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Full Length Research Paper

Ethanolic extracts of *Sophora moorcroftiana* seeds induce apoptosis of human stomach cancer cell line SGC-7901 *in vitro*

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The seeds of *Sophora moorcroftiana* are used in Chinese traditional medicine for the treatment of verminosis, infectious diseases, and anti-inflammation. To investigate the antitumor and induction of apoptosis activity of *Sophora moorcroftiana* seeds, the ethanolic extracts from the seeds was prepared and added into the culture of human stomach cancer cell line SGC-7901 *in vitro*. The proliferation and apoptosis of cells treated with the ethanolic extracts were assessed by tetrazolium salt reduction (MTT) assay, fluorescence microscopy, transmission electron microscopy, flow cytometry and agarose gel electrophoresis of DNA fragmentation. The results showed that the growth of SGC-7901 cells was strongly inhibited by the ethanolic extracts at the concentration ranging between 0.31-5.00 mg/ml, and the apoptosis of treated cells was induced at the concentration of 1.25, 2.50, and 5.00 mg/ml *in vitro.* This suggested that the ethanolic extracts from *S. moorcroftiana* seeds contain potent antitumor fraction(s) on human stomach cancer SGC-7901 cells.

Key words: *Sophora moorcroftiana*, human stomach cancer, MTT, Apoptosis.

INTRODUCTION

Medicinal plants play a key role in human health care. About 80% of the world population relies on the use of traditional medicine, which is predominantly based on plant materials (WHO, 1993). Scientific studies indicate that the promising phytochemicals can be developed from the medicinal plants for many health problems (Dahiru et al., 2006). Moreover, the herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness. These drugs are invariably single plant extracts or fractions thereof or mixtures of fractions/extracts from different plants, which

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have been carefully standardized for their safety and efficacy (Dahiru et al., 2006).

Sophora moorcroftiana (Wallich.) Benth. ex Baker is an endemic shrub species in Tibet, China, and mainly distributes in the wide valleys and the middle reaches of several main tributaries of Yalu Tsangbo River (Nianchu and Lhasa Rivers) (Xingming et al., 2004a). *S. moorcroftiana* seeds has been used for a long time in Chinese folk medicine. The decoction of the seeds is used for dephlogistication, detoxication, emetic, infectious diseases and verminosis (Xingming et al., 2004a). Alkaloids from *S. moorcroftiana* have a protoscolicidal, anti-inflammatory effect (Xingming et al., 2004b) and induced apoptosis of human stomach cancer cell line SGC-7901 *in vitro* (Xingming et al., 2004a). The sophorastilbene A and (+)-α-viniferin from *S. moorcroftiana* inhibited copper-induced protein oxidative modification *in vitro* (Toda and Shirataki, 2005). Prenylflavanones from *Sophora tomentosa* and *Sophora moorcroftiana* show tumor-specific cytotoxic activity and antimicrobial activity (Shirataki et al., 2001).

We have examined the antimicrobial activity and cytotoxicity of chloroform, 95% alcohol, 75% alcohol, and water extracts from *S. moorcroftiana* seeds on human stomach cancer cells *in vitro* (submitted). The most interesting discovery of the study was that the 95% ethanolic extracts is much more effective than other extracts of *S. moorcroftiana* seeds against human stomach cancer cell line SGC-7901 *in vitro*. In our present study, we further demonstrate the pertinent inhibitory mechanisms of the ethanolic extracts on human stomach cancer cell line SGC-7901 *in vitro*.

MATERIALS AND METHODS

Plant materials

The seeds of *Sophora moorcroftiana* were collected in October 2001 from the Bank of Yalu Tsangbo River, Tibet altiplano, and air dried at room temperature for 3 weeks. The identity of the plant was confirmed by Professor Hongyu Li, School of life Sciences, Lanzhou University, China. One herbarium voucher specimen (M2001-1005) of the plant has been preserved in our laboratory for future reference.

