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Research Article

The Alleviated Effect of α-Pinene on Amidst Alcoholic Liver Injury Pathology Through Autophagy Activation in Mice

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Abstract:

Background: Alcoholic liver damage is caused by long-term and heavy alcohol consumption, which leads to many diseases and even cancer. α-Pinene has been shown to have antioxidant and anti-inflammatory activity, however, it is still unclear the relationship between α-Pinene and alcohol-induced liver injury. The potential molecular mechanisms of α-pinene in reducing alcohol-induced liver injury in mice were investigated in this study.

Materials and methods: The C57BL/6 mice were randomly divided into five groups, which were the control groups (physiological saline, 0.2mL per days), alcohol group (50% alcohol, 5 mL/kg bw/day), alcohol with low/medium/high dosage α-pinene treatment group ((7.2 mg/kg bw, 14.4 mg/kg bw, 28.8 mg/kg bw, dissolved in 50% alcohol, Separately). The dosing method for mice is via oral gavage. After 8 weeks of experimentation, mouse serum and liver were collected for further testing.

Result: The increased antioxidant enzyme activities demonstrated the alleviated effect of α-pinene against alcohol-induced mouse liver injury. Moreover, in liver tissues, α-pinene promoted nucleus translocation of nuclear factor-erythroid-2-related factor 2 (NRF2) and transcription of antioxidant target genes, including heme oxygenase 1 (HMOX-1 / HO-1), NAD[P]H quinone dehydrogenase 1 (NQO-1), and glutathione S-transferase alpha 1 (Gsta-1). Meanwhile, α-pinene promoted the protein expression of autophagy-related proteins and inhibited the increase of inflammatory factors caused by chronic alcohol intake. Furthermore, α-pinene partially inhibited the activation of apoptotic signaling pathways by increasing the expression of Bcl-2 and decreasing Bax and cleaved caspase-3 proteins.

Conclusion: Taken together, our results indicated that α-pinene might alleviate alcoholic liver injury by reducing lipid accumulation, enhancing anti-oxidative stress and anti-inflammatory, activating autophagy, and inhibiting cell apoptosis.

Key words: alcoholic liver injury; α-pinene; antioxidation; autophagy; inflammatory

1. Introduction

As a global health problem, alcoholic liver disease (ALD) is an important risk factor for metabolic diseases of the liver [1]. ALD mainly includes alcoholic hepatitis (AH), alcoholic fatty liver (AFLD), and chronic ALD [2]. ALD could further result in liver fibrosis, cirrhosis, and liver cancer[1]. More and more studies revealed that lipid accumulation, free radical accumulation, and endotoxin lipopolysaccharide (LPS)-induced inflammatory damage were closely relevant to the development of these diseases [2]. Meanwhile, Betaine, cannabidiol, and inulin, which played a role as an antioxidant, autophagy activator, anti-inflammatory agent, and anti-apoptotic respectively, have been used for the treatment of ALD and enhanced the liver's defence ability to improve alcoholic liver injury [3-5]. Therefore, like betaine, cannabidiol inulin, medicines developed

from natural plants have become the focus for the prevention and treatment of ALD.

The α-pinene, with the chemical name of 2,6,6-trimetheylbicyclo [3.1.1] hept-2-ene, is a bicyclic monoterpene molecule found in various plants, including camphor, rosemary, and pine [6, 7]. Recently, α-pinene was also been found to be presented in the highland barley Baijiu [8]. Mahdieh et al. revealed that α-pinene could significantly reduce cerebral edema and infarct range, and improve neurobehavioral function by playing a protective role through restoring superoxide dismutase, catalase, glutathione peroxidase, and reducing MDA concentration (malondialdehyde), NO, and IL-6 in the hippocampus, cortex, and striatum [6]. In addition, α-pinene could significantly protect IEC-6 cells from cellular toxicity induced by aspirin and increased the ratio of cell survival by elevating GSH (glutathione) activity and reducing MDA [9]. the KEAP1 (Kelch-like ECH-associated protein 1)-NRF2 (activity of Nuclear factor-erythroid-2-related factor 2) pathway responds to oxidative stress [10]. Under normal conditions, NFE2L2 is negatively regulated by the cysteine-rich protein KEAP1 through proteasome degradation mediated by the CUL3 (cullin 3)-E3 ubiquitin ligase RBX1 (ring box 1) complex [11]. However, NRF2 is disassociated from KEAP1 and recruited into the nucleus when oxidative stress occurs. Furthermore, nucleus-translocated NRF2 activated the transcription of its target genes, including *HMOX-1 / HO-1* (heme oxygenase 1), *NQO-1* (NAD[P]H quinone dehydrogenase 1), and *Gsta-1* (glutathione S-transferase alpha 1), which play an important role in the decrease of reactive oxygen species (ROS) level[12-14]. Further study revealed that oxidative stress and autophagy, which is a major intracellular degradation system that depends on lysosomes, have a mutual influence. For instance, the PtdIns3-phosphatase MTMR3 interacts with mTORC1 and suppresses its activity [15]Even though the antioxidant, anti-inflammatory, neuroprotective and analgesic effects of α-pinene have been discussed [7, 16], few studies focused on the exploration of a its effect on ALD.

This study investigated the possible protective effect of α-pinene on amidst ALD pathology in a mouse model of chronic liver injury. Our results suggest that α-pinene protects against alcoholic liver injury by reducing lipid accumulation, enhancing anti-oxidative stress and anti-inflammatory, activating autophagy, and inhibiting cell apoptosis.

Materials and methods

Chemicals and reagents

α-pinene (purity>97%) purchased from Thermo Fisher Scientific Commercial kits were used to determine the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT). The primary antibodies against rabbit LC3, P62, and GAPDH were purchased from Cell Signaling Technology. The primary antibodies against mouse BCL-2 and the primary antibodies against rabbit NRF2, INOS, COX-2, NF-κB, caspase-3, and Lamin B2 were purchased from Proteintech Group. The secondary horseradish peroxidase (HRP)-labeled goat-anti-rabbit antibody was purchased from Cell Signaling Technology. All of the other chemicals used in this study were analytical grade.

