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Original Research

Effects of ethyl acetate extract of peony (*Paeonia suffruticosa*) seed coat on the proliferation and apoptosis of oral squamous carcinoma cells through miR-424-3p/STAT3/Survivin pathway

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Abstract: Oral squamous cell carcinoma is one of the most common high malignant tumors. This experiment aimed to investigate whether ethyl acetate extract of peony (*Paeonia suffruticosa*) seed coat could affect the proliferation and apoptosis of oral squamous carcinoma cells by regulating the miR-424-3p/STAT3/Survivin pathway. For this purpose, oral squamous cell carcinoma cell CAL27 was cultured in vitro, and cells were treated with ethyl acetate extract of peony seed coat at different concentrations. MTT was used to detect cell proliferation. Flow cytometry was used to detect apoptosis. qRT-PCR was used to detect the expression level of miR-424-3p. The miR-424-3p mimics and anti-miR-424-3p were transfected into CAL27 cells respectively, and the cell proliferation and apoptosis were detected by the above method. Western blot method was used to detect the expression of PCNA, Bcl-2, Bax, p-STAT3 and Survivin protein. Results showed that ethyl acetate extract of peony seed coat could reduce cell proliferation rate and the protein levels of PCNA, Bcl-2, p-STAT3, Survivin and the expression level of miR-424-3p ($P<0.05$), increase apoptosis rate and the protein level of Bax ($P<0.05$). After transfection with anti-miR-424-3p, the cell proliferation rate, the protein levels of PCNA and Bcl-2 were significantly reduced ($P<0.05$), the apoptosis rate and the protein level of Bax were significantly increased ($P<0.05$), while the effect of miR-424-3p mimics was the opposite. Transfection of miR-424-3p mimics could significantly reduce the regulatory effect of ethyl acetate extract of peony seed coat on CAL27 cell proliferation, apoptosis and STAT3/Survivin pathway. It concluded that ethyl acetate extract of peony seed coat could inhibit the activation of the STAT3/Survivin signaling pathway by down-regulating the expression of miR-424-3p, thereby inhibiting the proliferation of oral squamous carcinoma cells and inducing apoptosis.

Key words: *Paeonia suffruticosa*; Seed coat; Ethyl acetate extract; MiR-424-3p; STAT3/Survivin signaling pathway; Oral squamous cell carcinoma.

Introduction

Oral squamous cell carcinoma (abbreviated as OSCC) is one of the most common oral malignant tumors with high malignancy. Due to local invasion and metastasis of the tumor, patients' survival rate is reduced, seriously threatening the safety of human life. Surgery and radiotherapy are the main clinical treatment approach for the time being, but the incidence and mortality of the disease have not been effectively improved (1). Therefore, it is of great significance to seek therapeutic drugs against oral squamous cell carcinoma. Natural plant extracts have anti-tumor effects, which can also inhibit tumor cell proliferation, migration and invasion, and regulate apoptosis (2). However, the effect of natural plant extracts on oral squamous carcinoma cells has not been elucidated. MicroRNA (miRNA) can regulate biological processes such as proliferation and apoptosis of tumor cells via targeted regulation of downstream gene expressions (3). However, whether miRNA mediates the proliferation and apoptosis of oral squamous carcinoma cells induced by natural plant extracts has not been elucidated. Peony (*Paeonia suffruticosa*) is one of the flowers in China. Peony seed oil containing unsaturated fatty acids has certain medicinal values, and peony seeds can resist tumor cell proliferation and metastasis (4). However, the effect of ethyl acetate extracts of peony seed coat on the proliferation and apoptosis of oral squamous carcinoma cells has not been elucidated. microRNA-424-3p (miR-424-3p) has an increased expression level in prostate cancer, which may promote the occurrence and development of prostate cancer (5). Transcription activator 3 (STAT3)/apoptosis inhibitor gene (Survivin) has a signaling pathway closely related to tumor cell proliferation and apoptosis, which can promote tumorigenesis and development (6). However, the regulatory role of ethyl acetate extract of peony seed coat and miR-424-3p/STAT3/Survivin pathway in the proliferation and apoptosis of oral squamous carcinoma cells remains unknown. Therefore, this study mainly investigates the effect of ethyl acetate extract of peony seed coat on the proliferation and apoptosis of oral squamous carcinoma cells, and explores its regulation on miR-424-3p/STAT3/Survivin pathway during this process, with a view to providing potential targets for the treatment of oral squamous cell carcinoma.

