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Gnetum montanum extract ameliorates ethanol-induced hepatic injury and metabolic dysfunction *via* inhibition of xanthine oxidaseHong-Linh Tran¹, Thuy-Duong Nguyen¹, Thu-Hang Nguyen¹, Hai-Nam Nguyen², Duc-Vinh Pham¹✉¹Department of Pharmacology, Hanoi University of Pharmacy, Hanoi, Vietnam²Department of Medicinal Chemistry, Hanoi University of Pharmacy, Hanoi, Vietnam

ABSTRACT

Objective: To investigate the effects of a crude extract from *Gnetum montanum* Markgr. on ethanol-induced hepatotoxicity and metabolic disorders.

Methods: Alcoholic liver disorder was induced in mice by administering increasing doses of ethanol *via* oral gavage. Biomarkers of liver injury and oxidative stress were assessed at the end of the study. Liver tissue damage and fat deposition were evaluated using hematoxylin and eosin and oil red O staining, respectively. In addition, key biomarkers were examined in acetaldehyde-treated HepG2 cells.

Results: Ethanol consumption induced characteristic pathological changes, including elevated serum markers of liver injury, hepatic lipid accumulation, and oxidative stress in liver tissues. Oral administration of *Gnetum montanum* extract (175 and 350 mg/kg) decreased serum aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transferase, and bilirubin levels in ethanol-treated mice. The extract also lowered triglyceride levels in serum and liver tissue in a dose-dependent manner. Furthermore, it mitigated malondialdehyde levels, preserved reduced glutathione levels, and enhanced catalase activity and total antioxidant capacity in liver tissue homogenates. Additionally, ethanol-induced hyperuricemia was suppressed by *Gnetum montanum* extract by inhibiting xanthine oxidase activity. Similar effects were observed in *Gnetum montanum* extract-treated HepG2 cells.

Conclusions: This study demonstrates that *Gnetum montanum* extract alleviates ethanol-induced hepatic injury by alleviating oxidative stress and inhibiting xanthine oxidase activity.

KEYWORDS: Alcoholic liver disease; *Gnetum montanum*; Hepatic steatosis; Hepatoprotective effect; Xanthine oxidase

1. Introduction

Alcohol-associated liver disease (ALD) is a major cause of liver-related morbidity and mortality worldwide[1]. The global incidence of alcohol-associated hepatitis has increased in recent years, especially in the young population and women[2]. ALD develops through several stages classified according to the pathological features of the liver. In the early stage, chronic excessive alcohol consumption disrupts lipid metabolism, evidenced by an imbalance in the synthesis, esterification, and oxidation of fatty acid within the liver, resulting in fat accumulation in hepatocytes (hepatic steatosis)[3]. At this stage, ALD can be alleviated by alcohol abstinence. However, liver injuries may become irreversible upon continuous heavy alcohol

Summary

Question: Does *Gnetum montanum* extract effectively ameliorate ethanol-induced hepatic injury and metabolic disorders?

Findings: *Gnetum montanum* extract exhibited hepatoprotective effects by reducing liver cell damage, alleviating oxidative stress, and improving hepatic lipid metabolism in an animal model of alcohol-associated liver disease and HepG2 cells, primarily through the inhibition of xanthine oxidase activity.

Meaning: *Gnetum montanum* extract mitigates ethanol-induced hepatic injury and metabolic disorders.

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consumption[4], in which hepatic steatosis may gradually progress to severe liver disorders, including hepatitis, cirrhosis, and even hepatocellular carcinoma[5]. Alcoholic hepatitis is commonly treated with anti-inflammatory agents like corticosteroids and pentoxifylline[6]. Nevertheless, no specific treatment is currently available for alcoholic fatty liver disease, fibrosis, or cirrhosis, apart from alcohol cessation. In this context, novel approaches, including the use of plant-derived agents, have been investigated as potential options for the prevention and treatment of ALD-associated complications[7].

Alcohol-induced liver injury is linked to reactive oxygen species (ROS) generation, resulting in the onset of oxidative stress in hepatocytes[8]. Both alcohol dehydrogenase and CYP2E1 convert alcohol to acetaldehyde during chronic alcohol consumption. Acetaldehyde is a highly reactive metabolite forming adducts with DNA to promote tissue damage[9]. In addition, ROS can also sensitize hepatocytes to lipopolysaccharides released from the gut and tumor necrosis factor- α , which, in turn, generate more ROS[9,10]. Given the strong link between oxidative stress and ALD, antioxidant agents capable of disrupting this pathological cycle have been explored as potential interventions to prevent ALD-related complications.

In the context of oxidative stress and ALD, xanthine oxidase (XO), a key enzyme involved in the conversion of xanthine/hypoxanthine into urate, has emerged as a promising pharmacological target for ALD. XO plays a crucial role in the metabolism of purines by catalyzing the oxidation of hypoxanthine to xanthine and then to uric acid. During this process, XO generates ROS, including superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), which are implicated in oxidative stress and tissue damage[11]. Increasing evidence suggests that XO plays a role in the pathology of both alcoholic and non-alcoholic liver diseases by contributing to oxidative stress, inflammation, and hepatic fat accumulation[5,10,12,13]. Therefore, it has been speculated that medicinal plants with XO inhibitory activity may serve as potential therapeutic interventions for ALD prevention and treatment[14,15].

Among XO inhibitors, *Gnetum montanum* (*G. montanum*) Markgr., a woody vine from the Gnetaceae family, has recently attracted increasing attention. Notably, compounds isolated from the stems and roots of *G. montanum* have been shown to possess potent XO modulatory activity[16]. In Vietnamese traditional medicine, the roots and lianas of this plant have been used to treat rheumatism, bone pain, gout, menstrual disorders, snakebites, and certain hepatic disorders. Phytochemical analyses from studies in China and Vietnam have revealed that the lianas and roots of *G. montanum* contain stilbenoids, lignans, flavonoids, phenolics, and alkaloids[16–18]. While scientific reports on the biological activities of *G. montanum* remain limited, recent studies have primarily focused on its antioxidant, anti-tumoral, and XO inhibitory

properties, particularly in crude extracts and isolated compounds from the stems and roots[16,18,19]. However, its potential role in the treatment of liver-related disorders has yet to be explored.

