Submitted: 13 July, 2023 Accepted: 12 October, 2023 Published: 30 November, 2023 DOI:10.22514/jomh.2023.119

ORIGINAL RESEARCH

JOMH Journal of Men's Health

Esculentoside A inhibits ethyl alcohol-induced lipid accumulation and oxidative stress in hepatocytes by activating the AMPK pathway

Zhipeng Tang[1], Peng Zhang[2], Lu Li[1], Yang Guo[1], Yan You[1], Yong Liao[1,*]

[1]Department of Pharmacy, Affiliated Renhe Hospital of China Three Gorges University, 443001 Yichang, Hubei, China
[2]Department of Stomatology, Affiliated Renhe Hospital of China Three Gorges University, 443001 Yichang, Hubei, China

***Correspondence**
ly28368@163.com
(Yong Liao)

Abstract

Alcoholic fatty liver disease (AFLD) is a liver illness resulting from excessive alcohol consumption. Esculentoside A (EsA) possesses various properties, including antioxidative and anti-inflammatory capabilities, but its role and mechanism in AFLD have remained unclear. In this study, we aimed to elucidate the functions of EsA in AFLD. We utilized ethyl alcohol-induced Alpha Mouse 12 (AML-12) cells as a model to mimic AFLD conditions. Cell viability was evaluated utilizing the Cell Counting Kit-8 assay. Lipid accumulation was quantified *via* Oil Red O staining. The expression levels of key genes associated with lipid accumulation were determined using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and the contents of triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), reactive oxygen species (ROS) and superoxide dismutase (SOD) activity were quantified using commercially available assay kits. Additionally, western blot was performed to determine the levels of p-AMP-activated protein kinase (AMPK) Thr172/AMPK and peroxisome proliferator-activated receptor-alpha (PPARα). Our findings demonstrate that EsA effectively mitigated the damage induced by ethanol (EtOH) in AML-12 cells. Notably, EsA exhibited significant inhibitory effects on EtOH-induced lipid accumulation and oxidative stress in AML-12 cells. Importantly, our data suggest a potential connection between EsA-mediated effects and the activation of the AMPK pathway in EtOH-induced damage to AML-12 cells. In conclusion, EsA demonstrates promise in attenuating ethyl alcohol-induced lipid accumulation and oxidative stress in hepatocytes, likely through the activation of the AMPK pathway.

Keywords

Esculentoside A; Alcoholic fatty liver disease; Lipid accumulation; Oxidative stress; AMPK pathway

1. Introduction

Excessive alcohol consumption, or intemperance, is linked with an enhanced risk of various health issues, with alcoholic liver disease (ALD) being the primary cause of death among those who engage in heavy drinking [1]. Alcoholic fatty liver disease (AFLD), which represents the original stage of ALD, is distinguished by the gathering of triglycerides (TG) within liver cells and can progress to more serious conditions like alcoholic steatohepatitis and alcoholic cirrhosis [2]. The relationship between lipid accumulation, oxidative stress and AFLD is closely intertwined. When alcohol enters the liver, it inhibits the oxidation of fatty acids, thereby impairing the liver's ability to efficiently break down and metabolize lipids. This lipid accumulation primarily takes the form of triglycerides, resulting in an abnormally high-fat content within the liver and the progress of fatty liver [3]. Oxidative stress plays a key role in the advancement of AFLD [4] as it can facilitate the transition from steatosis to steatohepatitis in AFLD [5]. Prolonged alcohol abuse induces oxidative stress, distinguished by extreme manufacture of reactive oxygen species (ROS) in the body that surpasses the antioxidant defense system's capacity to eliminate them. In AFLD, this excessive generation of ROS leads to oxidative damage to the liver cell membranes, further exacerbating lipid accumulation and triggering inflammatory responses within the liver [6]. Lipid accumulation and oxidative stress interact synergistically, collectively promoting the development and progression of AFLD [7]. Given that men tend to consume more alcohol than women, they are at a higher risk of developing AFLD [8]. Currently, the most effective management strategy is alcohol restriction, as there are few drugs available for the direct management of AFLD [9]. Consequently, there is a crucial and pressing need for the advance of novel and effective drugs to improve the treatment outcomes of patients with AFLD.

