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# Synthesis and antitumor activity evaluation of oleanolic acid saponins bearing an acetylated L-arabinose moiety

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ABSTRACT

A series of oleanolic acid derivatives bearing acetyl-substituted L-arabinose moiety has been synthesized and screened *in vitro* for cytotoxicity against ten cancer cell lines and four normal cell lines. The antiproliferative evaluation indicated that synthetic derivatives showed excellent selectivity, as they were toxic against only A431 cell line. Among them, the compound **6** possesses the best inhibitory activity. A series of pharmacology experiments showed that compound **6** significantly induced A431 cells apoptosis and cell cycle arrest, which could serve as a promising lead candidate for further study.

## 1. Introduction

Cancer has become a common and frequently occurring disease that seriously threatens human health [1]. Traditional chemotherapy drugs were once considered as the killers of cancer. However, the drug resistance of cancer cells and the side effects of these treatments on normal tissues are becoming increasingly prominent [2,3]. Therefore, the development of a new generation of antitumor drugs to improve the antitumor efficacy is urgently needed.

Naturally occurring products are important sources of potential drug components with multifaceted effects and targets for cancer therapy. Oleanolic acid (OA) (3β-hydroxyolean-12-en-28-oic acid) is a biologically active pentacyclic triterpenoid found in terrestrial plants such as forage crops, a variety of foods and traditional herbs [4]. Studies have reported that OA and its derivatives exhibit superior and diverse biological and pharmacological activities, including antitumor, anti-inflammatory, anti-HIV and other activities [4–9]. Interest in OA synthesis has been driven by difficulties in isolating and purifying these useful compounds.

The introduction of various glycosyl fragments can enhance the bioactivity of OA [10]. To date, many oleanane-type triterpenoid saponins bearing a disaccharide or trisaccharide containing an α-L-arabinose moiety at the 3-OH position, such as β-hederin [11–13], Raddeanin A [14–16], and hederacolchiside $A_1$ [17], exhibit significant antitumor activities (Fig. 1), which prove that α-L-arabinose moiety may act as a potential antitumor pharmacophore. However, to best of our knowledge, the activity of a single α-L-arabinose moiety at the 3-OH position of OA has rarely been reported. 3-*O*-(4′-*O*-acetyl)-α-L-arabinopyranosyl oleanolic acid **1** is a triterpenoid saponin originally isolated from the resin of *Garcinia hanburyi*. Our interest in this molecule stems from the cytotoxicity it includes in various human tumor cell lines, such as HL60, NB4, U937 and K562 cells [18]. Thus, we postulated that an L-arabinose or acetylated L-arabinose moiety could enhance the tumor cytotoxic of OA. Herein, to investigate the role of the α-L-arabinose moiety and the structure-activity relationship (SAR) of acetyl residues in **1**, we report the design, synthesis, and bioactivity of structural analogues **2**–**7** (Fig. 2).

## 2. Results and discussion

### 2.1. Chemistry

First of all, we chose a more practical route to prepare compound **2,** using 2,3,4-*O*-benzoyl-arabinopyranosyl trichloroacetimidate **10** [19] as the sugar donor. Benzyl esterification of oleanolic acid followed by glycosylation with **10** under the promotion of TMSOTf afforded compound **11** in excellent yield. The debenzoylation of **11** in NaOMe/MeOH without influencing the benzyl ester at C-28 produced the key intermediate **12** [20]. Compound **7** was directly produced by the debenzylation of **12** through catalytic hydrogenation with 10% Pd–C under

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reflux conditions. The acetylation of all three hydroxyl groups of **12** by $Ac_2O$-pyridine and the removal of the benzyl group through catalytic hydrogenation with 10% Pd–C afforded compound **2** in 65% yield. 2, 2-Dimethoxypropane was successfully used to selectively shelter the two hydroxyl groups at C-3 and C-4 of the arabinose residue to yield intermediate **14** [21]. The acetylation of the left hydroxyl groups at C-2 on arabinose followed by deprotection of the isopropylidene group using TsOH was performed to yield compound **16**. The removal of the final benzyl group yielded saponin **3** (Scheme 1).

The selectivity of the 3-OH and 4-OH positions of arabinose has always been of interest in carbohydrate research [22,23], and herein, we describe the two different methods used to achieve the selective acetylation of these positions in the preparation of compounds **4** and **6**. Intermediate **14** was treated with BnBr in the presence of NaH followed by deprotection of the isopropylidene to afford compound **18**. To prepare the intermediate **19**, $Bu_2SnO$ was employed to protect both the 3-OH and 4-OH groups on the arabinose, and then, the one equivalent of AcCl was added [24,25]. Because of the selective activation of the hydroxyl group in the equatorial position of the 1,2-diol by $Bu_2SnO$, the compounds produced were mainly 3-OAc intermediate **19** and a small amount of the by-product 4-OAc, which were separated by silica gel column chromatography. Similarly, removal of the benzyl group of **19** afforded the target compound **4** (**Scheme 2**). The synthesis of compound **5** was based on **18**, in which the acetylation of two hydroxyl groups by $Ac_2O$ afforded **20**, followed by the removal of two benzyl groups (**Scheme 2**). To prepare compound **6,** diol **16** was formed into a cyclic ortho ester with $CH_3C(OEt)_3$ and then cleaved in 50% aq HOAc enable the monoacetylation of the axial 4-OH intermediate **21** in yield of 80.2%, over two steps [26,27]. The debenzylation of **21** directly afforded compound **6** (Scheme 2).

### 2.2. Cytotoxic activity

Natural saponin **1** and its analogues **2**–**7** were screened for their antiproliferation activities against ten human cancer cell lines (leukaemia HL60 cells, epidermal carcinoma A431 cells, liver cancer HepG2 cells, fibrosarcoma HT1080 cells, lung cancer A549 cells, colon cancer HCT116 cells, malignant melanoma A375S2 cells, cervical carcinoma HeLa cells, breast adenocarcinoma MCF-7 cells and leukaemia U937 cells) *in vitro* by the standard MTT method. 5-Fluorouracil (5-FU) was co-assayed as the positive control. The $IC_{50}$ values are presented in Table 1.

The experimental results revealed that compounds **2**, **5** and **6** exhibited moderate to high cytotoxic activity against HL60 cells with $IC_{50}$ values of 13.64, 15.58 and 5.17 μM, respectively; however, compounds **3**, **4** and **7** showed no activity against HL60. This result demonstrated that 4-*O*-acetyl substitution on the arabinose residue can be crucial for activity against HL60. Compounds **2**–**6** with acetyl substitutions on different positions of arabinose induced high cytotoxic activity in A431 cells, which exhibited excellent activities on A431 cells with IC50 values from 2.67 to 14.28 μM, while compound **7**, without acetyl substitution, induced no cytotoxic activity in any of the tested cell lines ($IC_{50}$ > 50 μM). The activity differences suggested that acetyl substitutions were important to the cytotoxic activity in A431 cells.