Given its traditional uses and known bioactivities, we hypothesized that a crude ethanol extract from the stems of *G. montanum* (GME) might exert protective effects against ethanol-induced liver damage and metabolic dysfunction. Therefore, this study aimed to explore the role of GME in normalizing hepatic lipid metabolic dysfunction, attenuating hepatic injury, and reducing oxidative stress. Furthermore, the mechanisms underlying the hepatoprotective effects of GME were investigated.

2. Materials and methods

2.1. Collection and preparation of plant extract

Lianas of *G. montanum* Markgr., including stems, branches, leaves, and flowers, were collected in Dien Bien, Vietnam, during the flowering season in April 2022. The plant was authenticated by Dr. Tran The Bach at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (IEBR-VAST). A voucher specimen (specimen number: DL2) was deposited at IEBr-VAST.

The collected plant materials were thoroughly cleaned, sliced, and dried to a constant weight in a hot-air oven at 50 °C, then ground into a fine powder. The powder (3×2000 g) was extracted three times with 95% aqueous ethanol by reflux for 3 h. The combined extracts were concentrated at 50 °C under reduced pressure, followed by oven drying at 50 °C to obtain a solid residue (1008 g, with a 21.5% drying loss). The yield of the extract was 13.2% relative to the dried liana powder. The obtained extract appeared as a thick, brown paste with a characteristic medicinal aroma. The quality of GME was assessed by quantifying its total polyphenol content (24.87%) using the Folin-Ciocalteu colorimetric method[20].

For *in vitro* experiments, the extract was dissolved in dimethyl sulfoxide to a stock concentration of 50 mg/mL, followed by serial dilution in a culture medium to achieve the desired concentrations. For *in vivo* administration, the extract was suspended in 0.5% sodium carboxymethyl cellulose (NaCMC) at concentrations of 15.5 and 35.0 mg/mL. The aqueous extract preparations were stored at 4 °C and renewed every three days.

2.2. Chemicals and reagents

Silymarin, allopurinol, glutathione, xanthine, XO from bovine milk, 5,5'-dithiobis (2-nitrobenzoic acid), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), oil red O, ammonium molybdate, thiobarbituric acid, and tetramethoxypropane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute

ethanol was procured from Samchun Chemicals (Seoul, Korea). All solvents and reagents used for plant extraction and characterization of plant extract were obtained from Merck (Darmstadt, Germany).

2.3. Cell culture and treatments

HepG2 cells were acquired from American Type Culture Collection (Rockville, MD, USA). Cells were routinely cultured in high-glucose Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS) and 1× penicillin/streptomycin and maintained at 37 °C in a humidified incubator with 5% CO₂. All reagents used for cell culture were purchased from PAN-Biotech GmbH (Aidenbach, Germany).

To examine the effects of GME on cellular lipid accumulation, cells were pretreated with GME at 3 and 10 µg/mL (concentrations selected based on preliminary experiments), followed by incubation with 1 mM oxidized ethanol (acetaldehyde) for 48 h in a complete medium. Lipid accumulation and peroxidation were assessed *via* oil red O staining, triglyceride quantification, and malondialdehyde (MDA) measurement.

2.4. Establishment of a mouse model of ethanol-induced hepatotoxicity

Eight-week-old Swiss mice were allocated into five groups ($n=8$ per group), including the normal control, the ethanol control, the positive control, and two tested groups. The positive control received 100 mg/kg of silymarin while the tested groups were treated with GME at doses of 175 and 350 mg/kg. Instead, mice in the normal and ethanol control groups received 0.5% NaCMC orally. All these treatments were administered once daily at 9 a.m. To induce hepatic injury, mice were administered ethanol orally once daily with increasing doses: 3 g/kg/day during the first week, 4 g/kg/day during the second week, and a single dose of 5 g/kg on the fifteenth day. Ethanol was administered *via* gavage two hours after the respective treatments. In a separate experiment, a group of mice were cotreated with allopurinol (10 mg/kg, orally) and ethanol following the same schedule.

Nine hours after the final ethanol dose, blood samples were collected from the retro-orbital sinus. Mice were then sacrificed, and livers were dissected. Blood samples were used to separate serum for aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), bilirubin, uric acid, and triglyceride analyses. Liver tissues were fixed in 10% formalin for histological examination or immediately stored at –80 °C for subsequent homogenate preparation. Liver homogenates were analyzed for total antioxidant capacity, triglyceride, reduced glutathione (GSH), MDA, catalase, and XO activity.

2.5. Determination of serum biochemical markers

Blood samples obtained from mice at the end of the study were settled at 4 °C and then centrifuged (3000 rpm for 10 min) to separate serum. Serum levels of AST, ALT, GGT, bilirubin, triglyceride, and uric acid were measured using commercial assay kits from BioSystems (Barcelona, Spain).

2.6. Measurement of oxidative stress indicators

Liver tissues (100 mg) were homogenized in ice-cold phosphate buffer (100 mM, pH 7.4, 10% *w/v*) using a Daihan Scientific homogenizer (Gangwon-do, Korea). After centrifugation (14000 rpm, 4 °C, 30 min), the supernatant was collected, stored at –80 °C, and used for further biochemical analysis. Total protein levels were normalized using the BCA method (Thermo Scientific, Waltham, MA, USA).

2.6.1. Total antioxidant capacity

The total antioxidant capacity of liver tissue homogenates was estimated using the ABTS radical scavenging assay. The reaction mixture consisted of 5 µL of tissue homogenate and 195 µL of ABTS⁺ reagent (containing 7 mM ABTS and 2.45 mM potassium persulfate). After a 2-minute incubation at room temperature, the absorbance of the mixture was measured at 734 nm. Antioxidant capacity was calculated based on the reduction in absorbance compared to the negative control (ABTS reagent only).

