Coumarins from *Angelica dahurica* and Their Antitumor Activities in Human MG-63 Osteosarcoma Cells

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Abstract: A new coumarin, angedahurin A (**1**), and seven known analogues (**2−8**), were isolated from the roots of *Angelica dahurica*. Their structures were identified by extensive NMR, IR, and HR-ESIMS spectroscopic analyses. The cytotoxicities of coumarins **1−8** against MG-63 human osteosarcoma cell lines were screened. Compound **1** showed significant cytotoxic effects against MG-63 with an IC_{50} value of 7.2 μM，for comparison, the positive control, 5-FU, had an IC_{50} value of 32.4 μM. Morphological features of apoptosis activities were evaluated in **1**-induced MG-63 cells and the results confirmed MG-63 cell apoptosis in a dose-dependent manner.

1. Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumours and is most prevalent in teenagers and young adults [1]. According to the US National Cancer Institute Surveillance, Epidemiology, and End Results program, the frequency of OS has increased by 0.3% per year over the last decade [2]. Currently, the most common treatment of osteosarcoma is combined use of chemotherapy and surgery. Despite major improvements in chemotherapeutic regimens, the survival rate of patients with osteosarcoma still lags behind the overall survival rates of patients with cancer in that age group, mainly because of the intrinsic or acquired drug resistance. Discovering novel preventive agents is thought to be an available strategy, which is mainly focused on the discovery of precise molecular targets for OS chemoprevention [3].

Angelica dahurica is a plant that belongs to the *Angelica* genus and is distributed in Northern and Northeastern China. The roots of *A. dahurica* called Bai-Zhi in traditional Chinese medicine that classified as a sweat-inducing drug able to counter harmful external influences on the skin, such as

cold, heat, dampness and dryness [4]. *A. dahurica* mainly contains coumarins, volatile oils, polysaccharides, and trace elements as the bioactive components [5]. Many of these coumarins showed significant cytotoxic, anti-inflammatory, antioxidant, and tyrosinase inhibitory activities [6]. As part of our screening program to explore antineoplastic compounds from Chinese herbs [3], in this study, the petroleum ether (PE) extract of *A. dahurica* showed a cytotoxic inhibitory activity. Subsequent chemical investigation led to the isolation of eight coumarins, including a new coumarin, angedahurin A (**1**), and seven known analogues (**2**−**8**) (Figure 1). The cytotoxicities of coumarins **1**−**8** against MG-63 human osteosarcoma cell lines were screened. Compound **1** showed significant cytotoxicities against MG-63 cell lines with an IC_{50} value of 7.2 μM. The positive control 5-FU against the MG-63 with an IC_{50} value of 32.4 μM. Herein, details of the isolation, structural elucidation, and cytotoxic activities of these compounds are described.
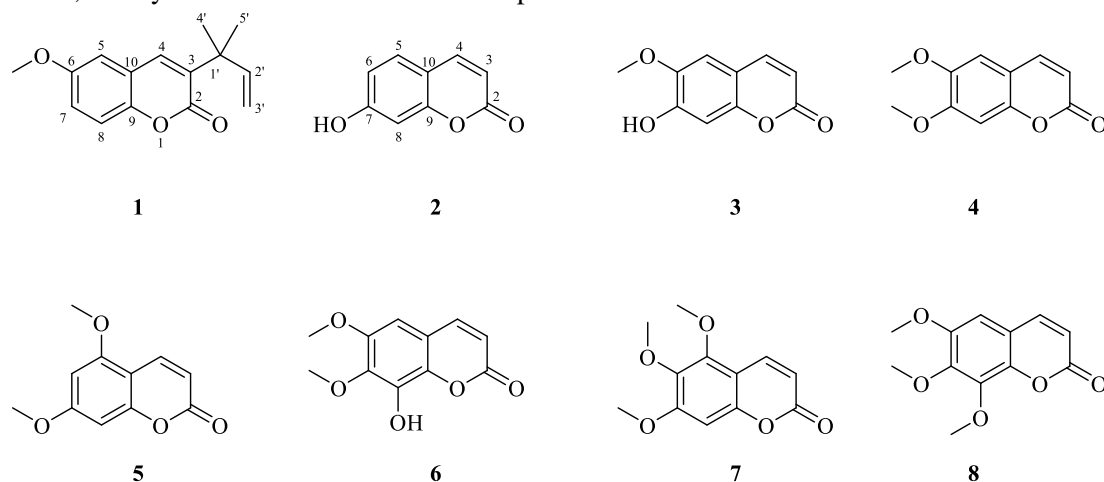

