Original Research Article

Protective effect of pomegranate seed oil against lead acetate-induced toxicity on the hippocampus and bone marrow in rats

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Abstract

Objective: Lead poisoning is one of the oldest occupational and environmental diseases in the world. It can enter the body by being absorbed in water, air, and food. Oxidative stress is one of the mechanisms responsible for lead toxicity. Anti-inflammatory and antioxidant properties are the primary effects of pomegranate seed oil (PSO). This research is designed to determine the impact of PSO on damage to the hippocampus, bone, and bone marrow in rats triggered by lead acetate.

Materials and Methods: Thirty-two adult male rats were subjected to this study. The animals were divided into four groups at random after they had acclimated. The control group received 1 ml/kg of normal saline for 21 days. Animals in the Pb group received 500 ppm of lead acetate in drinking water for 21 days. Pb+ PSO 0.4 ml/kg and Pb+ PSO 0.8 ml/kg received 0.4 or 0.8 ml/kg of PSO intraperitoneally, concomitant with exposure to lead acetate for 21 days. Blood, bone, bone marrow, and hippocampus samples were taken after the treatment for measuring malondialdehyde (MDA), thiol content and superoxide dismutase (SOD).

Results: Our results revealed that 0.8 ml/kg of PSO significantly decreased malondialdehyde in bone marrow, serum, and hippocampus. It also could increase thiol in serum and superoxide dismutase in bone marrow.

Conclusion: PSO could protect against lead-induced damage in bone, bone marrow, and hippocampus of treated animals through reduction of oxidative stress.

Introduction

Lead (Pb) is the heaviest stable element in the periodic table. Lead in all stages of extraction and production can cause environmental toxicity that endangers human health if not protected. Lead poisoning is one of the oldest occupational and environmental diseases in the world. It can enter the body by being absorbed

through water, air, and food (Kumar et al. 2020).

Lead poisoning is one of the problems of advanced societies. The highest accumulation of lead in bone tissue has been reported, with a half-life of about 20 years (Monir et al. 2010).

Treatment algorithms focus on reducing exposure and initiating chelation therapy. Chelation agents such as dimercaprol, edetate disodium calcium (CaNa$_2$EDTA), succimer, and D-penicillamine commonly treat metal toxicity (Schroder, Tilleman and Desimone 2015).

One of the mechanisms involved in lead toxicity is oxidative stress. The increase in the production of free radicals causes the reduction and depletion of antioxidant enzymes (Chen et al. 2022). Combination therapy with a chelating agent and an antioxidant led to improved patient outcomes (Kim, Kim and Kumar 2019).

The Middle East is the origin of the Pomegranate, *Punica granatum* L, which exhibits several medicinal properties including antioxidants (Boroushaki, Mollazadeh and Afshari 2016), anti-inflammatory (Adams et al. 2006), anti-neoplastic and antimicrobial effects (Goertz and Ahmad 2015). Pomegranate seed oil (PSO), a rich source of conjugated octadecadienoic fatty acids, contains 12-20% of the total seed weight (Vroegrijk et al. 2011). Punicic acid, which is found in conjugated linolenic acids, accounts for the antioxidant and anti-inflammatory abilities of pomegranate seed oil (PSO) (Carvalho Filho 2014).

We found previously that PSO has protective potential against various toxins, especially mercury (Boroushaki et al. 2014a; Boroushaki et al. 2016; Boroushaki et al. 2014b). The main goal of this project is to evaluate the effects of PSO on the bone marrow and hippocampus damage induced by lead acetate in rats.

Materials and Methods
Materials

TBA (2- thiobarbituric acid), DTNB (2,2′-dinitro-5,5′-dithiodibenzoic acid), NaCl (sodium chloride), n-butanol, NaOH (sodium hydroxide), phosphoric acid, HCl (hydrochloric acid), KCl (potassium chloride) and lead (II) acetate trihydrate obtained from Merck Company (Darmstadt, Germany). Pomegranate seed oil (production license number: 12/11616) was given as a thoughtful gift by Urom Narin Co. (Uromia, Iran).

Animals

The Animal House at the School of Medicine in Mashhad, Iran, provided 32 adult male rats that weighed 180-200 g for this project. The animals were kept in pathogen-free cages, under temperature and humidity-controlled (23±2°C and 30%, respectively), having free access to water and food during a 12-hr light/dark cycle.

After acclimating to the environment, four groups were formed by random selection (8 rats per group):

Control: The control group received 1 ml/kg intraperitoneal normal saline for 21 days.

