α -Pinene inhibits the growth and induces the apoptosis of cervical cancer cells through regulating miR-34a/Bcl-2 signaling axis

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Abstract

Purpose α -pinene was a chemical compound which was extracted from pine needles oil, and it exerted effects on various diseases. However, the effect of α -pinene on cervical cancer had not been reported. The goal of this study was to explore the anti-tumor role of α -pinene.

Methods Methyl thiazolyl tetrazolium (MTT) method was used to detect cytotoxicity of α -pinene. Flow cytometry was used to quantify the cell cycle and apoptosis. TUNEL staining was also performed for the revalidation of apoptosis. QRT-PCR and western blot was implemented to detect the expression levels of apoptosis genes and miR-34a-5p. Tumor-bearing nude mouse models was adopted to assess the anti-tumor action of α -pinene *in vivo*.

Results The results displayed α -pinene restrained proliferation of Hela cells in G1 phase and induced Hela cell apoptosis, which was related to up-regulating expressions of Bax, Bid, Caspase-9, Caspase-3, miR-34a-5p and down-regulating the expression of Bcl-2. Afterwards, α -Pinene could regulate miR-34a-5p/Bcl-2 pathway. Furthermore, α -pinene treatment also induced apoptosis in xenografts tumor models. The fluorescence intensity of Bax, Bid, Caspase-9, Caspase-3 increased and fluorescence intensity of Bcl-2 decreased.

Conclusions Our research demonstrated α -pinene could restrain the development of cervical cancer growth, and it might be an effective chemical compound for therapy of cervical cancer.

Introduction

Cervical cancer (CC) was a prevalent malignant disease for women all around the world [1]. The incidence and mortality of CC was increasing in developing countries [2]. In China, the five years survival rate of CC patients was only about 36.9% [3]. Chemotherapy is an effective treatment measure to prolong the life length of patients after they had accepted surgical operation [4]. However, chemotherapy resistance seriously affects the effectiveness of treatment and leads to recurrence and metastasis of CC patients [5]. Because of this reason it is important to explore the new and effective chemotherapeutic drugs for CC therapeutic program.

α -pinene was a bicyclic monoterpene compound which was extracted from turpentine. Recently, lots of medicinal values of α -pinene were found in treatment of various diseases, including sleep, inflammatory and cancer [6-8]. It had been found that α -pinene had anti-cancer effect on lung cancer by promoting apoptosis in lung cancer cells [9]. There was another study which showed that α -pinene inhibited prostate cancer growth in a xenografts model [10]. However, the potential effects of α -pinene and its treatment relative mechanisms in CC were not investigated. So we proceeded biological activity experiments to explore the effect of α -pinene on CC.

It was well known that microRNAs were conserved small non coding RNAs and they could regulate the transcription and translation process of the target genes in different types of cancers [11,12]. Many

studies have proved miR-34a could restrict growth and migration of various malignant tumor cells such as osteosarcoma, lung cancer and prostate cancer by controlling c-Met, CDK6 and LEF1 gene [13-15], And miR-34a was also reported as a novel molecular biomarker associated with cervical cancer [16,17]. And it was clear that miR-34a acted as a tumor suppressor gene by downregulating Bcl-2[18]. The synthetic function of miR-34a provided a new way to analyze the mechanism of α -pinene functions.

In our study, we evaluated the therapeutic effect of α -pinene in cervical cancer using Hela cell lines and tumor bearing nude mice models. And anti-cancer mechanism of α -pinene was explained basing on the miR-34a/Bcl-2-mediated regulation functions.

Materials And Methods

2.1. α -pinene purity and structural confirmation

Pine pneumatizing powder of the pulverized screen is soaked for a certain period of time, and the water vapor distillation method extracts the pine needle oil in the pine needle, and the fractionation is separated from the extracting high purity α -pinene. Analysis of product purity and structures by gas chromatography instrument, mass spectrometer.

2.2. Cell culture

Hela cell line, the human cervical carcinoma cell, was acquired from Suyan Biotechnology Co. (Guangzhou, Guangdong, China). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Los Angeles, CA, USA), mixed with 10% fetal bovine serum (Gibco-BRL, Los Angeles, CA, USA) and 1% streptomycin/-penicillin (Solarbio Corp., Beijing, China). The cells were applied to constant-temperature incubator with 5% CO₂ at 37°C.

