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Antiproliferative and apoptotic sesquiterpene lactones from *Carpesium faberi*

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ABSTRACT

Four new (**1**–**4**) and 13 known (**5**–**17**) sesquiterpene lactones along with two known diterpenes (**18**, **19**) were isolated from the whole plant of *Carpesium faberi*. The new structures were elucidated by means of spectroscopic techniques and some chemical transformations to be pseudoguaian-1α(H)-8α,12-olide-4β-*O*-β-D-glucopyranoside (**1**), 4β,10α-dihydroxy-5α(H)-1,11(13)-guaidien-8α,12-olide (**2**), 4β,10β-dihydroxy-5α(H)-1, 11(13)-guaidien-8β,12-olide (**3**), and (4*S*)-acetyloxyl-11(13)-carabren-8β,12-olide (**4**). All isolates were tested against MCF-7 human breast cancer cells using the MTT assay. Among them, the sesquiterpene lactones (except tomentosin **17**) possessing an α-methylene-γ-lactone moiety were found to have in vitro antiproliferative activities, with IC_{50} values of 3.0–38.8 μg/mL. The effects of four selected sesquiterpene lactones (guaianolide **2**, carabranolide **4**, pseudoguaianolide **9**, eudesmanolide **13**) on the cell cycle were examined using flow cytometry (FCM).

The genus *Carpesium* (Compositae) consists of about 20 species worldwide, and most of them are distributed in Eastern Asia.[1,2] It has been well documented that sesquiterpene lactones with different carbon frameworks are characteristic for *Carpesium*.[2,3] *Carpesium faberi* Winkl. has been used as a folk medicine in southwestern China due to its hemostatic, vermifugal, anti-inflammatory, and detoxifying properties.[1,4] However, this plant has not yet been phytochemically and pharmacologically investigated. As a part of our ongoing project towards the discovery of novel antitumor agents from plants,[5] four pseudoguaianolides (**1**, **9**–**11**), five guaianolides (**2**, **3**, **7**, **8**, **12**), three carabranolides (**4**–**6**), three eudesmanolides (**13**–**15**), a germacranolide (**16**), and one xanthanolide (**17**) together with an acyclic (**18**) and a labdane-type (**19**) diterpene (Fig. 1), were obtained from the whole plant of *C. faberi*.[6,7] Comparing their MS, NMR data, and physical properties with those of literature, the known compounds were identified as carabrol (**5**),[3g,8] carabrone (**6**),[3h,9] 4β,10β-dihydroxy-5α(H)-1,11(13)-guaidien-8α,12-olide (**7**),[3c] 4α,10β-dihydroxy-5α(H)-1,11(13)-guaidien-8β,12-olide (**8**),[10] 2-desoxy-4-*epi*-pulchellin (**9**),[2a,11] carabrolactone B (**10**),[2a] 2,3-dihydroaromaticin (**11**),[12] 4-*epi*-isoinuviscolide (**12**),[8] telekin (**13**),[3f] ivalin (**14**),[13] 2α,5α-dihydroxy-11α(H)-eudesma-4(15)-en-8β,12-olide (**15**),[3d] carabrolactone A (**16**),[2a] tomentosin (**17**),[8,9] *trans*-phytol (**18**),[14] and 8α,15-dihydroxy-13*E*-labdene (**19**).[15] We herein report the isolation and structure determination of the new compounds, as well as their antiproliferative and apoptotic activities against MCF-7 human breast cancer cells.

The molecular formula of compound **1**[16] was determined to be $C_{21}H_{34}O_8$ from the positive mode high resolution electrospray ionization mass spectra (HR-ESIMS), which gave an $[M+Na]^+$ ion peak at *m/z* 437.2147 (calcd 437.2146). Its IR spectrum indicated the presence of hydroxyl (3405 cm^{-1}) and γ-lactone (1771 cm^{-1}) groups. In the highfield ^1H NMR spectrum of **1**, one methyl group singlet at δ 0.94 (3H, s) and two methyl group doublets at δ 1.16 (3H, d, J = 7.8 Hz) and 0.95 (3H, d, J = 6.4 Hz) were observed (Table 1). The ^{13}C and DEPT NMR spectra of **1** showed 15 carbon signals classified as three methyls, four methylenes, six methines (two oxygenated at δ 93.3 and 84.2), and two quaternary carbons (one carbonyl at δ 182.9), in addition to six typical oxygen-bearing carbons attributed to a glucopyranosyl unit [δ 106.1 (CH, C-1′), 75.3 (CH, C-2′), 78.2 (CH, C-3′), 71.6 (CH, C-4′), 77.8 (CH, C-5′), 62.8 (CH_2, C-6′)] (Table 1). These data suggested that **1** should be a sesquiterpene glycoside with an aglycone structurally related to the known pseudoguaianolide **9**.[2a,11]

The planar structure of **1** was determined by detailed 1D and 2D NMR (COSY, HSQC, and HMBC) spectroscopic analysis. In the COSY NMR experiment (Fig. 2) of **1**, a spin system [–CH(O)$CH_2$$CH_2$-CHCH($CH_3$)$CH_2$CH(O)CH($CHCH_3$)$CH_2$–] in the aglycone was found between H-4 at δ 3.69 and H_2-3 at δ 1.99/1.58, between H_2-3 and H_2-2 at δ 1.72/1.45, between H_2-2 and H-1 at δ 1.63, between H-1 and H-10 at δ 1.74, between H-10 and Me-15 at δ 0.95, between H-10 and H_2-9 at δ 2.27/1.25, between H_2-9 and H-8 at δ 4.47, between H-8 and H-7 at δ 2.48, between H-7 and H-11 at δ

