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Original article

The Role of *Allium saralicum* Extract on Prevention of Acetaminophen-Induced Hepatic Failure: an Experimental Study

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Abstract

Background and objectives: Acetaminophen (APAP) is a common analgesic medicine whose overdose leads to severe hepatic dysfunction. Due to the known antioxidant properties of *Allium* species, the present study aimed to evaluate the protective effects of *Allium saralicum* plant on APAP induced liver toxicity. **Methods:** The hydro-alcoholic extract of *A. saralicum* was prepared by maceration and ultrasonic methods. Forty-two rats in seven groups were treated by gavage as follows: groups 1 and 2 received normal saline, groups 3 received 400 mg/kg of *A. saralicum* hydro-alcoholic extract, and the groups 4-7 were treated with 50, 100, 200 and 400 mg/kg of *A. saralicum* extract, respectively. After two consecutive weeks, the therapeutic groups, as well as the positive control (APAP) group, were administered a single dose of APAP (2 g/kg). After 48 hours, the animals were anesthetized, and blood and liver samples were collected for histological and biochemical examinations. **Results:** Our findings indicated that APAP caused a significant rise in ALT ($p<0.001$), AST ($p<0.001$), ALP ($p<0.001$) and LDH ($p<0.001$) serum levels, total and direct bilirubin ($p<0.001$), hepatic lipid peroxidation (LPO; $p<0.001$) and nitric oxide (NO; $p<0.001$). In addition, APAP let to the decreasing of the total antioxidant capacity (TAC; $p< 0.001$), total thiol molecules (TTM; $p<0.001$), and structural changes in the hepatic tissue. Following administration of *A. saralicum* extract, a remarkable improvement was observed in the functional and oxidative stress indices of liver tissue alongside histopathologic alterations. **Conclusion:** Our results showed that *A. saralicum* extract significantly improved APAP-induced hepatic failure through inhibition of oxidative/nitrosative stress.

Keywords: acetaminophen; *Allium saralicum*; hepatotoxicity; oxidative stress

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Introduction

Paracetamol (APAP; N-acetyl-p-aminophenol) is an analgesic and antipyretic medication that has been used as an over-the-counter medication [1,2]. Despite being safe at 1-4 g daily doses and widely accessible, APAP intoxication is prevalent in all ages [2-5]. APAP toxicity is associated with malaise, vomiting, and diarrhea; if untreated, it may lead to jaundice and severe liver injury [6]. The studies have indicated that annually about 6% of adults are exposed to APAP at the doses of more than 4 g/day; also, 30,000 patients are referred to hospitals for APAP-induced toxic effects [7].

Therapeutic doses of APAP are mainly

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metabolized by the liver through conjugation with sulfate and glucuronide conjugates [8]. Higher doses of APAP are metabolized by hepatic cytochrome P450-2E1 leading to the production of a highly reactive and electrophile product known as N-acetyl-p-benzoquinoneimine (NAPQI) [9]. Oxidative stress-induced by NAPQI may be partly due to cellular glutathione (GSH) depletion and mitochondrial proteins adduction leading to mitochondrial oxidant stress. Several studies on inducers and inhibitors of cytochrome P450 have indicated the role of ROS generated by P450-mediated metabolism in APAP hepatotoxicity [10,11].

Allium saralicum, native to Iran, is a member of the Amaryllidaceae family [12]. It contains various compounds including hexanedioic acid, γ-tocopherol, eicosane, hexatriacontane, vitamin E, 2-phenyl-5-methylindole, phytol, methyl ester and linolenic acid possessing various pharmacologic effects [13,14]. Previous studies have expressed the therapeutic properties of *A. saralicum* on a wide variety of disorders. For instance, this herbal medicine has been consumed for its antibacterial, anti-inflammatory, antioxidant, anti-hyperlipidemic and hepatorenal protective effects in some Middle Eastern countries [15,16]. Regarding the role of oxidative stress in the pathophysiology of APAP poisoning and also due to the known antioxidant properties of *Allium* species and using of *Allium saralicum* by local people as food, we evaluated the effect of *A. saralicum* hydro-alcoholic extract as a potential antioxidant on APAP induced acute liver injury.

Materials and Methods

Ethical considerations

The animal experiments were approved by the Ethics Committee of Hamadan University of Medical Science (HUMS), Hamadan, Iran with ethical code of (ID: IR.UMSHA.REC.1396.364), in accordance with the guideline of the Research Ethics Committee of the Health and Medical Education, Iran (2019), based on the Helsinki Protocol (Helsinki, Finland, 1975).