Preparation of extracts

The seeds was powdered (422.8 g) using a blender and dissolved in 5000 ml of absolute chloroform at room temperature for 5 days. The vacuum dried residues (401.0 g) were dissolved in 4000 ml of 95% ethanol for 5 days at room temperature, and then filtrated through filter paper (Whatman No. 2). The extracts were evaporated in vacuum to give the fraction (10.6 g). The dry ethanolic extracts of *S. moorcroftiana* seeds were dissolved in 0.1% dimethyl sulphoxide (DMSO) into the needed concentrations (0.31, 0.63, 1.25, 2.50, 5.00 mg/ml).

Cell culture

Human stomach cancer cell line SGC-7901 was obtained from the Medical Experiment Center, Lanzhou University, China. SGC-7901 cells were maintained in RPMI 1640 medium (Sino-American Biotechnology Company, China) supplemented with heat inactivated fetal calf serum (FCS, 10%) (Sino-American Biotechnology Company, China), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were grown at 37ºC in a humidified 5%, CO_2-95% air atmosphere.

MTT assay

The inhibitory effect of the ethanolic extracts on human stomach cancer cells was determined by MTT assay (Hussain et al., 1993). The MTT test is based on the enzymatic reduction of the tetrazolium salt MTT in viable/metabolically active cells. SGC-7901 cells in the logarithmic growth phase were collected, washed once with PBS, and resuspended in fresh medium. The cell suspension (1.5×10^4/well) was placed in a 96-well tissue culture plate. After logarithmic growth phase of cells was reached, the supernatant was discarded. Then 200 µl of the ethanolic extracts with final con-

centration of 0.31, 0.63, 1.25, 2.50, and 5.00 mg/ml was added. The cells treated with Etoposide (vp–16) (Qindao Haier Pharma Limited Company, China), a cytotoxic drug, at 0.08 µmol/L, were used as positive control, and cells treated with 0.1% DMSO as negative control. After 24, 48, and 72 h, MTT assay was performed for determining the inhibitory effect of the ethanolic extracts on SGC-7901 cells. The absorbance of formazan dye was read using ELISA plate reader at 570 nm. The whole procedure was repeated for three times. The inhibitory rate of cell growth was calculated as following formula: % Growth inhibition = (1 - OD extract treated) / OD negative control × 100.

Morphological study of apoptosis by acridine orange

Acridine orange (AO) is a membrane-permeable fluorescent dye. It is specific for apoptotic cell death and does not significantly stain necrotic cells. The morphological changes of the apoptotic cells were assessed with fluorescent microscope (JSM-6380LV, Japan) (Xin et al., 2001). Briefly, SGC-7901 cells (10^6/well) were seeded in a 24-well tissue culture plate. When logarithmic growth phase of cells was reached, the ethanolic extracts with final concentration of 1.25, 2.50, and 5.00 mg/ml or 0.1% DMSO (negative control) was added, respectively. After 48 h, cells were collected by centrifugation at 800 ×g for 5 min, and fixed in methanol: glacial acetic acid (3:1) for 30 min at room temperature, washed once with PBS, and then stained with 0.01% acridine orange for 5 min. The stained cells were viewed under a fluorescence microscope.

Transmission electron microscopies (TEM)

Cell-apoptosis was assessed by TEM (Xin et al., 2001). SGC-7901 cells (10^6/well) in logarithmic phase were treated with the ethanolic extracts (1.25, 2.50, and 5.00 mg/ml) as described above, harvested by trysinization, fixed in 3% glutaraldehyde for 1 h. After removal of the primary fixative, cells were washed three times with MOPS buffer, post fixed in 1% osmium tetroxide (OsO_4), dehydrated in graded alcohol, and embedded in epoxy resin. Ultra thin sections were double-stained with lead citrate/uranyl acetate before being examined by JEM-100CX transmission electron microscope (Japan).