Animals and experimental design

Male C57BL/6 mice (18–22 g body weight, bw) were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China). The Ethics Committee approved the present study of Joekai Biotechnology Co., Ltd. (JK (2021)-W-003, Beijing, China). The mice were individually housed in standard cages under a given temperature (23 ± 2°C) and humidity (60 ± 5%) with a 12-h light/dark cycle and were allowed free access to food and water. All protocols were carried out in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, enacted by the Ministry of Science and Technology of China. After one week adaptation period, 50 mice were stochastically divided into five experimental groups, which were the control group, alcohol group, alcohol and low/medium/high dosage α-pinene treatment group [17]. The detailed information was shown in Table 1 (n=10).

Groups	Treatments
Control	Vehicle-treated mice
Alcohol	Alcohol-treated mice (5 mL/kg bw/day (2.0 g/kg bw/day))
AαL (Alcohol+ low dose α-pinene)	Alcohol-treated mice receiving low-dose α-pinene (5 mL/kg bw/day (7.2 mg/kg bw, dissolved in 50% alcohol)) intragastrically
AαM (Alcohol+ medium dose α-pinene)	Alcohol-treated mice receiving medium-dose α-pinene (5 mL/kg bw/day (14.4 mg/kg bw, dissolved in 50% alcohol)) intragastrically
AαH (Alcohol+ high doseα-pinene)	Alcohol-treated mice receiving high-dose α-pinene (5 mL/kg bw/day (28.8 mg/kg bw, dissolved in 50% alcohol)) intragastrically

Table 1: Experimental groups and treatments in 9 weeks.

Each group was given a gavage at the dose listed above. The dose of gavage in mice was based on the WHO[18], and National Institute on Alcohol Abuse and Alcoholism [21] recommends 12.0–15.0 g of pure alcohol for an adult male (60–70 kg/body weight [BW]). The specific dosage was further screened by the results of using cck-8 detection and then were calculated the appropriate dose according to the peripheral blood volume (as shown in Supplementary Figure). After the 8-week experiment, the mice were anesthetized and sacrificed after 12 h of fasting. Blood samples were collected and centrifuged after blood clot formation determination of serum marker enzymes and lipid levels. After resecting the liver, the organ was washed with cold saline and weighed. Two small sections of the same liver lobe were fixed in each animal for pathological observation. The remaining liver tissue was frozen in liquid nitrogen and stored at -80°C until analysis[19].

Biochemical analysis and pathological evaluation

The level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), malondialdehyde (MDA) and the activities of superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) were determined according to the manufacturer's protocol (Nanjing Jiancheng

Bioengineering Institute, Nanjing, China). First, for pathological evaluation, the liver tissue was fixed with 4% paraformaldehyde. Then, the tissue (5 μm) was cut, trimmed, dehydrated, embedded, sectioned, stained, mounted, and finally microscopically inspected, and stained with hematoxylin-eosin (HE). Finally, the slides of livers were observed and photographed using an optical microscope[19].

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

The total RNA was isolated from the hippocampus using Eastep® Super Total RNA Extraction Kit. cDNA was obtained using a reverse transcription kit and analyzed using the ABI7500 Real-Time fluorescence quantitative PCR system with a fluorescent dye[19]. GAPDH was used as an internal reference, and the relative gene expressions of *AMPK-α2, SREBP-1c, Hmox-1, Gsta-1, Nqo-1, LC3, P62, Beclin-1, TNF-α, NF-κB, IL-1β, IL-6, Bax, Bcl-2* were measured according to the $2^{-\Delta\Delta Ct}$ formula[20]. The primers used are listed in **Table 2**.

	Forward Primers	Reverse Primers
AMPK-α2	GCTACCTATTTCCTGAAGACCCCTC	CTTGGTTCATTATTCTCCGATTGTC
SREBP-1c	TAGAGCGAGCGTTGAACTGTATTG	CCATGCTGGAGCTGACAGAGAA
NRF2	TCTCCTCGCTGGAAAAAGAA	AATGTGCTGGCTGTGCTTTA
Hmox-1	TGCAGGTGATGCTGACAGAGG	GGGATGAGCTAGTGCTGATCTGG
Gsta-1	TGGGAATTTGATGTTTGACC	CAGGGCTCTCTCCTTCATGT
Nqo-1	CAGCCAATCAGCGTTCGGTA	CTTCATGGCGTAGTTGATGATGTC
LC3	GACGGCTTCCTGTACATGGTTT	TGGAGTCTTACACAGCCATTGC
P62	TGTGGAACATGGAGGGAAGAG	TGTGCCTGTGCTGGAACTTTC
Beclin-1	GTGCGCTACGCCCAGATC	GTGCGCTACGCCCAGATC
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
NF-κB	GAGGCACGAGGCTCCTTTTCT	GTAGCTGCATGGAGACTCGAACA
IL-1β	CTCCATGAGCTTTGTACAAGG	TGCTGATGTACCAGTTGGGG
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
BAX	AGCAAACTGGTGCTCAAGGC	CCACAAAGATGGTCACTGTC
Bcl-2	AGTGGTATAGACAGGTCTGTTGG	CCCCACCGAACTCAAAGAAGG
GAPDH	CTCAACTACATGGTCTACATGTTCCA	CCATTCTCGGCCTTGACTGT
AMPK-α2	GCTACCTATTTCCTGAAGACCCCTC	CTTGGTTCATTATTCTCCGATTGTC
SREBP-1c	TAGAGCGAGCGTTGAACTGTATTG	CCATGCTGGAGCTGACAGAGAA
NRF2	TCTCCTCGCTGGAAAAAGAA	AATGTGCTGGCTGTGCTTTA

Table 2: The sequence of primers used for qRT-PCR assay in this study.

Western blot analysis

The liver tissues were lysed with Protein Extract A containing protease inhibitor cocktails and centrifuged at 12,000 g for 15 min at 4°C. The total protein concentration from the supernatant was determined with a BCA protein assay kit. The proteins in the nucleus were passed through the nuclear protein extraction cassette. Samples were heated at 98°C for 10 min and then centrifuged at 13,800 g for 10 min. Next, 10 μL of supernatant was loaded onto each sample's 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% milk for 1 h at room temperature in TBS/T (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated overnight with primary antibodies at 4°C[21]. The membrane was washed three times with TBST for 10 minutes, incubated with secondary antibodies for 40 minutes, and developed with the Chenmi Scope 6200 Touch Chemiluminescence imaging system.

