α-Pinene inhibits the growth of cervical cancer cells through its proapoptotic activity by regulating the miR-34a-5p/Bcl-2 signaling axis

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Abstract
Among gynecological tumors, cervical cancer (CC) has the second-highest prevalence and mortality rate. α-Pinene is a bicyclic monoterpene compound extracted from pine needles that carried promising anticancer properties. Nevertheless, its effect on CC and the underlying mechanism has not yet been elucidated. Therefore, we investigated the effect of α-Pinene on apoptosis in CC via in vitro assays of flow cytometry (FCW), terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay, quantitative real-time polymerase chain reaction (qRT-PCR), and Western blot. Following that, we detected the proapoptotic function of α-Pinene on HeLa cells in vivo by TUNEL assay and immunofluorescence staining. Our results displayed that the α-Pinene inhibited the growth of HeLa cells and stalled the cells in the G0/G1 phase. Interestingly, we also detected that α-Pinene induced HeLa cells to apoptosis. The results investigated that α-Pinene induced HeLa cells apoptosis along with up-regulating the expression of Bax, Bid, caspase-9, caspase-3, miR-34a-5p, and down-regulating the expression of Bcl-2 in vitro. At the same time, the expression levels of target genes in vivo were consistent with those in vitro. Our experiment proved that α-Pinene promoted apoptosis, which will be used to hopefully maximize the therapeutic strategies in clinical studies in CC.

KEYWORDS
α-Pinene, apoptosis, HeLa cells
INTRODUCTION

Cervical cancer (CC) is the second most common cancer among females worldwide, with a disproportionately high burden in low/middle-income countries of the world. Although with the rapid development of the human papillomavirus (HPV) vaccine, it was seen that the incidence and mortality of CC did not decrease and it was still regarded as a severe public health problem. Moreover, rates of HPV vaccination have not been popularized in less developed countries, resulting in missed or delayed vaccinations of most females—Xia et al. (2019). It was estimated that the mortality of CC in less-developed regions was still 13.2 per 100,000 women and resulted in nearly 604,127 new cases and 341,831 deaths globally recorded annually (Hoque et al., 2021). Unfortunately, the incidence and mortality rates are increasing year by year and the population is getting younger (Pistritto et al., 2016). Notwithstanding these therapeutic and preventive measures of the HPV vaccine, surgery, radiotherapy, and chemotherapy, women’s health continues to be jeopardized by the ongoing burden of the disease. Chemotherapy is used primarily to prolong the life expectancy of patients, including recurrence and metastatic disease. However, its resistance severely compromises treatment causing relapse and metastases for CC patients. Meanwhile, the drugs for CC including 5-FU, cisplatin, paclitaxel, and platinum are expensive and have several negative effects and high recurrence rates. For the reasons stated above, there is consequently an urgent need for seeking new therapeutic drugs with effective, cheap, and fewer side effects for CC.

Nowadays, a substantial body of evidence suggests that natural materials may be a good source for the development of a new generation of anticancer drugs. Natural compounds have the ability to include anticancer capacity, the potential to overcome drug resistance, and safety. α-Pinene, extracted from pine needles, possessed powerful anticancer and anti-inflammation activities (Chen, Liu, et al., 2015; X. J. Li et al., 2016). We and some previous mechanistic investigations demonstrated that α-Pinene was able to block the cell cycle in cancers (Chen, Liu, et al., 2015; Chen, Solomides, et al., 2015; Zhao et al., 2018). Recently, interest in exploring the anticancer mechanisms of α-Pinene appears to be rising. Nevertheless, to date, little is known about the effect and mechanism by which α-Pinene regulates CC.

To the best of our knowledge, miRNAs can accelerate the degradation and/or block the translation of their target genes, induce posttranscriptional gene expression, and thus engage in the regulation of various biological processes such as cell proliferation, metabolism, and apoptosis (J. Xu et al., 2012), as well as in tumor cell development. miR-34a-5 is one of the important miRNAs. Several studies have shown that upregulation of miR-34a-5 inhibits cell proliferation and promotes apoptosis (Hu et al., 2021; E. Ma et al., 2020; Xiao et al., 2020). There was however, it remains unclear whether α-Pinene has a regulatory effect on miR-34a-5 in HeLa cells.

