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# Chemical Constituents of *Siegesbeckia orientalis* and Their Anti-Proliferative Activity

**Rongxian Li [#], Di Ge [#], Wenying Yin , Yongqi Zhang , Mengjia Liu , Han Xue , Xianshou Zhao and Na Liu [*]**

*School of Biological Science and Technology, University of Jinan, Jinan 250022, China Jinan, Shandong 250000, P. R. China*

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**Abstract:** A new germacrane-type sesquiterpenoid compound (**1**) and four known compounds (**2-5**) were isolated from *Siegesbeckia orientalis*. Chemical structures of these compounds were elucidated using 1D and 2D NMR spectroscopic data, HR-ESIMS, electronic circular dichroism (ECD) and compared with the literature. Their anti-proliferative effects were evaluated on human triple-negative breast cancer (TNBC) cell line MDA-MB-231. The results demonstrated that compound **2** significantly inhibits the proliferation of MDA-MB-231 cells by inducing apoptosis.

**Keywords:** *Siegesbeckia orientalis*; germacrane-type sesquiterpenoids; anti-proliferative activity. 

## 1. Introduction

Breast cancer ranks as one of the most prevalent malignant neoplasms affecting women worldwide [1]. Clinically, breast cancer is typically classified into four subtypes based on types of receptors: luminal A-like, luminal B-like, HER-2, and triple-negative breast cancer (TNBC) [2]. TNBC is a highly invasive subtype of breast cancer, showing a tendency to recurrence and metastasis [3,4]. Due to the lack of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor-2 (HER-2), TNBC does not respond to traditional endocrine therapy and targeted therapy [5]. Currently, chemotherapy is the primary effective option for TNBC in clinical treatment. However, chemotherapy drugs will develop drug resistance and dose tolerance, causing tumor recurrence and metastasis [6]. Therefore, the discovery and development of new drugs are necessary for TNBC treatment. Natural products with minimal side effects and potent medicinal effects, provide promising and valuable source for developing novel cancer treatments.

Cell death is a fundamental biological process, crucial for development, maintenance of homeostasis, and response to stress in multicellular organisms [7]. The major forms of cell death include apoptosis, necrosis and autophagy [8]. Apoptosis, often referred to as programmed cell death, is a highly regulated process that involves the activation of caspases, resulting in characteristic cellular changes [9,10]. Increasing evidence supports the idea that inducing apoptosis is a key target in cancer

[*] Corresponding author: E-Mail: mls_liun@ujn.edu.cn; Phone:15169062614
[#] These authors contributed equally to this work

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chemotherapy [11,12]. Apoptosis is still the primary mechanism of cytotoxicity of anticancer drugs [13].

*Siegesbeckia orientalis* L. belongs to the genus *Siegesbeckia* in the family Asteraceae, mainly distributed in the tropical regions. The aerial parts of the plant are commonly used in the traditional Chinese medicine, "Xi-Qian-Cao", which have multiple pharmacological properties including heat detoxification, antibacterial and antioxidant [14,15]. In the previous study, we have reported the isolation of twenty-seven germacrane-type sesquiterpenoids from *Siegesbeckia orientalis*, showing that compounds sigesbeckialide A and orientalide had significant cytotoxicity against A549 cells and MDA-MB-231 cells [16]. However, the antitumor aspects of germacrane-type sesquiterpenoid remains largely unknown. To explore this, we further isolated one new germacrane-type sesquiterpenoid and four known compounds from the species and detected their activities against cancer cells, of which compound **2** was found to inhibit MDA-MB-231 tumor cell proliferation by inducing apoptosis. This study therefore provides a detailed analysis of compound separation, their structural elucidation, and anti-proliferative activities.