Materials and Methods

Materials and reagents

Peony seeds were purchased from Mudan District Peony Development Co., Ltd. in Heze City; oral squamous carcinoma cell CAL27 cells were purchased from ATCC, USA; DMEM medium and Trizol reagent were purchased from Beijing Solarbio Science & Technology Co., Ltd.; trypsin, reverse transcription and fluorescence quantification detection kits were purchased from Thermo Fisher, USA; fetal bovine serum was purchased from ExCell Biology, USA; miR-424-3p oligonucleotide mimics (miR-424-3p mimics) and the negative control mimic NC sequence (miR- NC), miR-424-3p specific oligonucleotide inhibitor (anti-miR-424-3p) and its negative control (anti-miR-NC) were purchased from Guangzhou RiboBio Co., Ltd.; MTT detection kit was purchased from Wuhan AmyJet Scientific Inc.; Annexin V-FITC/PI double-staining apoptosis detection kit was purchased from Sigma, USA; rabbit anti-human PCNA, Bcl-2, Bax antibodies were purchased from CST, USA; rabbit anti-human p-STAT3 and Survivin antibodies were purchased from Santa Cruz, USA; horseradish peroxidase (HRP) labeled goat anti-rabbit IgG secondary antibody was purchased from Beijing Zhongshan Glodenbridge Biotechnology Co., Ltd.

Experimental processing and grouping

Ethyl acetate extract of peony seed coat: 50 g of peony seed was weighed and ground into powder, added with 60% ethanol, extracted with flash extractor, centrifuged at 3000 r/min for 10 min at 4°C. The supernatant was taken, subject to reduction vaporization (to remove ethanol), added with ultrapure water to mix thoroughly and dissolved with ultrasound. The aqueous suspension was collected and placed in a separatory funnel, added with 200 μL ethyl acetate to let it stand at room temperature to separate layers. The ethyl acetate layer (upper layer) was extracted, freeze-dried to obtain ethyl acetate extract. 28 g drug was weighed, added to 0.1% DMSO solution to prepare mother liquor with a concentration of 10 mg/mL, which was diluted according to experimental requirements to obtain culture solutions with concentrations of 2 mg/ml, 4 mg/ml, and 8 mg/ml, respectively (7).

CAL27 cells were cultured in a medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, incubated in an incubator, and subcultured when the cell growth and fusion reached 80%. Logarithmic growth phase cells were seeded into 96-well plates (1×10^4 cell/well), added with ethyl acetate extracts of peony seed coat with different concentrations (2 mg/ml, 4 mg/ml, 8 mg/ml respectively) for 24 h reaction, which was recorded as 2 mg/ml ethyl acetate extract group of the peony seed coat, 4 mg/ml ethyl acetate extract group of the peony seed coat, and 8 mg/ml ethyl acetate extract group of the peony seed coat. Subsequent experiments were divided into a miR-NC group (miR-NC transfected to CAL27 cells), miR-424-3p group (miR-424-3p mimics transfected into CAL27 cells), anti-miR-NC group (anti-miR-NC transfected to CAL27 cells), anti-miR-424-3p group (anti-miR-424-3p transfected to CAL27 cells), ethyl acetate extract of peony seed coat + miR-NC group (miR-NC transfected to CAL27 cells, added with medium containing 8 mg/ml ethyl acetate extract of peony seed coat for 24 h reaction), ethyl acetate extract of peony seed coat + miR-424-3p group (miR-424-3p mimics transfected to CAL27 cells, added with medium containing 8 mg/ml ethyl acetate extract of peony seed coat for 24 h reaction).

Cell proliferation detection by MTT

Logarithmic growth phase CAL27 cells were seeded into 96-well plate (5×10^3 cell/well), grouped according to "1.2.1". 20 μL MTT solution was added to each well, incubated at room temperature for 4 h. After the supernatant was discarded, 150 μL DMSO was added, incubated at room temperature with shaking for 10 min, followed by detection of absorbance value (OD) of each well at a wavelength of 490 nm using a microplate reader. Cell proliferation rate (%) = experimental group OD value / control group OD value × 100%.