Pb: received lead acetate a dose of 500 ppm orally, which was added to the animals' drinking water for 21 days (Mehrjerdi et al. 2024; Zare Mehrjerdi et al. 2020).

Pb+ PSO 0.4 ml/kg: received 0.4 ml/kg of PSO intraperitoneally, concomitant with exposure to lead acetate 500 ppm orally for 21 days.

Pb+ PSO 0.8 ml/kg: received 0.8 ml/kg of PSO intraperitoneally, concomitant with exposure to lead acetate 500 ppm orally for 21 days (Boroushaki et al. 2016).

After the treatment was completed, all animals were anesthetized by intraperitoneal injection of ketamine/xylazine (60 mg/kg and 6 mg/kg, respectively), and blood samples were obtained using heart puncture. Bone marrow from femur and hippocampus samples were obtained from all groups. Some samples were stored in the freezer for biochemical tests. The activity of

superoxide dismutase (SOD) and the level of malondialdehyde (MDA) and thiol content were assayed in blood, bone marrow, and hippocampus samples.

For biochemical tests, 20 mg of tissue was homogenized in phosphate buffer, including protease inhibitor, using a tissue homogenizer device. Then the level of protein was determined using the BCA method according to the manufacturer's instructions (Pars Tous, Iran). For histological studies, the remaining samples were fixed in formalin.

Measurement of lipid peroxidation

Thiobarbituric acid reactive substance (TBARS) is a red color species produced when TBA reacts with malondialdehyde (MDA), a primary product of lipid peroxidation. This species shows a peak absorbance at 532 nm. Here, 1 ml of TBA (0.6%) and 3 ml of phosphoric acid (1%) were combined with 0.5 ml of each sample homogenate. The tubes were immersed in a hot water bath for 45 min. After vortexing the mixture for 1 min and adding 4 ml of n-butanol, the mixture was centrifuged at 20,000 rpm for 20 min until it cooled down. The supernatant's absorbance was measured at 532 nm. Then, MDA was determined using nanomoles/ gram tissue. Using the standard curve and a concentration range of 0-40 mg/L, the amount of MDA was measured (Alavi et al. 2022).

Measurement of sulfhydryl (thiol) groups

The standard coloring reagent, 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB), was employed to measure the thiol content in all sample homogenates using a spectrophotometer (Hosseini et al. 2022b).

This reagent generates a quantifiable yellow-colored result upon reaction with sulfhydryl. The absorbance measurement at 412 nm was achieved by combining 50 µL of homogenate with 1 mL of Tris-EDTA buffer (pH 8.6) in a test tube. (A1). Then, 20 mL of 10 mM DTNB was added to the mixture. The absorbance were tested once more after 15 min at lab temperature (A2). The absorbance of the DTNB reagent was represented by the blank (B).

The following equation was used to calculate the total SH groups.

Total thiol concentration
$$= \frac{(A1 - A2 - B) \times 1.07}{0.05 \times 13.6}$$

Superoxide dismutase activity assay

Calorimetric analysis (Madesh and Balasubramanian method) was used to measure the activity of superoxide dismutase (SOD). This method relies on inhibiting the formation of superoxide anion by pyrogallol auto-oxidation. Enzyme activity decreases the reaction of 4-5-dimethylthiazole-2-yl, 2-5-diphenyl tetrazolium (MTT) and superoxide anion (Hosseini et al. 2023). SOD activity was measured as U/mg protein.

Histopathological examinations

Hippocampus, bone marrow, and femur samples were fixed in a formalin solution of 10%, processed, and embedded in paraffin before sectioning for histological studies. The slides were stained with Hematoxylin and Eosin (H&E) and then, analyzed by a light microscope.

Statistical analysis

The mean±SD is used to present the data. The Tukey–Kramer post-hoc test was used for statistical analysis following conducting a one-way analysis of variance (ANOVA). The achievement of statistical significance was possible when the p-values <0.05.

Results
PSO decreased MDA levels in a rat model of lead toxicity

As shown in Figures 1A-C, MDA amounts in the bone marrow (0.208 ± 0.074 *vs.* 0.058 ± 0.011 µmol/µg), serum (0.299 ± 0.092 *vs.* 0.095 ± 0.061 µmol/µg), and

hippocampus (0.162 ± 0.048 *vs.* 0.049 ± 0.011 µmol/µg) samples of the Pb group were significantly higher in comparison to the control group (p<0.001).

