

## HPLC determination of ursolic acid in *Patrinia scabiosifolia* Fisch. ex Trevir. and its inhibitory effect on hela cells.

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### Abstract

To establish a method for determining ursolic acid content in *Patrinia scabiosifolia* Fisch. ex Trevir.; and to investigate the inhibitory effect of *Patrinia scabiosifolia* Fisch. ex Trevir. extract on *in vitro* cultured human cervical carcinoma Hela cells. HPLC with Agilent XBP-C18 column (4.6 mm × 250 mm, 5 μm) was used. Mobile phase was acetonitrile-methanol-0.5% ammonium acetate solution (68:10:22), while detection wavelength was 210 nm. Human cervical carcinoma Hela cells were cultured *in vitro* and treated with 0.125-2.0 mg/ml of *Patrinia scabiosifolia* Fisch. ex Trevir. extracts for 24-72 h. Then, cell proliferation was assayed by MTT assay, apoptosis was detected by FCM, and morphology was observed by TEM. Methodological results showed that regression equation was  $Y=4.7659x+17.933$ ,  $R^2=0.9998$ . Ursolic acid presented a good linearity within a 5.21-130.25 μg/ml concentration range. Average recovery was 99.9%, RSD=2.06%. *Patrinia scabiosifolia* Fisch. ex Trevir. inhibited Hela cells in a dose- and time-dependent manner, with 24 h IC<sub>50</sub> being 1.231 mg/ml. FCM revealed increased apoptosis of cells after treatment with *Patrinia scabiosifolia* Fisch. ex Trevir., while TEM observation found early apoptotic changes in partial cells. With advantages such as simplicity, fastness and good reproducibility, the method proposed can be used for quantitative determination of ursolic acid in *Patrinia scabiosifolia* Fisch. ex Trevir. *Patrinia scabiosifolia* Fisch. ex Trevir. has a pronounced inhibitory effect on Hela cells, and induction of apoptosis may be one of its mechanisms.

**Keywords:** *Patrinia scabiosifolia* Fisch. ex Trevir., Ursolic acid, HPLC, Content determination, Cervical carcinoma, Apoptosis.

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### Introduction

*Patrinia scabiosifolia* Fisch. ex Trevir. is a perennial herb, whose whole plant is used as medicine. Originally recorded in the Shen Nong's Herbal Classic, the herb is pungent and bitter, which has heat-clearing, detoxifying, stasis-removing and pus-exPELLING functions and is widely used in clinical practice, with prominent efficacy for chronic appendicitis. In some parts of China, *Patrinia scabiosifolia* Fisch. ex Trevir. seedlings and tender leaves are used for edible purposes [1,2]. *Patrinia scabiosifolia* Fisch. ex Trevir. contains triterpenoids, volatile oils, saponins, etc. [3-5]. Modern medical research has shown that *Patrinia scabiosifolia* Fisch. ex Trevir. has antitumor, anti-allergic dermatitis, antioxidant and immunoenhancing effects [6-9]. According to recent studies, *Patrinia scabiosifolia* Fisch. ex Trevir. appears in Chinese compound prescriptions and can be used for treating pelvic inflammatory disease, cervical erosion, cervicitis, cervical cancer, etc. [10-12], without side effects of chemotherapy drugs. Thus, the anti-cervical cancer effect of *Patrinia scabiosifolia* Fisch. ex Trevir. has gained

increasing attention. However, there are scarce reports on the cervical cancer inhibiting effect of *Patrinia scabiosifolia* Fisch. ex Trevir. at present. This study preliminarily investigated the inhibitory effect of *Patrinia scabiosifolia* Fisch. ex Trevir. extract on *in vitro* cultured human cervical carcinoma Hela cells. To control the quality of crude drug and extracts, we used reversed-phase HPLC to determine the ursolic acid content in *Patrinia scabiosifolia* Fisch. ex Trevir. and its extracts, in order to provide reference for the industrial drug production.

### Material and Methods

*Patrinia scabiosifolia* Fisch. ex Trevir. was collected from the West Tianmu Mountain in Zhejiang, which was identified by Professor Zhang Min of the Department of Pharmacy, Nanjing Pharmaceutical University as the whole plant of *Patrinia scabiosifolia* Fisch. ex Trevir. in the genus *Patrinia* of the family Valerianaceae. Fresh *Patrinia scabiosifolia* Fisch. ex Trevir. was dried in the shade, ground into a fine powder and sifted for later use.

