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Chemical constituents of *Euphorbia tibetica* and their biological activities

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[ABSTRACT]

AIM: To investigate the chemical constituents and their biological activities of the aerial parts of *Euphorbia tibetica*.

METHOD: Compounds were isolated and purified by various chromatographic methods, and their structures were elucidated through the use of extensive spectroscopic techniques including 2D-NMR, the structures of compounds **5** and **6** were confirmed by single-crystal X-ray crystallography. Bioactivities of all the isolated compounds were evaluated by the MTT method on A549 and anti-angiogenesis assay.

RESULTS: One new compound, methyl 4-*epi*-shikimate-3-*O*-gallate (**1**), together with twenty-three known constituents (**2–24**) were isolated from the aerial parts of *E. tibetica*.

CONCLUSION: Compound **1** is new, and the other compounds were isolated from this plant for the first time. Compounds **5–7**, **9**, **11**, **17**, **18** and **20** exhibited inhibitory effects on the growth of human lung cancer cell A549 and compounds **5**, **7**, **12**, **13**, **17** and **19** showed anti-angiogenic effects in a zebrafish model.

[KEY WORDS] *Euphorbia tibetica*; Euphorbiaceae; Shikimate derivatives; Diterpenes; Cytotoxicity

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Introduction

Most species of the genus *Euphorbia* (Euphorbiaceae) are sources of various secondary metabolites with interesting chemical structures and significant bioactivities [1]. *Euphorbia tibetica* Boiss. is a perennial herb which is distributed in China's Tibetan Plateau and Xinjiang Uygur Autonomous Region [2]. The unique geographical and climatic conditions of the Tibetan Plateau may have created special biological resources. In order to find new bioactive compounds and promote the rational utilization of bio-resources, *E. tibetica* was examined phytochemically, and one new (**1**) and twenty-three known (**2–24**) compounds were characterized. Structures of the isolated compounds were identified through various spectral data analyses, and compounds **5** and **6** by single- crystal X-ray crystallography. In addition, bioactive evaluation of the isolates on the human lung cancer cell line A549 and in a zebrafish model was conducted. Some of the isolates exhibited antiproliferative activity or anti-angiogenic effects.

Results

Compound **1** was obtained as light yellow oil, and its formula was determined to be $C_{15}H_{16}O_9$ by HREI-MS. The IR spectrum of **1** showed the presence of hydroxyl (3 427 cm^{-1}) and carbonyl groups (1 703 cm^{-1}) and an aromatic ring (1 615, 1 536 cm^{-1}). The ^{13}C NMR and DEPT spectra showed 13 carbon signals attributed to one methyl, one methylene, five methane (including three sp^3 and two sp^2), and six quaternary carbons (six sp^2). Comparison of the NMR spectra of **1** with those of 3-*O*-galloyl-shikimic acid (**2**) [3], revealed that compounds **1** and **2** have a similar skeleton, except for the presence of a methoxy group (δ_C 52.1) in **1**. The functional groups were assigned on the basis of HMBC and ^1H-^1H COSY studies (Fig. 1). The methoxy group located at C-7 in **1** was deduced from the HMBC correlations of the proton signals (δ_H 3.70) with C-7. In addition, the

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HMBC correlations of H-3 with C-7' indicated that the galloyl group was located at C-3. ROESY correlations were observed between H-2*α* and H-3, H-4, and between H-3 and H-4, which showed that the hydroxyl group attached to C-4 was *β*-oriented (Fig. 2). Therefore, the structure of **1** was determined to be methyl 4-*epi*-shikimate-3-*O*-gallate.

