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Original Article

Inula britannica ameliorates alcohol-induced liver injury by modulating SIRT1-AMPK/Nrf2/NF-κB signaling pathway

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ABSTRACT

Objective: *Inula britannica* is a traditional Chinese medicinal and functional food with various effects such as anti-liver injury, hypoglycemia, antioxidants, and anti-tumor. The aim of this study was to investigate the protective effects and mechanisms of the ethanolic extract of *I. britannica* (EEIB) on alcohol-induced liver injury in mice.

Methods: Fifty-six female C57BL/6 mice were randomly divided into seven groups: control group (Con), ethanol feeding model group (EtOH), Silibinin positive treatment group (EtOH + Silibinin 100 mg/kg), EEIB treatment group (EtOH + EEIB 100, 200, and 400 mg/kg), and EEIB control group (EEIB 400 mg/kg). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) ethanol-feeding model was used to study the effects of EEIB on alcohol-induced lipid metabolism, inflammation, oxidative stress, and fibril formation in mice by histopathological evaluation, immunofluorescence staining, Western blotting analysis and molecular docking.

Results: EEIB reduced liver indices to different degrees to normal levels and improved liver morphology in mice. EEIB inhibited alcohol-induced liver injury by activating the sirtuin 1 (SIRT1)-adenosine monophosphate-activated protein kinase (AMPK) signaling pathway in the liver of alcohol-fed mice, in which sesquiterpenes may be the potential active ingredients, and also down-regulated the expression of alpha-smooth muscle actin (α-SMA), collagen alpha (Collagen I), tumor necrosis factor-alpha (TNF-α) and attenuated alcohol-induced liver injury. In addition, EEIB also activated the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, which alleviated alcohol-induced liver injury at the level of oxidative stress. Notably, the EEIB control group demonstrated that EEIB had no toxic effects in mice. EEIB reduced alcoholic liver injury in a dose-dependent manner. Its therapeutic efficacy was comparable to, if not better than, that of Silibinin when administered at a dose of 400 mg/kg.

Conclusion: EEIB showed significant therapeutic effects on alcohol-induced liver injury in mice, and its mechanism of action was related to the SIRT1-AMPK, nuclear factor-kappa B (NF-κB), and Nrf2 signaling pathways, in which sesquiterpenes may be the potential active ingredients.

1. Introduction

Alcoholic liver disease (ALD) is a liver injury disease caused by excessive alcohol intake. Its pathogenesis involves multiple aspects, such as direct liver toxicity of alcohol and its metabolites, lipid peroxidation, oxidative stress, and immune inflammation (Cheng et al., 2023; Martinez-Castillo et al., 2023; Park, Lee, Sim, Seo, & Seo, 2022; Yan et al., 2023). According to data from the World Health Organization, more than three million people die each year due to drinking worldwide (Zhang et al., 2022), and alcoholism has a higher mortality rate than diabetes, tuberculosis, and acquired immune deficiency syndrome (AIDS) deaths. In general, alcohol is catalytically metabolized by acetaldehyde dehydrogenase (ALDH) and ethanol dehydrogenase (ADH) and finally oxidized in the body to carbon dioxide and water (Alharshawi & Aloman, 2021). However, this process induces fatty acid synthesis and reduces fatty acid oxidation, leading to the accumulation of triglycerides and the formation of a fatty liver (Namachivayam & Valsala Gopalakrishnan, 2021). Fatty liver is the early stage of ALD progression, which will further develop into steatohepatitis with inflammation in the middle stage, and collagen deposition in the late stage will lead to liver fibrosis and cirrhosis (Liu et al., 2018).

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Steatosis is the earliest stress response of the liver to alcoholism (Gao & Bataller, 2011). Chronic alcohol consumption induces disturbances in lipid metabolism and increases fatty acid synthesis in the liver, leading to the production of large amounts of fat. Adenosine 5′-monophosphate-activated protein kinase (AMPK), a major regulator of lipid metabolism, inhibits lipid synthesis and increases fatty acid oxidation (Steinberg & Kemp, 2009). However, long-term alcohol consumption reduces AMPK activity in rodents (Garcia-Villafranca, Guillen, & Castro, 2008). Attenuation of AMPK plays a central role in regulating fatty acid oxidation and synthesis (Tang, Huang, Lee, Tang, & Wang, 2013). AMPK is considered to be a critical master switch that regulates lipid metabolism by modulating gene transcription in muscle, liver, and fat (Wang, Liu, Zhai, Zhang, & Tian, 2018). In addition, large amounts of reactive oxygen species (ROS) are produced during alcohol metabolism (Sun, Zhong, Zhang, & Zhou, 2016). Under normal physiological conditions, the liver's antioxidant defense system can clear ROS to resist oxidative stress. However, excessive alcohol consumption leads to a significant increase in ROS levels in tissues and a decrease in the activity of antioxidant levels, leading to liver oxidative stress injury. ROS are mainly scavenged by glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) (Kalyanaraman, 2013). Chronic alcohol consumption decreases the activity of antioxidant enzymes in the body and disrupts the oxidative balance. (Leung & Nieto, 2013). Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential regulator of adaptive antioxidant responses to oxidative stress *in vivo* (Xue et al., 2016). Studies have shown that drinking alcohol can reduce the expression of Nrf2 in the liver, leading to hepatic lipid accumulation, and the activation of Nrf2 can successfully prevent alcohol-induced fatty liver (Lamle et al., 2008; Wu, Liu, & Klaassen, 2012). Notably, lipid accumulation and oxidative stress damage caused by continuous alcohol consumption can induce inflammation in the liver. Continuous alcohol intake can disrupt the normal intestinal microbial status, increase the permeability of the intestinal mucosa, and allow endotoxins to enter the blood circulation (Wang et al., 2015). Endotoxin can damage liver cells and, at the same time, activate Kupffer cells to activate the NF-κB signaling pathway and generate inflammation factors like IL-6, TNF-α, etc., thereby aggravating liver injury (Liu et al., 2022). Therefore, ALD may be ameliorated by modulating lipid metabolism and oxidative stress, and attenuating inflammatory responses.

Inulae Flos (Xuanfuhua in Chinese) usually refers to the dry inflorescence of *Inula britannica* L., a plant of the genus *Inula* in the family Asteraceae. It is mainly distributed in the Europe, Asia, Africa, and other places (Hong et al., 2012). There are more than 20 varieties in China. It has been active as a medicinal and edible food in people's lives in China for a long time. A large number of studies on *I. britannica* at home and abroad have found that *I. britannica* contains a variety of functional components, such as flavonoids, sesquiterpenes, and sterols (Khan et al., 2010; Qin et al., 2009; Yang, Wang, & Jia, 2003). Modern pharmacological studies have shown that sesquiterpenoids and flavonoids have shown suitable biological activities in anti-liver damage (Dong et al., 2013), anti-tumor (Li et al., 2013), anti-oxidation (Kim, Lee, Song, & Kim, 2020) and neuroprotection (Tang et al., 2021). Due to the great potential of *I. britannica* in medicinal use, it has attracted extensive attention from scholars at home and abroad. It is of great significance for developing medicinal resources and extracting natural active substances for the medical and health industries.

In this paper, the protective mechanism of ethanolic extract of *I. britannica* (EEIB) on alcoholic liver injury was evaluated by establishing an alcohol-induced liver injury model in mice, and the active ingredients and potential mechanisms of *I. britannica* to exert anti-alcoholic liver injury were explored.