Statistical analysis

All of the data were expressed as mean ± standard error of means (SEM) and analyzed by SPSS 25 software. Significant differences among groups were tested by one-way analysis of variance (ANOVA) with $P < 0.05$ regarded as significant.

Results

α-pinene alleviated pathology of alcoholic liver injury

As general indicators, ALT and AST in serum were used for the evaluation of liver injury. The results shown in Figure 1A, the level of serum ALT ($p<0.001$) and AST ($p<0.05$) in the alcohol group was much higher than that of the control group. However, the levels of these enzymes in AαL, AαM, and AαH groups with the treatment of α-pinene were significantly decreased ($p<0.001$, for each protein). Further study revealed that serum TC, TG and LDL levels in the alcohol group were significantly higher than those observed in the control group ($p<0.01$, $p<0.05$, $p<0.05$, respectively) (Figure. 1B–1C). On the other hand, administration of α-pinene could significantly suppress the elevation of

serum TC (AαL, AαM, AαH, p<0.001, p<0.05, p<0.001, respectively), TG (AαM, AαH, p<0.01, p<0.01, respectively) and LDL (AαL, AαH, p<0.01, p<0.01, respectively). Interestingly, a statistically significant increase was observed in the high-dose α-pinene treatment group compared no matter with the control group or alcohol group (AαH, p<0.001).

Then, HE staining photomicrographs of liver histology were conducted and the results were shown in Figure. 1D. Compared with control animals, daily alcohol intake caused more steatosis (fat accumulation) and greater inflammatory injury (Kupffer cell activation) in mice liver. Meanwhile, treatment with α-pinene alleviated the harmful effects of chronic alcohol intake and reduced the infiltration of Kupffer cells and hepatocytes in liver tissue, the same as biochemical analyses which also showed a potentially protective effect

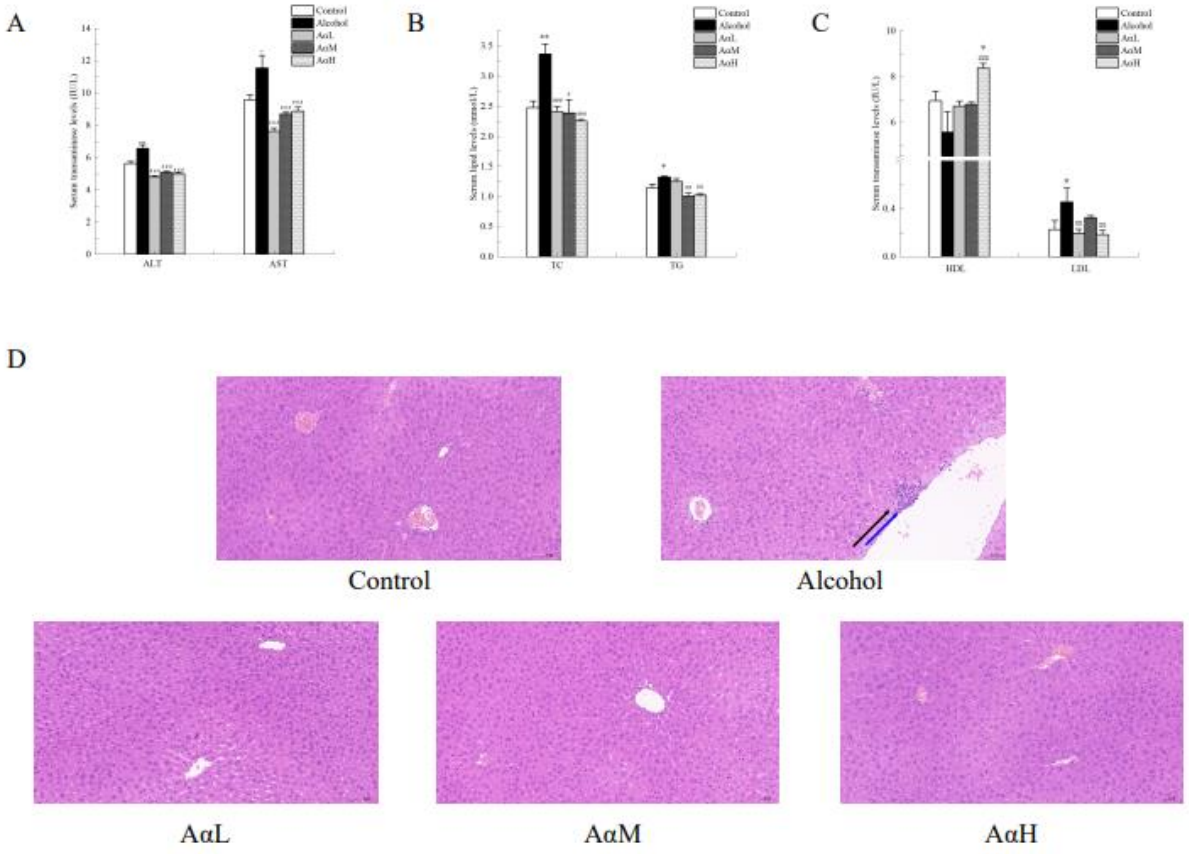

Figure 1: α-pinene alleviated alcoholic liver injury. Effect of α-pinene on (A) serum marker enzymes and (B) lipids. (C) Representative photomicrographs of HE staining of liver sections. Alcohol treatment induced Hepatic steatosis (black arrow) and focal infiltration of lymphocytes (blue arrow). Values are expressed as the mean ± SEM (n = 10, *p< 0.05, **P < 0.01, ***P < 0.0001 compared with the control group. #P < 0.05 and ##P < 0.01, ###p< 0.001 compared with the model group).

α-pinene is involved in the regulation of lipid peroxidation and multiple enzyme activities

The hepatic MDA, a marker of lipid peroxidation, and oxidative stress were detected in this study and the results were shown in Figure. 2A. MDA level was significantly increased after chronic alcohol feeding compared with the control group (p<0.001). However, the MDA content in AαL, AαM, and AαH groups (p<0.001, p<0.001, p<0.001) were dramatically decreased. Moreover, chronic alcohol intake significantly attenuated total hepatic GSH level (p<0.05), SOD activity (p<0.01,) and CAT activity (p<0.05) (See Figure. 2B–2D). On the contrary, the activity of these indicators including GSH level, SOD, and CAT activity (AαL, AαH, p<0.05, p<0.05, respectively) were significantly increased in the groups administrated with α-pinene. These results suggested that α-pinene protects liver tissues from alcohol-consuming via downregulation of lipid peroxidation and upregulation of GSH level and SOD and CAT activities.

