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ORIGINAL ARTICLE

Determination of Eupatilin in *Folium artemisiae* Argyi and Its Inhibitory Effect on Hepatoma Cells

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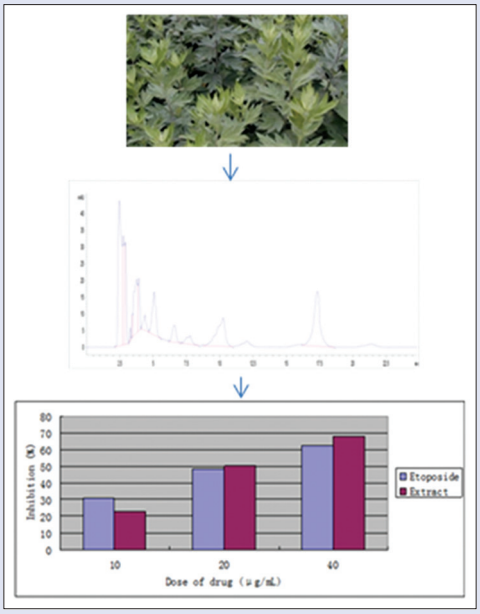
ABSTRACT

Aim: The aim of this study is to establish a method for determination of eupatilin in *Folium artemisiae* Argyi and observe the inhibitory effect of *Folium artemisiae* Argyi extract on human hepatoma SMMC-7721 cells. **Methods:** High-performance liquid chromatograph system with 2910 pump, 2930 UV detector, and N2000 workstation was used for determination of eupatilin in *Folium artemisiae* Argyi. Human hepatoma SMMC-7721 cells were cultured and cell proliferation was measured using the MTT assay. The expression protein levels of p53, Topo II, and bcl-2 were detected using Western blotting. **Results:** Eupatilin exhibited a linearity range of 0.5–3.0 μg/mL and a recovery of 100.72%, relevant standard derivation = 2.28%. *Folium artemisiae* Argyi extract had marked cytostatic and cytotoxic effects on SMMC-7721 cells, inhibited the SMMC-7721 colony formation in a dose-dependent manner. *Folium artemisiae* Argyi extract already possessed delayed effect after treating SMMC-7721 cells for 8 h, which became obvious at 12 h from treatment. After drug withdrawal, cells still tended to apoptosis. *Folium artemisiae* Argyi extract could inhibit p53, Topo II, and bcl-2 expressions in tumor cells. The present method for determination of eupatilin is simple, fast, accurate, sensitive, and reproducible. **Conclusion:** Hepatoma SMMC-7721 cells are quite sensitive to *Folium artemisiae* Argyi extract, which may be associated with its suppression of p53, Topo II, and bcl-2 expressions.

Key words: Antitumor, eupatilin, *Folium artemisiae Argyi,* high-performance liquid chromatograph, SMMC-7721

SUMMARY

- The study aimed to establish a method for determination of eupatilin in *Folium artemisiae* Argyi and observe the inhibitory effect of *Folium artemisiae* Argyi extract on human hepatoma SMMC-7721 cells. The results suggested that the present method for determination of eupatilin is simple, fast, accurate, sensitive, and reproducible. Hepatoma SMMC-7721 cells are quite sensitive to *Folium artemisiae* Argyi extract, which may be associated with its suppression of p53, Topo II, and bcl-2 expressions.



Abbreviations used: HPLC: High-performance liquid chromatograph; OD: Optical density; RSD: Relevant standard derivation; IC_{50}: Inhibitory 50% concentration.