The level of MDA was lower in the bone marrow (0.095 ± 0.039 µmol/µg, p<0.05) and hippocampal (0.101 ± 0.031 µmol/µg, p<0.05) samples of the Pb+ PSO 0.4 ml/kg group compared to the Pb group.

In animals that received Pb+ PSO 0.8 ml/kg, the levels of MDA were notably decreased in bone marrow (0.062 ± 0.016 µmol/µg, p<0.001), serum (0.171 ± 0.017 µmol/µg, p<0.01), and hippocampus (0.056 ± 0.013 µmol/µg, p<0.001) in comparison to the Pb group.

PSO altered thiol levels in a rat model of lead toxicity

As shown in Figures 2A-C, the thiol contents of bone marrow (1.824 ± 0.827 *vs.* 4.646 ± 1.486 µg/µg, p<0.001), serum (0.375 ± 0.074 *vs.* 0.581 ± 1.460 µg/µg, p<0.05), and hippocampus (0.377 ± 0.067 *vs.* 0.603 ± 0.096 µg/µg, p<0.001) were significantly decreased in the Pb group, compared to the control group. Administration of PSO 0.4 ml/kg had no significant effects on thiol content in comparison to the Pb group. PSO 0.8 ml/kg increased thiol content only in the serum sample compared to the Pb group (p<0.05).

PSO increased SOD activity in a rat model of lead toxicity

Data showed (Figures 3A-C) that the amount of SOD in bone marrow (0.015 ± 0.002 *vs.* 0.026 ± 0.004 U/µg), serum (0.005 ± 0.002 *vs.* 0.012 ± 0.003 U/µg), and hippocampus (0.004 ± 0.002 *vs.* 0.011 ± 0.005 U/µg) samples was significantly reduced in the Pb group which is comparable by the control group (p<0.01). Administration of PSO 0.4 ml/kg had no significant effects on SOD activity in comparison to the Pb group. Administration of PSO 0.8 ml/kg increased SOD level only in bone marrow sample (0.024 ± 0.004 U/µg) in comparison with the Pb group (p<0.05).
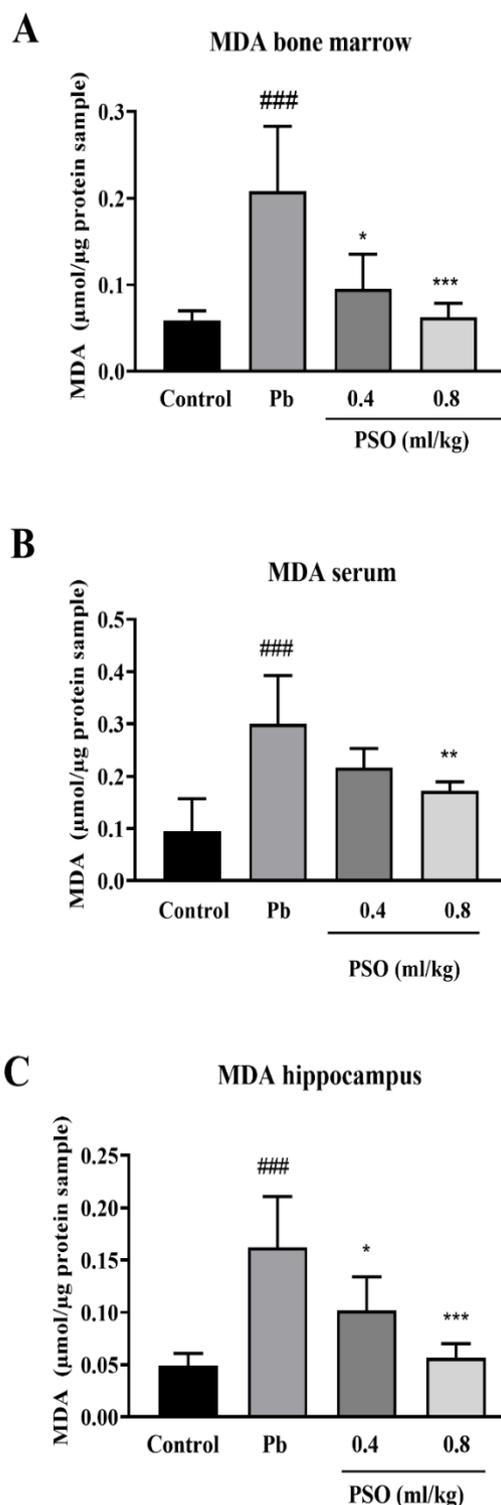