Chemicals

All chemicals were purchased from Merck (Darmstadt, Germany) unless otherwise stated. 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), N-(1-naphthyl) ethylenediamine dihydrochloride (NED), 2-thiobarbituric acid (TBA), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) and acetaminophen powder linear formula (CH3CONHC6H4OH) were obtained from Sigma-Aldrich Chemical Company (USA).

Plant material

Collection and extraction procedure

The aerial parts of *Allium saralicum* were gathered from Saral, Kurdistan, Iran in May 2018. It was identified at the Herbarium of School of Pharmacy, Hamadan University of Medical Sciences (HUMS), Hamadan, Iran with the code number (NO: 275). Maceration and ultrasonic methods were applied for extraction. The coarsely powdered plant (300 g of aerial parts) was kept in contact with 1500 mL of methanol and water (1:1) for 72 h at 25 °C. The obtained mixture was then filtered using filter paper. A vacuumed rotary evaporator with a controlled temperature of 50 °C was used for condensation of the extract. The whole process was repeated for three times. Finally 78 g dark brown extract was kept in a sterile vial in a dark and cool place 4 °C.

In the next stage, different fractions of hydroalcoholic extract including n-hexane, chloroform, ethyl acetate, and methanol fractions were prepared by column chromatography. For this purpose, a column with dimensions of 8 × 17 cm was used with a silica gel (70-30 mesh) as the solid phase. A portion of hydroalcoholic extract (70 g) was fractionated on a silica gel column eluted with 500 mL of n-hexane, chloroform, ethyl acetate, and methanol of increasing polarity to give four fractions. The fractions were then concentrated in vacuum at 40 °C using a rotary evaporator to produce n-hexane, chloroform, ethyl acetate, and methanol fractions Finally, the antioxidant properties of each fraction were determined using ferric reducing antioxidant power methods (FRAP) [17]. Also, the thiol levels were assessed using DTNB reagent as described by Hu and Dillard [18]. According to the results, the methanolic fraction as an effective fraction of hydro-alcoholic extract was selected and maintained at the temperature of 4°C.

Experiment design

Male Wistar rats (250±25 g) were obtained from the animal house of HUMS, Hamadan, Iran. The animals were kept under controlled conditions of laboratory (12 h light/dark cycles, 50% humidity and temperature of 22-25 °C) and supplied with

standard diet and water ad libitum for one week before the study.

Forty-two rats were divided into seven groups of six each and treated for two weeks by gavage as follows: groups 1 and 2 received normal saline, groups 3 received 400 mg/kg of *A. saralicum* extract, groups 4-7 were treated with 50, 100, 200 and 400 mg/kg of ASHE, respectively. After two consecutive weeks, except for groups 1 and 3, animals were administered a single dose of APAP (2 g/kg). After 48 h, the animals were anesthetized with ether and blood samples were collected from their carotid artery which were centrifuged at 2500 rpm (for 15 min) to separate serum for biochemical assays. Furthermore, a part of hepatic tissue was kept at the temperature of -80 °C for preparation of liver homogenate (10%, w/v). The other portion of the liver was fixed in 5% buffered formalin for histopathological experiments.

Serum biochemical analysis

Glucose, triglyceride, direct and total bilirubin as well as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in serum samples were assayed by Pars Azmun kits, Iran.

Measurement of total antioxidant capacity

The total antioxidant capacity was measured by detecting its ability to reduce Fe^{+3} to Fe^{+2} by ferric-reducing antioxidant power (FRAP) technique [17]. Briefly, FRAP reagent was prepared, freshly, via mixing ten volumes of 300 mM acetate buffer (pH 3.6), one volume of 20 mM $FeCl_3$, and also one volume of 10 mM TPTZ in 40 mM HCl. Then, 200 µL of reagent and 10 µL sample were mixed and incubated at 37 °C for 30 min, the absorbance was detected at 593 nm. Different concentrations of ferric sulfate (125, 250, 500 and 1000 µM) were used for plotting the calibration curve.