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INTRODUCTION

Folium artemisiae Argyi is the dried leaves of asteraceous plant *Artemisia argyi* Levi. Et. Vant., which has antipyretic, analgesic, channel warming, and hemostatic functions and is used internally for warming channels, arresting bleeding, dispelling cold, relieving pain, and externally for eliminating dampness and relieving itching.[1] *Folium artemisiae* Argyi contains a variety of chemical constituents, such as flavonoids, terpenoids, phenolic acids, and volatile oils.[2,3] According to modern pharmacological studies, *Folium artemisiae* Argyi has antioxidant, antitumor, and immunosuppressive effects.[4-6] Eupatilin is an active constituent of *Folium artemisiae* Argyi. In the 1980s, eupatilin was found to inhibit the growth of tumor cells; it can also cure gastric ulcers, relieve spasm, regulate blood glucose level, etc.[7] This paper selects highly

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malignant hepatoma cell lines to preliminarily study the inhibitory effect of *Folium artemisiae* Argyi extract on *in vitro* cultured tumor cells. Meanwhile, *Folium artemisiae* Argyi extract is subjected to reverse-phase high-performance liquid chromatograph (HPLC) to determine eupatilin content in *Folium artemisiae* Argyi, thus providing scientific basis for the standardization of quality control of the traditional Chinese drug.

Instruments and reagents

HPLC system with 2910 pump, 2930 UV detector, and N2000 workstation (Shanghai Tianpu Scientific Instruments Co., Ltd.) and KQ-250D ultrasonic cleaner (Nanjing Kaiqi Experimental Instruments Co., Ltd.) were used in this study. Forma CO_2 incubator was purchased from Thermo Scientific; XDS-500C inverted microscope was purchased from Caikon Optical Instruments Company; PCR-2A ultra clean bench was purchased from Sujie Purifying Equipment Factory; XW3000 transmission electron microscope was purchased from Hefei Xiangshi Electronic Technology Co., Ltd.; and 96-well, 24-well culture plates were purchased from Costar.

Eupatilin reference (purchased from Chengdu Best Reagent Co., Ltd.; batch No. 20150106A); HPLC grade acetonitrile (Tianjin Kemiou Chemical Reagent Co., Ltd.); self-prepared deionized water; and other reagents were all of analytical grade. *Folium artemisiae* Argyi samples were collected from Hubei Province, which was identified by Professor Yang Fang of the Qingdao University of Traditional Chinese Medicine as the leaves of composite plant *Artemisia argyi* Levl. et Vant. Human hepatoma cell line SMMC-7721 was provided by the First Affiliated Hospital of Qingdao University.

RPMI-1640 medium was purchased from GIBCO; FBS, IHC kit, SABC, and DAB kits were purchased from Qingdao Taihua Biotechnology Co., Ltd.; MTT and anti-DIG-biotin were purchased from SIGMA; TUNEL *in situ* end labeling kit was purchased from Promega; DIG-dUTP was purchased from BM Company; and etoposide was purchased from Jiangsu Hengrui Medicine Co., Ltd.

METHODS

Chromatographic conditions

Column: TIANHE C_{18} reverse-phase column (4.6 mm × 250 mm, 5 μm); mobile phase: acetonitrile: water: glacial acetic acid (64:36:2); flow rate: 1.0 mL/min; detection wavelength: 254 nm; column temperature: 30°C; and injection volume: 10 μl. Eupatilin in the sample separated well from other components [Figure 1].

Preparation of reference solutions

About 5 mg of eupatilin reference was accurately weighed, placed in a 10 mL amber bottle, and dissolved and diluted to the mark with 95% ethanol. About 1 mL was precisely drawn, placed in a 10 mL volumetric flask, mixed well, diluted to the mark, and shaken well to give the stock reference solution. The stock solution was then added with acetonitrile to prepare 0.5, 5, 10, 20, and 30 μg/mL reference solutions.

Preparation of sample solution

Selection of extractant

Five aliquots of each 0.5 g of *Folium artemisiae* Argyi samples (passed no. 4 sieve) were taken, placed in stoppered Erlenmeyer flasks, accurately weighed, added separately with methanol, 80% methanol, anhydrous ethanol, and 95% ethanol, and ultrasonically extracted for 45 min, followed by determination. The results showed that the methanol yielded the best extraction efficiency with few impurities, so methanol was selected as the extractant.

Selection of extraction time

Five aliquots of each 0.5 g of *Folium artemisiae* Argyi samples (passed No. 4 sieve) were taken, placed in stoppered Erlenmeyer flasks, accurately weighed, added with 50 mL of methanol, and ultrasonically extracted for 30, 45, 60, and 90 min, respectively. The results showed that samples could be extracted completely in 60 min, so extraction time was selected as 60 min.

