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Protective Effect Hydroalcoholic Extract *Allium Jesdianum Boiss* against Ccl4-Induced Hepatotoxicity in Male Mice

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A B S T R A C T

Allium Jesdianum Boiss *(Allium)* has many pharmacological beneficial effects. However, its protective effects against hepatotoxicity induced by carbon tetrachloride (CCl4) have not been explained. The present study was undertaken to investigate the protective effects of *Allium* on liver oxidative stress in male mice treated to CCl4. Forty-two male mice were randomly divided into six groups. Control group, sham group, CCl4 group, *Allium* 500 mg/kg + CCl4, *Allium* 1000 mg/kg + CCl4, *Allium* 2000 mg/kg + CCl4. Results showed that CCl4 significantly increased serum levels of alanine aminotransferase, aspartate aminotransferase, lipid peroxidation and decreased catalase activity and glutathione level, and also caused inflammation, coagulative necrosis, degeneration of hepatocytes, fatty change, and dilated sinosouids in liver tissue. While administration of *Allium* at the all doses used significantly altered all examined endpoints that induced by CCl4. Overall, our findings suggest that *Allium* possesses hepatoprotective effects against CCl4-induced hepatotoxicity by suppressing oxidative stress and improving the antioxidative defense system.

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Introduction

Many factors such as oxidative stress and inflammation are involved in the pathogenesis of the liver damage. Liver damage associated with oxidative stress can be arbitrated by reactive oxygen species (ROS) generation, lipid peroxidation of hepatocyte membranes, hepatic inflammation along with the proinflammatory cytokines inducible such as *tumor necrosis factor-α* **(*TNF-α*)** [1], interleukin-1β (IL-1β) and interleukin-6 (IL-6) [2, 3].

Carbon tetrachloride (CCl4) is a chemical that is used for the induction of hepatic injury including oxidative stress, inflammation and fibrosis in experimental models [4]. CCl4 in the liver by cytochrome P450 enzymes is metabolized to highly toxic trichloromethyl (•CCl3) and trichloromethyl peroxyl (•OOCCl3) free radicals that these free radicals cause damage to hepatocytes which results in necrosis and fibrosis [5].

The liver is the largest organ of the body which it is responsible for detoxification drugs and foreign agents and controls numerous metabolic functions. Drugs which used for the treatment of liver have therapeutic effects sometimes along with adverse effects [6]. So, research for finding of new drugs and agents for restrictive hepatic injury recently is on consideration. In recent years, herbal medicines because of their safety action and lack of side effects have gained more attention for the treatment of liver diseases [7].

Many studies have shown that natural antioxidants [8] can prevent hepatic damage by scavenging free radicals [9]. Previous studies have demonstrated that hepatotoxicity induced by CCl4 leads to induce oxidative stress and inflammation [10, 11]. *Allium* is the largest and most important plant genus of alliaceae family that its isolated compounds and extract have many pharmacological effects. A number of studies have shown some biological properties of *Allium* species such as anti-oxidant [12], anti-inflammatory [13], anti-proliferative [14], anti-bacterial [15], anti-parasitic [16], anti-carcinogenic [17], anti-fungal [18], anti-protozoa [19], anti-lipid [20] and anti-diabetic [21].

In the present study, we further investigated the antioxidant activity and hepatoprotective effect of *Allium* by biochemical, oxidative stress and histopathological parameters against CCl4-induced acute liver injury.

Materials and methods

Materials

Thiobarbituric acid (TBA), CCl4, trichloroacetic acid (TCA), ammonium molybdate and GSH were purchased from Sigma-Aldrich (St Louis, Missouri, USA). 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany). AST and ALT kit were obtained from biochemical assay kits (Pars Azmoon, Iran). *Allium Jesdianum* Boiss was purchased from the local herbal market in Ahvaz. Iran. And other chemicals were the highest grade commercially available.

