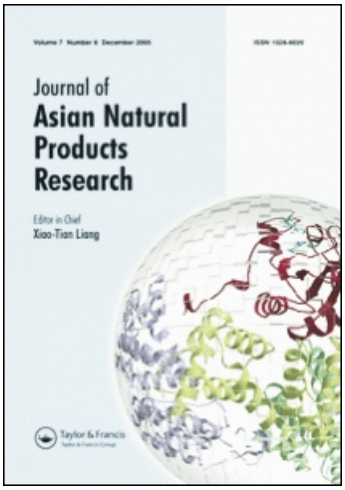

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Lin-Tao Han[a]; Juan Li[b]; Fang Huang[a]; Shang-Gong Yu[a]; Nian-Bai Fang[a]
[a] Key Laboratory of Traditional Chinese Medicine Resource and Compound Prescription, Hubei College of TCM, Wuhan, China [b] Guangxi Institute of Botany, Chinese Academy of Sciences, Guilin, China

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Lin-Tao Han[a], Juan Li[b], Fang Huang[a]*, Shang-Gong Yu[a] and Nian-Bai Fang[a]

[a]*Key Laboratory of Traditional Chinese Medicine Resource and Compound Prescription, Hubei College of TCM, Wuhan, China;* [b]*Guangxi Institute of Botany, Chinese Academy of Sciences, Guilin, China*

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Five triterpenoid saponins were isolated from *Anemone flaccida* Fr. Schmidt. Their structures were identified as glycoside St-I4a (**1**), glycoside St-J (**2**), anhuienoside E (**3**), hederasaponin B (**4**), and flaccidoside II (**5**). Compounds **1**–**2** were isolated from Anemone family for the first time, and compounds **3**–**4** were isolated from this plant for the first time. The inhibitory effects of saponins on proliferation of HeLa cells were studied by MTT assay, the apoptosis-induction activity was observed by cell-cycle analysis and caspase-3 expression assay. The antitumor activities of the saponins were ranked in the following order: **5** > **3** > **4** > **1** > **2**. The data presented here indicated that naturally occurring triterpenoid saponins can be regarded as excellent structures for the potential development of new anticancer agents.

Keywords: *Anemone flaccida*; triterpenoid saponins; antitumor; apoptosis

1. Introduction

Anemone family is an important source of bioactive triterpenoid saponins [1–6]. Some saponins have shown very strong antitumor activity. For example, the saponins from *Anemone david* could inhibit the growth of subcutaneous tumor S_{180} in mice [7]. *Anemone flaccida* is widely distributed in China and has been used as folk medicine to cure rheumatism and neuralgia. Six triterpenoid saponins have been reported from the rhizome of this plant [1,2]. The anti-proliferative activity of triterpenoid saponins from this plant on tumors has never been demonstrated. This paper describes the isolation, purification, and antitumor testes *in vitro* of saponins from *A. flaccida* in order to find new anticancer compounds and realize the effective chemotherapy of cancer.

2. Results and discussion

The present work described the extraction and purification of triterpenoid saponins from *A. flaccida.* Compounds **1**–**5** were investigated for their antiproliferative effects on HeLa cell. All saponin displayed cytotoxicity against HeLa cell line. The cytotoxic activity was listed as follows: **5** > **3** > **4** ⩾ **1** > **2**. When compared with compounds **3**–**5**, compounds **1** and **2** showed less activity. Since the reduction of cytotoxic activities in compounds **1** and **2** may be due to the presence of C-3 position glucuronic acid moiety in the molecules. The C-3 position glycosidic chain's polarity was essential for the growth inhibition in tested cancer cell line (Table 1 and Figure 1).

The apoptosis-induction potential rather than necrosis induction was accepted as a key feature of a potential antitumor drug [8].