Preparation of sample solution

Folium artemisiae Argyi extract was obtained as per the above extraction method. About 50 mg of the extract was accurately weighed, placed in a stoppered Erlenmeyer flask, added with 50 mL of methanol, weighed, ultrasonicated for 60 min, let cool, then weighed again. After replenishing the weight loss with methanol, the solution was shaken uniformly and filtered to give the sample solution [Figure 2].

Linearity investigation

Reference solutions were taken and determined according to the above chromatographic conditions. Standard curves were plotted with concentrations of eupatilin reference solutions as the abscissa and corresponding peak areas as the ordinate. Linear equation was obtained as follows: $y = 28745x - 792.8$, $r = 0.9997$, which indicated good linearity of eupatilin between 0.5 and 3.0 μg/mL.

Accuracy test

The same sample solution was injected five consecutive times according to the above chromatographic conditions, and relevant standard derivation (RSD) of peak areas was measured to be 0.98%.

Stability test

The same sample solution was taken and determined according to the above chromatographic conditions at 0, 2, 4, 6, 12, and 24 h, respectively.

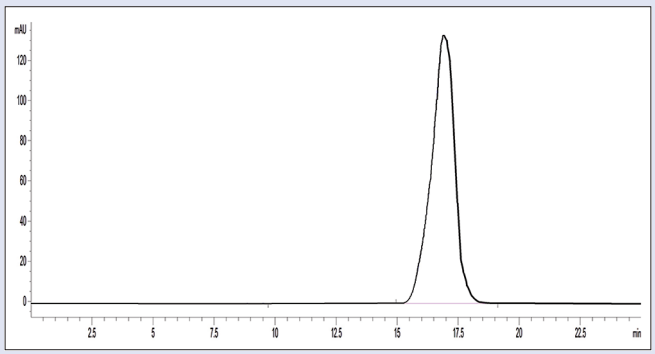

Figure 1: Eupatilin reference

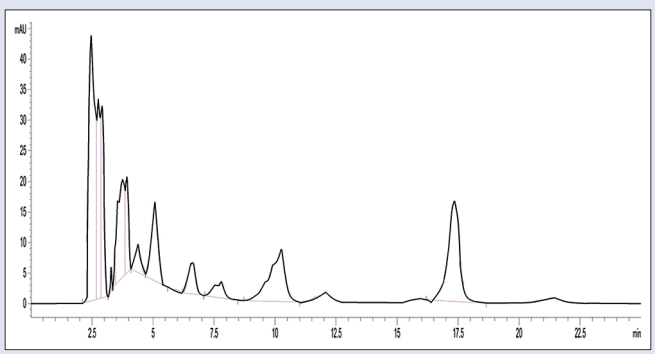

Figure 2: *Folium artemisiae* Argyi sample

RSD of eupatilin content in sample was found to be 1.56% (n = 7), indicating stability of the sample solution within 24 h.

Reproducibility test

Folium artemisiae Argyi sample was prepared in parallel into five aliquots as per the method for "Preparation of sample solution," then determined under the above chromatographic conditions. RSD was calculated to be 2.09%.

Recovery test

About 50 mg of the same *Folium artemisiae* Argyi extract with known contents was accurately weighed, added separately with equivalent amounts of eupatilin reference, prepared as per the method for "Preparation of sample solution," and determined for contents, followed by calculation of recoveries. The results are shown in Table 1.

Cell culturing

Human hepatoma SMMC-7721 cells were cultured routinely in complete RPMI-1640 medium and passaged once every 2–3 days in a 37°C, 5% CO_2 incubator. Exponential phase cells were harvested for experiments.