Measurement of total thiol molecules

Total thiol molecules were assessed using DTNB reagent [18]. Briefly, 200 µL of Tris-EDTA buffer containing 0.25 M Tris base and 20 mM EDTA (pH 8.2) was mixed with sample (10 µL) and its optimum absorbance was measured at 412 nm (A1). Then, 10 µL of DTNB solution (10 mmol/L in methanol) was added and incubated at 37 °C for 15 min. The absorbance of the samples (A2) and DTNB blank (B) was detected again at 412 nm. The level of thiol molecules was calculated by reduced glutathione as the standard.

Measurement of nitric oxide

Hepatic nitric oxide was measured by the colorimetric method using Griess reagent (1% sulfanilamide, 0.1% NED, and 2.5% phosphoric acid) [19]. Briefly, 100 µL of reagent was mixed with sample (100 µL) and its optimum absorbance was measured at 520 nm. sodium nitrite was used as the standard.

Measurement of lipid peroxidation

The lipid peroxidation was assayed in liver tissue using the thiobarbituric acid reactive substances (TBARS) method [20]. Briefly, a reagent was prepared containing TBA (0.2%) in H_2SO_4 (0.05 M). Then, 100 µL of homogenate sample and 500 µL reagent were mixed and heated in boiling water bath for 30 min, the absorbance was detected at 532 nm. Different concentrations of malondialdehyde (2, 4, 8 and 16 µM) were used for plotting the calibration curve.

Measurement of protein

At the end of each experiment, protein content was assayed in the crude homogenate of hepatic tissue using the Bradford method [19].

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The data were analyzed by SPSS, version 16.0 (SPSS, Inc., Chicago, IL, USA) using analysis of variance (ANOVA) and Tukey's post hoc test were used if variables were normally distributed. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

In the current study, the yield of n-hexane, chloroform, ethyl acetate, and methanolic fractions were 7% (4.9 g), 10% (7.0 g), 12% (8.4 g) and 40% (28 g), respectively. Regarding the bioassay guided fractionation; the methanolic fraction was used in the animal study. APAP could induce liver injury and increase levels of ALT (figure 1A; $p<0.001$), AST (figure 1B; $p<0.001$), ALP (figure 1C; $p<0.001$), LDH (figure 1D; $p< 0.001$), total bilirubin (figure 1E; $p<0.001$) and direct bilirubin (figure 1F; $p<0.001$) which is in line with other reports [21-23].