Apoptosis is generally regarded as one of the prominent signal pathways that triggered cancer cell death, and it played a pivotal part in both drug sensitization and drug resistance in cancer Zong et al. (2019). Apoptosis also could be triggered in cancer cells through death receptors and mitochondrial pathways, as well as caspase-mediated extrinsic or intrinsic pathways, and so on. The Bcl-2 family proteins, pivotal regulators of mitochondria-dependent apoptosis, have been sorted as pro-and antiapoptotic factors based on their different features (Z. W. Ma & Liu, 2018). Bcl-system, which includes oncoproteins affecting proapoptotic (Bax, Bid, Bim, Bak) and antiapoptotic (Bcl-2, Bcl-xL, Ral, McI-1) is a key factor in the regulation of the apoptotic processes Pal'tsev et al. (2000). A mounting number of investigations demonstrated that the Bcl-2 signaling pathway exerts a considerable contribution to cell proliferation and apoptosis (Ashkenazi et al., 2017; Berrak et al., 2016). Moreover, Bcl-2 is an antiapoptotic gene and its downregulation favors the reduction of tumor cell resistance to radiotherapy and the induction of apoptosis. The Bax gene belongs to the same family as the Bcl-2 gene and shares 21% of the amino acid sequence with Bcl-2. The Bax gene not only accelerates apoptosis directly but also antagonizes to some extent the function of the Bcl-2 gene in inhibiting apoptosis Gahl et al. (2016). Bid proteins are relevant in checking mitosis and maintaining genomic stability, among other things, and are characterized by enhanced apoptosis Yin (2000). Thus, our study explored how the expression of proapoptotic proteins (e.g., Bax and Bid), and antiapoptotic proteins (e.g., Bcl-2) in HeLa cells after α-Pinene treatment. Caspases 3, 6, 8, 9, 10, and so on are among the 14 caspases involved in cell apoptosis (Pistritto et al., 2016). Caspase-3 is a downstream apoptosis execution factor in the caspase protein family, and its elevated activity induces irreversible apoptosis (Huang et al., 2011). Caspase-9 is an important indicator of the mitochondrial pathway that induces apoptosis. When activated, caspase-9 induces apoptosis by initiating the downstream caspase-3 pathway (Wei et al., 2020). Consequently, we also examined whether the caspase family (caspase 9, 3) was affected in HeLa cells after treatment with α-Pinene.

Our discoveries exhibited that α-Pinene induced apoptosis in CC by activating death signals including miR-34a-5p, Bcl-2 family members, and caspases in vitro and in vivo, which could provide theoretical support for the fight against CC with α-Pinene and thus enhance patient survival. This study will provide greater insight into the causes of α-Pinene-induced HeLa cell apoptosis.

MATERIALS AND METHODS

2.1 Cell culture and cell treatment

Human cervical cancer HeLa cells were acquired from Suyan Biotechnology and cultured in RPMI-1640 medium (Gibco-BRL) mixed with 10% fetal bovine serum, and 1% streptomycin/penicillin (Solarbio Corp.). The cells were cultured in a constant-temperature incubator with 5% CO₂ at 37°C.

2.2 Cell viability

The cell viability of the HeLa cells was assessed using the methyl thiazolyl tetrazolium (MTT) assay. 5-FU is widely used in the
treatment of cervical cancer, and was known to downregulate the Bcl-2 family and induce caspases (Yim et al., 2004), so we used it as a positive control and set the concentration at 50 μmol/L according to Yi et al. (2020). For drug treatment experiments, the HeLa cells (4 × 10^3 cells/well) were firstly seeded into 96-well plates according to the report by P. Zhang et al. (2019), then treated with α-Pinene (10, 20, 40, 80, 160, 320, 640 μmol/L) according to our previous studies of Q. Xu et al. (2018) and Ye et al. (2020) for 24 h. After that, an MTT assay was conducted. Finally, the automated microplate reader (Sunrise) was utilized to detect the absorbance at 570 nm.

