

Cite this: *RSC Adv.*, 2017, 7, 38557

Cimifrigines A–G, cytotoxic triterpenes with an oxime group from the flowers of *Cimicifuga frigida*†

Yin Nian, Hui Yan, Xiao-Nian Li, Lin Zhou and Ming-Hua Qiu*

Seven new dahurinol-type triterpene derivatives, including three aglycones, cimifrigines A–C (1–3), and four glycosides, cimifrigines D–G (4–7), were purified from the flowers of *Cimicifuga frigida*. These triterpenoids are characterized by an oxime group at C-15. Spectroscopic analyses and X-ray crystallography were used to determine the new structures. In the *in vitro* cytotoxicity screening, glycosides (4–7) exhibited more noticeable activities than the aglycones (1–3) against human HL-60, SMMC-7721, A549, MCF-7, and SW-480 cell lines. Interestingly, compounds 5 and 7, bearing a 2'-O-acetyl moiety on the sugar unit, showed comparable cytotoxicities to the positive control, cisplatin (IC₅₀: 0.5 to 5.4 μM). Whereas, analogues 4 and 6, without the 2'-O-acetyl group, indicated weaker activities with IC₅₀ values ranging from 8.9 to 14.3 μM.

Received 1st July 2017
Accepted 25th July 2017

DOI: 10.1039/c7ra07275a

rsc.li/rsc-advances

Introduction

Plants of the *Cimicifuga* genus (Ranunculaceae) are time-honored herbal medicines worldwide.^{1–3} *C. racemosa*, namely black cohosh, is a popular food supplement in Europe and the United States for relief of menopausal disorders.^{4,5} In East Asia, several *Cimicifuga* spp., for instance, *C. foetida*, *C. dahurica*, *C. heracleifolia*, *C. simplex*, and *C. japonica*, have been used to alleviate fever, pain, and inflammation since ancient times.^{6,7}

Extensive efforts have been attracted to investigate chemical constituents of this genus. 9,19-Cycloartane triterpenoids (CTs) is the major chemical component of *Cimicifuga* spp. and more than 300 CTs^{2,3,7–27} have been reported (among them, our research group reported over 100 ones^{2,3,8–24}). Pharmaceutical studies revealed these CTs possessed various bioactivities, for instance, antiosteoporotic,²⁸ cytotoxicity,^{2,14} anti-AIDS,²⁹ anti-Alzheimer,³⁰ and immunosuppression.³¹ Nevertheless, aforementioned CTs were mainly from the rhizomes or roots of *C. racemosa*, *C. foetida*, *C. dahurica*, *C. heracleifolia*, and *C. simplex*.^{7–27} Therefore, we carried out successive investigations on the roots, aerial parts, and fruits of *C. yunnanensis*, an indigenous species in southwest China.^{2,3,15} Consequently, a number of bioactive CTs were discovered. Among them, cimiyunnin A, with an unprecedented carbon skeleton from the

fruits of this plant, was considered as an anti-angiogenic leading structure. It showed comparable *in vitro* and *ex vivo* activities to those of a first-line clinical medicine, sunitinib.³ Therefore, aforementioned studies exemplified that expansion of research objects in genus *Cimicifuga* may led to discovery of novel active CTs.

The taxonomic treatment of the Sino-Himalayan species *C. frigida* has a very long and controversial history.^{32,33} However, recent cytological evidence showed that this plant is the only tetraploid ($2n = 32$) currently known in the genus, which together with morphological features confirmed its independent species status.³⁴ Thus, it is of interest to explore whether there are novel bioactive compounds in this plant. In the present study, we initially carried out a study on the flowers of *C. frigida* from Litang County, Sichuan Province. Consequently, seven new 9,19-cycloartane triterpene derivatives (Fig. 1), cimifrigines A–G (1–7), were isolated. Their structures were elucidated by the help of MS, NMR, and single-crystal X-ray diffraction techniques. The unique characteristic of these analogues is that containing an oxime group at C-15 based on the dahurinol-skeleton. In addition, the isolated compounds showed potent to weak cytotoxicities against human HL-60, SMMC-7721, A549, MCF-7, and SW-480 cell lines.

Results and discussion

Cimifrigine A (1), purified as colorless crystals. The HREIMS ion peak at m/z 559.3864 [M]⁺ (calcd 559.3873) determined its molecular formula as C₃₃H₅₃NO₆, requiring 8 double-bond equivalents. The ¹H NMR spectrum (Table 1) showed characteristic cyclopropane methylene signals at δ_{H} 0.29 and 0.52 (each 1H, brs), a secondary methyl signal at δ_{H} 0.88 (d, $J = 5.9$

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. E-mail: mhchiu@mail.kib.ac.cn

† Electronic supplementary information (ESI) available: 1D and 2D NMR spectra, HRESIMS spectra of new compounds 1–7, and X-ray crystallographic data of 1. CCDC 1545247. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c7ra07275a



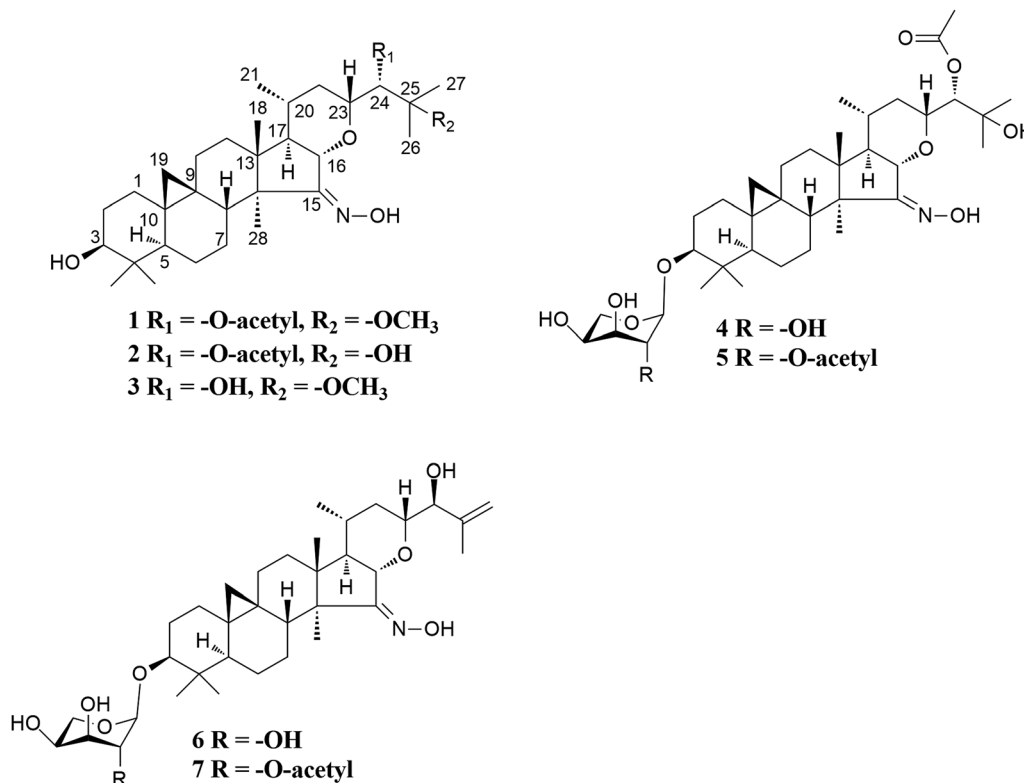


Fig. 1 Structures of compounds 1–7.