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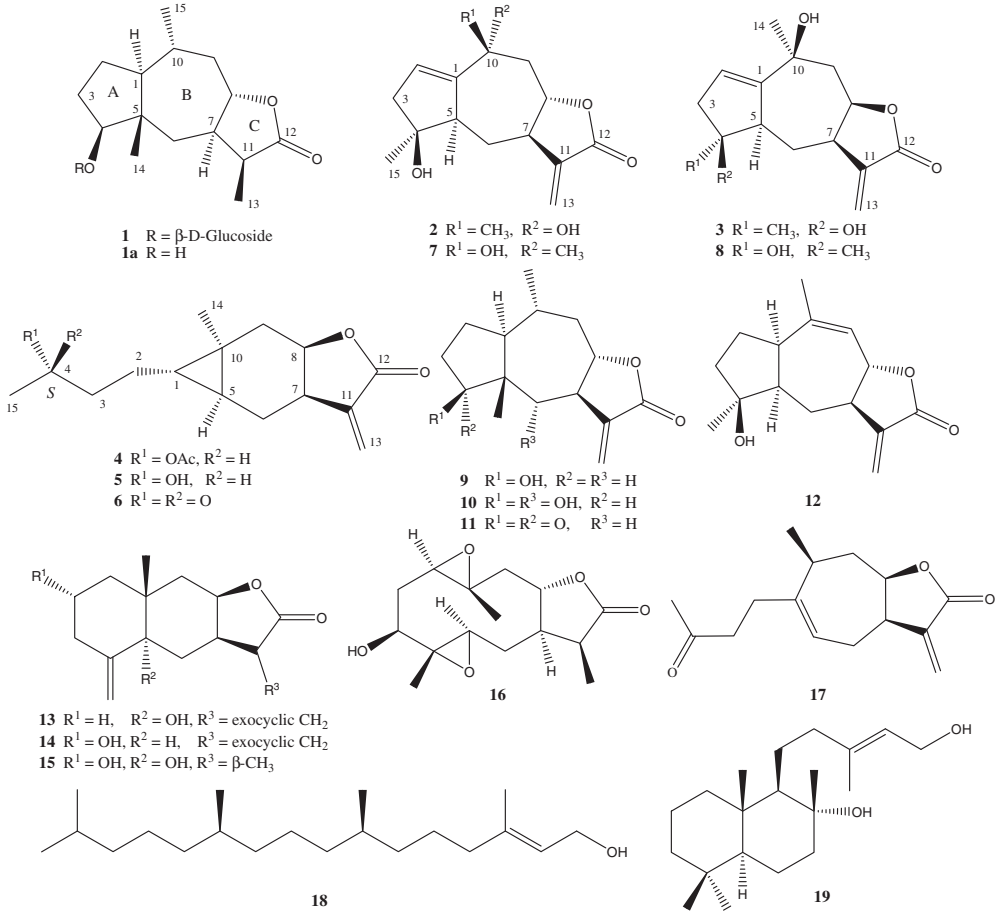


Figure 1. Structures of compounds **1**–**19**.

2.62, between H-11 and Me-13 at δ 1.16, as well as between H-7 and H_2-6 at δ 1.94/1.51. The relative stereochemistry of the chiral centers in the aglycone was deduced by extensive analysis of the coupling patterns of the protons bound to the seven-membered ring and by the NOE correlations in the NOESY NMR experiment. Clear NOE correlations (Fig. 2) were observed between H-1 at δ 1.63 (br dd, J = 11.1, 10.2 Hz) and H-9_{ax} at δ 1.24 (br dd, J = 12.2, 12.0 Hz), between H-1 and H-4, between H-1 and H-7, between H-1 and Me-15, between H-9_{ax} and H-7, between H-9_{ax} and Me-15, between H-6_{ax} at δ 1.51 (dd, J = 14.8, 12.7 Hz) and H-8 at δ 4.47, between H-6_{ax} and Me-13, between H-6_{ax} and Me-14, between H-7 and H-6_{eq} at δ 1.94 (1H, dd, J = 14.8, 5.6 Hz), between H-8 and Me-14, between H-8 and H-9_{eq} at δ 2.27 (ddd, J = 13.2, 3.6, 3.6 Hz), as well as between H-9_{eq} and H-10 at δ 1.74. These data revealed that H-1, H-4, H-7, H-11, and Me-15 were all α-oriented, whereas H-8, Me-13, and Me-14 were in the β-orientation (Figs. 1 and 2). Therefore, the A/B/C rings in the aglycone of **1** were in turn *trans*-fused.

The glycosidic linkage position at C-4 was determined by the HMBC NMR experiment (Fig. 2). A clear ^3J correlation were found between the anomeric proton at δ 4.30 (1H, d) and C-4 resonating at δ 93.3. The β-glycosidic linkage was deduced based on the observed coupling constant (7.8 Hz) of the anomeric proton. In addition, the enzymatic hydrolysis[17] of **1** with β-glucosidase gave the aglycone **1a**[18] (Table 1) and glucose. The relative stereochemistry of **1a** was further analyzed by its own NOESY NMR experiment, which was in full agreement with those of **1**. The D-configuration of the glucose was identified by direct comparison with an authentic sample using HPLC analysis and optical rotation [purified sugar: $[\alpha]_D^{22}$ +52 (*c* 0.09, H_2O)].[17] Thus, compound **1** was elucidated to be pseudoguaian-1α(H)-8α,12-olide-4β-*O*-β-D-glucopyranoside.