2.3. MTT assay

The α -pinene dilution was dissolve with dissolved in the DMSO. The proliferation of Hela cells was detected by MTT assay after they were treated with α -pinene. The Hela cells were inoculated into 96 well plate by adjusting cell accounts to about 4×10^3 cells/well with 100 μ L. Different concentration of α -pinene (10, 20, 40, 80, 160, 320, 640 μ mol/L,) was administered to each well after the cells adhered to the wall completely and each group contained six parallel wells. 5-FU was used as positive control group (40 μ mol/L). The control group was cultured in an equal volume of serum RPMI-1640 culture medium containing cells. After 24hours, the supernatant was discarded, and the medium containing 10 μ L of 5 mg/mL MTT reagent (Meilun Biotechnology Co., Dalian, Liaoning, China) was put into well and incubated in dark at 37 °C for 4 h. After removal of the MTT, cells were treated with 100 μ L DMSO, and shaken on an oscillator for 15minutes. OD was measured at 490nm using Automated Microplate Reader (Sunrise, Tecan, Switzerland) .Cell survival rate = (OD value of drug group-OD value of blank group)/ OD value of

control group -OD value of blank group) $\times 100\%$. Each experiment was repeated three times. The IC_{50} value of α -pinene in Hela cells was calculated with the help of Graphpad Prism 8.0 software.

2.4. Cell cycle analysis

1×10^6 Hela cells were inoculated into 6-well plate for each well. When the cultured cell confluency reached at about 60%, the drug was administered into cultured cells. According to IC_{50} calculated by MTT data, α -pinene groups were designed to three different concentration groups: low concentration group ($25 \mu\text{mol/L}$), medium concentration group ($50 \mu\text{mol/L}$) and high concentration group ($100 \mu\text{mol/L}$). After incubation for 24 hours, the cells were collected and mixed with 70% alcohol at 4°C for at least 4 hours. According to protocol provided in cell cycle kit, the cells were labeled with $50 \mu\text{g/mL}$ PI staining at 37°C for 30 minutes. Then the samples were measured by Flow Cytometry. The test data was analyzed by ModFit software.

2.5. Cell apoptosis proportion detection

The apoptosis of Hela cells treated with α -pinene was assessed by Annexin V-FITC/PI apoptosis detection kit (BestBio, Biotechnology Co., Shanghai, China). After they were incubated with α -pinene for 24 h, the Hela cells were gathered and cleaned by PBS. Cells were mixed with $5 \mu\text{L}$ Annexin V-FITC dyesolution and $5 \mu\text{L}$ PI dye solution away from light for 10min, the condition of cell apoptosis was tested by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

2.6. TUNEL staining

Cell apoptosis was also gauged by TUNEL staining kit (Beyotime, Shanghai, China). Hela cells were inoculated into climbing films and incubated with different concentration of α -pinene for 24h. Then the cells were fixed by 1 mL 4% paraformaldehyde for 30 minutes. Thereafter, the cells were dipped into PBS containing 0.3% TritonX-100. Then the cells of climbing flake were incubated with $50 \mu\text{L}$ of TUNEL detection solution in dark for 60 minutes at 37°C . The fluorescence pictures were observed and captured by fluorescence microscope (Olympus, Tokyo, Japan), then the fluorescence density value was analyzed by Image J software.

2.7. qRT-PCR

The cells were handled according to the previous concentration and action time of α -pinene. Total RNA was isolated from the cells of each group by use of Trizol agent (Invitrogen, USA). According to the operating manual of the reverse transcription kit primescript[™] RT Master Mix reagent Kit (TaKaRa Bio, Beijing, China), the acquired RNA was reverse transcribed to cDNA. TB Green Premix Ex TaqII kit (TaKaRa Bio, Beijing, China) was used to measure relative gene cDNA amplification. The primers were devised by NCBI website and then synthesized by bioengineering Co. (Shanghai, China). The upstream and downstream primer sequences of Bcl-2, Bax, Bid, Caspase-9, caspase-3 and GAPDH are shown in Table 1. The two-step procedure was: 95°C , 5s (thermal denaturation); 60°C , 30s (primer annealing), The Cycle is

repeated for 40 times. As for microRNA detection, PrimeScript™ RT reagent Kit (TaKaRa Bio, Beijing, China). Total RNA was extracted 24 hours after medicine administration. MiR-34a-5p was reverse transcribed by cervical loop method according to PrimeScript™ RT reagent Kit rules (TaKaRa Bio, Beijing, China). The reverse transcription primer sequences were 5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACACAACCAG-3', primers of miR-34a-5p and U6 were also shown in **Table 1**. Expression levels of miR-34a-5p in Hela cells were measured by qPCR. The relative quantity was calculated by means of $2^{-\Delta\Delta Ct}$ method.

