RESEARCH ARTICLE

Inhibitory Effects of α-Pinene on Hepatoma Carcinoma Cell Proliferation

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Abstract

Background: Pine needle oil from crude extract of pine needles has anti-tumor effects, but the effective component is not known. Methods: In the present study, compounds from a steam distillation extract of pine needles were isolated and characterized. Alpha-pinene was identified as an active anti-proliferative compound on hepatoma carcinoma BEL-7402 cells using the MTT assay. Results: Further experiments showed that α-pinene inhibited BEL-7402 cells by arresting cell growth in the G2/M phase of the cell cycle, downregulating Cdc25C mRNA and protein expression, and reducing cycle dependence on kinase 1(CDK1) activity. Conclusion: Taken together, these findings indicate that α-pinene may be useful as a potential anti-tumor drug.

Keywords: α-pinene - hepatoma carcinoma - proliferation - cell cycle proteins

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Introduction

Primary hepatic carcinoma is the most common type of malignant liver tumor and the second most likely type to lead to mortality. Most PHC cases are diagnosed at an advanced stage of cancer. Present conventional medicines for treatment of primary hepatic carcinoma are limited and possess severe toxic side effects. Therefore, it is very important to develop liver-targeted anti-cancer medicines with low toxicity and high efficacy. Chinese herbal medicines have proven to be a rich source of natural products from which the active components have been isolated in an attempt to develop bioactive drugs. Pine needle oil is a transparent, yellow, aromatic solution extracted from the leaves of pine needle plants using a steam distillation method (Wajs et al., 2010). Studies have shown that pine needle oil has anti-inflammatory and antiblastic effects and can significantly inhibit . biosynthesis (Pichette et al., 2006; Jeong et al., 2007). In another study, a pine needle extract solution was found to inhibit the rate of growth of a mouse transplantable tumor by more than 40% (Loizzo et al., 2010). However, the crude extract of pine needle oil contains a variety of components, and the components with anti-tumor activities remain unclear. In the present study, pine needle oil was extracted from the pine needle stem and leaves of Pinus massoniana Lamb from Guangzhou, China and the main components of pine needle oil were isolated. We also conducted *in vitro* cell experiments to investigate the inhibitory effects of α-pinene, a primary component of pine needle oil, on the proliferation of human hepatoma carcinoma BEL-7402 cells, and the influence of α-pinene on cell cycle distribution and cell cycle-related genes and proteins.

Materials and Methods

Main reagents

Methyl thiazolyl tetrazolium (MTT), propidium iodide (PI) and RNase A were purchased from Amresco (Solon, OH); RPMI-1640 culture medium and new-born calf serum from Gibco (GrandIsland, NY); fluorescent quantitative reverse transcription-PCR kit from Takara (Tokyo, Japan); mouse anti-human cell cycle depend on kinase 1 (CDK1), Cyclin B1 and Cdc25C monoclonal antibodies from Millipore (Billerica, MA); actin and rabbit anti-human CDK1 p34 (Tyr 15) phosphorylated polyclonal antibodies from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA); and enhanced chemiluminescence kit from Pierce (Rockford, IL).

Extraction and isolation

Pine needle stem and leaves of Pinus massoniana Lamb from Guangzhou were dried naturally in the shade, and cut into pieces of 2 cm to obtain a crude extract of pine needle oil using a self-designed steam distillation device. The extracts were stored at low temperature. α-Pinene was extracted from the pine needle oil using a

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steam distillation method. The extracted α-pinene was a transparent, yellow solution with aromatic odor and cold preserved. The extracted α-pinene was used as the test sample, and α-pinene (CAS-785-70-8) purchased from Sigma was used as a standard, and analyzed using a gas chromatograph-mass spectrometer. Gas chromatogram conditions were as follows: chromatographic column: HP-5 quartz capillary column (30 m×0.25 mm×0.25 μm); column temperature was set to 50°C, increased to 160°C by 3°C/minute for 2 minutes, and further increased to 280°C by 10°C/minute; the temperature was maintained until completion of analysis; sampling amount was 0.2 μL; carrier gas highly pure He (0.99999); carrier gas flow rate 1.0 mL/minute; and split ratio, 30:1. Mass spectrometry conditions were as follows: ion source temperature, 220°C; hand hole temperature, 240°C; electron energy, 70 eV.