2.6.2. GSH

Hepatic GSH content was determined by the Ellman method, as previously described[21]. Briefly, the tissue homogenate was mixed with 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) in phosphate buffer. The resultant colored product was quantified by measuring its optical density at 412 nm.

2.6.3. Catalase activity

Catalase activity in liver tissue homogenates was determined by measuring the rate of H₂O₂ decomposition. Tissue homogenates (5 µL) were incubated with 50 µL of 50 mM H₂O₂ for 3 min at 37 °C, followed by the addition of 200 µL of ammonium molybdate to quench the residual H₂O₂. The absorbance of the resultant yellowish complex was measured at 374 nm. Catalase activity was calculated as described previously[22].

2.6.4. MDA

MDA production was estimated by measuring thiobarbituric acid reactive substances in cell lysates or tissue homogenates, as previously described[21]. The reaction mixture, consisting of cell lysates or tissue homogenates and 0.25% thiobarbituric acid in acetic acid (pH 2.4–2.6), was heated at 95 °C for 60 min. The

resultant red-pink adduct was extracted using *n*-butanol, and its absorbance was measured at 532 nm. MDA levels were extrapolated from a standard curve constructed using tetramethoxypropane and normalized to total protein content.

2.7. Measurement of triglyceride content

Cellular and tissue triglyceride levels were determined using the Amplex Red TG Assay Kit (Beyotime Biotech Inc., Shanghai, China). Cells or tissues were homogenized in ice-cold isopropanol (1:10 *w/v*) using a tissue homogenizer. The resulting lysates were diluted with assay buffer and then incubated with lipase for 20 min at 37 °C. Afterward, equal volumes of assay reagent containing cofactor, enzyme mix, and Amplex Red in assay buffer were added. The reaction mixture was further incubated for 60 min at 37 °C, and fluorescence intensity was measured at Ex/Em = 560/590 nm using a multimode microplate reader (Varioskan Lux, Thermo Scientific).

2.8. Determination of XO activity

XO activity was measured in cell or tissue lysates using the xanthine oxidation method[23]. Cell or tissue lysates were prepared in cell lysis buffer and preincubated at 37 °C for 15 min, followed by mixing with xanthine (final concentration: 75 µM) in phosphate buffer (70 mM, pH 7.5). The absorbance of the mixture was immediately measured at 290 nm for 20 min. XO activity was calculated based on the change in optical absorbance over time and normalized to protein content.

2.9. H&E and oil red O staining

Liver tissues and cells were fixed with 4% paraformaldehyde at 4 °C for 24 h and 20 min, respectively. Frozen sections (10-µm thickness) were prepared from the fixed tissue samples using a microtome. To visualize lipid droplets, cells or tissue sections were incubated with 0.3% oil red O solution in 60% isopropanol for 15 min. After washing with 60% isopropanol, cells or tissue sections were counterstained lightly with hematoxylin. For H&E staining, tissue sections were sequentially stained with hematoxylin and eosin using an H&E staining kit (Beyotime Biotech Inc.), as previously described[24].

2.10. RNA isolation and real-time-quantitative polymerase chain reaction (RT-qPCR)

After the indicated treatments, cells were collected and lysed in Qiazol lysis reagent (Qiagen, Hilden, Germany). Total RNA was isolated and purified according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from the purified RNA using the GoScript™ Reverse Transcriptase system (Promega

Corporation, Madison, WI, USA). Finally, RT-qPCR was performed to examine the relative expression of genes, as previously described[25]. Primer sequences used in this study are provided in Table 1.

Table 1. Primer sequences used in RT-qPCR experiment.

Gene	Primer sequence
<i>GAPDH</i>	Forward: 5'-GTCTCCTCTGACTTCAACAGCG-3'
	Reverse: 5'-ACCACCCTGTGCTGTAGCCAA-3'
<i>SREBF1</i>	Forward: 5'-ACTTCTGGAGGCATCGCAAGCA-3'
	Reverse: 5'-AGGTTCCAGAGGAGGCTACAAG-3'
<i>FASN</i>	Forward: 5'-TTCTACGGCTCCACGCTTCC-3'
	Reverse: 5'-GAAGAGTCTTCGTCAGCCAGGA-3'
<i>PPARG</i>	Forward: 5'-AGCCTGCGAAAGCCTTTTGGTG-3'
	Reverse: 5'-GGCTTCACATTCAGCAAACCTGG-3'

2.11. Statistical analysis

Data was expressed as mean ± standard error of the mean (SEM). Statistical differences among groups were assessed using one-way analysis of variance (ANOVA) in GraphPad Prism 8.02 (GraphPad Software, San Diego, CA, USA). A *P*-value < 0.05 was considered statistically significant.

2.12. Ethical statement

All animal procedures were conducted following the guidelines of the Animal Center, Hanoi University of Pharmacy. The experimental protocol was reviewed and approved by the Scientific Committee of Hanoi University of Pharmacy (Approval No.: 1081/QD-DHN; date: 31/12/2021).

3. Results

3.1. GME protects mice from ethanol-induced liver injury

To investigate whether *G. montanum* prevents ethanol-caused hepatic injury, GME was administered to mice for 15 d concurrently with increasing doses of ethanol. Hepatotoxicity was assessed by measuring serum levels of hepatic enzymes, including AST, ALT, and GGT, as well as serum bilirubin levels. As shown in Figure 1, ethanol noticeably enhanced serum levels of AST (Figure 1A), ALT (Figure 1B), GGT (Figure 1C), and bilirubin (Figure 1D) (*P*<0.001). GME at doses of 175 and 350 mg/kg significantly attenuated these ethanol-induced increases in hepatic enzymes and bilirubin (*P*<0.05). Histopathological analysis (H&E staining) revealed characteristic features of ethanol-induced hepatotoxicity, including hepatocyte disorganization, necrosis, and ballooning degeneration. However, GME markedly alleviated these morphological abnormalities (Figure 1E). Taken together, these findings suggest that GME exerts hepatoprotective effects against ethanol-induced hepatotoxicity.