Apoptosis rate detection by flow cytometry

CAL27 cells of each group were collected, added with PBS, centrifuged at 3000 r/mi for 6 min at 4 °C. After the supernatant was discarded, centrifugation was performed under the same conditions to collect the supernatant. The cell pellet was collected, added with 500 μL binding buffer to resuspend the cells. 5 μL Annexin V-FITC and 5 μL PI were added, mixed well, incubated at room temperature in the dark for 10 min, with apoptosis rate of each group detected by flow cytometry.

Detection of miR-424-3p expression level in cells by real-time fluorescence quantitative polymerase chain reaction (qRT-PCR)

CAL27 cells of each group were collected, and the total RNA in the cells was extracted using the Trizol method. Nanodrop2000c ultra-micro spectrophotometer was used to detect RNA concentration. Reverse transcription was performed to synthesize cDNA. miR-424-3p forward primer 5'-AGGTAGAAGGTGGGGAGCTA-3', reverse primer 5'-TCGCTGCACTGACTGACTACGTC-3'; U6 forward primer 5'-GCTTCGGCAGCACATATACT-3', reverse primer 5'-GTGCAGGGTCCG AGGTATTC-3'. The primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. PCR amplification reaction system: SYBR Green Master Mix 10 μL, forward and reverse primers 0.8 μL, cDNA 2 μL, RNase-Free ddH_2O complemented system to 20 μL; reaction conditions: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, a total of 40 cycles. The relative expression level of miR-424-3p was calculated using the $2^{-\Delta\Delta Ct}$ method with U6 as the internal reference.

Western blot detection of PCNA, Bcl-2, Bax, p-STAT3, Survivin protein expression

CAL27 cells of each group were collected, with total protein extracted using protein extraction kit and detected for protein concentration. Proteins were separated by SDS-PAGE electrophoresis, the separated protein gel was transferred to PVDF membrane and blocked with 5% skim milk for 2 h, added with protein primary antibody dilution (1:100), incubated at 4°C for 24 h,

washed with TBST, added with secondary antibody dilution solution (1:2000), incubated at room temperature for 1 h, washed with TBST, exposed and developed in a dark room, followed by gray value analysis of each band using ImageJ software.

Statistical processing

SPSS21.0 statistical software was used to analyze the data. The measurement data was expressed in ($\bar{x}\pm s$) and all conformed to normal distribution. An independent sample *t*-test was used for comparison between two groups, and one-way analysis of variance was used for comparison between multiple groups. $P<0.05$ suggests a statistically significant difference.

Results

Effect of ethyl acetate extracts of peony seed coat at different concentrations on the proliferation of CAL27 cells

Compared with NC group, 2 mg/ml ethyl acetate extract group of the peony seed coat, 4 mg/ml ethyl acetate extract group of the peony seed coat, 8 mg/ml ethyl acetate extract group of peony seed coat have significantly decreased cell proliferation rates ($P<0.05$), significantly reduced PCNA protein level ($P<0.05$). 8 mg/ml ethyl acetate extract of peony seed coat was selected for subsequent experiments, as shown in Figure 1 and Table 1.

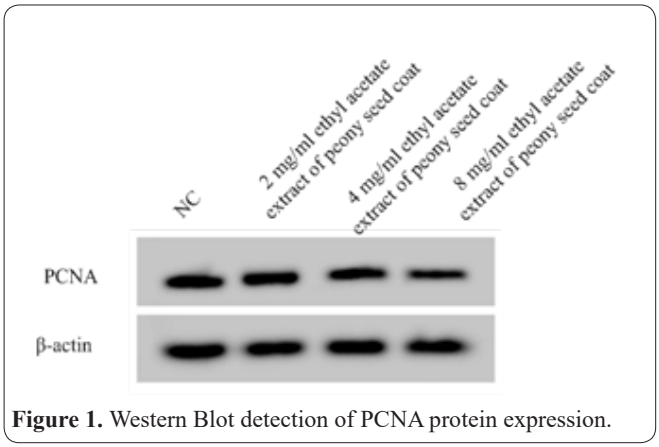

Figure 1. Western Blot detection of PCNA protein expression.

The effect of ethyl acetate extract of peony seed coat on apoptosis of CAL27 cells

Compared with NC group, ethyl acetate extract group of peony seed coat has significantly increased apoptosis rate ($P<0.05$), significantly increased Bax protein level ($P<0.05$), and significantly reduced Bcl-2 protein level ($P<0.05$), as shown in Figure 2, Table 2.

The effect of ethyl acetate extract of peony seed coat on the miR-424-3p expression

Compared with the NC group, the ethyl acetate extract group of peony seed coat has significantly reduced miR-424-3p expression level ($P<0.05$), as shown in Table 3.

