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Article

Protective effect of *Rheum ribes* extract against lead-induced hepatotoxicity in male rats

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The effects of *Rheum ribes* on lead acetate levels and hepatic biochemical factors due to lead acetate toxicity were investigated. Forty male Wistar rats were designated into four groups: Control; lead acetate (receiving in drinking water at 0.6 g/L, daily); hydroalcoholic extract groups (200 and 400 mg/kg doses, gavage, once daily). Treatments were conducted for 10 days. On the 11th day, blood samples were collected to measure lead acetate levels and biochemical factors. Liver tissue samples were examined for histopathological changes. Lead serum levels were increased in lead acetate-treated rats ($p<0.001$). Lead acetate treatment was associated with a significant increase in liver tissue damage ($p<0.001$), while *R. ribes* extract prevented liver tissue damage ($p<0.05$). The levels of alanine aminotransferase and aspartate aminotransferase were significantly lower in the groups lead acetate + extract (two doses) than in the lead acetate group ($p<0.001$ and $P<0.01$, respectively), but alkaline phosphatase level, prothrombin time, partial thromboplastin time and international normalized ratio were not different between the lead acetate + extract groups and the lead acetate group. The results showed the inhibitory role of *R. ribes* on lead-induced hepato-toxicity. The results make Rhubarb a good candidate to protect against the deleterious effect of chronic lead intoxication after complementary studies.

KEYWORDS: Hepatotoxicity. Lead acetate. Rat. *Rheum ribes* L.

INTRODUCTION

Lead is a potentially hazardous metal and an environmental pollutant used in a variety of colors, lacquers, and colored inks, as well as in gasoline as an element of combustion (Sharma, Sharma, Kansal, 2010; Abdel Moneim, 2016). Therefore, lead poisoning occurs more frequently around paint and battery factories, lead extraction mines, and oil industry facilities (Sandhir, Gill, 1995). Lead can accumulate immediately after intestinal absorption in organs and systems of the body, including the liver, and cause certain changes in the structure and function of the liver (Kilikdar *et al.*, 2011). Lead acetate has significant detrimental effects on the body, including glutathione and glutathione- associated enzymes. This decrease in glutathione seems to be, at least in part, responsible for lead-induced toxicity (Kilikdar *et al.*, 2011). Studies have shown that lead can cause adverse effects such as physiological, biochemical, neurological, behavioral, and reproductive disorders in laboratory animals and humans (Patrick, 2006; Abdallah, El-Sayed, Abo-Salem, 2010). The presence of more than 0.3 mg/kg of lead in blood can lead to toxicity through several mechanisms (Sandhir, Gill, 1995). Lead-induced oxidative stress is one of the most important mechanisms of damage to the organs of the body. Accordingly, the presence of antioxidants in treatment remedies can increase the efficacy of treatment and reduce lead toxicity (Mansouri, Cauli, 2009; Attia *et al.*, 2013). Rhubarb, botanically referred to as *Rheum ribes* L. (Polygonaceae), consists of 70 species and is native to certain Asian regions, including Iran, Pakistan, India, and China. The rhizome, stem, leaf, and flower of this plant are edible both raw and cooked. *R.*

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ribes leaf contains plenty of oxalate whose consumption, in large amounts, either as raw or as cooked, leads to certain complications, especially in the kidney (Öztürk *et al*., 2007). *R. ribes* contains flavonoids and vitamins A and E, which have antioxidant activities. This genus and some others from this family, such as *Rheum palmatum* L., are frequently used for the treatments of chronic liver diseases. In addition, many animal, cellular, and molecular experiments have revealed that this plant has a protective effect against hepatic injury (Arosio *et al*., 2000; Jin *et al*., 2005; Zhao *et al*., 2009). In the present study, we investigated the protective effect of *R. ribes* extract on lead acetate levels and liver biochemical factors in the blood and histopathologic liver damage due to lead acetate-induced toxicity in male Wistar rats.