Ursolic acid standard (batch No.: 332749-2015098) was provided by the National Institutes for Food and Drug Control. Chromatographically pure acetonitrile and methanol; chemically pure ethanol, methanol and other reagents (purchased from Tianjin Nansuo Chemical Reagents Co., Ltd.). Cervical carcinoma Hela cells were gifted by the Research Center of the Second Affiliated Hospital of Nanjing Medical University.

### Instruments

Agilent 1200 LC system; Mettler-Toledo MS104TS analytical balance; Kunshan Shumei KQ5200E ultrasonic unit (Shanghai Chongfeng Scientific Instruments Co., Ltd.); 0.45  $\mu$ m aqueous and organic phase microporous membrane (Nanjing Senfei Laboratory Supplies Co., Ltd.). FBS, DMEM medium (Gibco); MTT and DMSO (Sigma, USA).

### Chromatographic conditions

Octadecyl silane-bonded silica column Agilent XBP-C18 (4.6 mm  $\times$  250 mm, 5  $\mu$ m) was used for chromatography. Mobile phase was acetonitrile-methanol-0.5% ammonium acetate solution (68:10:22), detection wavelength was 210 nm, flow rate was 1.0 ml/min, column temperature was 35°C, and injection volume was 20  $\mu$ l.

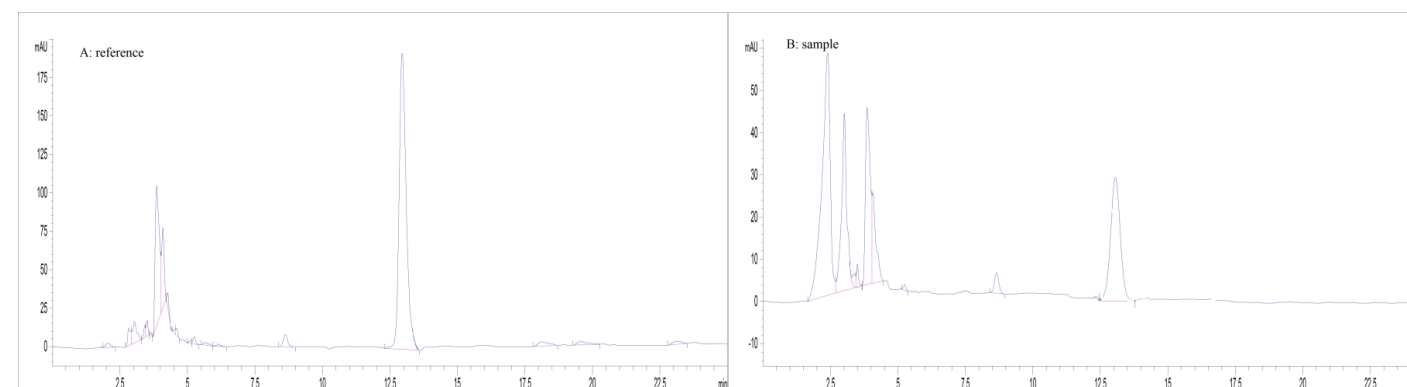


Figure 1. HPLC chromatograms of ursolic acid in the reference (a) and sample (b).

### Cell culturing

Cervical carcinoma Hela cells were routinely thawed and passaged in a 37°C, 5% CO<sub>2</sub> incubator with 10% FBS-containing DMEM medium.

### Preparation of *Patrinia scabiosifolia* Fisch. ex Trevir. extract

*Patrinia scabiosifolia* Fisch. ex Trevir. crude drug was pulverized, soaked overnight in a 6-fold volume of 80% aqueous ethanol, then extracted for 2 h three times. After filtration, solvent was removed under reduced pressure to give the 80% aqueous ethanol extract, which was then dried into a powder (0.5 kg). The extract powder was fully dissolved in water, injected onto the D101 macroporous resin column and eluted sequentially with water and 30%, 60%, 90% aqueous ethanol. The 60% aqueous ethanol eluted fraction was gradient

### Preparation of reference solutions and plotting of standard curves

10 mg of ursolic acid was accurately weighed, added with 75% methanol and shaken uniformly to give a 0.25 mg/ml reference stock solution. Appropriate amounts of the stock solution were accurately weighed and diluted serially with 75% methanol to give reference solutions with concentrations of 7.81, 15.63, 31.25, 62.50 and 125.00  $\mu$ g/ml, respectively. Each 20  $\mu$ l of the reference solutions was injected for HPLC analysis. Peak areas were recorded, and standard curves were plotted with concentration as the abscissa and peak area as the ordinate.