*Corresponding author. Email: hello_tracy@163.com

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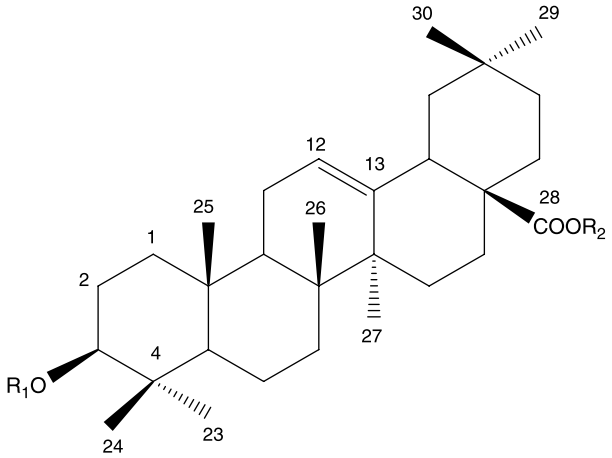
Table 1. The antitumor activities of compounds **1**–**5**.

	HeLa cell line		
Sample	IC_{50} (μmol/l)[a]	Apoptosis rate (%)[b]	Caspase-3 expression rate (%)[c]
Vehicle control	–	0.24 ± 0.15	7.8 ± 1.11
1	15.22	7.26 ± 0.42[d]	15.8 ± 1.34[d]
2	16.34	4.63 ± 0.37[d]	9.7 ± 1.03[e]
3	4.28	8.11 ± 0.49[d]	22.1 ± 1.44[d]
4	3.99	10.95 ± 0.74[d]	24.5 ± 1.36[d]
5	1.21	23.73 ± 0.53[d]	33.8 ± 1.79[d]

[a] Evaluation: strongly active ($IC_{50} < 0.1$ μmol/l); active (0.1 μmol/l $< IC_{50} <$ 1.0 μmol/l); weakly active (1 μmol/l $< IC_{50} <$ 10.0 μmol/l); inactive ($IC_{50} > 10.0$ μmol/l).
[b] Concentration: 5 μmol/l, mean ± SD, $n = 3$.
[c] Concentration: 5 μmol/l, mean ± SD, $n = 6$.
[d] $P < 0.01$ vs. vehicle control group.
[e] $P < 0.05$ vs. vehicle control group.

Accordingly, in the next set of experiment, compounds **1**–**5** were subjected to additional *in vitro* assays in order to verify their apoptosis-inducing potential. In the view of fluorescence microscope, HeLa cells treated with all tested compounds exhibited characteristic apoptotic features: cell shrinkage, nuclear chromatin condensation, and aggregation inside the nuclear envelope. Their inducing apoptosis activities were ranked in the following order: **5** > **3** > **4** ⩾ **1** > **2**. The clear parallelism of cytotoxicity assay and apoptosis analysis assay allowed the suggestion that the apoptosis induction played a crucial role in the cytotoxic mechanism of these triterpenoid saponins (Table 1 and Figure 2).

Caspase proteinases drive apoptotic signaling and execution by cleaving critical cellular proteins solely after aspartate residues. Once activated, initiator caspases in



No.	R_1	R_2
1	-glcA	-glc(6-1)glc(4-1)rha
2	-glcA	-glc(6-1)glc
3	-glc(2-1)rha	-glc(6-1)glc(4-1)rha
4	-ara(2-1)rha	-glc(6-1)glc(4-1)rha
5	-xyl(2-1)rha	-glc(6-1)glc(4-1)rha