Observation of *Folium artemisiae* Argyi extract's effect on SMMC-7721

Logarithmic phase SMMC-7721 cells were seeded into 96-well plates at 100 μL/well (cell concentration of 1 × 10^4/mL). *Folium artemisiae* Argyi extract and etoposide were diluted separately with RPMI-1640 medium and added dropwise into the 96-well plates at 100 μL/well 24 h after cell seeding. Treatment group contained Artemisiae Argyi extract + SMMC-7721; positive control group contained etoposide + SMMC-7721; and negative control group contained CM + SMMC-7721. Three replicate wells were set up for each group. A volume of 200 μL of complete culture medium was used for zero adjustment. After culturing in a 37°C, 5% CO_2 incubator for 1, 3, 5, and 7 day, 5 mg/mL MTT was added (20 μl/well), and the cells were incubated for an additional 4 h. Then, supernatant was aspirated, and 150 μl of DMSO was added to each well and dissolved by shaking, followed by measurement of optical density (OD) at 490 nm. Curve was plotted with drug concentration as the abscissa and cell growth inhibition rate as the ordinate.

Inhibition rate (E) = $(1 - OD_{treatment}/OD_{control}) \times 100\%$.

Soft agar colony formation assay

50°C 5% agar and 37°C RPMI-1640 were added into a small beaker at a ratio of 1:9, mixed well, added into a 24-well culture plate (each well contained 0.8 mL of 0.5% agar medium), and let stand at room temperature until solidification of agar to obtain the bottom agar. Cells in the 48 h-treated group were adjusted to a concentration of 600 cells/mL, 9.4 mL of which was then taken, placed into a small beaker, added with 0.6 mL of 50°C 5% agar, and mixed well to give the top agar. Top agar was poured into the 24-well plate layered with bottom agar at 0.8 mL/well. The assay comprised four groups with each group containing four replicate wells. SMMC-7721 served as the normal control (negative control), while 10 μg/mL (IC_{30}), 20 μg/mL (inhibitory 50% concentration [IC_{50}]), and 40 μg/mL (IC_{70}) *Folium artemisiae* Argyi extracts constituted the treatment groups. After culturing in a CO_2 incubator at 37°C, 5% CO_2 with saturated humidity for 2 w, colony formation was observed under an inverted microscope. Colonies were counted, and colony-forming efficiency and colony-forming equivalent rate were calculated.

Colony-forming efficiency = number of colonies/number of seeded cells × 100%.

Colony-forming equivalent rate = (number of colonies in treatment group/number of colonies in control group) × 100%.

Observation of drug delayed effect

Logarithmic phase SMMC-7721 cells were taken, adjusted to a concentration of 1 × 10^6/mL, and seeded separately into four 25 mL culture flasks a, b, c, and d at 4 mL/flask. Twenty-four hour later, cells in flask a were routinely replaced with medium to serve as the control group, while cells in the flasks b, c, and d treatment groups were decanted of medium, added with *Folium artemisiae* Argyi extract (40 μg/mL) at 4 mL/flask, and treated for 4, 8, and 12 h, respectively. Then, drug solutions were removed, and the cells were washed twice with the medium, added with medium at 4 mL/flask, and cultured for an additional 72 h, then observed and photographed.

Cellular p53, Topo II, and bcl-2 detection

After routine growth on slides for 24 h, cells were added with 40 μg/mL *Folium artemisiae* Argyi extract and treated for 48 and 72 h. Cells without treatment with *Folium artemisiae* Argyi extract served as controls. After termination of culturing, cells were rinsed twice with ice PBS, fixed in 95% ethanol, and stored at 4°C. Backsides of cell sheets were stuck onto the slides using transparent glass glue with cells facing upward. After drying at 37°C, slides were rinsed with PBS for 5 min, 0.3% Triton X-100 for 10 min, 0.01 mol/L PBS for 2 × 5 min, 3% hydrogen peroxide at 37°C for 10 min, and PBS for 3 × 3 min, then added with each 20 μl of primary antibodies bcl-2, p53, and Topo II, let stand overnight at 4°C, and reacted at 37°C for 30 min. After washing with PBS for 3 × 5 min, slides were added with secondary antibodies and reacted at 37°C for 30 min, washed with PBS for 3 × 5 min, added with ABC complex and reacted at 37°C for 30 min, then washed with PBS for 3 × 5 min, added dropwise with DAB solution at 15 μL per slide and stained for 5–15 min (DAB: 5 mg was dissolved in 10 mL of PBS, paper-filtered, and then added with 10–15 μL of 30% hydrogen peroxide). After microscopic observation and coloration, the glass slides were placed in distilled water to terminate the coloration. Due to nonnuclear staining of bcl-2 slides, they were further subjected to nuclear staining: HEMATOXYLIN for 1 min, rinsing with tap water, hydrochloric-alcohol solution for 2–5 min, washing with water, dilute ammonia solution for 15–20 s, and washing with water. All the slides were dehydrated through graded alcohol,