Agarose gel electrophoresis of DNA fragmentation

Upon completion of treatment, cells were processed for DNA fragmentation analysis by agarose gel electrophoresis as described (Herrmann et al., 1994). The treated SGC-7901 cells were harvested by trysinization, washed twice with PBS, then used for DNA isolation. DNA extraction was performed using Quick Genomic DNA Extraction Kit (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd., China) according to the manual instructions. The DNA pellet was resuspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) prior to loading (10 µl) onto a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. Electrophoresis was conducted at 35 V for 4 h. DNA fragments were visualized and photographed under UV illumination. DNA marker (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd., China) was used to estimate the size of DNA fragment.

Flow cytometry analysis

Following treatment, cells were rinsed with PBS, trypsinized by 0.25% trypsin/0.02% EDTA solution, and collected by centrifugation at 1000 rpm at 4ºC for 5 min. The cell pellets were fixed in 70% ethanol at 4℃ for at least 1 h. The fixed cells were washed twice with PBS, resuspended in PBS containing 50 g/L RNase A and 50 mg/L of propidium iodide (Xin et al., 2001). The suspension was incubated at 37℃ for 30 min, filtered through 200 µm nylon mesh, and analyzed by flow cytometer (Coulter EPICS XL, USA). The apoptotic proportion was identified as cells appeared in sub-G_1 phase. Multicycle software was used for data analysis.

RESULTS AND DISCUSSION

Although the alkaloids and prenylflavanones from *S. moorcroftiana* show an antitumor efficacy *in vitro* (Shirataki et al., 2001; Xingming et al., 2004a), no

Table 1. The inhibitory effect of the ethanolic extracts from *Sophora moorcroftiana* seeds on SGC-7901 cell line *in vitro*.

Group	Concentration	Growth inhibition (%)		
		24 h	48 h	72 h
Extract (mg/mL)	0.31	17±4.6	19±3.7	22±7.2
	0.63	21±7.4	34±3.8	43±4.7
	1.25	53±1.9	65±1.7	68±3.5
	2.50	60±9.1	71±2.7	88±4.9
	5.00	87±1.7	89±1.1	93±1.7
Vp-16 (µmol/L)	0.08	16±2.8	23±8.8	33±4.6

Values are mean ± SD (n = 9).

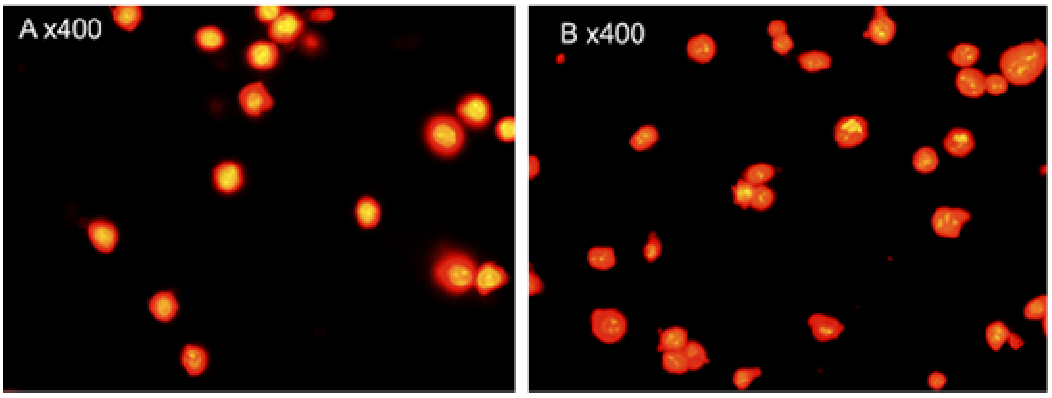

Figure 1. Fluorescence micrographs of SGC-7901 control cells (A) and cells treated with the ethanolic extracts from *Sophora moorcroftiana* seeds for 48 h at 1.25 g/ml (B).