Figure 1. Effect of pomegranate seed oil (PSO) on the malondialdehyde level (MDA) level in bone marrow (A), serum (B), and hippocampus (C) in a rat model of lead toxicity. Data are mean ± SD (n = 6). ###p<0.001 vs. control, *p<0.05 and **p<0.01 and ***p<0.001 vs. Pb. Pb: lead acetate.
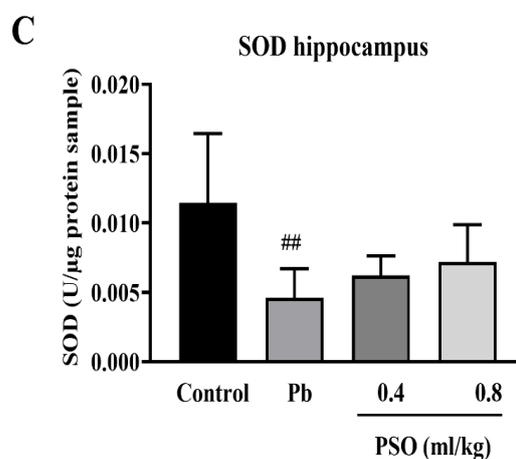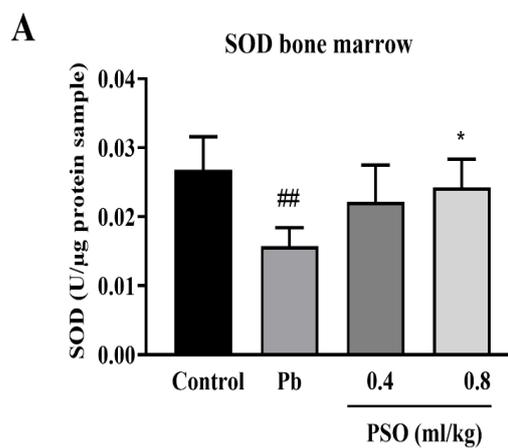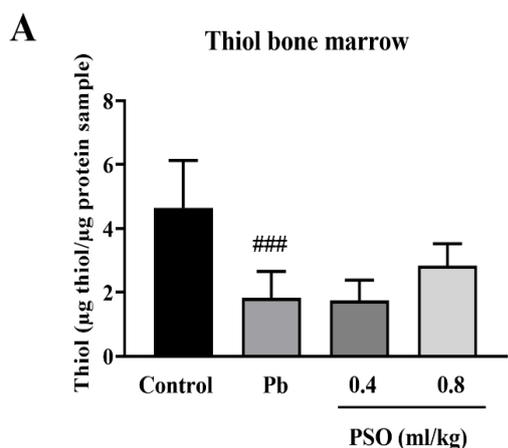
Figure 2. Effect of pomegranate seed oil (PSO) on the thiol level in bone marrow (A), serum (B), and hippocampus (C) in a rat model of lead toxicity. Data are mean±SD (n = 6). #p<0.05 vs. control, *p<0.05 vs. Pb. Pb: lead acetate.

Figure 3. Effect of pomegranate seed oil (PSO) on the activity of superoxide dismutase (SOD). In bone marrow (A), serum (B), and hippocampus (C) in rat model of lead toxicity. Data are mean±SD (n = 6). ##p<0.01 vs. control, *p<0.05 vs. Pb. Pb: lead acetate.
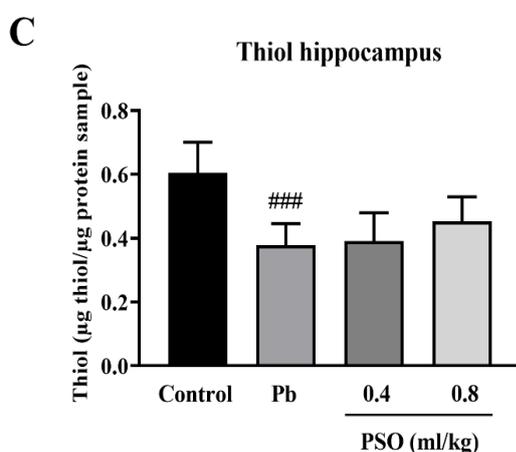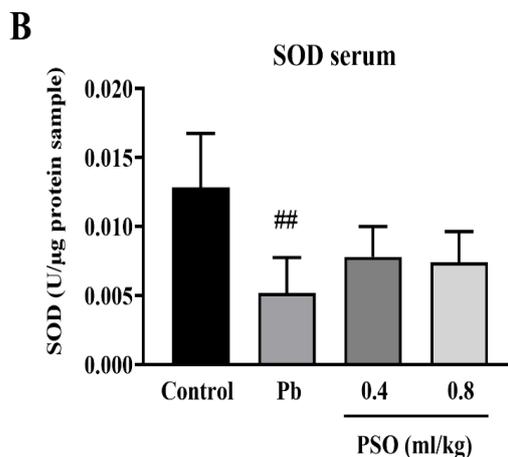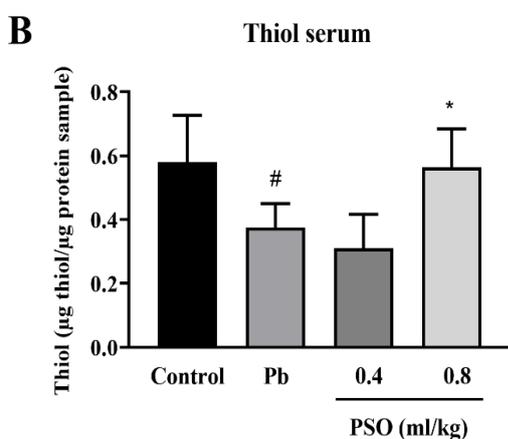
PSO improved histopathological alterations in a rat model of lead toxicity