Table 1: The experiment of adding recoveries and relevant standard derivation

Sampling amount (g)	Eupatilin content in sample (mg)	Reference addition (mg)	Measured amount (mg)	Recovery (%)	Average recovery (%)	RSD (%)
50.02	0.3247	0.3	0.6334	102.9	100.72	2.28027
49.09	0.3259	0.3	0.6212	98.4333		
51.42	0.3214	0.3	0.6156	98.0667		
50.74	0.3231	0.3	0.6283	101.733		
50.13	0.3244	0.3	0.6318	102.467		

RSD: Relevant standard derivation

cleared with xylene for 15 min, and mounted. Results interpretation: under a light microscope, cells with yellow to brown-stained nuclei were p53- and Topo II-positive cells, while those with pale yellow-stained cytoplasm were bcl-2-positive cells. At low magnification, three areas with mostly clear background and mostly satisfying DAB contrast were selected. Then, the number of positive cells per 500 cells was calculated separately at high magnification. Average number of positive cells in the three areas was taken as positive rate. Weak positivity (+): <20%; strong positivity (+++): >70%; moderate positivity (++): fell in between the two.

RESULTS

Dose-dependent inhibitory effect of *Folium artemisiae* Argyi extract on hepatoma SMMC-7721 cells

Folium artemisiae Argyi's inhibitory effect on SMMC-7721 growth was determined by MTT assay. As shown in Figure 3, *Folium artemisiae* Argyi extract had marked cytostatic and cytotoxic effects on SMMC-7721 cells. With increasing dose of *Folium artemisiae* Argyi extract, the inhibitory effect was also enhanced, showing a positive correlation ($r = 0.968$, $P < 0.01$). Statistical regression calculation after curve linearization showed that the IC_{50} of *Folium artemisiae* Argyi extract was 20 μg/mL; $IC_{50(}$Folium Artemisiae $_{Argyi)} < IC_{50(etoposide)}$ ($t = -10.73$, $P < 0.01$), indicating prominently superior SMMC-7721 inhibiting effect of 20 μg/mL *Folium artemisiae* Argyi extract to etoposide.

Effect of *Folium artemisiae* Argyi extract on hepatoma SMMC-7721 cell colony formation in soft agar

Colony-forming efficiency is a common measure for screening antitumor drugs, which is used mainly for observation of antitumor drugs' ability to kill tumor stem cells. Table 2 shows that *Folium artemisiae* Argyi extract can markedly inhibit SMMC-7721 colony formation, indicating its strong cytostatic and cytotoxic effects against tumor stem cells. Furthermore, the cytostatic effect was enhanced with increasing dose, exhibiting a good dose-effect relationship ($\chi^2 = 48.58$, $P < 0.01$; $r = 0.4$, $P < 0.01$).

Delayed effect and time dependence of the effect of *Folium artemisiae* Argyi extract

After treating the tumor cells for a certain time, drug was removed and culturing was continued with complete medium, to observe whether the drug had a delayed effect on tumor cells. In this set of experiments, cells in the control group grew normally. As for the *Folium artemisiae* Argyi extract (40 μg/mL) 4-h withdrawal group, only a small number of cells exhibited morphological changes, while most cells maintained normal adherent growth, without presenting morphological abnormalities. In the 8 h group, a large number of cells underwent morphological changes, so deformed cells were seen along with basically normal adherent cells. In the 12 h group, the majority of cells underwent significant morphological changes, with some being elongated and tapered, and some being rounded and detached.