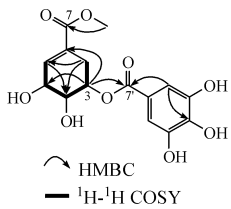


Fig. 1 Key ^{1}H-^{1}H COSY and HMBC correlation of 1

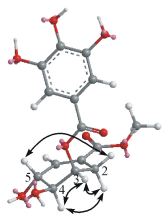


Fig. 2 Key ROESY correlation of 1

Compound 1 Light yellow oil; $[\alpha]_D^{25}$ –125.2 (*c* = 0.27, MeOH); UV (MeOH) λ_{max} (log *ε*) 218 (4.46) nm; IR (KBr) ν_{max} = 3 427, 2 954, 2 923, 2 853, 1 703, 1 615, 1 536, 1 446, 1 320, 1 234, 1 096, 1 033, 952, 767, 747 cm^{-1}; ^{1}H NMR (acetone-d_6, 400 MHz) δ: 7.07 (2H, s, H-2', 6'), 6.82 (1H, br s, H-6), 5.32 (1H, dd, *J* = 4.8 Hz, 11.2 Hz, H-3), 4.48 (1H, br s, H-5), 4.01 (1H, dd, *J* = 4.8 Hz, 6 Hz, H-4) , 3.70 (3H, s, OMe), 2.78 (1H, dt, *J* = 2.4 Hz, 18.4 Hz, H-2*β*), 2.38 (1H, dd, *J* = 4.0 Hz, 18.4 Hz, H-2*α*); ^{13}C NMR (acetone-d_6, 100 MHz) *δ*: 167.2 (s, C-7), 166.1 (s, C-7'), 146.1 (s, C-3', 5'), 139.3 (s, C-4'), 138.9 (d, C-6), 128.9 (s, C-1), 121.5 (s, C-1'), 109.8 (d, C-2', 6'), 71.0 (d, C-3), 68.8 (d, C-4), 66.8 (d, C-5), 52.1 (q, OMe), 28.0 (t, C-2); positive ESIMS *m/z* = 363 [M + Na]$^+$; HREI *m/z* 340.080 0 [M]$^+$ (Calcd. for $C_{15}H_{16}O_9$, 340.079 4).

Compound 2 Light yellow oil. ^{1}H NMR (acetone-d_6, 500 MHz) δ: 7.09 (2H, s, H-2', 6'), 6.91 (1H, s, H-6), 5.37 (1H, m, H-3), 4.59 (1H, m, H-5), 4.11 (1H, m, H-4), 2.87 (1H, dd, *J* = 2.4 Hz, 18.4 Hz, H-2*β*), 2.40 (1H, dd, *J* = 4.6 Hz, 18.4 Hz, H-2*α*); ^{13}C NMR (acetone-d_6, 100 MHz) δ: 168.7 (s, C-7), 166.6 (s, C-7'), 145.6 (s, C-3', 5'), 138.8 (s, C-4'), 138.7 (d, C-6), 129.3 (s, C-1), 121.1 (s, C-1'), 109.9 (d, C-2', 6'), 70.8 (d, C-4), 68.9 (d, C-5), 66.8 (d, C-3), 28.1 (t, C-2). It was characterized as 3-*O*-galloyl-shikimic acid by comparison of the spectral data with the literature [3].

Compound 3 Colorless oil, $C_{10}H_{18}O_2$, ESI-MS *m/z* 193 [M + Na]$^+$. ^{1}H NMR (acetone-d_6, 500 MHz) δ: 5.93 (1H, dd, *J* = 10.8 Hz, 17.3 Hz, H-2), 5.63 (2H, m, H-5, 6), 5.17 (1H, dd, *J* = 1.8 Hz, 17.3 Hz, H-1b), 4.93 (1H, dd, *J* = 1.8 Hz, 10.8 Hz, H-1a), 2.17 (2H, m, H-4), 1.22 (6H, s, H-8, 9), 1.18 (3H, s, H-10); ^{13}C NMR (acetone-d_6, 125 MHz) δ: 146.8 (d, C-2), 143.1 (d, C-6), 122.3 (d, C-5), 111.2 (t, C-1), 72.7 (s, C-3), 70.1 (s, C-7), 46.3 (t, C-4), 29.9 (q, C-8, 9), 27.4 (q, C-10). It was characterized as 3, 7-dimethyl-octa-1, 7-diene-3, 6-diol by comparison of the spectral data with the literature [4]. This is the first isolation of this type of compound from this genus.