Extraction

Plant after collection was identified by botanist from the Division of Pharmacognosy, AJUMS (number voucher: A-0138). The leaves of *Allium* were dried and ground. After powdering, were soaked in 70% aqueous-ethanol for three days. The solvents were well mixed, filtered by a filter (Whatman No. 2) and then vacuumed in a rotary evaporator to remove solvents and dryness.

Animals

Forty two male NMRI mice weighing 20-25 g and at 8-9 weeks of age were obtained from the animal facility of Ahvaz Jundishapur University of Medical Sciences (AJUMS). All procedures were conducted in accordance with the guidelines of Animal Ethics Committee of AJUMS (IR.AJUMS.REC.1395.04). They were kept in cages made of polycarbonate in suitable conditions with humidity of 10%, room temperature 25 ± 2°C, 12h light/ 12h dark cycle and free access to food and water.

Experimental Design

Mice were randomly divided into 6 groups; each group consisted of 7 animals.

. Group I: received normal saline as control group (NS group).
. Group II: received olive oil as sham group (Oil group).
. Group III: the CCl4 group received a single dose of CCl4 (1 ml/kg, i.p) (CCl4 group).
. Group IV: *Allium* 500 + CCl4 group were treated with *Allium* 500 mg/kg and a single dose of CCl4 (1 ml/kg, i.p).
. Group V: *Allium* 1000 + CCl4 group were treated with *Allium* 1000 mg/kg and a single dose of CCl4 (1 ml/kg, i.p).
. Group VI: *Allium* 2000 + CCl4 group were treated with *Allium* 2000 mg/kg and a single dose of CCl4 (1 ml/kg, i.p) [22].
Allium extract was dissolved in normal saline and given to the animals by gavage for 5 days. CCl4 was dissolved in olive oil and administrated a single dose on the fifth day. Twenty-four hour after administration of CCl4, the mice of each group were anesthesized by ketamine (100 mg/kg) and xylazine (10 mg/kg) and blood samples collected by heart puncture for biochemical analyzes. The liver of each mouse was removed. A part of the liver was kept in %10 formalin solutions for histopathology studies and the other part was kept at −70 °C for oxidative stress studies.

Liver Serum Assessments

Liver function indicators including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using standard diagnostic kits (Pars Azmoon Kit. IRI) and clinical spectrophotometer (UV-1650 PC, Shimadzu, Japan).

Preparation of Homogenized Liver Tissue

Liver tissue 1:10 with phosphate buffer (1 ml, 1 mM, pH 7.4) was homogenized using glass homogenizer, then homogenate was centrifuged at 12,000×*g* for 30 min at 4 ◦C. The supernatant was separated from sediment and used for GSH, MDA and CAT assessment [23].

Determination of CAT Activity in Liver Tissue

CAT activity was determined by a slightly modified version of L. Goth; Accordingly, tris-HCl (500 µl, 0.05 mmol) was added to H_2O_2 (1 ml) and supernatant (50 µl) then were mixed and incubated for 10 min, after then ammonium molybdate (500 µl, 4%) was added and absorbance was measured at 410 nm. The result was expressed as U/g tissue [24].

Lipid Peroxidation Measurement in Liver Tissue

Lipid peroxidation in the liver was measured based on the MDA level. The reaction of MDA with TBA produce a purple color with maximum absorbance at 532 nm [25]. For this assessment, supernatant (1 ml) was added to 2 ml TBA and placed in 100 °C for 15 min. After cooling, it was centrifuged (3000 rpm, 10 min) and the supernatant separated. The absorbance was read by spectrophotometer. Ultimately, the MDA level was reported as mol/g tissue [26].

Evaluation GSH Level in Liver Tissue

GSH level in liver tissue was measured with Ellman's reagent (dithionitrobenzoic acid (DTNB)) as an indicator. In concisely, DTNB (0.04%) was added to each tube of supernatant, and the absorbance of produced yellow color was read by a spectrophotometer at 412 nm (UV-1650PC, Shimadzu, Japan). GSH concentration was expressed as mol/g tissue [27, 28].