3.2. GME attenuates ethanol-induced hepatic steatosis in mice

Ethanol administration significantly increased triglyceride levels in both serum and liver tissue ($P < 0.001$) (Figure 2A and 2B). Additionally, ethanol markedly promoted lipid droplet formation

in hepatocytes (Figure 2C). Interestingly, GME dose-dependently reduced triglyceride levels in both serum and liver homogenates. Furthermore, GME inhibited hepatocellular lipid accumulation, as evidenced by reduced lipid droplet formation (Figure 2C). Overall, these findings indicate that GME effectively mitigates ethanol-induced disruptions in hepatic lipid metabolism.

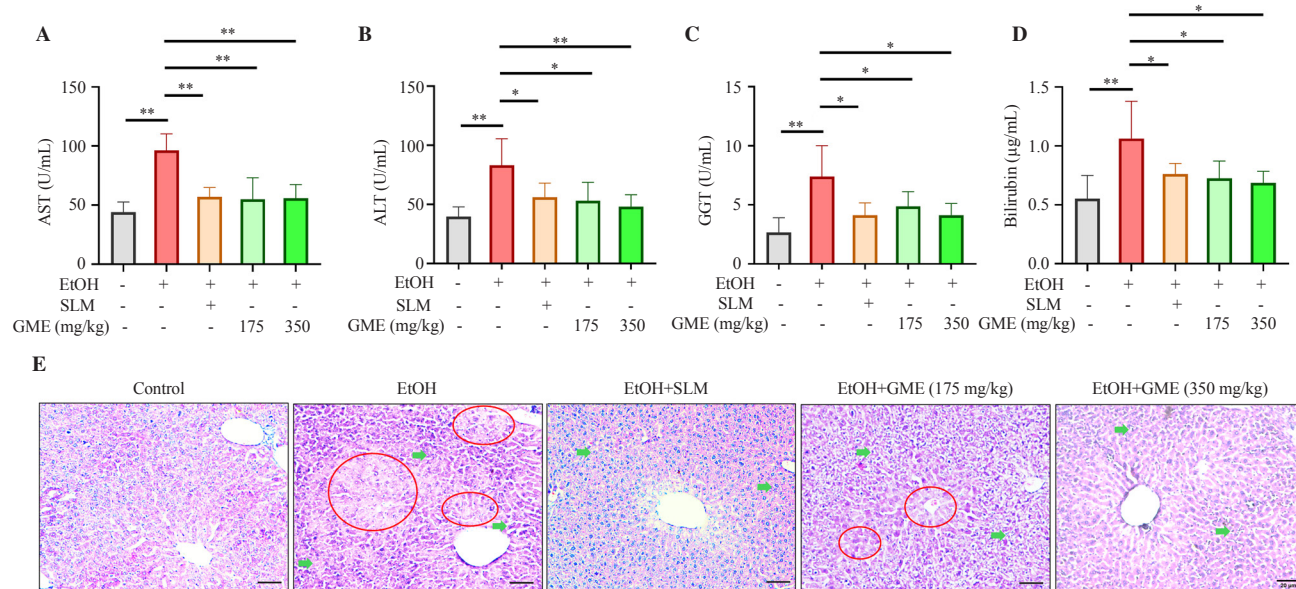


Figure 1. Effects of *Gnetum montanum* (*G. montanum*) extract on serum levels of AST (A), ALT (B), GGT (C), and bilirubin (D) in mice with ethanol-induced hepatocellular injury. (E) Representative histological images of liver tissues in mice (H&E, scale bar: 20 μm, magnification: 100×). The pathological alterations including necrosis and loss of hepatic architecture, as well as hepatocellular degradation, are indicated by red ovals and arrows, respectively. The data are expressed as mean ± standard error of the mean (SEM) ($n = 8$). * and ** denote $P < 0.05$ and 0.001, respectively. GME: *G. montanum* extract, SLM: silymarin.

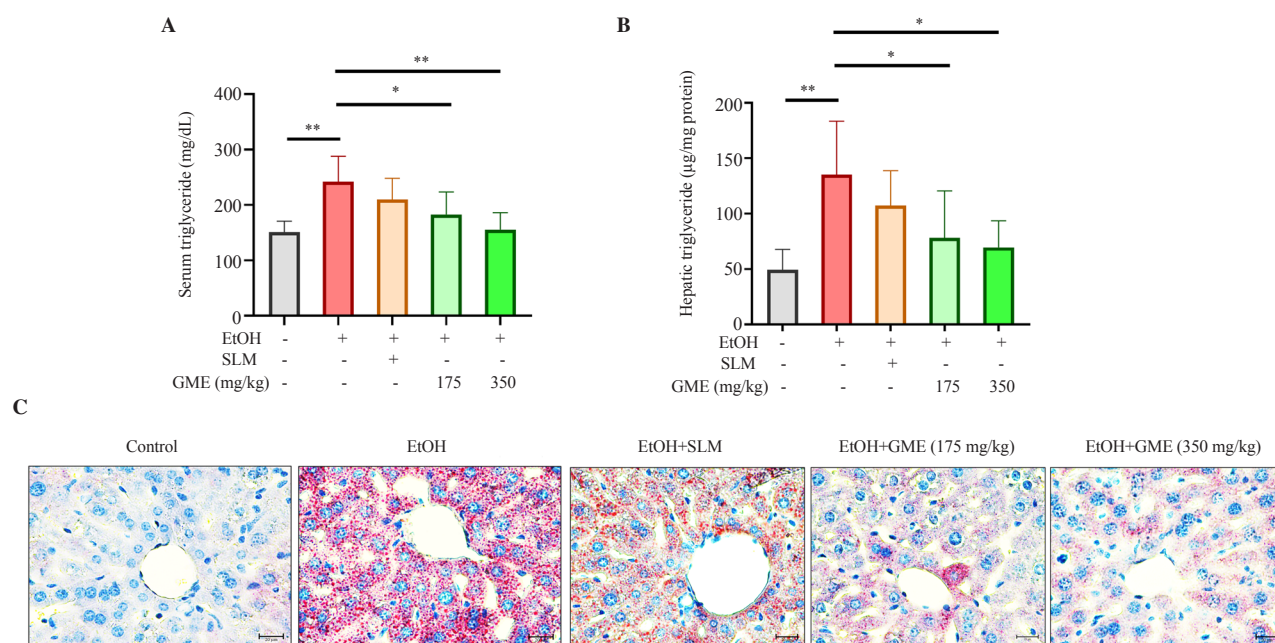


Figure 2. Effects of *G. montanum* extract on (A) serum and (B) tissue triglyceride levels in mice with ethanol-induced hepatic steatosis. (C) Frozen liver sections were stained with oil red O, and lipid droplet accumulation was observed under a light microscope (scale bar: 20 μm; magnification: 400×). The data are expressed as mean ± SEM ($n = 8$). * and ** denote $P < 0.05$ and 0.001, respectively.