Compound 4 Colorless oil, $C_{13}H_{24}O_4$, ESI-MS *m/z* 243 [M − H]$^-$. ^{1}H NMR (acetone-d_6, 500 MHz) δ: 6.13 (1H, d, *J* = 16.0 Hz, H-7), 5.80 (1H, dd, *J* = 6.1 Hz, 16.0 Hz, H-8), 4.31 (1H, m, H-9), 4.05 (1H, m, H-3), 2.04 (1H, dd, *J* = 4.4 Hz, 8.7 Hz, H-4*eq*), 1.64 (1H, dd, *J* = 8.7 Hz, 11.9 Hz, H-4*ax*), 1.41 (1H, dd, *J* = 4.5 Hz, 12.1 Hz, H-2*eq*), 1.25 (1H, m, H-2*ax*), 1.20 (3H, d, *J* = 6.3 Hz, H-10), 1.19 (3H, s, H-13), 1.10 (3H, s, H-11), 0.84 (3H, s, H-12); ^{13}C NMR (acetone-d_6, 125 MHz) δ: 136.1 (d, C-7), 130.3 (d, C-8), 78.3 (s, C-6), 77.2 (s, C-5), 68.6 (d, C-9), 64.3 (d, C-3), 46.9 (t, C-4), 46.3 (t, C-2), 40.3 (s, C-1), 27.4 (q, C-11), 27.3 (q, C-12), 26.1 (q, C-10), 24.5 (q, C-13). It was characterized as (3*S*, 5*R*, 6*R*, 7*E*, 9*R*)-3, 5, 6, 9-tetrahydroxy-7-megastigmene by comparison of the spectral data with the literature [5].

Compound 5 White powder, ^{1}H NMR (acetone-d_6, 500 MHz) δ: 5.51 (1H, dd, *J* = 2.3 Hz, 4.8 Hz, H-7), 4.41, 4.40 (2H, *AB*, d, *J* = 1.0 Hz, H-16*α* and H-16*β*), 4.05 (1H, d, *J* = 5.2 Hz, H-3), 3.68 (1H, d, *J* = 5.2 Hz, H-14*β*), 3.48 (1H, t, *J* = 5.1 Hz H-9), 2.85 (1H, s, H-14*α*), 2.47 (2H, s, H-1), 1.12 (3H, s, H-18), 1.05 (3H, s, H-17), 0.86 (3H, s, H-19), 0.75 (3H, s, H-20); ^{13}C NMR (acetone-d_6, 125 MHz) *δ*: 215.3 (s, C-15), 210.9 (s, C-2), 134.6 (s, C-8), 123.6 (d, C-7), 82.6 (d, C-3), 64.6 (t, C-16), 52.2 (d, C-5), 51.9 (t, C-1), 49.4 (d, C-9), 46.4 (s, C-4), 45.0 (s, C-10), 42.9 (s, C-13), 42.0 (t, C-14), 32.8 (t, C-12), 28.8 (q, C-18), 24.0 (t, C-6), 20.1 (t, C-11), 18.9 (q, C-17), 16.8 (q, C-19), 15.8 (q, C-20). Accordingly, the structure of **5** was assigned as *ent*-3*β*,16-dihydroxyisopimar-7-ene-2,15-dione [6]. The NMR data of this compound are reported for the first time.

Crystallographic data for 5 $C_{20}H_{30}O_4$, M = 334.44, orthorhomic, space group $P2_12_12_1$, a = 9.106 0(17) Å, b = 10.115(2) Å, c = 9.6826(18) Å, $\alpha = \gamma = 90°$, $\beta = 96.975(6)°$, V = 885.2(19) Å^3, Z = 2, d = 1.255 g cm^{-3}, crystal size 0.30 mm × 0.60 mm × 0.60 mm, was used for measurements on a Bruker APEX DUO diffractometer with a graphite monochromator, Cu K*α* radiation. The total number of independent reflections measured was 2 518, of which 2 496 were observed ($|F|^2 \geqslant 2\sigma|F|^2$). Final indices: R_1 = 0.056 0, wR_2 =0.159 7 (w = $1/\sigma|F|^2$). The crystal structure of **5** were solved and refined by the direct method SHELX-97 (Sheldrick, GM, University of Gottingen, Gottingen, Germany, 1985). Crystallographic data for the structure of **5** has been deposited in the Cam-

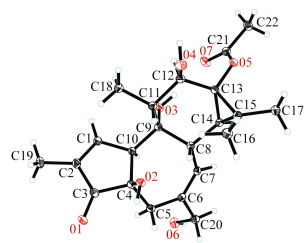


Fig. 3 X-ray structure of 6

bridge Crystallographic Data Centre (deposition number: CCDC 909190). Copies of this data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK; Fax: (+44) 1223-336-033; or deposit@ ccdc.cam.ac.uk).