Selective killing of cancer cells without affecting normal human cell growth is an important feature that must be considered in cancer chemotherapy. Therefore, the most active compound **6** was estimated for possible cytotoxicity towards human normal cells lines (brain microvascular endothelial cells HBMEC, liver cells L-O2, immortalized keratinocyte cells Hacat, and bronchial epithelial cells BEAS-2B). The growth of all four cell lines were not significantly influenced by compound **6** (Table 2), suggesting that compound **6** can selectively inhibit

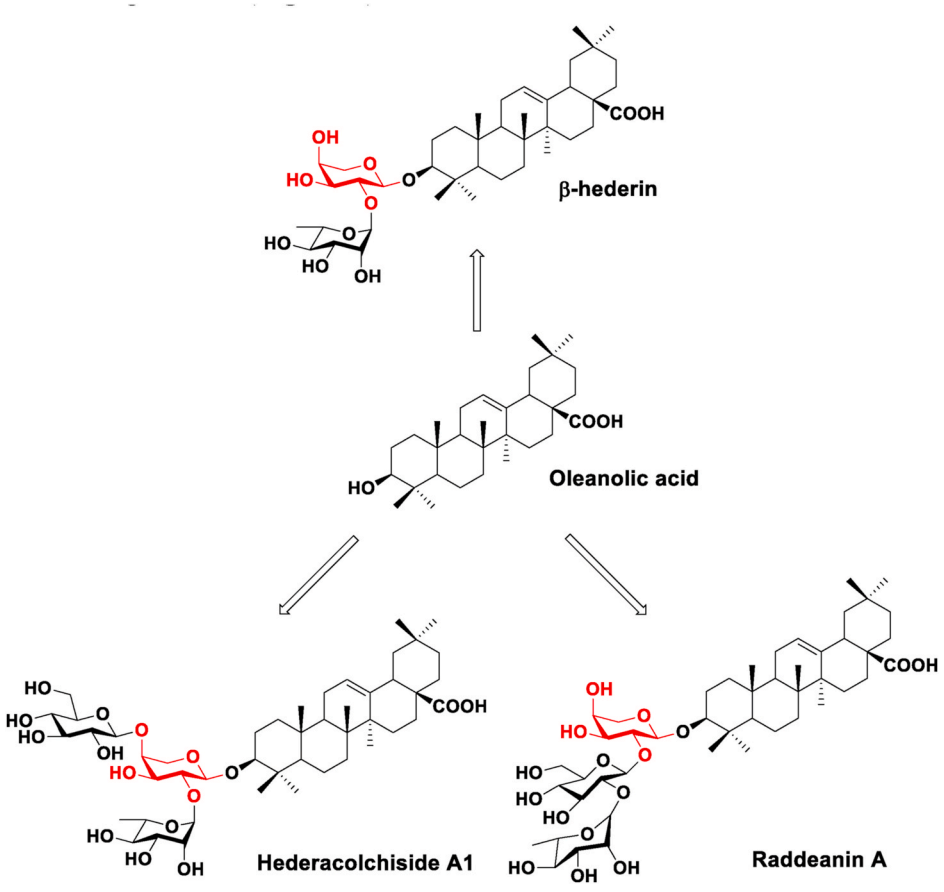



**Fig. 1.** Examples of natural oleanolic acid saponins bearing an L-arabinose moiety at 3-OH position.

the growth of cancer cells. Therefore, the most promising compound **6** was chosen for further exploration of the preliminary mechanism of A431 cell antiproliferation.

### 2.3. Analysis of apoptosis by annexin V-FITC/PI and TUNEL staining

To investigate whether the compound **6** exerts an antitumor effect on A431 cells by inducing apoptosis, an annexin V-FITC/PI binding assay was performed. A431 cells were treated with vehicle or various concentrations (1.5, 4.5, and 7.5 μM) of compound **6** for 48 h and then stained with FITC-annexin V and propidium iodide (PI). The percentages of apoptotic A431 cells were determined by flow cytometry. The results are shown in Fig. 3A, Compound **6** inducing apoptosis in a dose-dependent manner. The percentages of total apoptotic cells (Q1-UR + Q1-LR) were 14.78% (1.5 μM), 21.19% (4.5 μM), 45.15% (7.5 μM), respectively. To observe the apoptosis more intuitively, TUNEL staining was also performed, and the results are shown in Fig. 3B. The number of cells that emitted red fluorescence (apoptotic cells) increased as the administration concentration increased. In addition, obvious changes in cell morphology were also observed. These results demonstrated that compound **6** can induce A431 cell apoptosis and thus exert an antitumor effect.

### 2.4. Cell cycle analysis

Cell cycle arrest is also one of the key factors inhibiting the proliferation of tumor cells. The cell cycle distribution was determined using flow cytometry to determine whether the inhibition of proliferation induced by compound **6** was associated with the abnormal regulation of the cell cycle. As shown in Fig. 4, when A431 cells were treated with compound **6** at 0, 1.5, 4.5 and 7.5 μM for 48 h, there was an increase in the percentages of cells in the G0/G1 phase of the cell cycle, from 55.37% to 62.03%, 66.64% and 75.19%, respectively. The cells in the S and G2/M phases showed a downward trend compared with the vehicle control. These results revealed that compound **6** can arrest A431 cells at the G0/G1 phase.

### 2.5. Changes in mitochondrial membrane potential

Mitochondria play votal roles in the induction and suppression of apoptosis. Cell apoptosis is frequently accompanied by changes in mitochondrial membrane potential (MMP). To probe the role of mitochondria in compound **6**-induced A431 cell apoptosis, we explored mitochondrial membrane potential changes by flow cytometric analysis using JC-1 staining. As shown in Fig. 5., the flow cytometry studies revealed a concentration-dependent decrease in MMP after cells were treated with 0–7.5 μM of compound **6**, and the proportion of A431 cells with depolarized mitochondria was increased (10.26%, 18.71% and 50.72%, respectively) compared to the control group (1.27%).

### 2.6. Western blotting

Cell apoptosis is an active process involving the activation, expression and regulation of a series of proteins. Based on the JC-1 staining results, we further analyzed the expression of proteins related to apoptosis using a Western blot assay with β-actin was used as an internal control. As shown in Fig. 6., the expression levels of Bcl-2 and Bax, which are related to the mitochondrial membrane potential (MMP) were inversely correlated. Compound **6** significantly downregulated the expression of Bcl-2 (from 0.53 to 0.27) and upregulated the expression of Bax (from 0.40 to 1.03), which caused the mitochondrial membrane potential to decrease and cytochrome C to be released. As expected, the levels of cytochrome C increased in a dose-dependent manner in the presence of compound **6**, from 0.27 to 0.78. Since an excess of cytochrome C can enhance caspase activation to stimulate apoptosis, the expression of caspase-3 in A431 cells was also detected. Obviously, the level of caspase 3 increased notably, from 0.37 to 0.98, upon treatment with various concentrations (1.5, 4.5, and 7.5 μM) of compound **6**. The loss of MMP and the results of the Western blot assay indicated that compound **6** induces A431 cell apoptosis via the mitochondrial apoptotic pathway.