MATERIAL AND METHODS

Extraction of plant

Fresh *R. ribes* was purchased from a local market in April 2016. After the identification of the plant by an expert botanist (Shirmardi Hamzeh Ali, PhD., Research Center of Agriculture and Natural Resources, P.O. Box 415, Shahrekord, Iran); Therefore, a specimen of the plant was deposited in the Herbarium Unit of Shahrekord University of Medical Sciences (Herbarium No. 527). The aerial parts of the plant were washed, dried, pulverized, and extracted by maceration method. For this purpose, the powder was mixed with 70% ethanol and stored at room temperature for 48 h in the dark. Then it was filtered using Whatman filter papers grade 1 and concentrated in a rotary evaporator. The concentrated extract was incubated at 37°C to dry completely (Soofiniya, 2011). Then, 200 and 400 mg/kg concentrations of *R. ribes* extract were prepared by the addition of normal saline.

Measuring antioxidant capacity

To measure the antioxidant capacity of the *R. ribes* extract, 50 µL of this extract, dissolved in DMSO, was added to 200 µL of DPPH (2 and 2-dimethyl-1-picreyl hydrazyl), dissolved in methanol). After 15 min of incubation at room temperature, the absorbance was measured by a spectrophotometer at 517 nm (Rahimi-Madiseh *et al*., 2017). The percentage of inhibition was calculated by the formula below:

$$\text{Percentage of inhibition} = (\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}}) \times 100$$

Measurement of total phenolic and flavonoid contents

The total flavonoid content of the extract was measured using the aluminum chloride colorimetric method in accordance with the rutin standard. In this method, at first, the solutions of 25, 50, 100, 250, and 500 ppm of rutin were prepared in 60% methanol, and 1 mL of each resulting solution was transferred to the test tube. Then, 1 mL of 2% aluminum chloride solution and then 6 mL of 5% potassium acetate solution were added to the test tubes. After 40 min, the absorbance of the samples was read at 415 nm. Then, 0.01-0.02 g of dry extract was dissolved in 60% methanol, and the resulting solution was concentrated to a volume of 10 mL. Then, according to the aluminum chloride colorimetric method, the total flavonoid content of the extract was calculated and expressed as rutin/g dry weight of extract (Rahimi-Madiseh *et al*., 2017).

The total phenolic compounds were measured according to the Folin-Ciocalteu colorimetric method and expressed as mg gallic acid per gram dry weight of the extract. At first, standard gallic acid concentrations (12.5, 25, 50, 62.5, 100, and 125 ppm) in 60% methanol solution were prepared. Then, 0.1 mL of each concentration was transferred to the test tube. In the next step, 0.5 mL of Folin-Ciocalteu solution (10%) and, 3-8 min later, 0.4 mL of sodium carbonate solution (7.5%) were added to each tube. The tubes were left at laboratory temperature for 30 min, and then the absorbance was read by a spectrophotometer at 765 nm. Then, 0.01-0.02 g of dry extract was dissolved in 60% methanol to a final volume of 10 mL. Total phenolic content was determined by the Folin-Ciocalteu method according to the above protocol. The only difference was that instead of the standard solution, 0.1 mL of the extract was added. The total phenolic content of the extract was expressed as mg gallic acid per gram dry weight of extract (Rahimi-Madiseh *et al*., 2017).

Identification and measuring of flavonoid and phenolic acid compounds by HPLC

To determine the type and value of flavonoids and phenolic acid compounds contained in the extract, reversed-phase high-performance liquid chromatography was used. Identification and measurement were performed using an automatic gradient HPLC system (KNAVER Auto Sapler), which included a pump 1000, detector (UV PDA 2800), column (waters C18 EPHERISORB) with dimensions of 250×4.6 mm, particle size of 5 microliters at the room temperature. Detector wavelength was set at 372 (apigenin) 290 (naringin), 320 (caffeic acid), 339 (quercetin), 350 (luteolin), and 355 (rutin). The mobile phase, including solvent A (methanol and 0.05% Terry fluoro acetic acid), solvent B (water and 0.05 fluoro acetic acid), and washing protocol were as follows: 0-10 min 20% solvent A and 80% solvent B (V/V linear gradient), 10- 40 min 30% A and 70% B (V/V) B, 40-45 min 80% solvent A, and B 20% (V/V), 45- 50 100% A (V), 50-55 100% A (V), 55- 60 80% A and 20% B (V), and a flow rate of 0.5 mL per min. Identification and determining the values of flavonoids and phenolic acids were performed according to the Tarnawski *et al.* (2006) procedure with a little change (Tarnawski *et al.*, 2006).