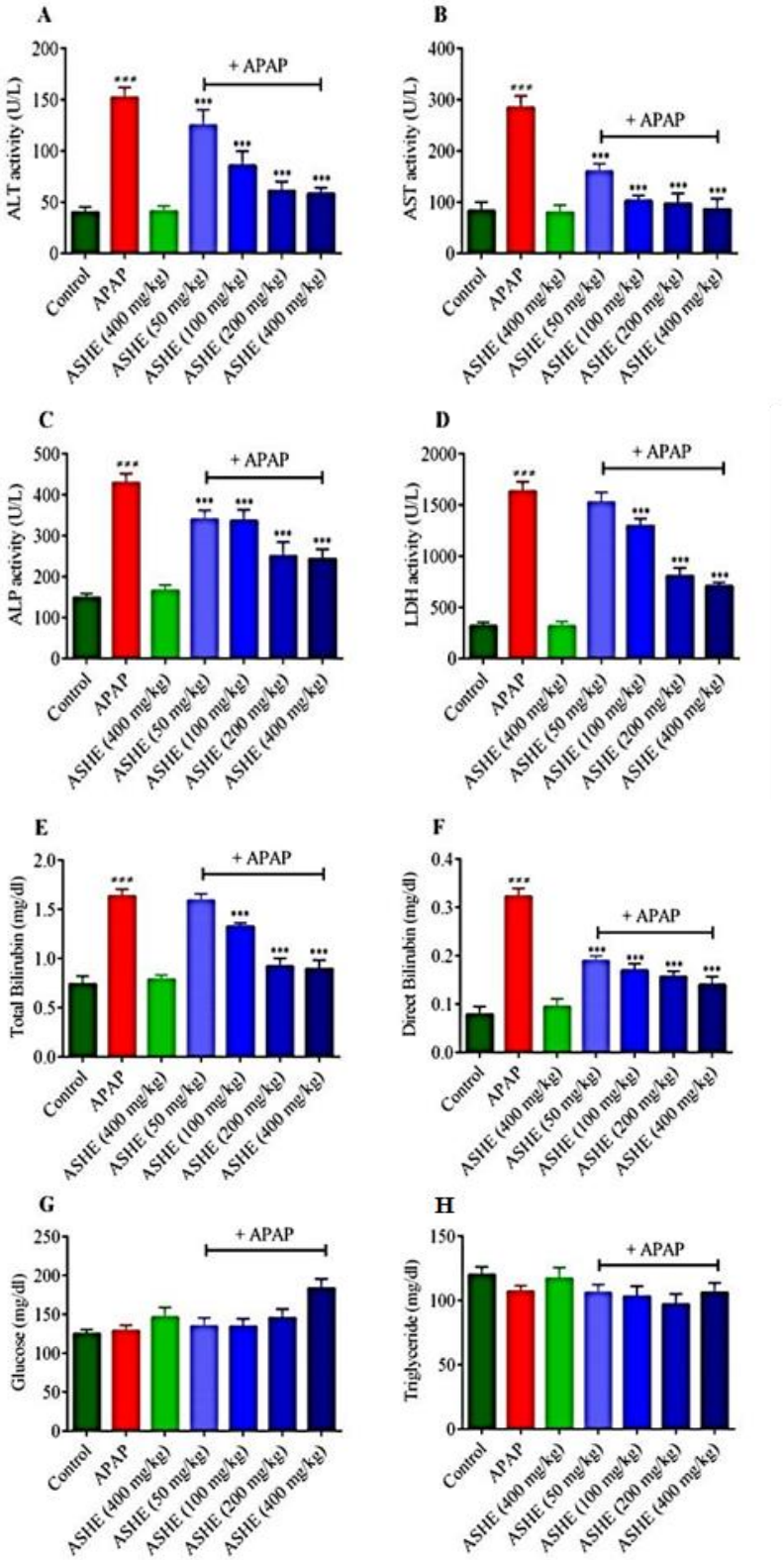

Figure 1. Effects of *Allium saralicum* hydro-alcoholic extract (ASHE) on hepatic serum enzymes levels in acetaminophen (APAP)-exposed Wistar rat. Statistical analysis used one-way ANOVA with Tukey's test. Values have been expressed as means ± SD, n=6 for each group. $^{\#\#\#}P < 0.001$ vs control group; $^{***}P < 0.001$ vs APAP group. ALT: alanine aminotransferase (A); AST: aspartate aminotransferase (B); ALP: alkaline phosphatase (C); LDH: lactate dehydrogenase (D); Total bilirubin (E); Direct bilirubin (F); Glucose (G) and Triglyceride (H). APAP: acetaminophen (equal 2 g/kg).

Table 1. Body and liver weight for different groups

Groups	Initial body weight (g)	Final body weight (g)	Liver weight	Relative liver weight (g/100g bw)
Control	250.3 ± 2.9	298.1 ± 2.9	12.2 ± 0.3	4.0 ± 0.2
APAP (2 g/kg)	249.6 ± 2.5	274.5 ± 6.4	12.2 ± 0.6	3.7 ± 0.3
ASHE (400 mg/kg)	243.6 ± 2.9	299.1 ± 5.6	12.7 ± 0.6	4.2 ± 0.4
APAP + ASHE (50 mg/kg)	252.1 ± 3.9	290.6 ± 7.3	13.2 ± 0.3	4.5 ± 0.2
APAP + ASHE (100 mg/kg)	235.1 ± 3.6	273.8 ± 7.7	12.9 ± 0.6	4.7 ± 0.3
APAP + ASHE (200 mg/kg)	241.3 ± 4.6	275.5 ± 6.7	11.8 ± 0.7	4.4 ± 0.4
APAP + ASHE (400 mg/kg)	262.6 ± 6.5	262.6 ± 6.5	11.3 ± 0.6	4.3 ± 0.3

Data are expressed as mean ± SD; n=6 for each group; ASHE: *Allium saralicum* hydro-alcoholic extract; APAP: acetaminophen (equal 2 g/kg).

APAP administration led to a distorted pattern of hepatic cords and nuclear pyknosis of hepatocytes as well as sinusoidal dilation in addition to necrosis and vacuolation of hepatocytes (figure 3). Although the weight of animals and relative liver weight increased, the changes were not significant (table 1).

Overall, loss of cell membrane integrity leads to ALT, AST and LDH leak into the bloodstream and their levels increase in blood [24-26]. Also, increased levels of ALP may also indicate biliary tract injury and increased biliary pressure [27]. According to the results, we observed no changes in the serum levels of glucose and triglyceride. It seems that changing in the level of these markers might occur with progressive liver dysfunction.

