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Isolation of Chemical Constituents from the Aerial Parts of *Verbascum thapsus* and Their Antiangiogenic and Antiproliferative Activities

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Phytochemical investigation of *Verbascum thapsus* led to the isolation and identification of one new iridoid compound named verbathasin A, along with ten known compounds. The structure and relative stereochemistry of verbathasin A were elucidated by analysis of spectroscopic data. All the isolates except 10-deoxyeucommiol and ajugol were tested for antiangiogenic and antiproliferative activities, and compounds luteolin and 3-*O*-fucopyranosylsaikogenin F showed promising antiproliferative activities, with an obvious effect of inducing apoptosis of A549 lung cancer cells.

Key words: *Verbascum thapsus*, Iridoid, NMR, Antiangiogenic activities, Antiproliferative activities, A549

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INTRODUCTION

Verbascum thapsus L. (Scrophulariaceae) is a species of mullein widely distributed throughout Europe, Asia, North America and North Africa (Mitsuhashi, 1988). It is a biennial plant that grows frequently in dry and rocky parts of the temperate Himalayan region (altitude 5000-12,000 ft). Traditionally, this plant is used as ethnomedicine for the treatment of inflammatory disease, asthma, spasmodic coughs, and migraine (Chopra et al., 1956; Ramachandran et al., 1986). Previous studies regarding the chemical constituents of *V. thapsus* L. revealed the presence of iridoid glucosides (Khuroo et al., 1988; Warashina et al., 1991; Kalpoutzakis et al., 1999; Hussain et al., 2009), phenylethanoid and lignan glycosides (Warashina et al., 1992), flavonoids (Mehrotra et al., 1989), and sesquiterpenes (Hussain et al., 2009).

In order to better use traditional ethnomedicine in Southwest China and to search for bioactive metabolites from *V. thapsus*, we collected plant samples from Northwest Yunnan and carried out an investigation of the chemical constituents, which led to the isolation of a new compound named verbathasin A (**1**) and ten additional known compounds (**2**-**11**). In the present paper, we report the isolation and structural elucidation of the a compound (**1**) and the antiproliferative activities using A549 lung cancer cells of all isolates except compounds **3** and **6**.

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MATERIALS AND METHODS

General experimental procedure

1D and 2D NMR experiments were performed on a Brucker AM-400 or DRX-500 spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the solvent signals. Mass spectra were recorded on a VG

Autospec-300 spectrometer under 70 eV. Optical rotation was measured with a Horiba SEPA-300 polarimeter. A Bio-Rad FTS-135 spectrophotometer was used for scanning IR spectroscopy of compounds with KBr pellets. Column chromatography was performed on silica gel (200-300 mesh, Qingdao Marine Chemical), silica gel H (10-40 μm, Qingdao Marine Chemical) and MCI gel CHP20P (75-150 μm, Mitsubishi Chemical Corporation). Fractions were monitored by TLC and spots were visualized by heating plates sprayed with 15% H_2SO_4 in EtOH.

Plant material

The aerial parts of *V. thapsus* were collected from Heqing county which had an altitude of 2200 meters, latitude 26.55 and longitude 100.18, Yunnan province, Southwest China. The plant material was identified by Prof. Yongping Yang. A voucher specimen (No. yyp20070456) was deposited at Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

The dry aerial parts of *V. thapsus* (8 kg) were powdered and extracted with 70% aqueous acetone (3 × 10.0 L) for 24 h at room temperature. The solvent was concentrated *in vacuo* to yield a crude extract. The extract was then dissolved in H_2O and partitioned with EtOAc. The EtOAc portion was subjected to column chromatography over MCI gel eluting with 95% EtOH and concentrated *in vacuo*. The residue (126 g) was subjected to column chromatography over a silica gel (80-100 mesh), eluted with petroleum ether-Me_2CO (from 1:0 to 1:1) to generate fractions I-V. Fraction II (20.1 g) was applied to RP-18, eluted with a 45%-100% MeOH-H_2O gradient system to generate six fractions. Fraction II-2 (2.0 g) gave the compounds **3** (2 mg) and **5** (2 mg), after being subjected to chromatography over silica gel developed with petroleum ether-EtOAc (6:4). Fraction II-3 (1.5 g) was purified over silica gel (petroleum ether-Me_2CO, 6:1) to generate compounds **1** (30 mg) and **2** (7 mg). Fraction II-4 was subjected to repeated chromatography over silica gel, RP-18 and Sephadex LH-20 (MeOH) to yield compounds **4** (5 mg), **6** (2 mg), and **11** (36 mg). Fraction III (17.9 g) was subjected to chromatography over a silica gel column and eluted with $CHCl_3$-MeOH (20:1, 10:1, 5:1, 2:1, 1:1) to afford five fractions. Fraction III-2 (3.1 g) was purified by recrystallization and subjected to repeated chromatography over silica gel, RP-18 and Sephadex LH-20 (MeOH) columns to yield compounds **7** (35 mg), **8** (38 mg), **9** (7 mg), and **10** (8 mg).