Standard solution

Approximately 10 mg of each standard by weight precision and in a volumetric flask with 1:1 water and methanol ratio was dissolved to obtain stoke standards. To obtain linear range and sequence regression, the concentrations of 1:56, 3:12, 25:50 mL per liter with diluting stoke standards in methanol and water in a ratio of 1:4 was obtained. Calibration standard curves were plotted from the mean of areas obtained from the injection of standard solution in different concentrations.

Sample preparation and injection

Of each sample, 2.5 mg was dissolved in 1 mL methanol and water (in a ratio of 1:4) to provide a concentration of 2500 ppm, and 20 µL of each prepared sample was injected into the HPLC injector.

Laboratory animals

The animals used in the current study were 40 male Wistar rats weighing 250-300 g. The rats were purchased from the Razi Institute of Tehran (Iran) and kept at the Animals House of Shahrekord University of Medical Sciences at 23 ± 2°C under 12 h light/dark cycles. Animals had unlimited access to water and a standard rat diet. The rats were kept in the test environment for one week to acclimatize to it before the implementation of the tests. The rats were monitored for general physical health during the study.

The whole protocol and all stages of the study were carried out in accordance with the University's regulations and the Guide for the Care and Use of Laboratory Animals of the National Institute for Laboratory Animal Research (ethics code: IR.SKUMS.REC.1395.222).

Interventions

The rats were randomly assigned into four groups of 10 animals: Control group (receiving normal saline); lead acetate group (receiving lead acetate in drinking water at 0.6 g/L daily) for induction of subchronic intoxication; and extract groups (receiving lead acetate as well as R. ribes extract at 200 and 400 mg/kg doses, by gavage for 10 days) (Asgharian *et al.*,. 2018).

After completion of the treatment, the mice were killed under general anesthesia with ketamine and xylazine, and then their blood samples with anticoagulant were collected from the heart to measure lead concentrations and biochemical factors. The liver tissue was surgically removed immediately after the rats were killed and fixed in a formaldehyde-containing container for the preparation of histopathology specimens (Carleton, Drury, Wallington, 1980).

To conduct examinations using optical microscope, the lams were numbered and examined by a person who was blind to the grouping of the samples. Inflammatory activity and fibrosis of each lam were checked, and semi-quantitatively scored as 1 to 5. Normal tissue without injury was scored as zero.

Measurement of serum lead acetate concentration

The measurement of lead acetate was carried out by the FLAM method using an atomic absorption spectrometer (Varian 220AA).

Measuring liver biochemical factors

The levels of alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT), and alkaline phosphatase (ALP), as well as prothrombin time (PT), partial thromboplastin time (PTT) and international normalized ratio (INR) were investigated. The enzymes were measured using standard enzyme kits (Pars Test, Iran) (Setorki *et al*., 2013).

Statistical analysis

Data were analyzed using the SPSS version 16. The results were expressed as Mean ± Standard Error of Mean (SEM). Comparisons of groups were conducted by one-way ANOVA followed by Tukey's test. The significance level (p) was considered at < 0.05.

RESULTS

Identification of phenolic acid and flavonoid compounds

The types of phenolic acid and flavonoid compounds of *R. ribes* extract were determined by chromatography of the samples (Table I). According to the results of our HPLC, there was no special examined flavonoid (Rutin, Quercetine, Luteolin, or Apigenin examined in our study) in the extract of *R. ribes*. However, various other types of flavonoids can be present in this extract. The flavonoid and phenolic contents of the plant are presented in Table I.