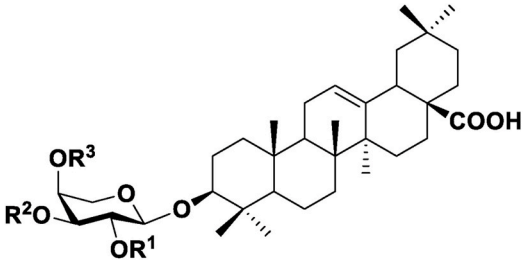
## 3. Conclusion

In conclusion, six analogues of natural saponin **1** with acetyl substitution on different positions of α-L-arabinose have been efficiently and practically synthesized. The use of two different methods to selectively acetylate the 3-OH and 4-OH on α-L-arabinose circumvented the use of the tedious protection-deprotection method. *In vitro* anticancer activity tests of a panel of ten human cancer cell lines and four human normal cell lines using an MTT assay showed that the synthesized saponins with acetyl substituents **2**–**6** demonstrated excellent activities for HL60 and A431 cell lines. The acetyl groups of these compounds were beneficial in terms of both anticancer activity and selectivity. Further investigations on the bioactivity mechanism of the most promising compound **6** indicated that the derivative could induce cell cycle arrest and apoptosis via mitochondrial pathway to exert antitumor effects, which could be used as a valuable lead compound for further study.

## 4. Experimental

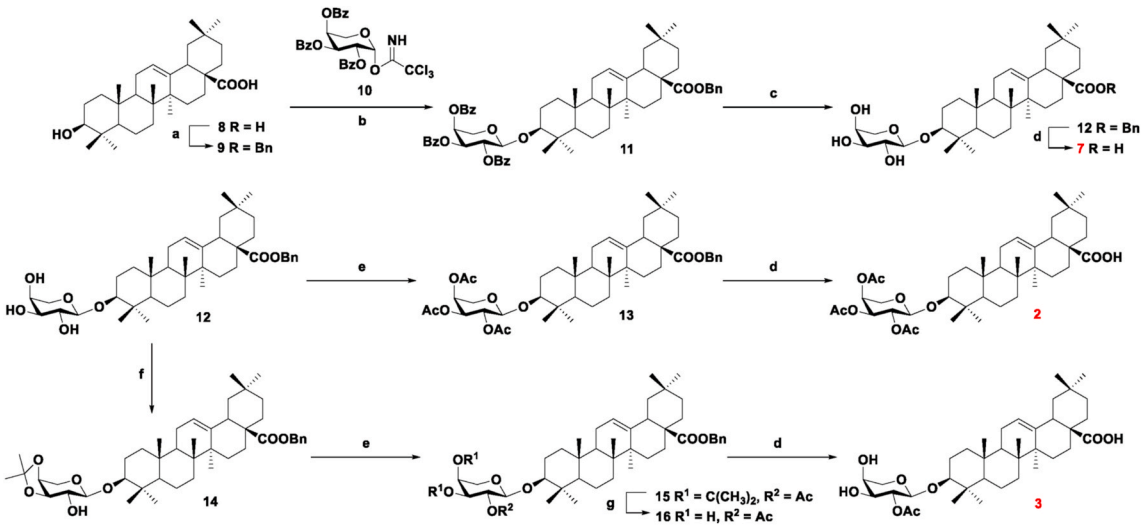
### 4.1. General chemistry experimental information

All the commercial reagents were used without further purification unless otherwise specified. Solvents were dried and redistilled prior to use in the usual manner. Analytical TLC was performed with silica gel HF254. Preparative column chromatography was performed with silica gel H. Melting points were detected with BÜCHI Melting Point B-540. Optical rotations were measured at the sodium D-line at room temperature with a Perkin–Elmer 241 MC polarimeter. $^{1}H$ NMR and $^{13}C$ NMR spectrum recorded on a Bruker ARX 600 MHz spectrometer, using $Me_4Si$ as the internal standard if not specially mentioned. *J* values are given in hertz. ESI-MS were obtained on an Agilent ESI-QTOF instrument.

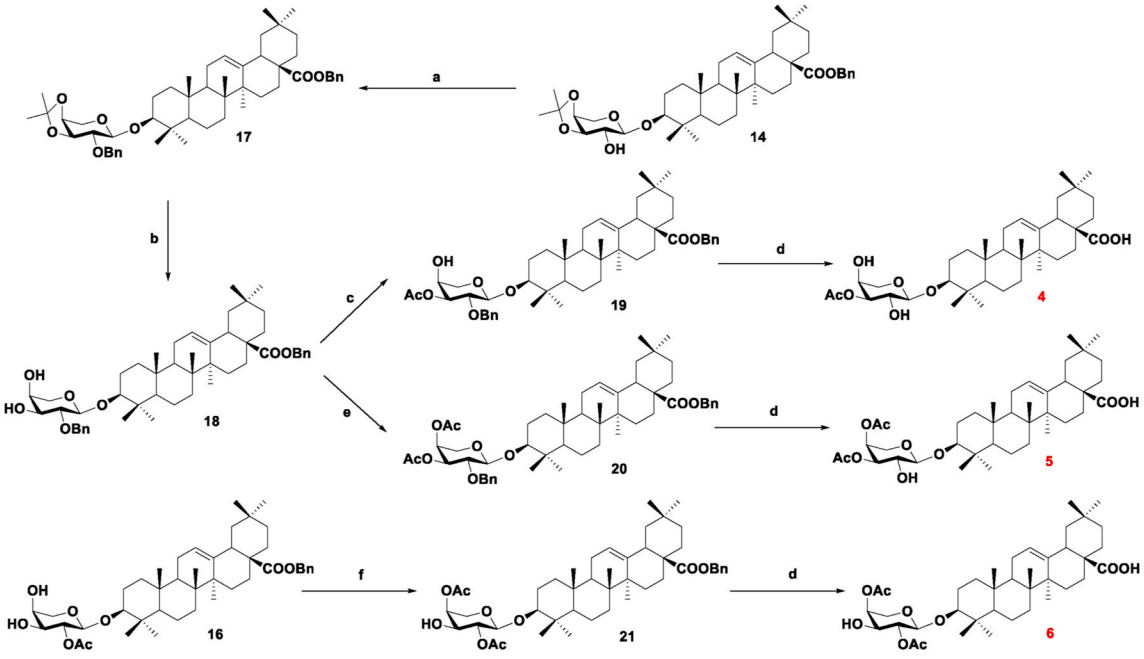



| | | | |
|---|---|---|---|
| **1** | $R^1$ = H | $R^2$ = H | $R^3$ = Ac |
| **2** | $R^1$ = Ac | $R^2$ = Ac | $R^3$ = Ac |
| **3** | $R^1$ = Ac | $R^2$ = H | $R^3$ = H |
| **4** | $R^1$ = H | $R^2$ = Ac | $R^3$ = H |
| **5** | $R^1$ = H | $R^2$ = Ac | $R^3$ = Ac |
| **6** | $R^1$ = Ac | $R^2$ = H | $R^3$ = Ac |
| **7** | $R^1$ = H | $R^2$ = H | $R^3$ = H |