Preparation of α-pinene dilution

The α-pinene dilution was prepared as previously described (Singh et al., 2006). Tween-80 (1%) and serum-free RPMI-1640 culture medium were used to prepare the α-pinene solutions at 50, 80 and 100 mM.

Cell culture

The human hepatoma carcinoma BEL-7402 cell line was cultured in RPMI-1640 complete culture medium containing 10% new-born calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated in a 5% CO_2 incubator with saturated humidity at 37°C. After several passages, cells in the logarithmic phase were collected.

Cell proliferation inhibition test

Cells in logarithmic phase were collected and the cell concentration was adjusted to 5×10^4/mL. Cells were seeded into a 96-well culture plate, 90 μL per well. Treatment group cells were cultured with 10 μL α-pinene at different concentrations (terminal concentrations were 5 mM, 8 mM and 10 mM), and the control group was cultured in an equal volume of serum-free RPMI-1640 culture medium containing tween-80 (1%). Each group contained five parallel wells, and a blank control well was also used. Cells were respectively harvested at 24, 48 and 72h after culture, and 10 μL MTT (5 mg/mL) was added to each well for an additional incubation of 4h. The supernatant was discarded, and 100 μL dimethyl sulfoxide was added to each well, and shaken on an oscillator for 15 minutes. Absorbance (A) at 570 nm (reference wavelength 450 nm) was measured using a microplate reader. The experiments were performed in triplicate. The inhibition ratio was calculated using the following:

Cell proliferation inhibition rate (%)=(1-Atreatment group/Acontrol group)×100%.

Annexin V-FITC/PI detection for cell apoptosis

Cells were collected after treatment with 8.4 mM α-pinene for 48h and washed twice with PBS. Cells, 3×10^5, were resuspended in 500 μL binding buffer, mixed with 5 μL Annexin V-FITC, and 5 μL PI, and the reaction was allowed to proceed at room temperature for 10 minutes in the dark. Within 1h, the cells were analyzed by flow cytometry to determine the apoptosis ratio.

Cell cycle test

Cells were collected after treatment with 8.4 mM α-pinene for 24, 48 and 72h, centrifuged at 1000 rpm for 5 minutes, washed with PBS after discarding the supernatant, centrifuged to discard the PBS, mixed with pre-cooled 70% alcohol, rapidly pipetted, and fixed at 4°C for at least 24h. The mixture was centrifuged to discard the alcohol, washed twice with PBS, centrifuged to remove PBS, then stained with 0.5 mL PI (50 μg/mL, containing 100 μg/mL RNase A, 0.1 mmol/L EDTA, 0.1% Triton X-100) for 30 minutes in the dark. DNA content was determined using flow cytometry to analyze cell cycle distribution. Five parallel tubes were used for each group.

Fluorescent quantitative reverse transcription-PCR

Cells were cultured in a 6-well culture plate, and treated with 8.4 mM α-pinene for 24, 48 and 72h. After the supernatant was discarded, cells were mixed with 1 mL Trizol and total RNA was extracted according to the manufacturer's instruction. Each total RNA was reverse transcribed using Oligo (dT) primers at 37°C for 15 minutes according to the instructions for the fluorescent quantitative reverse transcription-PCR kit, and then the reverse transcriptase was deactivated at 85°C for 5 s.

Relative quantitative PCR was used, with β-actin as the internal reference. The primers of β-actin were self-designed, and Cdc25C, CDK1, cyclin B1 primers were designed as previously described (Le Gac et al., 2006; Russo et al., 2006) and synthesized by Invitrogen, Shanghai, China (Table 1).