3.3. GME inhibits ethanol-induced oxidative stress in liver tissues

To assess oxidative stress, we measured MDA, total antioxidant capacity, GSH levels, and catalase activity in liver tissues of ethanol-administered mice. Ethanol administration significantly elevated MDA levels, depleted GSH, reduced catalase activity, and impaired total antioxidant capacity ($P < 0.001$) (Figure 3A-D). Treatment with GME (175 and 350 mg/kg) led to a dose-dependent decrease in

MDA levels (Figure 3A). Notably, GME at a higher dose (350 mg/kg) restored hepatic GSH and catalase activities, and improved total antioxidant capacity ($P < 0.05$). Collectively, these findings suggest that GME effectively alleviates ethanol-induced hepatocellular oxidative stress.

3.4. GME inhibits XO activity

We investigated whether modulation of XO activity contributes

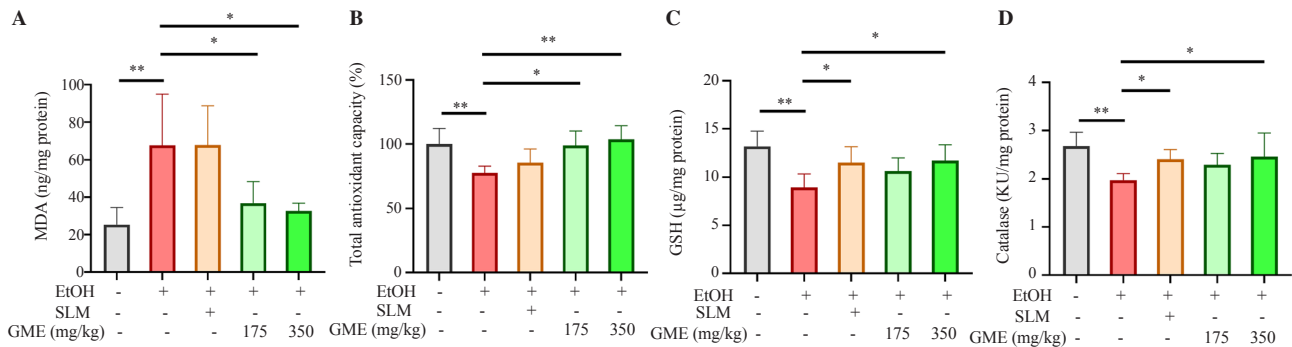


Figure 3. Effects of *G. montanum* extract on (A) MDA, (B) total antioxidant capacity, (C) GSH and (D) catalase levels in mice with ethanol-induced oxidative stress. The data are expressed as mean $\pm$ SEM ($n = 8$). * and ** denote $P < 0.05$ and 0.001 , respectively. MDA: malondialdehyde; GSH: reduced glutathione.