Compound 6 White powder. ^{13}C NMR (acetone-d_6, 100 MHz) δ: 208.5 (s, C-3), 174.8 (s, C-Ac), 159.9 (d, C-1), 142.3 (s, C-6), 133.2 (s, C-2), 129.2 (d, C-7), 78.4 (s, C-9), 76.9 (d, C-12), 74.4 (s, C-4), 69.2 (s, C-13), 67.7 (t, C-20), 57.5 (d, C-10), 45.9 (d, C-14), 39.8 (d, C-8), 38.4 (t, C-5), 36.1 (d, C-11), 26.3 (s, C-15), 24.1 (q, C-16), 21.1 (q, C-Ac), 17.4 (q, C-18), 15.5 (q, C-17), 10.2 (q, C-19). It was characterized as phorbol-13-acetate by comparison of the spectral data with the literature [7]. This is the first report of the ^{13}C-NMR data of this compound.

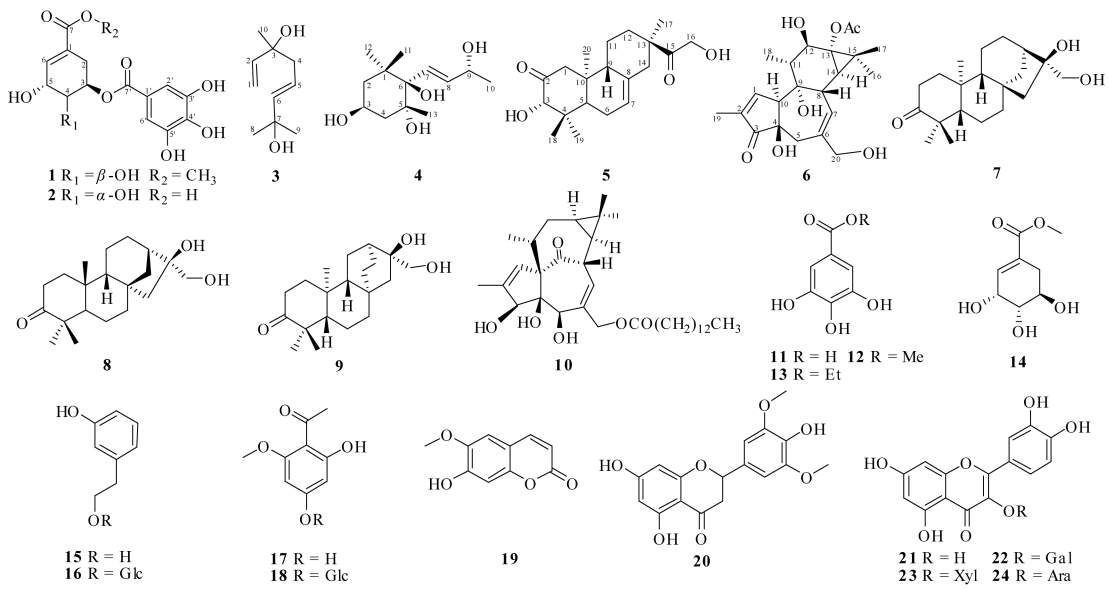
Crystallographic data for 6. $C_{22}H_{30}O_7$, M = 406.46, orthorhomic, space group $P2_12_12_1$, a = 9.883 5(6) Å, b = 11.354 4(6) Å, c = 17.4532(9) Å, $\alpha=\beta=\gamma=90°$, V = 1 958.62(19) Å^3, Z = 4, d = 1.378 g cm^{-3}, crystal size 0.16 mm × 0.25 mm × 0.60 mm, was used for measurements on a Bruker APEX DUO diffractometer with a graphite monochromator, Cu Kα radiation. The total number of independent reflections measured was 3449, of which 3442 were observed ($|F|^2 \geq 2\sigma|F|^2$). Final indices: R_1 = 0.029 0, wR_2 = 0.074 7 ($w = 1/\sigma|F|^2$). The crystal structure of **6** (Fig. 3) were solved and refined by the direct method SHELX-97 (Sheldrick, GM, University of Gottingen, Gottingen, Germany, 1985). Crystallographic data for the structure of **6** has been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 903118). Copies of this data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223-336-033; or deposit@ ccdc.cam.ac.uk).