2. Materials and methods

2.1. Materials and reagents

The medicinal material *I. britannica* was purchased from Anguo City, Hebei Province, China. Silibinin (Sil) was purchased from Sigam (St. Louis, USA). Standard Lieber-DeCarli alcohol liquid feed and Lieber-DeCarli control liquid feed were purchased from Nantong Trofee Feed Technology Co., Ltd. (Nantong, China). Glutathione Peroxidase (GSH-Px), alanine aminotransferase (ALT), glutathione peroxidase (SOD), aspartate aminotransferase (AST), malondialdehyde (MDA), total cholesterol (TC), and other kits were purchased from Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, China). Hematoxylin staining solution, eosin staining solution and oil red O staining solution were purchased from Wuhan Sevier Biotechnology Co., Ltd. (Wuhan, China). Interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) ELISA kits were purchased from Shanghai Enzyme Biotechnology Co., Ltd. (Shanghai, China). Rabbit polyclonal antibody, such as Nrf2, heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase quinone 1 (NQO1), SIRT1, phosphorylated AMPK (p-AMPK), AMPK, acetyl-CoA-carboxylase (ACC), Collagen I, phosphorylated ACC (p-ACC), α-SMA, inhibitory subunit kappa B alpha (IκB-α), phosphorylated IκB-α (p-IκB-α), transcription factor p65 (P65), TNF-α, and horseradish peroxidase-labeled goat anti-mouse/rabbit IgG (1: 1000) were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China). All other reagents were of the highest grade available.

2.2. Animal and experimental design

2.2.1. Drug preparation

The medicinal herb *I. britannica* was identified by Professor Jincai Lu, Department of Medicinal Plants, School of Chinese Materia Medica, Shenyang Pharmaceutical University, and the plant specimens (No. 20210126) were stored in this laboratory. The weighing balance weighed 50 g of *I. britannica* air-dried product, the material-liquid ratio was 1: 15, 70% ethanol was refluxed for 2 h, the filtrate was taken, and the concentration was reduced pressure to obtain EEIB.

2.2.2. National Institute on alcohol abuse and alcoholism (NIAAA) mouse model establishment

Fifty-six female C57BL/6 mice weighing about 20–25 g were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Shenyang, China). The routine operation of animal experiments was by the regulations on the Administration of Laboratory Animals. All animal experiments in this study were approved by the Laboratory Animal Ethics Committee of Shenyang Pharmaceutical University on 22 November 2021 [license number: SCXK (Liao) 2020–0001]. Adaptive feeding for one week before the experiment.

Mice were randomly divided into seven groups: control group (Con), ethanol feeding model group (EtOH), positive drug group (EtOH + Silibinin 100 mg/kg), model + EEIB group (EtOH + EEIB 100, 200, 400 mg/kg), EEIB group (EEIB 400 mg/kg). Eight animals per group were liquid-fed in the first five days. Then, as shown in Fig. 1A, it is administered for 10 d. Lieber-DeCarli alcoholic liquid feed and control liquid feed were prepared by Table S1. After the treatment, the mouse was sacrificed after blood was taken from the orbit, the liver was dissected, weighed and photographed to observe the liver morphology, and then part of the liver was fixed with formalin, and part of it was directly frozen at −80 °C for subsequent experiments.

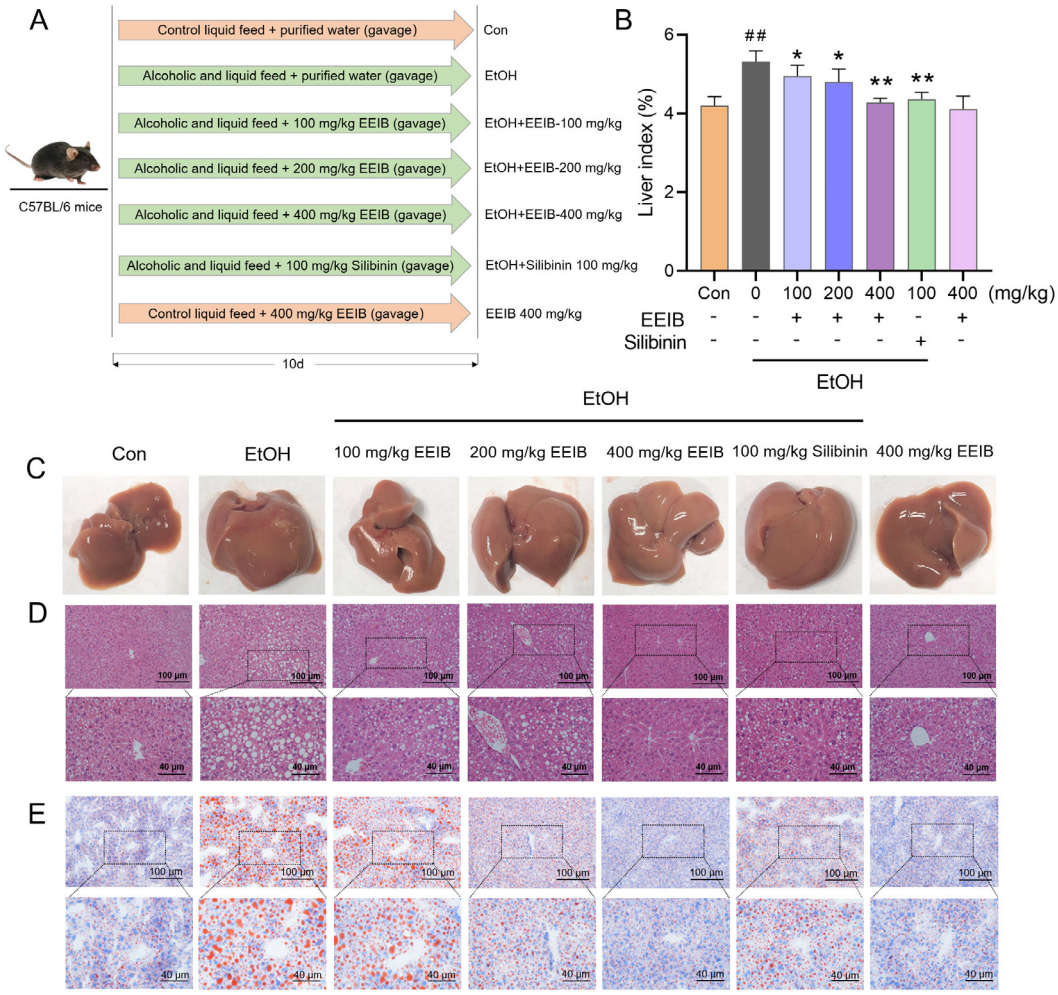

Fig. 1. Effects of EEIB on alcohol-induced liver and liver morphology in mice. (A) Animal model flow diagram; (B) Mouse liver index (mean ± SD, n = 8); (C) Liver morphology of mice; (D) H&E staining; (E) Oil Red O staining (magnification: × 20, scale bar = 100 μm; magnification: × 40, scale bar = 40 μm). $^{\#\#}P < 0.01$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* EtOH group.

2.3. Liver index

Mice's body weight was recorded daily during the experiment before gavage administration. After sacrificing the mouse, the liver was weighted to calculate the liver index. Liver index (%) = liver weight (g) / mouse body weight (g) × 100.