Hz), five tertiary methyl groups at δ_H 1.04–1.59, and an active hydrogen signal at δ_H 12.32, respectively. In the ^{13}C NMR spectrum (Table 2), an olefinic (or a carbonyl) carbon signal at δ_C 164.34 (C-15), and five oxygenated carbons at δ_C 78.01 (C-3, d), 80.55 (C-16, d), 78.76 (C-23, d), 77.13 (C-24, d), and 77.53 (C-25, s) were also observed. Aforementioned data suggested that **1** was a highly oxygen-bearing 9,19-cycloartane triterpenoid with a six-ring carbon skeleton.

Study of the 1H - 1H COSY (Fig. 2) spectrum of **1** revealed the existence of partial structures C1/C-2/C3 ($-CH_2-CH_2-CH-$), C-5/C-6/C-7/C8 ($-CHCH_2CH_2CH-$), C-11/C-12 ($-CH_2CH_2-$), and C-16/C-17/C-20(C-21)/C-22/C-23 ($-CHCHCH-(CH_3)CH_2CH-$), which consistent with typical rings A–E of a dahurinol-type triterpene.^{11,13,35,36} Based on HMBC correlation from H-16 at δ_H 4.24 to the oxygenated methine at δ_C 78.76 (C-23), and the existence of the spin system C-16/C-17/C-20(C-21)/C-22/C-23, the ring E was established as shown. 1H - 1H COSY association between H-23 (δ_H 4.11) and H-24 (δ_H 5.34) indicated the linkage of C-23 and C-24. In addition, the connection of C-24 and C-25 was elucidated from the HMBC correlation of H-24 (δ_H 5.34) to quaternary carbon resonance at δ_C 77.53 (C-25). Similarly, an acetoxy group was attached to C-24, and CH_3 -26 (δ_H 1.59) and CH_3 -27 (δ_H 1.56), and the methoxy group at δ_H 3.24 were linked to C-25 based on the HMBC correlations (Fig. 2). Thus, the side chain of **1** was constructed. Further analyses of HMBC cross-peaks from H-16 (δ_H 4.24) to C-14 (δ_C 51.67), and the olefinic (or carbonyl) carbon signal (C-15) at δ_C 164.34; H-17 (δ_H 1.60) to C-13 (δ_C 42.50) and C-14 (δ_C 51.67); CH_3 -18 (δ_H 1.07) to C-13 (δ_C 42.50), C-14 (δ_C 51.67) and C-17 (δ_C 54.60); CH_3 -28 (δ_H 1.14) to C-

14 (δ_C 51.67) and C-15 (δ_C 164.34), suggested the presence of a five-membered ring D with an oxime unit at C-15 (Fig. 2). In the HMBC spectrum, the active hydrogen (δ_H 12.32) coupled with C-15 (δ_C 164.34) further supported this elucidation. Thus, the planar structure of **1** was established as shown (Fig. 2).

The orientations of core structure of **1** was established by the ROESY correlations (Fig. 2) between H-5 (biogenetically α -oriented) and H-3, Me-28 (biogenetically α -oriented) and H-17, Me-18 (biogenetically β -oriented) and H-16, Me-18 and H-20, and H-23 and H-16. In addition, due to its similar coupling constant of H-24 (brs) as that of isodahurinyl-type molecules (<2 Hz), the configuration of C-24 of **1** was deduced as *S* (the coupling constants of H-24 of dahurinyl-type compounds is around 6–9 Hz).^{11,13,35,36} Finally, X-ray diffraction analysis (Fig. 3) allowed to confirm the oxime group at C-15, the relative configurations, and the stereochemistry of *S* at C-24 of **1** due to the Hooft parameter 0.13(8) for 1536 Bijvoet pairs.³⁷ Therefore, the structure of **1** was constructed as shown.

The molecular formulas of cimifrigines B (**2**) and C (**3**) were determined as $C_{33}H_{53}NO_6$ and $C_{31}H_{51}NO_5$ by the HREIMS ($[M]^+$ m/z 545.3723, calcd 545.3716, and $[M]^+$ m/z 517.3776, calcd 517.3767, respectively). The NMR spectra (Tables 1 and 2) of **2** and **3** resembled to those of **1**. The main differences were the substituent groups at C-25 and C-24, respectively. For compound **2**, the methoxy group was replaced by a hydroxyl group at C-25. This elucidation was supported by 14 Da less of the molecular weight of **2**, and the upfield shift of C-25 by 4.42 ppm. Therefore, a hydroxy group instead of an acetoxy unit at C-24 in compound **3** was determined by similar analyses. ROESY associations of H-3/



Table 1 ^1H NMR data of compounds 1–7 in pyridine- d_5 at 500 MHz (δ in ppm, J in Hz)

