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Influences of total alkaloids in Caulis Mahoniae on proliferation and apoptosis of cervical cancer cells and the caspase-3 expression

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ABSTRACT

The study aimed to investigate the influences of the active ingredient in Caulis Mahoniae, total alkaloids, on the proliferation and apoptosis of cervical cancer cells and the caspase-3 expression. The total alkaloids were extracted in vitro from Caulis Mahoniae, and cervical cancer HeLa cell lines were used as experimental objects. The half inhibitory concentration (IC_{50}) of total alkaloids on HeLa cell lines was detected via the preliminary experiment, the influences of total alkaloids at different concentrations on the proliferation of HeLa cell lines were detected via methyl thiazolyl tetrazolium (MTT) assay, and the cell growth curve was plotted. Moreover, the cell cycle and apoptosis after treatment with total alkaloids at different concentrations were detected via flow cytometry, and the caspase-3 gene and protein expressions were detected via reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The IC_{50} of total alkaloids in Caulis Mahoniaeon HeLa cell lines was 12.5 μg/mL. With the gradual increase of concentration of total alkaloids in the treatment of cervical cancer cells, the inhibitory rate on cancer cells was gradually increased, and the proportion of cells in the G0/G1 phase was gradually decreased, while that in S and G2/M phases was gradually increased. Besides, with the increase in the concentration of total alkaloids, the apoptotic rate of cervical cancer cells was gradually increased, and both caspase-3 gene and protein expressions were also gradually increased. The total alkaloids extracted from Caulis Mahoniae can effectively inhibit the proliferation and promote the apoptosis of cervical cancer HeLa cells, which may be realized by promoting the expression of apoptosis-related factor caspase-3.

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Introduction

Cervical cancer is a kind of common malignant tumor of the genital tract in women, and there are approximately 600,000 new cases every year in the world, which has higher morbidity and mortality rates, seriously threatening the patient's health (1). Surgery and chemoradiotherapy are dominant in the traditional treatment of cervical cancer. However, the limitations of traditional therapeutic methods are gradually exhibited with the extension of time, such as drug resistance and side effects (2, 3). Therefore, searching for new anti-cancer drugs is of great clinical significance. Traditional Chinese medicine, as a traditional treasure of the Chinese nation, has overcome the shortcomings of long-term chemoradiotherapy. Caulis Mahoniae has the effects of clearing heat and drying dampness, purging fire and removing toxins (4). It has been found in modern research that Caulis Mahoniae has antibacterial, anti-inflammatory, anti-viral, anti-hypertensive and anti-hyperglycemic effects, and can inhibit growth and promote apoptosis of tumor cells (5, 6). Moreover, it has a significant inhibitory effect in gastric cancer MGCS03 and BGC823 cell lines (7), esophageal cancer (8) and nasopharyngeal cancer CNE-2 cell lines (9). Hu W et al (10) studied and found that the total alkaloids in Caulis Mahoniae can exert an inhibitory effect on tumor cells, which are expected to be a novel anti-cancer drug with high efficiency and low toxicity. In this study, the total alkaloids were extracted in vitro from Caulis Mahoniae, the half inhibitory concentration (IC_{50}) of total alkaloids on cervical cancer HeLa cells was detected in vitro, and the inhibitory efficiency of total alkaloids at different concentrations on cancer cells was observed, the cell cycle and apoptotic rate were detected, and the expression of apoptosis-related

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factor caspase-3 was detected, so as to provide basic theoretical support for the development of clinical novel drug of Caulis Mahoniae.

