




A Stereological and Biochemical Examination: Hepatoprotective Activity of *Anthemis odontostephana* Boiss. Ethanol Extract Against CCl₄-Induced Hepatotoxicity in Mice

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Abstract

Background and objectives: In Iranian traditional medicine, *Anthemis odontostephana* Boiss has been used in treating gastric ulcers, diabetes, and inflammatory diseases. In the present study, hepatoprotective activity of *A. odontostephana* ethanol extract (AOEE) on carbon tetrachloride (CCl₄)-induced hepatotoxicity in male mice has been evaluated. **Methods:** In the present experimental study, Sixty male mice were divided into six groups (n=10); Group I was considered as control, received 1 mL/kg olive oil intraperitoneally and 0.5 mL distilled water through gavage. Group II was considered as untreated group, received 1 mg/kg CCl₄ mixed with olive oil in the ratio of 1:1, intraperitoneally and 0.5 mL distilled water orally. Group III, IV, V and VI received CCl₄ mixed with olive oil in the ratio of 1:1 intraperitoneally and 20, 40, 80 and 160 mg/kg of AOEE through gavage for 45 continuous days. On the last day, the animals of all groups were euthanized and blood and liver were collected for assessing biochemical and histological parameters. The data were analyzed using one-way ANOVA and post-hoc Duncan's tests. **Results:** Different doses of AOEE (especially AOEE160) could significantly (p<0.05) decrease the raised volumes of liver sub compartments and the raised levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, low-density lipoprotein (LDL) and increased high-density lipoprotein (HDL), superoxide dismutase (SOD) and catalase (CAT) as compared to the untreated group. **Conclusion:** According to the obtained results, AOEE can regulate the biochemical parameters and inhibits hepatic damages in CCl₄-induced hepatotoxicity in mice.

Keywords: *Anthemis odontostephana* Boiss.; ethanol extract; hepatoprotective activity; mice

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Introduction

Liver is the main detoxifying organ in the body and possesses a high metabolic rate and is subjected to many insults potentially oxidative stress [1,2]. Most of the hepatotoxic chemicals blemish liver [1,2]. Carbon tetrachloride (CCl₄), a colorless, clear, volatile, heavy and nonflammable liquid is a potent environmental hepatotoxin that causes steatosis, necrosis and cirrhosis [3-5]. In addition, several documented case studies have established that CCl₄ produces hepatic disease with a changed antioxidant status in humans [6]. Findings from the screening of several ethno medicinal plants describe their antioxidant effects and demonstrate that they could protect liver against CCl₄-induced oxidative stress by altering the levels of antioxidant enzymes [7,8]. Some medicinal plants are rich of antioxidant compounds such as triterpenes, tannins, saponins, naphthaquinone, alkaloids and flavonoids [9-13], so they can decrease the quality and rate of hepatotoxicity.

Anthemis odontostephana Boiss (Asteraceae family) widely grows in the western parts of Iran. [14]. It has been cultivated from the earliest times and is economically important as a garden vegetable [14]. It is an edible plant that has generated a lot of interest throughout human history as a medicinal plant [15]. In Iranian traditional medicine, it has been used in treating various gastric ulcers, diabetes and inflammatory, parasitic, bacterial, viral and fungal diseases [15]. In the present study, we have prepared *A. odontostephana* ethanol extract (AOEE) and have attempted to investigate the probable protective effect of this extract on hepatic structural and biochemical changes in CCl₄-induced hepatotoxicity in male mice.

Material and Methods

Ethical considerations

Animal studies were approved by Local Research Ethics Committee of Razi University, Kermanshah, Iran with the ethical code of 397-3-001.