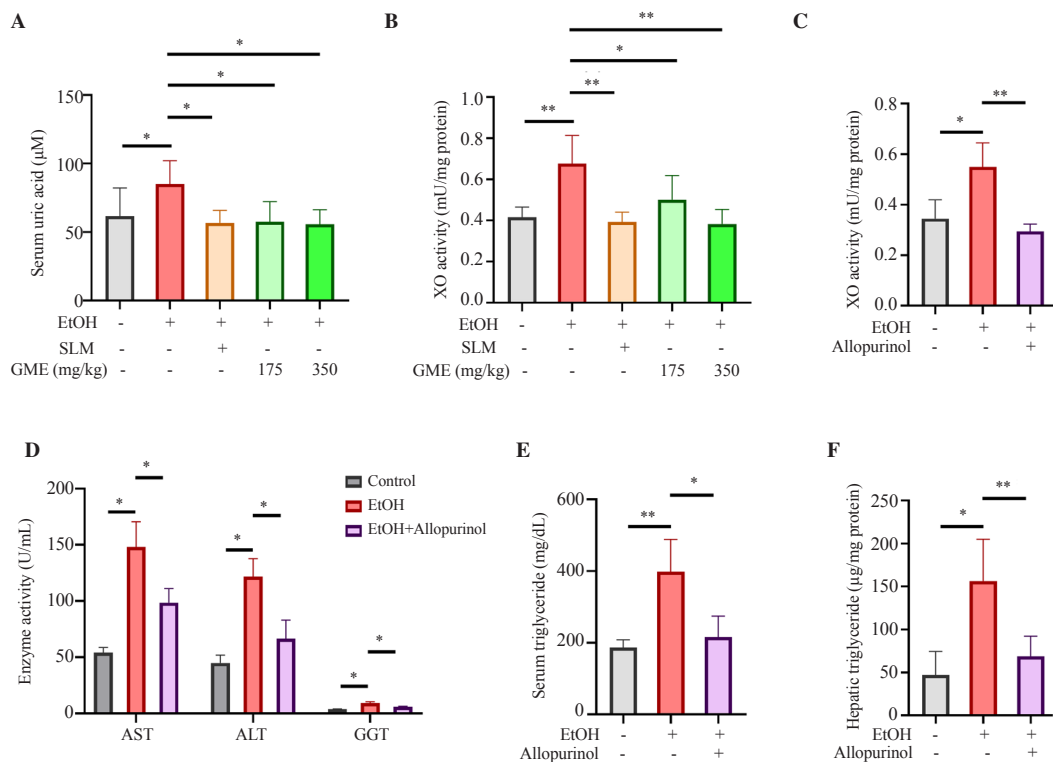


Figure 4. Involvement of xanthine oxidase (XO) in the hepatoprotective effect of *G. montanum* extract in mice with ethanol-induced hepatocellular injury. (A, B) Mice were treated with ethanol for 15 days in combination with GME (175 and 350 mg/kg). Serum uric acid levels and hepatic XO activity were measured. (C-F) Mice were treated with ethanol for 15 days with or without co-treatment with 10 mg/kg allopurinol, a pharmacological XO inhibitor. Hepatic XO activity (C), serum levels of hepatic enzymes (D), and triglyceride levels in serum and liver homogenates (E and F) were determined. The data are expressed as mean $\pm$ SEM ($n = 8$ for figure A and B or 5 for figure C-F). * and ** denote $P < 0.05$ and 0.001 , respectively.

to the hepatoprotective effects of GME against ethanol-induced toxicity and metabolic dysregulation. As expected, oral ethanol administration significantly increased serum uric acid levels (Figure 4A) and hepatic XO activity ($P < 0.05$) (Figure 4B). In contrast, GME dose-dependently suppressed ethanol-induced elevations in uric acid and XO activity ($P < 0.05$).

To further explore the role of XO inhibition in hepatoprotection, we examined whether XO inhibition alone was sufficient to confer protective effects against ethanol-induced pathological alterations. Herein, mice were cotreated with ethanol and allopurinol, a pharmacological XO inhibitor. As expected, allopurinol significantly inhibited hepatic XO activity ($P < 0.001$) (Figure 4C). Importantly, allopurinol effectively suppressed ethanol-induced hepatotoxicity, as evidenced by decreased serum AST, ALT, and GGT levels ($P < 0.05$) (Figure 4D). Furthermore, allopurinol treatment reversed ethanol-induced dysregulation of lipid metabolism by reducing both serum and hepatic triglyceride levels ($P < 0.05$) (Figure 4E and 4F). Given that allopurinol exerted hepatoprotective effects comparable to those of GME, and GME suppressed XO activity in ethanol-treated mice, modulation of XO activity is likely a key mechanism underlying the beneficial effects of GME against ethanol-induced hepatotoxicity

and metabolic disorders.

3.5. GME reduces acetaldehyde-induced XO activation and steatosis in HepG2 cells

Using a mouse model of ethanol-induced metabolic dysfunction, we demonstrated that GME protects hepatocytes from oxidative stress and steatosis, potentially through XO inhibition. To further validate these findings under well-controlled *in vitro* conditions, we examined the effects of GME in HepG2 cells. HepG2 cells were treated with oxidized ethanol (acetaldehyde) to mimic ethanol-induced metabolic dysfunction. As shown in Figure 5A, acetaldehyde significantly increased XO activity in HepG2 cells ($P < 0.05$). However, pretreatment with GME dose-dependently inhibited XO activity ($P < 0.05$). Additionally, acetaldehyde promoted intracellular lipid accumulation, as indicated by increased lipid droplet formation (Figure 5B and 5C). However, GME pretreatment effectively suppressed lipid droplet accumulation. Moreover, acetaldehyde significantly elevated intracellular triglyceride levels, but GME pretreatment reversed this effect ($P < 0.05$) (Figure 5D). Acetaldehyde also markedly increased MDA production, a final