Materials and Methods

Materials and reagents

Caulis Mahoniae (article No.: 121460-200401, Shanghai Yaji Biotechnology Co., Ltd.), D152 weakly acidic cation exchange resin (article No.: KS34103, Shanghai Keshun Biotechnology Co., Ltd.), cervical cancer HeLa cell lines (article No.: l111080, Shanghai Beinuo Biotechnology Co., Ltd.), Dojindo cell counting kit-8 (CCK-8) kit (article No.: CK04, Shanghai Xuanling Biotechnology Co., Ltd.), ANXN V FITC APOPTOSIS DTEC KIT I 100TST (article No.: 556547, BD, Shanghai), caspase-3 primary antibody (article No.: YT152-FHC, Beijing Biolab Technology Co., Ltd.), total ribonucleic acid (RNA) extraction kit (article No.: ZY-F1182, Shanghai Zeye Biotechnology Co., Ltd.), SYBR® Premix Ex Taq™ II (TliRNaseH Plus) (article No.: RR820A, Shanghai Shanran Biotechnology Co., Ltd.), RPMI 1640 basic medium (article No.: XY-391-00723, Shanghai Xiyuan Biotechnology Co., Ltd.), Gibco fetal bovine serum (FBS) (article No.: 16000-044, Shanghai Yaoyun Biotechnology Co., Ltd.), Sigma 0.25% trypsin-Ethylene DiamineTetraacetic Acid (EDTA) solution (article No.: T4049-500ML, Shanghai Beinuo Biotechnology Co., Ltd.), Sigma bicinchoninic acid (BCA) protein quantification kit (article No.: BCA1-1KT, Shanghai Beinuo Biotechnology Co., Ltd.), real-time fluorescence quantitative polymerase chain reaction (PCR) instrument (model: ABI7500), flow cytometer (BD, USA), and ELX800 microplate reader (Bio-TEK, USA).

Methods

Preparation of total alkaloids

The total alkaloids were prepared strictly according to the extraction method of Zhang et al (11). The Caulis Mahoniae was ground and extracted twice with ethanol at a concentration of 50%, respectively. The extract obtained via extraction twice was mixed and concentrated, and total alkaloids were obtained via treatment with D152 weakly acidic cation exchange resin.

The preliminary experiment of total alkaloids

The IC_{50} of total alkaloid anti-tumor activity was screened via methyl thiazolyl tetrazolium (MTT) assay. Cervical cancer HeLa cells in the logarithmic growth phase were taken, prepared into the suspension (4×10^4/mL) and inoculated into a 96-well plate. Seven concentrations were set for the total alkaloid solution, and the solution was diluted at 1:10 and inoculated for 12 h. After drugs were added, cells were incubated for 24 h, 48 h, 72 h and 96 h, and the supernatant was discarded. Then 100 μL complete cell culture solution and 20 μLMTT solutions at a concentration of 5 g/L were added, followed by incubation at 37°C for 4 h. The medium was discarded, and 150 μL dimethylsulfoxide (DMSO) solution was added and vibrated on a shaking table for 15 min. Then the optical density (OD) value was detected at a wavelength of 570 nm using a microplate reader, and the inhibitory rate and IC_{50} of drugs were calculated. It was found that IC_{50} declined with the prolongation of the action time of drugs, and IC_{50} (12.5 μg/mL) was similar at 72 h and 96 h.

Effect of total alkaloids on the proliferation of cervical cancer HeLa cells

Cervical cancer HeLa cells in the logarithmic growth phase were taken, prepared into the suspension (1×10^5/mL) and inoculated into the 96-well plate. 90 μL cell suspension and 10 μL total alkaloids were contained in each well at final concentrations of 12.5 μg/mL, 25.0 μg/mL, 50.0 μg/mL and 100.0 μg/mL. An equal amount of culture solution was added in the negative control group and 5 repeated groups were set. The 96-well plate was placed in an incubator with 5% CO_2at 37°C, and 20 μL MTT solution was added after 48 h for incubation for another 4 h. After the supernatant was discarded, 150 μL DMSO solution was added and vibrated, and the OD value was detected at a wavelength of 570 nm.

Inhibitory rate = $(OD_{\text{negative control group}} - OD_{\text{experimental group}})/OD_{\text{negative control group}} \times 100\%$.