**Fig. 2.** The structures of compound **1** and its analogues **2**–**7**.



**Scheme 1.** Reagents and conditions: (a) BnBr, $K_2CO_3$, TBAB, $H_2O$, $CH_2Cl_2$, overnight, 91%; (b) TMSOTf, $CH_2Cl_2$, 4 Å MS, 0 °C,$N_2$, 30 min, 86%; (c) MeONa, MeOH–$CH_2Cl_2$ 2:1, v/v, 10 min, 89%; (d) 10% Pd–C, $H_2$, EtOAc, r.t., 4 h; (e) $Ac_2O$, pyridine, 12 h; (f) $Me_2C(OMe)_2$, PTSA, $CH_2Cl_2$, 2 h, 89%; (g) PTSA, MeOH–$CH_2Cl_2$ 2:1, v/v, 6 h, 85%.



**Scheme 2.** Reagents and conditions: (a) NaH, DMF, BnBr, 0 °C ~ rt, 24 h; (b) TsOH, MeOH–$CH_2Cl_2$ 2:1, v/v, 6 h; (c) $Bu_2SnO$, toluene, reflux; then AcCl, rt; 32% for three steps; (d) 10% Pd–C, $H_2$, EtOAc, r.t., 4 h; (e) $Ac_2O$, pyridine, 12 h; (f) $CH_3C(OEt)_3$, TsOH, $CH_2Cl_2$, 5 h then 50% HOAc-$H_2O$, overnight, 80%.

**Table 1**
$IC_{50}$ values (μM/L) of compound **1**–**7**, OA and 5-FU against ten human cancer cell lines.

| Comp | HL60 | A431 | HepG2 | HT1080 | A549 | HCT116 | A375S2 | HeLa | MCF-7 | U937 |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 4.70 | 5.88 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| **2** | 13.64 | 9.61 | >50 | >50 | >50 | >50 | >50 | 16.68 | >50 | >50 |
| **3** | >50 | 14.28 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| **4** | >50 | 9.44 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| **5** | 15.58 | 3.53 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| **6** | 5.17 | 2.67 | >50 | >50 | >50 | >50 | >50 | 9.13 | >50 | >50 |
| **7** | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| OA | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| 5-FU | 27.43 | 37.18 | >50 | 15.25 | >50 | 38.38 | 35.28 | 26.18 | 26.14 | 18.22 |

**Table 2**
$IC_{50}$ Values (μM/L) of compound **6** against four human normal cell lines.

| Comp | HBMEC | L-O2 | Hacat | BEAS-2B |
|---|---|---|---|---|
| **6** | >50 | >50 | >50 | >50 |

### 4.2. Synthesis and characterizations of target compound

1 Oleanolic acid 3-*O*-(2′,3′,4′-tri-*O*-acetyl)-*α*-L-arabinopyranoside (2)

To a solution of compound **12** (300 mg, 0.44 mmol) in pyridine (10 mL) was added acetic anhydride. The mixture was stirred for 12 h followed by addition of water (10 mL). The reaction mixture was extracted with $CH_2Cl_2$ (15 mL) for three times. The organic layer was separated and successively washed with water, diluted HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $Na_2SO_4$ and concentrated to afford **13**. The mixture of compounds **13** (170 mg, 0.21 mmol) and 10%Pd–C (20 mg) in EtOAc (20 mL) was stirred under $H_2$ for 4 h. Pd–C was removed through filtration, and the filtrate was concentrated and purified by a silica gel column chromatography (3:1, petroleum ether-acetone) to afford **2** (110 mg, 73%) as a white foam. [α]25 D = +64.8 (*c* 1.08, $CHCl_3$); $R_f$ = 0.3 (3:1, petroleum ether-acetone); Mp 193.0–197.2 °C; $^1$H NMR ($CDCl_3$, 600 MHz): 5.27 (br s, 1H, H-12), 5.24–5.23 (m, 2H, H-2′, H-3′), 5.04 (dd, 1H, *J* = 9.6, 3.6 Hz, H-4′), 4.45 (d, 1H, *J* = 7.2 Hz, H-1′), 4.00 (dd, 1H, *J* = 13.5, 3 Hz, H-5′-1), 3.60 (br d, 1H, *J* = 12 Hz, H-5′-2), 3.08 (dd, 1H, *J* = 11.4, 4.8 Hz, H-3), 2.81 (dd, 1H, *J* = 13.8, 3.6 Hz, H-18), 2.13, 2.06, 2.02 (s, 3 × 3H, CH3CO), 1.12, 0.93, 0.92, 0.90, 0.90, 0.75, 0.73 (s, 7 × 3H, Me); $^{13}$C NMR (CDCl3, 150 MHz):183.9, 170.5, 170.3, 169.4, 143.6, 103.4, 90.2, 77.3, 77.0, 69.6, 67.9, 65.6, 63.3, 55.5, 47.6, 41.5, 40.9, 39.3, 38.9, 38.4, 36.8, 33.1, 32.6, 32.4, 30.7, 29.7, 27.3, 27.7, 25.9, 23.6, 23.4, 22.9, 21.0, 20.7, 19.2, 18.1, 17.1, 16.2, 15.3, 14.1, 13.7. ESI-MS(*m/z*): 737.6 [ $(M + Na)^+$ ], 753.5 [ $(M + K)^-$ ], 713.1 [ $(M - H)^-$].

