

Comparison of cytotoxic activities of extracts from *Selaginella* species

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ABSTRACT

Background: *Selaginella* species are resurrection plants, which are known, possess various molecular bioactivities depending on species, but only a few species have been detailed observe in the advanced research. **Objective:** The objective of the following study is to compare the chemical profiles of different species of *Selaginella* and to investigate cytotoxicity and induction of apoptosis activities of some species of *Selaginella*. **Materials and Methods:** The high-performance liquid chromatography (HPLC) method was developed for chemical analysis. Ethyl acetate, ethanol and water-soluble extracts from seven *Selaginella* species were submitted to 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, flow cytometry, deoxyribonucleic acid (DNA) laddering analysis and caspase-3 expression using Bel-7402, HT-29 and HeLa cells. **Results:** The HPLC analysis revealed two major common peaks, which were identified as amentoflavone and robustaflavone and another three main peaks in their chromatograms. The results showed that *S. labordei*, *Selaginella tamariscina* and *Selaginella uncinata* had relatively stronger activities on Bel-7402 and HeLa cells and *Selaginella moellendorffii* had moderate antiproliferation activities, but *Selaginella remotifolia* and *Selaginella pulvinata* had almost no inhibitory activities. The main active components were in the ethyl acetate extracts which had abundant biflavonoids. The effects of these extracts on cell proliferation and apoptosis in different cells were not the same, they were more apparent on HeLa cells than on HT-29 cells. The assay of DNA laddering analysis and caspase-3 expression further confirmed that inducing cell apoptosis was one of antitumor mechanisms and antitumor activities of *Selaginella* species were related to apoptosis induced by caspase family. **Conclusion:** *S. labordei*, *S. tamariscina* and *S. uncinata* would be potential antitumor agents.

Key words: Amentoflavone, antitumor, apoptosis, *Selaginella*

INTRODUCTION

The genus *Selaginella* is composed of more than 700 described species, which is the only extant genus in the family *Selaginellaceae*. It is mostly distributed in the subtropics and the tropics and there are about 60 species in China. *Selaginella* has been used widespread for the treatment of many kinds of diseases, including diabetes,^[1] cardiovascular diseases,^[2] a variety of inflammatory diseases^[3] and some kinds of cancer.^[4] The largest usage is conducted by

Chinese, especially for *Selaginella tamariscina*, *S. doederleini*, *S. moellendorffii*, *Selaginella uncinata* and *S. involvens*.^[3]

Selaginella species has a large number of bioactive compounds, which contains alkaloid, phenols, sterol, aliphatic acid and terpenoid. Biflavonoids, such as amentoflavone, sumafavone, robustaflavone, ginkgetin, hinokiflavone and isocryptomerin, are the most important valuable natural products of *Selaginella* and show various pharmacological activities including antioxidant, anti-inflammatory and antitumor.^[5-9]

Selaginella are known possess various pharmacological activities depending on species, but only a few species has been comprehensively studied. *S. tamariscina*, *S. delicatula*, *Selaginella moellendorffii*, *et al.* have been reported to show antitumor and antioxidant properties. The antitumor

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mechanisms are related to induction of apoptosis through deoxyribonucleic acid (DNA) fragmentation and nucleus clotting^[10] or through blockade of fatty acid synthesis,^[8] inducing expression of p53 and G1 arrest,^[11] inhibiting transactivation of iNOS and COX-2 through inactivate NF- κ B and prevent translocation of p65.^[12]

However, few studies on comparing antitumor activities of *Selaginella* species have been conducted. The present study is designed to investigate cytotoxicity and inducing apoptosis activities of some species of *Selaginella*. Determination of the content of amentoflavone (the most common chemical of *Selaginella*) in each species is used to assess the quality and survey the relationship between amentoflavone content and antitumor activity.

MATERIALS AND METHODS

Materials

The samples of *Selaginella* species, which was listed in Table 1, were identified by authors. Specimens of these plants were deposited in the herbarium, Hubei College of Traditional Chinese Medicine, China.^[13,14]

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) were purchased from GIBCO (BRL, Gaithersburg, MD). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), RNaseA, penicillin, streptomycin were obtained from Sigma Chemical Co (St Louis, MO, USA). *In situ* hybridization kit for caspase-3 and apoptotic DNA laddering kit were supplied by Wuhan Boster Biological Technology Co., Ltd. For the preparation of all samples and solutions distilled water deionized with Milli-Q Water Purification System (Millipore, Bedford, Massachusetts, USA) was used.