Animals

Male Balb/c mice weighing between 38-40 g were procured from laboratory animal center of Kermanshah University of Medical Sciences, Kermanshah, Iran. The animals were housed in an air-conditioned room (22±2 °C) with 12 h light/dark cycle and had free access to standard

pellet diet (metabolism energy: 2860 kcal/kg, crude protein: 21.5 %, crude fiber: 3.55 %, calcium: 1.05 %, phosphor: 0.5 %, sodium: 0.17 %, chlorine: 0.23 %, methionine (digestible): 0.59 %, methionine + Cysteine (digestible): 0.92 %, lysine (digestible): 1.2 %, Arginine (digestible): 1.33 %, threonine (digestible): 0.82 %, linoleic acid: 1.5 %, dry matter: 88 %) and water.

Plant extraction

Anthemis odontostephana at maturity was collected from around of Kermanshah, Iran during April 2017. Dried leaves of the plants were ground and about 150 g of the acquired powder was extracted with 1500 mL of ethanol for 6 h in oven (at 40 °C) with continuous shaking. The extract was left for 24 h at room temperature then it was filtered through watman paper no. 2. In rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan), the extract was concentrated, then lyophilized.

Experimental design

Sixty male mice were used. The mice were divided into six groups of ten mice each; group I served as control, received 1 mL/kg olive oil intraperitoneally and 0.5 mL distilled water through gavage; group II served as untreated group, received 1 mg/kg CCl₄ mixed with olive oil in the ratio of 1:1, intraperitoneally and 0.5 mL distilled water orally; group III, IV V and VI received CCl₄ mixed with olive oil in the ratio of 1:1 intraperitoneally and 20, 40, 80 and 160 mg/kg of AOEE (dissolved in water) through gavage, respectively.

The animals were treated twice a week for 45 consecutive days. At the end of the 45th day treatment, the animals of all groups were euthanized by ketamine HCl (40 mg/kg) and xylazine (5 mg/kg). Immediately blood samples were drawn from animals' heart and inserted in serum bottles [for determination of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL)]. All above parameters were measured by available commercial kits (Pars Azmun CO, Iran) according to the procedures. All biochemical measures were done in duplicate [7,8].

Determination of the activity of endogenous antioxidant enzymes

The whole liver (n=5) from each mice were removed after sacrificing the animal, and were rinsed in normal saline and immediately stored in freezer. Tissues were homogenized in 10 parts in ice-colds potassium phosphate buffer (pH 7.4) using mortar and pestle. The homogenate was centrifuged at 3000 g for 15 min and the supernatant was collected. Protein concentration of the sample was evaluated by Biuret method using bovine serum albumin as standard [16,17]. The ability of the extracts to boost the capacity of antioxidant enzymes was assessed by determining the activity of two endogenous antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] as follows:

Superoxide dismutase

SOD activity was assessed according to the method described by Martin et al. [16]. Briefly, 920 µL of assay buffer was added into clean test tube containing 40 µL of sample, mixed and incubated for 2 min at 25 °C, followed by adding 40 µL of hematoxylin solution, mixed quickly and the absorbance was evaluated immediately at 560 nm.

Catalase

CAT activity was evaluated using the procedure reported by Abei [17]. Ten µL of sample was added to test tube containing 2.8 mL of 50 mmol/L phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1 mL of fresh 30 mmol/L H₂O₂ and the decomposition rate of H₂O₂ was assessed at 240 nm for 5 min in a spectrophotometer. A molar extinction coefficient of 0.0411 mmol/L/cm was used to compute CAT activity.

Histological study

The whole liver of each group (n=5) was dissected out and washed with ice cold saline to remove blood. The livers were weighed and immersed in 10% neutral buffered formaldehyde. After 72 h fixation, the livers were cut using orientator method. Totally, 7-10 slabs were collected from each liver. The slabs were embedded in paraffin and sections (5 µm thicknesses) were prepared and stained by hematoxyline and eosin stain.

Volume density of liver structures including hepatocytes, sinusoids, central veins, portal veins,

hepatic arteries, and bile ducts were measured with point counting rule briefly as follow: one section from each liver was used. The images of microscopical fields from each section were projected on point probe (frame 15 cm×15 cm) by video projector via microscope equipped with a camera (Dinocapture ver.5, dino-lit.com 30.5 mm) connected to the computer.