Table I. The NMR Data of compound **1**[a]

Position	δ_H [mult., J (Hz)][b]	δ_C
1a	4.35 (dd, 11.8, 4.1)	67.6 (t)
1b	4.23 (dd, 11.8, 3.8)	
3	4.13 (m)	59.8 (t)
4	5.70 (m)	129.9 (d)
5		147.0 (s)
6	4.39 (m)	82.8 (d)
7a	2.82 (m)	29.7 (t)
7b	2.47 (m)	
8	3.30 (m)	44.0 (d)
9	2.65 (m)	44.3 (d)
10		174.2 (s)

[a]^{1}H- and ^{13}C-NMR spectra were tested in $(CD_3)_2CO$ and obtained at 400 and 125 MHz, respectively. Coupling constants were presented in Hertz, δ in ppm.

Verbathasin A (1)

White powder; m.p. 198~199°C; $[\alpha]_D^{24.0}$ –13.81 (*c* 0.20, CH_3OH); UV λ_{max} (MeOH) (log ε) 203 (2.61) nm; IR ν_{max} (KBr) 3386, 2902, 1737, 1659, 1432, 1249, 1162, 1007, 716 cm^{-1}; negative FAB *m/z* $[M-H]^-$ 183; HRESIMS: *m/z* 183.0433 $[M-H]^-$ (calcd $C_9H_{11}O_4$ for 183.0424). For ^{1}H- and ^{13}C-NMR see Table I.

Antiangiogenesis bioassay

Stock solutions (10 mg/mL) of all samples were prepared by dissolving the test compounds in 100% DMSO. These solutions were diluted in sterile salt water (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$) 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 (GFP) 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 (Li et al., 2009).

Antiproliferative bioassay

A549 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 penicillin/streptomycin. The cells were seeded onto 96-well plates and treated with compounds at 3, 10, 30 and 100 μg/mL for 48 h, respectively. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium) assay according to He et al. (2007). The light absorption was measured at

570 nm using Spectra MAX 190 microplate spectrophotometer (GMI Co.). Inhibition rate was calculated by the formula:

$$\text{Inhibition (\%)} = 100\% - (OD_{treatment} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$$

The cells were incubated with compounds (100 μg/mL) for 48 h, and stained with 0.1 mg/mL of acridine orange (AO) at room temperature for 5 min. Then the cells were observed and photographed using a fluorescent stereo microscope (Olympus) (He et al., 2007).

RESULTS AND DISCUSSION

From the 70% aqueous acetone extract of dry aerial parts of *V. thapsus*, one new compound (**1**) and ten known compounds (**2-11**) were isolated. The known isolates including ningpogenin (**2**) (Yue et al., 2001), 10-deoxyeucommiol (**3**) (Gouda et al., 2003), Jioglutolide (**4**) (Morota et al., 1989), 6β-Hydroxy-2-oxabicyclo [4.3.0]$\Delta^{8\text{-}9}$-nonen-1-one (**5**) (Valladares and Rios, 2007), ajugol (**6**) (Boros and Stermitz, 1990), 8-cinnamoylmyoporoside (**7**) (Qi et al., 2006), harpagoside (**8**) (Qi et al., 2006), luteolin (**9**) (Wagner et al., 1976), apigetrin (**10**) (Takeda et al., 1993), and 3-*O*-fucopyranosylsaikogenin F (**11**) (Ding et al., 1986), were identified by comparison of physical and spectroscopic data from previously published reports. The structures of compound **1** were established by means of MS and extensive NMR spectra as follows.