Chromatographic condition

The detailed method of high-performance liquid chromatography (HPLC) analysis could be seen in

literature.^[15] Briefly, HPLC analysis was performed on a Dionex HPLC system with P680 Pump, a Diamonsil™ C18 column (250 × 5.6 mm, 5 μ m) and a UVD 170 U variable wavelength UV-Vis detector. Data were collected and processed using ““Chromeleon version 6.0”” software (Dionex Corp.). The mobile phase consisted of acetonitrile (A) and water (B). The gradient program was as follows: 25% A in 0-5 min, 25-35% A in 5-12 min, 35-45% A in 12-17 min, 45-50% A in 17-25 min, 50-55% A in 25-40 min, 55-70% A in 40-45 min, 70-100% A in 45-50 min, 100% A in 50-55 min, 100-25% A in 55-60 min respectively. The flow rate was 1.0 mL/min and column temperature was maintained at 30°C. The injection volume was 10 μ L. The detector was set at 330 nm for acquiring chromatograms.

Each of the finely powdered samples (0.5 g) was accurately weighed into a Soxhlet extractor and 50 mL of chloroform was added. The mixture was heated to reflux for 4 h and the extract was filtered, the solvent evaporated. Then 50 mL methanol was added and heated for 10 h in a water bath. The original solvent weight was restored and the extract was filtered. The solution was filtered through a syringe filter (0.45 μ m). Each filtrate (10 μ L) was injected for HPLC analysis.

The precision of the chromatographic method was assessed by performing multiple injections of the same concentration of a solution of sample 4 [Table 1]. The repeat abilities of the extraction procedure and analysis were validated by repeating the extraction and HPLC analysis procedure 5 times on the same sample of 4. The stability test was performed using the same sample that was stored at room temperature and analyzed at 0, 2, 4, 8 and 12 h. Relative retention time of each characteristic peak against internal standard peak (amentoflavone) and percent of total peak area were calculated.

Quantitative HPLC analysis was performed under the same condition. Peak areas obtained at 330 nm were used for the construction of calibration curves and 0.1 mg/mL amentoflavone was used as the standard.

Extracts preparation

The dried and powdered samples (50.0 g) were extracted sequentially with ligarine (500 mL, 3 times), ethyl acetate (500 mL, 3 times), 95% ethanol (500 mL, 3 times) and water (500 mL, 3 times). Although ligarine was used for degreasing, not for further experiment. The extracts were dried and concentrated under reduced pressure to yield ethyl acetate extract, ethanol extract and water-soluble extract. All extracts were dissolved in DMSO and stored at -20°C for the assay on cell proliferation and apoptosis. Final concentrations of extracts for different experiments were prepared by diluting with solvent. The black control received the carrier solvent.

Table 1: The amentoflavone content of seven *Selaginella* species

Species	Province	Content (mg/g)
<i>Selaginella uncinata</i> (Desv.) Spring	Yichang, Hubei	2.03
<i>Selaginella tamariscina</i> (Beauv.) Spring	Yichang, Hubei	11.46
<i>Selaginella remotifolia</i> Spring	Yichang, Hubei	10.99
<i>Selaginella delicatula</i> (Desv.) Alston	Yichang, Hubei	4.23
<i>Selaginella moellendorffii</i> Hieron	Yichang, Hubei	13.33
<i>Selaginella pulvinata</i> (Hook. et Grev.) Maxim	Yichang, Hubei	16.41
<i>Selaginella labordei</i> Hieron. ex Christ	Mt. lu, Jiangxi	2.95

Cell culture and morphological observation

Bel-7402 human liver cells, HT-29 human colon adenocarcinoma cells and HeLa human cervical carcinoma cells were obtained from China Center for type culture collection of Wuhan University. All cells were maintained in DMEM medium supplemented with 10% FBS and 100 ng/mL penicillin and streptomycin for each at 37°C in a humidified atmosphere containing 5% CO₂.

For morphological study, Bel-7402, HT-29, HeLa cells were plated at 4×10^4 cells/60 mm plate and exposed to herbal extracts at the concentration of 50 µg/mL and incubated for 12, 24, 48, or 72 h. Then were examined under light microscopy (Olympus).