TABLE I - Phenolic acid and flavonoid compounds of *R. ribes* extract

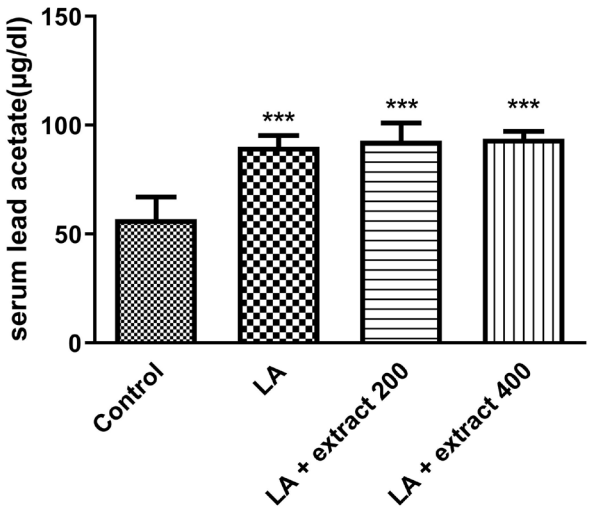
Phenolic acid concentration (µg/0.02 g dry extract)					
Syrginic acid	Gallic acid	3,4dihydrobenzoic acid	Ferulic acid	Caffeic acid	Chlorogenic acid
7.70±0.01	53.79±0.01	3.34±0.03	4.50±0.01	2.44±0.03	5.01±0.01

Antioxidant capacity and total phenolic and flavonoid contents of *R. ribes* extract

The antioxidant capacity (IC50) of *R. ribes* extract was measured as IC50 = 46.74 µg/mL. The total flavonoid and phenolic contents of this extract were 20 mg equivalent rutin/g dry weight of extract and 223.13 mg equivalent gallic acid per g dry weight of extract, respectively.

Serum lead level

According to the results of this study, the level of lead acetate was significantly higher in the lead acetate group than in the control group and the extract groups (90±1.66; $p < 0.001$), while the level of lead acetate was not significantly different between the lead acetate + extract (200 and 400 mg/kg) groups and the lead acetate group (Figure 1).



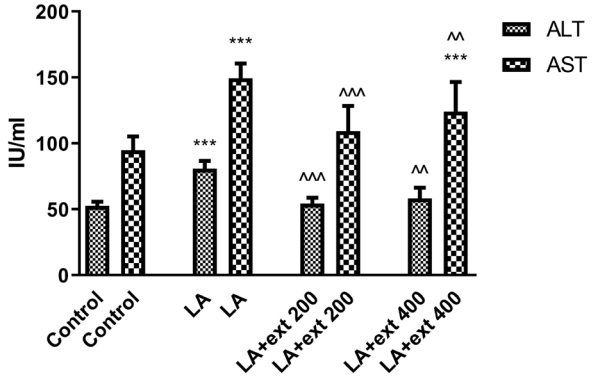
*** P<0.001; compaired with control group

FIGURE 1 - Effect of *Rheum ribes* extract (ext) on serum lead acetate levels in rats treated with lead acetate (LA).

Levels of ALT, AST, and ALP

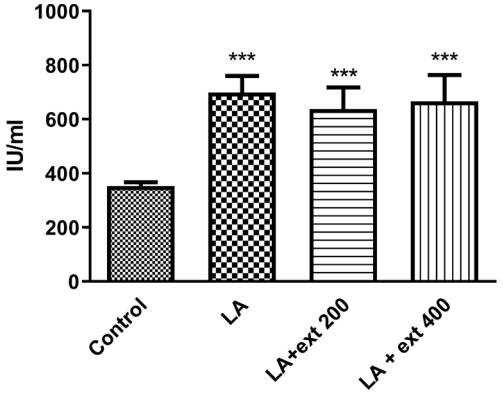
Serum ALT and AST levels were significantly higher in the lead acetate + extract (200 and 400 mg/kg) groups than in the lead acetate group (54.2±1.6; 109.3±6.1; 58.25±2.9; 124.1±7.5; $p < 0.001$ and 0.01, respectively). These levels were not significantly different between the lead acetate + extract (200 and 400 mg/kg) groups and the control group (Figure 2). Also, serum ALP levels were not significantly different between the lead acetate + extract (200 and 400 mg/kg) groups and the lead acetate group (Figure 3).