Histopathological Studies

The liver sections of each mouse were stained with H&E (Hematoxylin and Eosin) and assessed for histological criteria including degeneration of hepatocytes, accumulation of inflammatory cells, fatty change, coagulative necrosis, and dilated sinosouids. The average percentage of each feature was determined. (-) indicates normal, (+) indicates

mild, (++) indicates moderate, (+++) indicates severe, (++++) indicates extremely severe.

Statistical Analysis

The results of each group of animals were considered as mean ± SEM. Comparisons different between groups were calculated by variance one-way ANOVA. The difference between mean and the significance of difference groups were determined by Tukey's Post- hoc test at level of $p < 0.05$. All data were analyzed using statistical package program Prism 5.0 (Inc., La Jolla, CA).

Results

Biochemical

As (Fig. 1) shows CCl4 significantly increased the levels of hepatic enzymes. Serum levels of ALT and AST indicate as a reflex of hepatic function. When mice were exposed to CCl4 (1 mg/kg) significantly increased serum levels of ALT and AST compared with the control and sham groups ($p<0.001$). Pretreatment with 500, 1000 and 2000 mg/kg Allium showed a significant decrease in serum levels of ALT and AST compared with the CCl4 group ($p<0.001$).

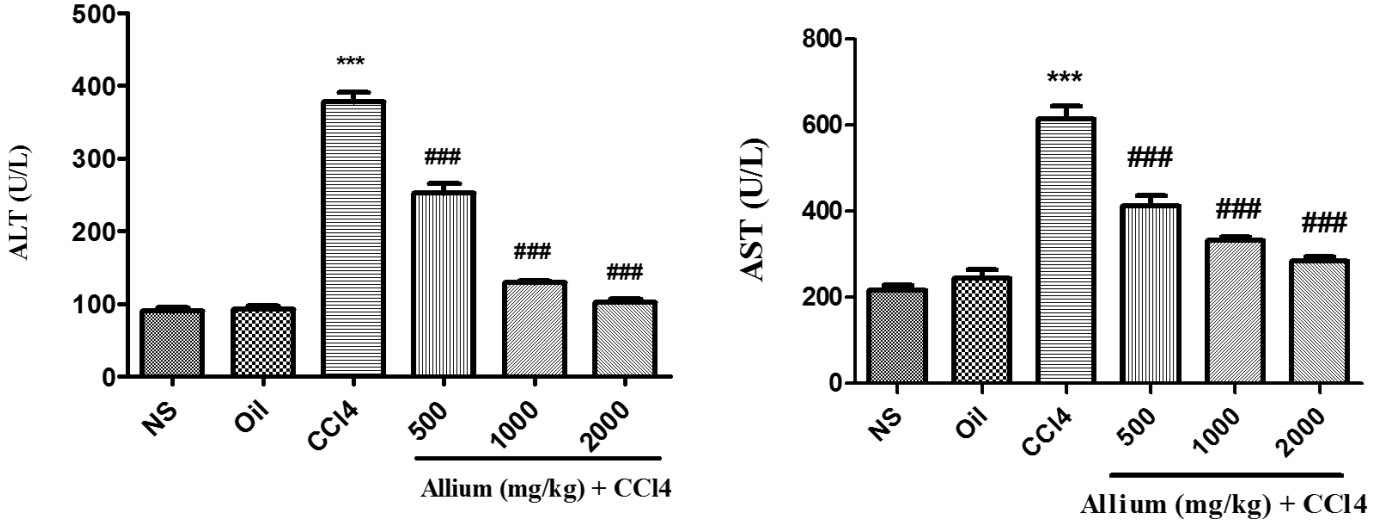

Fig. 1. Effect of Allium and CCl4 on serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). (Mean ± SEM; n = 7).
* * *P < 0.001 versus the control and sham groups.
#P < 0.01 versus the CCl4 group.