The effect of miR-424-3p on the proliferation and apoptosis of CAL27 cells

Compared with the miR-NC group, the miR-424-3p

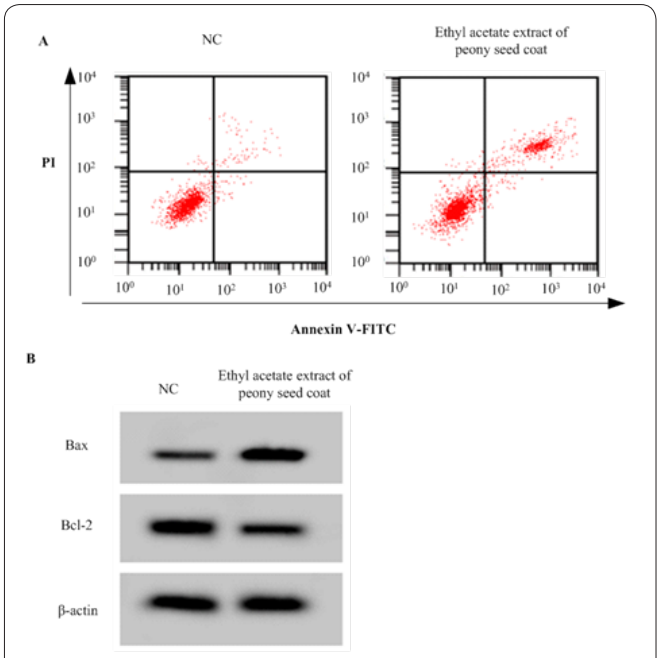

Figure 2. The effect of ethyl acetate extract of peony seed coat on apoptosis of CAL27 cells. A: Flow cytometry detection of apoptosis. B: Western Blot detection of Bax, Bcl-2 protein expression.

Table 1. The effect of ethyl acetate extracts of peony seed coat at different concentrations on the proliferation of CAL27 cells($\bar{x}\pm s$, n=9).

Group	PCNA	cell proliferation rate (%)
NC	0.83±0.08	100.28±9.24
2 mg/ml ethyl acetate extract of peony seed coat	0.72±0.07*	80.24±7.35*
4 mg/ml ethyl acetate extract of peony seed coat	0.52±0.05*	54.25±4.27*
8 mg/ml ethyl acetate extract of peony seed coat	0.40±0.04*	42.27±4.01*
F	87.643	140.684
P	0.000	0.000

Note: Compared with NC group, *$P<0.05$.

Table 2. Effect of ethyl acetate extract of peony seed coat on apoptosis of CAL27 cells($\bar{x}\pm s$, n=9).

Group	Bax	Bcl-2	Apoptosis rate (%)
NC	0.35±0.03	0.84±0.08	7.35±0.62
Ethyl acetate extract of peony seed coat	0.78±0.07*	0.42±0.04*	23.54±2.08*
t	16.939	14.087	22.378
P	0.000	0.000	0.000

Note: Compared with NC group, *$P<0.05$.

Table 3. The effect of ethyl acetate extract of peony seed coat on miR-424-3p expression ($\bar{x}$±s, n=9).

Group	miR-424-3p
NC	1.00±0.10
Ethyl acetate extract of peony seed coat	0.38±0.03*
t	17.816
P	0.000

Note: Compared with NC group, *P<0.05.

Table 4. The effect of miR-424-3p on the proliferation and apoptosis of CAL27 cells ($\bar{x}$±s, n=9).

Group	miR-424-3p	PCNA	Bax	Bcl-2	cell proliferation rate (%)	apoptosis rate (%)
miR-NC	1.00±0.09	0.82±0.08	0.37±0.03	0.88±0.08	100.53±10.24	7.35±0.62
miR-424-3p	2.68±0.20*	1.10±0.10*	0.11±0.01*	1.24±0.11*	135.48±12.37*	4.21±0.32*
anti-miR-NC	1.01±0.11	0.83±0.07	0.39±0.03	0.85±0.07	99.76±9.24	7.24±0.62
anti-miR-424-3p	0.47±0.04*	0.34±0.03#	0.76±0.07#	0.45±0.04#	58.96±5.10#	20.27±1.76#
F	537.282	162.095	378.485	150.192	95.428	463.881
P	0.000	0.000	0.000	0.000	0.000	0.000

Note: Compared with the miR-NC group, *P<0.05; compared with the anti-miR-NC group, *P<0.05.