Position	1	2	3	4	5	6	7
1	1.52 ^a 1.12 ^a	1.57 m 1.21 ^a	1.55 m 1.18 ^a	1.54 m 1.18 m	1.52 m 1.15 ^a	1.58 m 1.23 m	1.51 m 1.16 m
2	1.96 m 1.82 m	2.02 m 1.91 m	1.97 m 1.89 m	2.36 m 1.94 m	2.29 m 1.87 m	2.42 m 1.99 m	2.27 m 1.87 m
3	3.51 m	3.57 dd (11.2, 3.4)	3.54 m	3.49 dd (11.6, 4.2)	3.39 dd (11.6, 4.4)	3.54 dd (11.6, 4.3)	3.37 dd (11.2, 3.8)
4							
5	1.27 m	1.31 dd (12.4, 4.1)	1.32 dd (12.5, 4.3)	1.30 dd (12.6, 4.3)	1.30 dd (12.4, 4.1)	1.37 dd (12.5, 4.2)	1.31 m
6	1.52 ^a 0.71 m	1.56 m 0.73 q (12.7)	1.59 ^a 0.76 m	1.50 m 0.65 q (12.4)	1.51 m 0.68 q (12.7)	1.59 m 0.75 q (12.5)	1.55 m 0.73 m
7	2.54 m 1.01 ^a	2.57 m 1.07 ^a	2.60 m 1.08 ^a	2.53 m 1.10 ^a	2.55 m 1.02 m	2.60 m 1.13 m	2.59 m 1.08 ^a
8	1.82 m	1.84 m	1.86 m	1.80 m	1.80 m	1.87 dd (12.6, 3.5)	1.84 m
9							
10							
11	1.99 m 1.03 ^a	2.04 m 1.07 ^a	2.03 m 1.06 ^a	1.98 m 1.10 ^a	1.97 m 0.99 m	2.05 ^a 1.05 ^a	2.00 ^a 1.00 m
12	1.52 ^a (2H)	1.58 m (2H)	1.55 m (2H)	1.55 m (2H)	1.54 m (2H)	1.62 m (2H)	1.58 m (2H)
13							
14							
15							
16	4.24 d (10.1)	4.41 brd (10.3)	4.37 d (10.2)	4.23 brd (10.4)	4.27 d (10.4)	4.40 brd (10.1)	4.38 d (9.9)
17	1.60 ^a	1.66 brd (11.0)	1.58 ^a	1.62 m	1.63 m	1.65 m	1.62 m
18	1.07 s	1.14 s	1.09 s	1.07 s	1.09 s	1.09 s	1.08 s
19	0.52 brs 0.29 brs	0.56 d (3.3) 0.34 d (3.8)	0.55 d (3.7) 0.32 d (4.0)	0.48 d (3.3) 0.26 d (3.9)	0.45 d (3.5) 0.23 d (3.9)	0.53 d (3.4) 0.30 d (3.9)	0.47 d (3.3) 0.24 d (3.8)
20	1.77 m	1.84 m	1.80 m	1.80 m	1.80 m	1.76 m	1.75 m
21	0.88 d (5.9)	0.93 d (6.3)	0.91 d (6.1)	0.89 d (6.3)	0.90 d (6.3)	0.93 d (6.3)	0.92 d (6.3)
22	1.65 m 1.42 m	1.66 brd (11.0) 1.50 ^a	1.84 m 1.62 ^a	1.65 m 1.41 m	1.68 m 1.44 m	1.68 m 1.32 ^a	1.65 m 1.28 ^a
23	4.11 d (11.1)	4.39 brd (10.1)	4.25 brd (11.3)	4.23 brd (10.4)	4.26 m	4.02 m	3.99 m
24	5.34 brs	5.26 brs	3.64 s	5.55 brs	5.59 s	4.37 d (6.3)	4.34 d (6.3)
25							
26	1.59 s	1.68 s	1.57 s	2.02 s	2.05 s	5.30 s 5.04 s	5.27 s 5.01 s
27	1.56 s	1.50 s	1.61 s	2.09 s	2.13 s	2.05 s	2.02 s
28	1.14 s	1.19 s	1.16 s	1.14 s	1.15 s	1.29 s	1.27 s
29	1.04 s	1.09 s	1.08 s	1.00 s	0.96 s	1.05 s	0.96 s
30	1.16 s	1.21 s	1.20 s	1.27 s	1.09 s	1.32 s	1.08 s
3-Ara							
1'				4.79 d (7.1)	4.75 d (7.7)	4.83 d (7.1)	4.74 d (7.5)
2'				4.45 t (7.5)	5.95 dd (9.5, 7.8)	4.50 t (7.9)	5.92 t (8.1)
3'				4.16 dd (8.8, 3.2)	4.21 m	4.20 dd (8.8, 3.0)	4.19 dd (9.6, 3.0)
4'				4.32 brs	4.30 brs	4.35 brs	4.29 brs
5'				4.29 m	4.29 brd (11.4)	4.34 m	4.28 m
				3.78 d (11.4)	3.77 d (11.3)	3.83 d (10.9)	3.77 d (11.3)
24-OCOCH ₃	2.16 s	2.23 s		2.22 s	2.13 s		
25-OCH ₃	3.24 s		3.29 s				
2'-OCOCH ₃					2.24 s		2.11 s
C=N-OH	12.32 s	12.73 s	12.31 s	12.43 s	12.43 s	12.56 s	

^a Signals overlapped.

H5, H-16/CH₃-18, H-20/CH₃-18 and H-16/H-23 both in **2** and **3** suggested the α , β , β , and β orientations of H-3, H-16, H-20, and H-23, respectively. The *S* configuration of C-24 of **2** and **3** was proposed by the same way as that of **1**. Accordingly, the structures of **2** and **3** were established as shown.

Cimifrigine D (**4**) had the molecular formula C₃₇H₅₉NO₁₀ as determined by HREIMS (m/z 677.4129 [M]⁺, calcd 677.4139). In the ^1H NMR spectrum, resonances for an anomeric proton at δ_{H} 4.79 (H-1', 1H, d, $J = 7.1$ Hz), and a cyclopropane methylene at

δ_{H} 0.20 (6H, d, $J = 3.9$ Hz) and 0.48 (1H, d, $J = 3.3$ Hz) were observed. These data indicated **4** was a 9,19-cycloartane triterpene substituted with a sugar unit. Analyses of NMR spectroscopic data revealed that, structurally, the aglycone part of **4** was identical to **2**. The sugar unit in **4** was located to C-3 on the basis of HMBC correlation between the anomeric proton at δ_{H} 4.79 ($J = 7.1$ Hz) and the methine signal at δ_{C} 88.59 (C-3). In addition, by comparing its TLC and specific rotation with a standard after acid hydrolysis, the sugar was determined as l -arabinose. ROESY



Table 2 ^{13}C NMR data of compounds 1–7 in pyridine- d_5 at 150 MHz (δ in ppm)

