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Original Article:

Effects of *Aloysia citriodora* Hydroalcoholic Extract on Ethanol-induced Hepatotoxicity in Male Wistar Rats

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ABSTRACT

Background: Chronic ethanol consumption presents toxic effects on liver tissue by inducing oxidative stress and inflammation. The antioxidant and anti-inflammatory properties of lemon verbena were established.

Objectives: The present study aimed to evaluate the protective effects of the hydroalcoholic extract of *Aloysia citriodora* (*A. citriodora*) on ethanol-induced hepatotoxicity in male rats by evaluating inflammatory and oxidative stress factors.

Methods: The study animals were randomly divided into 7 groups, (6/group) including control, extract alone (400mg/kg), ethanol 10 mg/kg, vitamin C 500 mg/kg + ethanol 10 mg/kg, and the fifth, sixth and seventh groups respectively received an intraperitoneal injection of 100, 200, and 400 mg/kg of *A. citriodora* extract plus ethanol once a day for 6 weeks. Oxidative stress parameters, such as glutathione content, lipid peroxidation, protein carbonyl, and reactive oxygen species were measured. Furthermore, inflammation parameters (nitric oxide) and liver damage were evaluated by determining the levels of liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and histopathological examinations.

Results: In the liver tissue of the ethanol-receiving group, a significant increase ($P<0.001$) was observed in ALT, AST, and ALP levels and pathological changes, compared to the control group. There was also a significant increase in the levels of oxidative stress and inflammatory factors. Interestingly, *A. citriodora* extract could inhibit ethanol-induced liver damage by suppressing oxidative stress and inflammation.

Conclusion: *A. citriodora* extract significantly attenuated inflammation and oxidative stress caused by ethanol. Therefore, it can be suggested as a beneficial supplement for treating ethanol-induced hepatotoxicity.

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Introduction

Ethanol consumption is increasing worldwide [1]; depending on the dose and duration of exposure, it can exert toxic effects on the body. Ethanol metabolism mainly occurs in the liver [2]. Ethanol consumption, in addition to hypoglycemia, acidosis, and glycogen depletion can cause psychological and behavioral changes [1]. At the cellular scale, ethanol can cause mitochondrial damage, free radical production, the inhibition of insulin signals, and eventually cell death [3-8]. Ethanol-Induced liver damage can be associated with steatosis, inflammation, necrosis, fibrosis, and cirrhosis [1, 6, 8]. Several factors are involved in ethanol pathogenicity, like mitochondrial damage; oxygen-free radical production appears to be induced by ethanol metabolism [1, 6-9].

The lemon verbena, scientifically named *Aloysia citriodora* (AC), belongs to the Verbenaceae family and is native to South America [10]. This plant consists of high amounts of phenols and flavonoids and other effective compounds. Additionally, AC has properties, such as sedative effects [11, 12], anticonvulsant [13], reduce intestinal irritability [14], antioxidants and cellular protection [15-17], analgesic, and anti-inflammatory properties [18-20], and so on. Some studies revealed that 0.5 mg/kg of AC extract in pregnant animals is safe and without teratogenic effects [21]. In another study, the toxic dose of lemon verbena in mice was estimated to be 1 g/kg/day [22].

This study aimed to evaluate the protective effects of the hydroalcoholic extract of AC on ethanol-induced hepatotoxicity in male rats by evaluating inflammatory factors and oxidative stress.

Materials and Methods

The leaves of the lemon verbena were obtained by a botanist with pharmaceutical market code AE1-36-341 after species approval. Ethanol was collected from Merk Company (Germany). The assay kit for NO was a product of Jahad Daneshgahi Company (Iran). All other chemicals and reagents used in this study were of analytical grade. Market code AE1-36-341 after species approval and then, deposited in herbarium of faculty of pharmacy. Mazandaran University of Medical Sceinces

Initial extraction was performed with 100 g of crushed and dried leaves of the plant with hexane by maceration method to remove fat and pigment compounds. Extraction was performed by repeated maceration in 80% methanol solvent for 48 hours and repeated 3 times. The hydroalcoholic extract was concentrated under reduced pressure by a rotary evaporator and dried to a fine powder with a freeze-drier.

The standardization of *A. citriodora* leaves extract was performed based on total phenol content concerning Gallic acid using folin-ciocalteu reagent and flavonoid content using aluminum chloride respecting quercetin by spectrophotometry and drawing a standard curve [23].