Compounds **7-24** isolated from this plant were identified as (16*S*)-16,17-dihydroxy-*ent*-kauran-3-one (**7**) [8], (16*R*)-16, 17-dihydroxyphyllocladan-3-one (**8**) [9], *ent*-16α, 17- dihydroxyatisan-3-one (**9**) [10], ingenol-20-myristinate (**10**) [11], gallic acid (**11**)[12], methyl gallate (**12**) [13], ethyl gallate (**13**) [13], methyl shikimate (**14**) [14], 3-hydroxyphenyl alcohol (**15**)[15], 1-(3-hydroxy)-phenylethyl-*O*-β-D-glucopyranoside (**16**) [16], 2,4-dihydroxy-6-methoxy-acetophenone (**17**) [17], 4-*O*-glycosy-oxy-2-hydroxy- 6-methoxyacetophenone (**18**) [18], scopoletin(**19**) [19], tricin (**20**) [20], quercetin (**21**) [21], quercetin-3-*O*-β-D-galactoside (**22** [22], quercetin-3-*O*-β-D-xylopyranoside(**23**) [23], and quercetin-3-*O*-α-L-arabinofuranoside (**24**) [24], respectively, by comparison of the spectral data with those reported in the literature. Compounds **2–24** were isolated from *E. tibetica* for the first time.

Experimental

Apparatus and Reagents

1D- and 2D-NMR experiments were recorded on a Bruker AM-400 or a DRX-500 spectrometer with TMS as internal standard. Chemical shifts (δ) are expressed in ppm with reference to the solvent signals. Optical rotations were taken on a Horiba SEPA-300 polarimeter. UV data were obtained on a Shimadzu UV-2401PC spectrophotometer. IR spectra were recorded on a Bio-Rad FTS-135 spectrometer with KBr pellets. ESI-MS were recorded using a Finnigan MAT 90 instrument, and HREIMS was performed on a Waters AutoSpec Premier P776 instrument. Column chromatography was performed on silica gel (48–75 μm, Qingdao Marine Chemical Ltd., Qingdao, China), Rp-18 gel (40–63 μm; Merck, Germany), Sephadex LH–20 (Amersham Biosciences, Chandle, USA) or MCI gel CHP20P (75−150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Semipreparative HPLC was performed on an Agilent instrument (column: Zorbax SB-C_{18}, 250 mm × 9.4 mm; DAD detector). Fractions were monitored by TLC, visualized by heating silica gel plates sprayed with 15% H_2SO_4 in EtOH.



Plant material

E. tibetica was collected in Linzhi Prefecture of the Tibetan Autonomous Region, China, and identified by Prof. YANG Yong-Ping, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. YangYP–20080917) was deposited in the Herbarium of Kunming Institute of Botany.

Extraction and isolation

The dried and powdered aerial parts of *E. tibetica* (10 kg) were extracted with 70% acetone three times and concentrated under reduced pressure to yield a residue, which was partitioned between H_2O and EtOAc. After decolorization by MCI gel, the EtOAc extract (870 g) was subjected to column chromatography over silica gel (80–100 mesh), eluting with $CHCl_3$-acetone (from 1 : 0 to 1 : 1), to afford 3 fractions (Fr. 1−3). Fr. 2 was repeatedly purified by column chromatography on silica gel ($CHCl_3$–EtOAc or $CHCl_3$–MeOH gradient eluting), Sephadex LH-20 ($CHCl_3$–MeOH eluting), Rp-18 gel (MeOH–H_2O gradient eluting) and Semipreparative HPLC to yield compounds **1** (43 mg), **2** (50 mg), **3** (15 mg), **4** (31 mg), **5** (42 mg), **6** (5 mg), **7** (6 mg), **8** (3 mg), **9** (37 mg), **10** (8 mg), **11** (16 mg), **12** (15 mg), **13** (30 mg), **14** (6 mg), **15** (8 mg), **16** (35 mg), **17** (42 mg), **18** (16 mg), **19** (28 mg), **20** (17 mg), **21** (2 mg), **22** (9 mg), **23** (4 mg), and **24** (13 mg).

