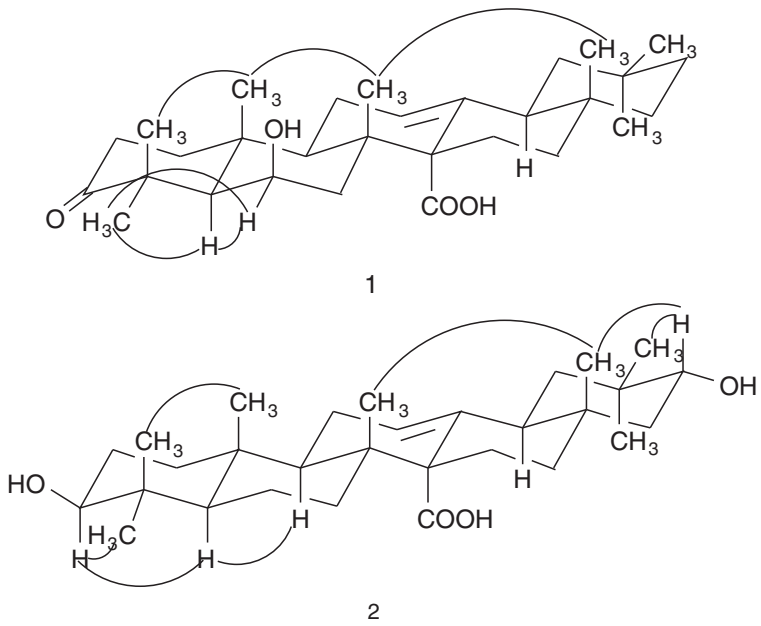

Fig. 3. Key ROESY correlations of compounds **1**–**2**.

The relative configuration of **2** was established on the basis of the ROESY spectrum (Fig. 3), H-3 was coupled with H-5 and H-23 (α-axial), which suggested that H-3 was α-axial, and consequently the hydroxyl group at C-3 was β-equatorial. A cross-peak between H-21 and H-28 (β-axial) proved the β-axial orientation of H-21 and consequently the α-equatorial orientation of hydroxyl group at C-21. On the basis of the above analysis, compound **2** was identified as 3β, 21α-dihydroxyolean-12-en-27-oic acid.

Compounds **3**–**16** were identified by comparing their spectrum data with literature values. In the anti-tumor test, compounds **1**, **3** and **4** showed strong activity against B16F10 and SP2/0 cells (with an IC_{50} value ranging from 13.1 to 31.5 μM) and moderate activities on Hep-G2 cells (with an IC_{50} ranging from 47.3 to 61.0 μM), while compound **2** was inactive. The other two triterpenoids (compounds **5** and **6**) were reported to exhibit considerable cytotoxicities against the K562 and HL-60 cells (with IC_{50} values ranging from 12.5 to 15.3 μM and from 12.3 to 13.3 μM, respectively), while oleanolic acid with a carboxyl group at C-28 was inactive ($IC_{50} > 100$ μM) [11]. Moreover, compound **6** was found to strongly inhibit the growth of various cancer cells such as stomach cancers, lung cancers, osteosarcomas and so on [7].

The research led to the discovery of a series of triterpenoids with carboxylic group on C-27 which were rarely found in natural sources. Combined with the previous works of literatures, the markable compounds may be used as a strong evidence in chemotaxonomy of the genus *Chrysosplenium*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2013.01.006.

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