Male rats weighing approximately 200 g were obtained from the Laboratory Animals Research Center, Mazandaran University of Medical Sciences, Sari City, Iran. They were housed under the standard conditions of temperature at 23±1°C with regular 12:12 h light/dark cycle, and 30%-40% humidity with free access to food and water. All experimental procedures were conducted according to the ethical standard and protocols approved by the Committee of Animal Experimentation of Mazandaran University of Medical Sciences, Sari, Iran, and consistent with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

The studied rats were randomly divided into 7 groups (n=6/group). Group 1 served as the control and group 2 received plant extract (400 mg/kg/day). Group 3 received ethanol (10mg/kg/day), and group 4-7 received ethanol with different doses of plant extract (100, 200, & 400 mg/kg/day) and vitamin C (as a known antioxidant, 500 mg/kg). All interventions were intraperitoneally administered for 6 consecutive weeks.

The day after receiving the last dose, the animals were anesthetized and a blood sample was obtained directly from the heart (centrifuged at 3000 rpm for 15 min). The serum was then stored in a freezer refrigerator until tested. Their livers were removed; one part of the liver was used for histological evaluation. For biochemical tests, the other parts of the liver were homogenated and centrifuged at 2000 rpm for 10 min. The supernatant was used for evaluating inflammatory and oxidative stress markers.

Protein content was determined in liver tissue by the Bradford method [24]. Bovine Serum Albumin (BSA) was used as standard and absorbance was determined at 595 nm by spectrophotometer (UV- 1601 PC, Shimadzu, Japan).

The radical scavenging properties of plant extracts were performed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) test with ascorbic acid as positive control and reported as IC50 [25]. The amount of carbonyl protein was measured

using a reagent (2,4-Dinitrophenyl-Hydrazine DNPH), lipid peroxidation by thiobarbituric acid [26]. Moreover, the amount of Reactive oxygen species (ROS) was measured using a 2'-7'dichlorofluorescin diacetate (DCFH-DA) reagent [27]. Also, glutathione (GSH) content was measured using DTNB at 412 nm [28].

Nitric oxide content was measured using the commercial kits based on the Griess reagent (Jahad Daneshgahi Company, Iran). In this method, Sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl) ethylenediamine, forming an azo dye that can be spectrophotometrically quantitated based on its absorbance at 548 nm [29].

Animal serum samples were provided to the laboratory for liver enzyme levels (including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP).

For light microscopic investigations, specimens from the liver were fixed in 10% phosphate buffer formalin, clarified in xylene, dehydrated in alcohols, and embedded in paraffin. Five-microns thick samples were stained with Hematoxylin and Eosin (H&E) for general histopathological examination. Slides were observed under a light microscope (Nikon Labophot, Japan).

The obtained data were reported as Mean±SD and evaluated by SPSS using one-way Analysis of Variance (ANOVA). The Tukey post hoc test was also used to compare differences between the study groups.

Results

After extracting and drying AC, 19.7 g of hydro-alcoholic, the extract powder was obtained from each 100 g of dried plant. The total yield of the extract respecting the total weight of AC dried plant equaled 19.7%.

The total phenolic content of the hydroalcoholic extract of *A. citriodora* leaves was determined by standard curve (y=0.005x+0.062); the relevant Mean±SD equaled 73.1±0.20 mg gallic acid per gram of extract.

The total Mean±SD flavonoid content of *A. citriodora* leaves extract was determined by standard curve (y=0.0064x+0.0076) to be 68.2±14.1 mg of quercetin per gram of extract.

The Mean±SD IC50 of the extract was calculated as 19.4±1.56 µg/mL and the Mean±SD IC50 of vitamin C was measured as 5.04±0.02 µg/mL.