### Preparation of sample solution

1 g of sample powder was accurately weighed, placed in a 10 ml stoppered Erlenmeyer flask, added with 10 ml of 75% methanol and ultrasonically extracted for 30 min, then let cool at room temperature, diluted to a constant volume with 75% methanol, shaken uniformly and filtered. The subsequent filtrate was then filtered through a 0.45  $\mu$ m microporous membrane to give the sample solution.

eluted with aqueous ethanol (30%, 60%, 90%) by column chromatography on MCI to give the 60% ethanol fraction, which was then rotary evaporated and lyophilized to obtain the *Patrinia scabiosifolia* Fisch. ex Trevir. extract powder.

### MTT assay of cell proliferation

Logarithmic phase Hela cells were plated in 96-well culture dishes at  $1 \times 10^4$  cells/well. After adherence, different concentrations (1.00 mg/ml, 0.50 mg/ml, 0.25 mg/ml) of *Patrinia scabiosifolia* Fisch. ex Trevir. extracts were added, where five replicate wells were set up for each concentration. Cells without drug addition served as control. Meanwhile, a blank control group was set up for zero adjustment. After culturing for 24, 48 and 72 h, respectively, cells were added with 20  $\mu$ l of 5 mg/ml MTT and incubated at 37°C for 4 h. Then, supernatant was discarded, each well was added with 200  $\mu$ l of DMSO and shaken to fully dissolve crystals,

followed by measurement of A values at 570 nm. Cell growth inhibition rate =  $(1 - \text{mean A value of treatment group} / \text{mean A value of control group}) \times 100\%$ . The experiment was repeated three times, and the values were averaged.

**FCM detection of apoptosis**

Hela cells were seeded in 6-well plates at  $1 \times 10^5$  cells/ml. 24 h later, culture medium was replaced with 1.0, 0.5 and 0.25 mg/ml *Patrinia scabiosifolia* Fisch. ex Trevir. extract-containing ones, respectively, while in the control group, the medium was replaced with drug-free medium. After treating for 48 h, cells in each group were digested and collected with 0.05% trypsin-EDTA, washed twice with PBS and centrifuged at 500 rpm for 5 min. After discarding the supernatant, cells were double stained with PI and Annexin V as per Annexin V-FITC kit (Bender MedSystem, Austria) instructions, followed by determination of the percentage of apoptotic cells in each sample with BECTON DICKINSON FACSCalibur flow cytometer. The experiment was repeated three times, and the values were averaged.

**TEM observation of the effect of *Patrinia scabiosifolia* Fisch. ex Trevir. extract on Hela cell ultrastructure**

Hela cells were treated with 0.5 mg/ml *Patrinia scabiosifolia* Fisch. ex Trevir. extract for 48 h, then scraped with a cell scraper, centrifuged, fixed in 2.5% glutaraldehyde and prepared routinely into TEM specimens, followed by ultrastructure observation under a HITACHI electron microscope.

**Results**

As shown in Figure 1, under identical chromatographic conditions, the sample and reference had the same peak appearance time ( $t=15.6$  min), where the peak was the ursolic acid peak. Under these chromatographic conditions, the peak of ursolic acid in sample had large separation from adjacent peaks, suggesting that the conditions were suitable for determination of ursolic acid in the sample.

**Preparation of reference solutions and plotting of standard curves**

Standard curves were plotted with concentration of ursolic acid as the abscissa and peak area as the ordinate. Regression equation was obtained as  $Y=4.76595X + 17.933$  ( $R^2=0.9998$ ), indicating good linearity of ursolic acid within a 7.81-125.00  $\mu\text{g/ml}$  concentration range (Figure 2).

**Stability test**

Each 20  $\mu\text{l}$  of sample solution was injected at 0, 1, 2, 4, 8, 12 and 24 h, respectively for measurement of peak areas and calculation of RSD values. The results showed that the peak areas of ursolic acid at 0, 1, 2, 4 and 8 h were 1894.41, 1843.70, 1851.99, 1819.65, 1879.83, 1824.81 and 1846.75,

respectively, with a RSD of 1.47%, indicating stability of ursolic acid in the sample within 24 h.

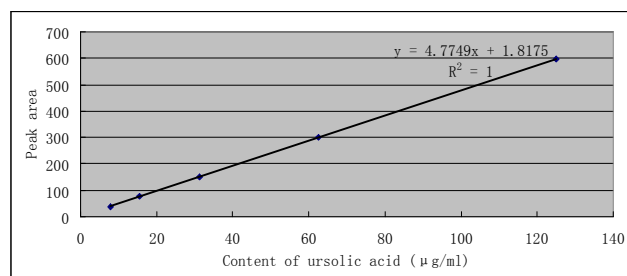


Figure 2. Standard curves of reference solutions.