GSH level

As (Fig. 2) shows GSH amount significantly decreased in liver tissue in the CCl4 group compared to the control and sham groups ($p<0.001$). While pretreatment with 500, 1000 and 2000 mg/kg Allium significantly increased GSH level in liver tissue when compared with the CCl4 group ($p<0.001$).

CAT Activity

As (Fig. 2) shows CAT activity in group treated with CCl4 significantly decreased compared to the control and sham groups ($P<0.001$). However, pretreatment with 500 ($P<0.01$), 1000 and 2000 mg/kg ($P<0.001$) Allium significantly increased CAT activity in the liver tissue when compared with the CCl4 group.

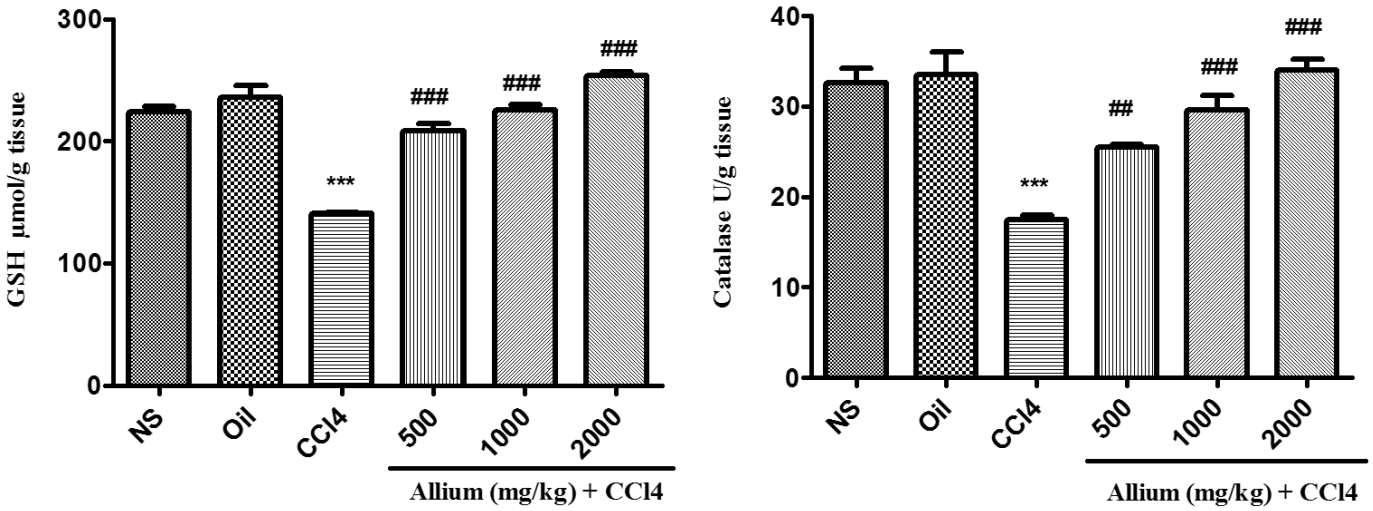

Fig. 2. Effect of Allium and CCl4 on glutathione (GSH) level and catalase activity. (Mean ± SEM; n = 7).
* * *P < 0.001 versus the control and sham groups.
#P < 0.01 and # # #P < 0.001 versus the CCl4 group.

Lipid Peroxidation

As (Fig. 3) shows results of lipid peroxidation in liver tissue. The MDA level in group received CCl4 significantly increased when compared to the control and sham groups (p<0.001). However, pretreatment with 500, 1000 and 2000 mg/kg Allium showed a significant decrease in the MDA level in liver tissue when compared with the CCl4 group (p<0.001).