According to Figure 1, administrating ethanol alone significantly increased the amount of oxygen free radicals in the liver tissue. Additionally, this amount was significantly ($P<0.05$) reduced by *A. citriodora* extract treatment. The highest effect was observed in the dose of 400 mg/

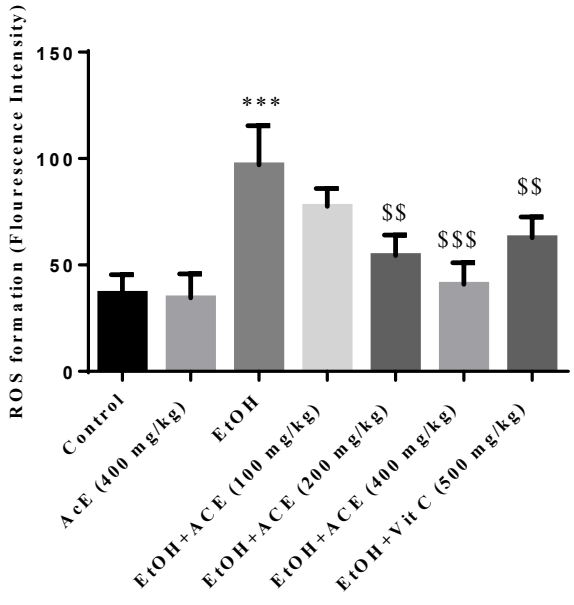

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Figure 1. The effects of ethanol and different doses of *A. citriodora* extract on ROS production in the liver tissue of male rats
***P=0.001, compared to the control group; $$P=0.01 and $$$P=0.001, compared to the ethanol group

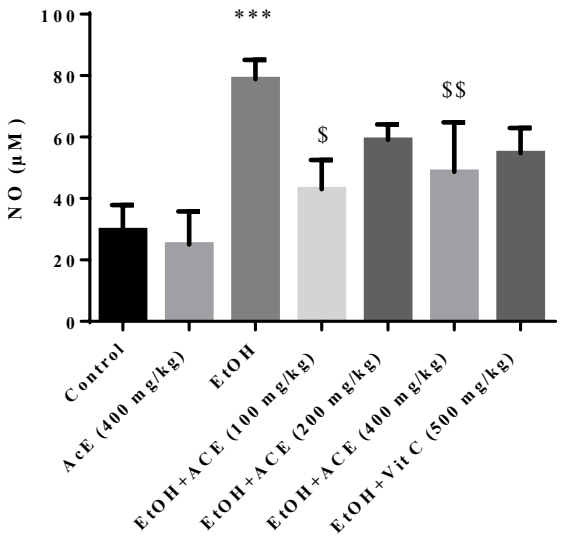

Figure 2. The effects of ethanol and different dos of *A. citriodora* extract on NO production in the liver tissue of male rats
***P=0.001, compared to the control group; $P=0.05 and $$P=0.01, compared to the ethanol group

kg of *A. citriodora* extract, i.e., less than the production of oxygen free radicals in the ethanol group (P<0.05).

According to Figure 2, administrating ethanol significantly (P<0.05) increased the amount of nitric oxide in the liver tissue. Moreover, AC extract at a dose of 400 mg/kg significantly (P<0.05) decreased the production of nitric oxide, compared to the ethanol group.

According to Figure 3, the liver GHS level test results signified that ethanol could significantly (P<0.05) reduce cellular GSH levels, compared to the control group. Furthermore, administrating *A. citriodora* extract (200 mg/kg) significantly increased the GSH, compared to the ethanol group (P<0.05).

According to Figure 4, lipid peroxidation was significantly (P<0.05) increased by the addition of ethanol, followed by MDA production. When *A. citriodora* extracts

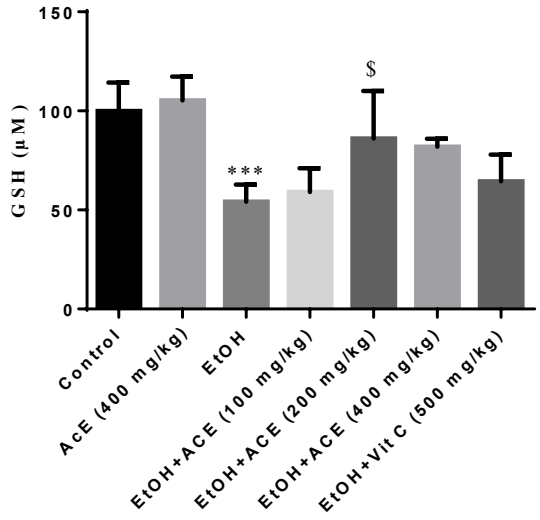

Figure 3. The effects of ethanol and different doses of *A. citriodora* extract on GSH levels in the liver cells of male rats
***P=0.001, compared to the control group; $P=0.05, compared to the ethanol group