AMP-activated protein kinase (AMPK) serves as a pivotal

regulator of energy homeostasis, promoting the oxidation of fatty acids while inhibiting their synthesis, which results in the suppression of fatty acid and cholesterol and triglyceride (TG) production [10]. The development of alcoholic liver disease (ALD) can be prevented by modulating lipid metabolism *via* AMPK and enhancing antioxidant systems [11]. Therefore, it is important to study the AMPK pathway to gain a deeper understanding of the regulatory mechanisms underlying AFLD. The presence of phosphorylated AMPK at threonine 172 (p-AMPK) serves as an indicator of the activated state of the AMPK pathway. Phosphorylation at the Thr172 site represents a critical step in AMPK activation, as it induces conformational changes in the AMPK protein, ultimately enhancing its catalytic activity [12]. Therefore, we focused on detecting the level of p-AMPK in this study.

Esculentoside A (EsA), the primary active component derived from Euphorbia saponins, possesses lots of pharmacological functions, containing antioxidant and immunomodulatory properties [13]. It can reduce apoptosis in 2, 4, 6-trinitrohydrosulfonic acid (TNBS)-tempted ulcerative colitis by constraining the nuclear translocation of nuclear factor kappaB (NF-κB) [14], activates AMPK and plays a protective role in Alzheimer's disease [15], has shown to possess a protective effect on liver damage, can resist acute liver failure caused by acetaminophen overdose *via* the AMPK/protein kinase B (Akt)/glycogen synthase kinase-3beta (GSK3β) pathway [16] and inhibits inflammation and oxidative stress to alleviate carbon tetrachloride and galactosamine/lipopolysaccharides-tempted acute liver injury in mouse [17]. However, the role and mechanism of EsA in AFLD remain indistinct.

Herein, we inspected the functions and underlying mechanisms of action of EsA in AFLD, and ethyl alcohol-induced AML-12 cells were utilized as a cellular model to simulate AFLD conditions. Overall, this research aimed to elucidate the modulation mechanism of EsA and propose a possible therapeutic strategy for the management of AFLD.

2. Materials and methods

2.1 Cell lines and culture

Alpha Mouse 12 (AML-12) cells, representing mouse normal hepatocytes, were sourced from the China Center for Type Culture Collection (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Solarbio, Beijing, China). The culture medium was enriched with insulin, transferrin, selenium, dexamethasone and 10% fetal bovine serum (FBS) (S9010, Solarbio, Beijing, China) at 37 °C in a 5% CO_2 environment. To replicate conditions akin to alcoholic liver disease (ALD) in a controlled *in vitro* setting, we followed established procedures involving ethyl alcohol (EtOH) induction [18]. Specifically, AML-12 cells were exposed to EtOH (250 mmol/L, Solarbio) and palmitate acid (PA) (0.25 mmol/L, Solarbio) for 24 h. EsA, with a purity exceeding 92.2%, was procured from Nanjing Spring and Autumn Biological Engineering Co., Ltd. (Nanjing, China). To investigate EsA's impact on ALD-like damage, varying concentrations of EsA (0, 10, 20, 40, 80 μM) were co-administered along with EtOH and PA, and the cells were coped with this mixture for 24 h. Additionally, to inhibit the AMPK signaling pathway, AML-12 cells were pretreated with compound C (25 μM) (Ribobio, Guangzhou, China) for 24 hours before exposure to EtOH and PA.

2.2 Cell Counting Kit-8 (CCK8) assay

AML-12 cells (2.0×10^5 cells per milliliter) were inoculated in 96-well plates and subjected to various treatments for 24 hours. Subsequently, a CCK-8 kit sourced (CA1210-100T, Solarbio, Beijing, China) was employed to assess cell viability in accordance with the provided protocol, and absorbance was determined at 450 nm *via* a microplate reader (Bio-Rad 680, Bio-Rad, Hercules, CA, USA).

2.3 Oil red O (ORO) staining

For the analysis of lipid content in AML-12 cells, we utilized the Oil Red O Stain Kit (ab150678, Abcam, Cambridge, MA, USA). After the respective treatments, the AML-12 cells were exposed to a 10% formalin solution (Solarbio) for 40 minutes. Afterwards, the cells were thoroughly washed with PBS (phosphate-buffered salin) to eliminate any residual formaldehyde and other contaminants. The cells were then treated with a 60% isopropanol solution (Solarbio) for 5 minutes. Following this step, the AML-12 cells were subjected to Oil Red O solution (Abcam) for 15 minutes. Finally, the stained cells were observed using a microscope (DM4, Leica, Wetzlar, Germany).