The results on PT, PTT, and INR showed that there were no significant differences in these variables between the lead acetate + extract (200 and 400 mg/kg) groups and the lead acetate group, but there was a significant difference between the control and lead acetate groups (Figures 4 and 5).



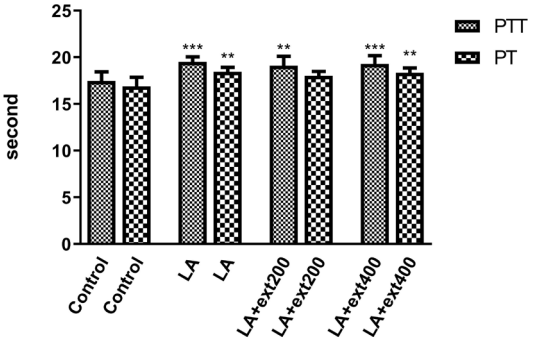
* P<0.001; compaired with control group
^^ P<0.01, ^^^ P<0.001; compaired with Lead acetate group

FIGURE 2 - Effect of *Rheum ribes* extract (ext) on the levels of alanine aminotransferase and aspartate aminotransferase in rats treated with lead acetate (LA).



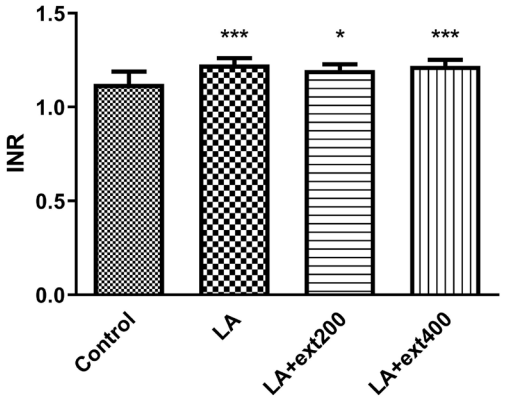
*** P<0.001; compaired with control group

FIGURE 3 - Effect of *Rheum ribes* extract (ext) on the levels of alkaline phosphatase in rats treated with lead acetate (LA).



** P<0.01, *** P<0.001; compaired with control group

FIGURE 4 - Effect of *Rheum ribes* extract (ext) on prothrombin time and partial thromboplastin time in rats treated with lead acetate (LA).

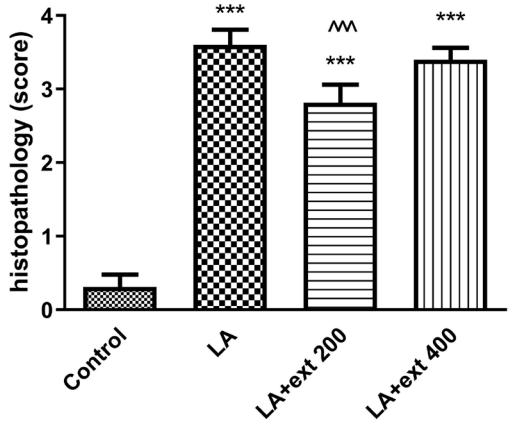


* P<0.05, *** P<0.00; compaired with control group

FIGURE 5 - Effect of *Rheum ribes* extract (ext) on the international normalized ratio in rats treated with lead acetate (LA).