Position	1	2	3	4	5	6	7
1	33.96 CH ₂	33.29 CH ₂	32.92 CH ₂	32.70 CH ₂	32.57 CH ₂	32.64 CH ₂	32.57 CH ₂
2	31.53 CH ₂	31.98 CH ₂	31.47 CH ₂	30.09 CH ₂	30.44 CH ₂	30.14 CH ₂	29.98 CH ₂
3	78.01 CH	78.37 CH	78.00 CH	88.59 CH	89.07 CH	88.51 CH	88.69 CH
4	41.16 C	41.58 C	41.14 C	41.35 C	41.47 C	41.34 C	41.06 C
5	47.69 CH	47.99 CH	47.67 CH	47.78 CH	48.02 CH	47.71 CH	47.67 CH
6	21.70 CH ₂	21.99 CH ₂	21.42 CH ₂	21.38 CH ₂	21.76 CH ₂	21.37 CH ₂	21.42 CH ₂
7	26.98 CH ₂	27.36 CH ₂	227.08 CH ₂	26.84 CH ₂	26.12 CH ₂	26.68 CH ₂	26.90 CH ₂
8	46.43 CH	46.74 CH	46.34 CH	46.33 CH	46.70 CH	46.21 CH	46.26 CH
9	20.06 C	20.38 C	20.02 C	20.03 C	20.46 C	19.98 C	20.13 C
10	27.16 C	27.59 C	26.99 C	26.84 C	27.25 C	26.85 C	26.70 C
11	25.83 CH ₂	26.30 CH ₂	25.75 CH ₂	25.66 CH ₂	26.02 CH ₂	25.55 CH ₂	25.62 CH ₂
12	30.99 CH ₂	31.26 CH ₂	30.94 CH ₂	30.89 CH ₂	31.21 CH ₂	30.83 CH ₂	30.93 CH ₂
13	42.50 C	43.01 C	42.44 C	42.48 C	42.85 C	42.61 C	42.52 C
14	51.67 C	51.93 C	51.65 C	51.62 C	51.98 C	51.62 C	51.71 C
15	164.34 C	164.63 C	164.64 C	164.10 C	164.53 C	164.37 C	164.50 C
16	80.55 CH	81.17 CH	80.09 CH	80.42 CH	80.83 CH	80.25 CH	80.55 CH
17	54.60 CH	54.61 CH	54.66 CH	54.36 CH	54.70 CH	54.88 CH	54.99 CH
18	19.86 CH ₃	20.36 CH ₃	19.83 CH ₃	19.77 CH ₃	20.27 CH ₃	19.80 CH ₃	19.86 CH ₃
19	31.44 CH ₂	31.85 CH ₂	31.42 CH ₂	31.36 CH ₂	31.62 CH ₂	31.20 CH ₂	31.19 CH ₂
20	33.28 CH	33.48 CH	33.23 CH	33.18 CH	33.57 CH	32.85 CH	32.93 CH
21	20.09 CH ₃	20.38 CH ₃	20.17 CH ₃	19.93 CH ₃	20.40 CH ₃	20.08 CH ₃	20.13 CH ₃
22	39.18 CH ₂	38.74 CH ₂	39.94 CH ₂	38.83 CH ₂	39.14 CH ₂	38.16 CH ₂	38.26 CH ₂
23	78.76 CH	80.00 CH	78.59 CH	78.77 CH	79.17 CH	81.89 CH	82.01 CH
24	77.13 CH	79.07 CH	78.34 CH	80.25 CH	80.65 CH	78.74 CH	78.82 CH
25	77.53 C	73.11 C	78.18 C	74.15 C	74.79 C	146.53 C	146.57 C
26	23.21 CH ₃	28.86 CH ₃	23.08 CH ₃	29.57 CH ₃	30.08 CH ₃	113.13 CH ₂	113.18 CH ₂
27	21.70 CH ₃	27.73 CH ₃	20.42 CH ₃	29.57 CH ₃	29.97 CH ₃	18.55 CH ₃	18.59 CH ₃
28	19.56 CH ₃	20.59 CH ₃	19.39 CH ₃	19.55 CH ₃	20.07 CH ₃	19.29 CH ₃	19.34 CH ₃
29	14.89 CH ₃	15.38 CH ₃	15.25 CH ₃	15.39 CH ₃	15.69 CH ₃	15.36 CH ₃	15.25 CH ₃
30	26.19 CH ₃	26.62 CH ₃	25.46 CH ₃	25.75 CH ₃	25.89 CH ₃	25.67 CH ₃	25.46 CH ₃
3-Ara							
1'				107.26 CH	105.01 CH	107.59 CH	104.50 CH
2'				72.92 CH	74.83 CH	72.93 CH	74.37 CH
3'				74.59 CH	72.89 CH	74.68 CH	72.52 CH
4'				69.39 CH	70.28 CH	69.61 CH	69.79 CH
5'				66.56 CH ₂	67.76 CH ₂	66.91 CH ₂	67.18 CH ₂
24-O $\underline{\text{C}}$ OCH ₃	170.83 C	171.74 C		170.62 C	170.52 C		
24-OCOC $\underline{\text{H}}$ CH ₃	21.05 CH ₃	21.99 CH ₃		20.75 CH ₃	21.78 CH ₃		
25-O $\underline{\text{C}}$ H ₃	49.27 CH ₃		49.23 CH ₃				
2'-O $\underline{\text{C}}$ OCH ₃					171.14 C		170.17 C
2'-OCOC $\underline{\text{H}}$ CH ₃					21.28 CH ₃		21.35 CH ₃

correlations of H-16/CH₃-18, H-20/CH₃-18 and H-16/H-23 suggested an α -orientation of the substituents at C-16, C-20 and C-23, respectively. Whereas, a β -orientation of the substituent at C-3

was established by the cross-peak of H-3/H-5. The configuration of C-24 was proposed as *S* by the same way as that of **1**. Therefore, the structure of **4** was elucidated as shown.

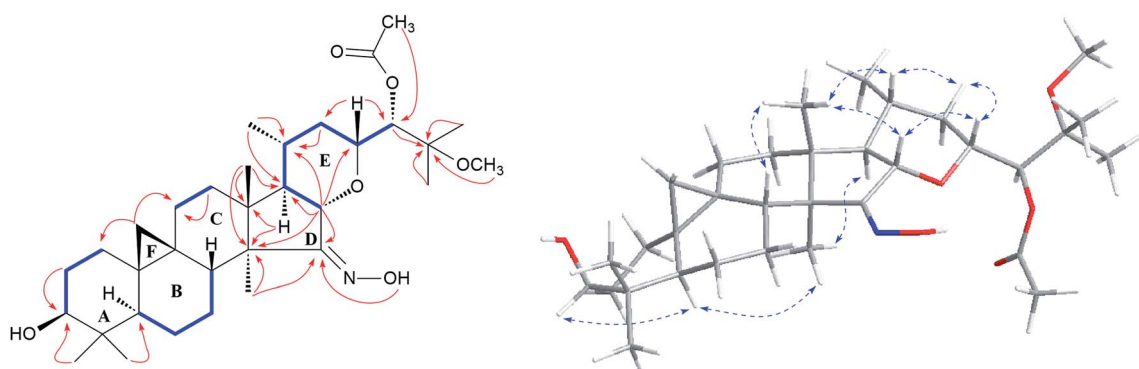


Fig. 2 Major HMBC (→), ^1H - ^1H COSY (→), and ROESY (←) correlations of compound **1**.