At total magnification of 2000×, points that hit desired structures were counted and volume density was computed using the following formula:

$$V_V = P_{\text{structure}}/P_{\text{reference}}$$

Where P_{structure} and P_{reference} were the number of points falling on the structure's profile and on the reference space, respectively. Ten to fourteen microscopic fields were examined in each liver. The absolute volume of the structures was measured by multiplying the fractional volume by the final volume of the liver to inhibit the reference trap [18,19].

Statistical analysis

The results were analyzed by SPSS-18 software using One-way Analysis of Variance (ANOVA) followed by Duncan's *post-hoc* test (p≤0.05).

Results and Discussion

Our findings in this study indicated that *A. odontostephana* showed strong liver protection effect. Ethno medicinal plants are popular remedies used by a majority of the world's population. The impression of ethno medicinal plants in prevention and treatment of diseases is irrecusable [20,21]. They have the immense potential for the management and remedy of every disease such as hepatotoxicity [22-24]. A list of medicinal plants that have been used for their hepatoprotective properties *Fagonia schweinfurthii* (Hadidi) M.Hall (whole plant), *Vitex glabrata* F.Muell. (whole plant), *Acacia nilotica* L. (aerial parts), *Abelmoschus manihot* (L.) Medik. (flower), *Daucus carota* L. (seed), *Moringa oleifera* Lam. (seed oil), *Feijoa sellowiana* (O.Berg) O.Berg (fruit and peel), *Garcinia indica* (Thouars) Choisy (fruit rind), *Melastoma malabathricum* L. (leaf), *Ficus religiosa* L. (leaf), *Cissus quadrangularis* (stem), *Feronia limonia* Swingle (stem bark), *Terminalia paniculata* Roth (bark), *Astragalus kahiricus* DC. (root) and *Zingiber officinale* Roscoe (rhizome) [25].

The data of the mean absolute volume of liver and its subcomponents in treated and untreated groups have been revealed in figures 1-5. The results indicated that the liver weight and volume enhanced 56 and 64 % ($p \leq 0.05$), respectively in the untreated mice when compared to the control ones. Administration of AOEE at all doses could significantly ($p \leq 0.05$) improve the liver weight and volume compared to the untreated group (figures 1,2).

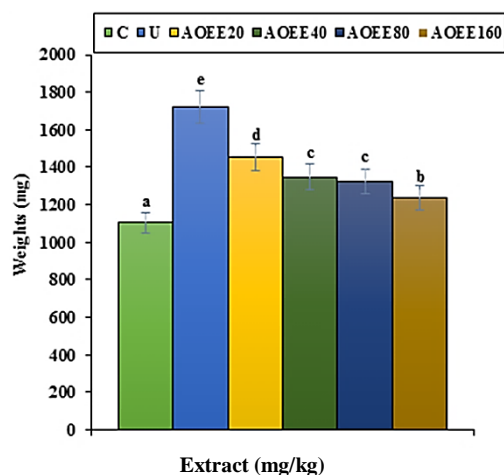


Figure 1. Weight of liver in all experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

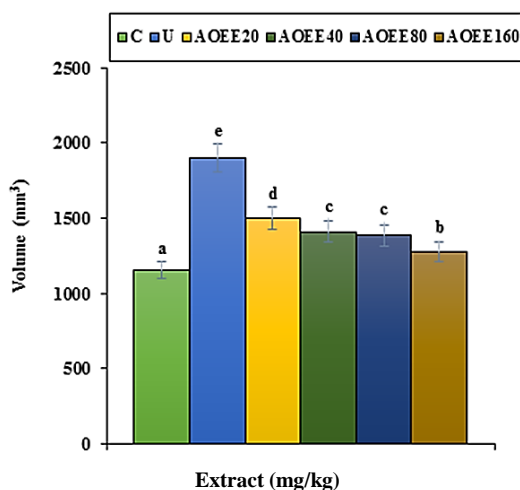


Figure 2. Volume of liver in all experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

The volume of hepatocytes, central veins, sinusoids, portal veins, hepatic arteries and bile ducts increased significantly ($p \leq 0.05$) in untreated diabetic mice compared to the control ones (figures 3-5). Administration of AOEE at all doses could significantly ($p \leq 0.05$) decrease the volume of the above structures in comparison with the untreated group.