Based on their HR-ESIMS, the molecular formula $C_{15}H_{20}O_4$ of both compounds **2**[19] and **3**[20] were determined to be the same as those of the known guaianolides **7**[3c] and **8**.[10] Compounds **2**, **3**, **7**, and **8**, each possessing an α-methylene-γ-lactone moiety, were found to have similar NMR spectral features (Table 2). The proton and carbon signals of the diastereoisomers **2** and **3** were unambiguously assigned by 2D NMR experiments (COSY, HSQC, and HMBC). Similar to **1**, the relative stereochemistry of **2** and **3** was deduced according to the coupling patterns of the protons bound to the seven-membered ring and the NOE correlations in their NOESY NMR experiments (Fig. 3). In the NOESY spectrum of **2**, clear NOE correlations were observed between H-7 at δ 3.07 and H-5 at δ 2.82, between H-7 and H-6_{eq} at δ 2.18 (ddd, J = 12.6, 4.6, 3.2 Hz), between H-7 and H-9_{ax} at δ 2.08 (dd, J = 13.8, 11.2 Hz), between H-8 at δ 3.83 and H-6_{ax} at δ 1.01 (dd, J = 12.1, 12.1 Hz), between H-8 and H-9_{eq} at δ 2.49 (dd, J = 13.8, 4.4 Hz), between H-8 and Me-14 at δ 1.58, between H-9_{eq} and Me-14, between H-5 and Me-15 at δ 1.39, as well as between H-6_{eq} and Me-15. These data showed that the γ-lactone ring is *trans*-fused at C-7 and C-8 in compound **2**. Therefore, **2** is the C-10 epimer of compound **7**. Similarly (Fig. 3), compounds **3** and **8** are epimers at C-4, and both compounds have a *cis*-fused γ-lactone ring at C-7 and C-8. Therefore, compounds **2** and **3** were elucidated to be 4β,10α-dihydroxy-5α(H)-1,11(13)-guaidien-8α,12-olide and 4β,10β-dihydroxy-5α(H)-1,11(13)-guaidien-8β,12-olide, respectively. Interestingly, the structure of **3** was once presented as a known compound by Hocine Dendougui et al.;[21] however, the structure (without any NMR data)

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of **1**, **1a**, and **4**[a]

Position	**1**[b]		**1a**[c]		**4**[c]	
	δ_H (mult, *J*, Hz)	δ_C (DEPT)	δ_H (mult, *J*, Hz)	δ_C (DEPT)	δ_H (mult, *J*, Hz)	δ_C (DEPT)
1	1.63 brdd (11.1, 10.2)	48.1 (CH)	1.58 brdd (10.3, 9.8)	47.6 (CH)	0.43 m	34.7 (CH)
2	1.72 m, overlapped	26.7 (CH_2)	1.73 m	25.3 (CH_2)	1.30 m (2H)	24.9 (CH_2)
	1.45 m		1.43 m, overlapped			
3	1.99 m	28.8 (CH_2)	1.97 m	28.9 (CH_2)	1.68 m	35.9 (CH_2)
	1.58 m		1.34 m		1.56 m	
4	3.69 dd (9.2, 8.9)	93.3 (CH)	3.74 dd (9.4, 8.3)	82.2 (CH)	4.88 m	70.7 (CH)
5		45.8 (C)		44.4 (C)	0.34 m	22.8 (CH)
6	1.94 dd (14.8, 5.6, H_{eq})	35.7 (CH_2)	1.83 dd (14.6, 5.7, H_{eq})	34.7 (CH_2)	2.35 dd (14.1, 6.9, H_{eq})	30.8 (CH_2)
	1.51 dd (14.8, 12.7, H_{ax})		1.41 m, overlapped		0.90 m	
7	2.48 m	44.2 (CH)	2.41 m	43.2 (CH)	3.15 dddd (11.8, 9.2, 3.1, 3.0)	37.8 (CH)
8	4.47 ddd (11.2, 10.5, 3.3)	84.2 (CH)	4.36 ddd (11.3, 10.3, 3.4)	83.4 (CH)	4.76 ddd (11.2, 9.2, 4.4)	75.6 (CH)
9	2.27 ddd (13.2, 3.6, 3.6, H_{eq})	46.1 (CH_2)	2.33 ddd (13.0, 3.7, 3.6, H_{eq})	44.9 (CH_2)	2.31 dd (13.8, 4.4, H_{eq})	37.3 (CH_2)
	1.24 br dd (12.2, 12.0, H_{ax})		1.23 br dd (12.2, 12.0, H_{ax})		0.94 dd (13.8, 11.2, H_{ax})	
10	1.74 m, overlapped	30.6 (CH)	1.69 m	29.7 (CH)		17.0 (C)
11	2.62 dq (7.9, 7.8)	41.4 (CH)	2.62 dq (7.9, 7.8)	39.8 (CH)		139.0 (C)
12		182.9 (C)		179.9 (C)		170.5 (C)
13	1.16 d (7.8)	11.8 (CH_3)	1.17 d (7.8)	11.6 (CH_3)	6.22 d (2.8), 5.54 d (2.3)	122.5 (CH_2)
14	0.94 s	18.5 (CH_3)	0.87 s	17.3 (CH_3)	1.05 s	18.2 (CH_3)
15	0.95 d (6.4)	20.9 (CH_3)	0.93 d (6.5)	20.5 (CH_3)	1.20 d (6.3)	21.3 (CH_3)
OAc					2.01 s	19.9 (CH_3)
						170.7 (C)
1′	4.30 d (7.8)	106.1 (CH)				
2′	3.19 dd (8.8, 7.8)	75.3 (CH)				
3′	3.34 dd (9.0, 8.8)	78.2 (CH)				
4′	3.29 dd (9.3, 9.0)	71.6 (CH)				
5′	3.24 ddd (9.3, 5.6, 2.1)	77.8 (CH)				
6′	3.83 dd (11.8, 2.1)	62.8 (CH_2)				
	3.66 dd (11.8, 5.6)					

[a] Assignments were made by a combination of 1D and 2D NMR techniques (COSY, HSQC, and HMBC).
[b] Recorded in CD_3OD.
[c] Recorded in $CDCl_3$.

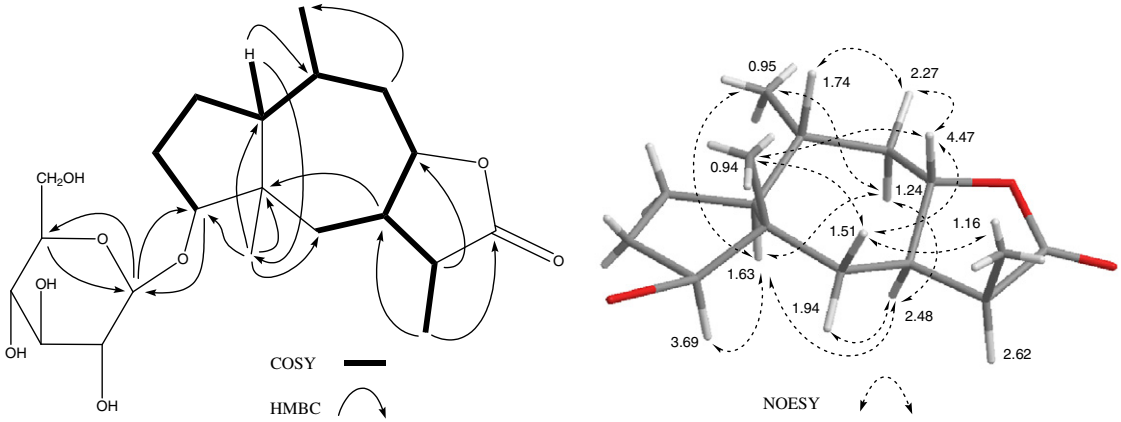


Figure 2. Observed key COSY, HMBC, and NOE correlations of compound **1**.

cited therein was not identical to the original one.[10,21] Thus, **3** is still a new naturally occurring compound.