Verbathasin A (**1**), $[\alpha]_D^{24.0}$ –13.81 (*c* 0.20, CH_3OH), isolated as a white powder, showed a quasi-molecular ion peak at *m/z* 183 $[M\text{-}H]^-$ in the negative FAB mass spectrum. The molecular formula of **1** was revealed as $C_9H_{12}O_4$ by HRESIMS (*m/z* 183.0433 $[M\text{-}H]^-$), corresponding to four unsaturation degrees. The ^{13}C-NMR spectrum of **1** showed signals for nine carbons, including one ester group, one quaternary carbon, four methines (an oxygenated one and an olefinic carbon), three methylenes (two oxygenated ones) (Table I). The data suggested that **1** may be a C_9 iridoid derivative. HMBC correlations of H_2-3 (δ_H 4.13, m) with C-4 (δ_C 129.9, d) and C-5 (δ_C 147.0, s) and of H-4 with C-5, C-6 (δ_C 82.8, d), and C-9 (δ_C 44.3, d), together with the ^1H-^1H COSY correlations of H-3/H-4 and H-6/H-7/H-8/H-9 determined the presence of ring A. In addition, observed HMBC correlations of H-7, H-8, and H-9 with C-10 (δ_C 174.2, s) and of H-1 with C-10, coupled

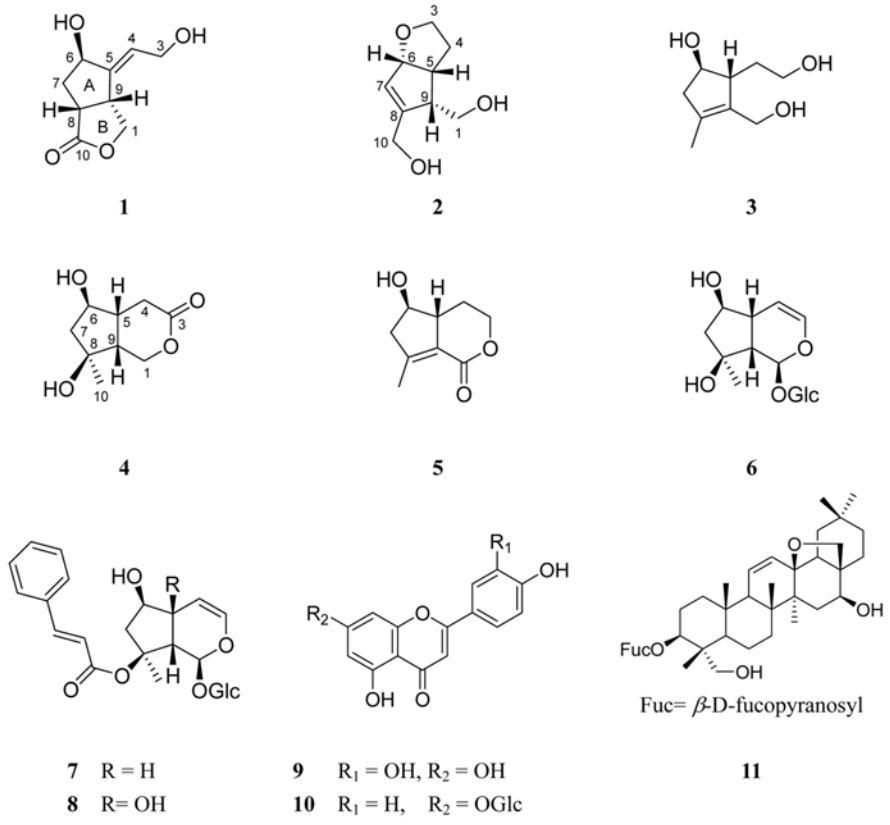

Fig. 1. The molecular structures of compounds **1-11**.

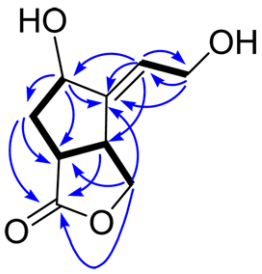

Fig. 2. Selected HMBC (→) and ^{1}H-^{1}H COSY (—) correlations of **1**.

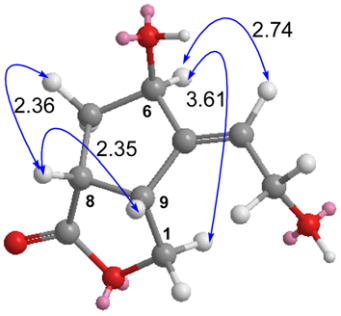

Fig. 3. Key ROESY correlations of **1** and corresponding interatomic distance (Å).