Bioactive Evaluation

Anti-angiogenesis Bioassay

Stock solutions (10 mg·mL^{-1}) of all samples were prepared by dissolving the test compounds in 100% DMSO. These solutions were diluted in sterile salt water (5 mmol·L^{-1} NaCl, 0.17 mmol·L^{-1} KCl, 0.4 mmol·L^{-1} $CaCl_2$, and 0.16 mmol·L^{-1} $MgSO_4$) to obtain solutions with the test compounds dissolved in 0.1% DMSO. These solutions were aliquoted into 96-well plates, and embryos at 24 hpf (hours post fertilization) were also transferred randomly into the above wells. After 24 h of treatment, the intersegmental vessels of embryos were visualized with green fluorescent protein labeling and endogenous alkaline phosphatase staining. The anti-angiogenic activities of compounds were calculated from the inhibition ratio of angiogenesis. PTK787 was used as the positive control.

Antiproliferative Bioassay.

A549 human lung cancer cells were cultured in RPMI 1640 medium at 37 °C with 5% CO_2 and 95% air, supplemented with 10% (*V/V*) bovine calf serum and 80 U·mL^{-1} penicillin/streptomycin. The cells were seeded into 96-well plates and treated with compounds at 50 and 100 μg·mL^{-1} for 48 h, respectively. Cell viability was determined by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium) assay according to Price *et al*. The light absorption was measured at 570 nm using Spectra MAX 190 microplate spectrophotometer (GMI Co., Belmont, USA). Inhibition rate was calculated by the formula:

Inhibition (%) =100%–($OD_{treatment}$–OD_{blank})/($OD_{control}$–OD_{blank}) ×100%

The cells were incubated with compounds (100 μg·mL^{-1}) for 48 h, and stained with 0.1 mg·mL^{-1} of acridine orange (AO) at room temperature for 5 min. Then the cells were observed and photographed using the fluorescent stereo microscope (Olympus, Tokyo, Japan).

Bioactivity Evaluation

The anti-angiogenic activities of all the isolated compounds were evaluated using a zebrafish model [25], with PTK787 as the positive control. The results showed that intersegmental vessels of embryos treated with compounds **5**, **7**, **12**, **13**, **17** and **19** (Table 1) were less than those of the control (0.1% DMSO in sterile salt water).

Table 1 Results of anti-angiogenisis assay

Compound	*c*/(μg·mL^{-1})	Intersegmental vessels (ISV)	Inhibition ratio/%	IC_{50}/(μg·mL^{-1})
5	40	12.6 ± 10.9	47.0	42.4
7	40	4.0 ± 7.1	83.2	31.1
12	80	13.4 ± 9.5	43.6	89.2
13	80	11.0 ± 8.6	53.7	75.1
17	20	10.2 ± 7.8	57.1	15.2
19	20	8.6 ± 6.3	63.8	12.3
Control[a]		23.8 ± 2.1		
PTK787	10	0**	100	0.15

Table 2 Results of cytotoxicity assay

Compound /(50 μg·mL^{-1})	Inhibition ratio/%
5	43.37 ± 2.20**
6	39.48 ± 1.52**
7	63.76 ± 3.76**
9	43.45 ± 7.55**
11	73.74 ± 6.40**
17	45.20 ± 4.96**
18	37.10 ± 3.15**
20	31.27 ± 3.66**
Doxorubicin	41.27 ± 2.56

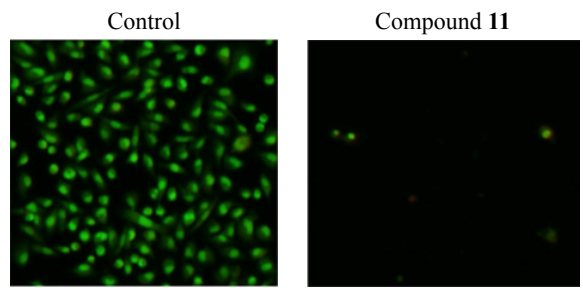


Fig. 4 Photos of acridine orange staining

The MTT method [25] was used to investigate the cytotoxic activity of the isolated compounds. The results show that compounds **5–7**, **9**, **11**, **17**, **18** and **20** (Table 2) exhibited inhibitory effects on the growth of human lung cancer cell A549 compared with the control group. From the photos of acridine orange staining (Fig. 4), compound **11** showed obvious effects of inducing apoptosis of A549 cells.

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