2.4 quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA from AML-12 cells was extracted using TRIzol reagent (9108Q, TaKaRa, Dalian, China). Subsequently, reverse transcription was adopted utilizing a PrimeScript RT Master Mix (RR036A; TaKaRa). The qRT-PCR was conducted with the SYBR® Premix Ex Taq™ quantitative kit (DRR041A, TaKaRa, Dalian, China), using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the reference gene. Relative gene content was computed utilizing the $2^{-\Delta\Delta Ct}$ method. The primers were delivered in Table 1.

2.5 TG quantification

Lipids from AML-12 cells were extracted using isopropanol. Subsequently, the lipids were reconstituted in a solution of 1% Triton X-100 in chloroform (Solarbio) and then dissolved in distilled water. TG levels were determined using the TG assay kit (ab65336; Abcam, Cambridge, MA, USA).

2.6 Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) analysis

AST and ALT activity assay kits (ab105135 and ab105134; Abcam, Cambridge, MA, USA) were used to assess their activities as per the provided protocol. Briefly, AML-12 cells were coped with AST or ALT assay buffer (200 μL; Abcam). Subsequently, the samples were centrifuged, and the resulting supernatants were collected. Then, a 10 μL aliquot of the supernatant was added to AST assay buffer (40 μL/per well)

TABLE 1. Primers for qRT-PCR.

Name	Primers for PCR (5′-3′)
SREBP-1c	
Forward	TGTGAGACCAACAGCCTGAC
Reverse	TGCGAGTGGTCTTCCATCAC
FAS	
Forward	TGCTTGCTGGCTCACAGTTA
Reverse	GAATCACTCCAACGGGCTGA
SCD1	
Forward	GAGTACCGCTGGCACATCA
Reverse	AAGCCCAAAGCTCAGCTACTC
GAPDH	
Forward	CCCTTAAGAGGGATGCTGCC
Reverse	TACGGCCAAATCCGTTCACA

Note: quantitative reverse transcriptase polymerase chain reaction: qRT-PCR; sterol regulatory element-binding protein-1c: SREBP-1c; fas cell surface death receptor: FAS; stearoyl-Coenzyme A desaturase 1: SCD1; glyceraldehyde-3-phosphate dehydrogenase: GAPDH.

or ALT assay buffer (10 μL/per well), and a reaction mix (100 μL/per well) was supplemented and hatched for 3 min in the dark. The absorbance at 450 nm (for AST) or 570 nm (for ALT) was estimated utilizing a microplate reader (Bio-Rad).

2.7 Reactive oxygen species (ROS) and Superoxide dismutase (SOD) measurement

For ROS level measurement in AML-12 cells, the 2′,7′-Dichlorodihydrofluorescein (DCF) assay was employed. AML-12 cells were exposed to a 5 μM DCF-DA (2′,7′-Dichlorodihydrofluorescein diacetate) working solution from Solarbio at 37 °C for 15 min. Subsequently, the cells were rinsed with PBS to remove unoxidized DCF-DA. The fluorescence signal within the cells was assessed employing a fluorescence microscope (Leica). The total SOD (U/L) activity in AML-12 cells was determined using an SOD assay kit (ab65354; Abcam, Cambridge, MA, USA). Specific experimental procedures were carried out following the instructions provided in the kit.

2.8 Western blot

Following the respective treatments, AML-12 cells were subjected to radio-immunoprecipitation assay (RIPA) buffer (Solarbio) for 30 minutes. Protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (MS3202, Solarbio, Beijing, China), the proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and they were transferred to a polyvinylidene fluoride (PVDF) membrane (Solarbio). After blocking, the blots were incubated overnight at 4 °C with primary antibodies: anti-p-AMPK Thr172 (8208; 1:1000; CST, Danvers, Massachusetts, USA), anti-AMPK (2532; 1:1000; CST), anti-peroxisome proliferator-activated receptor-alpha (PPARα) (ab178865; 1:1000; Abcam), and anti-β-actin (ab8226; 1:1000; Abcam). Following this, the membrane was probed with a goat anti-rabbit IgG secondary antibody (ab205718; 1:2500; Abcam) for 1 hour. Protein bands were visualized utilizing an Enhanced Chemiluminescence (ECL)-Plus reagent from Solarbio.

2.9 Statistical assay

All data were obtained from more than three independent tests, and the statistical analyses were performed utilizing SPSS 22.0 (IBM Corp., Armonk, NY, USA). Values are offered as mean $\pm$ standard deviation (SD). Paired and multiple comparisons were adopted using Student's *t*-test or analysis of variance (ANOVA), respectively. A significance level of $p < 0.05$ was statistically significant.