2 Oleanolic acid 3-*O*-(2′-*O*-acetyl)-*α*-L-arabinopyranoside (3)

To a solution of compound **14** (600 mg, 0.83 mmol) in pyridine (10 mL) was added acetic anhydride. The mixture was stirred for 6 h followed by addition of water (10 mL). The reaction mixture was extracted with $CH_2Cl_2$ (15 mL) for three times. The organic layer was separated and successively washed with water, diluted HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $Na_2SO_4$ and concentrated to afford crude **15**. TsOH (310 mg) was added to a solution of **15** in dry $CH_2Cl_2$–MeOH (1:2, 15 mL) and the solution was stirred at rt for 6 h. Then $Et_3N$ (0.5 mL) was added to quench the reaction followed by concentration under reduced vacuum to afford **16.** Compound **16** and 10% Pd–C (60 mg) in EtOAc (20 mL) was stirred under $H_2$ for 4 h. Pd–C was removed through filtration, and the filtrate was concentrated and purified by a silica gel column chromatography (2:1, petroleum ether-acetone) to afford **3** (240 mg) as a white foam. [α]25 D = +59.0 (*c* 1.07, $CHCl_3$); $R_f$ = 0.4 (1:1, petroleum ether-acetone); Mp 250.8–253.6 °C; $^1$H NMR ($CDCl_3$, 600 MHz): 5.24 (s, 1H, H-12), 5.02 (dd, 1H, *J* = 5.4, 4.2 Hz, H-2′), 4.38 (d, 1H, *J* = 4.2 Hz, H-1′), 3.85–3.82 (m, 2H, H-3′, H-5′-1), 3.65 (dd, 1H, *J* = 7.2, 3.6 Hz, H-4′), 3.56 (dd, 1H, *J* = 12, 3.6 Hz, H-5′-2), 3.09 (dd, 1H, *J* = 12, 4.8 Hz, H-3), 2.84 (dd, 1H, *J* = 13.8, 3.6 Hz, H-18), 2.06 (s, 3H, $CH_3CO$), 1.15, 0.96, 0.94, 0.93, 0.90, 0.81, 0.74 (s, 7 × 3H, Me); $^{13}$C NMR (DMSO-$d_6$, 150 MHz): 180.4, 170.6, 143.8, 122.2, 103.6, 89.3, 72.9, 71.3, 68.7, 55.5, 46.2, 41.5, 41.3, 39.2, 38.6, 38.2, 36.5, 33.5, 32.6, 32.4, 32.2, 30.2, 27.4, 27.0, 25.6, 23.1, 22.7, 22.6, 19.9, 18.0, 16.3, 15.6, 14.5. ESI-MS (*m/z*): 653.4 [ $(M + Na)^+$ ], 629.1 [ $(M - H)^-$].

3 Oleanolic acid 3-*O*-(3′-*O*-acetyl)-*α*-L-arabinopyranoside (4)

To a solution of compound **14** (360 mg, 0.50 mmol) in dry DMF (10 mL) was added NaH (200 mg) at rt. The mixture was stirred for 45 min, then benzyl bromide (0.40 mL) was added, and the reaction was stirred at 40 °C for further 24 h. The mixture was diluted by $CH_2Cl_2$ (10 mL) and washed by water. The organic layer was separated and successively washed with diluted HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $Na_2SO_4$, and concentrated to afford **17**. TsOH (310 mg) was added to a solution of **17** in dry $CH_2Cl_2$–MeOH (1:2, 15 mL) and the solution was stirred at rt for 6 h. Then $Et_3N$ (0.5 mL) was added to quench the reaction followed by concentration under reduced vacuum to afford **18.** The mixture of **18** and $Bu_2SnO$ (105 mg, 0.429 mmol) in dry toluene (8

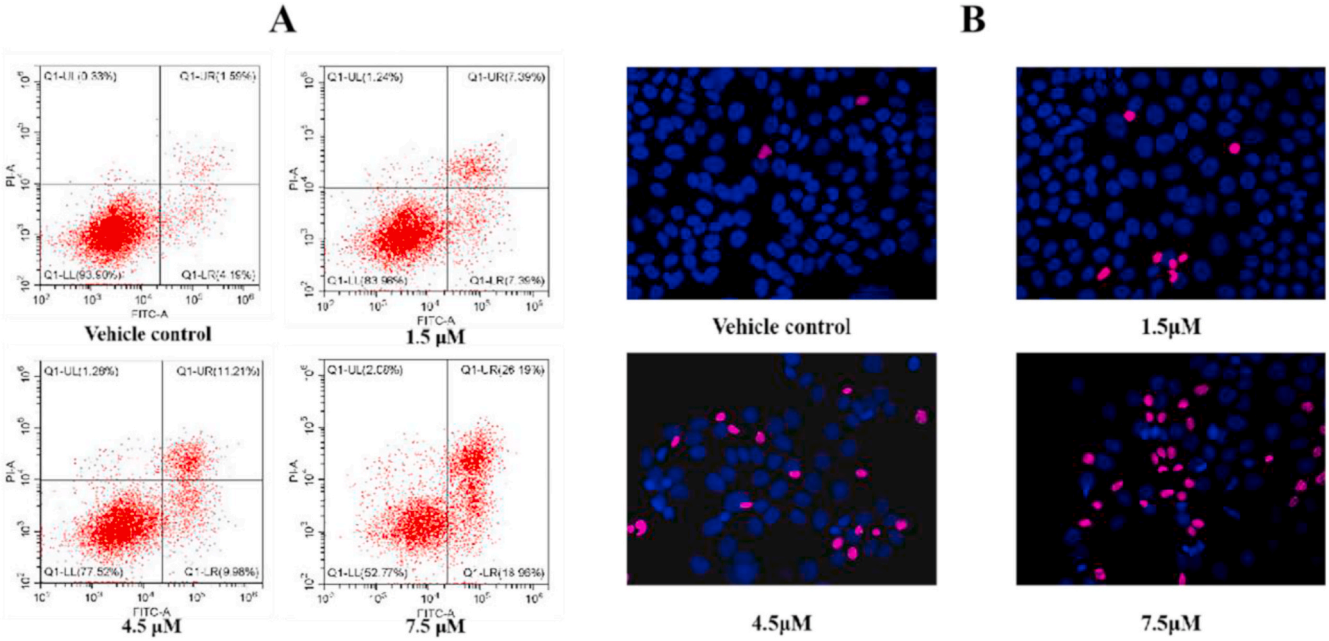



**Fig. 3.** Apoptosis effects on A431 cells induced by compound **6**. (A) Compound **6** induced apoptosis in A431 cells. A431 cells were incubated with varying concentrations of compound **6**. After 48 h of incubation, cells were collected and stained with Annexin V/PI, followed by flow cytometric analysis. (B) Cell morphological alterations and nuclear changes associated with A431 cells after treat with varying concentrations of compound **6** were assessed by TUNEL staining and visualized by fluorescence microscopy. Bars denote 100 $\mu$m.