2.3 Cell cycle analysis

For the cell cycle, HeLa cells were harvested by P. Zhang et al. (2019) and fixed in 75% ethanol overnight at 4°C, then resuspended in precooling phosphate-buffered saline (PBS) containing 10 μl RNase and 0.02 mg/ml propidium iodide (PI) for 30 min at 37°C. At last, stained cells were detected by flow cytometer (Beckman), and data analysis was performed with ModFit LT™ software (Beckman).

2.4 Cell apoptosis assay

Cell apoptosis was detected via Annexin V-FITC/PI Apoptosis Detection kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, the HeLa cells treated with α-Pinene were harvested and incubated in a solution mixed with 5 μl/ml Annexin V dye solution and 5 μl/ml PI dye solution at 4°C in the dark for 10 min. Immediately cell apoptosis was detected via flow cytometer (Beckman). Flowjo software (Beckman) was applied to analyze apoptotic cells.

2.5 Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay

TUNEL staining kit (Beyotime) was used to verify the α-Pinene induced apoptosis in HeLa cells (1 × 10^5 cells/well) following the manufacturer’s protocol and previously (Ye et al., 2020). The main steps are as follows, after 24 h incubation, the attached cells were fixed with 4% paraformaldehyde for 30 min. Next, cells were stained with 50 μl of TUNEL detection solution and incubated for 1 h at 37°C. Finally, the labeled cells were observed with a fluorescence microscope (Olympus; IX53), and the fluorescence density value was processed with Image J software.

2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was used to detect the messenger RNA expression of apoptosis-related genes. Total RNA was extracted from cells with Trizol reagent (Invitrogen). Then, qRT-PCR was performed using a sequence detection system with site-specific primers. The primer sequences were presented in (Table 1). qRT-PCR amplification was performed on a CFX96 real-time PCR detection system (Bio-Rad). Samples were normalized to the control and ploidy changes were calculated according to equation 2^−ΔΔCT.

2.7 Western blot analysis assay

Furthermore, the protein expression of the apoptosis-related molecules was detected by Western blot. HeLa cells (1 × 10^6 cells/well) were treated with 25, 50, and 100 μmol/L of α-Pinene for 24 h. The total cellular proteins were isolated using radio-immunoprecipitation assay buffer (Sigma-Aldrich) containing protease inhibitors (Roche). The protein concentration was detected using the bicinchoninic acid assay protein assay kit (Beyotime Biotechnology). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were blotted onto a polyvinylidene fluoride membrane (Millipore), the membrane was washed with tris buffered saline with tween (TBST, Millipore), then incubated with primary antibodies at 4°C overnight, washed three times with TBST for 15 min, and finally incubated with anti-rabbit/mouse immunoglobulin G antibodies (110,000 dilution) in 5% BSA buffer for 1 h at room temperature. The band intensity was visualized via ECL light-emitting liquid (Meilun Biotechnology Co.) and Image Lab™ Software (Bio-Rad).

| Gene name | Up sequence | Down sequence |
|-----------|-------------|---------------|
| GAPDH     | 5′-ACACATTTTGGTATCGTGGAAG-3′ | 5′-GCCATCACCAGCCACAGTTTC-3′ |
| Bid       | 5′-CCACACCGTGCTCTTCCA-3′ | 5′-GCACATCATTGCCAGTGCTC-3′ |
| Bcl-2     | 5′-TGGTGTATGAGTCTGGGCT-3′ | 5′-CTGATTTTATTTCGCCGGCTCC-3′ |
| Bax       | 5′-TCATCTAGGCGACTGAAGTCC-3′ | 5′-CTTTCCAGATGTTGAGTAGGCC-3′ |
| Caspase-3 | 5′-TGCTATTGTGAGGCCTTGT-3′ | 5′-CGTTCCATGTATGAGTTGCC-3′ |
| Caspase-9 miR-34a-5p U6 | 5′-GCTGAGCCAGACTGAGC-3′ | 5′-CTTTCTGCTGACATACCAAA-3′ |
|           | 5′-TCGGCAGTGGCAGTGCTTAG-3′ | 5′-TATCCAGTGCCTGTCG-3′ |
|           | 5′-CTCGCTTCGCAGCACA-3′ | 5′-AACGCTTTGCAATTTGCCG-3′ |