2.4. Liver histopathological assessment

After the liver tissue was stripped, it was fixed with 4% paraformaldehyde for 2 h and then embedded in paraffin after dehydration by gradient ethanol and xylene. After solidification, it was cut into a cross-sectional section of the liver with a thickness of 5 μm, which was used for subsequent pathological staining.

2.4.1. Hematoxylin and eosin (H&E) and oil red O staining

Liver sections were dewaxed in xylene, dehydrated in ethanol, and rinsed under running water. They were stained with H&E and oil red O and sealed with neutral glue. Finally, the morphology of the stained tissue was observed under × 20 and × 40 light microscopy and photographed and recorded.

2.4.2. Immunohistochemical staining detection

Liver slices were incubated with primary antibodies of Nrf2, α-SMA, Collagen I, and IL-1β at 4 °C overnight, and then incubated with coupled secondary antibodies of horseradish peroxidase (HRP) at room temperature and dark for 50 min. Sections were stained with hematoxylin and observed under a light microscope.

2.4.3. Immunofluorescence detection

After the liver slices were treated, rabbit anti-SIRT1 and NF-κB antibodies were added respectively, which were refrigerated at 4 °C overnight. The second antibody was added on the second day, incubated at room temperature and away from light for 2 h, and observed under a light microscope.

2.5. Detection of serum biochemical indexes

Orbital blood was taken and centrifuged in a centrifuge at 4 °C and 4 000 r/min for 30 min to aspirate the upper serum layer. The AST, ALT, TC, and TG levels were determined according to the kit instructions.

2.6. Detection of antioxidant indexes in liver tissue

The washed liver tissue was added nine times the amount of 0.9% normal saline to make 10% tissue homogenate, low-temperature centrifugation was conducted at 12 000 r/min for 10 min, and the supernatant was taken as the sample to be tested. MDA, GSH-Px, and SOD levels in liver tissue were measured using the kit.

2.7. Enzyme-linked immunosorbent assays (ELISA)

The liver tissue was thawed and then homogenized in cold phosphate buffered saline (PBS) (1: 9, mass to volume ratio). The liver homogenate was then centrifuged for 20 min, and samples were taken for testing. Detection of IL-6, IL-1β, and TNF-α was performed using ELISA kits.

2.8. Western blotting analysis

Liver tissue was lysed with lysate to obtain tissue protein extract. Protein concentrations were measured in all samples using the BCA kit and SDS loading buffer proteins were added. Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose (NC) membrane. The membrane was then blocked with 5% skim milk. Primary antibody incubation at 4 °C overnight and washing with tris buffered saline with Tween (TBST) were then performed. After incubation with the secondary antibody at room temperature for 1 h, after TBST washing, enhanced chemiluminescence (ECL) luminescent liquid (A: B = 1: 1) was added, and the exposure time was adjusted according to the luminescence situation. The protein band grayscale values were analyzed by Image J software.

2.9. Isolation and prediction of active ingredients

2.9.1. Isolation of active ingredients

Fifteen known compounds were isolated from EEIB using various chromatographic techniques such as silica gel column chromatography, Sephadex LH-20 gel column chromatography, high-performance liquid chromatography (HPLC) and preparative thin-layer chromatography (PTLC), and their structures were identified by high resolution electrospray ionization mass spectroscopy (HR-ESI-MS) as ayapin (Adfa, Yoshimura, Komura, & Koketsu, 2010) (IB-1), 2,3-dihydroaromaticin (Lee,Yang,Kim,Seo,& Mar, 2002) (IB-2), ergolide (Zeng et al., 2009) (IB-3), loliolide (De Marino et al., 2006) (IB-4), syringaldehyde (Kleczek, Malarz, & Kosecka-Strojek, 2019) (IB-5), 4H-tomentosin (Xin et al., 2020) (IB-6), bigelovin (Cheng et al., 2012) (IB-7), 1*β*-hydroxyalantolactone (Bohlmann, Mahant, Jakupovi, Rastogi, & Natu, 1978) (IB-8), inuviscolide (Mossa et al., 1997) (IB-9), 3-ethoxy-hydroxybenzoic acid (Harvala et al., 2002) (IB-10), scopoletin (Li, Wang, Lai, Feng, & Zhou, 2011) (IB-11), 1-*O*-acetyl-4*R*,6*S*-britannilactone (Je, Han, Lee, Mar, & Seo, 2004) (IB-12), 2*R*-acetoxy-4*R*-hydroxy-1*β*-guai-1 1(13),10(14)-dien-12,8α-olide (Nie et al., 2010) (IB-13), ent-16α,1 7-dihydroxy-kauran-19-oic acid (Sung et al., 2011) (IB-14), britannilactone (Xiang, Guo, Han, Gao, & Tang, 2016) (IB-15). The contents of the above 15 known compounds in mouse blood were determined by ultra performance liquid chromatography mass spectrometry (UPLC-MS) analysis. The structures of the compounds and the conditions for the determination of the blood content and the results were shown in Table S2–S3.

2.9.2. Molecular docking

Molecular docking studies were conducted using Discovery Studio (DS) 2016/LibDock software to explore the binding patterns of the active components of EEIB to AMPK signaling pathway proteins that play an essential regulatory role in the protection against liver injury. The three compounds with the highest blood levels in mice: IB-2, IB-12, IB-15, and ACC (PDBID: 1UYT) and SIRT1 (PDBID: 4IG9) were used as ligands and target proteins for molecular docking. The 2D structure of the ligands was drawn on ChemDraw software drawing and DS to generate stereoisomers of the small molecule ligand. The PDB structure format of the protein was downloaded from the RCSB PDB database (https://www.rcsb.org/) and imported it into the software for molecular docking. Interactions between IB and 2, IB-12, IB-15, and target proteins were determined by calculating LibDock scores.

2.10. Statistical analysis

Statistical analysis was completed using GraphPad Prism 9 (GraphPad Software, San Diego, USA). Comparisons between the two groups were made using the *t*-test, and multiple comparisons were made by One-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant. Data obtained were expressed as mean ± SD.

3. Results

3.1. Effects of EEIB on alcohol-induced liver and liver morphology in mice

Changes in liver indices of mice in seven groups of the alcoholic liver injury model were shown in Fig. 1B. The data results were significantly lower ($P < 0.01$) in the control group compared to the liver index in the model group, which indicated that alcohol intake could increase the liver weight, decrease the body weight of mice, and increase the liver index. Each treatment group's liver index was lower than that in the model group. It was illustrated that EEIB can improve the abnormality of the liver index. In addition, compared with the control group, the liver index of mice treated with EEIB alone did not change. The liver morphology of the model group was enlarged compared with that of the control group, and the liver morphology was improved after the administration of EEIB (Fig. 1C). H&E and Oil Red O (ORO) staining (Fig. 1D and E) showed that the liver tissue structure of normal mice was clear and the hepatocytes were evenly arranged, while some pathological phenomena, such as structural disorder, abnormal cell morphology, fat vacuolation, and inflammatory cell infiltration, in the model group were observed. Hepatic lipid droplet accumulation was reduced in the EEIB group, especially in the high-dose group, and there was a significant improvement in cell morphology and steatosis, suggesting a protective effect of EEIB against steatosis. There were no significant changes in the liver of the EEIB alone (non-alcohol-fed) group compared to the control group. The above results indicated that EEIB had no toxic effects on mice and that alcohol could cause damage to liver tissue. EEIB had a good protective effect against alcohol-induced liver damage.