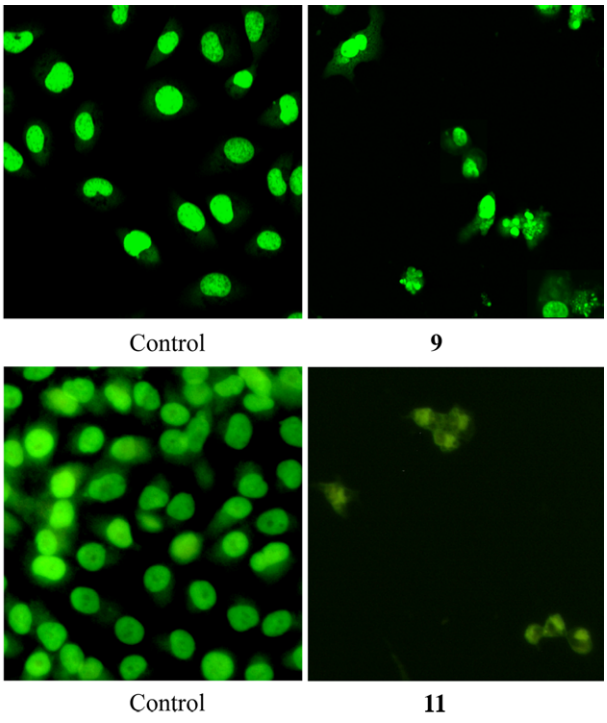

Fig. 5. Images of acridine orange staining at 48 h after being treated with 100 μg/mL **9** and **11**, respectively.

with ^{1}H-^{1}H COSY correlation of H-1/H-9 established the presence of ring B (Fig. 2). According to the molecular formula of **1** and the chemical shifts, two hydroxyl groups were attached at C-3 and C-6, respectively. Therefore, the planar structure of **1** was established. The relative stereochemistry of **1** was determined by its ROESY experiment (Fig. 3). C-9 was biogenetically considered to be β-oriented. Accordingly, C-1 was α-oriented. The strong ROESY correlation of H-8 with H-9 suggested H-8 was also β-oriented. ROESY correlation of H-1 with H-6 indicated H-6 was α-oriented and OH-6 was β-oriented. In addition, a computer-generated 3D structure was obtained by CHEM 3D ULTRA V 8.0, with MM2 forcefield calculations for energy minimization (Fig. 3). The calculated interatomic distances between H-1/H-6 (3.61 Å), H-8/H-9 (2.35 Å), H-4/H-6 (2.74 Å), and H-7/H-8 (2.36 Å) are all less than 4.00 Å; this further supported the well-defined ROESY correlations observed for each of these proton pairs.

The antiangiogenic activities of all compounds **1-11** except **3** and **6** were evaluated using a zebrafish model, in terms of the inhibition of the growth of intersegmental vessels, with PTK787 as a positive control (IC_{50} 0.15 μg/mL) (Li et al., 2009). No compounds showed obvious antiangiogenic activities. In addition, the antiproliferative activities of all compounds were evaluated using A549 lung cancer cells

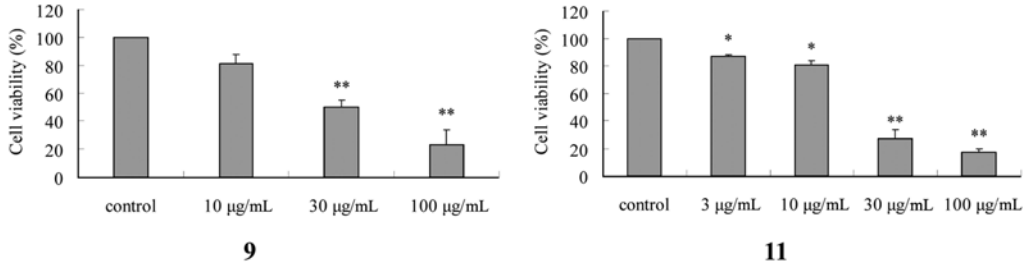

Fig. 4. Inhibitory effect of compounds **9** and **11** (48 h after treatment) on the proliferation of A549 lung cancer cells. *$p < 0.05$ and **$p < 0.01$, when compared with the control group.

by MTT assay (He et al., 2007). The results indicated that compounds **9** and **11** showed a certain extent of antiproliferative activity (Fig. 4.). From the images of acridine orange staining, compounds **9** and **11** exhibited an obvious effect of inducing apoptosis of A549 lung cancer cells (Fig. 5.). Other tested compounds did not show obvious antiproliferative bioactivities.

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