Figure 1. Structures of compounds **1**−**8** isolated from *Angelica dahurica*

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotations were measured on a Rudolph Autopol I automatic polarimeter. IR spectra were measured on a Bruker Tensor 37 infrared spectrophotometer. The NMR experiments were performed on a Bruker AM-400 spectrometer at 25°C. The mass spectra were recorded on an Agilent Q-TOF micro mass spectrometer. A YMC-Pack column (250 × 20 mm, 5 μm, Kyoto, Japan) was used to perform the HPLC separations. A YMC-pack ODS-A column (250 × 10 mm, 5 μm) were used for semi-preparative HPLC separation. Silica gel (200−300 mesh) was acquired from Qingdao Haiyang Chemical Co. (P. R. China), C18 reversed-phase silica gel (12 nm, S-50 μm, YMC Co., Ltd.), and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography (CC). Fractions were monitored by TLC with precoated glass plates GF254, and spots were detected by spraying with 10% H_2SO_4 in EtOH, followed by heating.

2.2. Plant Material

The roots of *A. dahurica* were purchased from Bozhou baisai Pharmaceutical Company (Bozhou, Anhui Province, China). Prof. Guokai Wang (School of Pharmacy, Anhui University of Chinese Medicine) identified the specimens; a voucher specimen (NO.20191012) has been deposited at department of medicine, Huangshan Vocational and Technical College.

2.3. Extraction and Isolation

The air-dried powder of the roots of *A. dahurica* (5 kg) was extracted with 3 L of 95% EtOH three times，following which the extract solution was filtered and concentrated in vacuo to yield the crude extract (510 g). The extract was suspended in water (3 L) and successively partitioned with petroleum ether (PE, 3×2 L) and EtOAc (3×2 L)， to afford petroleum ether (40.5 g)and EtOAc (56.1 g). The PE extract (40.5 g) was subjected to silica gel CC (10×160 cm) eluted with petroleum ether−EtOAc (100:1, 80:1, 50:1, 30:1, 10:1, 5:1, 2:1,1:1, and 0:1, v/v) to afford 5 fractions E1-E5. E1 (6.0 g) was loaded onto Sephadex LH-20 column and eluted with MeOH led to E1a–E1f. E1b were subjected to preparative HPLC (MeOH/H_2O, 4:1) to yield **2** (7.5 mg, t_R 16 min) and **3** (5.7 mg, t_R 18 min). E1c was separated by HPLC (MeOH/H_2O, 8:2, 3 mL/min) to afford **1** (8.9 mg, t_R 13 min) and **4** (9.1 mg, t_R 15 min). E1d was purified by a semi-preparative HPLC (Eclipse XDB-C_{18} column, MeOH/H_2O, 7:3, 3 mL/min) to yield **6** (8.3 mg, t_R 11.3 min), **7** (3.5 mg, t_R 13.5 min), and **8** (4.7 mg, t_R 16.1 min). E2 was chromatographed on Sephadex LH-20 column chromatography (MeOH) to yield 3 subfractions E2a-E2c, E2a was purified by preparative HPLC (CH_3CN/H_2O, 3:2, 3 mL/min) to provide **5** (7.3 mg, t_R 12.5 min).

2.4. Spectroscopic Data

*Angedahurin A (**1**)*: Colorless powder; $[\alpha]^{25}_D$ −12.2 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 322 (3.52), 204 (3.90) nm; IR (KBr) v_{max} 2928, 1740, 1697, 1615, 1365, 1241, 1005, 907, 823, 785, 629cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 245.1183 [M + H]$^+$ (calcd. for $C_{15}H_{17}O_3$, 245.1172).

2.5. Cell Culture

MG-63 human osteosarcoma cell line was obtained from the Cell Bank of the Chinese Academy of Sciences. The cells were maintained in DMEM containing 10% fetal bovine serum (Gibco BRL Co., Grand Island, NY, USA) at 37 °C in a humidified incubator containing 5% CO_2.

2.6. Cell Viability Assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate MG-63 cell viability as previously described [3]. Briefly, human MG-63 osteosarcoma cells were plated in 96-well plates (6×10^4 cells/well) for 24 h and then incubated with compound **1** in various concentrations with or without LPS (1.0 μg/mL) for an additional 24 h. Then, the prepared MTT solution (20 μL, 5 mg/mL) was added, and the cells were incubated for another 4 h. After the formazan that formed was fully dissolved in DMSO and the absorbance was read at 560 nm on a microplate reader.

2.7. Morphological Analysis

In this experiment, 6×10^4 MG-63 cells were seeded in 12-well plates and treated with compound **1** for 24 h. The cells were washed three times with phosphate-buffered saline (PBS). After the medium was replaced by nutrient-deprived medium (NDM) as a control or with various concentrations (1.8, 3.6, 7.2, 14.4, and 28.8 μM) of **1** added followed by incubation for an additional 24 h. Subsequently, the cells were stained with 100 μL of 200 μM AO reagent, and incubated for 5 min in the dark. Then, the cell morphology was observed under a fluorescence microscope ($20 \times$ objective).