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

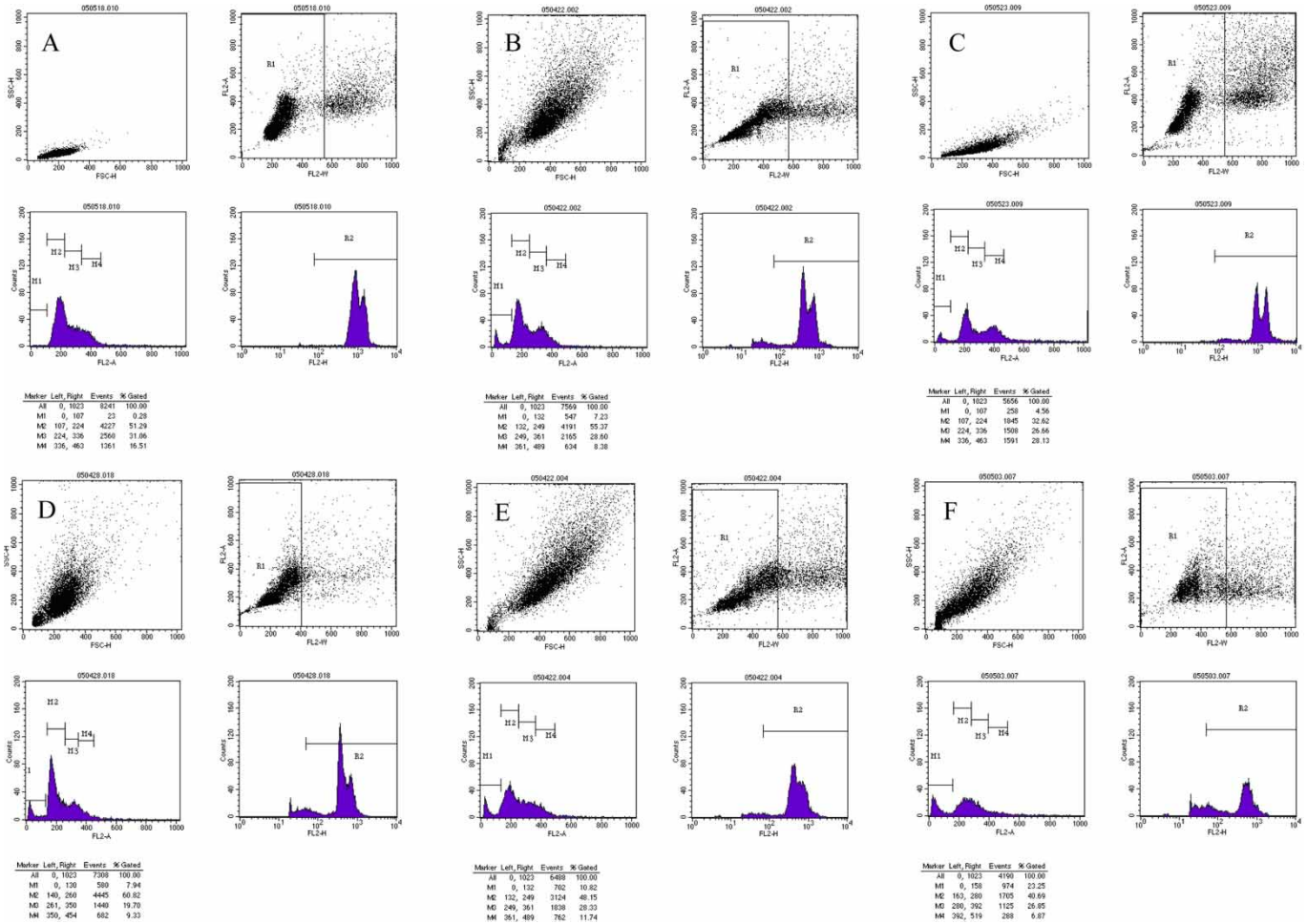


Figure 2. Apoptosis analysis assay of compounds **1**–**5**. Panel (A) HeLa cell treated with vehicle. Panels (B)–(F) HeLa cell treated with 5.0 μmol/l compounds **1**–**5**, respectively.

turn activate the executioner caspases, caspases-3, -6, and -7. The active executioners promote apoptosis by cleaving cellular substrates that induce the morphological and biochemical features of apoptosis. Although many proteinases may function in nuclear apoptosis, caspase-3 was the key determinant of DFF45/ICAD inactivation and apoptotic internucleosomal DNA fragmentation [9]. To ascertain the mechanism of the action of these triterpenoid saponins, the role of caspase-3 in the regulation of apoptosis was examined by SABC immunohistochemistry method. The increased expression percentage of caspase-3 protein compared with vehicle control demonstrated that compounds **1**–**5** could shift the antiapoptotic–proapoptotic balance of the cell toward apoptosis (Table 1 and Figure 3).

In summary, the results indicated that naturally occurring triterpenoid saponin compounds **1**–**5** from Anemone family may be used as starting structures for the potential development of novel cancer preventive and therapeutic agents.