## 2. Materials and Methods

### *2.1. General Experimental Procedures*

UV spectra were recorded using a Shimadzu UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan); Optical rotation was measured using a Rudolph VI rotameter (Derek Instrument, America); NMR spectra were recorded using an Agilent spectrometer (Agilent Technologies Inc, America); ESIMS and HRESIMS analyses were carried out on Agilent 6460 Triple Quad LC/MS and Agilent 6545 Q - TOF LC/MS spectrometers (Agilent Technologies Inc., Waldbronn, Germany); MCI gel CHP20P (Mitsubishi Chemical Corporation, Tokyo, Japan); Silica gel (Xinchanglai Silicone Co., Ltd, Qingdao, China); $C_{18}$ silica gel filler (Bkmam Biotechnology Co., Ltd, Changde, China); D101-macroporous absorption resin (Lijiang Bio-Technology Co., Ltd, Hebei, China); Thin layer chromatography GF254 plates (Merck KgaA, Germany); Semi-preparative high performance liquid chromatography (Agilent 1220, Agilent Technologies, America); ODS chromatographic column (Agilent Technologies Inc, America); The solvents used for the silica gel columns were all of analytical grade (Tianjin Fuyu Chemical Co., Ltd, Tianjin, China) and the chromatographic reagents used for HPLC (Zhaoxu Chemical Co., Ltd, Jinan, China).

Trypsin containing EDTA (Thermo Fisher Co., America); DMSO (Merck KgaA Co., Germany); SDS-PAGE and BSA (Beyotime Biotechnology Co., Ltd, Guangzhou, China); BCA protein concentration determination kit (Beyotime Biotechnology Co., Ltd, Guangzhou, China); Annexin V-FITC apoptosis kit (Enzyme-linked biotechnology Co., Ltd, Shanghai, China); Primary antibodies against poly (ADPribose) polymerase (PARP; 9542), goat anti-mouse IgG-HRP, primary antibodies against β-actin (47778) and secondary antibodies goat anti-rabbit IgG-HRP (Beyotime Biotechnology Co., Ltd, Guangzhou, China).

### *2.2. Plant Material*

*Siegesbeckia orientalis* was acquired in November 2022 from the Chinese herbal medicine market located in Mianyang City, Sichuan Province, and subsequently authenticated by Dr. Fengying Yang of Jinan University. The specimen was compared and recognized at the Institute of Botany of the Chinese Academy of Sciences, with a herbarium barcode of PE 01882664.

### *2.3. Cell Cultures*

The MDA-MB-231 cells were provided by the Wuhan Pricella Cell Bank of the Chinese Academy of Sciences. MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS) to provide essential nutrients and growth factors for cell growth.

*2.4. SRB Assay*

MDA-MB-231 cells in logarithmic growth phase at a density of about 6000 cells per well were seeded into 96-well plates. After 24 hours, when the cells grew to logarithmic phase, various concentrations of compound **2** (1, 5, 7.5, 10, 15 and 20μM) were added. The cells were then incubated in a 5% $CO_2$ incubator at 37 ℃ for 48 h. Next, cell viability was estimated using the SRB analysis. The cells were fixed with 10% trichloroacetic acid (TCA) at 4°C for one hour and subsequently washed five times with distilled water. After drying, stain with 0.4% SRB at room temperature for 5 minutes. Execss dye was washed away with 1% acetic acid. Subsequently, 150 μL of 10 mM Tris-based solution was added to each well. The absorbance was then measured at 540 nm using TECAN. The $IC_{50}$ value was calculated with GraphPad Prism 7.0. All assays were performed in triplicate across three independent experiments.

*2.5. Western Blot Analysis*

The procedures of cell lysis and protein extraction were carried out as decibed in the literature [17]. After treatment cell with the predetermined concentration or for the specified duration, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethylsulfonyl fluoride (PMSF) protease inhibitor at room temperature for 10 minutes and subsequently boiled for another 10 minutes. Equal amounts of total protein (30 μg) were separated with 14% SDS-PAGE and then transferred onto a PVDF membrane. The PVDF membrane was blocked with 5% (w/v) non-fat dry milk in PBS-Tween 20 (PBST; 0.05%) for one hour. The membrane was then incubated overnight at 4°C with the corresponding primary antibody (diluted 1:1000 in PBST). After three washes with PBST, the membrane was incubated at room temperature with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (at a dilution of 1:5000) for one hour. Immunoreactive bands were finally visualized using an enhanced chemiluminescence (ECL) detection system.

*2.6. Flow Cytometry*

TNBC cells in logarithmic growth phase were inoculated in a 60 mm small dish at $1\times10^5$ cells / mL. After 24 h, they were dosed. To collect celss including the bottom and supernatant of the dish, 500 μL of 1×Binding Buffer was added to resuspend the precipitates without EDTA trypsin. Next, 5 μL of Annexin V-PE and 5 μL of 7-AAD solution were added to each tube. The mixture was gently agitated and incubated in the dark for 15 minutes. Three replicates were performed in this experiment.