3.2. Effect of EEIB on serum biochemical indexes in mice

To verify the diagnostic and therapeutic effects of EEIB on ALD, some common biochemical indexes in the serum of mice were detected. TC and TG levels were significantly higher ($P < 0.001$) in the model than those in the control groups (Fig. 2A and B). In contrast, the serum TC and TG levels in the EEIB treatment group decreased gradually with the increase in dose. In addition, AST and ALT levels in model mice were significantly higher than those in control mice ($P < 0.001$) and decreased after treatment with EEIB (Fig. 2C and D). It was also found that EEIB alone had little effect on the contents of TC, TG, AST, and ALT in the serum of mice. These

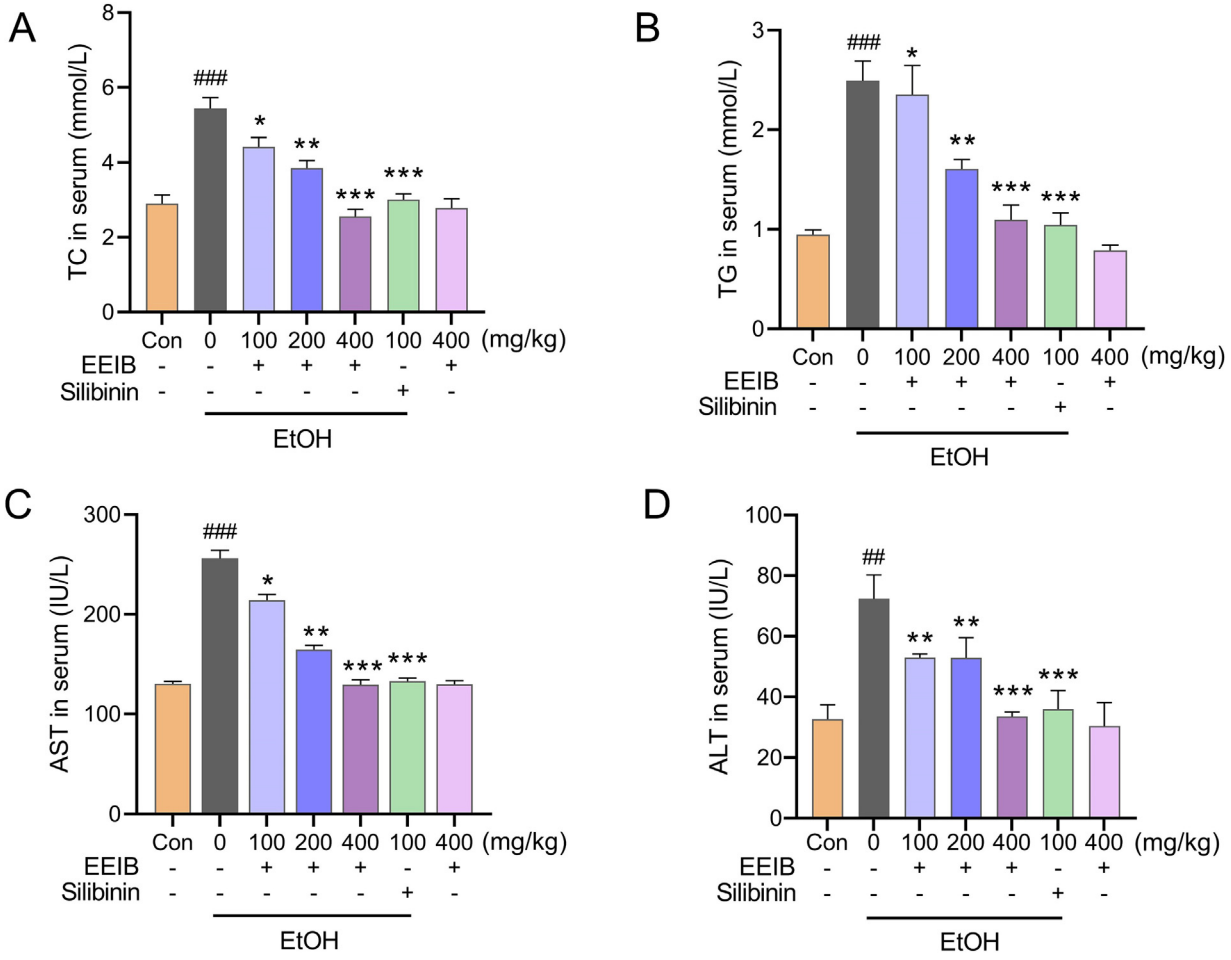

Fig. 2. Content of biochemical indicators of TC (A), TG (B), AST (C) and ALT (D) in serum of mice (mean ± SD, n = 6). $^{###}P < 0.001$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* EtOH group.

data indicated that EEIB treatment blocked the interference ability of alcohol on serum ALT, TG, AST, and TC in mice, demonstrating that EEIB can treat ALD.

3.3. Effect of EEIB on oxidative stress induced by alcohol in mice

Immunohistochemistry and Western blotting were used to examine the protein expression of Nrf2, HO-1 and NQO1. The expression of Nrf2, HO-1 and NQO1 was significantly decreased ($P < 0.001$) in the model group compared to the control group (Fig. 3A–E). In contrast, EEIB upregulated their expression in a dose-dependent manner. In addition, the positive drug group experimental results were similar to those of the EEIB group. These experiments suggested that EEIB activated the Nrf2 signaling pathway and regulated alcohol-induced oxidative stress imbalance in the liver. Also, to assess the effect of EEIB on antioxidant activity, the activity of antioxidant enzymes was assayed. MDA content is an important parameter reflecting the degree of resistance of the organism to lipid peroxidation (Wang, Yu, Xing, & Li, 2022). Compared with the control group, the level of liver MDA in the model group increased significantly ($P < 0.001$) and decreased significantly after EEIB treatment (Fig. 3F). On the contrary, the contents of GSH-Px and SOD decreased significantly after feeding alcohol and increased after administration of EEIB (Fig. 3G and H). These results suggested that EEIB reduced lipid peroxidation, increased the activity of antioxidant enzymes, and promoted antioxidant capacity. In summary, EEIB can enhance the body's antioxidant defense system by reducing lipid peroxidation, promoting antioxidant capacity, and activating the Nrf2 signaling pathway to prevent and improve alcohol-induced oxidative stress damage.

3.4. Effects of EEIB on alcohol-induced liver injury by regulating SIRT1-AMPK signal pathway

AMPK is a bioenergetic metabolism-regulating enzyme that regulates lipid metabolism (Townsend & Steinberg, 2023). EEIB treatment activated AMPK phosphorylated protein and ACC phosphorylated protein (Fig. 4A). Compared to the model group, the p-AMPK/AMPK ratio increased by 1.16-fold, 1.40-fold, and 1.47-fold ($P < 0.05$, $P < 0.01$) in the EEIB-treated group, respectively, and the p-ACC/ACC ratio also increased with increasing EEIB dose ($P < 0.01$). These data suggested that EEIB prevented ALD by regulating AMPK activation, promoting fatty acid catabolism, and attenuating fatty acid synthesis. SIRT1, an NAD^+-dependent histone and protein deacetylase, has been reported to protect against ALD by regulating inflammation, oxidative stress, and lipid metabolism. Furthermore, previous studies have shown that SIRT1 and AMPK had mutually regulated functions (Cheng et al., 2018; Yang et al., 2016). To assess whether SIRT1 activation is involved in EEIB-mediated protection against hepatic steatosis, inflammation, and oxidative stress, immunofluorescence staining of the *SIRT1* gene and protein expression of SIRT1 by Western blotting were examined. The results showed that SIRT1 protein expression levels were reduced in the model group of mice (Fig. 4B and C).