Table 5. miR-424-3p can reverse the effect of ethyl acetate extract of peony seed coat on proliferation and apoptosis of CAL27 cells ($\bar{x}$±s, n=9).

Group	miR-424-3p	PCNA	Bax	Bcl-2	CPR (%)	apoptosis rate (%)
Ethyl acetate extract of peony seed coat + miR-NC	1.00±0.10	0.40±0.04	0.76±0.07	0.38±0.05	43.57±4.15	22.87±2.13
Ethyl acetate extract of peony seed coat +miR-424-3p	2.38±0.21*	0.70±0.09*	0.45±0.0⁴*	0.72±0.08*	82.76±7.96*	11.31±0.94*
t	17.799	9.138	11.535	10.812	13.097	14.896
P	0.000	0.000	0.000	0.000	0.000	0.000

Note: Compared with the ethyl acetate extract of peony seed coat + miR-NC group, *P<0.05. CPR: cell proliferation rate.

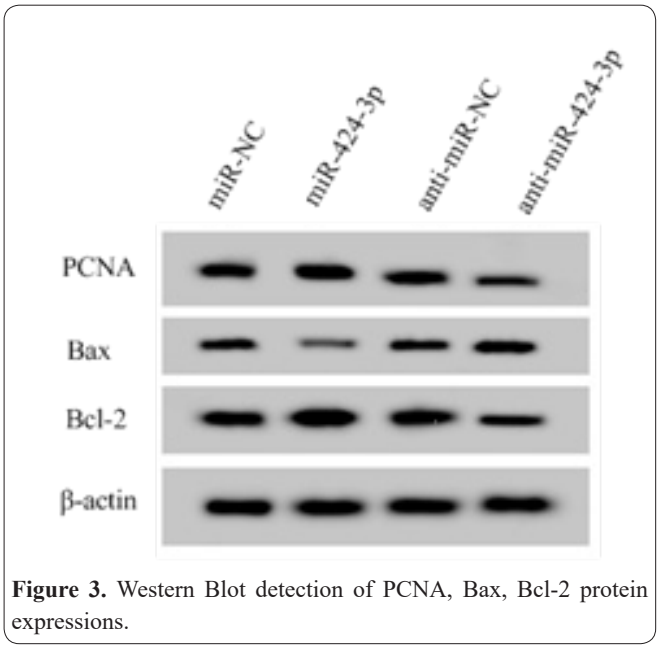

Figure 3. Western Blot detection of PCNA, Bax, Bcl-2 protein expressions.

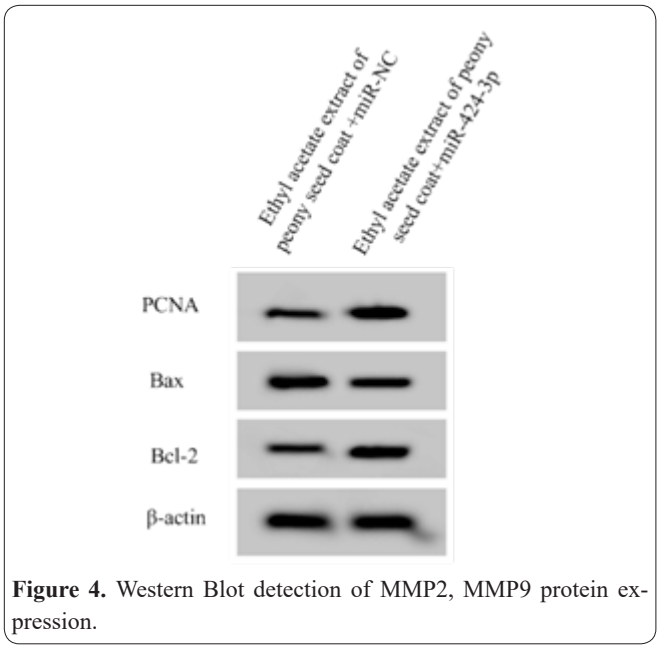

Figure 4. Western Blot detection of MMP2, MMP9 protein expression.