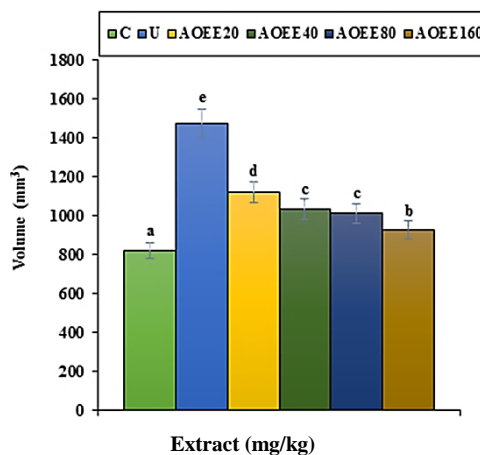


Figure 3. Volume of hepatocytes in all experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

Administration of AOEE160 could significantly ($p \leq 0.05$) decrease the volume of sinusoids, hepatic arteries and bile ducts similar to the control group. Alike AOEE160 and control groups, AOEE80 and AOEE40 could significantly ($p \leq 0.05$) decrease volume of hepatic arteries and bile ducts. Also AOEE20 could significantly ($p \leq 0.05$) decrease volume of bile ducts similar to AOEE40, AOEE80, AOEE160 and control groups.

A hepatic damage is evaluated by the elevated serum levels of cytoplasmic enzymes as well as by histological examination. The enhanced serum parameter levels such as ALP, AST, ALT, cholesterol and LDL have been attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into the circulation after cellular damage [26,27]. In this study, the measured values of the liver enzymes have been demonstrated in figures 6-8. CCl_4 - induced toxicity increased ALP, AST, ALT, cholesterol and LDL and decreased HDL, SOD and CAT significantly ($p < 0.05$) as compared to the control group.

