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Pinene Promotes Liver Cancer Cell Apoptosis by Regulating the Bcl-2/Bax/Caspase-3 Pathway

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Abstract:

This study investigated whether pinene promotes apoptosis of liver cancer cells by regulating the Bcl-2/Bax/Caspase-3 signaling pathway. In this study, the expression levels of Bcl-2/Bax/caspase-3 protein are detected by Western Blot, and this study adopts Annexin V-FITC/PI FACS to measure the cell apoptosis level and adopts vernier caliper to measure the xenograft tumor size. The paper tries to prove that pinene could enhance liver cancer cell apoptosis by up-regulating the expression level of Bax protein, down-regulating the expression level of Bcl-2 protein, and inducing the cleavage of caspase-3. This study will fill the research gap on the pinene's curative effect on liver cancer and provide significant insight into whether people could invent new drugs that treat liver cancer effectively with pinene extracted from Artemisia argyi.

Keywords: Pinene; Liver cancer; Apoptosis; Artemisia argyi; Bcl-2/Bax/caspase-3 pathway

1. Introduction

1.1 Liver Cancer

It is known to all that cancer is a major health issue globally nowadays, and liver cancer is one of the most dangerous malignancies to human life and health. The World Health Organization's International Agency for Research on Cancer (IARC) has released data on the global cancer burden for 2020. The data indicated that there are more than 900,000 diagnosed patients and more than 830,000 deaths worldwide [1]. The death rate is over 91.66%. The global incidence of liver cancer fifth and the mortality is third among all cancers. Finding effective methods or drugs to treat liver cancer is necessary.

1.2 Artemisia argyi

Artemisia argyi, a traditional Chinese herb, has long been used by Chinese people to treat liver-related diseases [2]. What's more, there is clinical research showing that injections of folium artemisiae argyi extract into the human body can relieve liver function gradually [3]. However, from the point of view of modern medicine, whether artemisia argyi extracts have the effect of treating liver-related diseases or even liver cancer is still unknown.

1.3 Existing Relevant Research

Hopefully, a relevant study found that artemisia argyi

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extract, which doesn't clearly clarify which component, exerts a modulatory inhibitory influence on the proliferation of liver cancer cells by curtailing the manifestation of the Bcl-2 gene and enhancing the expression of Bax and Caspase-3 genes [4]. There is also relevant research which found that Eupatilin, an extract from artemisia argyi , can induce effectively the apoptosis of hepatocellular carcinoma cell(HCC) and inhibit their proliferation by down-regulating the apoptosis-inhibiting protein Bcl-2, proliferation-related protein Topo2α, up-regulating the pro-apoptotic proteins p53 and Caspase-3, and activating the IRE1/JNK/MCP-1 signaling pathway [5]. What is more, many studies also confirmed that many extracts from artemisia argyi have the potential to treat liver cancer [3].

1.4 Pinene & Relevant Research

The extract of artemisia argyi is mainly composed of volatile oil. Pinene, as a component of volatile oils, is widely found in different varieties of artemisia [6]. What's more, many studies showed that pinene has many different pharmacological effects. In previous studies, in an animal model of Huntington's disease, the researchers found that alpha-pinene protects against 3-NP-induced motor dysfunction [7]. The α-pinene is capable of directly blocking the inflammatory signaling pathways in immune cells [8,9]. α-pinene has demonstrated neuroprotective properties in pre-clinical studies using models of various neurological diseases (in-vivo) [10]. Pinene is found to decrease migraine-related pain by controlling inflammation and vasoactive modulators [11]. Some indications suggest that pinene has anti-inflammatory effects and can prevent oxidative stress. It also shows potential as an antidepressant, anxiety-reducing agent, and anti-seizure treatment. Additionally, it provides neuroprotection in stroke and ischemia models, enhances cognition, and provides pain relief for inflammatory, migraine-related, and neuropathic pain in preclinical rodents or in vitro studies [12]. A remarkable discovery has shown that α-pinene can stimulate NK cells and enhance their ability to kill cancer cells, indicating its potential use as a compound in cancer immunotherapy [13]. However, there is a rare study proving pinene plays a role or has relevant pharmacological effects on liver cancer.