Effect of total alkaloids on the growth curve of cervical cancer HeLa cells

The standard growth curve of cervical cancer HeLa cells was plotted using the MTT method. HeLa cells in the logarithmic growth phase were prepared for the suspension. Each well in the 96-well plate was added with 2×10^3 cells (180 μL in volume) and 20 μL total alkaloids at different concentrations. An equal amount of culture solution was added to the negative control group. 5 repeated wells were set in each group, and a total of 5 plates were placed in the incubator with 5% CO_2 at 37°C. The OD value of one cell culture plate was detected every day, the corresponding number of cells was calculated combined with the standard curve, and the cell growth curve was plotted.

Detection of the cycle of cervical cancer HeLa cells using a flow cytometer

Cervical cancer HeLa cells in the logarithmic growth phase were collected, digested, centrifuged, washed and counted, and then they were adjusted into the suspension (4×10^4/mL) and inoculated into a 6-well plate. After 12 h, the total alkaloid solution at final concentrations of 12.5 μg/mL, 25.0 μg/mL, 50.0 μg/mL and 100.0 μg/mL was added, respectively. After 48 h, cells were collected, fixed in the 95% alcohol pre-cooled in a refrigerator and placed in the refrigerator at 4°C overnight. After the alcohol was removed via centrifugation, cells were washed, resuspended in phosphate buffered saline (PBS) and centrifuged to discard the supernatant. Then 500 μLpropidium iodide (PI) working solution was added for treatment in the refrigerator at 4°C for 30 min, and the cell cycle was detected on the machine.

Detection of apoptosis of cervical cancer HeLa cells using a flow cytometer

Cells were collected after treatment with total alkaloids at different concentrations according to the method in 1.2.4, and the binding buffer, Annexin V-FITC and PI were added and mixed evenly in each sample, followed by incubation in a dark place for 10 min and detection of apoptosis on the machine.

Detection of caspase-3 messenger RNA (mRNA) expression via RT-PCR

Primers were synthesized by HuameiRuikang (Beijing) International Institute of Biotechnology. Primer sequences are as follows: caspase-3: sense strand: 5'-GTGGAACTGACGATGATATGGC-3', antisense strand: 5'-CGCAAAGTGACTGGATGAACC-3'. The total RNA was extracted from cervical cancer HeLa cells according to the instructions of the kit, and complementary deoxyribonucleic acid (cDNA) was obtained via reverse transcription.

Reaction solution (10 μL): 2 μL 5 ×genomicDNA (gDNA) Eraser Buffer, 1 μLgDNA Eraser, 1 μg Total RNA and 6 μgRNase-Free dH_2O.

Reverse transcription system (20 μL): 4 μL 5 × PrimeScript Buffer, 1 μLPrimeScipt RT Enzyme Mix, 1 μLPrimeScript RT Enzyme Mix, 10 μL of the above reaction solution, and 4 μLRNase-Free dH_2O. Reverse transcription reaction: 37°C for 15 min, and 85°C for 5 s.

PCR system (25 μL): 12.5 μL SYBR Premix Ex Taq™ II, 1 μL forward primer, 1 μL reverse primer, 2 μLcDNA and 8.5 μL dH_2O. Reaction conditions: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 20 s, annealing at 58°C for 20 s, extension at 72°C for 30s, a total of 40 cycles. With β-actin as an internal control, the relative expression level of caspase-3 mRNA was automatically calculated using the RT-PCR instrument.

Detection of caspase-3 protein expression via Western blotting

The total protein was extracted according to instructions of the protein extraction kit, the protein concentration was detected using the BCA protein assay, and the protein was stored at -70°C for standby application. The gel was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the position of the gel where the protein was located was roughly determined according to the marker band. The protein was transferred onto a membrane for 35 min, sealed with 5% skim milk powder at 37°C for 90 min, and incubated with the primary antibody at 4°C overnight. The protein was washed with tris-buffered saline with Tween-20 (TBST) on a shaking table 3 times (15 min/time) and incubated with the secondary antibody at 37°C for 1 h. Then the protein was washed again with TBST on a shaking table 3 times (15 min/time), and the enhanced chemiluminescence solution was added in a dark room for color development, followed by exposure, image development and fixation. Finally, the image was scanned using the ChemiDocTMMPimaging system and analyzed using the Image J professional image analysis software, and the OD value was recorded, with β-actin as an internal control.