The molecular formula $C_{17}H_{24}O_4$ of compound **4**[22] was deduced from its positive mode HR-ESIMS, which resulted in an $[M+Na]^+$ ion peak at *m*/*z* 315.1587 (calcd 315.1567). Its ^{1}H and ^{13}C NMR data (Table 1) showed that **4** is an analog of **5**,[3g,8] with an acetoxyl group [δ_H: 2.01 (3H, s, H-17), δ_C: 19.9 (CH_3), 170.7 (C=O)] in place of the hydroxyl group at C-4 in the side chain of **5**. Alcoholysis[23] of **4** with MeOH/MeONa gave **4a**, which was found to be identical to compound **5** by comparing their spectroscopic data and physical properties. To the best of our knowledge, the stereochemistry of C-4 in **4a** (**5**) has not been previously assigned. Compound **4a** was then subjected to a modified Mosher's method[24,25] by treatment with (*S*)- and (*R*)-α-methoxy-α-trifluoromethylphenyl acetic acid (MTPA) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and 4-dimethylaminopyridine (4-DMAP) to give its 4-(*S*)-MTPA ester (**4b**)[26] and 4-(*R*)-MTPA ester (**4c**),[27] respectively. In their ^{1}H NMR spectra, the chemical shifts of Me-15 and H-4 in **4b** could be observed at higher fields ($\Delta\delta_H = \delta_S - \delta_R$: negative value), while the other protons resonated at a lower field ($\Delta\delta_H$: positive value) (Fig. 4) when compared with those of **4c**. This indicated an *S* configuration at C-4 in **4a**. Thus, compound **4** was elucidated as (4*S*)-acetyloxyl-11(13)-carabren-12,8β-olide. Consequently, the structure of **5** was updated as (4*S*)-acetyloxyl-11(13)-carabren-8β,12-olide. Compound **4** was detected in the MeOH extract of the title plant by TLC and HPLC analysis, which indicated that **4** is not an artificial product.

Table 2
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of **2**, **3**, and **7** (in $CDCl_3$)[a]

Position	**2**		**3**		**7**[b]
	δ_H (mult, *J*, Hz)	δ_C (DEPT)	δ_H (mult, *J*, Hz)	δ_C (DEPT)	δ_H (mult, *J*, Hz)
1		151.8 (C)		151.8 (C)	
2	5.68 br s	125.0 (CH)	5.57 br s	122.0 (CH_2)	5.88 dd (3.0, 1.6)
3	2.51 d (17.4)	45.9 (CH_2)	2.40 br s (2H)	46.7 (CH_2)	2.51 d (17.2)
	2.33 dd (17.4, 3.2)				2.31 dd (17.2, 3.2)
4		81.5 (C)		81.3 (C)	
5	2.82 dd (12.6, 4.8)	54.7 (CH)	2.70 br d (12.0)	55.4 (CH)	2.36 dd (12.6, 3.1)
6	2.18 ddd (12.6, 4.6, 3.2, H_{eq})	32.0 (CH_2)	2.06 ddd (13.2, 4.7, 4.4, H_{eq})	32.5 (CH_2)	2.22 ddd (12.6, 3.5, 3.5, H_{eq})
	1.01 br dd (12.2, 12.2, H_{ax})		1.34 br dd, overlapped		1.07 br dd (12.4, 12.2, H_{ax})
7	3.07 dddd (11.8, 9.2, 3.1, 3.0)	45.6 (CH)	3.39 m	42.2 (CH)	2.65 dddd (11.5, 9.8, 3.3, 3.0)
8	3.83 ddd (11.2, 9.2, 4.4)	81.8 (CH)	5.15 ddd (11.7, 4.5, 3.5)	77.5 (CH)	3.97 ddd (11.2, 9.8, 1.4)
9	2.49 dd (13.8, 4.4, H_{eq})	46.5 (CH_2)	2.29 dd (13.8, 4.0, H_{eq})	42.5 (CH_2)	2.50 dd (14.2, 1.3, H_{eq})
	2.08 dd (13.8, 11.2, H_{ax})		1.76 dd (13.8, 12.1, H_{ax})		2.07 dd (14.2, 11.3, H_{ax})
10		70.2 (C)		69.6 (C)	
11		139.8 (C)		140.4 (C)	
12		170.0 (C)		170.1 (C)	
13	6.21 d (3.0)	120.1 (CH_2)	6.26 d (2.8)	121.6 (CH_2)	6.17 d (3.4)
	5.52 d (3.0)		5.59 d (2.4)		5.46 d (3.1)
14	1.58 s	30.7 (CH_3)	1.54 s	29.9 (CH_3)	1.45 s
15	1.39 s	23.3 (CH_3)	1.36 s	24.5 (CH_3)	1.39 s

[a] Assignments were made by a combination of 1D and 2D NMR techniques (COSY, HSQC, and HMBC).
[b] Data were recorded in $CDCl_3$ for the first time.