**Precision test**

Each 20  $\mu\text{L}$  of reference solution (ursolic acid 0.25 mg/ml) was injected repeatedly five times to measure the peak areas of reference, which were found to be 2198.18, 2220.31, 2169.04, 2179.97 and 2282.51, respectively, with a RSD of 2.03%, indicating good precision of the instrument.

**Recovery test**

Six aliquots of *Patrinia scabiosifolia* Fisch. ex Trevir. sample with known ursolic acid content were accurately weighed, added separately with 0.7 mg of ursolic acid, extracted as per the method in the "Preparation of sample solution" and quantitatively determined. Average recovery of ursolic acid was 95.01%, with a RSD=1.92%. The results are shown in Table 1.

Table 1. Recovery test.

Sample amount (g)	Sample content (mg)	Added amount (mg)	Measured amount (mg)	Recovery (%)	Average recovery (%)	RSD%
0.2301	0.690	0.7	1.408	101.14	100.45	2.49
0.2312	0.694	0.7	1.390	98.57		
0.2303	0.691	0.7	1.376	96.57		
0.2310	0.693	0.7	1.410	101.43		
0.2308	0.692	0.7	1.409	101.29		
0.2309	0.693	0.7	1.426	103.71		

**Determination of ursolic acid content in extract**

*Patrinia scabiosifolia* Fisch. ex Trevir. extract was obtained as per the method in the "Preparation of *Patrinia scabiosifolia* Fisch. ex Trevir. extract". Five aliquots of 1 g of the extract were accurately weighed, placed in 10 ml stoppered Erlenmeyer flasks, added separately with 10 ml of 75% methanol, ultrasonically extracted for 30 min and let cool at room temperature. After dilution to a constant volume with 75% methanol, each 1 ml was accurately weighed, placed in 100 ml stoppered Erlenmeyer flasks, diluted to the mark with methanol, shaken uniformly and filtered. The subsequent

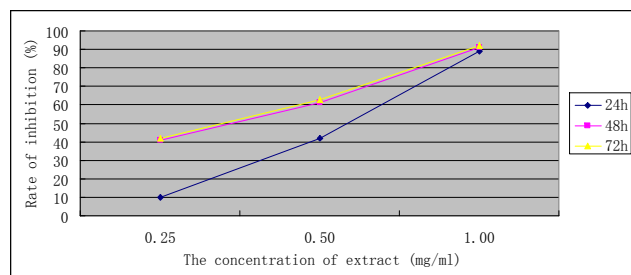
filtrates were then filtered through a 0.45  $\mu\text{m}$  microporous membrane to serve as the test solutions. Each 20  $\mu\text{l}$  of test solutions were injected into the HPLC system for measurement of peak areas, and ursolic acid contents in test samples were calculated by external standard method. The results are shown in Table 2.

**Table 2.** Sample content determination.

Extract No.	BJ01	BJ02	BJ03	BJ04	BJ05	Mean
Content (%)	30.23	31.06	32.71	30.10	31.20	31.06

### Growth inhibitory effect of *Patrinia scabiosifolia* Fisch. ex Trevir. extracts on Hela cells

0.25-1.00 mg/ml *Patrinia scabiosifolia* Fisch. ex Trevir. extracts all markedly inhibited the Hela cells, and the effects strengthened gradually with increasing extract concentration and prolonging incubation time. *Patrinia scabiosifolia* Fisch. ex Trevir. extracts inhibited the growth of cervical carcinoma Hela cells in a time- and dose-dependent manner (Figure 3).



**Figure 3.** Antiproliferative effect of *Patrinia scabiosifolia* Fisch. ex Trevir. extracts on Hela cells.

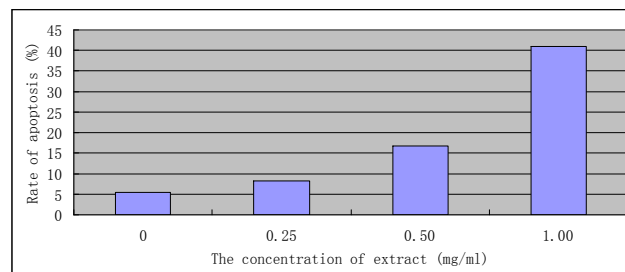
### Effect of *Patrinia scabiosifolia* Fisch. ex Trevir. extracts on apoptosis of Hela cells

After treating with 0.25, 0.50 and 1.00 mg/ml *Patrinia scabiosifolia* Fisch. ex Trevir. extracts for 48 h, apoptosis of Hela cells increased with increasing drug concentration, which were 5.66%, 8.25%, 16.64% and 41.02%, respectively. The results are shown in Figure 4.