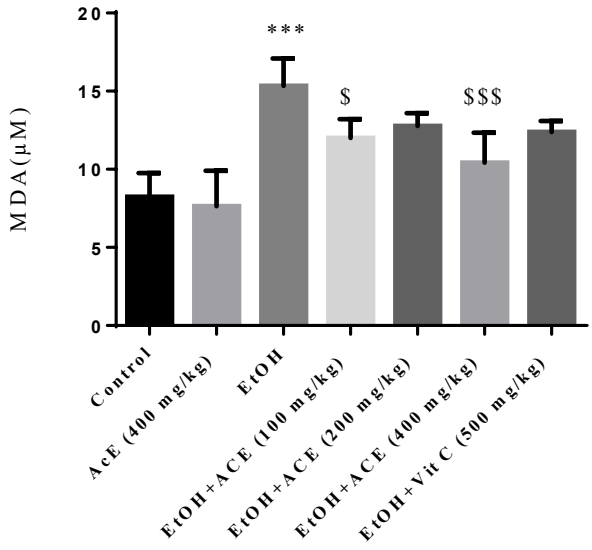

Figure 4. The effects of ethanol and different doses of *A. citriodora* extract on MDA concentration in the liver tissue of the examined male rats

***P=0.001, compared to the control group; $P=0.05 and $$$P=0.001, compared to the ethanol group

were administered to the explored rats after ethanol, MDA production, as a marker of lipid peroxidation, was significantly reduced (P<0.05) at a dose of 400 mg/kg of this extract. According to Figure 5, ethanol significantly (P<0.05) increased carbonyl protein in the liver tissue. However, the addition of *A. citriodora* extract reduced the amount of carbonyl protein in the liver supernatant. The significant effect of *A. citriodora* extract was observed at the doses of 200 mg/kg and 400 mg/kg, compared to the ethanol group (P<0.05).

According to Figure 6, adding ethanol significantly enhanced the liver enzymes. Administrating *A. citriodora* extract after ethanol significantly reduced the liver enzymes. Histopathological changes of all research groups are exhibited in Figure 7. In the control group, there was liver tissue with a normal structure (Figure

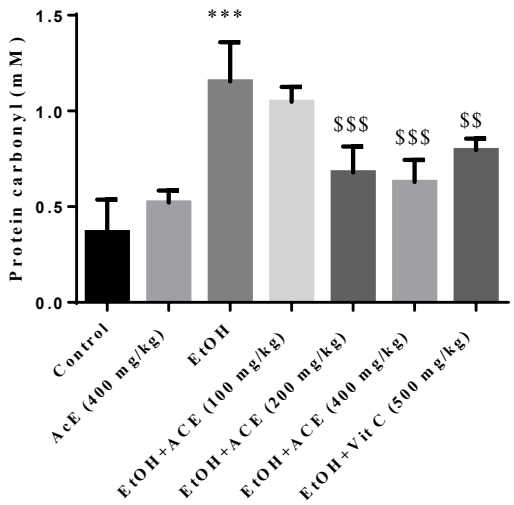

Figure 5. The effects of ethanol and different doses of *A. citriodora* extract on carbonyl protein content in the liver tissue of the explored male rats