TABLE 1 Primer sequences
2.8 | miR-34a-5p mimics and inhibitor transfection

miR-34a-5p mimics, inhibitors, and control were synthesized by Ruibo Biotechnology. HeLa cells (1 x 10^6 cells/well) in the logarithmic growth phase were cultured in six-well plates and cell transfection was performed when the confluent of cells reached 80%. Subsequently, miR-34a-5p mimics and inhibitors were transfected into HeLa cells by lipofectamine 3000™ (Invitrogen) following manufacturer protocol. After transfection of 24 h, cells were treated with α-Pinene, and qRT-PCR and Western blot assay were employed to detect the related molecular expression.

2.9 | Animal experiments

To determine the effect of α-Pinene in vivo, a xenograft nude mouse model was constructed. Experiments were carried out on female BALB/c nude mice (n = 30) weighing 20–22 g obtained from Guangdong Medical Experimental Animal Center. All animal use and operation procedures were approved by the Animal Care and Use Committee of Guangdong Pharmaceutical University (approval No. gdpulac2019055, Guangdong, China). HeLa cells (1.5 x 10^7 cells/well) were inoculated subcutaneously on the lower right back of each nude mouse. When the tumor volume approximately reached 0.2–0.3 cm³, the mice were arbitrarily divided into the control group (PBS group), 5-FU group (2 mg/ml), and α-Pinene group (2 mg/ml, n = 10 for each group). The concentration of 5-FU (20 mg/kg) was determined according to the report of Kavitha et al. (2021) and combined with the pre-experiment, which was converted from 20 to 2 mg/ml (200 μl) based on body weight of mice (20 ± 2 g). Similarly, we used the same approach of exploring 5-FU concentration and determined the effective concentration of α-Pinene (2 mg/ml, 200 μl) based on the published studies of Zhao et al. (2018) and Matsuo et al. (2011). The 5-FU and α-Pinene were intraperitoneally injected for 14 days.

On day 14, all the mice were killed and the collected tumors were taken for further experiments. Tumor volume was measured with calipers to calculate it using the following formula, \( V = \frac{a \times b^2}{2} \), where a and b represent the large and small diameters, respectively (Yang et al., 2019). TUNEL assay was to detect apoptotic xenogeneic tumor cells according to the manufacturer’s instructions and previously (Ye et al., 2020). Correspondingly, the proteins associated with cell apoptosis were detected through the immunofluorescence staining in tumor tissues.

2.10 | Statistical analysis

All the experiments were performed in triplicate. The data were presented as the mean ± standard deviation (SD). Differences between groups were evaluated by one-way analysis of variance. Statistical significance was defined as \( p < .05 \). The analyses were performed using GraphPad Prism 8.0 statistical analyses.

3 | RESULTS

3.1 | Effects of α-Pinene on cell viability

Preliminary, the MTT experiment (Figure 1) clearly showed that α-Pinene inhibited the proliferation of HeLa cells s with IC_{50} esteem at 46.87 ± 0.78 μmol/L, so we elucidated the impacts of treatment with α-Pinene (25, 50, 100 μmol/L) on the following studies.

3.2 | α-Pinene locked HeLa cells in the G0/G1 phase

The cycle distribution of HeLa cells was determined by flow cytometry following 24 h treatment with α-Pinene at concentrations (25, 50, 100 μmol/L). As shown in Figure 2, compared with negative control, the proportion of cells in the G0/G1 phase increases while the proportion of cells in the S phase decreases with the increase of concentrations for α-Pinene (Figure 2a). It was shown that the treatment of α-Pinene resulted in a higher percentage of G0/G1 cells and the number of cells was increased in a dose-dependent manner (Figure 2b).