The histopathologic analysis of bone marrow revealed the normal architecture of bone marrow cells in the control group. The administration of Pb reduced the amount of bone marrow, increased fat vacuole cells, and significantly increased the proliferation of erythroid cells compared with the control group. Treatment with PSO, in a dose-dependent manner, maintained the structural organization of bone marrow and significantly increased the number of bone marrow cells as compared with the Pb group (Figure 4 and Table 1).

In Figure 5, histopathologic analysis of femur bone revealed normal cortical bones with healthy marrow, normal bony spicules, and adequate marrow spaces in the control group. Administration of Pb caused significant structural damage, hypoplasia, and osteoporotic changes compared with the control group. In a dose-dependent manner, PSO maintained the structural organization of femur bone and significantly increased the number of bone marrow cells compared with the Pb group (Table 1).

In Figure 6, histological observations of the dentate gyrus region of the hippocampus of in control group revealed normal cytoarchitecture and characteristic appearance of the granular cells. The dentate gyrus region of the hippocampus in the Pb group revealed distortion in the cytoarchitecture of the hippocampus with the loss and degeneration of granular cells. Treatment with PSO, in a dose-dependent manner, maintained the structural organization of the hippocampus as compared with the Pb group.

Table 1. Morphological changes in rat bone marrow and femur sections.

Group	Bone marrow cells	Fat cells	Congestion and hemorrhage	Osteoporotic changes
Control	+++	+	0	0
Pb	+	+++	++	+++
Pb+PSO 0.4	++	++	+	++
Pb+PSO 0.8	+++	++	+	+

Changes are categorized and scored as follows: 0, +, ++, and +++, in increasing order of severity. Group 1: control; Group 2: Pb; Group 3: Pb+PSO 0.4; Group 4: Pb+PSO 0.8
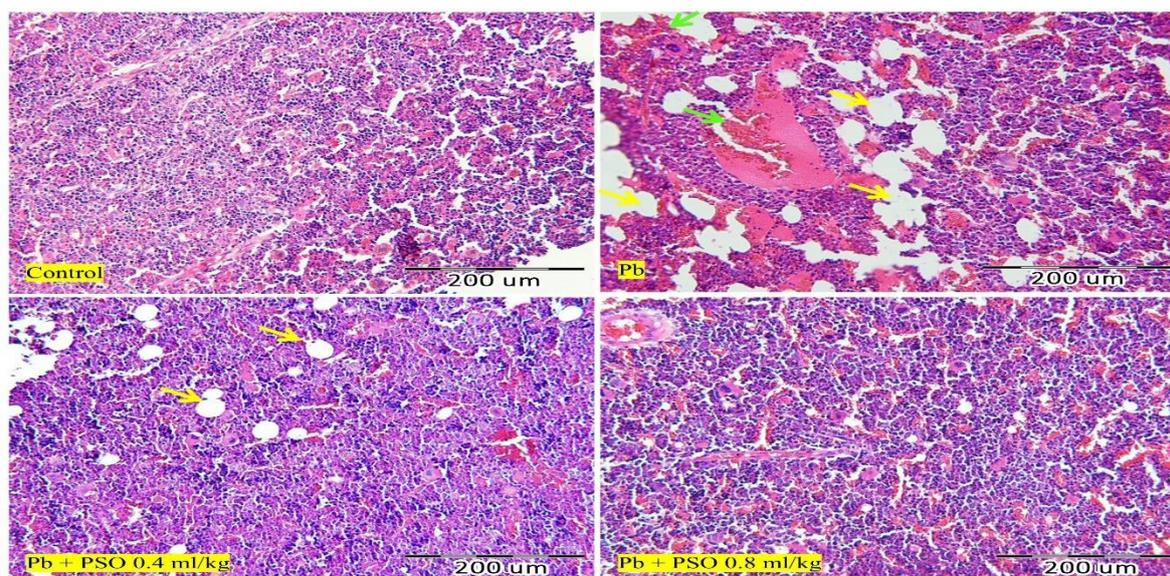