MTT assay

The effects of extracts on the viability of Bel-7402, HeLa and HT-29 cells were determined by MTT assay, which was performed according to Mosmann's method.^[16] Briefly, cells (1×10^4 /well) were seeded in 96-well plates. After 24 h, various concentrations of different extracts were added and cells were further cultured for 48 h at 37°C. Then, MTT solution (5 mg/mL) were added into each well after the removal of the medium and incubated for an additional 4 h. The formazan crystal formed was dissolved with DMSO. Absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay reader (TECAN SPECTRA, Wetzlar, Germany). Absorbance of cells treated with medium containing 0.2% DMSO was regarded as 100% cell survival and IC₅₀ represents the drug concentration causing 50% decrease in the survival rate. 5-fluorouracil was used as positive control. All experiments were repeated at least 3 times to ensure the reproducibility.

FLOW CYTOMETRIC ANALYSIS

The effect of extracts on the cell apoptosis was determined by flow cytometric analysis. Bel-7402 and HeLa cells (5×10^5 cells/mL) were incubated with 50 µg/mL different extracts for 12 h at 37°C. 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 30 min incubation at room temperature in the dark, cells were analyzed by FACSCalibur flow cytometry (FCM) (made by BD Company) using CELL Quest 3.0.1 software. The percentage of cells with sub-G1 DNA content was taken as a measure of apoptosis. In each experiment, 10,000 events per sample were recorded.

DNA fragmentation and caspase-3 expression assay

For DNA laddering analysis and caspase-3 expression assay, HeLa cells were cultured with 50 µg/mL extracts

for 12 h at 37°C respectively. DNA was extracted with an Apoptotic DNA Ladder Detection Kit according to the manufacturer's instructions and DNA sample was electrophoresed on a 1.5% agarose gel. The *in situ* hybridization protocol used for caspase-3 expression was performed as described in kit introduction.

Statistical analysis

The statistical analysis was evaluated by Student's *t*-test. All data were expressed as mean \pm standard deviation and the values of $P < 0.05$ were considered to be significantly different from each control.

RESULTS

The common peak HPLC analysis and determination of amentoflavone content

The HPLC method was developed to compare the chemical profiles of different species of *Selaginella*, which revealed two major common peaks (peaks 1 and 2 were identified as amentoflavone and robustaflavone) and another three main peaks in their chromatograms (peaks 6, 9 and 13) [Table 2]. Furthermore, some samples collected from a variety of sources and conditions were analyzed, including different cultivation areas, various cultivating environments, harvesting times. The results indicated that their chromatographic patterns of five main peaks (peaks 1, 2, 6, 9 and 13) were generally consistent although some other peaks were different. The more detailed results could be seen in literature.^[15] Interestingly, *S. tamariscina* and *Selaginella pulvinata* are both listed in the Chinese pharmacopoeia and were more similar than other species in HPLC analysis, lacking peak 6 and with high absorption intensity of peak 1. The amentoflavone content of *Selaginella* species could be seen on Table 1.

In vitro antitumor effects of *Selaginella* species

The inhibitory effects of extracts from some *Selaginella* species [Table 2] on the viability of Bel-7402, HeLa and HT-29 cells were determined by MTT assay and morphological observation. Compared with blank control, cells treated with ethanol extracts (50 µg/mL) had no significant change and cells treated with water extracts (50 µg/mL) had almost no morphological change. Although, cells treated with some ethyl acetate extracts, the cell number decreased significantly and the cells became shrinkage and round when compared with blank control. Given that ethyl acetate extracts were evidently the most potent active extracts of *Selaginella* species, therefore ethyl acetate extracts were chosen for further mechanistic studies to elucidate its growth inhibitory activity in tumor cells.

Ethyl acetate extracts of *S. uncinata*, *S. tamariscina* and *S. labordei* significantly decreased the viability of HeLa cells in a dose-dependent manner. As shown in Table 3,

the strength of their antitumor effects was shown to be in the following order: *S. labordei* > *S. tamariscina* > *S. uncinata* > *S. moellendorffii* > *S. delicatula* > *S. remotifolia* > *S. pulvinata*. Similar results were observed in Bel-7402, but HT-29 cells were less sensitive to ethyl acetate extracts than HeLa cells.