1.5 The Purpose of the Study

This study tries to link pinene with liver cancer and explore further whether pinene can promote apoptosis of liver cancer cells through a similar mechanism or pathway mentioned above. This study can provide a new and practical idea to design new drugs for liver cancer.

1.6 The Cell line Applied in this Study—SMMC-7721 Cells

SMMC-7721 is a human liver cancer cell line, which can form tumors in immunodeficient mice. The condition of culture is: RPMI-1640+10%FBS. The morphological characteristic of SMMC-7721 is epithelioid.

1.7 The Apoptosis Pathway Applied in this Study

Cell apoptosis pathway—Bcl-2/Bax/Caspase-3: The development of apoptosis can be summarized into three stages: apoptosis induction, execution, and effect. At least three pathways have been confirmed to be involved in apoptosis, including the mitochondrial pathway, death receptor pathway, and endoplasmic reticulum pathway. The mitochondria-dependent pathway is the main pathway, the JNK pathway is a major signal transduction pathway, Cyt-c plays a crucial role in the electron transport chain within mitochondria, and its liberation from mitochondria is a critical event in triggering apoptosis. The activation of Caspase-3, which is the main driver of apoptosis following the Caspase cascade, largely depends on the release of Cyt-c. Additionally, it is widely recognized that the Bcl-2 and Bax genes within the Bcl-2 family play a pivotal role as regulatory genes in apoptosis [14]. Releasing Cyt-c and other substances through the mitochondrial pathway can be facilitated by it [15]. Bcl-2 and Bax not only serve as the upstream regulatory mechanism for Caspase-3 and participate in regulating its activity, but also act as direct substrates of Caspase-3 downstream. They are interconnected and mutually constrained during the process of apoptosis [16].

1.8 Basic Experiment Design

SMMC-7721 cells are cultured in vitro and implanted in immunodeficient mouse models. With increasing pinene concentration and time, apoptotic protein Bcl-2 expression is down-regulated, pro-apoptotic protein Bax expression is up-regulated, and Caspase-3 is cleaved, thereby regulating the Bcl-2/Bax/Caspase-3 signaling pathway and inducing SMMC-7721 cell apoptosis. In addition, pinene reduces tumor size in mice transplanted with SMMC-7721 in vivo.

2. Materials and Methods

The SMMC-7721 cell cultured in vitro, their protein Bcl-2, Bax and caspase-3 are quantitatively analyzed by western blot, and the apoptosis is analyzed by Annexin V-FITC/PI FACS. The size of tumors in the xenograft mice model is measured by vernier caliper to judge the SMMC-7721 xenograft tumor growth.

2.1 Cell Lines and Cell Culture

This experiment will use the SMMC7721 cell line purchased from ATCC, which will be cultured on RPMI-1640+10%FBS complete medium purchased from Cobioer (in a 5% CO2 atmosphere at 37 ℃). The SMMC-7721 cell will be cultured by methods for 11 days and then begin to grow.

2.2 Animal Model

The SMMC-7726 cells (1×10^6 cells/mouse, five mice per group) are administered via subcutaneous injection into the right leg of male nude mice aged 4-6 weeks (Dossy, China). The mice are kept in an environment with a temperature of 24℃ and a light/dark cycle of 12 hours. The mice are monitored for 30 days after allograft implantation [17].

2.3 Targeted Reagent Preparation

Pinene will be extracted using the previously developed method [18].

2.4 Controls

Positive control is paclitaxel (PTX) for all experiments. Negative control are DMSO/PBS for experiments in vitro and normal saline for experiments in vivo.
The following three experiments adopt the same positive control and the two experiments conducted in vitro adopt the same negative controls.