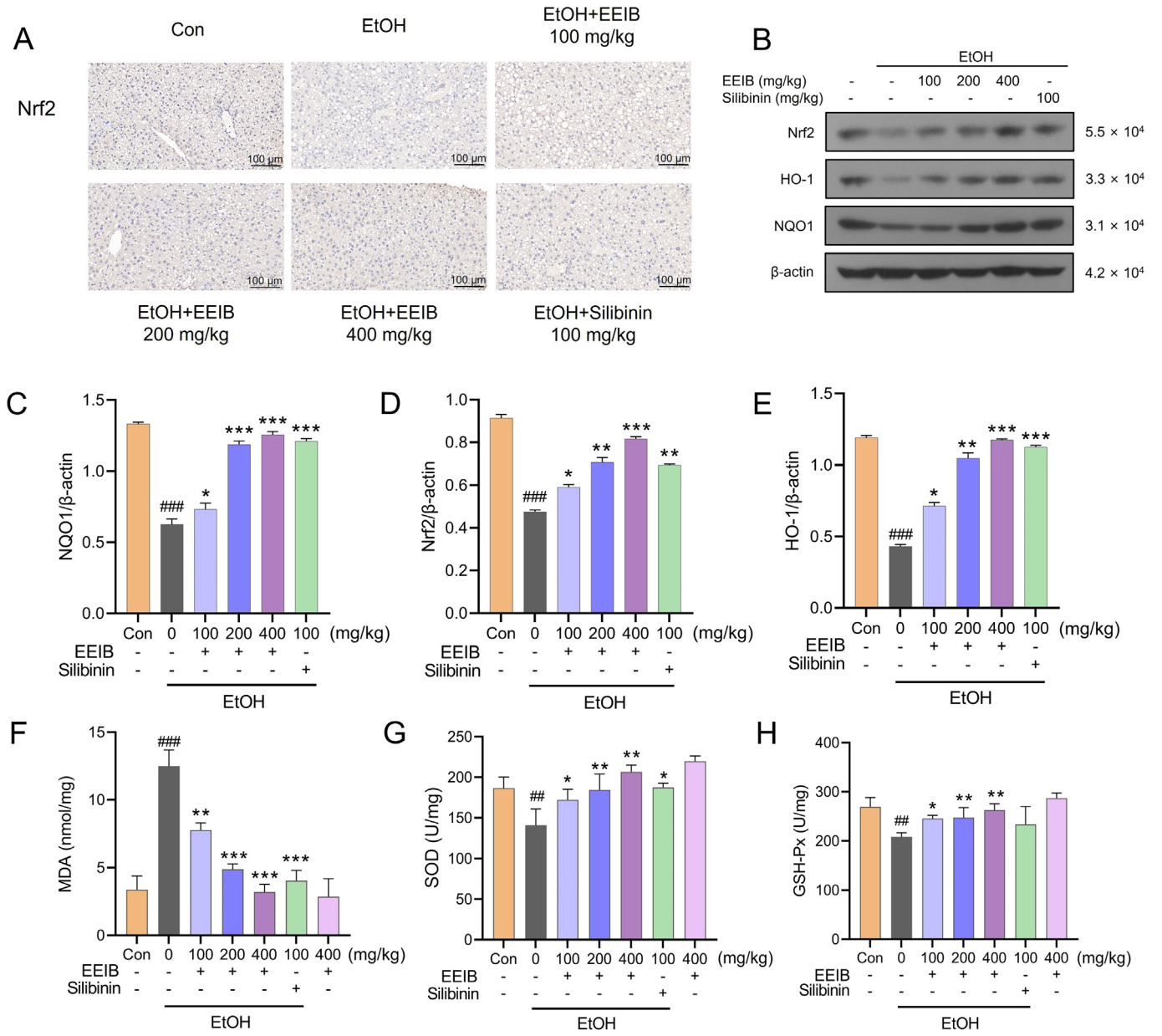

Fig. 3. Effect of EEIB on oxidative stress induced by alcohol in mice. (A) Immunohistochemical staining of Nrf2 (magnification: × 20, scale bar = 100 μm). (B–E) Effect of EEIB on expression of Nrf2, HO-1 and NQO1 proteins. (F) MDA content. (G) SOD content. (H) GSH-Px content. Data were presented as mean ± SD (n = 3 B–E, n = 6 F–H); $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* EtOH group.

However, SIRT1 expression levels were effectively restored by EEIB treatment. It was demonstrated that EEIB inhibited ALD by activating the SIRT1-AMPK signaling pathway in the liver of a mouse.

3.5. Effect of EEIB on alcohol-induced hepatic fibrosis in mice

Chronic heavy alcohol consumption is usually the cause of ALD and even alcoholic liver fibrosis. To assess whether the NIAAA model reaches the level of hepatic fibrosis and the effect of EEIB on alcohol-induced hepatic fibrosis in mice, experiments were performed to detect the expression of α-SMA and collagen I by immunohistochemistry and Western blotting (Meurer, Karsdal, & Weiskirchen, 2020; Sorensen et al., 2022). α-SMA and collagen I are indicators of hepatic fibrosis, and immunohistochemical staining showed that mice in the model group had increased α-SMA positive staining and collagen I accumulation in the liver compared to control mice, whereas the expression of both α-SMA and collagen I decreased after EEIB treatment (Fig. 5A). In addition, the Western blotting results were consistent with the immunohistochemical results (Fig. 5B). There was a significant increase in α-SMA and collagen I protein expression in the mouse model group, which was inhibited by EEIB treatment, whereas α-SMA and collagen I protein expressions were reduced in the liver of the mouse in the positive drug group. These data suggested that the NIAAA model allowed the development of mild hepatic fibrosis in the hepatic steatosis fraction and EEIB treatment attenuated alcohol-induced mild hepatic fibrosis in mice.