Table 2: Relative retention time-percent of total peak area in high-performance liquid chromatography analysis

Peak no.	Relative retention time	Percent of total peak area from sample 1-7 (%)						
		70.93	40.17	21.37	51.17	77.65	12.55	
1	1	7.55						
2	1.052	16.74	3.6	18.71	8.32	15.42	2.54	12.59
3	1.076	1.8		0.49				
4	1.094	0.85		0.52				0.4
5	1.116	0.35						0.6
6	1.169	3.35		0.45	4.68	1.35		3.9
7	1.208	0.38			0.37			1.73
8	1.233			2.13				
9	1.246	57.36	4.06		25.58	3.54	0.77	23.37
10	1.279	5.31						
11	1.308	0.43			0.5	0.89		
12	1.345				0.91			
13	1.368		4.6	13.13	3.15	1.98	5.9	3.74
14	1.382							
15	1.404			10.3	0.93			13.4
16	1.451			0.46	0.92			13.49
17	1.479							
18	1.55				0.73			
19	1.598	0.42			3.72			
20	1.624					0.53		
21	1.666							
22	1.662					2.48		
23	1.687							
24	1.747				0.42			
25	1.778		2.63		1.99		2.04	
26	1.862				0.63			1.49
27	2.019		1.09				1.56	0.67
28	2.207					1.82	0.39	
29	2.285		3.04	0.69	0.45			3.26
30	2.290							

Table 3: Effects of ethyl acetate extracts of *Selaginella* species on the proliferation of HeLa, Bel-7402 and HT-29 cells

No.	Growth inhibition, (IC ₅₀) (µg/mL)		
	HeLa	Bel-7402	HT-29
1	15.14±2.32	20.36±1.66	33.64±0.55
2	3.24±0.83	7.83±2.14	3.24±0.38
3	>100	>100	>100
4	92.52±3.42	76.29±3.21	>100
5	75.57±4.57	84.42±7.72	96.92±10.77
6	>200	>200	>200
7	1.93±0.69	1.07±0.61	4.93±0.96
5-fluorouracil	5.2±0.61	3.1±0.55	6.7±1.08

Determination of sub-G1 by FCM

To determine whether the decrease in cell viability was associated with apoptosis, PI-stained cells were analyzed by FCM. Similar with MTT assay, the effects of ethyl acetate extracts on HT-29 cell apoptosis were less significant than on HeLa and Bel-7402 cells. Treatment with ethyl acetate extracts of *S. uncinata* (29.81%) and *S. labordei* (18.96%) led to a high number of cellular populations in sub-G1 phase, relative to other species and blank control (3.97%) [Figure 1]. While treatments with higher concentrations of ethyl acetate extracts of *S. labordei* (100 µg/mL), cell cycles distribute irregularly. Ethyl acetate extracts of *S. labordei* led to a dose-dependent increase in cellular population in sub-G1 phase indicating apoptosis occurrence [Figure 2]. Similar data could be seen on Bel-7402 cells.

DNA ladder analysis

DNA ladder analysis was performed to evaluate the ability of *Selaginella* species to induce apoptosis. As shown in Figure 3, the DNA ladder bands of ethyl acetate extracts from *S. labordei* and *S. uncinata* had much clearer ladder-shaped changes.

Ethyl acetate extracts induces expression of caspase-3

The activation of the caspase cascade which induces apoptosis was examined in HeLa cells. By *in situ* hybridization assay, consistent with MTT, FCM and DNA ladder analysis assay, acetate extracts from *S. labordei* and *S. uncinata* led to a high number of caspase-3 activated cells [Figure 4].

DISCUSSION

Selaginella species are resurrection plants. Uniquely, its detached fronds have the ability to survive in nearly complete desiccation similar to that of the whole plant.^[17,18] Thus, in China some of these herbs are called longevity herbs and were thought to be beneficial for the human health. Studies have demonstrated that some *Selaginella* species have potent antitumor activities. The present study compared the antitumor activities of extracts from seven species of *Selaginella*. The assay of cell cycle analysis, DNA laddering assay and caspase-3 expression further confirmed that inducing cell apoptosis was one of the antitumor mechanisms.