Western blot assay

Cells were treated with 8.4 mM α-pinene for 48h, washed twice with cold PBS, mixed with pre-cooled lysate, collected, placed in an ice bath for 20 minutes, and centrifuged at 12,000×g at 4°C for 15 minutes. The supernatant was collected to quantify protein concentration. Protein samples were boiled for 5 minutes, and then subjected to 10% sodium dodecyl

Table 1. Primer Sequence

Primer	GeneBank accession	Forward primer sequence(5' - 3')	Reverse primer sequence (5' - 3')	Product (bp)
b-actin	NM_001101.3	CACCAACTGGGACGACAT(312-329) exon3	ACAGCCTGGATAGCAACG(483-500) exon4	189
Cdc25C	NM_001790	GAACAGGCCAAGACTGAAGC (934-953) exon8	GCCCCTGGTTAGAATCTTCC (1093-1112) exon9	179
CDK1	NM_001786	TGGGGTCAGCTCGTTACTCA (669-688) exon6	CACTTCTGGCCACACTTCATTAT (813-835) exon7	167
Cyclin B1	NM_031966	GGCCTCTACCTTTGCACTTCC (1073-1093) exon6	GGCCAAAGTATGTTGCTCGAC(1126-1146) exon7	74

*The PCR reaction conditions were as follows: SYBR® Premix Ex TaqTM (2×) 12.5 μL, upstream and downstream primers (10 μM) 0.5 μL each; template cDNA 2 μL, dH2O 9.5 μL for 25 μL in total. Reaction parameters: 95°C for 10 s to activate Taq polymerase, 95°C for 5 s, 60°C for 20 s, with 40 cycles in total.

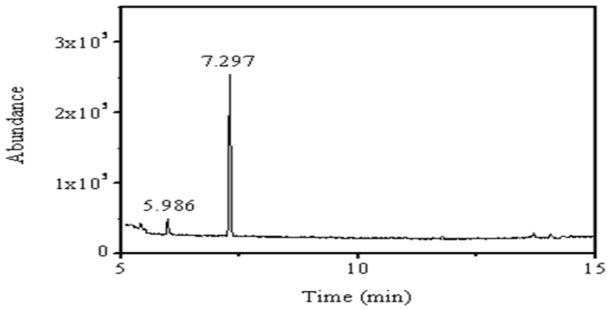

Figure 1. Total Ion Current of the α-Pinene sample

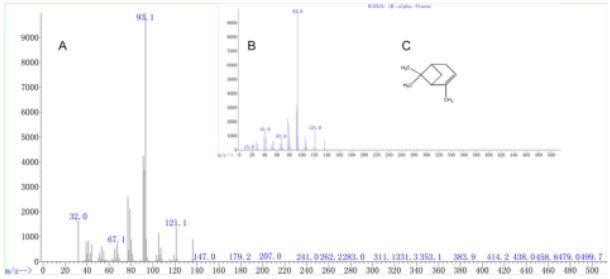

Figure 2. Mass Spectrum of the Main Peak of the α-Pinene Sample. A) ion peak mass spectrum at 7.297 minutes, **B)** reference mass spectrum of α-pinene **C)** structural formula of α-pinene

sulfate polyacrylamide gel electrophoresis. Isolated proteins were electrotransferred to polyvinylidene fluoride membrane, blocked at room temperature for 2h, and incubated respectively with actin, CDK1, cyclin B1, Cdc25C and CDK1 p34 (Tyr 15) phosphorylated antibodies at room temperature for 2 hours, followed by horseradish peroxidase-labeled secondary antibody at room temperature for 2 hours. The membrane was subjected to enhanced chemiluminescence, X-ray film exposed, developed and fixed. The integral optical density value ratio of CDK1, cyclin B1, Cdc25C and CDK1 p34 (Tyr 15) phosphorylated protein bands to the actin band was calculated and standardized, followed by semiquantitative analysis.

Statistical analysis

Experimental data were expressed as mean±SD and analyzed using SPSS 11.0 software (SPSS Inc., Chicago, IL) with one-way analysis of variance. A value of $p<0.05$ was considered to indicate a significant difference and a value of $p<0.01$ was considered to indicate a highly significant difference.