Previous studies implicated AMPK and SREBP as proteins involved in regulating lipid metabolism and synthesis [22, 23]. As shown in Figure. 2E–2F, chronic ingestion of alcohol dramatically increased the mRNA expression of SREBP-1c (p<0.001). However, administration of α-pinene significantly inhibited the over-expression of SREBP-1c (AαL, p<0.05; AαM, p<0.01; AαH, p<0.01, respectively). In addition, the expression of AMPK-α2 was significantly increased in the α-pinene groups (AαM, p<0.05; AαH, p<0.01, respectively) compared to the control group and alcohol group (AαH, p<0.01). Taken together, α-pinene not only activated various antioxidant enzymes but also enhanced lipid catabolism and inhibited lipid synthesis to regulate lipid peroxidation.

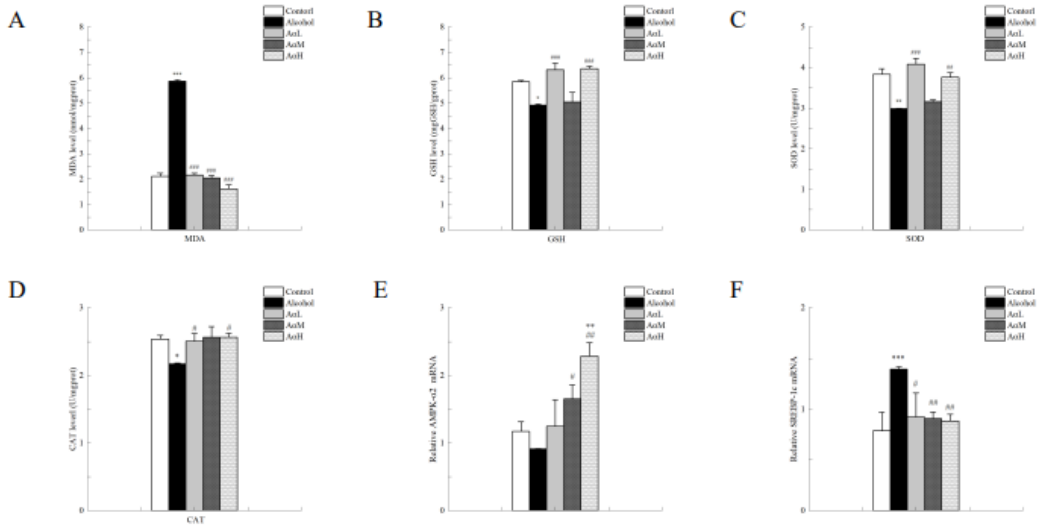

Figure 2. α-pinene treatment reduced lipid peroxidation and enhanced multiple enzyme activities. Effect of α-pinene on the levels of (A) MDA, and (B) GSH, (C) SOD activity, (D) CAT activity, and the expression of hepatic lipid relative mRNA levels of (E) AMPK-α2 and (F) SREBP-1c. Data are expressed as the mean ± SEM (n = 10, *p< 0.05, **P < 0.01, ***P < 0.0001 compared with the control group. #P < 0.05 and ##P < 0.01, ###p< 0.001 compared with the model group).

α-pinene protects mice liver tissue by inhibiting oxidative stress

It has been suggested that the KEAP1-NRF2 pathway played an important role in responding to oxidative stress. As a transcription factor, NRF2 was translocated into the nucleus and upregulated the related target genes, including *HMOX-1, Gsta-1, and NQO-1*, among others, when the antioxidant mechanism was activated [24]. Therefore, nuclear proteins were separated in this study from the total protein of the mouse liver, and western blotting was employed to analyze the nuclear-translocated NRF2. Compared with the control groups, nuclear-NRF2 decreased significantly in the alcohol group (p<0.01), whereas more NRF2 was observed in α-pinene treatment groups (AαL, p<0.05; AαM, p<0.01; AαH, p<0.001, respectively; see Figure. 3A–3B). Furthermore, the mRNA expression levels of *HMOX-1, Gsta-1, and Nqo-1* were dramatically increased in the α-pinene treatment group compared with the control and alcohol groups, which confirmed the expression level of the above indicator protein (see Figure. 3C–3E).

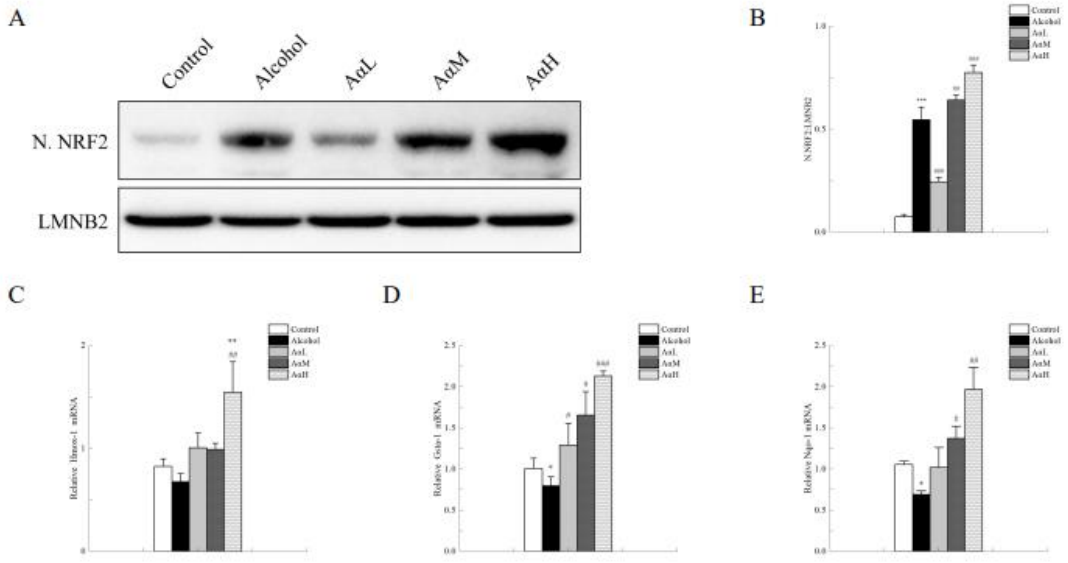

Figure 3. α-pinene promoted anti-oxidation. (A) The expression levels of NRF2 in the cell nucleus were detected by western blotting analysis. (B) Quantification of Nrf2 in cell nucleus expression. The mRNA levels of (C) HMOX-1, (D) Gsta-1, (E) Nqo-1. Data are expressed as the mean ± SEM (n = 10, *p< 0.05, **P < 0.01, ***P < 0.0001 compared with the control group. #P < 0.05 and ##P < 0.01, ###p< 0.001 compared with the model group).