Liver tissue damage

The results on liver pathology showed that liver necrosis was significantly higher in the lead acetate group than in the control group (3.6±0.07; 0.3±0.16; $p < 0.001$). Liver necrosis was significantly lower in the lead acetate + extract (200 mg/kg) group than in the lead acetate group (2.8±0.09; $p < 0.05$) (Figure 6). The liver tissue damage in the lead acetate + extract (400 mg/kg) group was lower than in the lead acetate group, but it was higher than in the lead acetate + extract (200 mg/kg) group (Figure 6).



*** P<0.001; compaired with control group
^^^ P<0.001; compaired with Lead acetate group

FIGURE 6 - Effect of Rheum ribes extract (ext) on liver tissue in rats treated with lead acetate (LA).

DISCUSSION

In the present study, treatment with lead acetate at 0.6 g/L by drinking water for 10 days significantly increased ALP, ALT, and AST levels and serum lead acetate level as well as PTT, INR, and PT. Lead acetate treatment was also associated with liver tissue necrosis. The effects of lead acetate exposure on liver tissue and function have been studied by a number of studies. A study reported that intraperitoneal injection of lead acetate at 15 mg/kg for seven consecutive days significantly increased the serum ALT and AST levels in the rats. In addition, lead acetate treatment caused liver tissue damage and hepatocyte necrosis in the rats (Kilikdar *et al.*, 2011). The study of Jarrar and Taib (2012) showed that chronic treatment with lead acetate (at 0, 0.25, 0.5, 1, and 2% in drinking water) for 1-12 months significantly increased the activity of ALP and α-glycerophosphate dehydrogenase in rat, which was associated with significant tissue changes in hepatocytes, trigeminal liver cells, and liver sinusoid. Treatment with lead acetate by oral gavage for 8 weeks also led to a significant increase in liver enzymes (AST, ALT, and ALP) and changes in liver tissue in rat (Mehana, Meki, Fazili, 2012). Oral treatment with lead acetate at 1.20, 1.40, and 1.60 LD_{50} for 14 weeks caused a significant increase in the activity of ALT, AST, and ALP in the albino rats (Carleton, Drury, Wallington, 1980). Treatment with lead [0.5% concentration in diet (w/w) for one month] has also been reported to be associated with a significant increase in the activity of AST, ALT, and ALP in mice (El-Ashmawy, El-Nahas, Salama, 2005). In our study, consistent with other studies, lead acetate significantly increased serum ALP, ALT, and AST levels as well as liver tissue damage. This toxicity indicates the need to seek out a solution for it.

Regarding the lead-induced hepatotoxicity mechanism, studies have reported that lead interacts with enzymes and proteins of interstitial liver tissue leading to impairment of the antioxidant defense system and production of reactive oxygen species (ROS). ROS are very active, trigger inflammatory processes, and cause oxidative stress. Inflammatory processes are characterized by increased levels of inflammatory markers, the genesis of inflammatory cells in the liver

tissue, oxidative stress induced by altered levels of antioxidant enzymes, and hepatocyte necrosis (Johar *et al*., 2004). Lead-induced oxidative stress is associated with the increased production of free radicals, including superoxide. Superoxide increases the production of H_2O_2, which in turn produces hydroxyl radicals in the presence of metal ions such as iron and copper ions. Hydroxyl radicals are highly reactive and lead to cell dysfunction and hepatocyte death by damaging macromolecules, including proteins, lipids, carbohydrates, and DNA (Sandhir, Gill, 1995).

Free radical-induced hepatocyte damage results in leakage of intracellular enzymes into the bloodstream, resulting in elevated serum levels of AST, ALT, and ALP (Haleagrahara *et al*., 2010). In the present study, treatment with *R. ribes* extract at 200 and 400 mg/kg doses significantly decreased serum ALT and AST levels and liver tissue necrosis in the lead acetate-receiving rats. Therefore, it can be argued that *R. ribes* extract can significantly prevent lead acetate-induced liver damage and dysfunction.