Results

Alpha-pinene extraction and identification

The main components of the extracted α-pinene samples detected by gas chromatography-mass spectrometry were compared with the NIST spectrum bank, and the α-pinene relative percent (total ion current in Figure 1) was calculated using an area normalization method. Results showed that the main component made up 91.0% (7.297 minutes) of the total sample, and mass spectrometry analysis confirmed that the component was α-pinene (Figure 2).

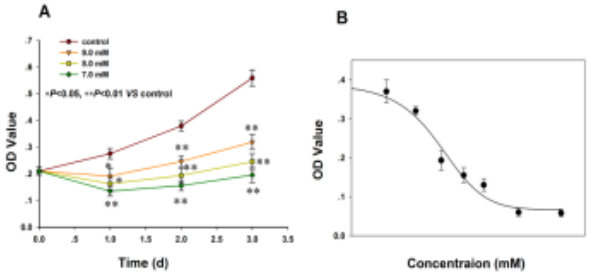

Figure 3. Inhibitory Effects of α-Pinene on the Proliferation of BEL-7402 Cells. A) Alpha-pinene time-dependently inhibited the proliferation of BEL-7402 cells. **B)** Alpha-pinene dose-dependently inhibited the proliferation of BEL-7402 cells with an IC_{50} of 8,4 mM

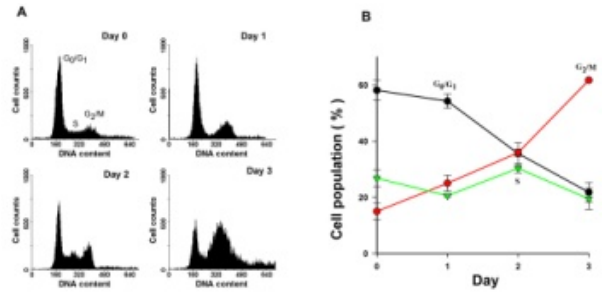

Figure 4. Influence of α-Pinene on the Cell Cycle of BEL-7402 Cells. A) Cell cycle distribution of BEL-7402 cells after treatment with 8.4 mM α-pinene for different times. **B)** Cell cycle distribution of BEL-7402 cells versus time of incubation

Alpha-pinene inhibited BEL-7402 cell proliferation

Alpha-pinene inhibited proliferation of BEL-7402 cells, and the inhibition increased with drug concentration and duration in a dose-dependent manner (Figure 3). The IC_{50} was 8.4 mM and this concentration was used in subsequent experiments.

Alpha-pinene induced growth retardation in the G2/M phase of BEL-7402 cells

Flow cytometry showed that after cells were treated with 8.4 mM α-pinene for 24, 48 and 72 hours, cell growth was arrested in the G2/M phase (Figure 4). At 24 hours, the percentage of cells in the G2/M phase increased to 12.1%, significantly greater than in the control group (7.7%; $p<0.05$). By 48 hours, the percentage of cells in the G2/M phase increased to 14.7%, nearly two times that in the control group (7.6%). At 72 hours, the percentage of cells in the G2/M phase further increased to 23.1%, nearly three times that in the control group (7.0%).

Alpha-pinene did not induce BEL-7402 cells apoptosis

A double-variable flow cytometry scatter plot (Figure 5) showed that the apoptosis rate was 6.3% in the control group, and 6.5% in cells treated with 8.4 mM α-pinene for 48 hours, indicating that α-pinene did not induce cell apoptosis.

Alpha-pinene downregulated Cdc25C mRNA expression in BEL-7402 cells

Fluorescent quantitative reverse transcription-PCR showed that after cells were treated with α-pinene for 24, 48 and 72 hours, CDK1 and cyclin B1 mRNA expression remained unchanged, indicating that the genes for CDK1