3. Results

3.1 EsA inhibits EtOH-induced hepatocyte damage

The impact of EsA was examined on the viability of AML-12 cells. The chemical structural formula of EsA is presented in Fig. 1A. When AML-12 cells were subjected to varying concentrations of EsA (0, 10, 20, 40 and 80 μM), we observed that only the highest concentration, 80 μM EsA, exhibited toxic effects on the cells. Consequently, for subsequent experiments, EsA concentrations of 0, 10, 20 and 40 μM were selected (Fig. 1B). Our investigations revealed that EtOH treatment led to a reduction in AML-12 cell viability. However, co-treatment with EsA resulted in a dose-dependent enhancement of cell viability (Fig. 1C). Among these concentrations, 40 μM EsA demonstrated the most pronounced effect in improving cell vitality. Therefore, 40 μM EsA was chosen for further experiments. In summary, our findings indicate that EsA effectively mitigates EtOH-induced damage to AML-12 cells.

3.2 EsA inhibits EtOH-induced lipid accumulation and oxidative stress in hepatocytes

Next, we investigated the role of EsA in lipid accumulation. When compared with the control group, cells induced by EtOH exhibited an increased red signal indicative of heightened neutral lipid accumulation. Nevertheless, this effect was mitigated by co-treatment with EsA (Fig. 2A,B). Furthermore, the levels of key lipid accumulation markers, namely sterol regulatory element-binding protein-1c (*SREBP-1c*), fas cell surface death receptor (*FAS*) and stearoyl-Coenzyme A desaturase 1 (*SCD1*), were elevated in EtOH-treated AML-12 cells, whereas these effects were attenuated by EsA co-treatment (Fig. 2C). In addition, EsA demonstrated an apparent reduction in EtOH-induced TG, AST and ALT accumulation in AML-12 cells (Fig. 2D). Furthermore, while EtOH treatment led to an increase in the content of reactive oxygen species (ROS), it simultaneously decreased the activity of SOD in AML-12 cells. In contrast, EsA effectively reduced ROS levels and boosted SOD activity in EtOH-exposed AML-12 cells (Fig. 3A,B). These results collectively illustrate that EsA effec-