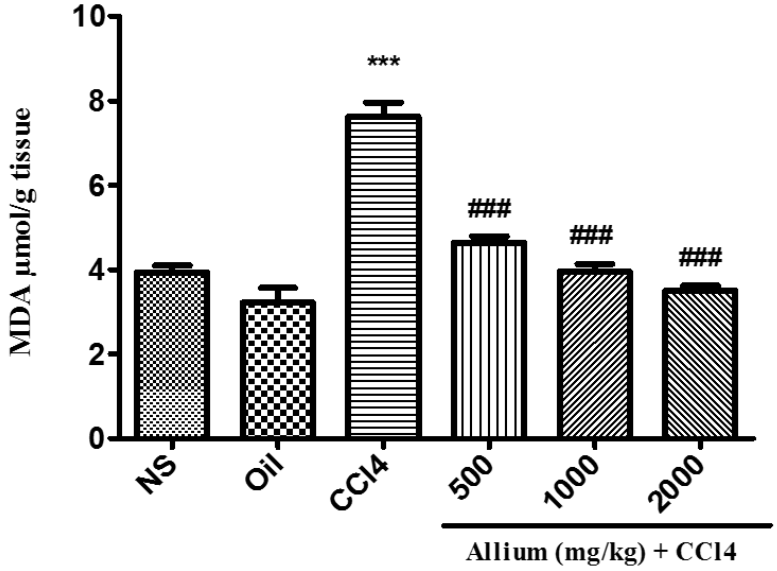

Fig. 3. Effect of Allium and CCl4 on malondialdehyde (MDA) level. (Mean ± SEM; n = 7).
* * *P < 0.001 versus the control and sham groups.
#P < 0.001 versus the CCl4 group.

Histopathological Analysis

As (Fig. 4) showed histopathological changes and also (Table 1) showed semi quantitative scoring of damage on histopathological examination of liver in the control and the CCl4-treated mice. The control and sham groups revealed entirely normal histological features. A number of hepatocytes were shown severe signs of necrosis, infiltration of inflammatory cells, fatty change, and dilated sinosouids in the CCl4 group. Examination of the liver sections of mice after pretreatment with 500 mg/kg Allium and CCl4 showed congestion and enlargement of liver blood vessels and bile canaliculated between 2 hepatocytes. Pretreatment with 1000 and 2000 mg/kg Allium plus CCl4 revealed an improvement in the histological appearance of the liver tissue compared with the CCl4 group.