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was isolated as a colorless powder with a molecular formula $C_{15}H_{16}O_3$, as deduced by a HRESIMS ion at *m*/*z* 245.1183 [M + H]$^+$ (calcd for $C_{15}H_{17}O_3$, 245.1172), corresponding to eight

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degrees of unsaturation. The IR spectrum exhibited absorption bands for carbonyl group (1740 cm⁻¹), phenyl (1615 and 1505 cm⁻¹), and terminal double bonds (1697 and 1005 cm⁻¹). The ^{1}H NMR spectrum exhibited signals for three methyl groups [δ_H 3.85 (3H, s) and 1.47 (3H × 2, s)], a set of olefinic protons of a terminal double bond [δ_H 6.17 (1H, dd, J = 17.1, 11.0 Hz), 5.08 (2H, dd, J = 17.1, 11.0 Hz)], four olefinic protons [δ_H 7.52 (1H, s), 7.34 (1H, d, J = 2.4 Hz), 6.81 (1H, dd, J = 8.5, 2.4 Hz) and 6.78 (1H, d, J = 8.5 Hz)]. The ^{13}C NMR spectrum, in combination with DEPT experiments, resolved 15 carbon resonances attributable to a carbonyl group (δ_C 160.0), one terminal double bond (δ_C 145.5 and 112.1), a phenyl bonds (δ_C 162.0, 155.0, 128.5, 112.9, 112.2, and 100.0), one trisubstituted double bond (δ_C 137.7 and 131.7), three methyls (including one methoxyl), and one sp³ quaternary carbon. As six of the eight degrees of unsaturation were accounted for by one carbonyl group, a phenyl group, and four double bonds, the remaining degrees of unsaturation required that **1** was dicyclic system. The stated information above, coupled with literature reports and biogenetic considerations [7], established the presence of a coumarin skeleton. Analysis of the ^{13}C NMR, HSQC and ^{1}H–^{1}H COSY spectra suggested a coumarin system with an additional C$_5$ moiety, which consisted of a terminal double bond, a quaternary carbon and two tertiary methyl groups. In the HMBC experiment, correlations from H$_3$C-4'/5' (δ_H 1.47, 3H × 2) to C-3 (δ_C 131.7) and H-2' (δ_H 6.17, 1H, dd, J = 17.1, 11.0 Hz) to C-3 (δ_C 131.7), respectively, indicated that the C$_5$ side-chain was located at C-3. The ^{1}H and ^{13}C NMR data (Table 1) showed high similarity to 3-(1',1'-dimethylallyl)-herniarin [8]. The differences between these two compounds were that the methoxy group at C-7 in 3-(1',1'-dimethylallyl)-herniarin was moved to C-6 in **1**. This deduction was verified by the HMBC correlation from H$_3$CO-C6 (δ_H 3.85) to C-6 (δ_C 162.0). The planar structure of **1** was further established by detailed interpretation of its 2D NMR data (Figure 2). Due to the structure of **1** does not involve chiral configurations, thus, new compound **1** was established as shown and named as angedahurin A.
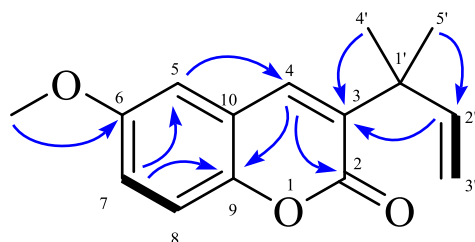

Figure 2. Selected ^{1}H–^{1}H COSY (—) and HMBC (→) correlations of **1**

Seven known coumarins, umbelliferone (**2**) [9], scopoletin (**3**) [10, 11], 6,7-dimethoxycoumarin (**4**) [12], 5,7-dimethoxycoumarin (**5**) [13], fraxidin (**6**) [14], 5,6,7-trimethoxy-2*H*-benzopyran-2-one (**7**) [11], 6,7,8-trimethoxycoumarin (**8**) [15] were also isolated from the *A. dahurica* extract. Those known compounds were identified by spectroscopic data comparison with published literature.