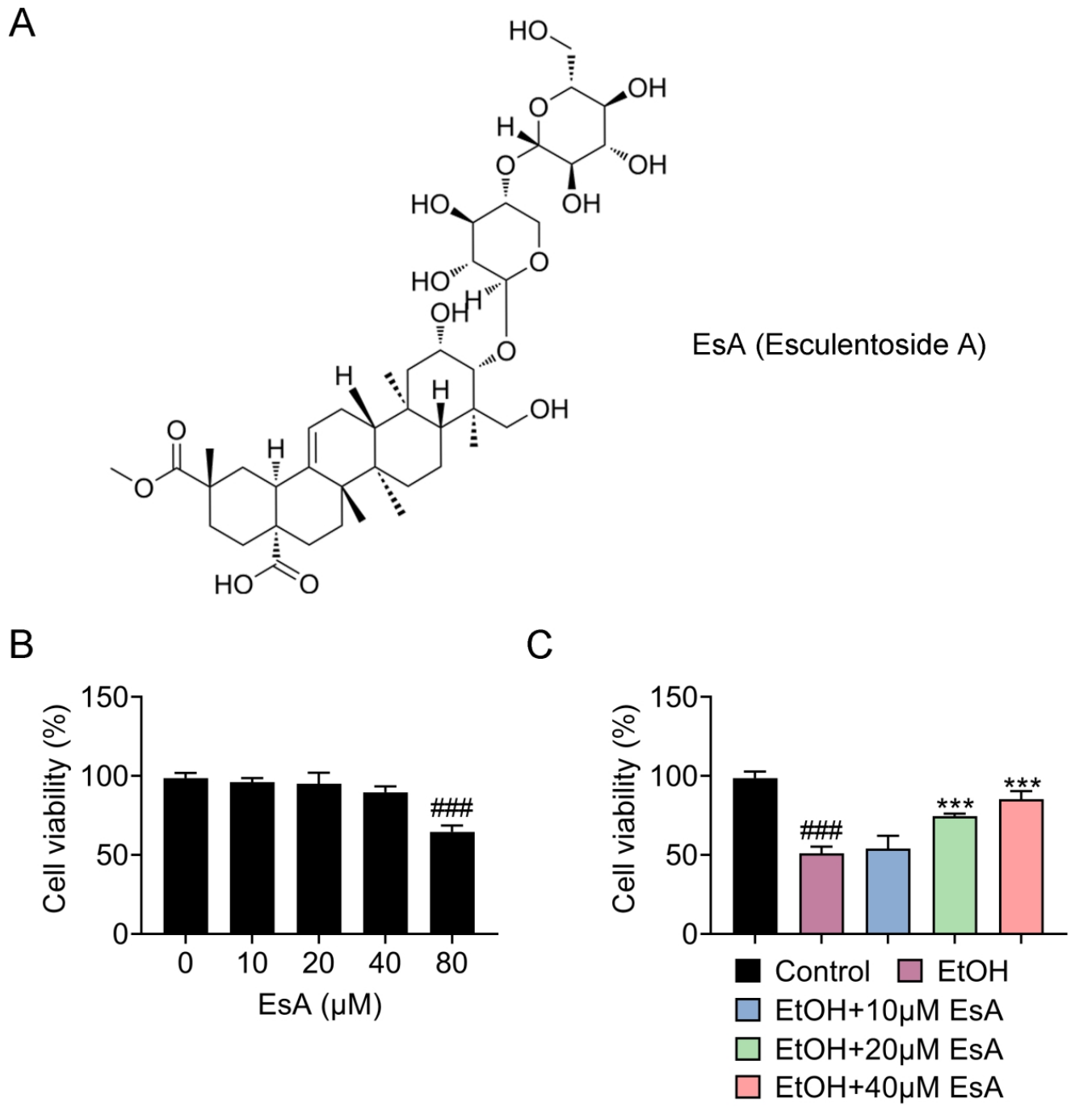

FIGURE 1. EsA inhibits EtOH-induced hepatocyte damage. (A) The chemical structural formula of EsA. (B,C) The AML-12 cell viability was assessed by CCK8 assay. $^{###}p < 0.001$ compared with the EsA 0 μM group or control group; $^{***}p < 0.001$ compared to the EtOH group. EtOH: ethanol.

tively inhibits EtOH-induced lipid accumulation and mitigates oxidative stress in AML-12 cells.

3.3 EsA relieves EtOH-induced lipid accumulation and oxidative stress by activating the AMPK pathway

Besides, we examined the specific pathway modulated by EsA in AFLD. We confirmed that the levels of p-AMPK Thr172/AMPK and PPARα were decreased following EtOH treatment but were elevated upon co-treatment with EsA (Fig. 4A). Furthermore, cell viability (Fig. 4B) in EtOH-induced AML-12 cells were enhanced in response to EsA treatment. However, these effects were diminished when compound C was co-administered. Moreover, EsA treatment led to a reduction in TG, AST and ALT accumulation and a enhancement in SOD activity in EtOH-exposed AML-12 cells (Fig. 4C,D), which could be counteracted when compound C was co-administered. These findings indicate that the activation of the AMPK pathway may be associated with the suppressive effects of EsA on EtOH-induced damage in AML-12 cells.