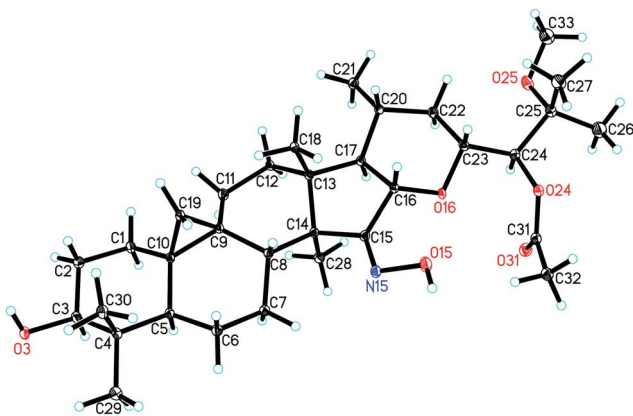


Fig. 3 X-ray crystal structure of 1.

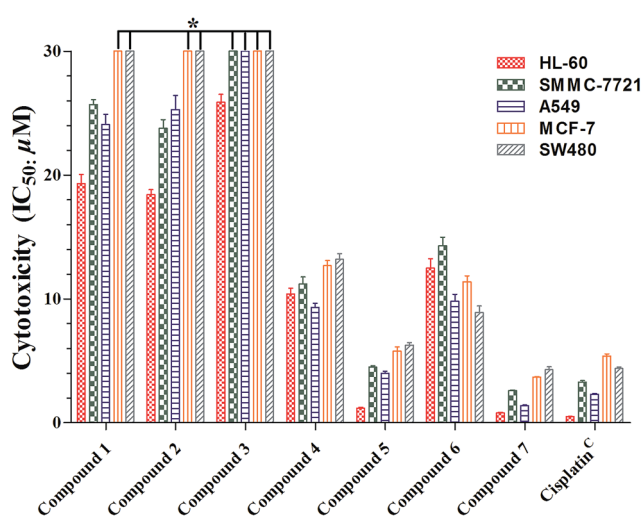


Fig. 4 Cytotoxicity of compounds isolated from the flowers of *C. frigida*. Results are expressed as the average ($n = 3$) of IC_{50} values (μM). 0.1% DMSO as the solvent control and did not show any cytotoxicity to those cell lines. ^cUsed as a positive control substance for the cytotoxicity assay. *The IC_{50} values are $>30 \mu M$.

On the basis of the HREIMS peak at m/z 719.4281 $[M]^+$ (calcd 719.4245), the molecular formula of cimifrigine E (5) was determined as $C_{39}H_{61}NO_{11}$. Comparison of NMR data of compounds 5 and 4 revealed these two compounds were structurally identical except for the sugar unit. The molecular weight difference between 5 and 4 was 42 Da, consistent with an acetyl unit. Besides, H-2' signal of 5 was shifted to downfield at δ_H 5.95, which together with the HMBC correlation between the acetoxy methyl group (δ_H 2.13) and C-2' (δ_C 74.83) located an acetoxy group at C-2' of 5. The sugar was identified as L-arabinose using the same way as that of 4. The orientations of H-3, H-16, H-20, and H-23 were determined as α , β , β , and β , respectively, by analyses of ROESY correlations. In addition, the configuration of C-24 was elucidated as *S* by comparison of coupling constant of H-24 of 5 with those of known isodahurinyll-type triteroids.^{11,13,35,36} Thus, the structure of 5 was determined as shown.

The spectroscopic features of cimifrigines F (6) and G (7) resembled to each other except for the sugar unit. The molecular formulas of 6 and 7 were determined as $C_{35}H_{55}NO_8$ and $C_{37}H_{57}NO_9$, respectively, by HREIMS ($[M]^+$ m/z 617.3943, calcd 617.3928, and $[M]^+$ m/z 659.4053, calcd 659.40337, respectively). Same to that of compounds 4 and 5, the molecular weight difference between 6 and 7 was 42 Da, which equivalent to an acetyl moiety. In addition, the acetoxy group in 7 was located to C-2' based on the HMBC correlation of the acetoxy methyl group (δ_H 2.11) and C-2' (δ_C 74.37). Structurally, the aglycone part of 6 and 7 was similar to that of 2 except that a terminal double bond was formed between C-25 and C-26 in 6 and 7. HMBC correlations from the olefinic protons at δ_H 5.30 (for 6) and 5.27 (for 7) and δ_H 5.04 (for 6) and 5.01 (for 7) to C-24 (δ_C 78.74 for 6 and 78.82 for 7), C-25 (δ_C 146.53 for 6 and 146.57 for 7), and CH₃-27 (δ_C 18.55 for 6 and 18.59 for 7) further supported this elucidation. The sugar obtained after acid hydrolysis was identified as L-arabinose by comparing its TLC and specific rotation with a standard. The α -orientation of the substituents at C-16, C-20 and C-23 were determined by ROESY correlations of H-16/CH₃-18, H-20/CH₃-18 and H-16/H-23. Whereas, correlation of H-3/H-5 indicated the β -orientation of the substituent at C-3. The coupling constant of H-24 for 6 and 7 was 6.3 Hz which consistent with those of dahurinyll-type compounds (6–9 Hz).^{11,13,35,36} Thus, the stereochemistry of C-24 was elucidated as *S* and the structure of 6 and 7 were determined as shown.

Cimifrigines A–G (1–7) were evaluated against human HL-60, SMMC-7721, A549, MCF-7, and SW-480 cell lines for their cytotoxicities. All compounds (1–7) showed different levels of activities with the glycoside derivatives (4–7) stronger than the aglycone analogues (1–3) (Fig. 4, Table S1[†]). Compounds 5 and 7 exhibited as potent activities as positive control cisplatin (IC_{50} : 0.5 to 5.4 μM) against all cell lines with IC_{50} values ranging from 0.8 to 6.3 μM . However, 4 and 6 indicated broad spectrum and moderate activities with IC_{50} values around 8.9 to 14.3 μM . Structurally, the C-2' position was substituted by an acetoxy unit in 5 and 7. While, a hydroxy group was located at this position in 4 and 6. Therefore, based on the aforementioned data, it may give the preliminary conclusion that sugar unit is critical to the cytotoxicities of this new type of CTs. Besides, hydrophobic groups like acetoxy at the sugar unit could enhance the activities as much as one order of magnitude.

Conclusions

As mentioned in the Introduction besides the roots of *Cimicifuga* spp., nontraditionally used part, such as fruits, also contained novel active CTs. Therefore, we expanded the research target to the flowers of *C. frigida*, a species of which the chemical constituents have not been reported yet. As a result, seven new dahurinyll-type analogues, cimifrigines A–G (1–7), with an oxime group at C-15, were isolated and identified. Significantly, all of the compounds had cytotoxic effect and two of them showed comparable activity as that of cisplatin, the positive control, in the *in vitro* cytotoxicity assay. Besides, preliminary structure–activity relationship also discussed in the study which afforded potential informations for further chemical



modifications. In summary, to the best of our knowledge, this is the first example of naturally occurred oxime group bearing cytotoxic CTs. In addition, on the basis of the delightful discoveries from the fruits and flowers of *Cimicifuga* spp., we assume that the more sophisticated parts of this genus, such as pollen and vegetative organ, may also contain novel active chemical constituents and deserved further investigations.