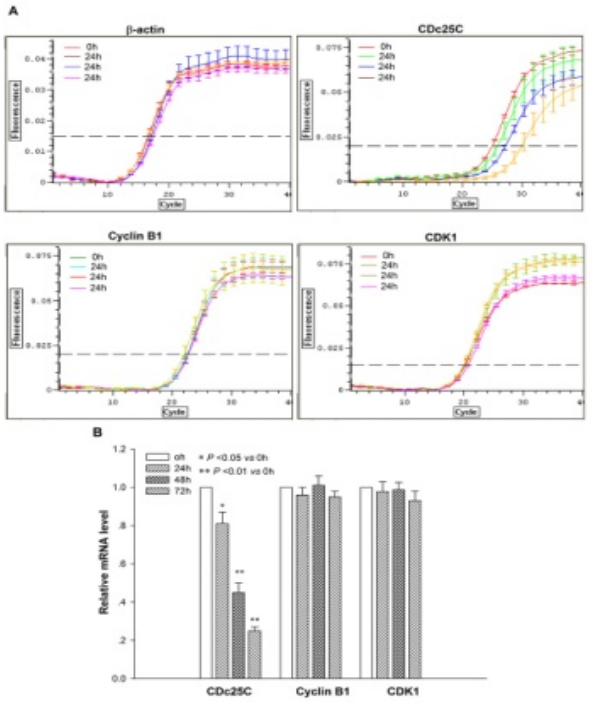

Figure 6. Influence of α-pinene on Cdc25C, cyclin B1 and CDK1 mRNA expression in BEL-7402 cells. **A)** Fluorescent quantitative PCR amplification curves of Cdc25C, cyclin B1, CDK1 and β-actin. **B)** Cdc25C, cyclin B1 and CDK1 mRNA expression in BEL-7402 cells treated with α-pinene

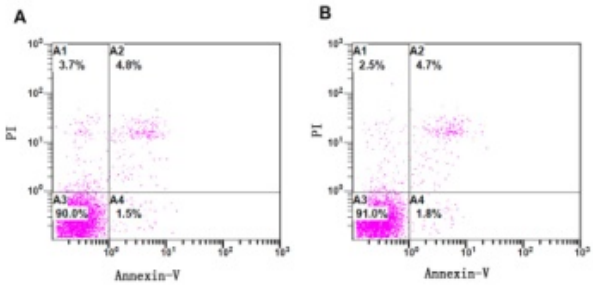

Figure 5. Influence of α-Pinene on the Apoptosis of BEL-7402 Cells. A) Control group**. B)** 8.4 mM α-pinene treatment group

and cyclin B1 are not involved in α-pinene-induced cell cycle arrest. Alpha-pinene downregulated Cdc25C mRNA expression (Figure 6), and the expression was gradually decreased with increasing time, indicating that Cdc25C may participate in α-pinene-induced cell cycle arrest in the G2/M phase.

Alpha-pinene downregulated Cdc25C protein expression and reduced CDK1 activity

Western blot analysis showed that cyclin B1, CDK1 and Cdc25C proteins were expressed in both the control and treated groups (Figure 7A). After the cells were treated with α-pinene for 48 hours, Cdc25C protein expression was downregulated, and cyclin B1 and CDK1 protein expression remained unchanged, but phosphorylated CDK1 (Tyr 15) protein expression was upregulated (Figure 7B), indicating that the degree of CDK1 (Tyr 15) protein phosphorylation was increased, activated CDK1 was reduced, and CDK1 activity was decreased. This indicated that α-pinene-induced cell cycle arrest in the

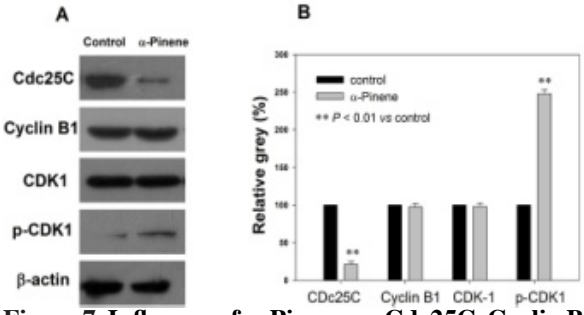

Figure 7. Influence of α-Pinene on Cdc25C, Cyclin B1, CDK1 and Phosphorylated CDK1 (Tyr 15) Protein Expression in BEL-7402 Cells. A) Western blot results. **B)** Image analysis results

G2/M phase may be related to an increased degree of phosphorylation of CDK1 protein and reduced Cdc25C protein expression.