previous literature was found showing the effectiveness of the ethanolic extracts from *S. moorcroftiana* seeds on cancer cells . However, our present study observed that the ethanolic extracts from *S. moorcroftiana* seeds significantly inhibited the proliferation of human stomach cancer cells and its activity was in dose- as well as time-dependent manner. While the cytotoxic agent, VP-16, at the concentration of 0.08 µmol/l, caused cytotoxicity at 16% (24 h), 23% (48 h), and 33% (72 h); the ethanolic extracts resulted in much higher cytotoxicity for SGC-7901 cells from the concentration 0.63 mg/ml to 5.0 mg/ml. The higher concentration of the ethanolic extracts and the longer time of treatment on cells, the more significant cytotoxicity was achieved (Table 1).

We utilized several methods to measure the effect of the ethanolic extracts on stomach cancer cell apoptosis. After treatment with the ethanolic extracts for 48 h, both morphologic study by acridine orange fluorescence staining and TEM detected the typical morphology of apoptosis from cultured SGC-7901 cells: cell pyknosis, chromatin condensation, and nuclear fragmentation (Figures 1 and 2). Meanwhile, the results of DNA agarose gel electrophoresis showed that typical "DNA ladder", which indicates the internucleosomal DNA fragmentation

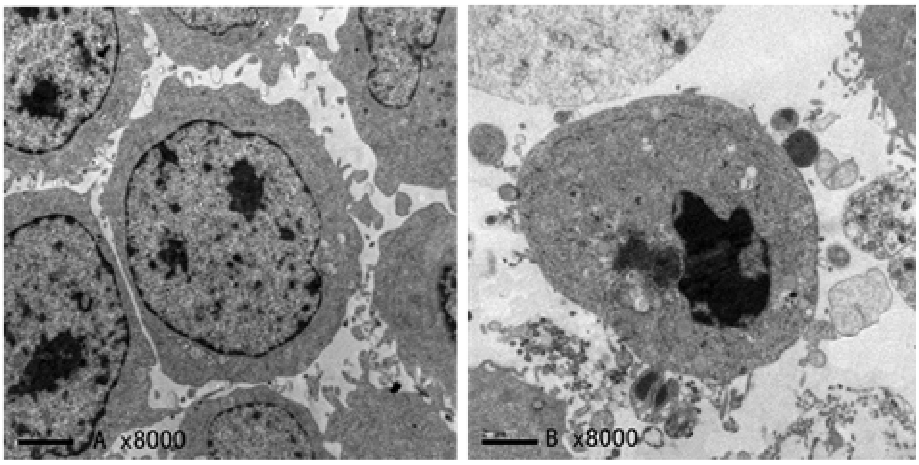

Figure 2. Electron micrographs of SGC-7901 control cells (A) and cells treated with the ethanolic extracts from *Sophora moorcroftiana* seeds for 48 h at 1.25 g/ml (B).