3.6. Effect of EEIB on alcohol-induced inflammation

To investigate the effect of EEIB on alcohol-induced inflammation in mice, the expression of TNF-α, IL-6, and IL-β in mouse

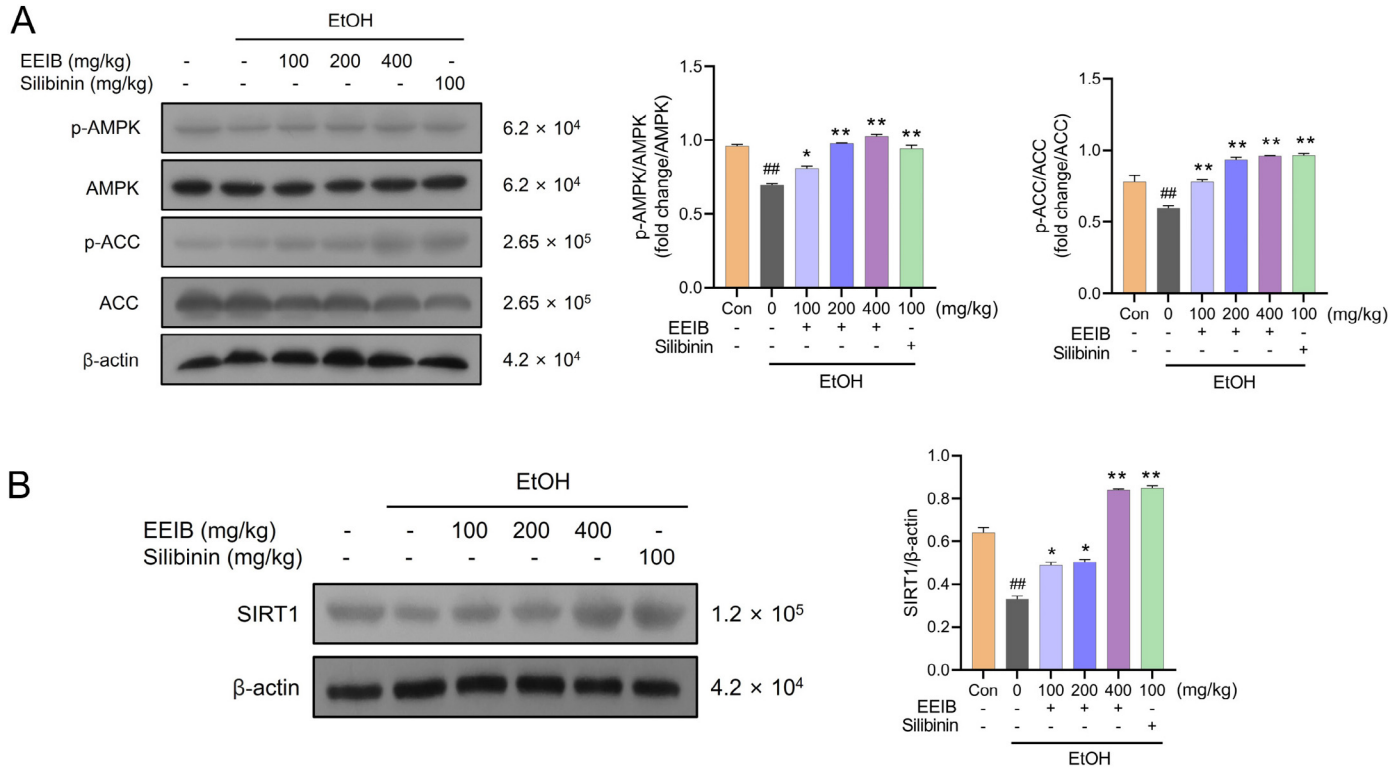

Fig. 4. Effect of EEIB on SIRT1-AMPK signaling pathway (mean ± SD, $n = 3$). (A) Effect of EEIB on protein expression associated with AMPK signaling pathway. (B) Effect of EEIB on protein expression of SIRT1. (C) Immunofluorescence staining of liver slices SIRT1 (magnification: × 40, scale bar = 40 μm). $^{\#\#}P < 0.01$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* EtOH group.

serum was detected by ELISA kits, and the expression of IL-1β in liver tissue sections was detected by immunohistochemical analysis. The study found that the levels of IL-1β, TNF-α, and IL-6 in the blood of mice were significantly elevated after feeding alcohol ($P < 0.01$), and the expression levels of these three factors were dose-dependently reduced after EEIB intervention (Fig. 6A–C). As seen in the graph of immunohistochemical staining results (Fig. 6D), alcohol induction increased the production of IL-1β, while its expression level was reduced by EEIB treatment. Notably, the intervention in the EEIB high-dose group reduced inflammatory factors in mice to normal levels, almost identical to the expression levels in the positive drug group. The inhibitory effect of EEIB on inflammatory mediators was further examined by immunofluorescence staining and Western blotting analysis. Compared to the control group, NF-κB immunofluorescence staining results showed increased positive staining for NF-κB (red) and DAPI (blue) in the model group, whose expressions were reduced by EEIB treatment (Fig. 6E). Western blotting experiments showed that the expression of IκB-α and P65 phosphorylated proteins was significantly elevated in mice fed alcohol (Fig. 6F). The ratio of p-IκB-α/IκB-α decreased after EEIB treatment ($P < 0.001$), and the ratio of p-P65/P65 also decreased with increasing EEIB dose. Also, EEIB decreased the protein expression of TNF-α. Taken together, these results supported that EEIB can attenuate the inflammatory response in alcohol-induced liver injury.

3.7. Molecular docking verification

The LibDock module of DS software was used to validate the molecular docking of the three compounds with the highest blood entry levels to the two key targets, and the molecular docking results for each ligand molecule and receptor protein were shown in Table 1. The results showed that the binding modes of ligand molecules and receptor proteins were mainly linked by hydrogen,

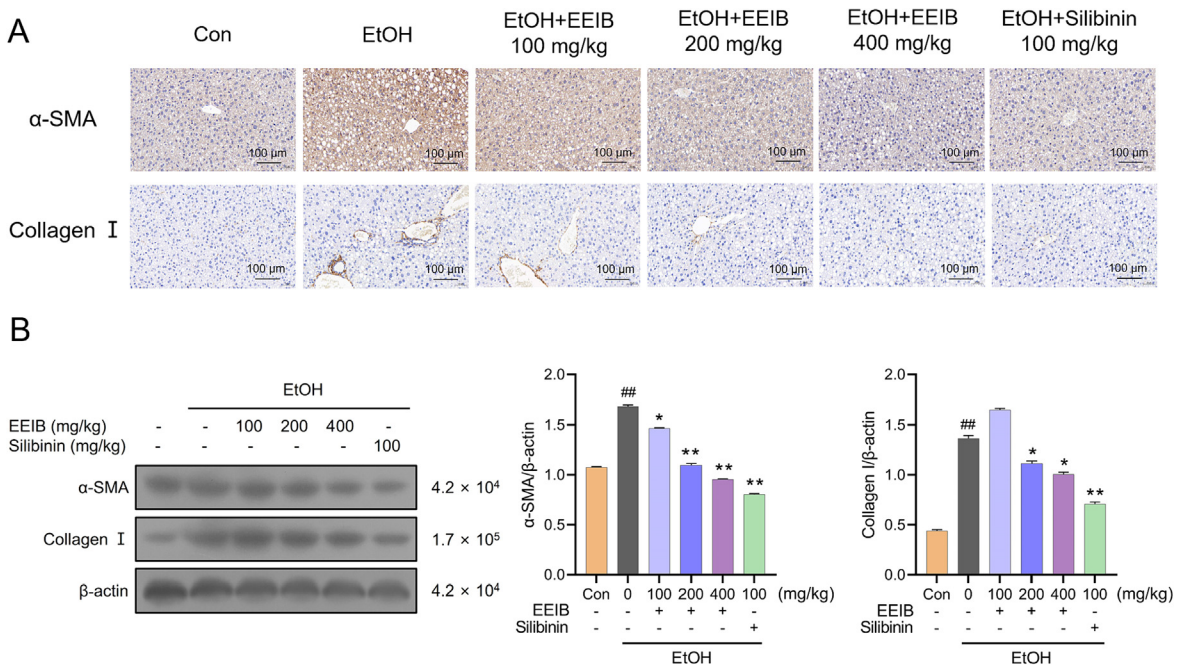

Fig. 5. Effect of EEIB on alcohol-induced hepatic fibrosis in mice (mean ± SD, n = 3). (A) Immunohistochemical staining of α-SMA and Collagen I. (B) Effect of EEIB on protein expression of α-SMA and Collagen I (magnification: × 20, scale bar = 100 μm). $^{\#\#}P < 0.01$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* EtOH group.