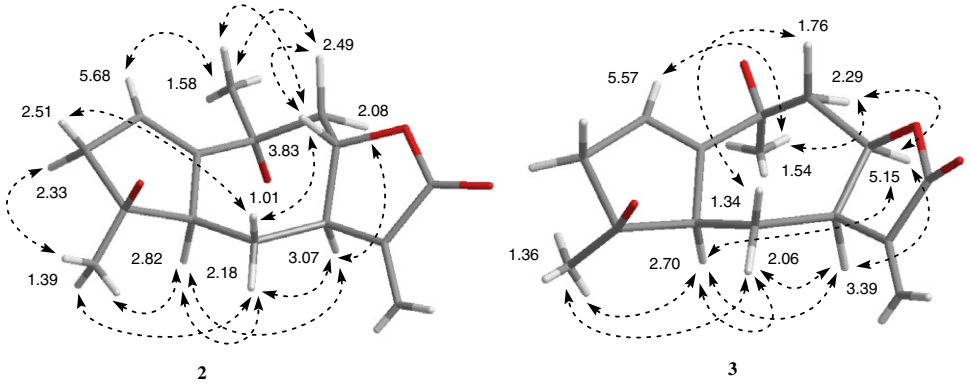
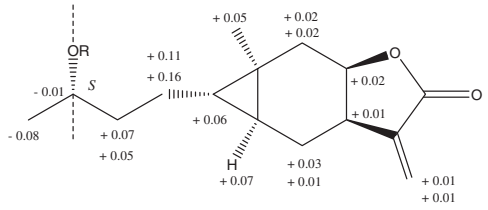


Figure 3. Observed key NOE correlations of compounds **2** and **3**.



4b: R = (*S*)-MTPA
4c: R = (*R*)-MTPA

Figure 4. Results with the modified Mosher's method ($\Delta\delta_H = \delta_S - \delta_R$).

The genus *Carpesium* is a rich source of sesquiterpene lactones.[2,3] From a chemotaxonomical point of view the isolation of different types of sesquiterpene lactones (**1**–**17**) from *C. faberi* may be of interest. Sesquiterpene lactone glycosides (e.g., **1**) have seldom been found from this genus.[3e] All isolated compounds (**1**–**19**) were evaluated for their antiproliferative activities (Table 3) against MCF-7 human breast cancer cells using the MTT assay.[5,28,29] The isolated sesquiterpene lactones (except xanthanolide **17**) with an α-methylene-γ-lactone moiety exhibited

Table 3
Effects of **1**–**19** against MCF-7 cells (mean ± SD, $n = 3$)

Compound	IC_{50} (μg/mL)
1	>50
2	31.7 ± 1.6
3	38.8 ± 0.5
4	9.4 ± 0.3
5	11.4 ± 0.3
6	10.6 ± 0.6
7	31.9 ± 1.6
8	26.5 ± 0.6
9	9.8 ± 0.4
10	10.7 ± 0.1
11	3.9 ± 0.2
12	16.3 ± 1.1
13	3.0 ± 0.1
14	12.5 ± 0.7
15	>50
16	>50
17	>50
18	>50
19	18.6 ± 0.4
5-Fluorouracil	14.1 ± 0.7
Daunorubicin	0.5 ± 0.1

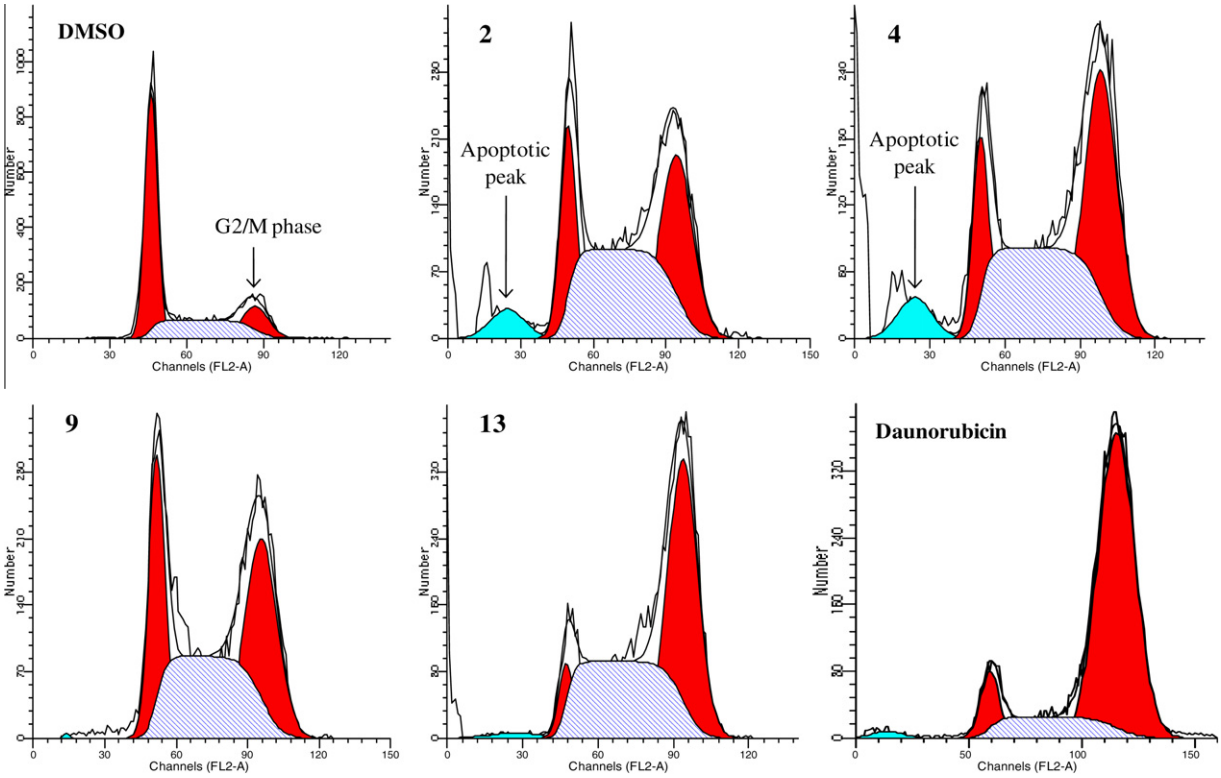


Figure 5. Cell cycle effects of compounds **2**, **4**, **9**, and **13** on MCF-7 cells.