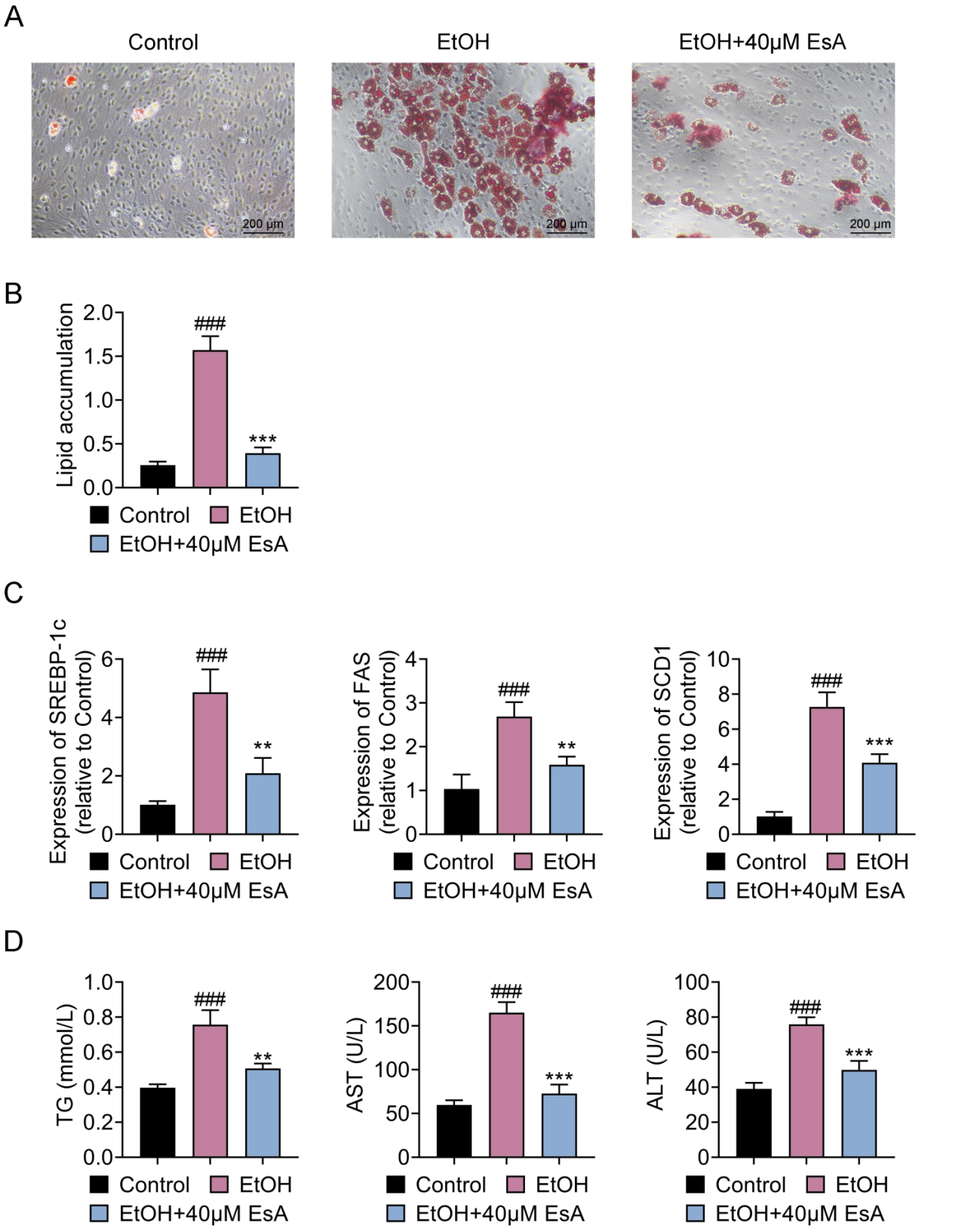

FIGURE 2. EsA hinders EtOH-tempted lipid accumulation in hepatocytes. (A) Oil red O (ORO) staining (scale bar = 200 μm). The lipid accumulation of AML-12 cells was determined by ORO staining. (B) Analysis of lipid accumulation in AML-12 cells. (C) The levels of SREBP-1c, FAS and SCD1 were determined by qRT-PCR. (D) The TG, AST and ALT contents in AML-12 cells were measured by corresponding commercial kits. $^{###}p < 0.001$ versus the control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ with respect to the control group EtOH group. EtOH: ethanol; EsA: Esculentoside A; TG: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; *SREBP-1c*: sterol regulatory element-binding protein-1c; *FAS*: fas cell surface death receptor; *SCD1*: stearoyl-Coenzyme A desaturase 1.

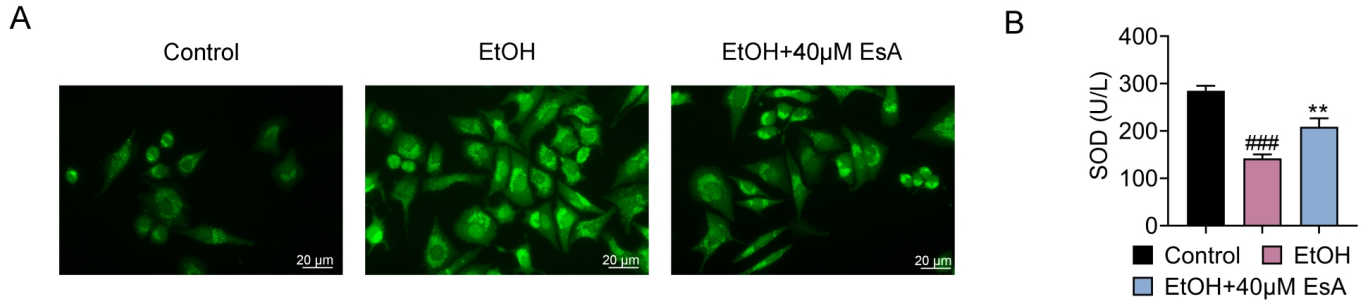

FIGURE 3. EsA constrains EtOH-tempted oxidative stress in hepatocytes. (A) DCF assay was employed to measure the level of ROS in AML-12 cells (scale bar = 20 μm). (B) The SOD content was assessed utilizing a commercial kit. $^{\#\#\#}p < 0.001$ compared with the control group; $^{**}p < 0.01$ compared with the EtOH group. EtOH: ethanol; EsA: Esculentoside A; SOD: superoxide dismutase.