3.3 | α-Pinene promoted apoptosis in HeLa cells

As shown in Figure 3, Annexin V/PI double staining assay results revealed that the proportion of apoptotic cells increased in α-Pinene-treated HeLa cells. After treatment for 24 h, the proportion of apoptotic cells increased from 5.29 ± 0.98% in the control to 33.04 ± 1.22%, 39.92 ± 1.49%, and 59.65 ± 2.3% in HeLa cells treated with 25, 50, and 100 μmol/L α-Pinene.

3.4 | α-Pinene markedly induced apoptosis in HeLa cells

The TUNEL assay was conducted to further confirm the effects of 24 h treatment with α-Pinene (25, 50, and 100 μmol/L) on apoptosis in HeLa cells.
cells. As shown in Figure 4a, TUNEL assay results illustrated that α-Pinene treatment increased the proportion of apoptotic cells. Interestingly, as illustrated in Figure 4b, the TUNEL assay results also demonstrated that increased the proportion of apoptotic cells increased in a concentration-dependent manner under α-Pinene administration. Hence, consistent with Annexin V-FITC/PI Apoptosis assay results, these findings demonstrated that α-Pinene promoted apoptosis in HeLa cells.

### 3.5 α-Pinene influenced the expression of apoptosis-related genes

The expression levels of five genes (Bcl-2, Bax, Bid, Caspase-9, and Caspase-3) were examined by quantitative polymerase chain reaction (qPCR) and Western blot to confirm whether α-Pinene could affect the expression changes of apoptosis-related genes. As shown in Figure 5a,
the qRT-PCR results showed that α-Pinene decreased the expression level of Bcl2, while the gene expression level of Bax, Bid, caspase-9, and caspase-3 in HeLa cells was increased. Moreover, the result of the Western blot was consistent with the qRT-PCR (Figure 5b,c).

3.6 | α-Pinene regulated the miR-34a-5p/Bcl-2 pathway

The data revealed that miR-34a-5p was highly expressed in HeLa cells after being treated with α-Pinene (Figure 6a). Moreover, we further predict the relationship between miR-34a-5p and Bcl-2 with online software. The results of bioinformatics analysis used the prediction tools miRBase (http://www.mirbase.org/) and TargetScan (http://www.targetscan.org/vert72/) showed that Bcl-2 harbored putative binding sites of miR-34a-5p, which indicated Bcl-2 as a target gene for miR-34a-5p. The qRT-PCR results indicated that the miR34a-5p was downregulated after intracellular transfection with the miR-34a-5p inhibitor, and correspondingly, the expression of Bcl-2 was increased (Figure 6b,c). In contrast, in the intracellular transfection with miR-34a-5p mimic, the expression of miR-34a-5p was upregulated, and accordingly, the expression of Bcl-2 was decreased (Figure 6d,e), which confirmed that Bcl-2 was a downstream target protein of miR-34a-5p. From these results, it could be summarized that α-Pinene regulated the miR-34a-5p/Bcl-2 pathway.

3.7 | α-Pinene induced HeLa cells apoptosis in vivo

The growth of xenogeneic tumors was inhibited after treatment (Table 2). The difference in tumor weight between groups was not statistically significant before treatment, whereas the tumor weight of α-Pinene and 5-FU groups was significantly smaller than that of the PBS group after treatment (Table 2). Additionally, compared to the PBS group, the treatment with α-Pinene and 5-FU showed a significant decrease in tumor volume on Day 14. Briefly, α-Pinene inhibited tumor growth, which was reflected in the reduction in tumor weight and volume. To elucidate the phenomenon of cell apoptosis in vivo, we proceeded with TUNEL staining in tumor tissue. Notably, the fluorescence intensity of the 5-FU group and the α-Pinene group was stronger than that of the control group (Figure 7a). It indicated that α-Pinene led to apoptosis of HeLa cells in vivo. This was consistent with the in vitro result, including Annexin V-FITC/PI Apoptosis and TUNEL assay. It was noteworthy that the expression levels of Bcl-2 in both the 5-FU group and α-Pinene group were significantly lower than those in the control group (Figure 7b). Conversely, the expression level of Bax, Bid, caspase-9, and caspase-3 in both the 5-FU group and α-Pinene group were significantly higher than those in the control group, which revealed their roles in α-Pinene induced HeLa cells apoptosis (Figure 7c–f).