*2.7. Extraction and Isolation*

The air-dried and powdered aerial parts of *Siegesbeckia orientalis* (6.0 kg) were extracted three times with 95% EtOH (3×90L) at room temperature. The crude extract (0.9 kg) was evaporated under reduced pressure and partitioned with EtOAc and $H_2O$. The EtOAc extract (450 g) was separated by CC over macroporous resin (D-101) column with EtOH/$H_2O$ system (30%, 50%, 85% and 95% v/v) to afford four fractions. The 85% fraction (35 g) was purified by using MCI gel column (MeOH/$H_2O$, 50%, 80%, 90% and 100%, v/v) to obtain six fractions (Fr. 1-Fr. 6).

Fr. 2 (10.3 g) was purified by RP-C18 silica gel (MeOH/$H_2O$ 30%, 50%, 60% and 70%, v/v) to give four fractions (Fr. 2.1-Fr. 2.4). Fraction 2.3 (580 mg) was eluted by a silica gel column with $CH_2Cl_2$/EtOAc (from 10:1 to 1:1) to obtain three fractions (Fr. 2.3.1 - Fr. 2.3.3). Fr. 2.3.1 (42 mg) was purified by semipreparative HPLC (MeOH/$H_2O$, 45: 55, v/v) to afford compound **2** (2.6 mg, $t_R$=16.5 min) and compound **1** (2.2 mg, $t_R$=17.3 min).

Fr. 3 (8.5 g) was separated by silica gel column chromatography ($CH_2Cl_2$/MeOH, 10:1 to 1:1, v/v) to give six subfractions (Fr. 3.1-Fr. 3.6). Fr. 3.4 (401 mg) was purified on a Sephadex column

($CH_2Cl_2$/MeOH, 1:1, v/v) to yield three fractions (Fr. 3.4.1–Fr. 3.4.3). Fr. 3.4.2 (68 mg) was purified by two recrystallizations to afford compound **4** (3.5 mg). Fr. 3.6 (280 mg) was separated by silica gel column (PE/EtOAc, from 10:1 to 1:1, v/v) to give five fractions (Fr. 3.6.1-3.6.5). Fr. 3.6.3 (29mg) was purified on HPLC (MeOH/$H_2O$, 55:45, v/v) to yield compound **3** (4.0 mg, $t_R$=29.2 min) and **5** (2.9 mg, $t_R$=35.1 min). The structures of compounds **1-5** are as follows (Figure 1).

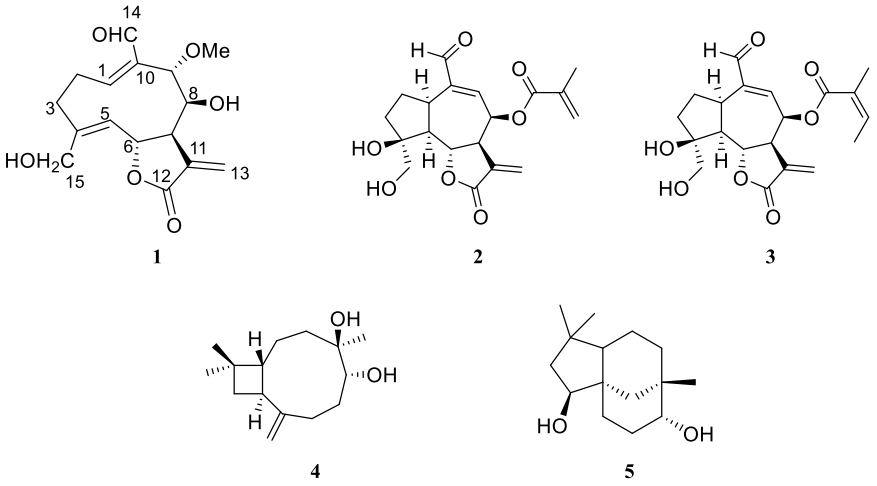


**Figure 1**. Structures of compounds **1**–**5**

*2.8. Spectroscopic Data*

*Orientalis A (**1**):* White powder, $[\alpha]_D^{20}$ -43.42 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 221 (4.19) nm; IR (KBr) $\nu_{max}$ 3441, 2926, 1632, 1595, 1348 $cm^{-1}$; ECD (MeOH) λ (Δε) 201 (+16.35), 225 (−16.72) nm. $^1H$ and $^{13}C$ NMR data, see Table 1. (+)-HR-ESIMS *m/z* 331.1154 $[M + Na]^+$ (calcd for $C_{16}H_{20}O_6Na$, 331.1152).