2.5 Western Blot

2.5.1 Antibody Preparation

To analyse the protein Bcl-2 expression level, adopting the primary antibody Bcl-2Antibody, and the secondary antibody Goat Anti-Rabbit IgM antibody.
To analyse the protein Bax expression level, adopting the Anti-Bax Antibody[E63] as its primary antibody and the Goat Anti-Rabbit IgM antibody as its secondary antibody.
To analyse the caspase-3 protein, the primary antibody is Anti-Cleaved Caspase-3 antibody[E83-77] and the secondary antibody is Goat Anti-Rabbit IgM antibody.

2.5.2 Treatment Concentration and Treatment Duration

For the experiment in vitro, the SMMC-7721 cells will be administered with gradient concentrations of pinene(0μM, 25μM, 50μM, 100μM, 200μM, 400μM) for (0h, 12h, 24h, 48h) (which is set based on a previous study [7]). The apoptosis-related protein will be analysed by Western Blot. The experiment will be conducted 3 times.

2.5.3 Procedure

The cells are rinsed twice with ice-cold PBS, followed by collection in a lysis buffer (containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 2.5 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium vanadate and 2 mM PMSF) supplemented with protease inhibitor cocktail (APExBIO, TX, USA) for half an hour. The lysates are then centrifuged at 13,000 rpm for fifteen minutes at a temperature of four degrees Celsius. Total protein concentrations are determined using a Bradford reagent. The resulting supernatants are combined with equal amounts of Laemmli sample buffer and boiled for ten minutes before subjecting to SDS-PAGE using either eight percent or ten percent gels. The proteins are subsequently transferred to a nitrocellulose membrane via electrophoresis and incubated with specific antibodies for three hours at room temperature or overnight at four degrees Celsius as indicated. After multiple ishing, the membrane is incubated with a secondary antibody bound with horseradish peroxidase (Biosharp, Beijing, China) for 60 minutes and ished 7 times for 5 minutes each time. Visualization of the proteins is achieved using Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology Rockford IL USA) [19,20,21].

2.6 Annexin V-FITC/PI FACS

2.6.1 Treatment Concentration and Treatment Duration

For experiments in vitro, the SMMC-7721 cells will be administered with gradient concentrations of pinene (0μM, 25μM, 50μM, 100μM, 200μM, 400μM) for (0h, 12h, 24h, 48h) (which is set based on a previous study [7]). The cell apoptosis will be analysed by Annexin V-FITC/PI FACS. The experiment will be conducted 3 times.

2.6.2 Procedure

The Annexin V-FITC/PI FACS apoptosis detection kit is utilized for the assessment of cell apoptosis. A total of 1×10^5 cells are harvested and then stained with 195 ml of Annexin V-FITC binding buffer, 5 ml of Annexin V-FITC, and 10 ml of PI, following the instructions provided by the manufacturer. Subsequently, after suspension and incubation, flow cytometry (Beckman, USA) is employed to analyze the stained cells [22,23].

2.7 Measure the Xenograft Tumor Size by Vernier Caliper

Mice are randomly assigned into the treatment group, positive control group, and negative control group. The treatment group mice are treated with 40 mg/kg pinene for 16 days referring to a previous relevant study [7]. The

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positive control group are treated with an equal volume of PTX for 16 days, and the negative control group mice are treated with an equal volume of normal saline for 16 days. After pinene treatment is completed, measurements are recorded, including tumor volume or dimensions, at specified time points. The allografted tumor sizes are externally assessed every two days, and the tumor volumes are obtained according to the equation Volume = ($L \times S^2$)/2, where L and S are the lengths of the major and minor axes, respectively. The experiment will be conducted 3 times.

The growth of tumor allografts is significantly suppressed by pinene, leading to a significant reduction in tumor weight in mice treated with 40 mg/kg of pinene compared to the control group. In BALB/c mice, pinene effectively inhibited the growth of CT-26 allografts, resulting in a 42.83% inhibition ratio compared to the control group. These findings suggest that pinene has inhibitory effects on tumor growth [24,25].

3. Statistical Analysis

All experiments are conducted three times. This study uses the one-sample T-test to analyze the data. The critical t-value can be determined from the t-distribution table or by using statistical software. Based on the selected significance level of α=0.05, the computed t-value is compared with the critical t-value to determine whether the difference between the control group and the experimental group is statistically significant.