Experimental section

General experimental procedures

Column chromatography (CC) was run on silica gel (200–300 mesh, Qingdao Marine Chemical, Inc.), and Lichroprep RP-18 (40–63 μm , Merck). Semipreparative HPLC was carried out on an Agilent 1100 liquid chromatography system using an YMC-Pack 10 mm \times 250 mm column (Pro C18 RS). Precoated TLC plates (200–250 μm thickness, silica gel 60 F₂₅₄, Qingdao Marine Chemical, Inc.) were used for thin-layer chromatography. The spots in TLC were visualized by heating after spraying with 10% aq. H₂SO₄. 1D and 2D NMR spectra were performed on Bruker DRX-500 and Avance III-600 MHz spectrometers (Bruker, Zürich, Switzerland) with solvent signal as internal reference. ESIMS and HRESIMS were run on a Shimadzu LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan) or an Agilent G6230 TOF MS (Agilent Technologies, Palo Alto, USA). Infrared spectra were tested on a Shimadzu IR-450 instrument with KBr pellets. A JASCO P-1020 digital polarimeter was applied to record optical rotations, using MeOH as solvent. X-ray diffraction was realized on a Bruker SMART APEX CCD crystallography system.

Plant material

The flowers of *Cimicifuga frigida* (1.0 kg) were collected from Litang County, Sichuan Province, China, in September 2012. Prof. Wang Zongyu, Kunming Institute of Botany, Chinese Academy of Sciences, identified the species. A voucher specimen (KUN no. 201209003) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, PR China.

Extraction and isolation

MeOH (4 L, 3 times, 7 days each) was used to extract the dried and milled flowers of *Cimicifuga frigida* (1.0 kg) at room temperature. MeOH was evaporated under vacuum at 50 °C to afford the extract (87.4 g). The extract gave fractions A (10.3 g), B (18.1 g), C (13.4 g), and D (12.3 g) by silica gel CC (2.5 kg, 10 \times 150 cm) eluted with CHCl₃–MeOH [100 : 0 (4 L), 50 : 1 (8 L), 10 : 1 (7 L), 0 : 100 (4 L)]. Another six sub-fractions (B.1–B.6) were obtained through RP-18 CC (500 g, 6 \times 50 cm), gradiently eluted with MeOH–H₂O from 60 : 40 to 100 : 0. Fraction B.3 (2.7 g) yielded compounds 1 (3.8 mg), 2 (3.2 mg), and 3 (1.9 mg) by silica gel CC (40 g, 3 \times 60 cm) eluting with CHCl₃–Me₂CO from 30 : 1 gradient to 10 : 1 and semipreparative HPLC (eluted with CH₃CN–H₂O, gradient from 65 : 35 to 85 : 15). Sub-fractions (C.1–C.4) were obtained by RP-18 CC (eluted with MeOH–H₂O,

gradient from 50 : 40 to 85 : 15) on fraction C. Consequently, 5 (3.3 mg), and 7 (2.8 mg) were purified from fraction C.2 (1.8 g) by silica gel CC (40 g, 3 \times 40 cm) eluted with CHCl₃–Me₂CO (gradient from 20 : 1 to 10 : 1), and then repeated semipreparative HPLC (eluted with CH₃CN–H₂O, gradient from 50 : 50 to 65 : 35). Fraction C.3 gave compounds 4 (2.5 mg) and 6 (2.7 mg) by successively silica gel CC (40 g, 3 \times 40 cm, eluted with CHCl₃–Me₂CO 10 : 1, 13 L), and semipreparative HPLC (eluted with CH₃CN–H₂O, gradient from 50 : 50 to 65 : 35).

(3 β ,16S,23R,24S)-16,23-epoxy-3-hydroxy-15-oximido-24-acetoxy-25-methoxy-9,19-cycloartane (1). Colorless crystals; $[\alpha]_{\text{D}}^{25} = +38.9$ (c0.09, MeOH); mp 315–317 °C; IR (KBr): ν_{max} 3438, 2967, 2867, 1740, 1628, 1456, 1374, 1241, 1068, 926 cm⁻¹; see Tables 1 and 2 for ¹H NMR (500 MHz, C₅D₅N) and ¹³C NMR (150 MHz, C₅D₅N) data; positive ESIMS [M + Na]⁺ *m/z* 582; positive HREIMS [M]⁺ *m/z* 559.3864 (calcd for C₃₃H₅₃NO₆, 559.3873).

(3 β ,16S,23R,24S)-16,23-epoxy-3,25-dihydroxy-15-oximido-24-acetoxy-9,19-cycloartane (2). White powder; $[\alpha]_{\text{D}}^{25} = -2.1$ (c0.03, MeOH); IR (KBr): ν_{max} 3442, 2932, 2869, 1734, 1631, 1452, 1384, 1248, 1098, 1028 cm⁻¹; see Tables 1 and 2 for ¹H NMR (500 MHz, C₅D₅N) and ¹³C NMR (150 MHz, C₅D₅N) data; positive ESIMS [M + Na]⁺ *m/z* 568; positive HREIMS [M]⁺ *m/z* 545.3723 (calcd for C₃₂H₅₁NO₆, 545.3716).

(3 β ,16S,23R,24S)-16,23-epoxy-3,24-dihydroxy-15-oximido-25-methoxy-9,19-cycloartane (3). White powder; $[\alpha]_{\text{D}}^{25} = +30.1$ (c0.10, MeOH); IR (KBr): ν_{max} 3397, 2928, 2865, 1724, 1632, 1459, 1376, 1144, 1081, 927 cm⁻¹; see Tables 1 and 2 for ¹H NMR (500 MHz, C₅D₅N) and ¹³C NMR (150 MHz, C₅D₅N) data; positive ESIMS [M + Na]⁺ *m/z* 540; positive HREIMS [M]⁺ *m/z* 517.3776 (calcd for C₃₁H₅₁NO₅, 517.3767).

(3 β ,16S,23R,24S)-16,23-epoxy-15-oximido-24-acetoxy-25-hydroxy-9,19-cycloart-3-O- α -1-arabinopyranoside (4). White powder; $[\alpha]_{\text{D}}^{25} = +38.4$ (c0.09, MeOH); IR (KBr): ν_{max} 3423, 2938, 2870, 1745, 1630, 1459, 1375, 1232, 1085, 1020, 937 cm⁻¹; see Tables 1 and 2 for ¹H NMR (500 MHz, C₅D₅N) and ¹³C NMR (150 MHz, C₅D₅N) data; positive ESIMS [M + Na + H₂O]⁺ *m/z* 718; positive HREIMS [M]⁺ *m/z* 677.4129 (calcd for C₃₇H₅₉NO₁₀, 677.4139).