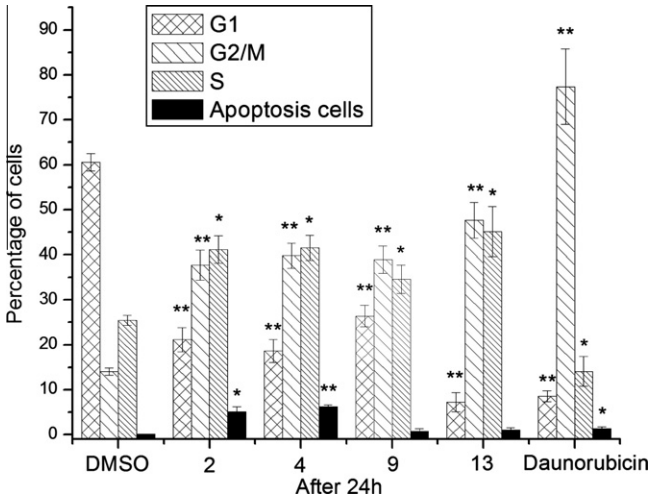


Figure 6. Effects of compounds **2**, **4**, **9**, and **13** on MCF-7 cells after 24 h exposure (mean ± SD, $n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$.

significant in vitro antiproliferative activities, and compounds **11** and **13** were the most potent with an IC_{50} value of 3.9 and 3.0 μg/mL, respectively. Cell cycle analysis[30] indicated that when MCF-7 cells were treated with four selected sesquiterpene lactones (guaianolide **2**, carabranolide **4**, pseudoguaianolide **9**, eudesmanolide **13**) at IC_{50} concentrations for 24 h, the percentage of cells in the G_2/M phase was significantly increased ($p < 0.01$) (Figs. 5 and 6), suggesting that the cancer cells were killed mostly at G_2/M phase of the cell cycle. Moreover, when treating MCF-7 cells with compounds **2** and **4** at IC_{50} concentrations for 24 h, obvious sub-G_1 peaks (apoptosis >5%) were observed (Figs. 5 and 6), demonstrating that these two new compounds may have apoptosis effects on MCF-7 cells.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.138.

References and notes

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6. *Plant material*: The whole plant of *C. faberi* was collected from Changshou County, Chongqing, PR China in August 2008. The plant was identified by

Professor Bao-Kang Huang (Department of Pharmacognosy, The Second Military Medical University, Shanghai, PR China). A voucher specimen (no. 090523) was deposited at the Herbarium of the Shanghai Key Laboratory of Brain Functional Genomics, East China Normal University.