4. Results

Table 1 The different combinations of all results for test groups

Combination of possible results (CR)	Pinene increases Bax, decreases BCL2 and caspase-3 by western blot?	pinene increases apoptosis by Annexin V-FITC/PI FACS?	pinene decreases xenograft tumor size by caliper?	Supporting hypothesis?
CR1	+	+	+	Fully
CR2	+	+	-	Partially
CR3	+	-	+	Partially
CR4	-	+	+	Partially
CR5	+	-	-	Partially
CR6	-	+	-	Partially
CR7	-	-	+	Partially
CR8	-	-	-	Fully Contradicts

Table 1 legend:

The "+" indicates that the phenomenon in each column heading is observed in the experiments and is significantly different from the negative control group, and is similar to the positive control group(PTX).

The "-" indicates that the phenomenon is not observed in the experiments, which may be due to the observation contradicting the hypothesis or being not significantly different from the negative control group.

4.1 Description of Each Combination

4.1.1 Combination of Possible Results 1 (CR1)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro treated by pinene shows that the expression of Bcl-2 is significantly decreased, the expression of Bax is significantly increased, and the cleavage of caspase-3 is facilitated. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro shows that cell apoptosis is enhanced. The vernier caliper measurement for the xenograft tumor size of immunodeficient mice treated with pinene shows that the pinene diminishes the xenograft tumor.

4.1.2 Combination of Possible Results 2 (CR2)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro treated by pinene shows that the expression of Bcl-2 is significantly decreased, the expression of Bax is significantly increased, and the cleavage of caspase-3 is facilitated. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro shows that cell apoptosis is enhanced. The vernier caliper measurement for the xenograft tumor size of immunodefi-

cient mice treated by pinene shows that the pinene doesn't obviously diminish the size of xenograft tumors.

4.1.3 Combination of Possible Results 3 (CR3)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro treated by pinene shows that the expression of Bcl-2 is significantly decreased, the expression of Bax is significantly increased, and the cleavage of caspase-3 is facilitated. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro shows that cell apoptosis isn't enhanced. The vernier caliper measurement for the xenograft tumor size of immunodeficient mice treated by pinene shows that the pinene diminishes the size of xenograft tumor.

4.1.4 Combination of Possible Results 4 (CR4)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro treated by pinene shows that pinene doesn't regulate the Bcl-2/Bax/Caspase-3 pathway as expected. Specifically, the proteins expression level on this pathway doesn't change a lot by the pinene treatment across the period. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro shows that cell apoptosis is enhanced. The vernier caliper measurement for the xenograft tumor size of immunodeficient mice treated with pinene shows that the pinene diminishes the size of the xenograft tumor.

4.1.5 Combination of Possible Results 5 (CR5)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro and treated by pinene shows that the expression of Bcl-2 is significantly decreased, the expression of Bax is significantly increased, and the cleavage of caspase-3 is facilitated. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro shows that cell apoptosis isn't enhanced. The vernier caliper measurement for the xenograft tumor size of immunodeficient mice treated with pinene shows that the pinene doesn't diminish the size of the xenograft tumor.

4.1.6 Combination of Possible Results 6 (CR6)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro treated by pinene shows that pinene doesn't regulate the Bcl-2/Bax/Caspase-3 pathway to decrease BCL-2 expression, increase Bax expression and the cleavage of caspase-3 isn't facilitated. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro shows that cell apoptosis is enhanced. The vernier caliper measurement for the xenograft tumor size of immunodeficient mice treated with pinene shows that the pinene doesn't diminish the size of the xenograft tumor.

4.1.7 Combination of Possible Results 7 (CR7)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro treated by pinene shows that pinene doesn't regulate the Bcl-2/Bax/Caspase-3 pathway to get that the expression of Bcl-2 is significantly decreased, the expression of Bax is significantly increased, and the cleavage of caspase-3 is facilitated. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro shows that cell apoptosis isn't enhanced. The vernier caliper measurement for the xenograft tumor size of immunodeficient mice treated with pinene shows that the pinene diminishes the size of the xenograft tumor.