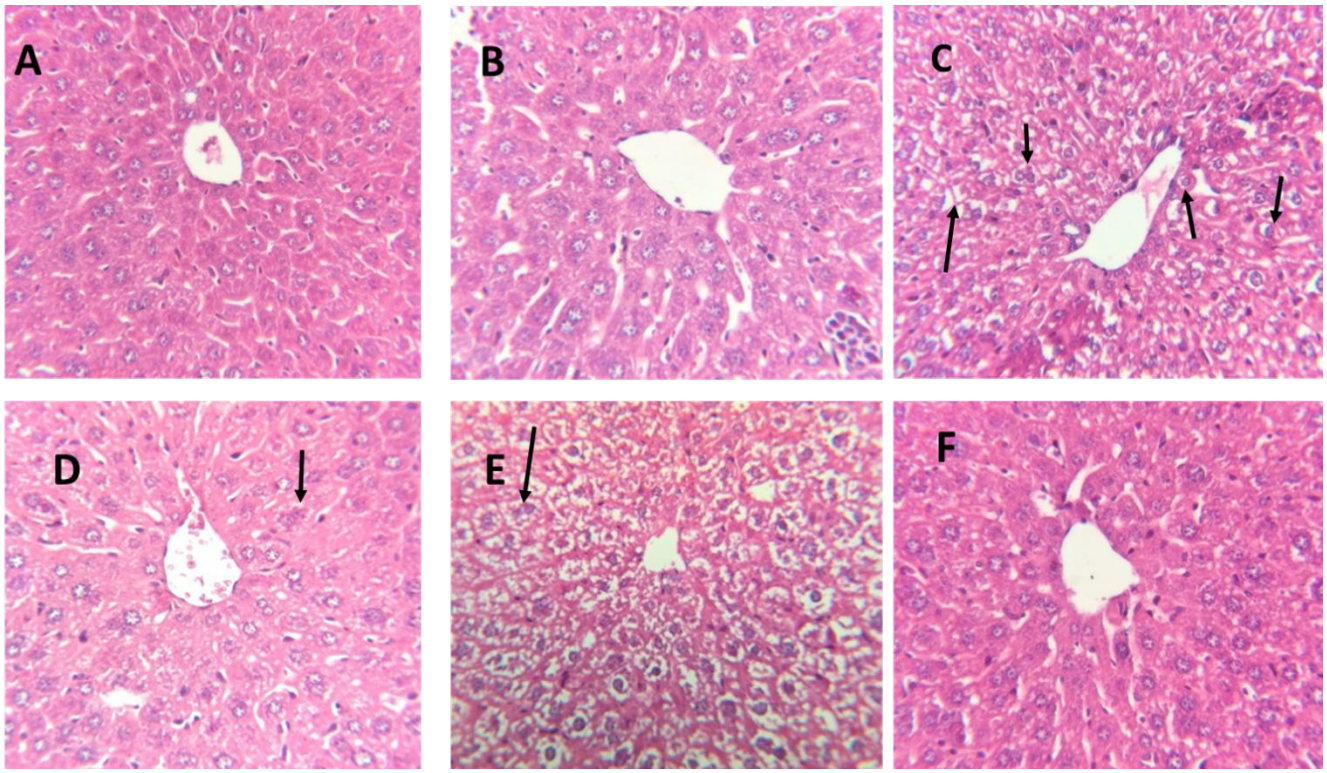

Fig. 4. Photomicrograph shown the histopathological sections of liver tissue from each group (original magnification: X 40). A: Control group; B: Sham group; C: CCl4 group (1 mg/kg, i.p); D: Allium 500 mg/kg + CCl4; E: Allium 1000 mg/kg + CCl4; F: Allium 2000 mg/kg + CCl4.

Table 1. Semi quantitative scoring of damage on histopathological examination of liver in all groups.

Histological criteria	Groups					
	Control	Sham	CCl4	Allium 500+CCl4	Allium 1000+CCl4	Allium 2000+CCl4
Degeneration of hepatocytes	-	-	++++	+++	+++	+
Infiltration of inflammatory cells	-	-	++++	+++	++++	++
Fatty change in hepatocytes	-	-	++++	++++	+++	+
Coagulative necrosis in hepatocytes	-	-	+++	+++	++	+
Dilated sinosouids	-	-	++	+	++	+

(-) indicates normal, (+) indicates mild, (++) indicates moderate, (+++) indicates severe, (++++) indicates extremely severe.