Autophagy was activated by α-pinene to protect mouse liver tissue

AMPK regulates fatty acids, glucose metabolism, and also an upstream key autophagy protein, mTOR [25]. To explore whether α-pinene can activate autophagy, the protein levels of two autophagy marker proteins, LC3 and P62, were detected. Compared with the control group, the LC3 was significantly decreased in the alcohol group ($p<0.05$), while the P62 was significantly increased ($p<0.001$; Figure. 4A–4C). This indicated that chronic consumption of alcohol suppressed autophagy in the liver. However, the LC3 in the α-pinene treatment group was significantly increased (AαM, $p<0.05$; AαH, $p<0.01$, respectively), and the P62 decreased dramatically (AαL, $p<0.01$; AαM, $p<0.01$; AαH, $p<0.001$, respectively). Moreover, the expression level of mRNA for LC3 and P62 was consistent with the level of protein in a dose-dependent manner (Figure. 4D–4F).

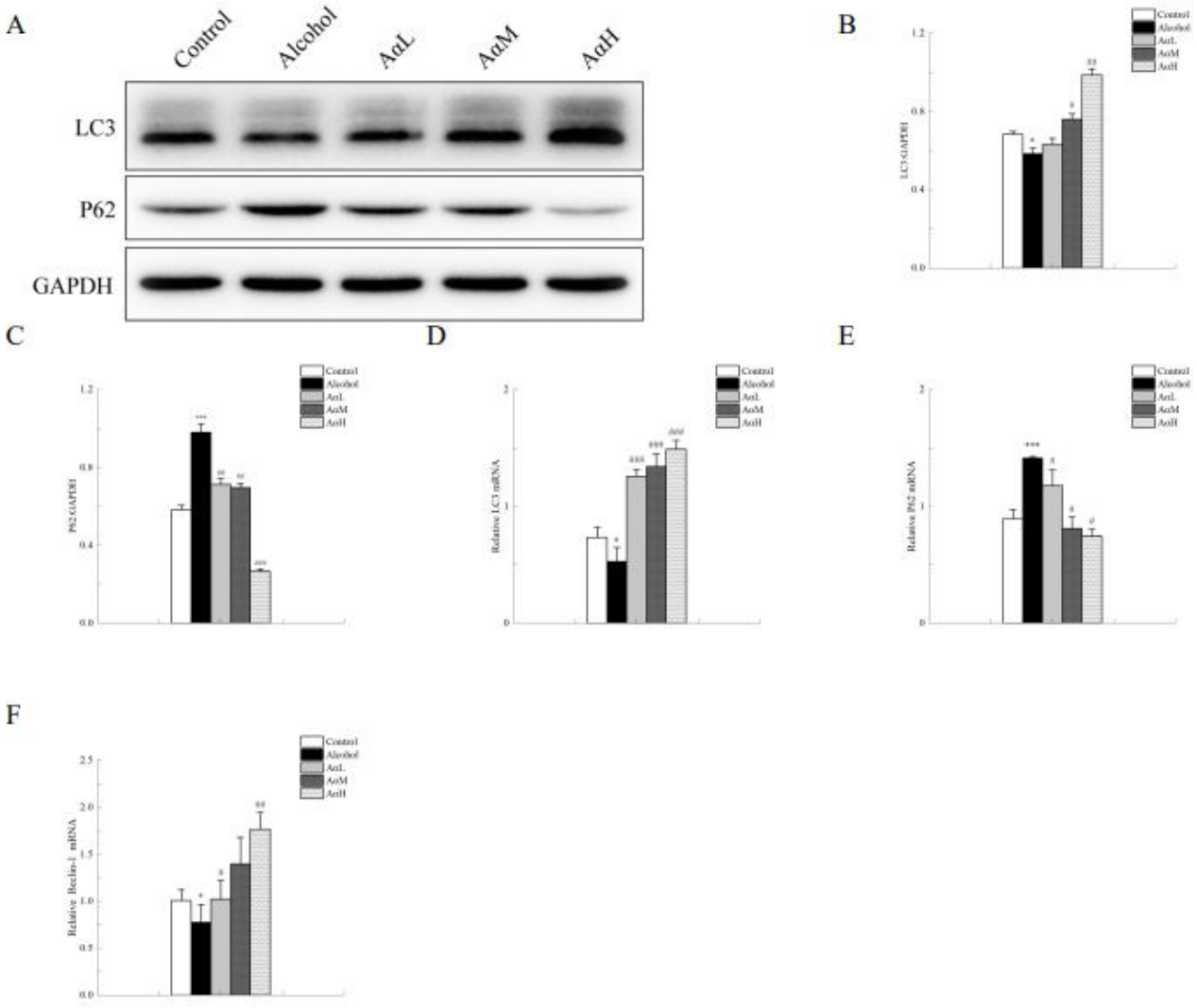

Figure 4: The effect of α-pinene on autophagy. (A) The expression levels of LC3 and P62 in mouse liver were detected by western blotting analysis. (B) Quantification of LC3/GAPDH. (C) Quantification of P62/GAPDH. The mRNA levels of (D) LC3, (E) P62, and (F) Beclin-1. Data are expressed as the mean ± SEM (n = 10, *p< 0.05, **P < 0.01, ***P < 0.0001 compared with the control group. #P < 0.05 and ##P < 0.01, ###p< 0.001 compared with the model group).