7. *Extraction and Isolation*: The air-dried and pulverized plant material (3.6 kg) was extracted with MeOH (5 L × 6) at room temperature (rt). After removal of the solvent under reduced pressure, the entire residue (260.0 g) was subjected to column chromatography (CC) over silica gel (column: 10 × 80 cm) with a petroleum ether (PE)–acetone (AC) gradient (15:1—AC neat, v/v) followed by AC–MeOH (10:1—MeOH neat, v/v) to yield five fractions (Fr.1–Fr.5). Fr.1 (PE–AC 15:1, 12.9 g) was applied to a silica gel column (4.0 × 25 cm) and eluted with PE–EtOAc (12:1) to give five subfractions (Fr.1.1–Fr.1.5). Fr.1.4 (108.8 mg) was purified by gel permeation chromatography on Sephadex LH-20 (2.5 × 90 cm) in CH_2Cl_2–MeOH (2:1) to afford compound **18** (57.8 mg). Fr.2 (PE–AC 3:1, 25.8 g) was chromatographed on an MCI gel column (5.5 × 35 cm) using a stepwise gradient elution with MeOH–H_2O (from 1:2 to MeOH neat, v/v) to generate five subfractions (Fr.2.1–Fr.2.5). Fr.2.2 (12.5 g) was subjected to a silica gel column (4.0 × 25 cm) eluted with PE–AC (5:1) and further purified by semi-preparative HPLC with an isocratic elution of MeOH–H_2O (68:32, v/v) over 20 min (flow rate: 3 mL/min) to afford compounds **11** (10.2 mg, t_R = 9.2 min) and **13** (55.2 mg, t_R = 14.8 min). Fr.2.3 (69.3 mg) was also purified by semi-preparative HPLC with an isocratic elution of MeOH–H_2O (52:48, v/v) for 30 min (flow rate: 3 mL/min) to afford compounds **6** (31.0 mg, t_R = 21.5 min) and **17** (6.9 mg, t_R = 29.1 min). Fr.2.4 (3.5 g) was subjected to CC over RP-C18 silica gel (column: 3.0 × 25 cm) eluted with a MeOH–H_2O gradient (from 1:1 to MeOH neat, v/v) to furnish compounds **5** (67.3 mg), **9** (161.9 mg), and **12** (96.0 mg). Fr.3 (PE–AC 1:1, 3.3 g) was applied to a silica gel column (2.5 × 25 cm) using CH_2Cl_2–AC (15:1) to give three subfractions (Fr.3.1–Fr.3.3). Compounds **4** (48.8 mg) and **19** (4.2 mg) were purified from Fr.3.1 (165.5 mg) by semi-preparative HPLC with an isocratic elution of MeOH–H_2O (68:32, v/v) over 25 min (flow rate: 3 mL/min; **4**: t_R = 18.5 min, **19**: t_R = 23.8 min). Fr.4 (AC–MeOH 10:1, 25.8 g) was subjected to an MCI gel column (5.5 × 35 cm) eluted with MeOH–H_2O (from 1:4 to MeOH neat, v/v) to afford six subfractions (Fr.4.1–Fr.4.6). Fr.4.2 (1.2 g) was chromatographed on a silica gel column (3.0 × 25 cm) with PE–AC (2:1) and purified by semi-preparative HPLC to afford compounds **7** (5.0 mg), **15** (5.6 mg), and **16** (7.1 mg). The method was an isocratic elution of MeOH–H_2O (23:77, v/v) over 15 min, followed by a linear gradient of MeOH–H_2O (23:77 → 30:70, v/v) for 20 min (flow rate: 3 mL/min; **7**: t_R = 18.4 min, **15**: t_R = 27.5 min, **16**: t_R = 15.8 min). Fr.4.3 (9.2 mg) was purified by semi-preparative HPLC with an isocratic elution of MeOH–H_2O (21:79, v/v) over 2 min, followed by a linear gradient of MeOH–H_2O (21:79 → 25:75, v/v) for 30 min (flow rate: 3 mL/min) to afford compound **8** (3.5 mg, t_R = 24.5 min). Fr.4.4 (2.3 g) was subjected to a silica gel column (3.0 × 25 cm) eluted with a CH_2Cl_2–MeOH gradient (20:1–5:1, v/v), and further purified by semi-preparative HPLC with an isocratic elution of MeOH–H_2O (50:50, v/v) over 20 min (flow rate: 3 mL/min) to furnish compounds **2** (2.9 mg, t_R = 14.8 min) and **3** (2.3 mg, t_R = 16.8 min). Compound **1** (15.6 mg) was purified from Fr.4.5 (30.5 mg) by semi-preparative HPLC with an isocratic elution of MeOH–H_2O (53:47, v/v) over 25 min (flow rate: 3 mL/min; **1**: t_R = 20.4 min). Fr.4.6 (120 mg) was applied to a silica gel column (2.0 × 25 cm), and purified by semi-preparative HPLC to afford compounds **10** (3.3 mg) and **14** (3.6 mg). The method was an isocratic elution of MeOH–H_2O (46:54, v/v) over 50 min, followed by an isocratic elution of MeOH–H_2O (95:5, v/v) for 10 min (flow rate: 3 mL/min; **10**: t_R = 30.3 min, **14**: t_R = 47.7 min).
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16. *Pseudoguaian-1α(H)-8α,12-olide-4β-O-β-D-glucopyranoside* (**1**): White amorphous power; $[\alpha]_D^{22}$ −54 (*c* 0.16, MeOH); IR ν_{max}^{KBr} 3405 (br), 2967, 2933, 2877, 1771, 1454, 1380, 1218, 1076, 1042, 994 cm^{-1}; HRESIMS *m/z* = 437.2147 $[M+Na]^+$ (calcd for $C_{21}H_{34}O_8Na$, 437.2146, Δ = −0.3 ppm); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.
17. *Enzymatic hydrolysis of compound* ***1***: A solution of **1** (8.0 mg) in 0.2 M acetic acid and sodium acetate buffer (pH 5.0, 1.5 mL) was treated with lyophilized almond β-glucosidase (28.2 mg), and was then stirred at 40 °C for 6 h. After cooling, the reaction mixture was partitioned into EtOAc. The aqueous layer was filtered and concentrated under reduced pressure. The residue was purified by gel permeation chromatography on Sephadex LH-20 (2.5 × 90 cm) in MeOH to afford a mono-sugar (2.1 mg), which was then analyzed by HPLC [column: Waters Sugar-pak 1 (300 × 6.5 mm); column temperature: 70 °C; detector: Sedex 80 (SEDERE, France) evaporative light-scattering detector (ELSD); mobile phase: H_2O; flow rate: 0.5 mL/min]. Comparison of the retention time (t_R) in the HPLC-ELSD chromatogram and the optical datum of the purified sugar with those of an authentic sample confirmed that the sugar unit in **1** was D-glucose. The EtOAc layer was also filtered and concentrated under reduced pressure to furnish the aglycone **1a** (3.8 mg), which was purified by preparative TLC (PE–AC 3:1, R_f = 0.6).
18. *Pseudoguaian-1α(H)-4β-hydroxyl-8α,12-olide* (**1a**): Colorless gum; $[\alpha]_D^{22}$ −15 (*c* 0.06, $CDCl_3$); HRESIMS m/*z* = 275.1625 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_3Na$, 275.1618, Δ = −2.5 ppm); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.
19. *4β,10α-Dihydroxy-5α(H)-1,11(13)-guaidien-8α,12-olide* (**2**): White amorphous power; $[\alpha]_D^{22}$ −19 (*c* 0.05, $CDCl_3$); UV (MeOH) λ_{max} (log ε) 217 (3.34); IR ν_{max}^{KBr} 3356 (br), 2958, 2924, 2856, 1766, 1648, 1457, 1374, 1263, 1165, 1061, 1002, 711, 623 cm^{-1}; HRESIMS *m/z* = 287.1243 $[M+Na]^+$ (calcd for $C_{15}H_{20}O_4Na$, 287.1254, Δ = 3.8 ppm); for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 2.