To investigate the role of oxidative stress in the hepatotoxicity of acetaminophen, we measured some oxidative stress indicators such as LPO, NO (as oxidative biomarkers) and thiol groups, (as antioxidant indices) in liver tissue. Our results demonstrated that LPO (figure 2A; $p<0.001$) and NO (figure 2B; $p<0.001$) significantly increased, while TAC (figure 2C; $p<0.001$) and TTM (figure 2D; $p<0.001$) significantly decreased in the APAP group. These results indicated the occurrences of oxidative stress which is in line with the other literature [28-30].

Generally, lipid peroxidation is considered an index of oxidative degradation of lipids that indicates increase in reactive radicals in hepatic tissue [31-33]. Also, massive level of NO indicates the role of the nitrosative stress pathway in APAP-induced hepatotoxicity. The high levels of NO can react with the superoxide anion, resulting in the generation of the highly reactive peroxynitrite anion ($ONOO^-$). The role of nitrosative stress in the development of hepatic failure is confirmed through inhibition of endogenous NO generation by the NOS inhibitor as described by Gardner et al. [34].

The results of the present study showed that *A. saralicum* extract up to 400 mg/kg produced no sign of hepatotoxicity (table 2).

Table 2. The effects of ASHE extract on liver function tests.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	36.44 ± 1.2	83.68 ± 3.55	148.80 ± 6.15
ASHE (50 mg/kg)	32.67 ± 1.21	73.81 ± 5.68	152.06 ± 5.52
ASHE (100 mg/kg)	33.38 ± 2.36	82.63 ± 3.7	153.69± 4.32
ASHE (200 mg/kg)	33.17 ± 1.45	71.91 ± 8.8	158.2 ± 4.48
ASHE (400 mg/kg)	36.72 ± 0.8	75.49 ± 5.38	166.91 ± 6.04

Data are expressed as mean ± SD; n=6 for each group; ASHE: *Allium saralicum* hydro-alcoholic extract

In addition, our results showed that *A. saralicum* extract could decrease LPO and NO hepatic levels along with enhanced total antioxidant capacity and total thiol molecules levels (figure 2) and improved liver function parameters (figure 1). At higher doses of *A. saralicum* extract, a decrease in the sinusoidal dilation and necrotic hepatocytes were observed (figure 3). It seems that the antioxidant properties of *A. saralicum* extract may be associated with the phytochemical compounds. For instance, Goodarzi et al, showed that *A. saralicum* contains antioxidant components such as linolenic acid-methyl ester, phytol, neophytadiene, 2-phenyl-5-methylindole, hexadecanoic acid, vitamin E, ethanol, 2-tetradecyloxy, n-tetracosane, hexatriacontane, γ-tocopherol, eicosane, n-ethyl-1,3-dithioisoindoline, 2-hexadecene, 3,7,11,15-tetramethyl, hexanedioic acid, and 1,4,8,11-tetraazacyclotetradecane [35]. Therefore, this plant has been used in the treatment of diabetic hepatopathy and fatty liver, and it was shown to make significant amelioration in liver oxidant/ antioxidant status [35,36]. In conclusion, the results showed that *A. saralicum* may prevent the hepatic oxidative damages induced by APAP through an increase in levels of thiol molecules and total antioxidant capacity. However, further studies need to evaluate the exact role of *A. saralicum* as a hepatoprotective agent.