3. Experimental

3.1 General experimental procedures

Melting points were determined on an XT-4 micro-melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer digital polarimeter. The UV spectra were recorded on a Shimadzu UV-2000 spectrometer. The IR spectra were determined on a Nicolet 170-SX infrared spectrometer in KBr pellets. The NMR spectra were taken with TMS as internal standard on a Varian Inova 400 FT-NMR spectrometer. ESI-MS were measured on a Finnigan LCQ DECA XP^{plus} spectrometer. Cell apoptosis was analyzed by FACS Calibur flow cytometry using CELLQuest 3.0.1 software.

3.2 Plant material

Rhizome of *A. flaccida* Fr. Schmidt was collected in September 2002 from Jiufeng County of Hubei Province, China. The sample was identified by Prof. Keli Chen, Department of Pharmacology, Hubei College of Traditional Chinese Medicine. A voucher specimen had been deposited in the Herbarium of the Hubei College of Traditional Chinese Medicine.

3.3 Extraction and isolation of compounds 1–5

The dried rhizome of *A. flaccida* (5 kg) was extracted with CH_3OH at room temperature. The extract was filtered, concentrated, and chromatographed on a D_{101} resin column using EtOH—H_2O (30:70, 50:50, 70:30, and 100:0) as eluents to yield four fractions. Residues weighing 55.0, 58.5, 29.7, and 20.5 g were obtained, respectively. The residue obtained from the 30% EtOH fraction was further separated on a low-pressure reversed-phase C_{18} column using CH_3OH—H_2O (20:80 → 90:10, gradient mixtures) as eluent to afford compounds **1** (50 mg) and **2** (40 mg). By similar procedures, compounds **3** (55 mg), **4** (30 mg), and **5** (35 mg) were obtained from the 50% EtOH fraction.

The structures of compounds **1**–**5** had been determined by chemical and spectral evidences as glycoside St-I4a (**1**) [3], glycoside St-J (**2**) [4], anhuienoside E (**3**) [5], hederasaponin B (**4**) [6], and flaccidoside II (**5**) [2]. Compounds **1**–**2** were isolated from Anemone family for the first time, and compounds **3**–**4** were isolated from this plant for the first time (Figure 1).

3.4 Cell culture

The cervix carcinoma cell line (HeLa) was obtained from China Center for Type Culture Collection. The cell line was maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA), containing 1% non-essential amino acids and 10% fetal calf serum. They were cultured in the humidified 5% carbon dioxide in air at 37°C.

3.5 Cytotoxicity assay

The inhibitory effects of compounds **1**–**5** on human cervix cancer cell line (HeLa) proliferation were tested by MTT method. HeLa cells