During this study, it is found that IC₅₀ value of some samples could not be calculated out. The cause may be that the color of the sample solution was dark and there were some interference caused by non-active compounds. Only those extracts which solution were apparent and with no interference could have a strong inhibitory effect on cell proliferation in a dosage-dependent manner. Thus, ligarine extract was removed, the medium are centrifugal more times and discard the supernatant before adding MTT solution.

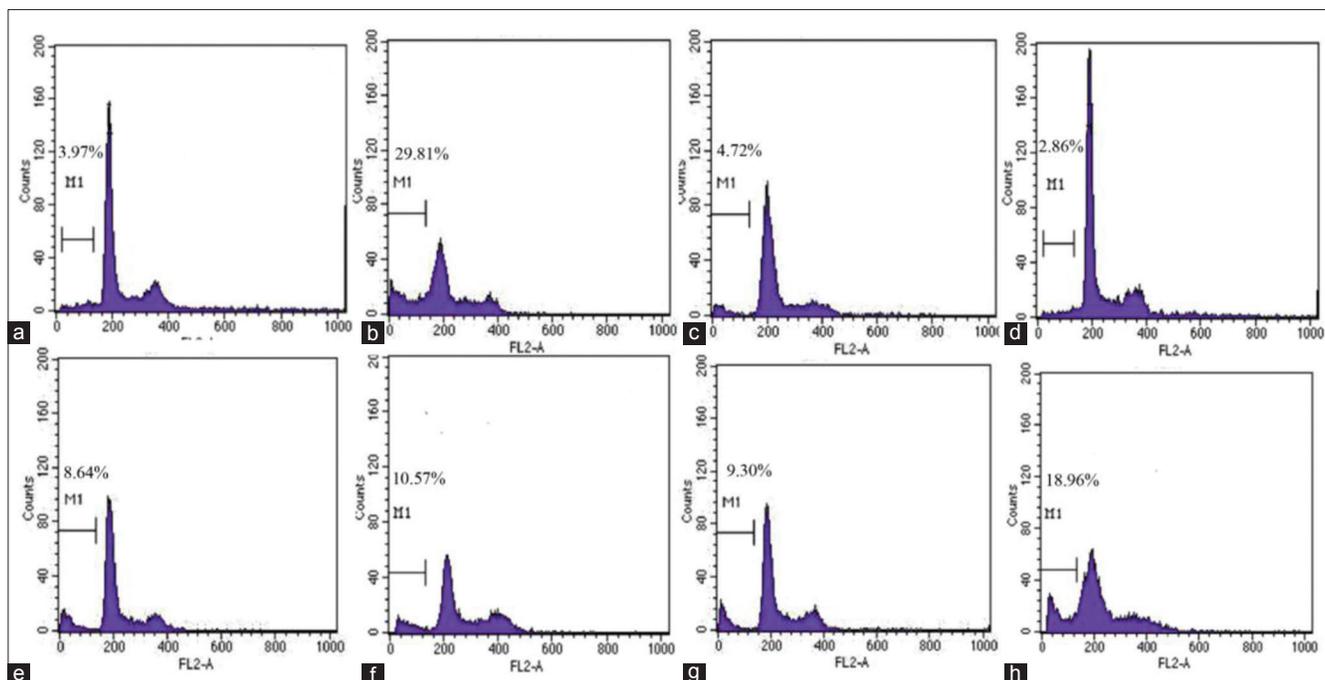


Figure 1: The apoptotic rate inducing by ethyl acetate extracts on HeLa cells with the concentration of 50 µg/mL. In each flow cytometry figure, M1 was apoptotic cells. (a) Blank control. (b) *Selaginella uncinata*. (c) *Selaginella tamariscina*. (d) *Selaginella remotifolia*. (e) *S. delicatula*. (f) *Selaginella moellendorffii*. (g) *Selaginella pulvinata*. (h) *S. labordei*