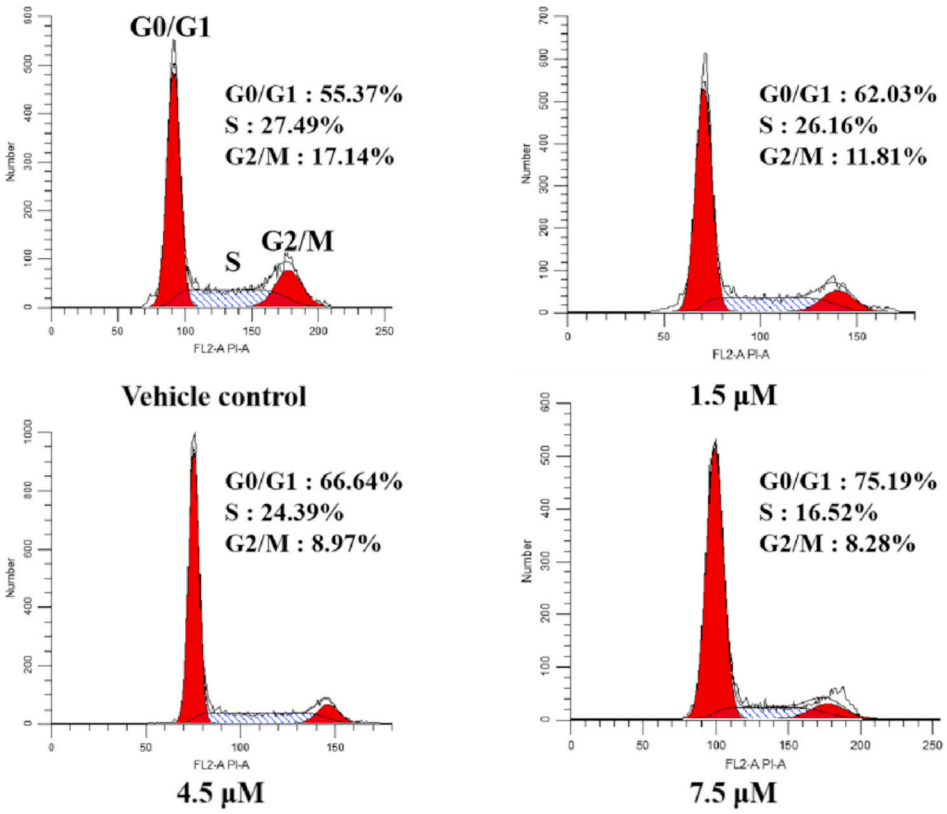



**Fig. 4.** Effects of compound **6** on the cell cycle distribution of A431 cells.

mL) was reflux for 1 h till the mixture was completely dissolved. Then the solution was cooled to r.t., and a solution of AcCl in toluene (10% v/v, 0.3 mL) was added dropwise. The resulting solution was stirred for 8 h, concentrated and purified through a silica gel column chromatography (10:1, petroleum ether-EtOAc) to afford **19** (100 mg, 32% for three steps) as a white foam. The mixture of compound **19** (200 mg, 0.25 mmol) and 10% Pd–C (20 mg) in EtOAc (20 mL) was stirred under $H_2$ for 4 h. Pd–C was removed through filtration, and the filtrate was concentrated and purified by a silica gel column chromatography (2:1, petroleum ether-acetone) to afford **4** (110 mg, 70.7%) as a white foam. [α]25 D = +61.0 (*c* 1.05, $CHCl_3$); $R_f$ = 0.4 (1:1, petroleum ether-acetone); Mp 270.5–274.0 °C; $^1$H NMR ($CDCl_3$, 600 MHz): 5.21 (br s, 1H, H-12), 4.81 (dd, 1H, *J* = 9.6 Hz, 3, H-3′), 4.25 (d, 1H, *J* = 7.2 Hz, H-1′), 3.96 (br s, 1H, H-4′), 3.79 (dd, 1H, *J* = 12.6, 2.4 Hz, H-5′-1), 3.51 (dd, 1H, *J* = 9.6, 7.2 Hz, H-2′), 3.11 (dd, 1H, *J* = 12, 4.8 Hz, H-3), 2.75 (dd, 1H, *J* = 13.8, 3 Hz, H-18), 2.10 (s, 3H, $CH_3CO$), 1.05, 0.93, 0.86, 0.85, 0.83, 0.74, 0.67 (s, 7 × 3H, Me); $^{13}$C NMR ($CDCl_3$, 150 MHz): 183.2, 170.7, 143.6, 122.6, 105.6, 104.9, 90.1, 89.8, 74.6, 70.2, 67.2, 65.9, 65.6, 55.5, 47.6, 46.5, 45.9, 41.2, 40.9, 39.3, 39.1, 38.4, 36.8, 33.8, 33.1, 30.7, 28.3, 27.7, 25.9, 23.6, 21.1, 18.1, 17.1, 16.6, 15.3. ESI-MS (*m/z*): 629.2 [ $(M - H)^-$].

4 Oleanolic acid 3-*O*-(3′,4′-di-*O*-acetyl)-α-L-arabinopyranoside (5)

To a solution of compound **18** (390 mg, 0.51 mmol) in pyridine (10 mL) stirred at rt was added acetic anhydride. The mixture was stirred for 12 h followed by addition of water (10 mL). The reaction mixture was extracted with $CH_2Cl_2$ (15 mL) for three times. The organic layer was separated and successively washed with water, diluted HCl, saturated $NaHCO_3$ and saturated NaCl, dried $Na_2SO_4$ and concentrated to afford crude **20** $R_f$ = 0.8 (2:1, petroleum ether-acetone). To a mixture of compounds **20** and 10% Pd–C (43 mg) in EtOAc (20 mL) was stirred under $H_2$ for 4 h. Pd–C was removed through filtration and the filtrate was concentrated and purified by a silica gel column chromatography (3.5:1, petroleum ether-acetone) to afford **5** (220 mg) as a white foam. [α]25 D = +54.2 (*c* 1.03, $CHCl_3$); $R_f$ = 0.4 (2:1, petroleum ether-acetone); Mp 251.2–254.2 °C; $^1$H NMR ($CDCl_3$, 600 MHz): 5.28 (t, 1H, *J* = 3.6 Hz, H-12), 5.24 (t, 1H, *J* = 1.8 Hz, H-4′), 4.95 (dd, 1H, *J* = 10.2, 3.6 Hz, H-3′), 4.33 (d, 1H, *J* = 7.2 Hz, H-1′), 3.98 (dd, 1H, *J* = 13.2, 1.8 Hz, H-5′-1), 3.87 (dd, 1H, *J* = 10.2, 7.8 Hz, H-2′), 3.62 (d, 1H, *J* = 12.6 Hz, H-5′-2), 3.18 (dd, 1H, *J* = 12.0, 4.8 Hz, H-3), 2.82 (dd, 1H, *J* = 13.8, 4.2 Hz, H-18), 2.12 (s, 3H, $CH_3CO$), 2.07 (s, 3H, CH3CO), 1.12, 1.01, 0.93, 0.92, 0.90, 0.83, 0.74 (s, 7 × 3H, Me); $^{13}$C NMR ($CDCl_3$, 150 MHz): 183.7, 170.4, 143.6, 122.6, 105.6, 90.1, 72.2, 70.0, 68.3, 64.2, 55.5, 46.5, 41.5, 40.9, 39.3, 39.0, 36.8, 33.1, 30.6, 29.7, 28.3, 26.0, 23.6, 22.7, 20.9, 20.8, 17.1, 16.6, 15.3, 14.1. ESI-MS (*m/z*): 695.7 [ $(M + Na)^+$ ], 711.7 [ $(M + K)^+$ ].