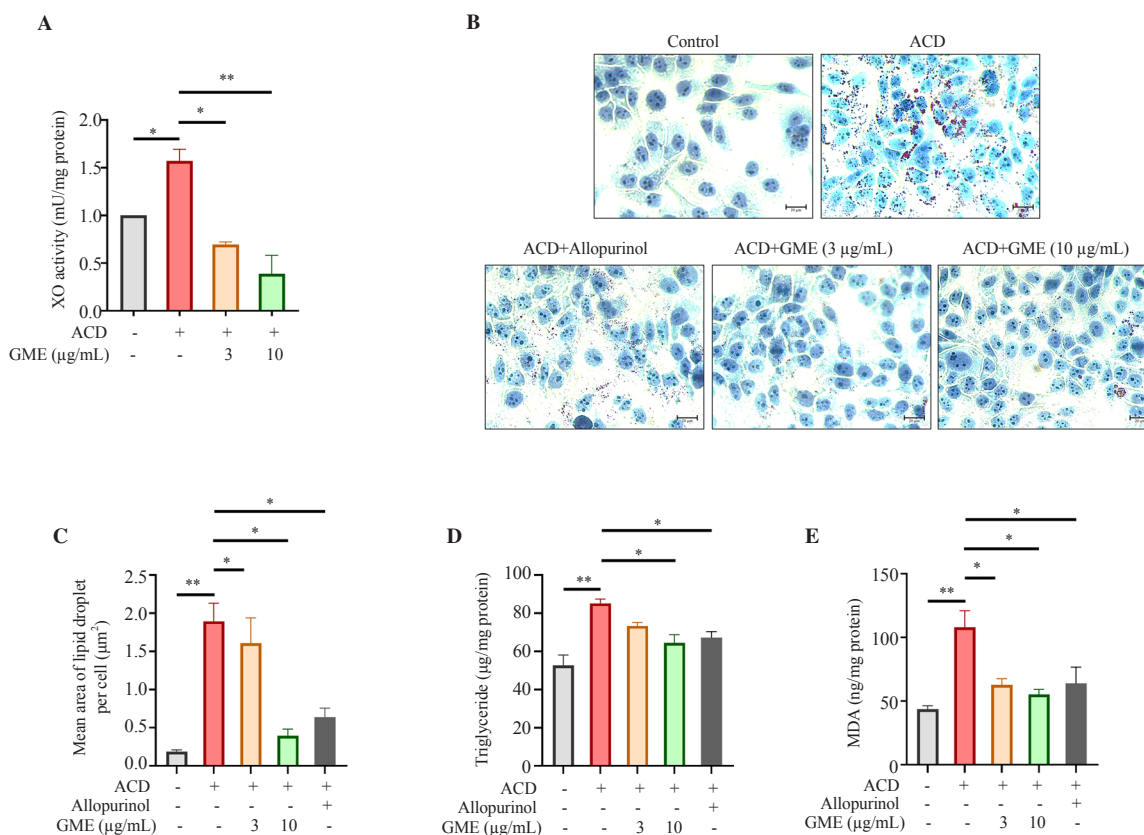


Figure 5. Effects of *G. montanum* extract on XO activity and lipid accumulation in acetaldehyde (ACD)-treated HepG2 cells. HepG2 hepatocytes were pretreated with 3 and 10 µg/mL of *G. montanum* extract, or 10 µM of allopurinol for 2 h, followed by incubation with 1 mM ACD for an additional 48 h. (A) XO activity was measured in cell lysates. (B) Cells were stained with oil red O, and images were captured using a microscope (scale bar: 20 µm; magnification: 400×). (C) Mean lipid droplet area per cell was quantified using ImageJ software. (D and E) Triglyceride and MDA levels in cell lysates were measured. The data are expressed as mean ± SEM ($n = 3$). * and ** denote $P < 0.05$ and 0.001 , respectively.

product of lipid peroxidation. As expected, GME significantly attenuated acetaldehyde-induced MDA formation ($P < 0.05$) (Figure 5E). Notably, allopurinol, a pharmacological XO inhibitor, exhibited hepatoprotective effects similar to those of GME, including reduced lipid accumulation and lipid peroxidation (Figure 5).

Given that GME suppresses acetaldehyde-induced hepatocellular lipid accumulation, we investigated whether its effect on lipid metabolism involves the modulation of lipogenesis. To address this, we examined the mRNA expression levels of lipogenesis-associated genes and found that GME downregulated the mRNA expressions of *SREBF1* (Figure 6A), *FASN* (Figure 6B), and *PPARG* (Figure 6C) in acetaldehyde-treated hepatocytes. Together, these results highlight the role of GME in modulating hepatocellular oxidative stress and metabolic dysfunction upon ethanol exposure. These findings further support XO inhibition as a key mechanism underlying the hepatoprotective effects of GME.

4. Discussion

G. montanum is an indigenous Vietnamese medicinal plant traditionally used to treat inflammation, pain, gout, and certain liver-associated disorders. However, little research has been conducted to elucidate the biological activities of this plant. Recent studies have shown that crude extracts and bioactive compounds derived from *G. montanum* exhibit anti-tumoral, antioxidant, and XO inhibitory activities[16,18,19]. Yet, its potential role in liver-associated disorders remains largely unexplored. To bridge the gap between experimental evidence and traditional medicinal use, we evaluated the hepatoprotective effects of GME using an animal model of ethanol-induced hepatic injury, along with *in vitro* studies in hepatocytes. Our findings indicate that GME protects mice from ethanol-induced hepatic damage, oxidative stress, and lipid metabolic dysfunction.

Interestingly, both *in vitro* and *in vivo* data consistently demonstrated that GME inhibits ethanol-induced XO activation, which may critically contribute to its hepatoprotective properties.

ALD is characterized by elevated serum triglycerides, excessive hepatic fat accumulation, oxidative stress, hepatocellular necrosis, and structural degradation[26,27]. In previous studies, ethanol administration in mice has primarily been achieved through an ethanol-containing diet[28]. However, this method is time-consuming and does not accurately reflect the pattern of ethanol consumption in humans. In contrast, in the present study, ethanol was administered intermittently *via* oral gavage. This approach successfully induced key pathological features of ALD, including elevated serum triglycerides, hepatic steatosis, increased lipid peroxidation, and hepatocellular oxidative stress and damage after 15 days of ethanol exposure. These findings support the applicability of this model for evaluating the hepatoprotective effects of GME against ethanol-induced toxicity. However, a limitation of this model is its relatively short duration of ethanol exposure, which may not fully replicate the chronic progression of ALD in humans. Future studies should employ a chronic ethanol exposure model to further validate these findings.

Chronic ethanol consumption disrupts lipid metabolism, leading to hepatic fat accumulation. Therefore, we investigated whether GME could normalize lipid metabolism in ethanol-treated mice. Interestingly, GME significantly reduced hepatic lipid accumulation, as evidenced by lower triglyceride levels and reduced intracellular lipid droplet formation. These findings were further supported by *in vitro* studies, in which GME abrogated acetaldehyde-induced lipid accumulation in HepG2 cells. Acetaldehyde, the primary metabolite of ethanol, was used instead of ethanol because HepG2 cells lack alcohol dehydrogenase activity, making them resistant to ethanol toxicity[29]. Using acetaldehyde allowed us to overcome this limitation while assessing the direct effects of GME. Importantly,

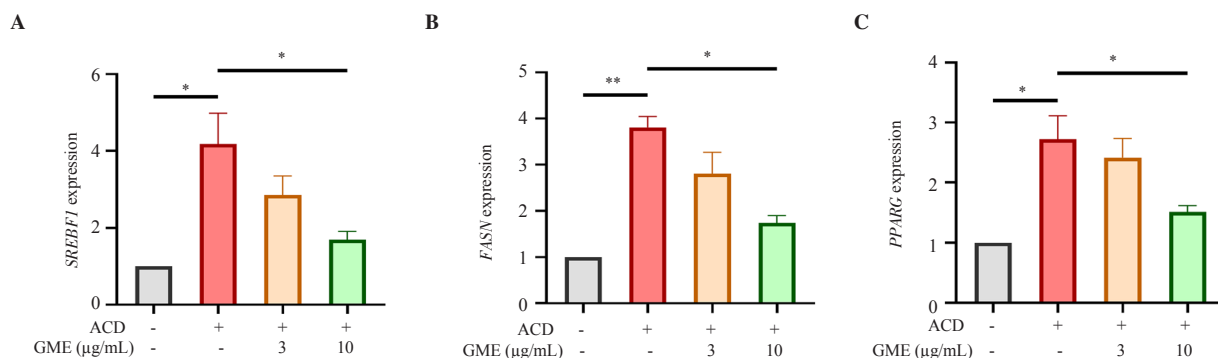


Figure 6. Effects of *G. montanum* extract on the expression of key lipogenesis-related genes in acetaldehyde-treated HepG2 cells. (A-C) HepG2 cells were treated with 3 and 10 $\mu\text{g/mL}$ of *G. montanum* extract for 2 h, followed by incubation with 1 mM ACD for further 48 h. The mRNA levels of *SREBF1* (A), *FASN* (B), and *PPARG* (C) were measured by RT-qPCR analysis. *GAPDH* was used as an internal control. The data are expressed as mean $\pm$ SEM of three independent experiments. * and ** denote $P < 0.05$ and 0.001 , respectively.