(3 β ,16S,23R,24S)-16,23-epoxy-15-oximido-24-acetoxy-25-hydroxy-9,19-cycloart-3-O-(2'-acetoxy)- α -1-arabinopyranoside (5). White powder; $[\alpha]_{\text{D}}^{25} = +28.1$ (c0.09, MeOH); IR (KBr): ν_{max} 3441, 2935, 2870, 1744, 1629, 1456, 1374, 1232, 1084, 936 cm⁻¹; see Tables 1 and 2 for ¹H NMR (500 MHz, C₅D₅N) and ¹³C NMR (150 MHz, C₅D₅N) data; positive ESIMS [M + Na + H₂O]⁺ *m/z* 760; positive HREIMS [M]⁺ *m/z* 719.4281 (calcd for C₃₉H₆₃NO₁₂, 719.4245).

(3 β ,16S,23R,24S)-16,23-epoxy-15-oximido-24-hydroxy-25-en-9,19-cycloart-3-O- α -1-arabinopyranoside (6). White powder; $[\alpha]_{\text{D}}^{25} = +10.2$ (c0.06, MeOH); IR (KBr): ν_{max} 3450, 2930, 2868, 1630, 1454, 1378, 1250, 1068, 1010, 973 cm⁻¹; see Tables 1 and 2 for ¹H NMR (500 MHz, C₅D₅N) and ¹³C NMR (150 MHz, C₅D₅N) data; positive ESIMS [M + Na]⁺ *m/z* 640; positive HREIMS [M]⁺ *m/z* 617.3943 (calcd for C₃₅H₅₅NO₈, 617.3928).

(3 β ,16S,23R,24S)-16,23-epoxy-15-oximido-24-hydroxy-25-en-9,19-cycloart-3-O-(2'-acetoxy)- α -1-arabinopyranoside (7). White powder; $[\alpha]_{\text{D}}^{25} = +15.0$ (c0.06, MeOH); IR (KBr): ν_{max} 3424, 2935, 2869, 1737, 1628, 1454, 1377, 1249, 1069, 1019, 935 cm⁻¹; see Tables 1 and 2 for ¹H NMR (500 MHz, C₅D₅N) and ¹³C NMR (150



MHz, C₅D₅N) data; positive ESIMS [M + Na]⁺ *m/z* 682; positive HREIMS [M]⁺ *m/z* 659.4053 (calcd for C₃₇H₅₇NO₉, 659.4033).

X-ray crystal structure analysis

A Bruker APEX DUO diffractometer equipped with an APEX II CCD was used to obtain the intensity data at 100 K, using Cu K α radiation. Bruker SAINT was applied for cell refinement and data reduction. The structure was determined by direct methods using SHELXS-97.³⁸ Refinements were performed with SHELXL-97, using full-matrix least-squares, with anisotropic displacement parameters for all the non-hydrogen atoms. The H atoms were placed in calculated positions and refined using a riding model. Molecular graphics were calculated with PLATON.

X-ray crystallography of compound 1

Colorless crystals of compound 1 (CCDC: 1545247†) was obtained by methanol extract of *Cimicifuga frigida*. The X-ray crystallographic data of 1: C₃₃H₅₃NO₆, monoclinic, space group *P*2₁, *a* = 9.5634(4) Å, *b* = 11.2799(5) Å, *c* = 14.9190(6) Å, α = 90.00°, β = 103.4570(10)°, γ = 90.00°, *V* = 1565.19(11) Å³, *Z* = 2, *D*_{calcd} = 1.188 g cm⁻³. The final *R*₁ values were 0.1056 (*I* > 2 σ (*I*)). The final *wR*(*F*²) values were 0.2898 (*I* > 2 σ (*I*)). The goodness of fit on *F*² was 1.492. Crystal size, 0.54 × 0.40 × 0.10 mm³. Flack parameter = 0.2(3). The Hooft parameter is 0.13(8) for 1536 Bijvoet pairs.

Hydrolysis and identification of the sugar units in compounds 4–7

The MeOH solution (3 mL) of each compound (1.5 mg) was refluxed with 0.5 N HCl (2 mL) for 2 h. CHCl₃ (3 × 10 mL) was used to extract the reaction mixture after diluting with H₂O. A monosaccharide was given by neutralizing each aqueous layer with Ag₂CO₃ and filtering the precipitate. The monosaccharide from compounds 4–7 had an *R*_f (EtOAc–CHCl₃–MeOH–H₂O, 3 : 2 : 2 : 1) and specific rotation of [α]_D²⁰ +63.4 (*c*0.06, MeOH) corresponding to those of L-arabinose (Sigma-Aldrich).

Cytotoxicity bioassay

The human tumor cell lines HL-60, SMMC-7721, A549, MCF-7, and SW-480 were used in the cytotoxic assay. These cell lines were obtained from ATCC (Manassas, VA, USA). DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA), was used to culture cells in 5% CO₂ at 37 °C. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method was applied to evaluate the cytotoxicity.^{39,40} Briefly, cells were seeded into each well of a 96-well cell culture plate. After 12 h of incubation at 37 °C, the test compound was added. Each tumor cell line was exposed to compounds 1, 2, and 3 at concentrations of 3, 10, 20, 50, and 100 μ M triplicates for 48 h, to compounds 4 and 6 at concentrations of 1, 3, 10, 30, and 50 μ M triplicates for 48 h, and to compounds 5 and 7 at concentrations of 0.1, 0.3, 1, 5, 10, and 20 μ M triplicates for 48 h, respectively. Cisplatin (Sigma, USA) was used as a positive control. After compound treatment, cell viability was detected

and a cell growth curve was graphed. IC₅₀ values were calculated by Reed and Muench's method.⁴¹ (All compounds were dissolved in DMSO as 100 mM stock and 0.1% DMSO was used as the solvent control).

Acknowledgements

This project was supported by the National Natural Science Foundation of China (81302670 and U1132604) and the Foundation of State Key Laboratory of Phytochemistry and Plant Resources in West China (P2015-KF03).