Statistical analysis

The data were analyzed using the SPSS23.0 software (provided by Beijing Xinmeijiahong Technology Co., Ltd.) and expressed as ($\chi\pm s$). One-way analysis of variance was used for the intergroup comparison, and the least significant difference (LSD) *t*-test was used for the pairwise comparison. $\alpha=0.05$ was set as the test standard.

Results and discussion

The inhibitory rate of total alkaloids on cervical cancer HeLa cells

The IC_{50} of total alkaloids on HeLa cells was 12.5 μg/mL. With the gradual increase in the concentration of total alkaloids, the inhibitory rate on HeLa cells was also gradually increased, and there was a statistically significant difference compared with the negative control group ($p<0.01$) (Table 1).

Effect of total alkaloids on the growth curve of cervical cancer HeLa cells

The total alkaloids had no significant influence on the proliferation of cervical cancer cells at 1 d in the negative control group and total alkaloid groups at different concentrations. Later, the cell proliferation rate in the negative control group was significantly higher than that in total alkaloid groups at different concentrations, and the increased rate of cell proliferation showed a decreasing trend with the increase in the concentration of total alkaloids (Figure 1).

Effect of total alkaloids on the cycle of cervical cancer HeLa cells

The proportions of cervical cancer HeLa cells in the G0/G1 phase in the four total alkaloid groups were significantly lower than that in the negative control group

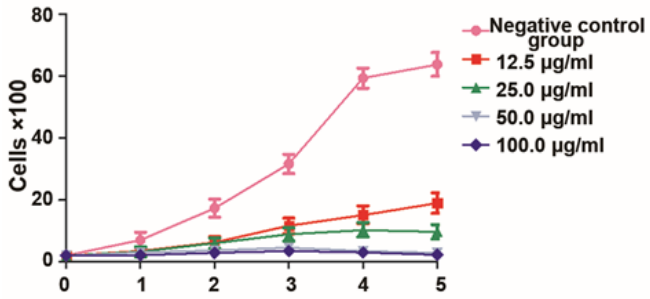

Figure 1. Effect of total alkaloids on the growth curve of cervical cancer HeLa cells.

Table 1. The inhibitory rate of total alkaloids on cervical cancer HeLa cells (n=5).

	OD value (570 nm)	Inhibitory rate (%)
Negative control group	1.03±0.03	0
12.5 μg/mL	0.56±0.06*	49.82
25.0 μg/mL	0.44±0.05*	58.96
50.0 μg/mL	0.25±0.04*	79.22
100.0 μg/mL	0.12±0.02*	90.29

Note: *$p<0.01$ vs. negative control group.

Table 2. Effect of total alkaloids on the cycle of cervical cancer HeLa cells (χ±s, n=5).

	G0/G1	S	G2/M
Negative control group	70.47±3.36	25.04±3.12	4.53±1.28
12.5 μg/mL	51.32±4.01*	34.68±3.77*	15.64±2.57*
25.0 μg/mL	44.28±3.89*	37.34±3.93*	18.93±2.88*
50.0 μg/mL	35.07±3.41*	40.95±4.38*	24.07±3.26*
100.0 μg/mL	30.64±3.05*	43.49±4.92*	27.67±5.25*

Note: *p<0.01 vs. negative control group.

(p<0.05), and it gradually decreased with the increase of the concentration of total alkaloids.

The proportions of cervical cancer HeLa cells in the S and G2/M phases in the four total alkaloid groups were significantly higher than that in the negative control group (p<0.05), and they gradually increased with the increase in the concentration of total alkaloids (Table 2).