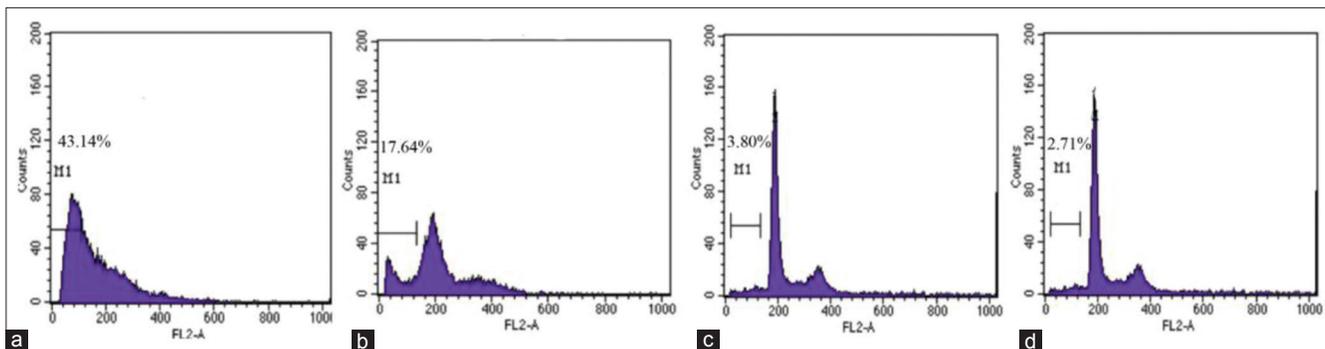


Figure 2: The apoptotic rate inducing by ethyl acetate extracts of *S. labordei* on HeLa cells in dose dependent manner. In each flow cytometry figure, M1 was apoptotic cells. (a) 100 µg/mL. (b) 50 µg/mL. (c) 25 µg/mL. (d) Blank control

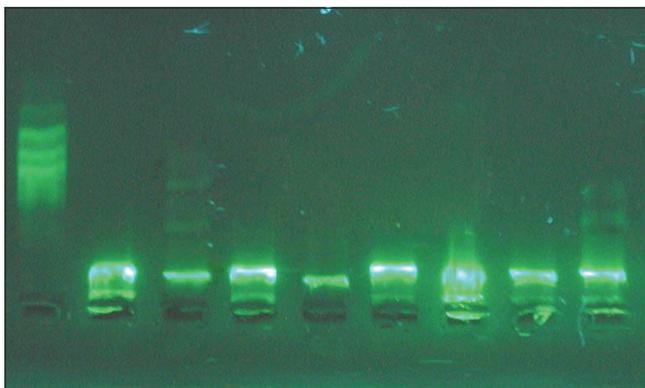


Figure 3: Effects of ethyl acetate extracts on deoxyribonucleic acid ladder analysis. From left to right: Marker, black control, *Selaginella uncinata*, *Selaginella tamariscina*, *Selaginella remotifolia*, *S. delicatula*, *Selaginella moellendorffii*, *Selaginella pulvinata*, *S. labordei*

Given that *S. labordei* was evidently the most potent active plant among *Selaginella* species in this study, *S. labordei* was chosen for further mechanistic studies to elucidate its growth inhibitory activity in cancer cells. We had found that *S. labordei* contains 4'-methylether robustaflavone, robustaflavone, eriodictyol and amentoflavone,^[19] it can down-regulate COX-2 gene expression in human colon adenocarcinoma CaCo-2 cells^[20] and some bisflavones had relatively stronger activities.^[21] However, further investigation is needed to clarify the chemicals and mechanisms.

S. tamariscina and *S. uncinata* also had good antitumor activities and *S. moellendorffii* had moderate activities on Bel-7402 and HeLa cell. *S. tamariscina* is the most powerful and the