4.1.8 Combination of Possible Results 8 (CR 8)

The Western Blot analysis for the SMMC7721 cell line cultured in vitro treated by pinene doesn't show that the expression of Bcl-2 is significantly decreased, the expression of Bax is significantly increased, and the cleavage of caspase-3 is facilitated. The Annexin V-FITC/PI FACS analysis for the SMMC7721 cell line cultured in vitro doesn't show that cell apoptosis is enhanced. The vernier caliper measurement for the xenograft tumor size of immunodeficient mice treated with pinene doesn't show that the pinene diminishes the size of the xenograft tumor.

4.2 Possible Results for the Variables of Concentration and Treatment Duration

Potential Factor 1: The expression level of protein Bcl-2, Bax, caspase-3, the level of cell apoptosis, and the xenograft tumor size exhibit a dependence on both concentration and duration. An escalation in the concentration of pinene and the protraction of the treatment period could potentially lead to a reduction or augmentation expression level of protein and apoptosis levels, as well as a fluctuation in xenograft tumor size.

Potential Factor 2: The expression level of protein Bcl-2, Bax, caspase-3, the level of cell apoptosis, and the xenograft tumor size are not solely dependent on concentration and time. Despite the escalation in pinene concentration and extended treatment duration, the levels of proteins, and apoptosis, the xenograft tumor size remain unchanged.

5. Discussion

5.1 Discussion of Each Combination

CR1 shows that in the Bcl-2/Bax/Caspase-3 pathway, the Bcl-2 protein expression decreases, the Bax protein expression increases and the caspase-3 is cleaved more. It indicates that pinene does have the regulating function to Bcl-2/Bax/Caspase-3 pathway as expected. What is more, in vitro, the SMMC-7721 cell apoptosis is promoted, and

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in vivo, the xenograft tumor size diminishes. Hence, it indicates that pinene enhances liver cancer cell apoptosis, causing the tumor size to be diminished by regulating the Bcl-2/Bax/Caspase-3 pathway. Therefore, it supports the hypothesis. And future research could try the new drug invented with pinene on clinical trials.

CR2 shows that pinene could regulate the Bcl-2/Bax/Caspase-3 pathway and cell apoptosis which accords with the hypothesis. However, in immunodeficient mice, the xenograft tumor doesn't diminish. The result partially supports the hypothesis. The probable explanation could be that pinene is broken by enzymes in vivo like aspergillus niger [26], or the Bcl-2 gene or the Bax gene or both the mutates in vivo, or the macrophages in tumor microenvironment phagocytoses the pinene making it unable to work [27], or exosomes in the tumor microenvironment increase the efflux of drugs in tumor cells, and the concentration of the intracellular drug is not sufficient to exert a pro-apoptotic effect [28]. Thereby causing tumor drug resistance [29]. Future research could focus on treating the nude mice with pinene encased in a shell that will not be broken down by Aspergillus niger and then observing the effect of treatment. Future experiments also could conduct PCR to detect whether the gene mutates or not.

CR3 shows that pinene regulates the Bcl-2/Bax/Caspase-3 pathway, and the tumor size diminishes as well. However, the SMMC-7721 cells apoptosis isn't be enhanced. The result partially supports the hypothesis. It indicates that cell apoptosis and tumor reduction couldn't be induced only by the Bcl-2/Bax/Caspase-3 pathway. It would be possible that the Bcl-2/Bax/Caspase-3 pathway and the external signaling pathway, Fas/Fasl pathway [30], play in concert to induce the SMMC-7721 cells apoptosis, and the whole liver cancer cell apoptosis process needs the histamine molecule in the internal environment [31]. Hence the experiment in vivo meets the expectation, but the experiment in vitro doesn't. Future research could focus on activating the pathway mentioned above together to regulate the SMMC-7721 cells apoptosis. Additionally, future experiments could also conduct the same experiment in vivo and analyse the expression of the Fas/Fasl pathway and analyse the change of the concentration of the histamine in blood or in lymph, in order to confirm the explanation.