Effect of total alkaloids on the apoptosis of cervical cancer HeLa cells

The apoptotic rates of cervical cancer HeLa cells in the four total alkaloid groups were obviously higher than that in the negative control group (p<0.01), and it gradually increased with the increase in the concentration of total alkaloids (Table 3).

Effect of total alkaloids on the caspase-3 mRNA expression in cervical cancer HeLa cells

The expressions of caspase-3 mRNA in cervical cancer HeLa cells in the four total alkaloid groups were obviously higher than that in the negative control group (p<0.01), and it gradually increased with the increase of concentration of total alkaloids (Figure 2).

Table 3. Effect of total alkaloids on the apoptosis of cervical cancer HeLa cells ($\bar{\chi}$±s, n=5).

	Apoptotic rate (%)
Negative control group	17.53±2.79
12.5 μg/mL	69.41±4.33*
25.0 μg/mL	77.92±4.65*
50.0 μg/mL	83.75±3.97*
100.0 μg/mL	89.45±5.51*

Note: *p<0.01 vs. negative control group.

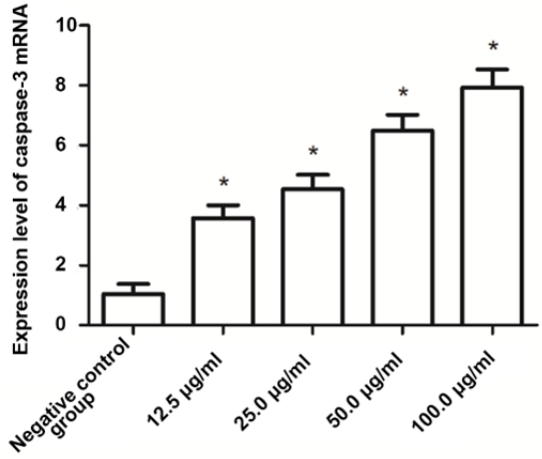

Figure 2. Detection of the caspase-3 mRNA expression in cervical cancer HeLa cells via RT-PCR.

Effect of total alkaloids on the caspase-3 protein expression in cervical cancer HeLa cells

The expressions of caspase-3 protein in cervical cancer HeLa cells in the four total alkaloid groups were remarkably higher than that in the negative control group (p<0.01), and it gradually increased with the increase of concentration of total alkaloids (Figure 3 and Table 4).

Currently, surgery and chemoradiotherapy are mainly applied in the clinical treatment of most solid tumors in vivo, so as to improve the survival rate and reduce the mortality rate of tumor patients. However, these conventional treatment means often cause damage to normal cells in vivo during the process of scavenging tumor cells, thus leading to immune dysfunction in tumor patients (12). Therefore, it is extremely important to develop novel anti-tumor drugs. Traditional Chinese medicine has been applied for thousands of years in China, and studies have found nowadays that the anti-cancer traditional Chinese medicine in vivo possesses the function of a bio-regulator (13). There are differences in the anti-tumor mechanism, and the same traditional Chinese medicine may have multiple independent or crossed anti-tumor mechanisms, or different types of traditional Chinese medicine may have the same anti-tumor mechanism (14). Moreover, the toxic and side effects of traditional Chinese medicine are weaker (15).

Caulis Mahoniae is the stem of Berberidaceae Mahonia bealei, Mahonia fortune Fedde or Mahonia japonica DC,

Table 4. Detection of the relative expression of caspase-3 protein via Western blotting ($\bar{\chi}$±s, n=5).

	Relative expression of caspase-3 protein
Negative control group	0.36±0.05
12.5 μg/mL	0.70±0.08*
25.0 μg/mL	0.97±0.11*
50.0 μg/mL	1.14±0.15*
100.0 μg/mL	1.35±0.17*

Note: *p<0.01 vs. negative control group.