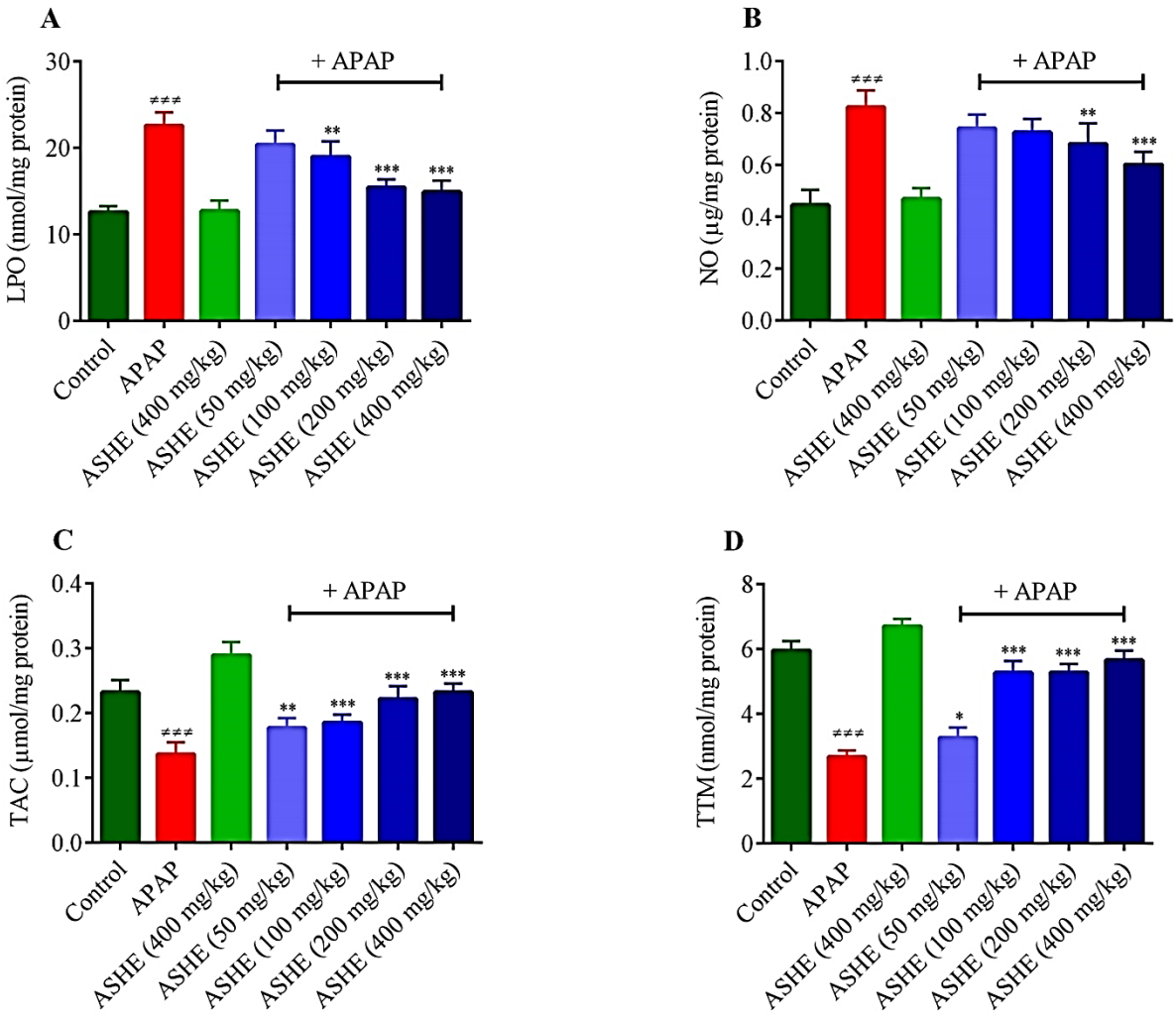

Figure 2. Effects of *Allium saralicum* hydro-alcoholic extract (ASHE) on hepatic oxidative stress biomarkers in acetaminophen (APAP)-exposed Wistar rats. Statistical analysis used one-way ANOVA with Tukey's test. Values have been expressed as means ± SD, n=6 for each group. $^{\#\#}P < 0.001$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs APAP group. LPO: lipid peroxidation (A); NO: nitric oxide (B); TAC: total antioxidant capacity (C); TTM: total thiol molecules (D). APAP: acetaminophen (equal 2 g/kg)