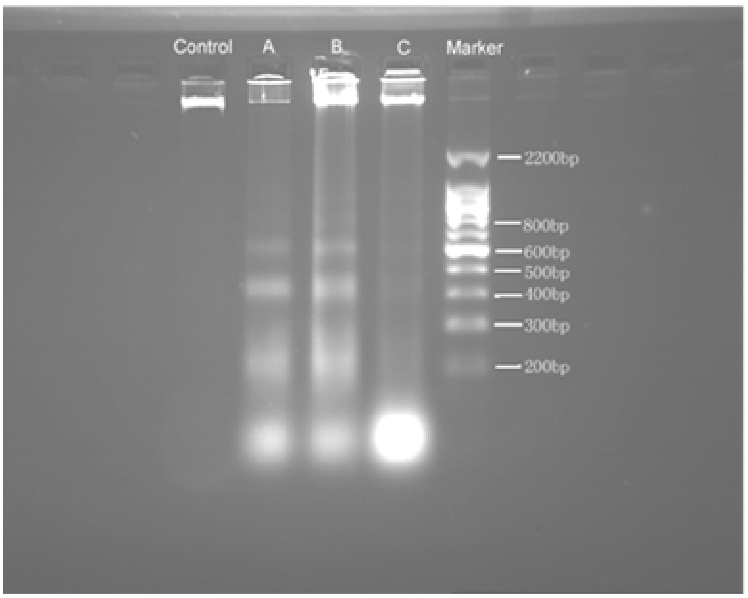

Figure 3. Agarose gel electrophoresis of DNA fragmentation extracted from SGC-7901 control cells and cells treated with the ethanolic extracts from *Sophora moorcroftiana* seeds for 48 h at the concentration of 5.00 mg/ml (A), 2.50 mg/ml (B), and 1.25 mg/ml (C).

in apoptotic cells occurred in SGC-7901 cells treated with the ethanol extracts from *S. moorcroftiana* seeds for 48 h at the concentration of 1.25, 2.50 and 5.00 mg/ml (Figure 3). These characteristics were further verified by FACScan analysis, which showed the presence of apoptotic peak in sub-G_1 phase. SGC-7901 cells treated with 1.25, 2.50, and 5.00 mg/ml of ethanolic extracts for 48 h displayed a dose-dependent accumulation of apoptotic cells, and the apoptosis percentage of cells at the sub-G1 phase was 14.2, 20.1 and 32.1%, respective-

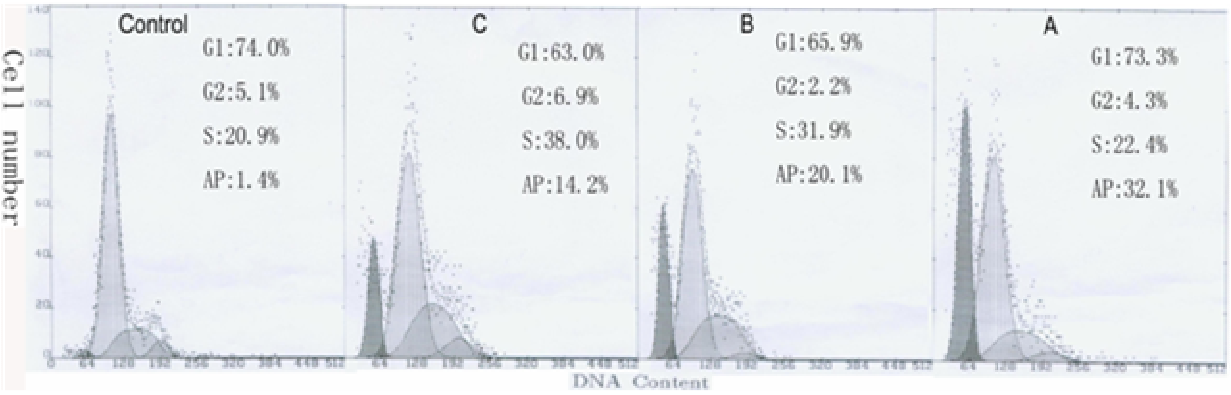

Figure 4. DNA content of SGC-7901 control cells and cells treated with the ethanolic extracts from *Sophora moorcroftiana* seeds for 48 h at the concentration of 5.00 mg/ml (A), 2.50 mg/ml (B), and 1.25 mg/ml (C). AP is apoptosis percentage.

ly, as compared to untreated control (1.4%) (Figure 4). These results suggest that ethanolic extracts may exert its anti-tumor effects associated with two fundamental processes: suppression of cell proliferation and induction of apoptosis on human stomach cancer cell *in vitro*. Therefore, it is concluded that the ethanolic extracts from *S. moorcroftiana* seeds possess potent antitumor fraction(s) on human stomach cancer SGC-7901 cells. However, the effective biochemical component of the ethanolic extracts and how it inhibits the proliferation and induces apoptosis of SGC-7901 cells remain unknown. Further studies are needed to address those important questions.

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