5 Oleanolic acid 3-*O*-(2′,4′-di-*O*-acetyl)-α-L-arabinopyranoside (6)

A solution of **16** (960 mg, 1.33 mmol), $CH_3C(OEt)_3$ (1.22 mL, 6.65 mmol) and TsOH (23 mg) in $CH_2Cl_2$ (50 mL) was stirred at rt for 5 h, then the solvent was removed in vacuum. The mixture was then dissoloved in 50% aq HOAc (10 mL) and vigorous stirred overnight. Coevaporation with toluene and purified by a silica gel column chromatography (3:1, petroleum ether-acetone) to afford **21** (810 mg, 79.7%) as a white foam. To a mixture of compounds **21** (690 mg, 0.90 mmol) and 10% Pd–C (69 mg) in EtOAc (50 mL) was stirred under $H_2$ for 4 h. Pd–C was removed through filtration and the filtrate was concentrated and purified by a silica gel column chromatography (6:1, petroleum ether-acetone) to afford **6** (420 mg, 69.0%) as a white foam. [α]25 D = +78.2 (*c* 1.10, $CHCl_3$); $R_f$ = 0.3 (3:1, petroleum ether-acetone); Mp 176.5–181.6 °C; $^1$H NMR ($CDCl_3$, 600 MHz): 5.27 (s, 1H, H-12), 5.03 (d, 1H, *J* = 6 Hz, H-4′), 5.00 (t, 1H, *J* = 8.4 Hz, H-2′), 4.56 (d, 1H, *J* = 8.4 Hz, H-1′), 4.05–3.88 (m, 2H, *J* = 12.6, 2.4 Hz, H-3′, H-5′-1), 3.57 (br d, 1H, *J* = 13.2 Hz, H-5′-2), 3.11 (dd, 1H, *J* = 11.4, 4.2 Hz, H-3), 2.82 (br d, 1H, *J* = 10.2 Hz, H-18), 2.14 (s, 3H, $CH_3CO$), 2.13 (s, 3H,$CH_3CO$), 1.26, 1.12, 0.97, 0.92, 0.90, 0.78, 0.74 (s, 7 × 3H, Me); $^{13}$C NMR ($CDCl_3$, 150

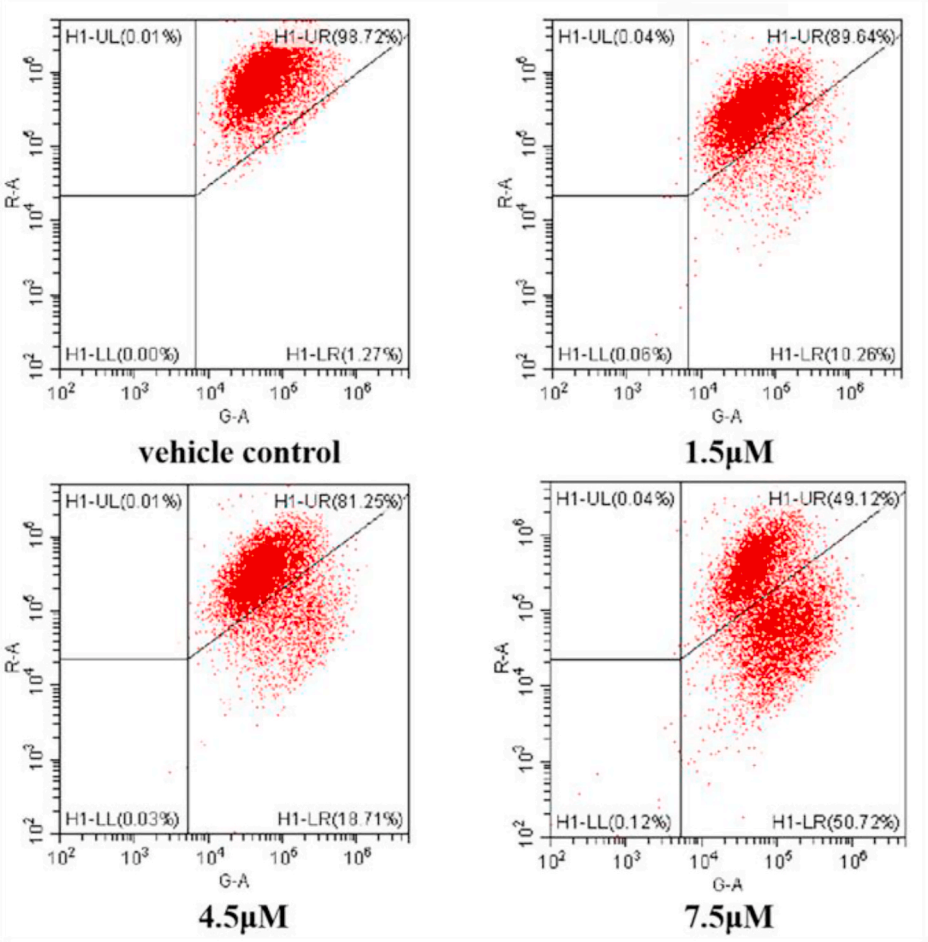



**Fig. 5.** Effects of compound **6** on A431 cells MMP.

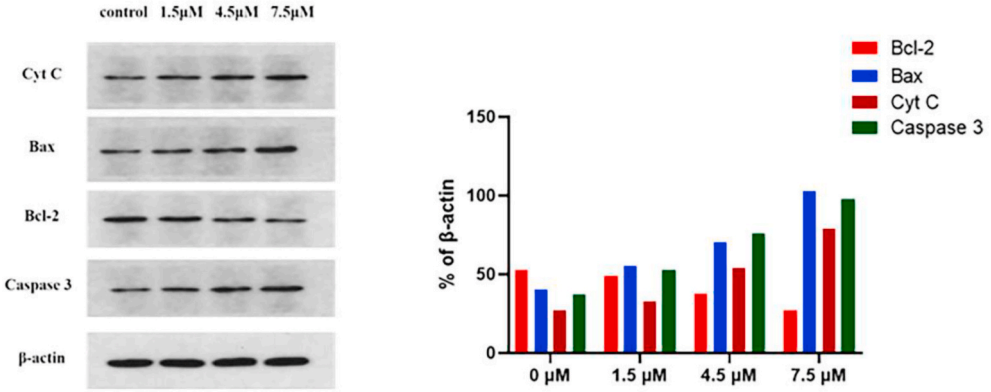



**Fig. 6.** Effects of compound **6** on the expression of related proteins of A431 cells.

MHz): 183.7, 170.7, 170.5, 143.6, 101.8, 90.4, 72.0, 69.5, 69.4, 60.4, 55.5, 47.6, 46.5, 45.9, 41.6, 40.9, 39.3, 38.9, 38.4, 36.8, 33.8, 33.1, 32.6, 32.4, 30.7, 27.9, 27.6, 25.9, 25.7, 23.6, 23.4, 22.9, 21.1, 21.0, 18.2, 17.0, 16.4, 15.3, 14.2. ESI-MS (*m/z*): 695.7 [ $(M + Na)^+$ ], 671.3 [ $(M - H)^-$].