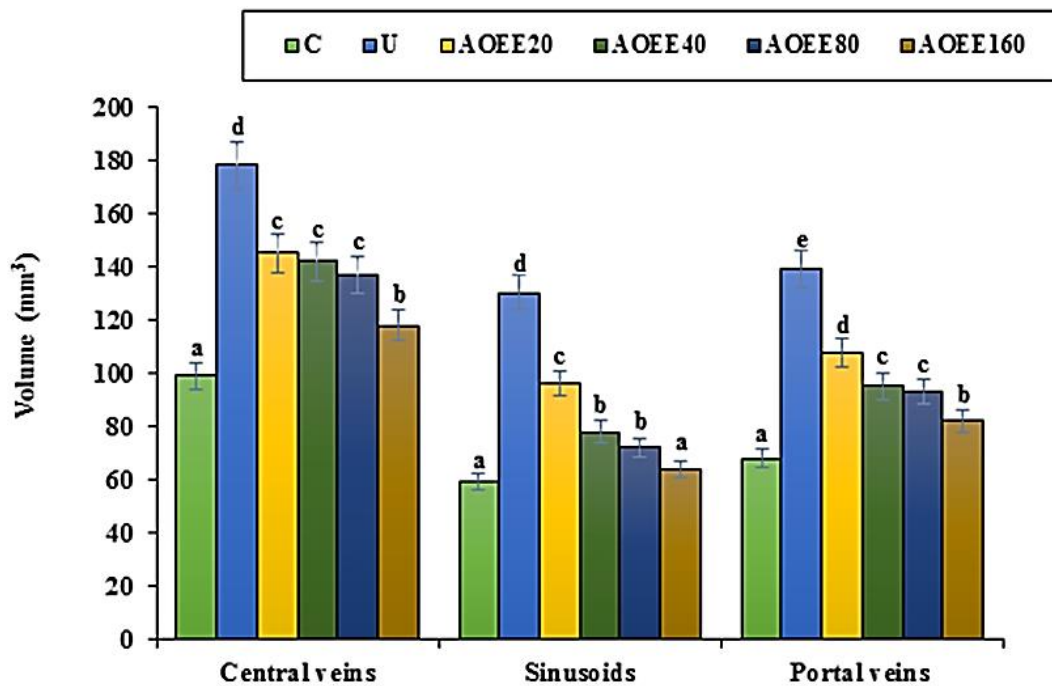


Figure 4. Volume of central veins, sinusoids and portal veins in all experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

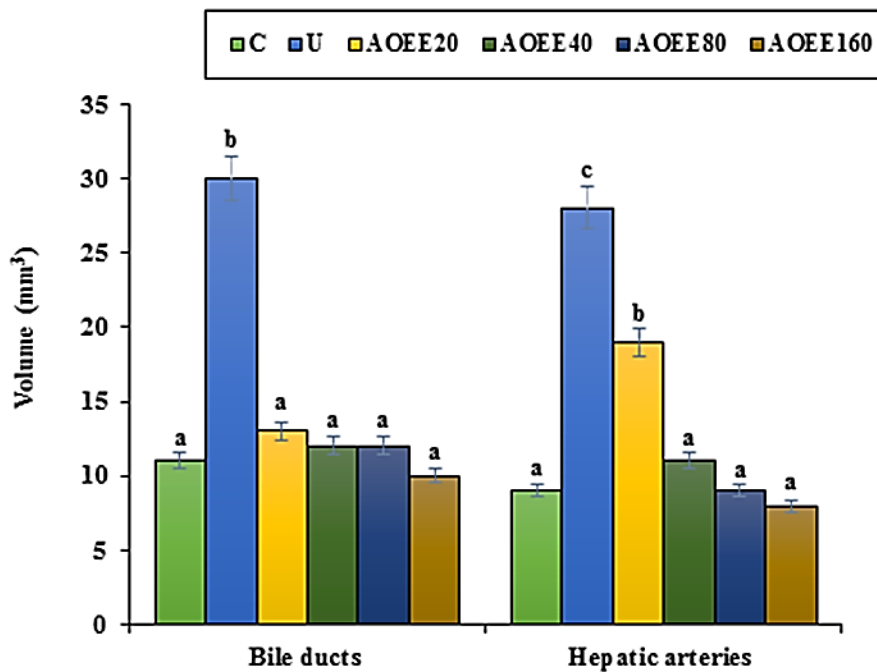


Figure 5. Volume of bile ducts and hepatic arteries in all experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