Pi-Alkyl, and hydrocarbon bonds. The compound-target scores were all greater than 100, indicating that the three monomeric compounds showed good binding to both ACC as well as SIRT1 target proteins, and they may be effective substances for activating the SIRT1-AMPK signaling pathway. This was consistent with the above conclusion that EEIB can inhibit ALD by modulating the SIRT1-AMPK signaling pathway in the liver of alcohol-fed mice. The molecular docking models of IB-3, IB-12, and IB-15 with target proteins ACC and SIRT1 were shown in Fig. 7 and Fig. S1. A review of the data revealed that sesquiterpenes, flavonoids, sterols, and other potent components were present in *I. britannica*, and IB-3, IB-12, and IB-15 were all sesquiterpenoids. Therefore, it was speculated that the sesquiterpenoids in EEIB may act through the SIRT1-AMPK signaling pathway to prevent and treat alcoholic liver injury and that sesquiterpenoids may be potential active substances for the prevention and treatment of alcoholic liver injury.

4. Discussion

This study demonstrated that the EEIB had an excellent therapeutic effect on ALD in mice, and its mechanism of action was to affect SIRT1-AMPK, Nrf2, and NF-κB signaling pathways. Lieber-DeCarli's liquid nutritious diet model can closely reproduce human drinking behavior and the pathogenesis of alcoholic liver injury (Bertola, 2020). Alcohol metabolism produces excess ROS, which can lead to abnormal oxidative stress and tissue damage in the body (Park et al., 2022). GSH-Px and SOD are the main antioxidant enzymes which have the function of scavenging ROS. Meanwhile, GSH-Px, MDA, and SOD are also common indicators of oxidative stress levels in the body. In our study, we found that EEIB was effective in increasing the activities of GSH-Px and SOD and decreasing alcohol-induced MDA in the liver by measuring the levels of GSH-Px, MDA, and SOD. In addition, Nrf2 is an essential factor in regulating oxidative stress *in vivo*. The protein expression of antioxidant defense-related genes *Nrf2*, *NQO1*, and *HO-1* was detected by Western blotting assay and immunohistochemical analysis. The study confirmed that EEIB activated the Nrf2 signaling pathway, regulated the balance of oxidative stress levels in mice, and alleviated alcohol-induced liver injury.

Abnormal lipid metabolism in the liver is one of the important features of alcoholic liver injury (Crabb & Liangpunsakul, 2006). Long-term sustained alcohol intake induces liver lipid metabolism abnormalities, producing large amounts of fat and forming a fatty liver. AMPK can inhibit fatty acid synthesis in the liver and promote fat oxidation by regulating ACC phosphorylation. Previous studies have shown that SIRT1 and AMPK have the function of regulating each other (Ruderman et al., 2010). We studied whether the activation of SIRT1-AMPK was related to the beneficial protective effect of EEIB on ALD. The results demonstrated that administration of EEIB increased phosphorylated AMPK and SIRT1 levels and promoted lipid metabolism. In addition, AMPK stimulated fatty acid oxidation by inactivating ACC and increasing the expression of fatty acid oxidation proteins, whereas EEIB significantly increased the levels of phosphorylated ACC. Immunofluorescence results of SIRT1 also indicated that alcohol-induced decrease in SIRT1 expression was effectively restored by EEIB treatment. At the same time, molecular docking also illustrated that the semi terpenes in *I. britannica* could bind to the target proteins in the SIRT1-AMPK signal pathway. The results suggested that the SIRT1-AMPK pathway mediated the protective effect of EEIB against alcohol-induced hepatic steatosis.

Oxidative stress imbalance and lipid accumulation due to chronic alcohol consumption induce liver damage and inflammatory responses. Continuous intake of alcohol will activate the NF-κB signal pathway and promote the secretion of inflammatory factors. IL-1β, TNF-α, and IL-6 expression in mice was determined using ELISA kits. It was found that alcohol induction significantly increased the level of inflammatory cytokines, while EEIB treatment decreased the expression of these three factors. The expression of NF-κB in liver tissue detected by immunofluorescence method showed that the positive staining of NF-κB increased in the model group and decreased after EEIB treatment. Mechanism