4 | DISCUSSION

CC is a great threat to women’s health worldwide and more than 30% of patients develop recurrence or metastasis after treatment worldwide. In China, the 5-year survival rate is a mere 36.9% (Saleh et al., 2020). Chemotherapy resistance is the most serious obstacle to cancer treatment which is also the most frequent factor leading to clinical failure in cancer therapy Zheng (2017). Therefore, it is essential to explore potential curative agents for CC to create new therapeutic tactics. Here, we investigated the effect of α-Pinene on
CC and its basic mechanistic aspects. Our study revealed that α-pinenne increased CC apoptosis by regulating the miR-34a-5p and Bcl-2 mediated apoptosis pathway.

α-Pinenne is a type of monoterpane that has been previously studied in several cancers. α-Pinenne is effective in the treatment of experimental metastatic melanoma, where it reduces tumor nodules in the lungs Matsuo et al. (2011). The combination of α-Pinenne and β-Pinenne can reduce serious side effects by reducing the dose of paclitaxel without affecting its effect on non-small cell lung cancer Z. Zhang et al. (2015). α-Pinenne, the main constituent of the essential oil, has been reported that stimulates the immune system and induce apoptosis in melanoma cancer (Hakkim et al., 2019). Our previous reports have indicated that α-Pinenne inhibited the propagation of HepG2 cells (Chen, Liu, et al., 2015). It was also shown that α-Pinenne enhances the anticancer activity of natural killer cells Jo et al. (2021). The present study suggested that α-Pinenne possesses G0/G1 cell cycle arrest on the HeLa cells line, which is different from other previous studies on cell cycle arrest in vitro. Based on our findings and by other investigators, it can be stated that α-Pinenne may be a good candidate as a potential anticancer natural drug or herbal medicine.

Of note, apoptosis is a process that regulates cell death and antitumor drugs acted principally through the induction of apoptosis in cancer cells (X. Xu et al., 2019). In the present study, results from Annexin V/PI double staining and TUNEL assay demonstrated that α-Pinenne induced apoptosis in HeLa cells in a dose-dependent manner. The apoptosis-promoting Bcl-2 family triggered mitochondria apoptosis following its interaction with mitochondria (Mortezaee et al., 2019). Bax, Bid, and Bcl-2 are activated when apoptosis happens (Tang et al., 2021). Typically, the content of intracellular Bid in the cytoplasm increased, which upregulated the Bax on the mitochondrial membrane and downregulated the Bcl-2 Michurina et al. (2019). Caspase-9 is known to be activated to form apoptotic bodies, which further activated caspase-3, a widely distributed effector molecule in the process of apoptotic cell death. In this study, we continued to analyze the molecular mechanism of

**Figure 5** α-Pinenne promoted the apoptosis of HeLa cells. (a) The effect of α-Pinenne on the gene levels of Bcl, Bax, Bid, caspase-9, and caspase-3 was detected by quantitative real-time polymerase chain reaction. (b) Protein levels of Bcl, Bax, Bid, caspase-9, and cleaved caspase-3 were determined by Western blot analysis. (c) The band intensities of Bcl, Bax, Bid, caspase-9, and cleaved caspase-3 protein were quantified by Image J software and normalized to the band intensity of nicotinamide adenine dinucleotide phosphate. Data and graphs were presented as mean ± standard deviation, n = 3. Significantly different at: *p < .05; **p < .01.
apoptosis by fluorescence qPCR, the results showed that the expression of Bid, Bax, caspase-3, and caspase-9 was upregulated and Bcl-2 was downregulated after α-Pinene administration. More importantly, caspase-3 is cleaved only at the time of apoptosis, cleaved caspase-3 is only present in cells that have undergone apoptosis and are undetectable in cells that have not undergone apoptosis or have become necrotic. Interestingly, the WB assay showed that after α-Pinene treatment, the expression of Bid, Bax, cleaved caspase-3, and caspase-9 increased, and Bcl-2 decreased. In vivo assays, the TUNEL fluorescence staining analysis of tumor tissues revealed that more apoptotic cells were found in the tumor tissues of the α-Pinene administration group than in the negative control group. Finally, immunofluorescence staining analysis revealed that α-Pinene could downregulate the expression of Bcl-2 protein and upregulate the expression of Bid, Bax, caspase-9, and caspase-3 protein in tumor tissues. Similar to the results from in vitro studies, in vivo results also suggested that α-Pinene triggered apoptosis by inhibiting the antiapoptotic factor Bcl-2 and activating the proapoptotic factors Bax, Bid, caspase-3, and caspase-9.