The expression levels of inflammatory mediators were suppressed by α-pinene

It is well known that cytokines play an important role in alcohol-induced mouse liver, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [26, 27]. Therefore, the expression level of INOS, COX-2, and NF-κB protein was detected. It was found that these cytokines in α-pinene treatment were significantly reduced compared to the alcohol groups (see Figure. 5A–5D). In addition, the mRNA expression levels of TNF-α, NF-κB, IL-1β, and IL-6 in mice livers were measured (see Figure. 5E–5H). Compared to the control group, these cytokines in the alcohol were significantly enhanced. However, these cytokines in α-pinene treatment were significantly reduced compared to alcohol groups.

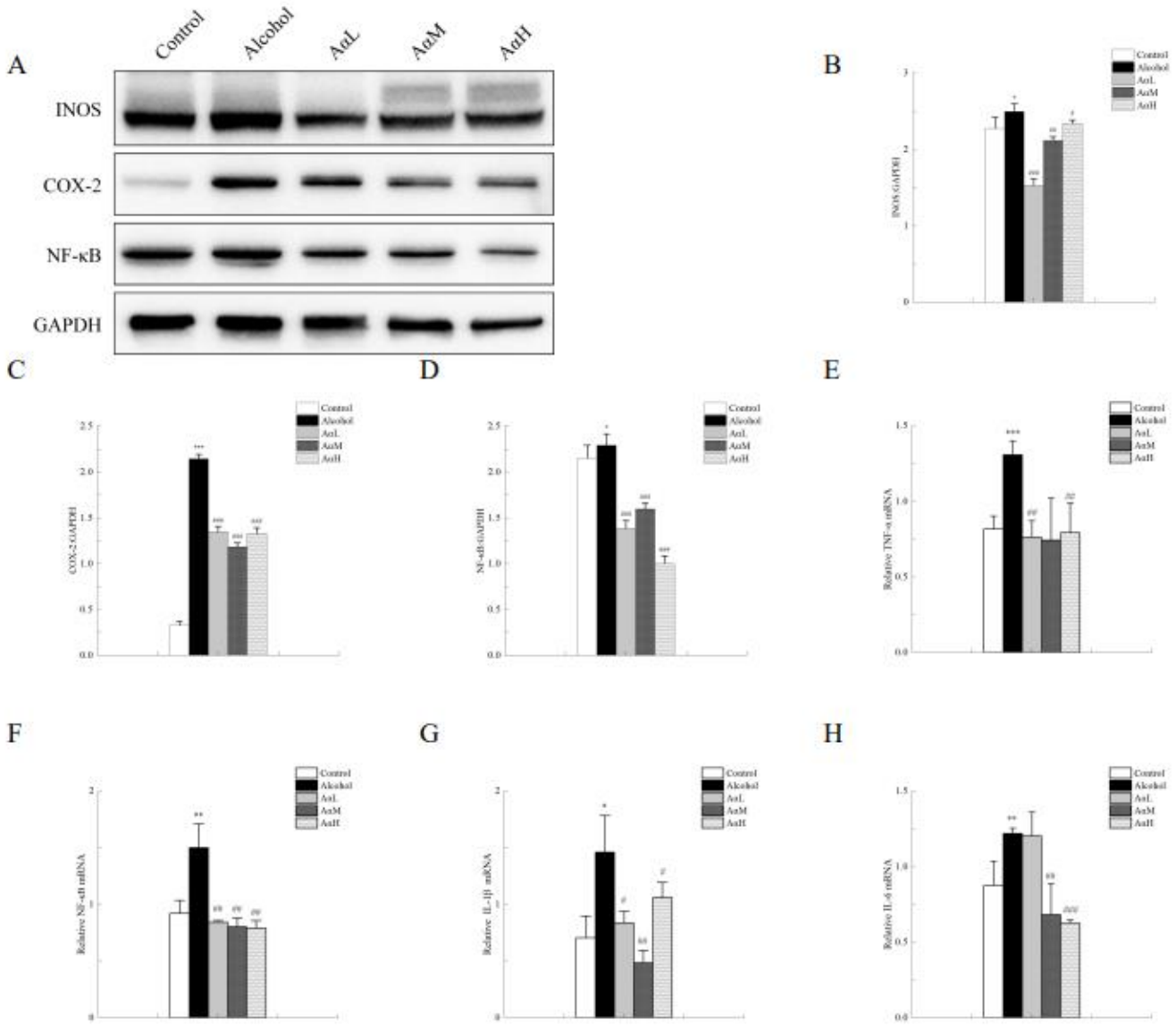

Figure 5: α-pinene reduced inflammatory response. (A) The expression levels of INOS, COX-2, and NF-κB in mouse liver were detected by western blotting analysis. (B) Quantification of INOS/GAPDH. (C) Quantification of COX-2/GAPDH. (D) Quantification of NF-κB/GAPDH. The mRNA levels of (E) TNF-α, (F) NF-κB, (G) IL-1β, (H) IL-6. Data are expressed as the mean ± SEM (n = 10, *p< 0.05, **P < 0.01, ***P < 0.0001 compared with the control group. #P < 0.05 and ##P < 0.01, ###p< 0.001 compared with the model group).

α-pinene protects mice liver tissue by inhibiting apoptosis

Caspase-3, a stable and inactive enzyme in normal cells, is processed into an active form when cells undergo apoptosis [28]. To investigate the effect of α-pinene on apoptosis, cleaved caspase-3 protein levels were monitored by western blot. The results showed that the expression of cleaved caspase-3 was significantly increased in the alcohol administration group compared with the control group ($p<0.01$; Figure. 6A–6B). However, α-pinene treatment reduced the cleaved caspase-3 level, which indicated that α-pinene could protect liver cells from apoptosis induced by chronic alcohol intake (AαL, AαM, AαH, $p<0.001$, $p<0.001$, $p<0.05$, respectively). In addition, it has been well demonstrated that the Bcl-2 family, including Bax and Bcl-2, plays a considerable role in the apoptotic pathway [29]. In this study, the expression levels of Bax and Bcl-2 in the α-pinene-treated group were significantly downregulated than the alcohol group (Figure. 6A,6C–6E). On the contrary, the expression levels of BCL-2 were significantly upregulated compared to the alcohol group. The above results indicated that α-pinene played an inhibitory role in apoptosis in mouse liver.