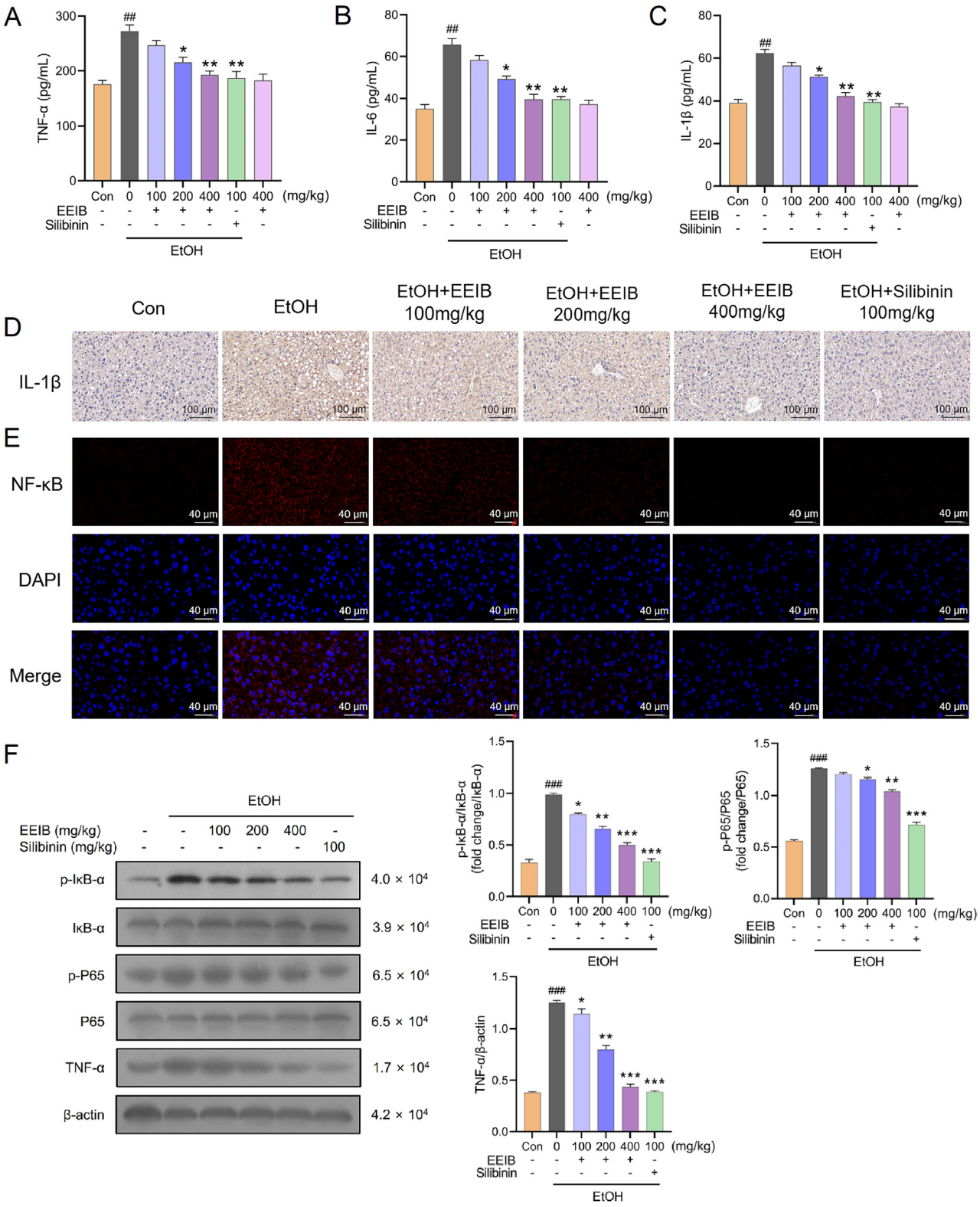

Fig. 6. Effect of EEIB on alcohol-induced inflammatory responses. (A–C) Levels of TNF-α, IL-6 and IL-1β (mean ± SD, n = 6). (D) Immunohistochemical staining of IL-1β (magnification: × 20, scale bar = 100 μm). (E) Immunofluorescence staining of NF-κB (magnification: × 40, scale bar = 40 μm). (F) Effect of EEIB on NF-κB signaling pathway protein expression (mean ± SD, n = 3). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* EtOH group.

studies have shown that EEIB can reduce alcohol-induced inflammation by down-regulating the expression of p-IκB-α, p-P65 and TNF-α.

In this study, the EEIB was investigated for liver injury protection in mice, which had certain limitations, such as predicting that sesquiterpenoids may be the main components of its efficacy

Table 1
Interaction between active substances and target proteins.

Entry	Proteins	PDB ID	LibDock scores	Interactions
1.1	ACC	1UYT	113.842	H bonds: VAL2163 Interacting residues: SER216, PRO2160, LEU1797, LEU1514
2.1	ACC	1UYT	121.568	H bonds: LYS2137 Interacting residues: GLN1944, PRO1817, LEU2130, PRO1486
3.1	ACC	1UYT	112.049	H bonds: ASN1815, VAL1818, Interacting residues: PRO1486, MET1816, LEU2130, PRO1817
1.2	SIRT1	4IG9	100.395	H bonds: GLN345 Interacting residues: ASN346, ALA262, ILE270, PHE273, LYS444, ARG274
2.2	SIRT1	4IG9	112.306	H bonds: ILE411, LYS408, SER365, GLN352 Interacting residues: GLU410, GLN421, PRO419, ILE360
3.2	SIRT1	4IG9	108.741	H bonds: ARG274, ILE347, VAL266 Interacting residues: SER265, ILE270, ASN346, ALA262, PHE273

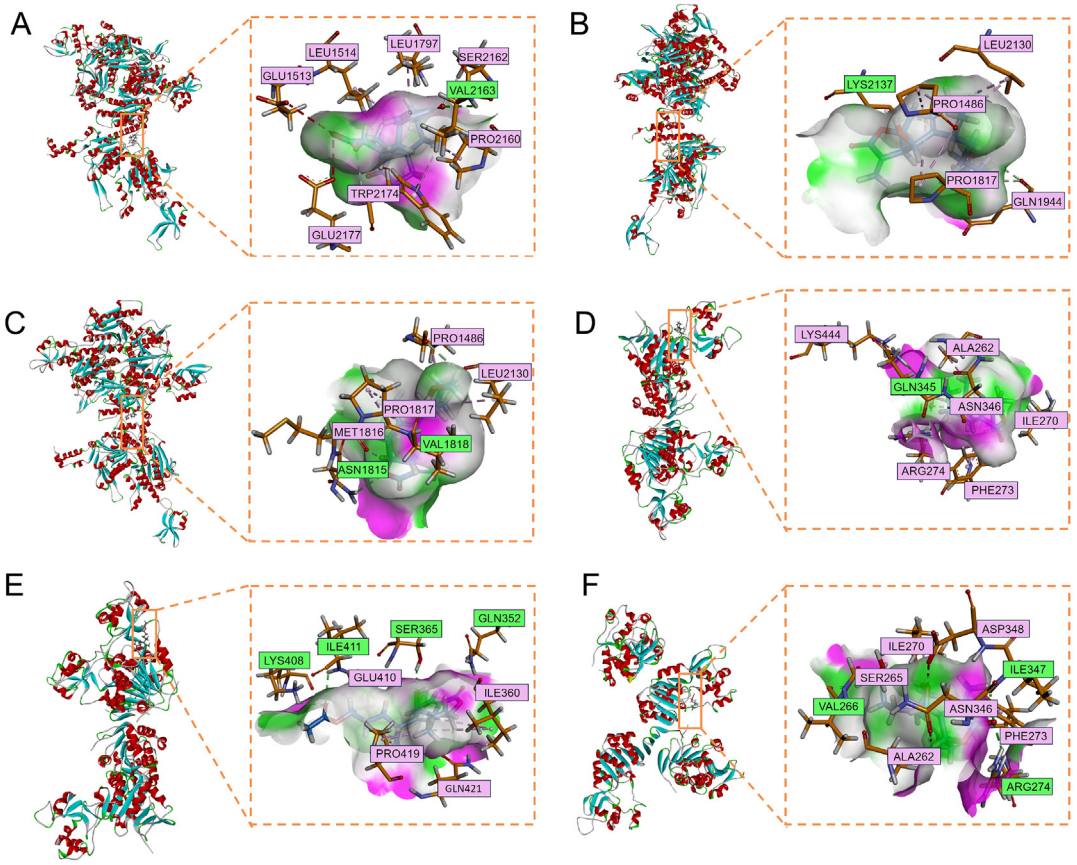

Fig. 7. A three-dimensional H-binding model showing interaction between active substances and target proteins. (A–C) IB-3, IB-12, IB-15 and ACC. (D–F) IB-3, IB-12, IB-15 and SIRT1.

through molecular docking while the specific bioactive components exerting efficacy in *I. britannica* were uncertain and needed to be further studied. In addition, the results of the study showed that the protective effect of EEIB on ALD was similar to or better than that of Silibinin, and EEIB, as a natural product, had the advantages of low toxicity, multi-targeting and low side effects.

5. Conclusion

The results showed that EEIB had an excellent therapeutic effect on alcohol-induced liver injury in mice. It ameliorated liver injury mainly through the SIRT1-AMPK/Nrf2/NF-κB pathway, thereby regulating lipid metabolism, reducing oxidative stress, and attenuating inflammatory responses. Sesquiterpenes may be potential components of EEIB with protective effects. Therefore, EEIB could have high development value as a potential drug for the treatment of alcoholic liver disease.

CRediT authorship contribution statement

Zhennan Meng: Writing – review & editing, Formal analysis. **Mengyuan Li:** Writing – review & editing, Formal analysis. **Xiaoli Wang:** Methodology. **Kuo Zhang:** Methodology. **Chunfu Wu:** Methodology. **Xiaoshu Zhang:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2023.12.006.

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