***P=0.001, compared to the control group; $$P=0.01 and $$$P=0.001, compared to the ethanol group

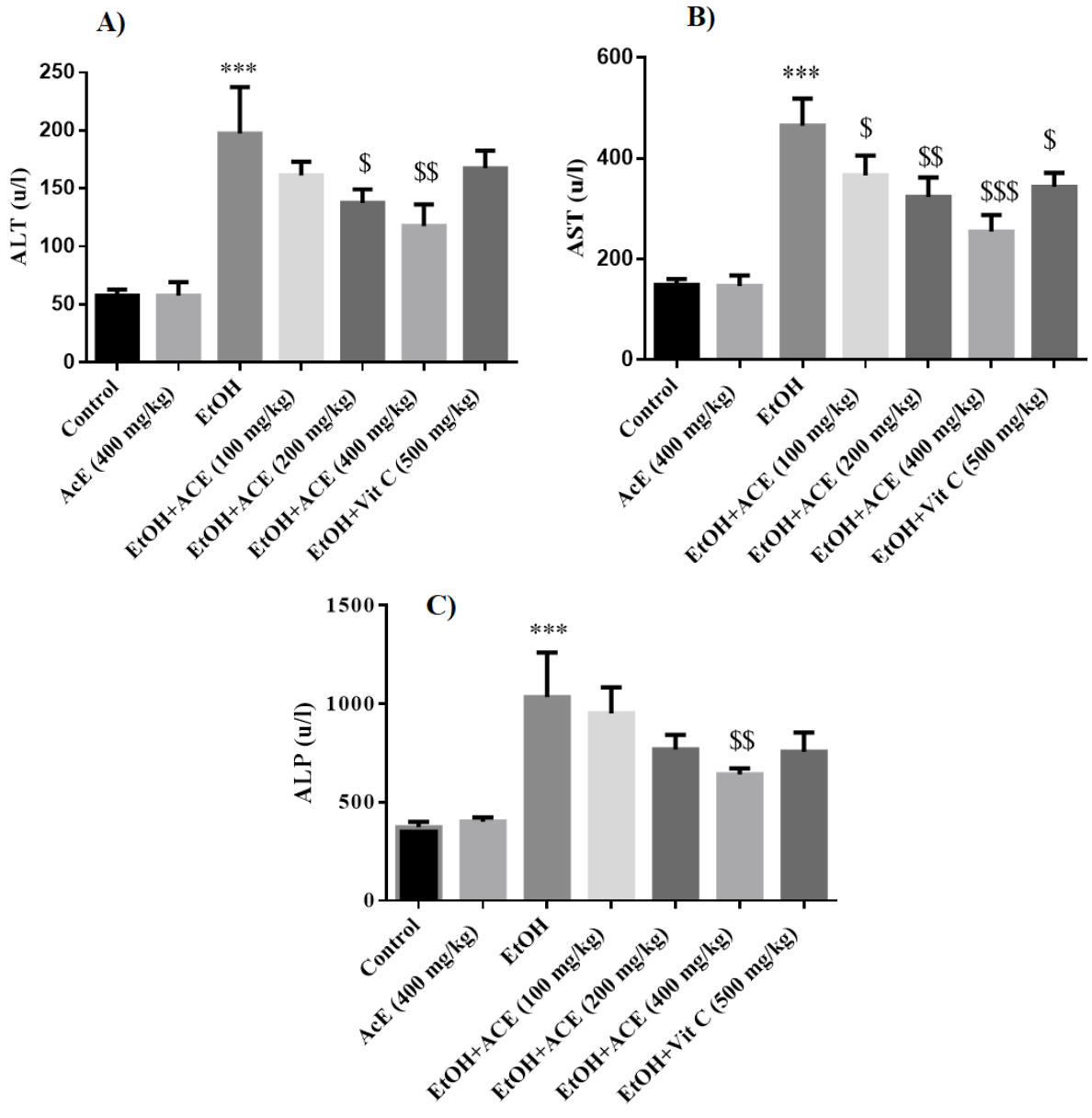

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Figure 6. The effects of ethanol and different concentrations of *A. citriodora* extract on liver enzymes in the studied male rats

The data were presented as Panel A) ALT, Panel B) AST Panel C) ALP. ***P=0.001, compared to the control group; $^{\$}$P=0.05 and $^{\$\$}$P=0.01, compared to the ethanol group

7A). In the examined rats receiving ethanol, severe liver damages, such as congestion, sinusoid dilation (black arrow), increased nuclear density (indicating the onset of necrosis), granulomatosis formation (white arrow), Kupffer cell proliferation, and hepatocyte eosinophilia (red arrow) were detected (Figure 7B). In the group that received the extract plus ethanol, the structure of liver tissue was relatively preserved and the examination of tissue sections suggested that further improvement was achieved by increasing the dose of the extract (Figures 7C, 7D, 7E).

Discussion

This study investigated the protective effects of the hydroalcoholic extract of *A. citriodora* on ethanol-induced hepatotoxicity in male rats by evaluating inflammatory factors and oxidative stress. Various studies indicated that ethanol can affect liver function [30-33]. Hepatic transaminases are evaluated to measure liver damage. When a hepatocyte dies, its intracellular enzymes are released into the blood. Some of these enzymes, i.e., tested for liver damage include ALT, AST, and ALP. Ethanol increases hepatic transaminases by inducing oxidative