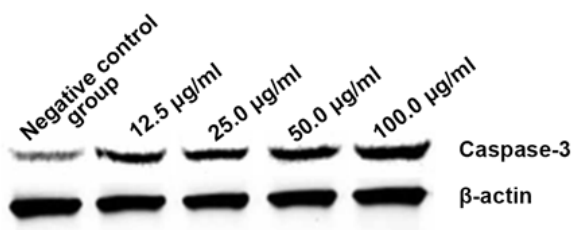

Figure 3. Detection of the caspase-3 protein expression in cervical cancer HeLa cells via Western blotting. Note: 1-5: negative control group, 12.5 μg/mL group, 25.0 μg/mL group, 50.0 μg/mL group and 100.0 μg/mL group.

which is commonly used in the heat clearance and detoxication and treatment of a common cold. It has been found in recent years that Caulis Mahoniae can inhibit the proliferation and promote the apoptosis of a variety of tumor cells (16). In this study, the total alkaloids were extracted in vitro from Caulis Mahoniae, and the IC_{50} of total alkaloids on the proliferation of cancer cells was detected via MTT assay. Results revealed that total alkaloids could exert a significant inhibitory effect on the proliferation of cervical cancer HeLa cells in a dose-dependent effect, and the IC_{50} was 12.5 μg/mL. The whole process of cell proliferation is completed by the cell cycle and strictly controlled by the cell cycle regulation mechanism. The most prominent feature of tumor cells in the body is the uncontrollable growth of cancer cells, and the intrinsic factor is that the cell cycle regulation mechanism is broken by external factors (17). Therefore, the regulation of cell cycle progression and cell proliferation may induce programmed death of cells, thereby producing an anti-tumor effect. The duration of the cell cycle in the G0/G1 phase determines the rate of cell proliferation. The shorter the cell cycle in the G0/G1 phase is, the higher the cell proliferation rate and the larger the number of cells in this phase will be. Results of this study manifested that cervical cancer cells in the negative control group were mostly in the G0/G1 phase, and the apoptotic rates on cervical cancer HeLa cells in total alkaloid groups at different concentrations were significantly increased. It is speculated that the treatment with total alkaloids may block the DNA synthesis and replication in cells and inhibit normal cell division, thus producing an inhibitory effect on the proliferation and increasing the apoptosis of tumor cells.

Caspase-3, as a member of the caspase family, plays a role in the execution stage of apoptosis (18), which can directly degrade the apoptotic substrate with the strongest degradation activity. Studies have demonstrated that caspase-3 is activated in the early stage of apoptosis, so that the substrates in the cytoplasm and nucleus can be cleaved, ultimately leading to apoptosis (19). However, when apoptosis is in the late stage or cells are dead, the activity of caspase-3 will significantly decline (20). Mittal A et al (21) also found that various pathways of caspase-3 in apoptotic signal transduction can play crucial roles. It was found in this study that both caspase-3 gene and protein expressions were significantly increased in cervical cancer HeLa cells after treatment with total alkaloids at different concentrations, and it was increased with the increase of concentration of total alkaloids, indicating that the expression activity of caspase-3 is increased. At the same time, it was also found that the apoptotic rate on cervical cancer cells was also obviously increased during this process, and it was also increased with the increase of concentration of total alkaloids.

In conclusion, the total alkaloids extracted from Caulis Mahoniae can effectively inhibit the proliferation and promote the apoptosis of cervical cancer HeLa cells, which may be realized through promoting the expression of apoptosis-related factor caspase-3, and are expected to provide a new idea for the development of new therapeutic drugs for cervical cancer

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions
GY wrote the manuscript. GY and MZ performed PCR and Western blot. KL and JL were responsible for the flow cytometer. NJ and BZ helped with the MTT assay. GF and DL contributed to the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the ethics committee of GuangXi University of Chinese Medicine.

Consent for publication
Not applicable.

Interest conflict
The authors declare that they have no conflict of interest.

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