group has significantly increased cell proliferation rate (P<0.05), significantly reduced apoptosis rate (P<0.05), and significantly increased PCNA and Bcl-2 protein levels (P<0.05), significantly reduced Bax protein levels (P<0.05); compared with the anti-miR-NC group, the anti-miR-424-3p group has significantly reduced cell proliferation rate (P<0.05), significantly increased apoptosis rate (P<0.05), significantly reduced PCNA and Bcl-2 protein levels (P<0.05), significantly increased Bax protein levels (P<0.05), as shown in Figure 3 and Table 4.

miR-424-3p can reverse the effect of ethyl acetate extract of peony seed coat on proliferation and apoptosis of CAL27 cells

Compared with the ethyl acetate extract of peony seed coat + miR-NC group, ethyl acetate extract of peony seed coat + miR-424-3p group has significantly increased cell proliferation rate (P<0.05), significantly decreased apoptosis rate (P <0.05), significantly increased PCNA and Bcl-2 protein levels (P<0.05), significantly decreased Bax protein levels (P<0.05), as shown in Figure 4 and Table 5.

Table 6. Protein expression of STAT3/Survivin signaling pathway ($\bar{x}$±s, n=9).

Group	p-STAT3	Survivin
NC	0.73±0.07	0.65±0.06
Ethyl acetate extract of peony seed coat	0.38±0.03*	0.30±0.02*
miR-424-3p	0.96±0.09*	0.88±0.08*
Ethyl acetate extract of peony seed coat+miR-NC	0.40±0.04	0.28±0.02
Ethyl acetate extract of peony seed coat+miR-424-3p	0.68±0.06#	0.55±0.05#
F	139.476	213.733
P	0.000	0.000

Note: Compared with NC group, *P<0.05; compared with ethyl acetate extract of peony seed coat+ miR-NC group, #P<0.05.

Protein expression of STAT3/Survivin signaling pathway

Compared with the NC group, ethyl acetate extract group of peony seed coat has significantly reduced p-STAT3 and Survivin protein levels (P<0.05), the miR-424-3p group has significantly increased p-STAT3 and Survivin protein levels (P<0.05). Compared with ethyl acetate extract of peony seed coat + miR-NC group, ethyl acetate extract of peony seed coat + miR-424-3p group has significantly increased p-STAT3 and Survivin protein levels (P<0.05), as shown in Figure 5, Table 6.

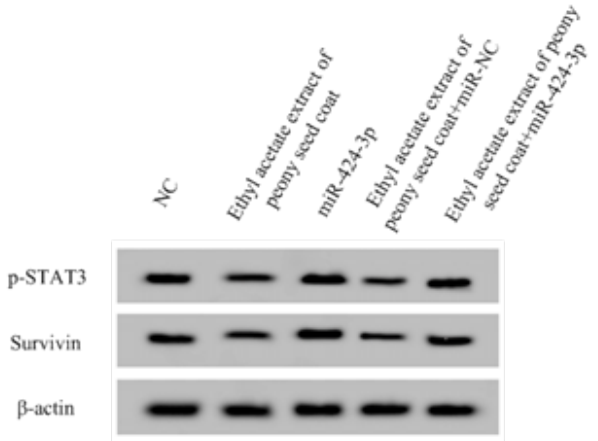

Figure 5. Western Blot detection of p-STAT3, Survivin protein expression.

Discussion

Oral squamous cell carcinoma has complicated pathogenesis, whose incidence may be caused by increased production of oxidative stress products after smoking and drinking. Usually treated by chemotherapy, it often has drug resistance, relapse after chemotherapy, which reduces therapeutic effects. Previous studies have shown that natural products have the advantages of low side effects and strong anti-tumor effects, and may inhibit the development of oral squamous cell carcinoma by regulating cell proliferation, apoptosis processes (8-10). Therefore, the search for high-efficiency natural products and investigation into their related mechanisms have become the research focus.

Paeonol, the main active ingredient in the peony seed coat, can inhibit tumor cell proliferation and promote apoptosis (11). Paeonoside A, one active component of the peony seed coat, belongs to galloyl paeonoside. Studies have shown that paeonoside A has an anti-oxidative stress effect, which can also inhibit the proliferation of non-small cell lung cancer cells and promote apoptosis (12). However, the mechanism of anti-tumor action of ethyl acetate extract of peony seed coat has not been elucidated. The results of this study indicate that ethyl acetate extract of peony seed coat significantly reduces the cell proliferation rate and promotes apoptosis rate, suggesting that ethyl acetate extract of peony seed coat can inhibit the proliferation of oral squamous cell carcinoma cells and promote apoptosis. Studies have shown that increased PCNA expression levels can promote cell proliferation by regulating the cell cycle (13). Bcl-2 is an anti-apoptotic protein, whose increased expression level can inhibit apoptosis, and its down-regulation could increase Bax expression level. Bax is a pro-apoptotic protein, which can promote the release of cytochrome C by mitochondria to activate caspase cascade, thereby inducing apoptosis (14). The results of this study indicate that after treatment with ethyl acetate extract of the peony seed coat, PCNA and Bcl-2 protein levels are decreased and Bax protein level is increased, further confirming that ethyl acetate extract of peony seed coat can inhibit the proliferation of oral squamous cell carcinoma cells and induce apoptosis.