In the time-dependent experiments, cells were incubated with drug for 1, 3, 5, and 7 d, respectively, to observe the effects of *Folium artemisiae* Argyi extract $IC_{30,}$ IC_{50} dose groups, and control group on SMMC-7721 cell viability. The results are shown in Figure 4. As can be seen, with the prolonging action of *Folium artemisiae* Argyi extract on SMMC-7721, its cell viability-suppressing effect became increasingly significant, showing marked time dependence ($r = -0.941$, $P < 0.05$).

Effects of *Folium artemisiae* Argyi extracts on SMMC-7721 cellular expressions of p53, Topo II, and bcl-2

p53, Topo II, and bcl-2 expressions were determined by immunohistochemistry [Table 3]. Topo II and p53 proteins were present only in the nuclei while not found in the cytoplasm. Besides, nuclei were stained yellow to brown. bcl-2 proteins were pale yellow in the cytoplasm while not found in the nuclei. In the normal control stained SMMC-7721 cells, all the three expressions were positive or strongly positive, but with prolonging action of *Folium artemisiae* Argyi extracts, all the three were markedly weakened.

DISCUSSION

During the investigation of eupatilin extracting solvent, we found that methanol yielded the best extraction efficiency. Ultrasonic

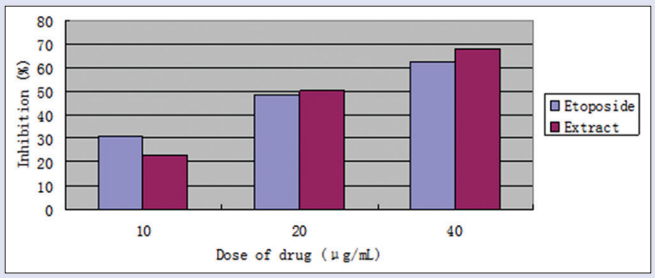

Figure 3: Inhibitory effect of *Folium artemisiae* Argyi extract on SMMC-7721

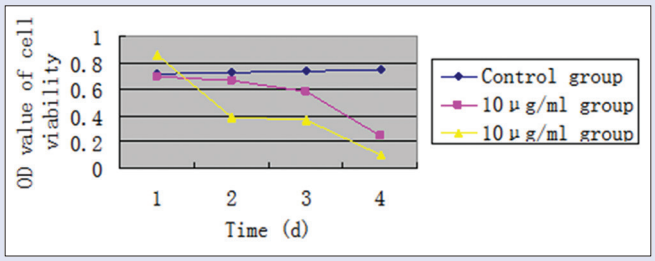

Figure 4: Time dependence of the effect of *Folium artemisiae* Argyi extract on SMMC-7721

Table 2: Effects of different concentrations of *Folium artemisiae* Argyi extracts on SMMC-7721 colony formation

Concentration of *Folium artemisiae* Argyi extract (μg/mL)	SMMC-7721		
	Number of colonies	Colony-forming efficiency (%)	Colony-forming equivalent rate (%)
Control	389	67.2	-
10	83	15.9	23.4
20	57	11	16.2
40	19	3.8	4.7

Table 3: Effects of *Folium artemisiae* Argyi extracts on bcl-2, p53, and Topo II expressions

Group	bcl-2	p53	Topo II
Control group	+++	++	++
48 h group	++	+	++
72 h group	+	+	+

Weak positivity (+): <20%; Strong positivity (+++): >70%; Moderate positivity (++): fell in between the two.

extraction time was investigated, and the results showed that 60 min of ultrasonication was the optimal option. Through three-dimensional scanning, absorption maximum of eupatilin was found at 254 nm. The eupatilin content determination method established herein is accurate, simple, rapid, sensitive, and reproducible, which can accurately determine eupatilin in *Folium artemisiae* Argyi.