Table 1

Primer sequences

Gene name	Up sequence	Down sequence
GADPH	5'-ACAAC TTTGGTATCGTGGAAGG-3'	5'-GCCATCACGCCACAGTTTC-3'
Bid	5'-CCACACCGTGGTCTTTCCA-3'	5'-GCACATCATTGCCAGTGCTC-3'
Bcl-2	5'-TGGTGATGTGAGTCTGGGCT-3'	5'-CTGATTTTATTTGCGCGGCTCC-3'
Bax	5'-TCACTGAAGCGACTGATGTCC-3'	5'-CTTCCAGATGGTGAGTGAGGC-3'
Caspase-3	5'-TGCTATTGTGAGGCGGTTGT-3'	5'-CGCTTCCATGTATGATGGTTCC-3'
Caspase-9	5'-GCTCAGACCAGAGATTCGCA-3'	5'-CTTTCTGCTCGACATCACCAA-3'
miR-34a-5p	5'-TCGGCAGGTGGCAGTGTCTTAG-3'	5'-TATCCAGTGCGTGTCGTG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

2.8. Western-blot

Proteins were extracted from Hela cells of every group through RIPA liquid containing 1% PMSF Beyotime Biotechnology Co., Ltd., Shanghai, China. After the protein concentration was measured by BCA method (Beckman Coulter, Brea, CA, USA). Each denatured protein specimen was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Watford, UK). Then PVDF membrane was blocked by 5% non-fat milk for 1h. According to the operation manual of the primary antibody, the diluted primary antibody (GADPH, Bcl-2, Bax, Bid, Caspase 9, Caspase 3 (Proteintech Group Inc., Wuhan, China) was added to the membrane bearing proteins at 4°C overnight. After it was washed by TBST, the PVDF membrane was incubated with goat anti-rabbit IgG secondary antibodies (1:5000, Proteintech Group Inc., Wuhan, China) for 1h. At last, the membrane was soaked in ECL light-emitting liquid (Meilun Biotechnology Co., Dalian, China) and then checked by Image Lab™ Software.

2.9. miR-34a-5p mimics and inhibitor transfection

MiR-34a-5p mimics (5'-UGGCAGUGUCUUAGCUGGUUGU-3') and miR-34a-5p inhibitor (5'-ACAACCAGCUAAGACACUGCCA-3') were obtained from RiboBio, (Guangzhou, Guangdong, China). According to operation rules of transient transfection, LipofectamineTM 3000 (Invitrogen, Carlsbad, CA, USA) and Opti-MEMTM (Gibco-BRL, Los Angeles, CA, USA) were added to help miR-34a-5p compounds affect cells. The experiment divided into four groups: negative control group (unrelated sequence transfection), miR-34a-5p mimics group, miR-34a-5p inhibitor group, miR-34a-5p mimics+50μmol/L α-pinene group). After 24 hours, total RNA was extracted for qPCR detection. Bcl-2 was looked as the target gene of miR-34a-5p, so combining with the results of previous apoptosis experiments, the expression of Bcl-2 was observed by western blot. Finally, the gray value of the stripe is calculated by ImageJ software, and then the statistical calculation was carried out.

2.10. Animal experiments

Thirty female BALB/C nude mice were obtained from Guangdong Medical Experimental Animal Center. The nude mice were raised in SPF environment. Then 1×10⁷/200μL Hela cells were injected at the right back of nude mice. When the volumes of tumors rose to 100mm³, the mice with tumors were divided into three groups: control group (PBS group), 5-FU group (15μmol/L) and α-pinene group (60μmol/L). The mice of each group were intraperitoneal injected 200μL corresponding drug every two days for 14 days. Immunostaining was performed on deparafnized tissue sections. Each sample was incubated with about 40μL transdermal solution at room temperature for 25 minutes. Subsequently, 40μL TUMEL reaction solution was added to the sample for 60 minutes at 37°C. Finally, the fluorescence photograph was captured by the fluorescence microscope. Furthermore, apoptotic genes detected in our previous study were detected in tumor tissues by immunofluorescence. 40μL goat serum was used to block each tissue section at 37 °C for 10 minutes. After that, every primary antibody were diluted to 1:1000-1:10000 and mixed with 0.2% Triton-100x, and added to each tissue overnight at 4°C. The fluorescent second antibody (dylight594, dylight488, Proteintech Group Inc., Wuhan, China) was diluted 1:100 and added to the tissue in dark for 3 minutes, and immediately washed with PBST. After the appropriate amount of anti fluorescence quenching liquid was covered on the tissue, the fluorescence image was observed by the fluorescence microscope (Olympus, Tokyo, Japan).