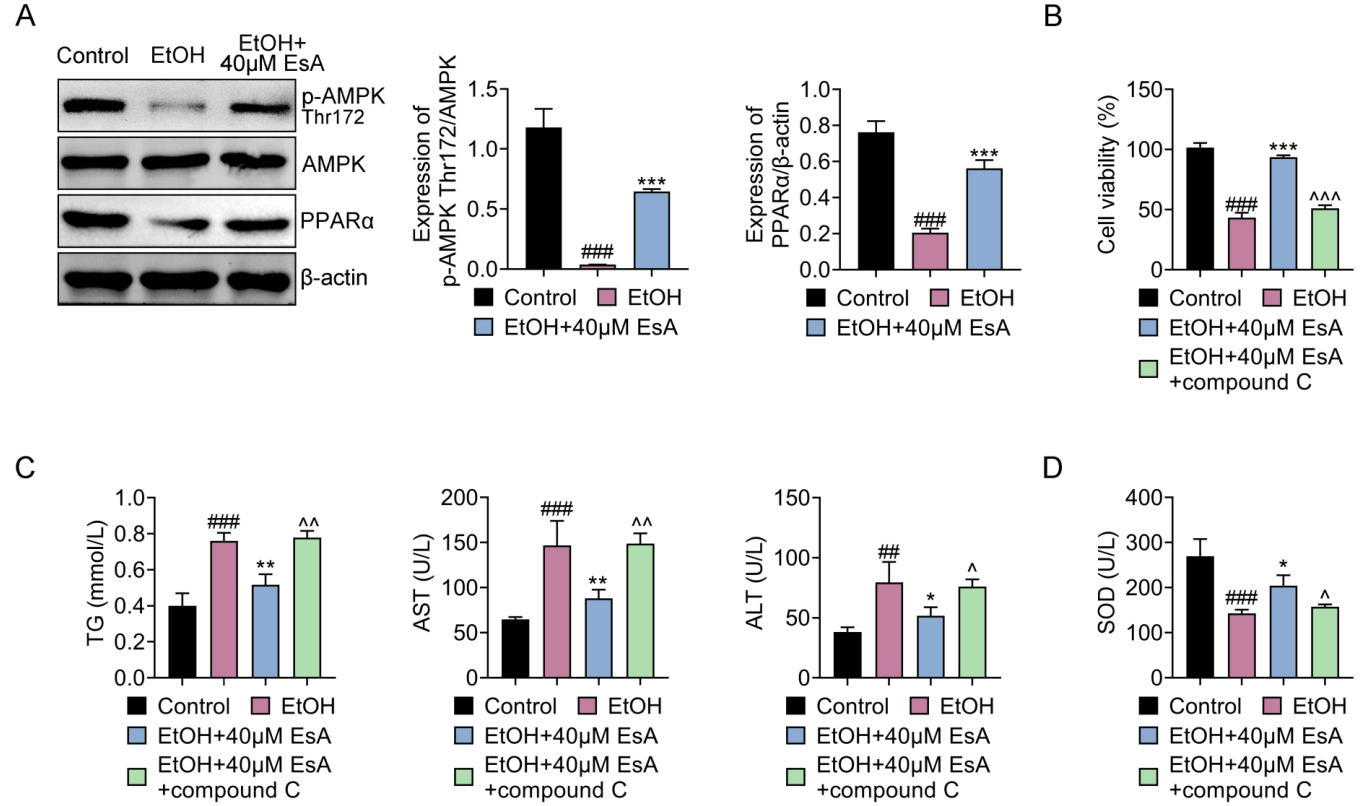

FIGURE 4. EsA relieves EtOH-tempted lipid accumulation and oxidative stress by activating the AMPK pathway. (A) The levels of p-AMPK Thr172/AMPK and PPARα in AML-12 cells were assessed by western blot. (B) The AML-12 cell viability was assessed by CCK8 assay. (C) The TG, AST and ALT contents in AML-12 cells were determined by corresponding commercial kits. (D) The SOD content in AML-12 cells was evaluated by a commercial kit. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ with respect to the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the EtOH group; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ and $^{\wedge\wedge\wedge}p < 0.001$ in contrast to the EtOH + 40 μM EsA group. EtOH: ethanol; EsA: Esculentoside A; AMPK: AMP-activated protein kinase; TG: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; SOD: superoxide dismutase; PPARα: peroxisome proliferator-activated receptor-alpha.