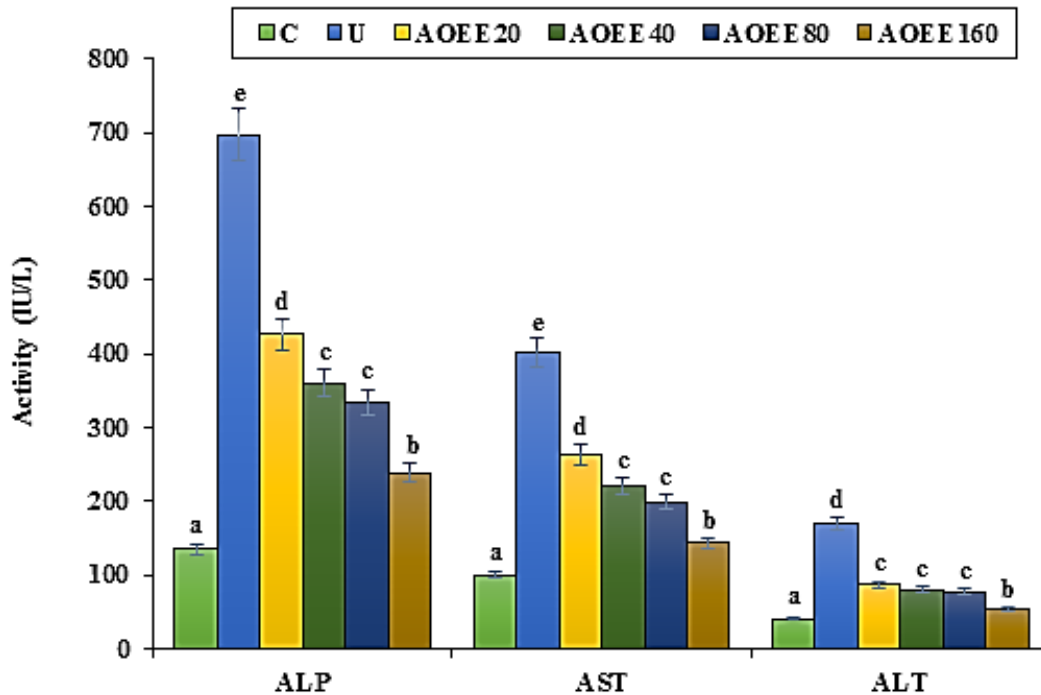


Figure 6. ALP, AST and ALT levels in all experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. ALP (Alkaline phosphatase), AST (Aspartate aminotransferase) and ALT (Alanine aminotransferase). Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

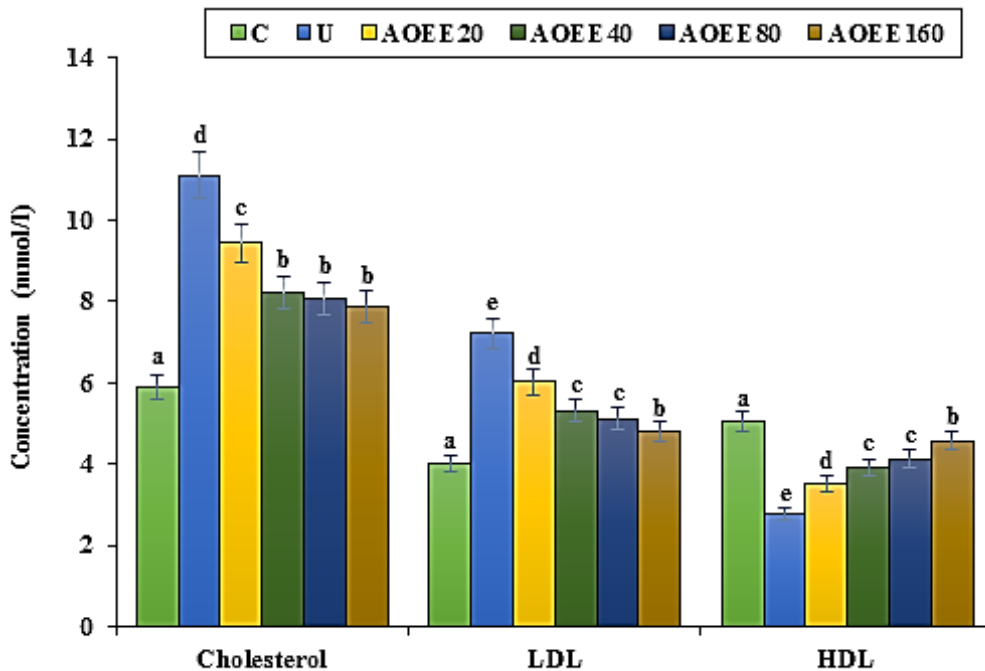


Figure 7. Cholesterol, LDL and HDL levels in experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. LDL (Low-density lipoprotein) and HDL (High-density lipoprotein). Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