MiR-424-3p expression level is increased in tumors like lung cancer, prostate cancer, and the progress of tumor development can be inhibited by inhibiting its expression (15, 16). The results of this study show that ethyl acetate extract of peony seed coat can reduce miR-424-3p expression levels in oral squamous cell carcinoma cells. Further analysis indicates that overexpression of miR-424-3p can increase cell proliferation rate and decrease apoptosis rate, which can also promote PCNA and Bcl-2 expressions and inhibit Bax expression. Inhibiting miR-424-3p expression can reduce the cell proliferation rate and increase the apoptosis rate, which can also promote Bax expression and inhibit PCNA, Bcl-2 expressions. It suggests that miR-424-3p may act as an oncogene in the occurrence and development of oral squamous cell carcinoma, while ethyl acetate extract of peony seed coat may resist oral squamous cell carcinoma by reducing miR-424-3p expression. In this study, oral squamous cell carcinoma cells were treated with miR-424-3p mimics and ethyl acetate extract of the peony seed coat. It turned out that the cell proliferation rate was significantly increased, the apoptosis rate was significantly reduced, the expression levels of PCNA and Bcl-2 were significantly increased, Bax expression level was significantly reduced, suggesting that miR-424-3p overexpression can reverse the effect of ethyl

acetate extract of peony seed coat on proliferation and apoptosis of oral squamous cell carcinoma cell. Studies have shown that Survivin is an apoptin inhibitor, which can inhibit cell apoptosis by promoting cell cycle progression. STAT3 is an essential transcription factor in Survivin's action. Increased STAT3 expression can promote tumorigenesis, while inhibition of STAT3 expression can reduce Survivin expression level and thereby promote apoptosis, and up-regulated STAT3 expression can increase Survivin expression level and then inhibit apoptosis (17, 18). The results of this study indicate that p-STAT3 and Survivin protein levels are significantly reduced in oral squamous cell carcinoma cells after treatment with ethyl acetate extracts of the peony seed coat, p-STAT3 and Survivin protein levels are significantly increased in oral squamous cell carcinoma cells after miR-424-3p overexpression, while p-STAT3 and Survivin protein levels are significantly increased in oral squamous cell carcinoma cells after co-treatment of miR-424-3p overexpression and ethyl acetate extract of the peony seed coat, suggesting that ethyl acetate extract of peony seed coat can inhibit the activation of STAT3/Survivin signaling pathway and then regulate the proliferation and apoptosis of oral squamous carcinoma cells, in which process, miR-424-3p plays an important regulatory role.

There are several reports of gene expression and ways to control it (19-25). Numerous genetic and non-genetic factors affect gene expression. In relation to non-genetic factors, the most optimal condition should be determined. (26-30). MicroRNAs play an important role in this regard (31). There are some factors that stimulate the expression of genes in plants, or by producing transgenic plants and using the engineering method to create metabolites (32-35). In this research, we investigate the effects of ethyl acetate extract of peony (*Paeonia suffruticosa*) seed coat on the proliferation and apoptosis of oral squamous carcinoma cells through miR-424-3p/STAT3/Survivin pathway.

To conclude, miR-424-3p can mediate the process of oral squamous cell carcinoma cell proliferation and apoptosis regulated by ethyl acetate extract of the peony seed coat, and its mechanism may be that miR-424-3p positively regulates STAT3/Survivin signaling pathway, and ethyl acetate extract of peony seed coat may inhibit the activation of STAT3/Survivin signaling pathway by inhibiting miR-424-3p expression, thus providing a potential target for targeted therapy of oral squamous cell carcinoma, which may further lay the experimental foundation for illustrating the molecular mechanism of ethyl acetate extract in its anti-tumor effect.

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