## 3. Results and Discussion

*3.1. Structure Elucidation of the Compounds*

Compound **1** was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{16}H_{20}O_6$ with seven degrees of unsaturation based on the (+)-HRESIMS (m/z 331.1154 $[M + Na]^+$, calcd 331.1152). The $^1H$ NMR data (Table 1) showed the presence of an aldehyde group [$\delta_H$ 9.48 (d, H-14)], four olefinic protons [$\delta_H$ 5.65 (d, *J* =3.1 Hz, H-13a), 6.34 (d, *J* =3.5 Hz, H-13b), 6.81 (dd, *J* =10.0, 7.6 Hz, H-1) and 4.99 (d, *J* =10.6 Hz, H-5)], two oxymethylene protons [$\delta_H$ 4.39 (s, $H_2$-15)] and three oxymethine protons [$\delta_H$ 5.28 (t, *J* =10.2 Hz, H-6), 5.08 (d, *J* =8.0 Hz, H-8) and 3.79 (dd, *J* =8.0, 2.2 Hz, H-9)]. The $^{13}C$ NMR spectrum (Table 1) showed 16 carbon resonances, which were categorized by DEPT and HSQC spectra as one methoxy group, four methylenes (one oxygenated at $\delta_C$ 61.2), six methines (three oxygenated at $\delta_C$ 73.1, 69.1, 80.2), one aldehyde group ($\delta_C$ 194.7) and four quaternary carbons (three double bonds, one ester carbonyl). Examination of the $^1H$-$^1H$ COSY spectrum (Figure 2) indicated two structural fragments: A (H-1/H-2/H-3) and B (H-5/H-6/H-7/H-8/H-9). In the HMBC spectrum, the correlations from H-14 to C-1, C-9 and C-10, from $H_2$-15 to C-3, C-4 and C-5 and from H-8 to C-6, C-7 and C-9 indicated the occurrence of a 10-membered ring from C-1 to C-10 with two hydroxy groups at C-8 and C-15, an aldehyde group at C-10. There is a methoxy group at C-9, which was validated by the HMBC correlation from 9-OMe to C-9 (Figure 2). In addition, the presence of a fused unsaturated lactone ring at C-6 and C-7 was established by the HMBC correlations from H-13a and H-13b to C-7, C-11 and C-12 and from H-6 to C-12. Thus, the planar structure of **1** was elucidated as shown, representing a new germacrane-type sesquiterpenoid, exhibiting particularly high similarity to compound **11**, which has been documented in existing literature 0. The only difference found was that compound **1** possesses a methoxy group instead of a methacryloxy group as in **11.**

The relative configuration of **1** was determined from the NOESY correlations (Figure 3). The *E*-configuration of the $\Delta^{1,10}$ double bond and the *Z*-configuration of the $\Delta^{4,5}$ double bond were deduced from the NOESY correlations of H-1/H-14 and H-6/H-15α. Then the cross-peaks of H-6/H-9, H-9/H-2*β* led to the assignment of *β*- orientation for H-6 and H-9, which was also consistent with the coupling constant of $J_{8,9}$ (8.0 Hz). While the cross-peaks of H-3*α*/H-5, H-5/H-7 and H-7/H-8 indicated that H-7, H-8 were *α*-orientations (Figure 3). The CD spectrum of compound **1** showed a positive cotton effect at 200.7 nm and a negative cotton effect at 224.7 nm, which was the same as that of the known compound **11**. Further analysis from the perspective of biogenetic relationships confirms that the absolute configuration of compound **1** was similar to compound **11** [16], therefore the absolute configurations of compound **1** can be determined as 6R, 7R, 8S, and 9S. Therefore, the structure of **1** was established as (1(10)E,4Z)-6α,8β,15-trihydroxy-9α-(methoxy)-14-oxogermacra-1(10),4,11(13)-triene-12-oic acid 12,6-lactone and named Orientalis A.