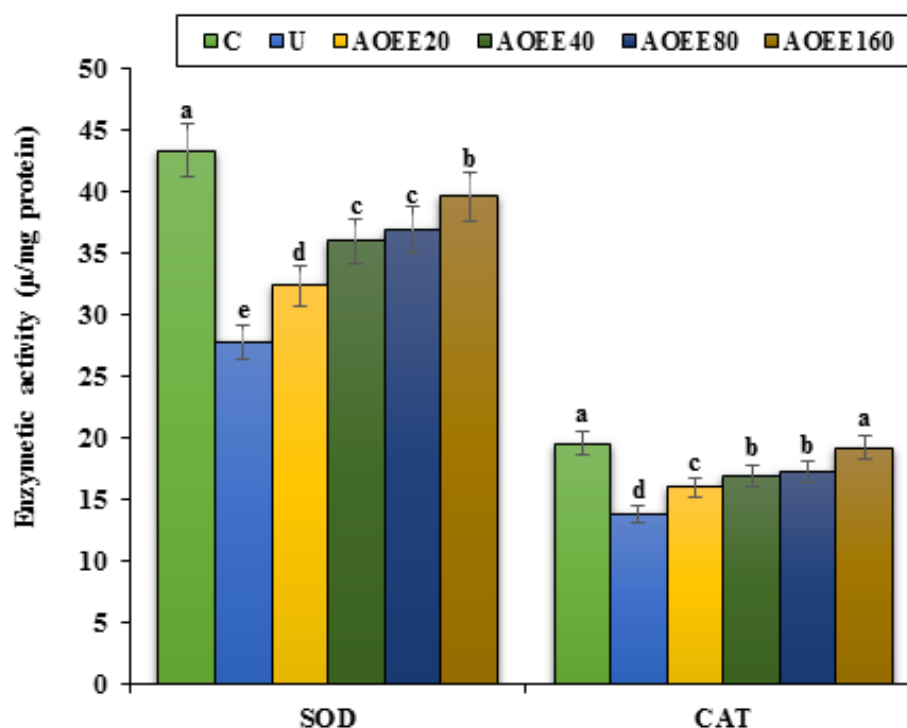


Figure 8. The level of SOD and CAT in liver in all experimental groups; C (control), U (untreated group), AOEE20, AOEE40, AOEE80 and AOEE160 were treated group with 20, 40, 80 and 160 mg/kg of *Anthemis odontostephana* ethanol extract, respectively. SOD (Superoxide dismutase) and CAT (Catalase). Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

Different doses of AOEE could significantly ($p < 0.05$) reduce the raised levels of ALP, AST, ALT, cholesterol and LDL and increased HDL, SOD and CAT significantly ($p < 0.05$) as compared to the untreated group. There weren't significant difference between AOEE160 and the control groups in CAT levels ($p < 0.05$). In previous studies the major components of AOEE were found to be spathulenol [14], ascorbic acid 2, 6-dihexadecanoate [29] and borneol [15] (anti-inflammatory and antioxidant components). The protective effect of AOEE against CCl_4 -induced hepatotoxicity in the recent study may be partly related to the anti-inflammatory and antioxidant constituents. The present research has shown the hepatoprotective activity of the AOEE, offering its possible use as a therapeutics supplement or medication. Additional clinical trial studies would be needed to justify and further assess the potential of the plant as a hepatoprotective agent in human.

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Author contributions

Mohammad Mahdi Zangeneh and Akram Zangeneh prepared the manuscript; Mohammad Mahdi Zangeneh and Reza Tahvilian performed the biochemical analysis; Mohammad Mahdi Zangeneh and Akram Zangeneh designed and performed the stereological plan; Mohammad Mahdi Zangeneh contributed in the statistical analysis; Hossein Amiri and Nassim Amiri were involved in animal handling and treatments; Reza Tahvilian and Rohallah Moradi prepared the plant extract

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

AOEE: *Anthemis odontostephana* ethanol extract; ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDL: low-density lipoprotein; HDL: high-density lipoprotein; SOD: superoxide dismutase; CAT: catalase