4. Discussion

Herein, we have demonstrated that EsA effectively inhibits EtOH-induced damage to AML-12 cells, EsA hinders EtOH-tempted lipid accumulation and oxidative stress in these cells and identified the involvement of the AMPK pathway in the protective effects of EsA. Overall, our study confirms that EsA mitigates EtOH-induced lipid accumulation and oxidative stress in hepatocytes by activating the AMPK pathway.

AFLD is a liver disease resulting from excessive alcohol consumption, which leads to the accumulation of lipids in the liver [18]. Excessive alcohol intake impairs the liver's ability to metabolize alcohol [19], and over time, this prolonged lipid accumulation can result in liver tissue damage and inflammation, eventually progressing to cirrhosis and liver failure [20–22]. It is known that AST and ALT are pivotal enzymes in

liver injury, and their levels are elevated in individuals with AFLD [5]. In this research, we utilized EtOH-induced AML-12 cells as a cell model to mimic AFLD, and our results showed a significant increase in lipid accumulation, TG, AST and ALT levels following EtOH treatment, confirming the successful establishment of the cell model.

AFLD typically does not manifest specific symptoms, but some individuals may experience nonspecific symptoms like fatigue and abdominal bloating. Severe liver damage may give rise to symptoms such as jaundice and liver pain. The diagnosis of AFLD involves liver function tests, liver ultrasound, and other medical examinations [23–25]. The primary approach to managing AFLD is to cease alcohol consumption. Patients with mild AFLD can often restore liver function through lifestyle modifications, including alcohol abstinence, adopting a healthy diet, and engaging in regular exercise. In cases of severe AFLD, medical intervention, surgical procedures, or even liver transplantation may be required [26, 27]. As a result, the discovery of new effective drugs remains crucial for the treatment of individuals with AFLD.

EsA is a natural product belonging to the triterpenoid saponin class of compounds, which is widely found in some plants, especially in some traditional Chinese medicines [28]. He *et al*. [29] reported that EsA exerts beneficial effects in the treatment of liver disease *via* multiple mechanisms, including alleviating liver oxidative stress, reducing inflammatory reactions, and inhibiting liver cell apoptosis. Wang *et al*. [16] confirmed that EsA can regulate the AMPK pathway to protect the liver. Although EsA has multiple biological activities, there is currently insufficient mechanism research to confirm its therapeutic effects in AFLD. Therefore, more exploration is required to evaluate the potential value of EsA in the management of AFLD. Herein, we found that EsA could inhibit EtOH-induced AML-12 cell damage, concordant with the study findings of He *et al*. [29]. Additionally, we are the first to confirm that EsA could inhibit EtOH-induced lipid accumulation in AML-12 cells. Moreover, we uncovered that EsA inhibited EtOH-induced oxidative stress, which was alike to the research of Zhang *et al*. [17]. Chen *et al*. [30] revealed that EsA could curb acute kidney injury by activating PPAR-γ (a crucial gene in the PPAR signaling pathway). Silencing PPARα stimulates lipid accumulation in the liver [31]. Herein, we found that EsA relieved AFLD by activating the AMPK pathway, which was comparable with the report of Wang *et al*. [16]. We also confirmed for the first time that EsA boosted the level of PPARα in EtOH-tempted AML-12 cells. However, there are still some limitations that should be clarified in this research. We only verified the effect of EsA in cell models, and validations in animal models and clinical data are required.

5. Conclusions

In summary, our findings revealed that EsA effectively mitigates EtOH-induced lipid accumulation and oxidative stress in hepatocytes by activating the AMPK pathway. This study contributes to our understanding of the molecular mechanisms underlying EsA's protective effects on liver cells and introduces a novel approach for the potential management of individuals with AFLD.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

ZPT and YL—designed the study and carried them out; prepare the manuscript for publication and reviewed the draft of the manuscript. ZPT, PZ, LL, YG and YY—supervised the data collection, analyzed the data, interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Zhipeng Tang, Peng Zhang, Lu Li, Yang Guo, Yan You, Yong Liao. Esculentoside A inhibits ethyl alcohol-induced lipid accumulation and oxidative stress in hepatocytes by activating the AMPK pathway. Journal of Men's Health. 2023; 19(11): 82-89. doi: 10.22514/jomh.2023.119.