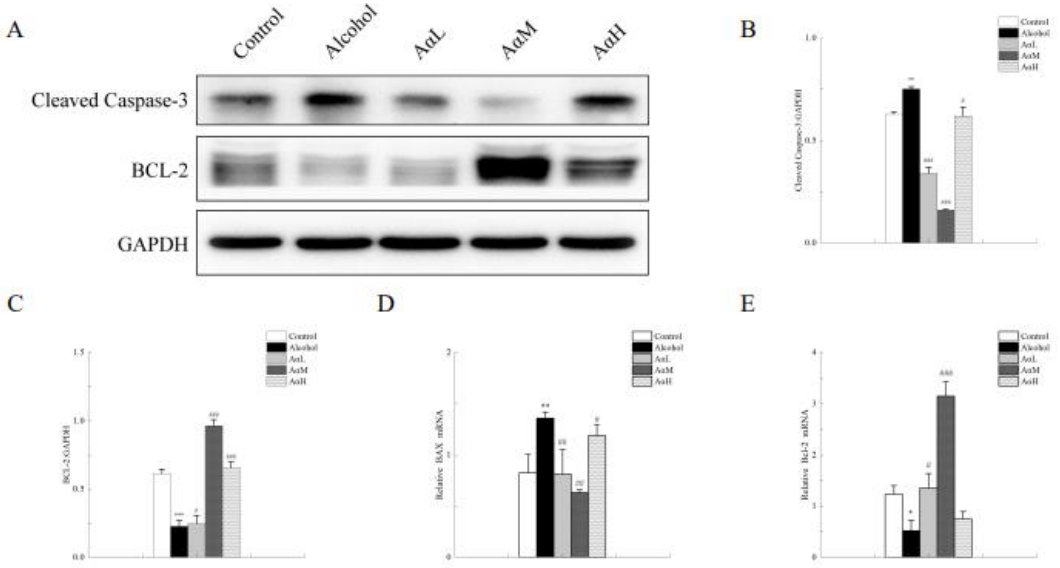

Figure 6: α-pinene declines apoptosis. (A) The expression levels of cleaved caspase-3 and BCL-2 in mouse liver were detected by western blotting analysis. (B) Quantification of cleaved caspase-3/GAPDH. (C) Quantification of BCL-2/GAPDH. The mRNA levels of (D) BAX and (E) BCL-2. Data are expressed as the mean ± SEM (n = 10, *p< 0.05, **P < 0.01, ***P < 0.0001 compared with the control group. #P < 0.05 and ##P < 0.01, ###p< 0.001 compared with the model group).

Discussion

Excessive alcohol consumption is a public health concern as it can lead to ALD, cirrhosis, and other related diseases [30, 31], which will further lead to the development of a variety of liver diseases, including fatty liver, AH, fibrosis, and cirrhosis [32]. Recent studies have shown that monoterpenoids have antioxidant and anti-inflammatory effects on protection cells [33, 34]. However, whether α-pinene played a similar role in chronic alcohol-induced liver injury should be investigated.

The study showed that long-term alcohol consumption caused liver injury as evidenced by elevation of serum ALT and AST activity, serum TC, TG, and LDL activity, as well as enlargement and fatty infiltration of hepatocytes, all of which reflected early biochemical and pathological changes in ALD [35]. However, after the treatment of α-pinene, the levels of these serum marker enzymes and lipids were close to normal or only slightly elevated, suggesting a protective role of α-pinene in liver cells (Figure. 1A–1C). In addition, histopathological liver changes induced by alcohol were also remarkably attenuated after α-pinene treatment (Figure. 1D). These results suggested that α-pinene was able to suppress the hepatotoxicity amidst chronic alcoholic liver injury[7, 9].

Chronic alcohol intake increases free radicals in the body, thereby leading to oxidative stress and alcoholic liver damage. Various pathways, including lipid peroxidation, the weakening of various antioxidant enzymes, the increase of lipogenesis, and the inhibition of fatty acid oxidation are thought to be involved in this process [36]. Our studies have confirmed that α-pinene can attenuate alcoholic liver damage and has a significant hepatoprotective effect, as evidenced by the significant reduction in lipid peroxidation marker MDA (Figure. 2A). In addition, the treatment of α-pinene also significantly increased the activities of antioxidant enzymes GSH, SOD, and CAT (Figure. 2B–D). Moreover, stimulation of AMPK can promote lipid metabolism and inhibit the expression of the SREBP-1c gene in mouse liver, which results in a decrease in adipogenesis and lipid accumulation [23]. However, α-pinene treatment significantly reduced the mRNA expression of SREBP-1c and significantly increased the expression of AMPK, following the decrease of TC and TG levels and the alleviation of liver histopathological changes. These results indicate that α-pinene can inhibit alcohol-dependent lipid accumulation by regulating the balance between lipid synthesis and catabolism in mouse liver cells, which may be mediated by the upregulation of AMPK and the downregulation of SREBP-1c expression (Figure. 2E–2F). Excessive lipid accumulation can induce oxidative stress [37]. Chang et al. suggested that enzyme-treated *Z. latifolia* extract protected cells from lipotoxicity by upregulating nuclear NRF2 levels and increasing the expression of downstream target genes *HO-1, NQO1, and GCLC* [38]. Our results showed that the expression level of NRF2 protein in the α-pinene treatment group increased in a concentration-dependent manner, with the maximum effect at AαH (Figure. 3A–3B) and it was consistent with previous research. In addition, after the treatment of α-pinene, the mRNA expression levels of target genes regulated by NRF2 increased in a concentration-dependent manner, with the maximum effect at AαH (Figure. 3C–3D). Therefore, the results of this study demonstrated that α-pinene could effectively exert an antioxidant effect and reduce the consequences of chronic alcohol ingestion to the mouse liver caused by oxidative damage.

In this study, changes in AMPK have been observed in lipid metabolism. Since the AMPK-mTOR pathway was a key pathway to regulating autophagy [39], we suspected that α-pinene could affect not only oxidative stress but also autophagy. Autophagy is a complex molecular mechanism in cells that can clean up damaged organelles and misfolded proteins in time [40, 41]. The expression levels of LC3-II and P62 were generally used to evaluate whether a certain substrate changes the autophagy flux of the cell. Typically, when autophagy is activated, the expression level of LC3-II increases, and the expression level of P62 decreases [42, 43]. In addition, Beclin-1 is an autophagy-related protein, its expression level will increase with the activation of autophagy [44]. The results showed that α-pinene treatment increased the mRNA expression level of LC3 and Beclin-1 in a concentration-dependent manner (Figure. 4A–4F). In contrast, the level of P62 decreased in a concentration-dependent manner. This result is consistent with our hypothesis that α-pinene protects the mouse liver by attenuating oxidative stress and activating autophagy (Figure. 4A–4F).