**Table 1.** NMR spectral data for compound **1** in $CDCl_3$ (600/150 MHz)

| No. | **1** | | | | |
|---|---|---|---|---|---|
| | $\delta_H$ (*J*, Hz) | $\delta_C$ | $^1$H-$^{13}$C HMBC | $^1$H-$^1$H COSY | $^1$H-$^1$H NOESY |
| 1 | 6.81 dd (10.0, 7.6) | 157.6 | C-3, C-9, C-10, C-14 | H-2 | H-2α, H-14 |
| 2α | 2.66 m | 27.7 | C-1,C-3,C-4 | H-1 | H-1, H-3α |
| 2β | 2.73 m | | | | H-9 |
| 3α | 2.04 m | 33.0 | C-1, C-2, C-5, C-15 | | H-2α, H-5, H-15α |
| 3β | 2.82 m | | | | |
| 4 | | 139.6 | | | |
| 5 | 4.99 d (10.6) | 130.0 | C-3, C-4, C-7, C-15 | H-6 | H-3α, H-7 |
| 6 | 5.28 t (10.2) | 73.1 | C-4, C-7, C-8 | H-5, H-7 | H-15β, H-9 |
| 7 | 2.45 m | 51.2 | C-5, C-6, C-8, C-13 | H-6, H-8 | H-5, H-8 |
| 8 | 5.08 d (8.0) | 69.1 | C-6, C-7, C-10, C-9 | H-7, H-9 | H-7 |
| 9 | 3.79 dd (8.0, 2.2) | 80.2 | C-1, C-8, C-10, C-14 | | H-2β, H-6 |
| 10 | | 140.9 | | | |
| 11 | | 135.3 | | | |
| 12 | | 169.9 | | | |
| 13a | 5.65 d (3.1) | 121.1 | C-7, C-11, C-12 | | |
| 13b | 6.34 d (3.5) | | | | |
| 14 | 9.48 d | 194.7 | C-1, C-9, C-10 | | H-1 |
| 15 | 4.39 s (2H) | 61.2 | C-3, C-4, C-5 | | H-3α<br>H-6 |
| -OMe | 3.22 s | 57.0 | C-9 | | |

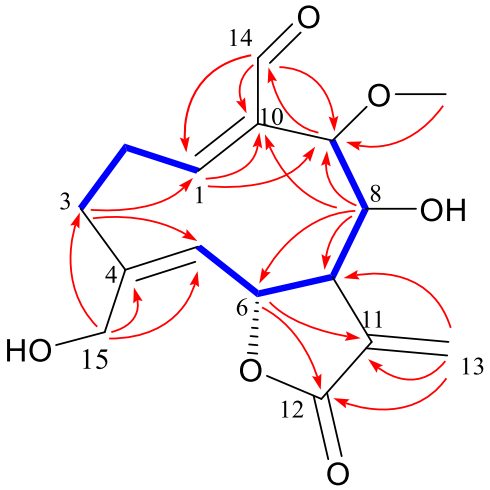


**Figure 2**. $^1$H–$^1$H COSY (—) and HMBC (H→C) correlations of compound **1**

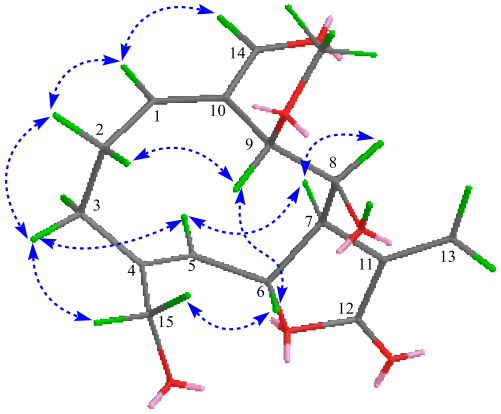


**Figure 3**. NOESY correlations of compound **1**

The known compounds isolated from *Siegesbeckia orientalis* were determined by comparing NMR data to literature values, which were identified as Glabrescone C (**2**) [18], 2-Butenoic acid, 2-methyl-(3a*R*,4*R*,6a*R*,9*S*,9a*S*,9b*S*)-6-formyl-2,3,3a,4,6a,7,8,9,9a,9b-decahydro-9-hydroxy-9-(hydroxymethyl)-3-methylene-2-oxoazuleno[4,5-*b*]furan-4-yl ester (**3**) [19], (1*R*,4*R*,5*R*,9*S*)-4,11,11-Trimethyl-8-ethylenebicyclo[7.2.0]undecane-4,5-diol (**4**) [20], Clovanediol (**5**) [21].