2.11. Statistical analysis

Graphpad prism 8.0 software was applied to draw the histogram and analyze the results. All of the data's were expressed as mean ± SD. Student-t test was used to analyze the comparison between the two samples, and one-way ANOVA was used to compare the multiple copies. The difference was significant when $p < 0.05$.

Results

3.1. Product purity and structure are confirmed

This experiment uses gas chromatography and mass spectrometry to analyze the product purity and structure. As shown in (Fig. 1A,) the normalized measured purity product is 91.0% (at 7.297minutes).The sample mass spectrum similarity to the reference spectrum retrieved by the National Institute of Standards and Technology (National Institute of Standards and Technology,NIST) spectrum library is 97%, as shown in (Fig. 1 B-C)

The mass spectrogram (B). mass spectrogram of product (C). mass spectrogram of α -pinene which was retrieved from NIST database.

3.2. α -Pinene controlled the proliferation of Hela cells

The MTT experiment showed that α -Pinene had obvious inhibition on Hela cells at different concentration gradient. And IC_{50} value for α -Pinene was ($46.49 \pm 0.78 \mu\text{mol/L}$ at 24h,) (Fig. 2). Therefore, 24 hours was selected as the administration time, and $25 \mu\text{mol/L}$, $50 \mu\text{mol/L}$ and $100 \mu\text{mol/L}$ were considered as the low, medium and high concentration gradient groups.

3.3. α -Pinene made Hela cells to remain at G1 phase

The cycle distribution in Hela cells was detected by flow cytometry following 24h treatment with α -Pinene at concentrations of $25 \mu\text{mol/L}$, $50 \mu\text{mol/L}$ and $100 \mu\text{mol/L}$. Compared with negative control (Fig. 3A), the proportion of cells in G1 phase increased while the proportion of cells in S phase decreased with increase of concentrations for α -Pinene (Fig. 3B-E). This result displayed that α -Pinene could restrain the proliferation of Hela cells through making the cells to stay at the G1 stage and decreasing the percentage of cells in S stage.

We measured the apoptosis in Hela cells after treatment with α -pinene using AnnexinV-FITC/PI staining and TUNEL fluorescent staining. Treatment with α -pinene increased the proportions of apoptotic Hela cells (Fig. 4A). TUNEL results also showed that in comparison with the control group, the fluorescence intensity of the α -pinene treatment group was significantly enhanced (Fig. 4B). These outcomes demonstrated that α -pinene treatment could lead to apoptosis of Hela cells. In order to further confirm if α -Pinene could influence expression change of apoptosis related genes, the operations of qPCR and western blot were conducted to detect expression levels of five genes (Bcl-2, Bax, Bid, Caspase 9, Caspase 3). In comparison with the control group, the outcomes demonstrated that the expression of Bcl-2 decreased, while the expressions of Bax, Bid, Caspase 9, Caspase 3 increased (Fig. 5).

3.5. α -Pinene regulated miR-34a-5p/Bcl-2 pathway

We further explored the molecular mechanism that α -Pinene induced apoptosis of Hela cells. MicroRNA were important for regulating expression change of apoptosis related genes. Bcl-2 was always considered as the target gene of miR-34a-5p. So we detected the expression of miR-34a-5p following treatment with α -Pinene. The data displayed that miR-34a-5p was high-expressed after Hela cells was treated by α -Pinene (Fig. 6A). After that,we tried to certify α -Pinene could regulate miR-34a-5p/Bcl-2

pathway to induce cells apoptosis. Transfection assay showed that compared with negative group, miR-34a-5p mimics could decrease expression of miR-34a-5p, while miR-34a-5p inhibitor increased the expression of miR-34a-5p, and α -Pinene plus miR-34a-5p mimics could obviously enhance expression of miR-34a-5p (Fig.6B). Meanwhile, compared with negative group, miR-34a-5p mimics could decrease expression of Bcl-2, while miR-34a-5p inhibitor increased the expression of Bcl-2. miR-34a-5p allied with α -Pinene induce obvious down-regulation of Bcl-2 (Fig. 6C, D). In conclusion, α -Pinene affected the expression of miR-34a-5p and Bcl-2, miR-34a-5p could influence expression of Bcl-2, so α -Pinene could regulate miR-34a-5p/Bcl-2 pathway.