GME remained effective against acetaldehyde-induced lipogenesis, suggesting that its hepatoprotective effects are independent of alcohol dehydrogenase inhibition.

Ethanol is known to deplete GSH, thereby exacerbating hepatocellular oxidative stress. Additionally, steatosis-induced lipotoxicity may further aggravate oxidative damage by promoting lipid peroxidation[30]. Although this study did not directly evaluate the impact of GME on ethanol oxidation and ROS generation, its ability to alleviate ethanol-induced oxidative stress suggests that its antioxidant properties, previously reported in the literature, may contribute to its protective effects. Additionally, the suppression of lipid accumulation by GME likely plays a role in restoring redox balance by reducing lipid peroxidation. These actions collectively protect hepatocytes from ethanol-induced injury, as indicated by reduced serum leakage of hepatic enzymes and preservation of hepatocyte integrity. Regarding the dosage of GME used in the study, the two doses (175 and 350 mg/kg) were selected based on preliminary studies and traditional medicinal usage. Interestingly, the lower dose (175 mg/kg) was nearly as effective as the higher dose in reducing hepatocellular necrosis, whereas the higher dose exhibited additional benefits in reducing hepatic lipid accumulation and oxidative stress. Future research should explore the dose-dependent mechanisms of GME's hepatoprotective effects.

Alcohol consumption is a major risk factor for hyperuricemia. Mechanistically, ethanol increases adenine degradation, resulting in an elevated supply of XO substrates (hypoxanthine and xanthine)[31]. Additionally, ethanol alters xanthine metabolism by increasing the O-form of XO over its D-form, further promoting uric acid production[32]. Alongside uric acid overproduction, XO-mediated metabolic reactions generate superoxide radicals, which contribute to oxidative stress and lipid peroxidation[33]. Recent reports have highlighted a strong correlation between XO activity and hepatic steatosis, with elevated serum XO activity identified as an independent risk factor for hepatosteatosis[12]. Moreover, XO inhibition has been shown to ameliorate diet-induced hepatic lipid accumulation, emphasizing its role as a key mediator in hepatic metabolic disorders[33]. Consistent with previous reports, ethanol exposure in this study led to hyperuricemia, at least in part *via* hepatic XO activation. Notably, GME suppressed XO activity and reduced ethanol-induced hyperuricemia, suggesting that XO inhibition contributes to its hepatoprotective effects. This hypothesis was further supported by our observation that allopurinol, a pharmacological XO inhibitor, alleviated ethanol-induced liver injury and steatosis. Interestingly, silymarin, a well-established hepatoprotective agent, also reduced serum uric acid and hepatic XO activity. Also, silymarin has previously been identified as a potent XO inhibitor and a modulator of serum urate levels[34,35]. These findings suggest that XO inhibition may represent a novel therapeutic

strategy for steatotic liver diseases, and this study demonstrates the protective effects of a natural XO inhibitor against ethanol-induced liver damage.

Phytochemical studies have shown that *G. montanum* is rich in stilbenes[16]. Notably, several stilbenes found in *G. montanum*, including (E)-2'-methoxy-3,5,5'-trihydroxystilbene, gnetol, gnetulin, and resveratrol, are known pharmacological inhibitors of XO[16,36]. Resveratrol, in particular, has been reported to protect the liver from ethanol-induced oxidative stress and lipid accumulation[37,38]. It is also worth noting that while resveratrol is a potent SIRT-1 activator, impaired SIRT-1 signaling is involved in the action of ethanol on hepatic lipid metabolism. For example, SIRT-1 deacetylates SREBP-1, which is a master regulator of *de novo* lipogenesis, and thereby inhibits SREBP-1 activation by ethanol[6,39,40]. Interestingly, febuxostat, a second-generation XO inhibitor, has been shown to upregulate SIRT-1 expression, suggesting a complex interaction between XO activity and SIRT-1 signaling[41]. To further explore this connection, we analyzed the effects of GME on lipogenesis-related genes in HepG2 cells exposed to ethanol. GME prevented ethanol-induced upregulation of key lipogenic genes, including *SREBF1*, *FASN*, and *PPARG*. These findings are consistent with previous reports linking XO activity to lipid metabolism[42]. Future studies should investigate whether SIRT-1 activation contributes to the hepatoprotective effects of GME and identify the specific bioactive compounds responsible for its actions.

In conclusion, the present study demonstrates the role of GME in protecting the liver from ethanol-induced toxicity and metabolic disorders. First, GME mitigates oxidative stress in liver tissues and thereby prevents hepatocellular damage. Second, GME suppresses hepatic fat deposition by improving lipid metabolism in hepatocytes and decreases serum triglyceride levels. Finally, GME attenuates ethanol-induced hyperuricemia. From a mechanistic perspective, the inhibition of XO activity might play a central role in the beneficial actions of GME. Future studies should focus on further elucidating the underlying molecular mechanisms as well as clinical translation of these findings.

Conflict of interest statement

The authors have no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

HLT performed the experiments, collected data, and drafted the manuscript. Both TDN and THN contributed to the experimental design and data analysis. TDN secured funding for the project. HNN and DVP critically revised the manuscript and were responsible for the final version. DVP conceived the idea and supervised the project.

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