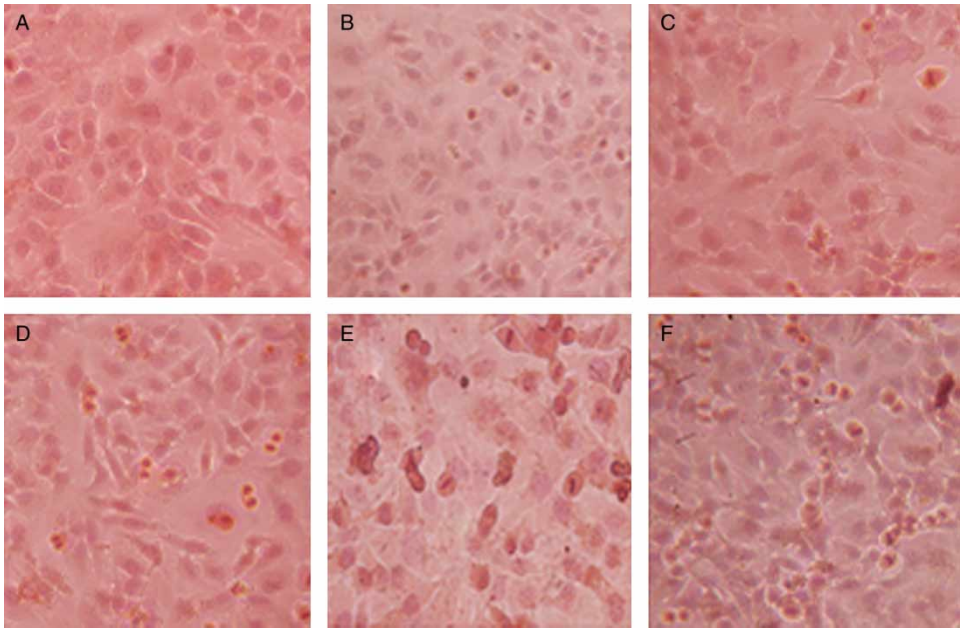


Figure 3. Caspase-3 expression assay of compounds **1**–**5**. Panel (A) HeLa cell treated with vehicle. Panels (B)–(F) HeLa cell treated with 5.0 μmol/l compounds **1**–**5**, respectively. Original magnification ×400.

(1×10^4 cells/ml) were seeded to DMEM supplemented with 10% heat-inactivated FBS and 100 ng/ml penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Test compounds **1**–**5** were added to this culture and incubated at 37°C a further 72 h without medium change. Afterward, the medium was discarded and 100 ml of the MTT solution (Sigma-Aldrich, Inc., St Louis, MA, USA, 500 mg/ml in one-fold DMEM) were added and incubated for 4 h at 37°C. The medium was carefully removed and 150 ml of DMSO were added. After gently shaking at room temperature for 10 min, optical absorbance at 570 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA, USA) [10]. The experiment was done in triplicate and repeated thrice.

The half-maximal inhibitory concentration values (IC_{50}) were listed as follows: **5** (1.21 μmol/l) < **4** (3.99 μmol/l) < **3** (4.28 μmol/l) ≪ **1** (15.22 μmol/l) < **2** (16.34 μmol/l). Compound **5** was proved to be an outstandingly potent cytotoxic agent against HeLa cells, while compounds **4** and **3** were moderately active and compounds **1** and **2** were weakly active (Table 1).

3.6 Apoptosis analysis assay

For the measurement of cellular DNA content, flow cytometric analysis was used [11]. HeLa cells were treated with 5.0 μmol/l test compounds and incubated for 24 h. Cells were washed and fixed overnight with cold 70% ethanol, and then stained with PI solution consisting of 45 μg/ml PI, 10 μg/ml RNase A, and 0.1% Triton X-100. After 1 h incubation at room temperature in the dark, the percentage of apoptotic cells was determined by FACS Calibur flow cytometry using CELLQuest 3.0.1 software. The experiment was done in triplicate.

Results showed that the apoptotic rate of compounds **1**–**5** was significant ($P < 0.01$), compared with the vehicle control group. Their inducing apoptosis activities were consisted with the cytotoxic activities: **5** > **4** > **3** ⩾ **1** > **2** (Table 1 and Figure 2).

3.7 Caspase-3 expression assay

The caspase-3 activation of compounds **1**–**5** was measured as an indicator of apoptosis induction since different upstream pathways leading to apoptosis depend on caspase-3 induction for final apoptotic execution. The caspase-3 assay was performed based on the method of Voss *et al.* [11]. HeLa cells seeded on coverslips were fixed in polyethylene glycol after treated with 5.0 μmol/l test compounds at 37°C. Assay for caspase-3 activity was measured as described in the SABC immunohistochemistry kit's introduction (Boster Biological Technology Ltd, Wuhan, China). The positive and negative expression cells were identified and counted under the light microscope.

There was significant increase in the caspase-3 protein expression with 5.0 μmol/l compounds **1**–**5**. Caspase-3 assay showed that the positive rate of compound **5** is the highest ($P < 0.01$), when compared with the vehicle control group. And then compound **4** ($P < 0.01$) and compound **3** ($P < 0.01$). And then compound **1** ($P < 0.01$) and compound **2** ($P < 0.05$). The results were the same with apoptosis analysis assays (Table 1 and Figure 3).

3.8 Statistical analysis

The statistical analysis was evaluated by Student's test. All data were expressed as means ± SD. And values of $P < 0.05$ were considered to be significant. $P < 0.01$ was considered to be obviously significant.

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