3.6. α -pinene inhibited the growth of xenotransplanted tumors

Anti-tumor effect of α -pinene *in vivo* was evaluated by xenotransplanted tumor model of Hela cells. To test phenomenon of cell apoptosis, we proceeded TUNEL staining in tumor tissue. The fluorescence intensity of 5-FU group and α -pinene group was stronger than that of control group (Fig. 7A). TUNEL staining showed that α -pinene could lead to apoptosis activation of tumor cells *in vivo*. The results concurred with the results of cell experiments *in vitro*. The proteins which were associated with cell apoptosis were detected through immunofluorescence method in transplanted tumor tissues. DAPI dye was used to dye the nucleus, and the expressed protein was stained green or red fluorescence. Fluorescence analysis showed exhibited lower expression of Bcl-2 in both 5-FU treatment group and α -pinene treatment group than the control group. In contrast, the wider expression of Bax, Bid, Caspase 9, Caspase 3 exhibited in 5-FU treatment group and α -pinene treatment group (Fig. 7B-F). The results of *in vivo* experiments were consistent with those of *in vitro*. These data further proved that α -pinene can induce apoptosis of HeLa cells by promoting cell apoptosis.

Discussion

Cervical cancer was one of the most prevalent gynecological malignancies, 85% cervical cancer cases appeared in developing countries [19]. Chemotherapy was the common treatment method for cervical cancer. Nowadays, more and more chemosynthetic antitumor drugs had severe toxicity and drug resistance [20]. So lots of natural anticancer drugs including Vincristin, Paclitaxel, Camptothecin were abstracted from plants and displayed effective anti-tumor activity [21-23]. α -pinene was separated and distilled from pine needles. In our previous studies, we found α -pinene inhibit the growth of hepatocellular cancer cells [24]. α -pinene also could showed the ability of promoting the apoptosis of prostate carcinoma cells [10]. In order to verify pinene was a broad spectrum anti-cancer drug, we evaluated the function of α -pinene in Hela cells, a cervical cell line.

As a result, cytotoxicity test *in vivo* showed that with the improvement of drug concentration, the growth of Hela cells and formed tumors were inhibited. We deeply found that α -pinene could restrain growth of Hela cells depending on affecting the distribution of cell cycle and regulating cell apoptosis. There were increased proportions of cells in G1 phase of the cell cycle after being treated with α -pinene, which suggested that α -pinene could make Hela cells to remain in G1 phase and affect the synthesis of DNA.

Apoptosis played an important role in controlling tumor progress, but apoptosis was often inhibited in cancer, so activating apoptosis was the main target of drug treatment in cancer. In this study, AnnexinV-FITC/PI staining and TUNEL fluorescent staining indicated α -pinene promoted cell apoptosis in a concentration dependent manner. And all the results of qPCR data, the western-blot and immunofluorescence staining in tumor tissues showed that the expression of Bax, Bid, caspase-9 and caspase-3 were up-regulated and the expression of Bcl-2 was down regulated after α -pinene administration. The expression change of apoptosis related proteins can fully explain the effect of α -pinene induced apoptosis through mitochondrial mediated endogenous apoptosis pathway. In this way, the content of intracellular Bid in cytoplasm increased, which promoted the expression of Bax on mitochondrial membrane and repressed the expression of Bcl-2. Finally, Caspase-9 and Caspase-3 was activated, which always led to apoptosis.

The abnormal expression of microRNAs in tumor tissues was closely related to tumor proliferation, apoptosis and metastasis. In the development of cervical cancer, miR-34a-5p could also hold up progress of cancer cells [17]. MiR-34a-5p was looked as one of the prognostic factors of cervical cancer patients [16]. We confirmed that α -Pinene increase expression of miR-34a-5p, and α -Pinene plus miR-34a-5p mimics could obviously enhance expression of miR-34a-5p. The result revealed α -Pinene could regulate miR-34a-5p. Bcl-2 was an important anti-apoptotic protein, which was proved to being regulated by miR-34a-5p in cancers [25]. In our experiments, when miR-34a-5p mimics was transfected the Hela cells, the expression standard of Bcl-2 reduced. On contrary, miR-34a-5p inhibitor could raise the expression standard of Bcl-2. These results proved that the controlling relationship between miR-34a-5p and Bcl-2. In addition, we also found that after α -pinene treatment, the expression of Bcl-2 was down-regulated. Meanwhile, our experimental information showed that in comparison with miR-34a-5p transfection group lonely, the expression of Bcl-2 protein was declined obviously after being transfected with miR-34a-5p plus α -pinene. We could speculate α -pinene induced Hela cell apoptosis by controlling miR-34a-5p/Bcl-2 pathway.