Numerous studies indicated that noncoding RNAs could act as a crucial part of the pathogenesis of multifarious cancers, including CC (Boija et al., 2021; Pan et al., 2021). For instance, miR-34a-5p mediated CC growth, migration, and other malignant behaviors through the regulation of CDC25A (Jiang & Cheng, 2021). Meanwhile, Wang et al. (2021) suggested that the lncRNA MEF2C-miR-592-RSPO1 axis was a potential pathway for CC treatment. Currently, miR-34a-5p has been shown to have important biological functions in tumors where it is often upregulated. It has been found that the expression of miR-34a is relatively lower in tumor tissues than in normal tissues B. Li et al. (2010). miR-34a-5p can also exert oncogenic effects by inhibiting the proliferation, activity, and invasion of cancer cells (Lv et al., 2019). Our study (Figure 6a) showed the expression of miR-34a-5p in HeLa cells was increased after 24 h of α-Pinene administration compared to the control group by qPCR, which suggested that α-Pinene may promote HeLa cells apoptosis through miR-34a-5p. In other words, exploring the function of miR-34a-5p in the regulatory control of HeLa cells will
Contribute to the development of effective drugs for CC treatment. Intriguingly, miR-34a-5p had previously been demonstrated to downregulate Bcl-2 (Qu et al., 2021). In this study, we used bioinformatics to confirm that Bcl-2 is the downstream target gene of miR-34a-5p. In cell transfection experiments, on one hand, the expression of miR-34a-5p was downregulated upon transfection of miR-34a-5p inhibitor, and correspondingly, the expression of Bcl-2 increased. On the other hand, intracellular transfection of miR-34a-5p mimics resulted in upregulation of miR-34a-5p expression and downregulation of Bcl-2 expression. Based on the results mentioned above, Bcl-2 is a target molecule of miR-34a-5p, and α-Pinene induced apoptosis in HeLa cells via the miR-34a-5p/Bcl-2 pathway.

To summarize, α-Pinene was a natural extract that is characterized by its low price, abundant sources, and small adverse effects. Our study demonstrated that α-Pinene induced cell cycle arrest and apoptosis in CC, which provided the theoretical foundation for treating CC with natural plant extract. Our finding highlighted the requirement of an apoptotic pathway to achieve induced cell death, a high concordance between observed in vitro and in vivo after treatment with α-Pinene.

5 CONCLUSION

Collectively, the current study provided evidence that α-Pinene induced CC apoptosis through activation of the miR-34a-5p/Bcl-2-dependent pathway both in vitro and in vivo. Accordingly, α-Pinene may be an effective therapy for CC. Nevertheless, the pharmacokinetics of α-Pinene in CC remains unclear so far, more specific work and in-depth discussion employing additional cellular and in vivo animal models or clinical experiments will be necessary to validate the potential of α-Pinene as a useful drug for CC treatment.

AUTHOR CONTRIBUTIONS

Weiqiang Chen: Designed study; funding acquisition; supervision. Chang Zhou: Conceptualization. Xiaosu Huang and Pei Yan: Formal analysis and investigation; writing – original draft preparation; writing – review and editing. Qiuxiang Xu: Formal analysis and investigation; writing – original draft preparation. Ming Li: Formal analysis and investigation. Lianbao Ye: Formal analysis and investigation. Wenqing Ding: Funding acquisition. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the author. Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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