Discussion

Recent studies have shown that pine needle oil may have many pharmacological actions. In particular, its anti-tumor effects are of interest. The main components of pine needle oil include α-pinene and β-pinene. Results from the present study showed that α-pinene inhibited hepatoma carcinoma BEL-7402 cell proliferation, and the inhibition dependent upon concentration and duration of incubation. A previous study reported that the tumor volumes from mice treated with α-pinene were about 40% smaller than those in the control mice, but α-pinene had no inhibitory effect on melanoma cell proliferation *in vitro* (Kusuhara et al., 2012; Bhattacharjee et al., 2013). These results were not consistent with our study. The difference may result from variations in the sensitivity of solid tumors to α-pinene. Alpha-pinene was also able to induce apoptosis as evidenced by the early disruption of the mitochondrial potential, production of reactive oxygen species, and increase in caspase-3 activity (Matsuo et al., 2011). Most importantly, α-pinene was very effective in the treatment of experimental metastatic melanoma, reducing the number of lung tumor nodules. These findings indicate that α-pinene may inhibit cell proliferation by inducing cell apoptosis (Catanzaro et al., 2012). However, results from the present study showed that α-pinene did not induce BEL-7402 cell apoptosis. Thus, it may inhibit cell proliferation through other mechanisms.

Abnormal regulation of the cell cycle is a contributing factor to excess cell proliferation and tumorigenesis (Kim et al., 2011). Loss of control at the start point of the cell cycle can induce abnormal proliferation by transfer from the G0/G1 phase to the G1/S and G2/M phase (Drouet et al., 2001; Meng et al., 2011). Flow cytometry detection showed that α-pinene induced cell cycle arrest in the G2/M phase. The G2/M phase restriction point is important for cell growth, and only completely replicated and uninjured cells can cross the G2/M phase restriction point, indicating that after α-pinene treatment, control of the cell cycle restriction point was lost. This affected DNA synthesis and therefore inhibited cell proliferation.

Cell cycle proteins play key roles in tumorigenesis, and

thus are ideal targets for the development of anti-tumor drugs. Cyclin B1 is a regulatory subunit of maturation promoting factor, binding with CDK1, the catalytic subunit of maturation promoting factor, to form cyclin B1/CDK1 composite. This functions in the G2/M phase to drive cell cycle phase transfer, thereby playing a key role in triggering mitosis (Bassermann et al., 2007). CDK1 Tyr15 and Thr14 are phosphorylated early in the M phase, which deactivates the composite (Ruiz et al., 2010). Thus, the G2/M phase monitoring point can arrest the cell cycle in the G2/M phase by reducing cyclin B1 and CDK1 expression or by reducing CDK1 activity to deactivate the composite. CDK1 activation requires regulation of Cdc25, a cell cycle regulatory protein. Increased Cdc25 activity can dephosphorylate Tyr15 and Thr14 in CDK1, hence activating it (Timofeev et al., 2010) to phosphorylate the substrate and enter the M phase. Human Cdc25 contains Cdc25A, Cdc25B and Cdc25C subtypes. Cdc25B and Cdc25C play roles in the G2/M control point (Lau et al., 2010). Fluorescent quantitative reverse transcription-PCR results from the present study showed that α-pinene cannot downregulate cyclin B1 or CDK1 mRNA expression, but does reduce Cdc25C mRNA expression with prolonged treatment time. Western blot results were consistent with PCR in that after α-pinene treatment, Cdc25C expression was reduced, which interferes with the activation of CDK1. Results also indicated that phosphorylated CDK1 (Tyr 15) protein expression was upregulated, indicating a reduction of CDK1 activity. Therefore, although cyclin B1 expression remained unchanged, the amount of activated cyclin B1/CDK1 composite was decreased, resulting in arrest in the G2/M phase.

In conclusion, α-pinene inhibits proliferation of BEL-7402 cells and treatment results in cell cycle arrest in the G2/M phase. The mechanism may be associated with reduced Cdc25C expression and decreased CDK1 activity. The results imply that α-pinene might be a useful structural motif from which to design and develop anti-tumor drugs.

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