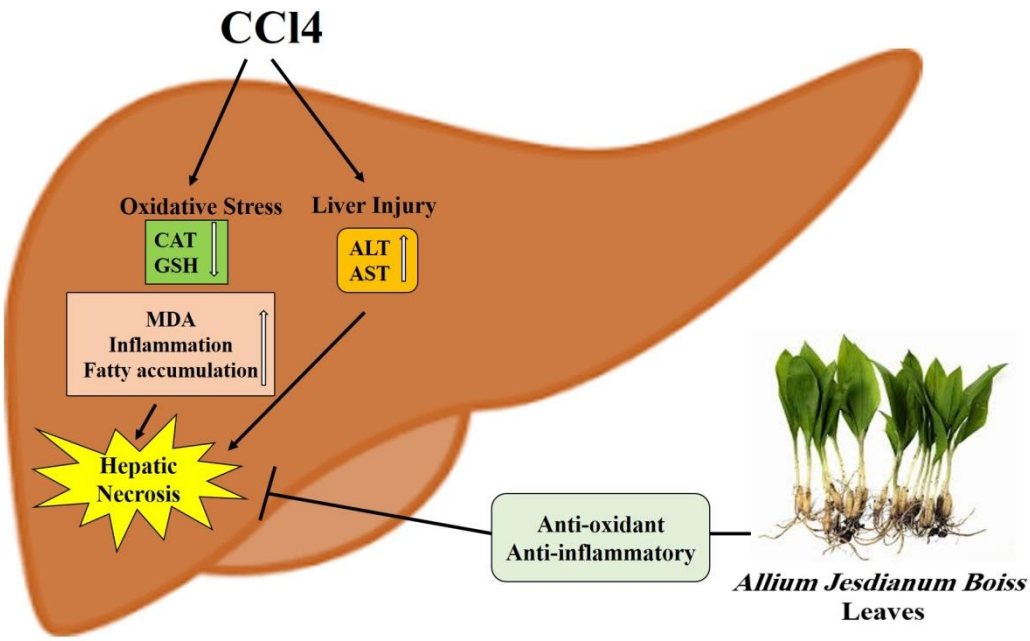

Fig. 5. The effects of Allium and CCl4 on mice liver. CCl4 in the liver metabolizes to highly toxic free radical that leads to oxidative stress generation and decreases the levels of glutathione (GSH), catalase (CAT) and increases the levels of malondialdehyde (MDA), inflammation and also leads to fatty change that resulted an increase in the levels of aspartate transaminase (AST) and alanine transaminase (ALT). Allium administration alters all examined parameters that induced by CCl4.

Discussion

The present study indicated that *Allium* inhibited liver oxidative stress induced by CCl4 possibility via enhancing antioxidant defense system in male mice.

Previous evidences have shown that CCl4 can cause liver injury [4, 29, 30]. The leakages of the liver enzymes including ALT and AST into the blood stream were associated with loss of hepatic architecture, degeneration of hepatocytes, inflammation, and necrosis. In the present study, CCl4 treatment significantly increased the levels of AST and ALT along with necrosis and infiltration of inflammatory cells in liver tissue. Free radicals produced from carbon tetrachloride metabolism induced reactive oxygen species and could activate inflammatory cells and stimulate the generation of lipid peroxidation thus cause liver damage and hepatocyte necrosis [31-33]. While pretreatment with *Allium* in a dose-dependent manner significantly prevent CCl4-induced liver damage in male mice by decreasing serum levels of AST and ALT and relieving histological changes caused by CCl4.

Previous studies have shown that extreme oxidative stress caused by CCl4 can stimulate monocytes of blood stream and tissue macrophages and lead to the release of a variety of proinflammatory cytokines [34] such as TNF-α and IL-1β that these are early response proinflammatory cytokines and have a main role in the expansion of inflammatory and associate with many liver diseases. The present study showed that CCl4 caused infiltration of inflammatory cells in the mouse liver [35]. Moreover, monocytes and macrophages are nonspecific response to stimulants in immune system. When liver damage occurs, white blood cells quickly migrate to sites of damage and initiate inflammatory responses. Therefore white blood cells infiltration was considered as an indicator of inflammatory response [36]. However, *Allium* inhibited liver inflammation. These results suggested that *Allium* could improve liver inflammation caused by CCl4 possibility through suppressing leukocytes infiltration in the mouse liver. Previous studies showed that *Allium* have anti-inflammatory activity by inhibiting phagocytosis via decreasing of nitro-oxidative stress [37]. *In vitro* studies also confirmed anti-inflammatory activity of *Allium* family [13].