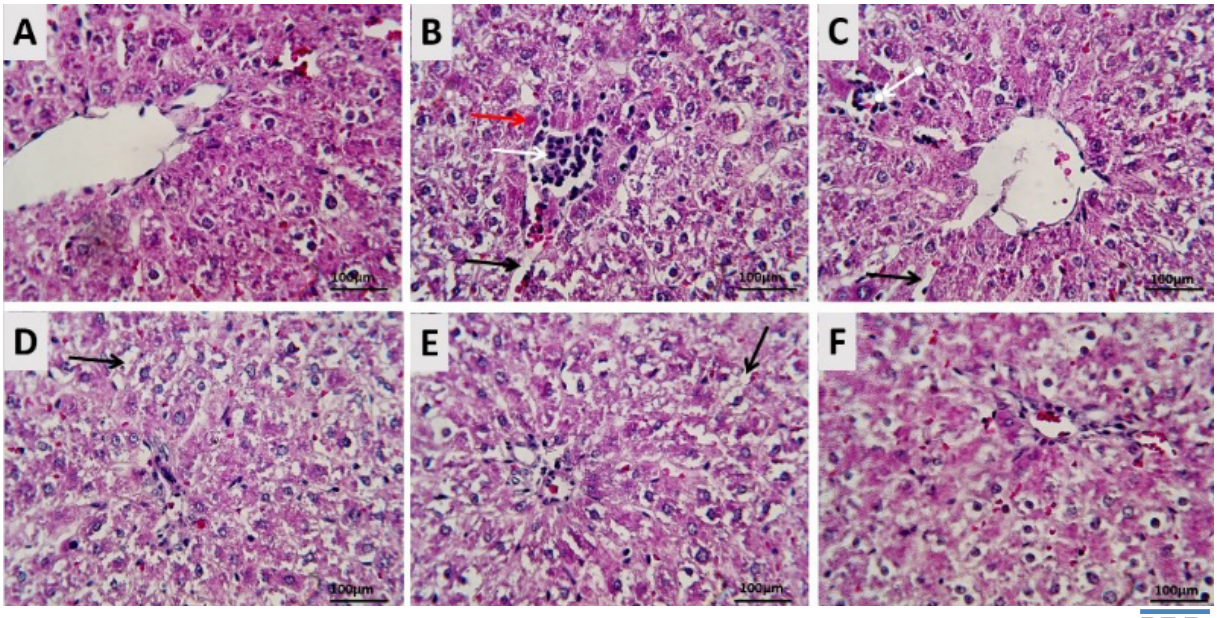

Figure 7. Liver tissue photomicrographs manifesting the effects of ethanol and plant extract on liver histoarchitecture in all research groups

A: Control, B: Ethanol, C: Ethanol and plant extract at a concentration of 100 mg/kg, D: Ethanol and plant extract at a concentration of 200 mg/kg, E: Ethanol and plant extract at a concentration of 400 mg/kg, F: Ethanol and vitamin C. Hand&E staining, magnification: 40×, scale bar= 100 µm

stress and increasing hepatocyte mortality [34-39]. Previous studies indicated that ethanol consumption leads to histopathological changes in the liver tissue, such as liver weight gain, focal necrosis, mild to moderate steatosis, fibrosis, and necrotic inflammation [40-42].

The histopathological and biochemical findings highlighted that ethanol could present a toxic effect on the liver, i.e., in line with the previous data. Additionally, chronic and acute ethanol consumption can cause oxidative damage in the liver tissue [43]. In the first stage of ethanol metabolism, it is converted to acetaldehyde by alcohol dehydrogenase; then, it oxidizes to acetate by the aldehyde dehydrogenase activity [44]. In this process, xanthine-oxidoreductase (one of the main sources of superoxide anion), is activated. Besides, ethanol can be oxidized to acetaldehyde via cytochromes P450system (CYP2E1) that results in ROS generation [44].

In this study, ethanol injection caused increased oxidative stress markers, such as lipid peroxidation, protein carbonyl, and the depletion of glutathione content in the liver tissue. Our data supported those of the previous studies reflecting that ethanol can cause oxidative stress in the liver [23, 40-42]. These data supported the link between ethanol-induced oxidative stress and liver toxicity. Therefore, medications and compounds with antioxidant effects may be beneficial in attenuating ethanol-induced liver oxidative damages.