6 Oleanolic acid 3-*O*-α-L-arabinopyranoside (7)

To a mixture of compounds **12** (320 mg, 0.47 mmol) and 10% Pd–C (32 mg) in EtOAc (20 mL) was stirred under $H_2$ for 4 h. Pd–C was removed through filtration and the filtrate was concentrated and purified by a silica gel column chromatography (10:1, $CHCl_3$–MeOH) to afford **7** (203 mg, 77%). $R_f = 0.56$ (5:1, $CHCl_3$–MeOH); $^1H$ NMR (pyridine-$d_5$, 600 MHz): 5.49 (br s, 1H, H-12), 4.78 (d, 1H, $J = 7.0$ Hz, H-1′), 4.45 (t, 1H, $J = 8.7$ Hz, H-2′), 4.36–4.30 (m, 1H), 4.18 (dd, 1H, $J = 8.8$, 3.1 Hz, H-4′), 3.92–3.78 (m, 1H), 3.37–3.27 (m, 2H, H-3′, H-18), 1.31, 1.29, 1.02, 1.01, 0.97, 0.96, 0.86 (s, 7 × 3H, $CH_3$); $^{13}C$ NMR (pyridine-$d_5$, 150 MHz): 181.1, 108.5, 89.6, 75.6, 73.9, 70.5, 56.8, 47.6, 43.1, 40.7, 40.5, 37.9, 34.2, 34.1, 31.9, 29.2, 27.1, 24.7, 18.3, 17.9, 16.4. ESI-MS: 611.5 [$(M + Na)^+$], 627.3 [$(M + K)^+$], 587.4 [$(M - H)^+$].

## 5. Biological experiments

### 5.1. Cell cytotoxicity assay

Cytotoxicity of test compounds against ten different tumor cell lines and four normal cell lines were evaluated using an MTT assay *in vitro*. All cells used for testing were maintained in Dulbecco Modified Eagle Medium (DMEM) containing 4.0 mM L-Glutamine and 4500 mg/L Glucose supplemented with 10% (v/v) foetalbovine serum (FBS) and 100 unites/mL penicillin/streptomycin at 37 °C in humidified atmosphere of 5% $CO_2$ and 95% air (In the following experiments, the cell culture conditions were the same). Cells were seeded into 96-well plates at a density of 5 × $10^4$ cells per well and stabilized at 37 °C with 5% $CO_2$ for 24 h. Compounds **1**–**7**, OA and 5-FU were added to each well at various concentrations, and then the cells were incubated for 48 h. The MTT solution (100 μL 0.5 mg/$mL^{-1}$) was added to each well, and the cells were incubated for another 4 h. Then, 150 μL DMSO was added to each well and the absorbance of samples was measured at 492 nm. The $IC_{50}$ values were calculated according to Logit method after getting the inhibitory rate.

### 5.2. Annexin V/PI staining assay

Cell apoptosis was assessed using Annexin V/PI staining assay. A431 cells were seeded into 6-well plates for 24 h, and then treated with compound **6** at various concentrations (0, 1.5, 4.5 and 7.5 μM) for 48 h. Then, cells were collected, washed with 500 μL annexin-binding buffer, and stained with 5 μL annexin V-FITC and 5 μL PI for 15 min at 25 °C. After that, the samples were analyzed by flow cytometry (Beckman Coulter cytoFLEX, USA).

### 5.3. TUNEL staining

The slides were immersed in 4% paraformaldehyde (pH 7.4) for 25 min at room temperature, and then washed with PBS for 3 times. The cells were immersed in 0.1% Triton X-100 solution prepared with PBS for 10 min (operation on ice), and then washed twice with PBS. Dilute 5 × equilibration buffer with deionized water in the ratio of 1:5.100 μL 1 × equilibration buffer was added to each climbing tablet to cover the sample area to be tested, and incubated at room temperature for 15 min. After the buffer solution of 1 μD was added to the buffer solution, most of the buffer solution was added to the buffer solution of 1 μD, and then the buffer solution was used to wash off the buffer. Place a paper towel soaked in water at the bottom of the wet box. The slides were placed in a wet box and incubated at 37 °C for 60 min. Wrap the wet box with aluminum foil to avoid light. Then wash 3 times with PBS. DAPI was dripped and incubated in dark for 5 min. The specimens were stained with nuclei. Water absorbent paper was used to absorb the liquid on the climbing sheet, and the sealing liquid containing anti fluorescence quenching agent was used to seal the film, and then the images were observed and collected under the fluorescence microscope.

### 5.4. Cell cycle analysis

Cell cycle was assessed using the PI staining assay. A431 cells were seeded into 6-well plates for 24 h, and then treated with compound **6** at various concentrations (0, 1.5, 4.5 and 7.5 μM) for 48 h. The cells were collected, washed with ice-cold PBS buffer, fixed with 70% alcohol at 4 °C for 12 h and washed with PBS. Then 100 μL RNase A was added, and the cells were incubated at 37 °C for 30 min. They were then stained with 400 μL PI at 4 °C for 30 min before analysis by measuring the absorbance at 488 nm wavelength using flow cytometry (Beckman Coulter cytoFLEX, USA).

### 5.5. JC-1 mitochondrial membrane potential assay

The mitochondrial membrane potential was assessed using JC-1 dye. A431 cells were plated at 1 × 106 cells per well in 24-well plates and incubated with compound **6** at various concentrations (0, 1.5, 4.5 and 7.5 μM) for 48 h. Subsequently, the cells were incubated with JC-1 dye and finally analyzed by flow cytometry (Beckman Coulter cytoFLEX, USA).

### 5.6. Western blotting assay

A431 cells were seeded at a density of 4 × 105 cells per well and treated with various concentrations of compound **6** (0, 1.5, 4.5 and 7.5 μM) for 48 h. After this, cells were collected and washed twice with ice-cold DPBS. The pellets were resuspended in a total protein extraction buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF) containing a protease inhibitor cocktail and incubated on ice for 30 min with intermittent mixing. The protein concentration was measured using the Bradford reagent (Bio-Rad Laboratories Inc, CA, USA). An equal amount (20 μg) of protein was loaded on 10% polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk, the membrane was incubated at 4 °C overnight with specific primary antibodies. The membrane was washed and incubated at room temperature for 1 h with secondary antibodies conjugated with horseradish peroxidase (HRP). Finally, the immunoblot was developed for visualization using a chemiluminescence kit. Primary antibodies for cytochrome C, caspase-3, Bax, Bcl-2, and β-actin and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

## Declaration of competing interest

The authors declare no conflict of interest.

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