*3.2. Anti-breast Cancer Activity*

The anti-breast cancer activities of compounds **1-5** against human TNBC cell line MDA-MB-231 at the concentration of 20 μM were determined using the SRB method, with adriamycin as the positive control. The results showed that compound **2** exhibited significant anti-proliferative activities. To further investigate the effect of compound **2** on cell proliferation, MDA-MB-231 cells were treated with various concentrations of compound **2** for 48 h (Figure 4). The data suggested that compound **2** markedly reduced the viability of MDA-MB-231 cells in a dose-dependent manner when compared with the untreated control group, with an $IC_{50}$ value of 13.73 μM.

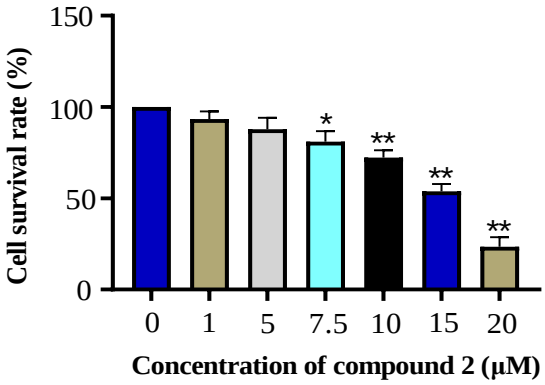


**Figure 4**. Compound **2** had cytotoxic effect on MDA-MB-231 cells.
MDA-MB-231 cells were exposed to different concentrations of **2** (1, 5, 7.5, 10, 15 and 20 μmol/L) for 48 h, and the cell viability was detected by SRB assay. The results are presented as means ± SD. *p < 0.05, and **p < 0.01 versus control group

**Table 2.** Anti-breast cancer activity of isolated compounds ($IC_{50}$ in μM)

| | **1** | **2** | **3** | **4** | **5** | Adriamycin |
|---|---|---|---|---|---|---|
| MDA-MB-231 | >20 | 13.73 ± 1.24 | >20 | >20 | >20 | 4.35 ± 1.08 |

### *3.3. Induced Apoptosis of MDA-MB-231 Cells*

Since compound **2** inhibits cell viability and reduces MDA-MB-231 cell survival, it likely induces cell death through mechanisms such as apoptosis. To investigate how compound **2** induces cell death, we tested several cell death inhibitors: namely 3-methyladenine (3-MA, a potent autophagy inhibitor), Necrostatin-1 (Nec-1, a potent necroptosis inhibitor), and Z-VAD-FMK (Z-VAD, a pan-caspase inhibitor). MDA-MB-231 cells were pretreated with 3-MA, NEC-1 and Z-VAD for 2 h, followed by treatment with different concentrations of compound **2** for 48 h. Cell survival was calculated and the results showed that the cell death induced by compound **2** was reversed only upon the addition of Z-VAD (Figure 5A-C). To further investigate the mechanism, the expression of apoptosis-related protein PARP was determined. Western blotting analysis indicated that compound **2** increased the cleavage of the apoptosis-related protein PARP in a dose-dependent manner (Figure 5D-E) compared to the control group.

To further confirm that validation that the anti-proliferative effect of compound **2** correlates with apoptosis, we employed flow cytometry to assess the proportion of apoptotic cells. MDA-MB-231 cells were incubated in the presence of various concentrations of compound **2** for a duration of 24 h. Results showed a dose-dependent increase in the rate of apoptotic cell death as the drug concentration increased (Figure 5F-G). These findings suggest that compound 2 induced cell death of MDA-MB-231 cells through the activation of apoptosis.