Inflammation is another factor that could cause liver disease, and its development would lead to the occurrence of many diseases. Long-term drinking can cause inflammation, and lead to an increasing level of inflammatory cytokines. Xiao-Jun Li et al. proved that α-pinene inhibits the inflammatory response and analgesia by inhibiting noxious stimulus-induced inflammatory infiltration and COX-2 over-expression [16]. Our study confirmed its anti-inflammatory effects by checking the protein expression of INOS, COX-2, NF-κB, and the mRNA expression levels of *TNF-α, NF-κB, IL-1β, and IL-6* (Figure. 5A–5H). The results showed that the protein expression of INOS, COX-2, and NF-κB, and the mRNA expression level of inflammatory cytokines treated with α-pinene was significantly downregulated, which is consistent with the previous study. Ultimately, α-pinene potentially protected the mouse liver by upregulating NRF2-mediated antioxidants, activating autophagy, and downregulating the TNF-α, NF-κB, IL-1β, IL-6, and inflammation pathways[7, 16].

There was the study showed that salvianolic acid α-pinene could prevent P13K/AKT/mTOR stimulation from being inhibited, and the expression of α-SMA, HYP, the proteins Bax and caspase-3/cleaved caspase-3 were all reduced. On the contrary, the expression of Bcl-2 was increased and thereby inhibited cell apoptosis [45]. Our results suggested that cleaved caspase-3 was decreased by the treatment of α-pinene (AαL, $p<0.001$; AαM, $p<0.001$; respectively). In addition, BAX was reduced in a dose-dependent manner (AαL, $p<0.01$; AαM, $p<0.01$, respectively). On the contrary, the expression level of Bcl-2 increased in a dose-dependent manner (AαL, AαM, $p<0.05$, $p<0.001$, respectively; Figure. 6A–6E).

Interestingly, however, in the AαH group, the cleaved caspase-3 expression was similar to the alcohol group. The previous study showed that, when the autophagy flux exceeds the cell's capacity, it will induce apoptosis [46-48]. Therefore, this phenomenon may be caused by excessive autophagy. In addition, the anti-apoptotic protein BCL2 was activated in the AαH, which is inconsistent with typical apoptosis results and therefore it also may relate to autophagy. Under normal circumstances, BCL-2 binds to Beclin-1 and inhibits autophagy [49]. Our results suggested that the expression of Beclin-1 was upregulated by the treatment of α-pinene. This may lead to the dissociation of BCL-2 from Beclin-1 and activate autophagy. This may result in the abundance of BCL-2 protein increasing [50].

In addition, Sirtuin 1, The role of the anti-aging gene, may be relevant to the effects of α-pinene on autophagy, apoptosis and the therapy for alcoholic liver injury [51, 52]. The previous studies shows that Sirtuin 1 is intimately tied to NRF2 and transcription of antioxidant target genes, including heme oxygenase 1 (HMOX-1 / HO-1), NAD[P]H quinone dehydrogenase 1 (NQO-1), AMPK, mTOR to limit oxidative stress, increase life span, improve insulin sensitivity and maintain mitochondrial function [53, 54]. Sirtuin 1 is critical to regulate autophagy and inflammation [55]. Activators and inhibitors of Sirtuin 1 may be important to alcoholic liver disease and α-pinene may be a Sirtuin 1 activator [56]. The research on Sirtuin 1 will be determined in the follow-up experiments.

Conclusion

This study demonstrated that α-pinene might protect the mouse liver from alcohol consumption by activating NRF2-mediated oxidative stress and autophagy, downregulating inflammatory cytokines, and inhibiting apoptosis. Thus, our results demonstrated the effects of α-pinene on autophagy and apoptosis, it provided a new perspective for treating an alcoholic liver injury. However, the molecular mechanism of α-pinene on autophagy needs to be further investigated.

Declaration of Competing Interest

We declare that we have no conflict of interest.

Data availability

The data used to support the results of this study are available from the corresponding authors upon request and permission.

Author contribution

Deliang Wang, Feike Hao, and Chunguang Luan conceived and designed the experiments. Yishu Li performed the experiments and statistical analysis and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval statement

The Ethics Committee approved the present study of Joekai Biotechnology Co., Ltd. (JK (2021)-W-003, Beijing, China). All protocols were carried out in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, enacted by the Ministry of Science and Technology of China.

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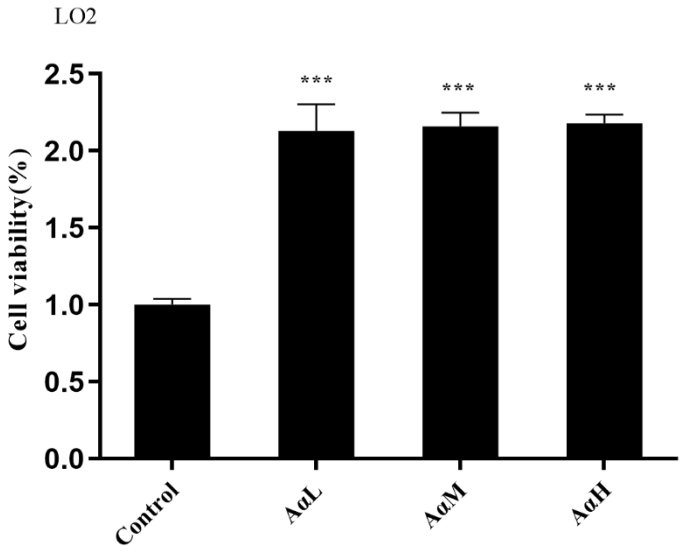
Supplemental material



Supplemental description. (A) The cell viability of LO2 cells was detected by the cck-8 kit. Data are expressed as the mean ± SEM (n = 10, *p< 0.05, **P < 0.01, ***P < 0.0001 compared with the control group.

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