In our study, CCl4 treatment significantly reduced the levels of GSH, the highest antioxidant in the liver as well as reduced CAT activity as an enzymatic antioxidant defense; while increasing the level of MDA, indicating that CCl4 has caused severe oxidative stress. The reduction of GSH as non-enzymatic antioxidant defenses is in agreement with our previous studies [38, 39]. GSH as a non-enzymatic antioxidant capacity in hepatocytes is used to prevent tissue damage against free radicals and the enzymatic antioxidant defense capacity is a valuable indicator for measuring antioxidant defenses, it is supposed that non-enzymatic and enzymatic antioxidant defenses are conceded by CCl4 in the liver mice. The reduction of GSH levels in liver mice can reveal increased ROS generation produced by CCl4. In the present study, the decrease in CAT activity represents an elevated level of H_2O_2 in the liver tissue. Because this enzyme involves in removing excesses of H_2O_2 in hepatocytes. The accumulation of H2O2 can cause the inhibition of the antioxidant system that can be aided by a decrease in CAT activity [40]. CCl4 by cytochrome P450 enzymes is metabolized into free radicals, including highly toxic trichloromethyl free radical (•CCl3) and trichloromethyl peroxyl (•OOCCl3) and leads to damage to hepatocytes. Both of these free radicals can bind to lipids and initiate lipid peroxidation processes and liver damage; which lipid peroxidation is considered to be the most important mechanism in the pathogenesis of hepatotoxicity induced by CCl4 [41].

In present study, one possible mechanism of GSH depletion against toxicity induced by CCl4 is based on the directly reacted CCl4 metabolites with GSH, which reduced the levels of GSH. Because it has been previously reported that the trichloromethyl free radical (•CCl3) has reacted with sulfhydryl groups of GSH and other protein thiols and changed the redox status of

hepatocytes. GSH is reflected as an endogenous important protection against lipid peroxidation damage for the removal of hydrogen peroxide and hydroxyl radicals. Therefore, the levels of GSH will be affected by enzymatic antioxidant defenses in the cells of the liver. It has been suggested that a decrease in the activity of primary antioxidant; CAT may be due to the accumulation of reactive oxygen species [42]. The excessive ROS disrupts the balance of the antioxidant defense system. ROS attacks to membrane lipids and generates MDA, which is an indicator of oxidative stress [43]. In our study, pretreatment with *Allium* at all doses used significantly increased the level of GSH and/ or its rate of synthesis, CAT activity and decreased MDA levels, which suggests enhanced protection against oxidative stress and lipid peroxidation. These results propose the protective effects of *Allium* against CCl4-induced oxidative stress, could be attributed to its high level of phenol and other antioxidants. These compounds could scavenge the free radicals of CCl4 generated through the P450 enzyme system thereby diminishing the oxidative injuries. Previous investigations *in vitro* from hydroethanolic extracts of various parts of the *Allium* spontaneous species (*Allium neapolitanum* Cyr., *Allium subhirsutum* L., and *Allium roseum* L.) showed that the *Allium* leaves had the best antioxidant activity, and the levels of polyphenol were generally directly correlated with antioxidant/antiradical activity [44]. Also one another study showed the antioxidant potential of *Allium vineale* flavones, isolated from water-soluble, that its antioxidant activity related to contain phenolic levels [45].

In the present study, histopathological findings support the biochemical and molecular findings. These findings show that major damage caused by CCl4 occurs in the liver tissue. This is because CCl4 is metabolized by the cytochrome P450 enzyme to free radicals in the liver tissue and leads to lipid peroxidation and subsequent tissue damage including necrosis, infiltration of inflammatory cells, fatty changes, and dilated sinosouids that these findings are confirmed by previous studies. [46, 47].

Conclusions

In conclusion, this study confirmed that oxidative stress plays an important role in CCl4 induced hepatotoxicity. Biochemical, oxidative stress, and pathological findings suggest that *Allium* in a dose-dependent manner protects the liver against CCl4 induced hepatotoxicity in male mice (Fig 5). This protective effect may involve the antioxidant potential of *Allium* that can be attributed to its high level of phenol and other antioxidants compounds. This study demonstrated scientific evidence for its pharmacological use in liver injury. However, more studies need to be conducted to prove the efficacy of this extract.

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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