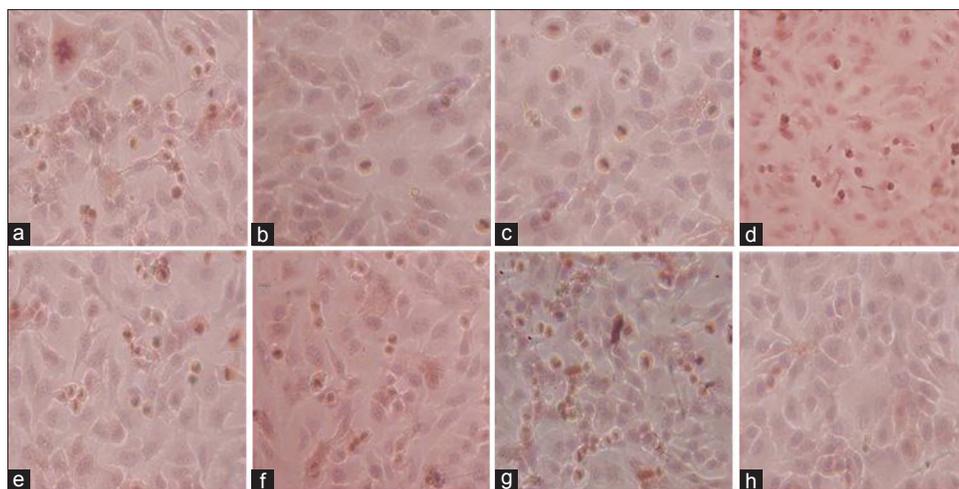


Figure 4: Effects of ethyl acetate extracts on caspase-3 expression. (a) *Selaginella uncinata*. (b) *Selaginella tamariscina*. (c) *Selaginella remotifolia*. (d) *S. delicatula*. (e) *Selaginella moellendorffii*. (f) *Selaginella pulvinata*. (g) *S. labordei*. (h) Blank control

most useful plant *Selaginella* in the world, which can inhibit growth of several types of cancer, such as lung, leukemia, gastric, breast;^[8,10,11,22] possesses an antimetastatic activity in osteosarcoma cells by down-regulating MMP-2 and MMP-9 secretions and increasing TIMP-1 and TIMP-2 expressions through p38 and Akt-dependent pathways.^[11,23,24] This property is mostly given by amentoflavone and isocryptomerin.^[3,8] Extract of *S. delicatula* that contained robustaflavone and amentoflavone or its derivatives is cytotoxic against cancer cell of P-388, HT-29,^[25] Raji, Calu-1, lymphoma and leukemia.^[7] Ginkgetin that extracted from *S. moellendorffii* can inhibit cancer cell growth of OVCAR-3, HeLa and FS-5^[26,27] and showed anti-metastasis activity in lung cancer cell;^[22] while amentoflavone and its derivatives, kayaflavone and podocarpusflavone A, have no this bioactivity.^[25] However, such study found that *S. remotifolia* and *S. pulvinata* had almost no inhibitory activities, also the antitumor activities of *S. remotifolia* and *S. pulvinata* were seldom reported yet. *S. uncinata* has activity as antitumor but generated by low amentoflavone content [Table 1]. In previous investigations of this plant, the chromone glycosides, namely uncinoside A and B, were reported to show antitumor and antiviral activity.^[28]

In our following studies, it was found that the active components of extracts of these *Selaginella* species were biflavonoids. Amentoflavone, the most common biflavonoid of *Selaginella*, has various biological and pharmacological effects, including antioxidant,^[29] anticancer.^[28,30] Thus, determination of amentoflavone content were used to get the message of their qualities. However, the results indicate that the content of amentoflavone isn't consistent with the antitumor activities. Because there are other biflavonoids, for example the amentoflavone content in *S. uncinata* (Desv.) Spring was very low, whereas other biflavonoids were abundant.

The IC₅₀ values of ethyl acetate extracts of *S. labordei*, *S. tamariscina* and *S. uncinata* <10 µg/mL as shown in Table 1, which had a dosage-dependent manner in inhibiting cell growth and inducing cell apoptosis. These herbs have widespread distribution and abundant resources. These results revealed these herbs would be potential antitumor agents.

CONCLUSION

The present study compared the chemical profiles of different species of *Selaginella*, which revealed two major common peaks (amentoflavone and robustaflavone) and compared the antitumor activities of extracts from seven species of *Selaginella*. The results showed that *S. labordei*, *S. tamariscina* and *S. uncinata* had relatively stronger activities on Bel-7402 and HeLa cells and *S. moellendorffii* had moderate moderate antiproliferation activities, but *S. remotifolia* and *S. pulvinata* had almost no inhibitory activities. The main active components were in the ethyl acetate extracts which had abundant biflavonoids. The action of cell proliferation and apoptosis of these extracts on different cells were not the same. It was more apparent on HeLa cells than on HT-29 cells. DNA laddering analysis and caspase-3 expression assays which further confirmed that inducing cell apoptosis was one of antitumor mechanisms, which was induced by caspase family. *S. uncinata* distribute widely and abundant resources are available. Thus these herbs could be developed to potential antitumor agents.

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