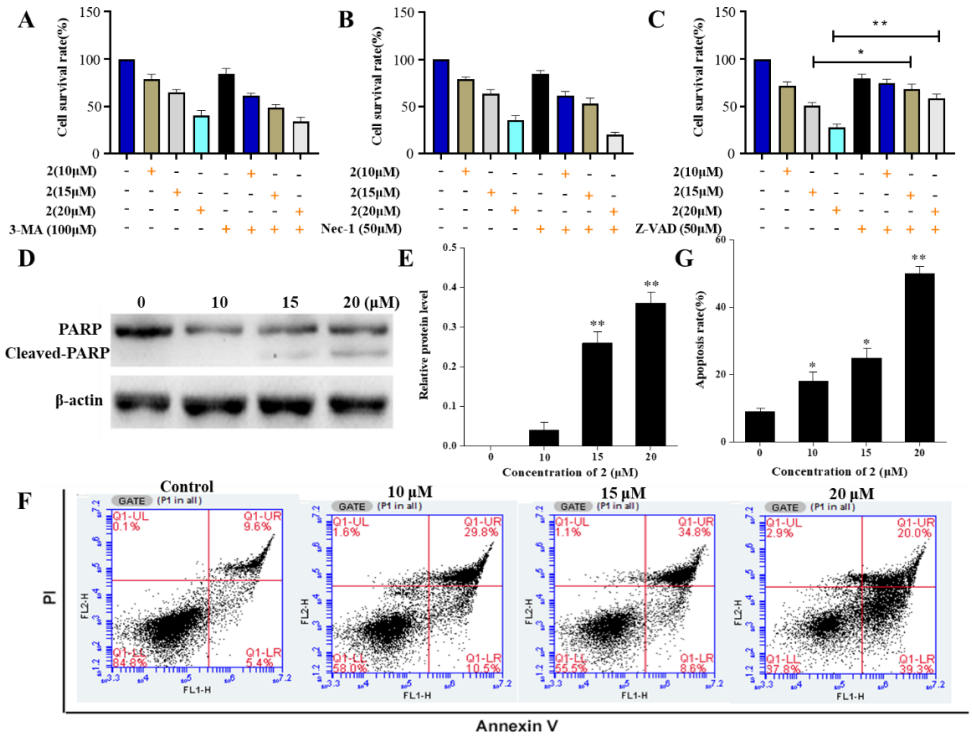


**Figure 5**. Apoptosis was induced by compound **2** in MDA-MB-231 cells.
(A): MDA-MB-231 cells were subjected to treatment with compound **2**, with or without the addition of 3-MA (a potent autophagy inhibitor) for a duration of 48 h, followed by an assessment of growth inhibition. (B): MDA-MB-231 cells were subjected to treatment with compound **2**, with or without NEC-1 (a potent necroptosis inhibitor) for a duration of 48 h to assess growth inhibition. (C): MDA-MB-231 cells were subjected to treatment with Z-VAD (a pan-caspase inhibitor) for a duration of 48 h to assess the effects on growth inhibition. The results are presented as means ± SD. *$p < 0.05$, and **$p < 0.01$ versus control group. (D-E): The cleavage effect of PARP on apoptotic target proteins was assessed using Western blotting following a 24 h treatment of MDA-MB-231 cells with compound **2**. β-Actin protein was used as internal reference. *$p < 0.05$, **$p < 0.01$ compared with control group cells. Data were obtained by at least three independent experiments. (F-G): The percentage of early and late apoptotic cells was assessed using flow cytometry after the treatment of MDA-MB-231 cells with compound **2** for 24 h. The corresponding histograms were shown on the right.

In conclusion, a new germacrane-type sesquiterpenoid and four known compounds were obtained from *Siegesbeckia orientalis*. Our previous research has shown that when the C-8 was replaced by methacryloyl group, the germacrane-type sesquiterpenoid compounds increase antitumor activity. In this study, compound **2** that has a methacryloyl group at C-8 and exhibited significant bioactivity by inhibiting cell proliferation through the mechanism of inducing apoptosis in MDA-MB-231 cells. Compound **2** was found to have the possibility of serving as a potential therapeutic agent in the treatment of tumor diseases.

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## Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

**ORCID**
Rongxian Li: 0009-0005-6386-1152
Di Ge: 0000-0001-8254-2281
Wenying Yin: 0009-0008-3628-3563
Yongqi Zhang: 0009-0002-5494-2506
Mengjia Liu: 0009-0007-6391-1636
Han Xue: 0009-0003-6322-6453
Xianshou Zhao: 0009-0003-6508-9704
Na Liu: 0000-0001-5730-4732

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