The effects of *R. ribes* extract in preventing lead acetate-induced liver damage may be related to the antioxidant properties of this plant. Considering the antioxidant and anti-inflammatory effects of *R. ribes* extract reported in several studies (Öztürk *et al*., 2007; Emen *et al*., 2013; Hamzeh *et al*., 2014) and confirmed in our study, it can be argued that *R. ribes* extract may prevent hepatocyte damage and dysfunction by decreasing the levels of inflammatory cytokines, inhibiting free radicals and ROS, and increasing the antioxidant capacity of the tissue and serum. Antioxidant properties are the result of compounds with different functional groups, such as hydroxyl group or O2. This property is caused in plants by several secondary metabolites such as phenols, flavonoids, anthocyanins, sulfur compounds, etc. The specific purpose of this study was not to identify the specific compound responsible for the occurrence of this property. However, *R. ribes* contains flavonoids, vitamin A, vitamin E, high levels of vitamin C, oxalic acid, cinnamic acid, rhein, and β-glucogalin. Most of these components have antioxidant activity (Öztürk *et al*., 2007). This plant has antioxidant properties that can increase the antioxidant capacity at the serum and tissue levels, as shown in our previous study. Also, based on HPLC analysis, syrginic acid, gallic acid, 3,4 dihydro benzoic acid, ferulic acid, caffeic acid, and chlorogenic acid are present as phenolic compounds in Rhubarb extract. In previous studies, phenolic compounds in medicinal herbs have been identified as antioxidant compounds (Balasundram, Sundram, Samman, 2006; Zu *et al*., 2006). The study of the effects of chlorogenic acid on liver toxicity induced by acetaminophen showed that this phenolic compound had a protective effect on hepatotoxicity by acetaminophen by activating Nrf2 antioxidative signaling pathway (Wei *et al*., 2017). A study confirmed that chlorogenic acid significantly inhibited CCl_4-induced liver fibrosis (Shi *et al*., 2013). Various studies have confirmed the ability of gallic acid to reduce oxidative stress (Lu *et al*., 2006). The protective effect of this phenolic compound on the oxidative stress induced by sodium fluoride has previously been confirmed (Nabavi *et al*., 2013). The results of a study showed that this phenolic compound reduced the damages to neuronal cells by preventing the production of reactive oxygen species (Ban *et al*., 2007). Also, in a study by Lee *et al*. (2008), the protective effect of caffeic acid on the CCl_4-induced hepatotoxicity in mice was confirmed. This study showed that lipid peroxidation caused CCl_4-induced hepatotoxicity. Antioxidant enzymes such as catalase, SOD, and GST are deactivated by lipid peroxides or reactive oxygen species and, as a result, the activity of these enzymes is reduced against the toxicity of CCl_4. Caffeic acid protects the liver by prevention of the reduced activity of these enzymes. Also, in a study ferrulic acid reduced oxidative damage of liver tissue induced by CCl_4 poisoning (Kim *et al*., 2011).

Lead acetate also increases lipid peroxidation in liver tissue which is significantly reversed by antioxidants such as vitamins C and E (Patra, Swarup, Dwivedi, 2001). Given the presence of high levels of vitamins E and C and flavonoids, also, according to the results of HPLC and identification of high amounts of phenolic compounds with antioxidant activities in *R. ribes*, it can be argued that the extract of this plant could considerably prevent lead-induced liver damage and liver dysfunction by decreasing lipid peroxidation and strengthening the antioxidant defense system.

The results of our study showed that *R. ribes* extract could considerably prevent lead acetate-induced hepatotoxicity in rats probably by decreasing lipid peroxidation and strengthening the antioxidant defense system. According to our results, the lower dose of *Rheum ribes* extract seems to be more effective than the higher dose, but this is not discussed. The reason of these results is not clear. However, it has been found that antioxidants in some situations, such as high doses, may act as pro-oxidant. This may exacerbate the outcome.

Given the nutritional value of *R. ribes*, this plant proposes to be used in the diet of people exposed to lead acetate to prevent or reduce injuries. However, it is recommended that the extract of this plant be investigated in human subjects.

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DECLARATION OF INTEREST STATEMENT

The authors report no conflict of interest

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