### Morphological observation

Under an inverted microscope, untreated cells were well adherent and morphologically normal. In comparison, cells treated with *Patrinia scabiosifolia* Fisch. ex Trevir. extracts presented decreased nuclear-cytoplasmic (N/C) ratio with increasing drug dose, appearance of vacuoles of varying sizes within the cytoplasm, reduced cell viability and non-adherence of partial cells. Under TEM, untreated cells possessed the general characteristics of cancer cells, presenting large invaginated nuclei with much euchromatin and little heterochromatin, high N/C ratio, and a number of irregularly shaped large nucleoli with uneven electron density and frequent margination. No degenerated organelles were observed intracellularly, cytoplasmic matrices were uniform in

density, and cell surface microvilli were elongated and dense. In comparison, cells treated with 0.50 mg/ml *Patrinia scabiosifolia* Fisch. ex Trevir. extract for 48 h presented significantly shrunk nuclei; lowered N/C ratio; reduced, condensed or even disappeared nucleoli; markedly increased intranuclear heterochromatin with increased aggregation along the nuclear membrane margin; and thickened, partially blurred or disappeared nuclear membranes.



**Figure 4.** Effect of *Patrinia scabiosifolia* Fisch. ex Trevir. extracts on apoptosis of Hela cells.

Cytoplasm was filled with vacuoles of varying sizes, some of which were wrapped by membranes and were dilated smooth endoplasmic reticula, while others were not wrapped by membranes and were fat vacuoles or local vacuolar degeneration of mitochondria. Partial mitochondria were densely stained, with deposition of electron-dense granules. Focal lysis of cytoplasm was observed, and autophagosomes appeared, which contained phagocytosed degenerated organelles. Cell surface microvilli became thicker and shorter, decreased in number or even disappeared. Cell membranes were substantially intact.

### Discussion

MTT assay showed that *Patrinia scabiosifolia* Fisch. ex Trevir. extract could significantly inhibit the proliferation of cervical carcinoma Hela cells *in vitro*, and the effect increased with prolonging treatment time and increasing drug concentration, suggesting Hela cell inhibition by *Patrinia scabiosifolia* Fisch. ex Trevir. extract in a time- and dose-dependent manner. FCM revealed that 1.00 mg/ml and higher concentrations of *Patrinia scabiosifolia* Fisch. ex Trevir. extracts could increase the apoptosis of Hela cells, which became more significant with increasing drug concentration. Morphological observation also found early apoptotic changes in cell ultrastructure, which suggested that apoptosis may be one of the mechanisms to inhibit human cervical carcinoma Hela cells. Since *Patrinia scabiosifolia* Fisch. ex Trevir. extract could inhibit the proliferation of human cervical carcinoma Hela cells, it provided broader ideas for clinical treatment of cervical cancer. Activity analyses have already found that *Patrinia scabiosifolia* Fisch. ex Trevir. had WBC elevating and immunoenhancing effects, which demonstrated a wider clinical application value of the *Patrinia scabiosifolia* Fisch. ex Trevir. extract. Further research on *Patrinia scabiosifolia* Fisch. ex Trevir.'s inhibitory effect on Hela cells can provide more theoretical bases for cervical cancer treatment with the herb.

## Conclusion

Through HPLC methodology investigation, regression equation is obtained as  $y=4.7659X + 17.933$  ( $R^2=0.9998$ ), indicating good linearity of ursolic acid within a 5.21-130.25  $\mu\text{g/ml}$  concentration range. Besides, the sample is stable, and instrument precision and sample recoveries all meet the relevant requirements, which suggest that the method is sensitive, reliable and accurate. *Patrinia scabiosifolia* Fisch. ex Trevir. extracts are obtained by extraction and purification. Active analyses find that *Patrinia scabiosifolia* Fisch. ex Trevir. has a pronounced inhibitory effect on human cervical carcinoma Hela cells, and induction of apoptosis may be one of its mechanisms. This is the first report of the effect of *Patrinia scabiosifolia* Fisch. ex Trevir. on Hela cells inhibitory and HPLC was used to determine the content of the ursolic acid in *Patrinia scabiosifolia* Fisch. ex Trevir. The research has played a role in controlling the quality of extracts to lay the foundation for future industrial production or clinical application.

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