In conclusion, we found that α -pinene could inhibit the development of Hela cells both *in vitro* and *in vivo*. α -pinene played an anti-tumor role by causing cell cycle to stagnate in G1 phase and promoting apoptosis in Hela cells, along with up-regulating expression of Bax, Bid, Caspase-9, Caspase-3 and down-regulating expression of Bcl-2. Furthermore, α -pinene could down-regulate the expression level of Bcl-2 by regulating miR-34a-5p. These results confirmed α -pinene could exert anti-tumor efficacy in Hela cells, which provide theoretically strong for treating cervical cancer with natural plant extracting compound.

Declarations

Author Contributions

Huang Xiaosu: Conceptualization Methodology, Data curation, Writing-Original draft preparation, Writing-Reviewing and Editing **Yan pei:** Methodology, Data curation, Writing-Original draft preparation **Zhou**

chang: Conceptualization, Software. **Xu qiuxiang:** Visualization. **Li ming:** Software, Validation. **Ye lianbao:** Supervision. **Ding wenqing:** Validation. **Chen weiqiang:** Funding acquisition.

Conflicts of Interest

All authors have no conflict of interest in the publication of this manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Experimental Animal Ethics Review Committee of Guangdong Pharmaceutical University (gdpulac2019074)

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Figures

Figure 1

(A). gas chromatography of product.

Figure 2

Survival rate of Hela cells was detected from 24h, after treatment with α -pinene using the MTT array.

Figure 3

Cell cycle detection revealed an increase in G0/G1 phase of HeLa cells. A the negative control group; B 5-Fu control group; C α -pinene low concentration group; D α -pinene medium concentration group; E α -pinene high concentration group. F Statistical analysis graph of Cell cycle distribution. In comparison with the negative control group, * P < 0.05; ** P < 0.01. 3.4. α -Pinene induced apoptosis of Hela cells

Figure 4

α-pinene promoted the apoptosis of Hela cells. (A) Apoptotic cells were checked by Annexin V-FITC/PI apoptosis detection kit after treatment with α-pinene for 24h. a: the negative control group; b: 5-Fu control group; c: α-pinene low concentration group; d: α-pinene medium concentration group; e: α-pinene high concentration group. In comparison with the negative control group, *P < 0.05; **P < 0.01. (B) Apoptotic cells were also stained with TUNEL test kit, and cells with green fluorescence were observed by fluorescence microscopy. *P < 0.05, **P < 0.01.

Figure 5

α -pinene influenced the expression of apoptosis related genes in HeLa cells. The effect of α -pinene on expression of apoptosis related genes were detected by qRT-PCR (A) and Western blot (B) in HeLa cells. a: the negative control group; b: 5-Fu control group; c: α -pinene low concentration group; d: α -pinene medium concentration group; e: α -pinene high concentration group. In comparison with the negative control group, * $P < 0.05$, ** $P < 0.01$.

Figure 6

α -Pinene regulated miR-34a and Bcl-2 expression. (A) The influence of α -pinene on the expression of miR-34a-5p in Hela cells was evaluated using qRT-PCR. (B) The expression of miR-34a-5p was determined after miR-34a-5p mimics and inhibitor transfection. (C) The effect of miR-34a-5p mimics and inhibitor on Bcl-2 gene expression determined by qRT-PCR. (D) Protein expression of Bcl-2 was determined after miR-34a-5p mimics (b), miR-34a-5p mimics with α -pinene (c) and miR-34a-5p inhibitor transfection (d). In comparison with the negative control group (a), ** $P < 0.01$, **** $P < 0.001$.

Figure 7

α-pinene promoted the apoptosis of HeLa cells in vivo. (A) TUNEL staining was applied to test the affect of α-pinene on apoptosis of tumor cells. green: TUNEL- positive cells; blue; DAPI. (B-F) The expression of Bcl-2, Bax, Bid, Caspase 9, Caspase 3 protein was detected by immunofluorescence staining after α-pinene treatment. green: Bcl-2, Bid, Caspase 3; red: Bax, Caspase 9; blue: DAPI. (original magnification ×100)

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