20. *4β,10β-Dihydroxy-5α(H)-1,11(13)-guaidien-8β,12-olide* (**3**): Colorless gum; $[\alpha]_D^{22}$ −25 (*c* 0.04, $CDCl_3$); UV (MeOH) λ_{max} (log ε) 218 (3.40); IR ν_{max}^{KBr} 3412 (br), 2956, 2924, 2856, 1758, 1656, 1459, 1376, 1268, 1175, 1125, 1025, 996 cm^{-1}; HRESIMS *m/z* = 287.1239 $[M+Na]^+$ (calcd for $C_{15}H_{20}O_4Na$, 287.1254, Δ = 5.1 ppm); for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 2.
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22. *(4S)-Acetyloxyl-11(13)-carabren-8β,12-olide* (**4**): Colorless gum; $[\alpha]_D^{22}$ +72 (*c* 0.30, $CDCl_3$); UV (MeOH) λ_{max} (log ε) 220 (2.65); IR ν_{max}^{KBr} 3442 (br), 2935, 2865, 1763, 1733, 1661, 1461, 1373, 1246, 1145, 1035, 996, 950, 814 cm^{-1}; HRESIMS *m/z* = 315.1557 $[M+Na]^+$ (calcd for $C_{17}H_{24}O_4Na$, 315.1567, Δ = 3.1 ppm); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.
23. *Alcoholysis of compound* ***4***: A solution of **4** (20.8 mg) in 2 mL CH_2Cl_2–MeOH (1:20) was first treated with 5.5 mg MeONa and was then dehydrated by Molecular Sieve 4A. The mixture was stirred at room temperature (rt) for 12 h and then worked up as usual to give an oily residue of **4a** (12.9 mg), which was purified by preparative TLC (PE–AC 3:1, R_f = 0.4).
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25. *Preparation of 4-O-(S)-MTPA ester (**4b**) and 4-O-(R)-MTPA ester (**4c**) of **4a***: A solution of **4a** (3.8 mg) in dehydrated CH_2Cl_2 (1.5 mL) was treated with (*S*)-MTPA (44.7 mg) in the presence of EDC·HCl (37.9 mg) and 4-DMAP (24.7 mg). The mixture was stirred (rt) for 4 h and then worked up as usual to give an oily residue of **4b**. Compound **4b** (3.5 mg) was purified by prep. TLC (PE–AC 5:1, R_f = 0.4). Similarly, **4c** (2.5 mg) was subsequently generated from **4a** (2.7 mg) using (*R*)-MTPA (32.4 mg) in a CH_2Cl_2 (1.5 mL) solution containing EDC·HCl (41.7 mg) and 4-DMAP (37.2 mg).
26. *(4S)-11(13)-Carabren-8β,12-olide-4-O-(S)-MTPA ester* (**4b**): HRESIMS *m/z* = 489.1840 $[M+Na]^+$ (calcd for $C_{25}H_{29}O_5F_3Na$, 489.1859, Δ = 4.0 ppm); ^1H NMR ($CDCl_3$, 500 MHz) δ 7.52–7.54 (2H, m), 7.40–7.41 (3H, m), 6.25 (1H, d, *J* = 2.8 Hz, H-13), 5.56 (1H, d, *J* = 2.3 Hz, H-13′), 5.15 (1H, m, H-4), 4.78 (1H, m, H-8), 3.54 (3H, s, OCH_3), 3.15 (1H, m, H-7), 2.36 (1H, m, H-6), 2.32 (1H, m, H-9), 1.79 (1H, m, H-3), 1.67 (1H, m, H-3′), 1.39 (1H, m, H-2), 1.30 (1H, m, H-2′), 1.28 (3H, d, *J* = 6.3 Hz, Me-15), 1.04 (3H, s, Me-14), 0.95 (1H, m, H-9′), 0.89 (1H, m, H-6′), 0.43 (1H, m, H-1), 0.33 (1H, m, H-5).
27. *(4S)-11(13)-Carabren-8β,12-olide-4-O-(R)-MTPA ester* (**4c**): HRESIMS *m/z* = 489.1858 $[M+Na]^+$ (calcd for $C_{25}H_{29}O_5F_3Na$, 489.1859, Δ = 0.3 ppm); ^1H NMR ($CDCl_3$, 500 MHz) δ 7.53–7.54 (2H, m), 7.39–7.40 (3H, m), 6.24 (1H, d, *J* = 2.8 Hz, H-13), 5.56 (1H, d, *J* = 2.2 Hz, H-13′), 5.16 (1H, m, H-4), 4.76 (1H, m, H-8), 3.54 (3H, s, OCH_3), 3.14 (1H, m, H-7), 2.33 (1H, m, H-6), 2.30 (1H, m, H-9), 1.72 (1H, m, H-3), 1.62 (1H, m, H-3′), 1.28 (1H, m, H-2), 1.14 (1H, m, H-2′), 1.36 (3H, d, *J* = 6.3 Hz, Me-15), 0.99 (3H, s, Me-14), 0.93 (1H, m, H-9′), 0.88 (1H, m, H-6′), 0.37 (1H, m, H-1), 0.26 (1H, m, H-5).
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29. *Antiproliferative assay*: MCF-7 cells were obtained from the American Type Culture Collection (ATCC). The inhibition of the cell proliferation was assessed by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT)-based colorimetric assay. Briefly, MCF-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) medium containing heat-inactivated 10% (v/v) fetal bovine serum (FBS) (Bio-west, FRANCE), 100 unit/mL penicillin G sodium salt and 100 unit/mL streptomycin sulfate (Gibco, Grand Island, NY, USA), in a 37 °C incubator under an atmosphere of 5% CO_2. The cells in exponential growth were placed at a final concentration of 4.0 × 10^3 cells/well in a 96-well plate. After 24 h, the cells were treated with compounds at varying concentrations. In order to exclude phototoxicity, the process was kept away from bright light and the cells were incubated in a dark incubator. After 48 h, the cells were incubated in fresh cell culture medium and washed carefully. Then, 100 μL MTT (1 mg/mL) was added. After incubating for an additional 4 h, the supernatant was discarded and 150 μL DMSO (Sigma, USA) was added. After 0.5 h, the optical density (O.D.) of each well was measured at the wavelength of 570 nm using a Power Wave XS microplate reader (Bio-Tek, Germany). 5-Fluorouracil (Sigma, USA) and daunorubicin (Pharmacia Italia S.p.A., Italy) were used as the positive controls, whereas 0.5% DMSO was used as the negative control. The purity of the positive controls and tested compounds ranging from 95.0% to 99.9% was determined by analytical HPLC with ELSD detection. The IC_{50} (the drug concentration reducing by 50% the absorbance in treated cells, with respect to untreated cells) values were calculated from the curves generated by plotting the percentage of the viable cells versus the test concentration on a logarithmic scale using SigmaPlot 10.0 software.

30. *Flow cytometric (FCM) analysis*: MCF-7 cells were used to analyze the cell cycle effects of compounds **2**, **4**, **9**, and **13**. MCF-7 cells were seeded at 10×10^4 cells per well in six-well culture plates. After 24 h, the cells were treated with tested compounds at concentrations equivalent to their IC_{50} values, and incubated for an additional 24 h. Control samples included 0.5% DMSO and 1.8 μg/mL daunorubicin. The cells were then fixed with 70% ethanol at 4 °C overnight, then treated with RNAse (100 μg/mL) for 20 min, stained with propidium iodide (Sigma, USA) for 10 min, and finally analyzed using a FACS Calibur flow cytometer (Becton Dickson, USA). The percentages of cells in G_1, S, and G_2/M phases were determined by ModFit software (Verity Software House). All experiments were performed in triplicate and gave the similar results.