CR4 shows that pinene doesn't regulate the Bcl-2/Bax/Caspase-3 pathway as the hypothesis predicts. However, pinene truly enhances apoptosis and diminishes tumor size. The result partially supports the hypothesis. It indicates that pinene may achieve that by regulating another possible pathway such as PERK signaling pathway or IRE1 signaling pathway [32,33]. Future research could focus on conducting the same experiment and analyse the expression of the PERK signaling pathway and the IRE1 signaling pathway.

CR5 shows that pinene regulates the Bcl-2/Bax/Caspase-3 pathway as the hypothesis predicts, but apoptosis isn't be enhanced and the tumor size isn't diminished. The result partially supports the hypothesis. So just regulating the Bcl-2/Bax/Caspase-3 pathway is not enough for pinene to induce cell apoptosis. It may need to function together with the activated caspase-8 and caspase-10 or other caspase family proteases to jointly induce the SMMC-7721 cells apoptosis [34]. Future research could focus on conducting the same experiment and analyse the expression of the caspase-8 and caspase-10 to explore the mechanism further.

CR6 shows that pinene induces the cells in vitro to become apoptotic but not by the Bcl-2/Bax/Caspase-3 pathway. The result partially supports the hypothesis. It is possible that pinene achieves that by death receptor signaling pathway. And in vivo, such effect probably is removed by IL6 secreted by CAF in the tumor microenvironment [35], which can improve the survival rate of SMMC-7721 cells. Future research could focus on conducting the experiment and analysing the expression of the death receptor signaling pathway to explore the mechanism further and analysing the concentration of the IL6 in the tumor microenvironment to confirm the explanation.

CR7 shows that pinene couldn't regulate the Bcl-2/Bax/Caspase-3 pathway and induce cell apoptosis cultured in vitro. However, in vivo, pinene succeeds in achieving tumor diminishment. The result partially supports the hypothesis. The experiment in vivo truly shows that the pinene diminishes the tumor size and probably has the function of enhancing SMMC-7721 cell apoptosis, but the experiment in vitro doesn't detect the apparent cell apoptosis. The possible reason could be that the experiment in vitro is wrong, namely, the treatment concentration and duration for an experiment in vitro are not enough. Thus, future research could increase the 0μM, 25μM, 50μM, 100μM, 200μM, 400μM pinene solution to 0μM, 50μM, 100μM, 200μM, 400μM, 800μM, 1000μM pinene solution, and increase the 0h, 12h, 24h, 48h treatment duration to 0h, 24h, 48h, 96h treatment duration.

CR8 shows that none of the results meet the expectation. The result fully contradicts the hypothesis. So probably pinene doesn't have the function to induce liver cancer cell apoptosis. If so, this study verifies this, and it could reduce unnecessary attempts by other researchers.

5.2 Discussion of the Possible Results for the Variables of Concentration and Treatment Du-

ration

Potential cause 1 suggests that pinene concentration and treatment time have significant correlation with the levels of proteins and apoptosis, and the xenograft tumor size. Further experimental studies are needed to determine the optimal therapeutic concentration and duration of pinene to maximize its beneficial effects.

Potential cause 2 indicates that the levels of proteins and apoptosis, and the xenograft tumor size do not depend on concentration and time. This phenomenon may be attributed to insufficient pinene input concentration and insufficient treatment time or pinene doesn't have the effects. Further research could expand the range of pinene treatment concentrations and durations.

6. Conclusion

This study investigates the regulating mechanism to liver cancer cell apoptosis under pinene treatment. Upon confirming the function of pinene and developing a new drug, new insights into the treatment of liver cancer will be provided. The possible result combinations are discussed, and further experiments are suggested. Future research may focus on the other mechanisms of pinene to cell apoptosis and the effect of pinene on other subtypes of liver cancer.

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