Table 1. NMR Spectroscopic Data (^{1}H 400 MHz, ^{13}C 100 MHz, CDCl$_3$) for **1**

No.	δ_H	δ_C	No.	δ_H	δ_C
2		160.0, C	9		155.0, C
3		131.7, C	10		112.9, C
4	7.52, s	137.7, CH	1'		40.3, C
5	7.34, d (2.4)	128.5, CH	2'	6.17, dd (17.1, 11.0)	145.5, CH
6		162.0, C	3'	5.08, 2H, dd (17.1, 11.0)	112.1, CH$_2$
7	6.81, dd (8.5, 2.4)	112.2, CH	4'	1.47, s	26.1, CH$_3$
8	6.78, d (8.5)	100.0, CH	5'	1.47, s	26.1, CH$_3$
6-OCH$_3$	3.85, s	55.7, CH$_3$			

3.2. Cytotoxic Effects of the Isolates

The in vitro cytotoxicities of all the isolated compounds **1−8** against human MG-63 osteosarcoma cell lines were tested by the MTT assay. Compound **1** was found to be the most active compound, exhibiting an IC_{50} value of 7.2 μM toward MG-63 cells, for comparison, the positive control 5-FU against the MG-63 with an IC_{50} value of 32.4 μM (Figure 3).
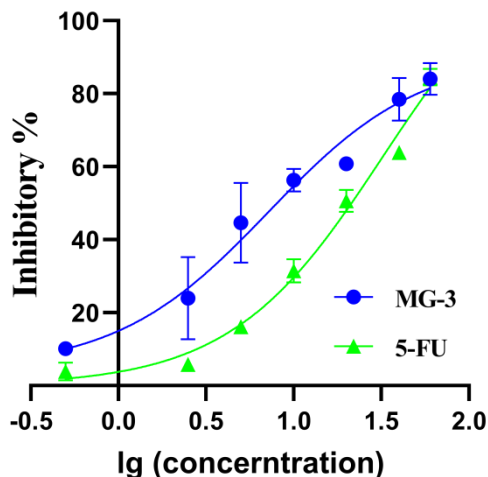
Figure 3. Inhibitory curves of compound **1** in human MG-63 osteosarcoma cells

3.3. Morphological Changes in MG-63 Cells Treated with angedahurin A (**1**)

To confirm the apoptosis-inducing effects of angedahurin A (**1**), a nuclear morphological evaluation of MG-63 cells was performed by fluorescence microscopy following staining acridine orange (AO) after treatment for 24 h with 1.8, 3.6, 7.2, 14.4, and 28.8 μM concentrations of **1**. Afterwards, the characteristic morphological features of apoptosis were examined under fluorescence microscope. Compared with the control group, treatment with **1** caused significant morphological changes, including the appearance of membrane blebbing and granular apoptotic bodies (Figure 4), with blue arrows highlighting apoptotic cells.
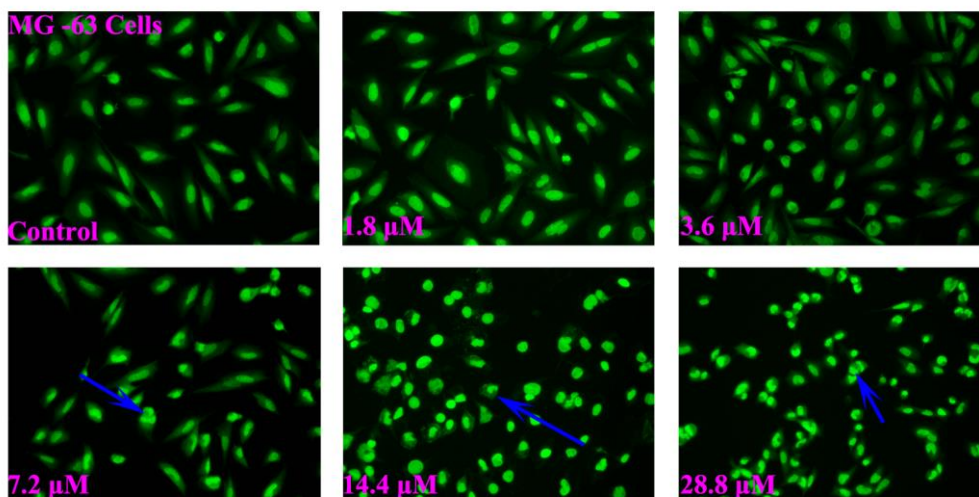
Figure 4. Evaluation of the effects of compound **1** on apoptosis

4. Conclusions

In this study, chemical investigation of petroleum ether extracts led to the isolation of eight coumarins including a new coumarin, angedahurin A (**1**), and seven known analogues (**2−8**). Their

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structures were identified by extensive 1D and 2D NMR, IR, and HR-ESIMS spectroscopic analyses. The cytotoxicities of all the coumarins (**1−8**) were evaluated against MG-63 human osteosarcoma cell line. We considered any compound exhibiting an inhibition rate of less than 50% at 50 μM to be inactive. The results showed that compound **1** exhibited significant cytotoxic effects against MG-63 with an IC$_{50}$ value of 7.2 μM, for comparison, the positive control, 5-FU against the MG-63 with an IC$_{50}$ value of 32.4 μM. The IC$_{50}$ values of other compounds were all more than 50 μM. Morphological features of apoptosis activities were evaluated in **1**-induced MG-63 cells and the results confirmed apoptosis in a dose-dependent manner.

Acknowledgments

Supporting Information

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

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