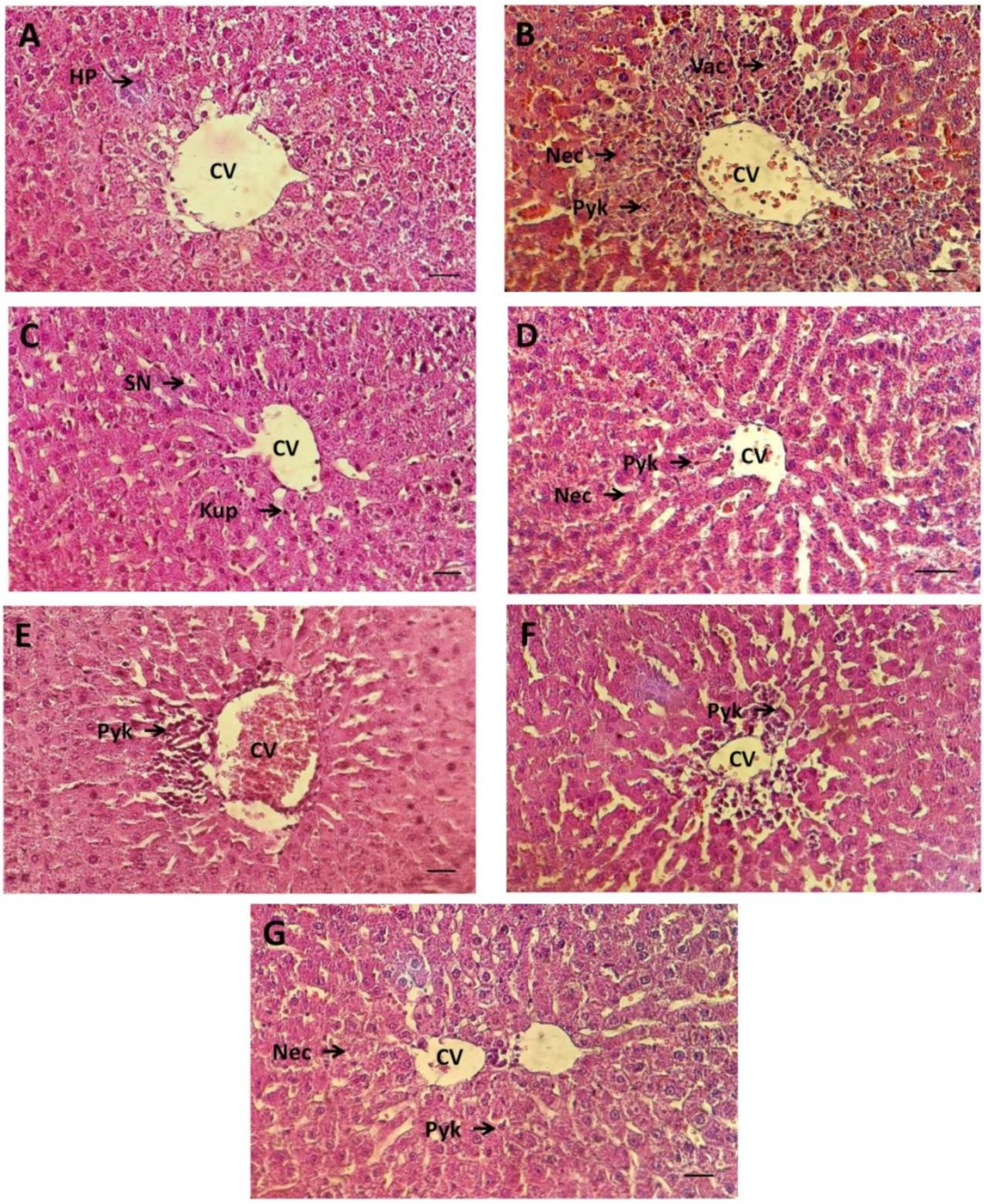

Figure 3. Photomicrographs of hepatic tissue in different groups: A, negative control group; B, acetaminophen (APAP) (2 g/kg); C, ASHE (400 mg/kg); D, ASHE (50 mg/kg) + APAP (2 g/kg); E, ASHE (100 mg/kg) + APAP (2 g/kg); F, ASHE (200 mg/kg) + APAP (2 g/kg); G, ASHE (400 mg/kg) + APAP (2 g/kg). Original magnification of all images is × 40. CV, central vein; HP, hepatocyte; Kup: Kupfer cells; Nec: necrosis; Pyk: pyknosis; SN: sinusoid; APAP: acetaminophen (equal 2 g/kg); ASHE: *Allium saralicum* hydro-alcoholic extract.

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Author contributions

Mahdi Alvandi helped performed the experimental parts and drafted the manuscript as his Pharm. D thesis. Dara Dastan was the co-supervisor to the thesis. Sara Soleimani Asl was

the advisers of the study. Amir Nili-Ahmadabadi conceived and supervised the study and edited the manuscript.

Declaration of interest

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

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Abbreviations

APAP: N-acetyl-p-aminophenol; NAPQI: N-acetyl-p-benzoquinoneimine; GSH: glutathione; FRAP: ferric reducing antioxidant power; ASHE: *Allium saralicum* hydro-alcoholic extract; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; TBARS: thiobarbituric acid reactive substances; TTM: total thiol molecules; TAC: total antioxidant capacity; NO: nitric oxide; LPO: lipid peroxidation