Notes and references

- 1 E. Liske and P. Wustenberg, *Menopause*, 1998, 5, 250–255.
- 2 Y. Nian, H. Zhu, W. R. Tang, Y. Luo, J. Du and M. H. Qiu, *J. Nat. Prod.*, 2013, 76, 896–902.
- 3 Y. Nian, J. Yang, T. Y. Liu, Y. Luo, J. H. Zhang and M. H. Qiu, *Sci. Rep.*, 2015, 5, 9026–9031.
- 4 D. J. McKenna, K. Jones, S. Humphrey and K. Hughes, *Altern. Ther. Health Med.*, 2001, 7, 93–100.
- 5 S. Nobuko, K. Mutsuo, T. Harukuni, M. Teruo, E. Fumio, N. Hoyoku, N. Masahiro, S. Yohiro and H. L. Kuo, *Bioorg. Med. Chem.*, 2005, 13, 1403–1408.
- 6 *Chinese Pharmacopoeia Commission, The Pharmacopoeia of Chinese People's Republic*, ed. Y. Li, The Chemical Industry Publishing House, Beijing, China, 2010, vol. 1, pp. 68–69.
- 7 J. X. Li and Z. Y. Yu, *Curr. Med. Chem.*, 2006, 13, 2927–2951.
- 8 N. M. Bao, Y. Nian, W. H. Wang, X. L. Liu, Z. T. Ding and M. H. Qiu, *Phytochem. Lett.*, 2015, 12, 200–202.
- 9 W. H. Wang, Y. Nian, Y. J. He, L. S. Wan, N. M. Bao, G. L. Zhu, F. Wang and M. H. Qiu, *Tetrahedron*, 2015, 71, 8018–8025.
- 10 N. M. Bao, Y. Nian, G. L. Zhu, W. H. Wang, L. Zhou and M. H. Qiu, *Fitoterapia*, 2014, 99, 191–197.
- 11 Y. Nian, H. Y. Wang, L. Zhou, J. Su, Y. Li and M. H. Qiu, *Planta Med.*, 2013, 79, 60–69.
- 12 Y. Nian, H. Y. Wang, J. Su, L. Zhou, G. Feng, Y. Li and M. H. Qiu, *Tetrahedron*, 2012, 68, 6521–6527.
- 13 Y. Nian, X. M. Zhang, Y. Li, Y. Y. Wang, J. C. Chen, L. Lu, L. Zhou and M. H. Qiu, *Phytochemistry*, 2011, 72, 1473–1481.
- 14 Y. Nian, Y. L. Zhang, J. C. Chen, L. Lu, C. Qing and M. H. Qiu, *J. Nat. Prod.*, 2010, 73, 93–98.
- 15 Y. Nian, J. C. Chen, L. Lu, X. M. Zhang and M. H. Qiu, *Helv. Chim. Acta*, 2009, 92, 112–120.
- 16 L. Lu, J. C. Chen, Y. Li, C. Qing, Y. Y. Wang, Y. Nian and M. H. Qiu, *Chem. Pharm. Bull.*, 2012, 60, 571–577.
- 17 H. Y. Wang, Y. Nian, C. Y. Ma, Y. B. Song, L. Zhou and M. H. Qiu, *Chin. J. Chem.*, 2012, 30, 1265–1268.
- 18 D. S. Li, Y. Nian, Y. Sun and M. H. Qiu, *Helv. Chim. Acta*, 2011, 94, 632–638.
- 19 L. R. Sun, J. Yan, L. Zhou, Z. R. Li and M. H. Qiu, *Molecules*, 2011, 16, 5701–5708.
- 20 L. Lu, J. C. Chen, H. J. Song, Y. Li, Y. Nian and M. H. Qiu, *Chem. Pharm. Bull.*, 2010, 58, 729–733.
- 21 L. Lu, J. C. Chen, Y. Nian, Y. Sun and M. H. Qiu, *Molecules*, 2009, 14, 1578–1584.



- 22 L. R. Sun, J. Yan, Y. Nian, L. Zhou, H. J. Zhang and M. H. Qiu, *Molecules*, 2008, **13**, 1712–1721.
- 23 L. R. Sun, C. Qing, Y. L. Zhang, S. Y. Ji, Z. R. Li, S. J. Pei, M. H. Qiu, M. L. Gross and S. X. Qiu, *Beilstein J. Org. Chem.*, 2007, **3**, 1–6.
- 24 L. R. Sun, J. Yan, L. Lu, S. J. Pei, Z. R. Li, L. Zhou, X. M. Zhang and M. H. Qiu, *Helv. Chim. Acta*, 2007, **90**, 1313–1318.
- 25 Z. Ali, S. L. Khan, R. S. Pawar, D. Ferreira and I. K. Khan, *J. Nat. Prod.*, 2007, **70**, 107–110.
- 26 C. Dan, Y. Zhou, Y. Deng, S. L. Peng, L. S. Ding, M. L. Gross and S. X. Qiu, *Org. Lett.*, 2007, **9**, 1813–1816.
- 27 J. C. Gao, F. Huang, J. C. Zhang, G. Y. Zhu, M. S. Yang and P. G. Xiao, *J. Nat. Prod.*, 2006, **69**, 1500–1502.
- 28 J. X. Li, J. Liu, C. C. He, Z. Y. Yu, Y. Du, S. Kadota and H. Seto, *Maturitas*, 2007, **58**, 59–69.
- 29 N. Sakurai, J. H. Wu, Y. Sashida, Y. Mimaki, T. Nikaido, K. Koike, H. Itokawa and H. Lee, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1329–1332.
- 30 M. A. Findeis, F. Schroeder, T. D. McKee, D. Yager, P. C. Fraering, S. P. Creaser, W. F. Austin, J. Clardy, R. Wang, D. Selkoe and C. B. Eckman, *ACS Chem. Neurosci.*, 2012, **3**, 941–951.
- 31 J. H. Lee, T. D. Cuong, S. J. Kwack, J. H. Seok, J. K. Lee, J. Y. Jeong, M. H. Woo, J. S. Choi, H. K. Lee and B. S. Min, *Planta Med.*, 2012, **78**, 1391–1394.
- 32 J. A. Compton, *Plantsman*, 1992, vol. 14, pp. 99–115.
- 33 Editorial Committee of Chinese flora, *Flora of China*, ed. W. C. Wang, China Science Press, Beijing, China, 2004, vol. 27, pp. 93–103.
- 34 C. Ren, Q. Yuan and Q. E. Yang, *Nord. J. Bot.*, 2012, **30**, 585–595.
- 35 Y. Shao, A. Harris, M. F. Wang, H. J. Zhang, G. A. Cordell, M. Bowman and E. Lemmo, *J. Nat. Prod.*, 2000, **63**, 905–910.
- 36 G. Kusano, Y. Murakami, N. Sakurai and T. Takemoto, *Yakugaku Zasshi*, 1976, **96**, 82–85.
- 37 R. W. W. Hooft, L. H. Straver and A. L. Spek, *J. Appl. Crystallogr.*, 2008, **41**, 96–103.
- 38 G. M. Sheldrick, *SHELXL97*, University of Göttingen, Germany, 1997.
- 39 T. Mossmann, *Immunol. Methods*, 1983, **65**, 55–63.
- 40 M. C. Alley, D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski and D. L. Fine, *Cancer Res.*, 1988, **48**, 589–601.
- 41 L. J. Reed and H. Muench, *Am. J. Hyg.*, 1938, **27**, 493–497.