Dose-dependent experimental results showed that *Folium artemisiae* Argyi extract could significantly inhibit the proliferation of hepatoma SMMC-7721 cells, and the inhibitory effect was enhanced with increasing dose, showing a positive correlation ($P < 0.01$). IC_{50} of *Folium artemisiae* Argyi extract was calculated to be 20 μg/mL. In this set of experiments, etoposide's cytotoxic effect on SMMC-7721 cells was observed. The results revealed that the *Folium artemisiae* Argyi extract's SMMC-7721 inhibiting effect was slightly greater than etoposide ($IC_{50(SJ)} < IC_{50(etoposide)}$, $P < 0.01$), indicating that hepatoma SMMC-7721 cells were more sensitive to the *Folium artemisiae* Argyi extract than to etoposide and that *Folium artemisiae* Argyi extract had the potential to be developed as novel antihepatoma drugs.

Colony formation assay is a commonly used method for screening antitumor drugs.[8,9] Colony-forming cells are stem cells, which possess infinite proliferative capacity. Therapeutic purpose of antitumor drugs is to kill the proliferative tumor stem cells, so colony formation assay is a feasible index for assessing the antitumor effect of drugs.[10,11] Our experimental results showed that *Folium artemisiae* Argyi extract can markedly inhibit SMMC-7721 colony formation compared to the control groups, which indicating that strong cytostatic and cytotoxic effects against tumor stem cells. Furthermore, the cytostatic effect was enhanced with increasing dose, which exhibiting a good dose-effect relationship ($\chi^2 = 48.58$, $P < 0.01$; $r = 0.4$, $P < 0.01$), and this result displayed that *Folium artemisiae* Argyi extract had the ability to inhibit and kill the SMMC-7721 stem cells.

In the drug delayed effect experiment, *Folium artemisiae* Argyi extract exhibited delayed effect after treating SMMC-7721 cells for 8 h, which became obvious at 12 h from treatment. After drug withdrawal, cells still tended to apoptosis, indicating that the tumor cell-inhibiting effect of *Folium artemisiae* Argyi extract was irreversible. *Folium artemisiae* Argyi extract may activate some apoptotic mechanisms of tumor cells after treating cells for 8–12 h.

The root cause of tumorigenesis is changes in gene regulation while apoptosis is an automatic cell death process controlled by genes. Apoptosis involves a cascade of gene expression, which plays a very important role in different stages of tumorigenesis. It can be induced by a variety of biological, physical, and chemical factors at the genetic level and prevent tumor development and progression by independently removing malignant or possibly malignant cells. Bcl-2 gene, an oncogene, encodes bcl-2 protein, which can promote cell survival and prolong the life of cells. This study found that in the SMMC-7721 apoptosis induced by *Folium artemisiae* Argyi extract, bcl-2 gene expression was significantly reduced at the protein level, suggesting that downregulated bcl-2 expression played a certain role in the apoptotic process of SMMC-7721 cells induced by *Folium artemisiae* Argyi extract. This also disproved the antiapoptotic effect of bcl-2 from the gene expression perspective. Wild-type p53 gene, a tumor suppressor gene, has a role in controlling cell proliferation and transformation, which regulates cell proliferation (G_0 phase monitoring function) by preventing the G_0 phase of cell cycle from entering the S phase and promotes terminal differentiation and programmed death of cells. Mutant p53 gene, on the other hand, loses tumor-suppressing function, which causes tumorigenesis instead by promoting cell transformation and excessive proliferation.[12] The present immunohistochemistry showed that the p53 protein expression in SMMC-7721 cells was positive (++) in the control group, suggesting the association between rapid proliferation of human hepatoma SMMC-7721 cells and overexpression of p53 protein. After treating with *Folium artemisiae* Argyi extract, abnormal p53 protein expression weakened (+), indicating that the antitumor mechanism of the drug may be associated with the inhibition of abnormal p53 protein expression.

In conclusion, the results of this study showed that reduced bcl-2, p53 gene, and Topo II expressions played important roles in the *Folium artemisiae* Argyi extract-induced apoptosis of SMMC-7721 cells. However, correlation between bcl-2 and p53 and their correlations with Topo II still need further study and investigation as well as how *Folium artemisiae* Argyi extract suppresses the bcl-2, p53 gene expressions and whether there are other apoptosis-related factors and other apoptosis-inducing pathways.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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