Phenolic compounds are part of the plant's secondary metabolites; the production of which can vary depending on the culture and maturity of the plant [45-48]. Flavonoids are polyphenolic structures with anti-inflammatory, antimicrobial, cellular protection, and antineoplastic properties [49, 50]. *Aloysia citriodora* belongs to the Verbenaceae family. It contains high amounts of phenols and flavonoids and other effective compounds [10]. Various studies addressed flavonoids in the leaves of the lemon plant, including 6-hydroxy luteolin, acastin-7-diglucuronide, apigenin, apigenin 7-Diglucuronide, Chrysoeriol, Chrysoeriol-7-diglucuronide, Cirsiliol, Cirsimaritin, Diosmetin, Eupafolin, Upaturin, Hispidolin, Jaceosidin, Luteolin, Neptin, Nepitrin, and Pectolinarigenin [51-53].

DPPH is a stable nitrogen-free radical that produces a yellow color during the scavenging process. Substances that can cause this discoloration are known as antioxidants and free radical scavengers [54, 55]. Furthermore, 83.4% of phenols and flavonoids manifest good antioxidant activity [56]. *A. citriodora* extract, with the levels of flavonoids and phenols, can trap free radicals. We found that *A. citriodora* extract can reduce ethanol-induced ROS. Therefore, the presence of phenolic and flavonoid compounds in the structures of this plant can be respon-

sible for their antioxidant activity. Various studies highlighted that *A. citriodora* extract can reduce the amount of ROS; thus, it declines oxidative stress by decreasing the translation of nuclear factor kappa B (NF-κB) and elevating the translation of adiponectin [57] and free radical scavenging [58], as well as activating antioxidant enzymes, such as such as Superoxide dismutase (SOD and Glutathione-S-transferase (GST) [59, 60].

The obtained results indicated that *A. citriodora* extract can reduce NO produced by ethanol. Studies suggested that the 3 compounds of luteoline-7-O-diglocronide, verbascoside, and verbascoside iso in *A. citriodora* extract can affect inflammation by trapping nitric oxide [61]. The combination of Verbascoside in this plant also prevents the production of further NO by inhibiting activating AP-1 [62, 63].

Glutathione is a well-known non-enzymatic antioxidant that plays a main role in the detoxification of free radicals and electrophilic metabolites [64, 65]. The present research results revealed that *A. citriodora* extract could prevent ethanol-induced GSH reduction in the rat's liver. Various studies signified that the extract of this plant, with its antioxidant compounds, can help increase GSH levels by trapping free radicals [66-69].

A main undesirable effect of ROS is presented on cellular macromolecules, such as lipids and proteinlipid that can lead to cell membrane oxidative damages [70, 71]. We found that *A. citriodora* extract can reduce lipid peroxidation and ethanol-induced protein oxidation. Furthermore, *A. citriodora* extract can prevent lipid peroxidation and protein oxidation by increasing SOD activity [72-74]. This can also be explained by the strong antioxidant properties of this extract described earlier.

Additionally, ROS-mediated cell toxicity adversely influences organ function. Thus, assessing the plasma levels of liver enzymes represents the impairment of liver function [75]. The present study also found that *A. citriodora* extract could reduce the serum levels of liver enzymes (ALP, AST, ALT), i.e., increased with ethanol. Various studies have attributed this effect to the antioxidant properties of this extract and the presence of verbascoside [76-78].

The current study results indicated that the hepatoprotective effects of *A. citriodora* extract largely resulted from its ability to decrease oxidative stress and preserve antioxidant activity by stabilizing antioxidant defense systems, ROS scavenging activity, reducing lipid peroxidation, and protein oxidation. It can also protect the liver from damages caused by ethanol.

Conclusion

Overall, we found that *A. citriodora* presents beneficial effects on hepatotoxicity and oxidative injury following ethanol toxicity in rats. Therefore, A. cirtiodora would be considered as a supplement for protection against ethanol-induced liver injuries.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Mazandaran University of Medical Sciences (Ethical Code: IR.MAZUMS.REC.1398.949).

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Authors' contributions

Conceptualization and Supervision: Fatemeh Shaki and Dr.Emran Habibi; Methodology: Fatemeh Shaki, Emran Habibi, Fereshteh Talebpour Amiri; Investigation, Writing – original draft, and Writing – review & editing: All authors; Data collection: Mahboobeh Feyzi Gharehsou and Mehdi Mokhtari; Data analysis: Fatemeh Shaki, Emran Habibi, and Fereshteh Talebpour Amiri; Funding acquisition and Resources: Fatemeh Shaki and Emran Habibi.

Conflict of interest

The authors declared no conflict of interest.

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