Figure 4. Photomicrograph of rat bone marrow in different treatment groups. The administration of Pb reduced the amount of bone marrow, increased fat vacuole cells (yellow arrow), and significantly increased the proliferation of erythroid cells (green arrow). The structural organization of bone marrow was maintained by PSO in a dose-dependent manner (H&E ×200).
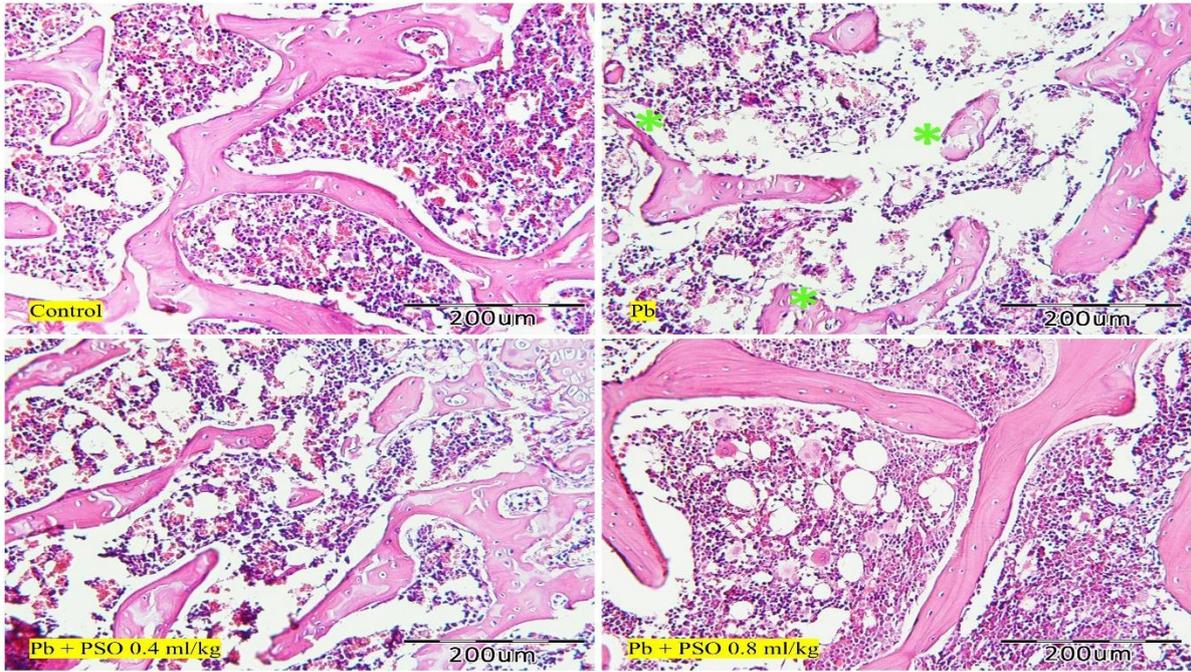
Figure 5. Photomicrograph of femur bone in different treatment groups. The histopathologic analysis of femur bone revealed normal bony spicules with adequate marrow spaces in the control group. The administration of Pb resulted in significant structural damage to bone, hypoplasia, and osteoporotic changes (asterisk). PSO maintained the structural organization of bone depending on the dose compared to the Pb group (H&E ×200).

Figure 6. Photomicrograph of rat hippocampus in different treatment groups. The dentate gyrus region of the hippocampus revealed cell loss and degeneration of granular cells (black arrow) in the Pb group. Treatment with PSO, in a dose-dependent manner, preserved the structural organization of the hippocampus (H&E ×200).

Discussion
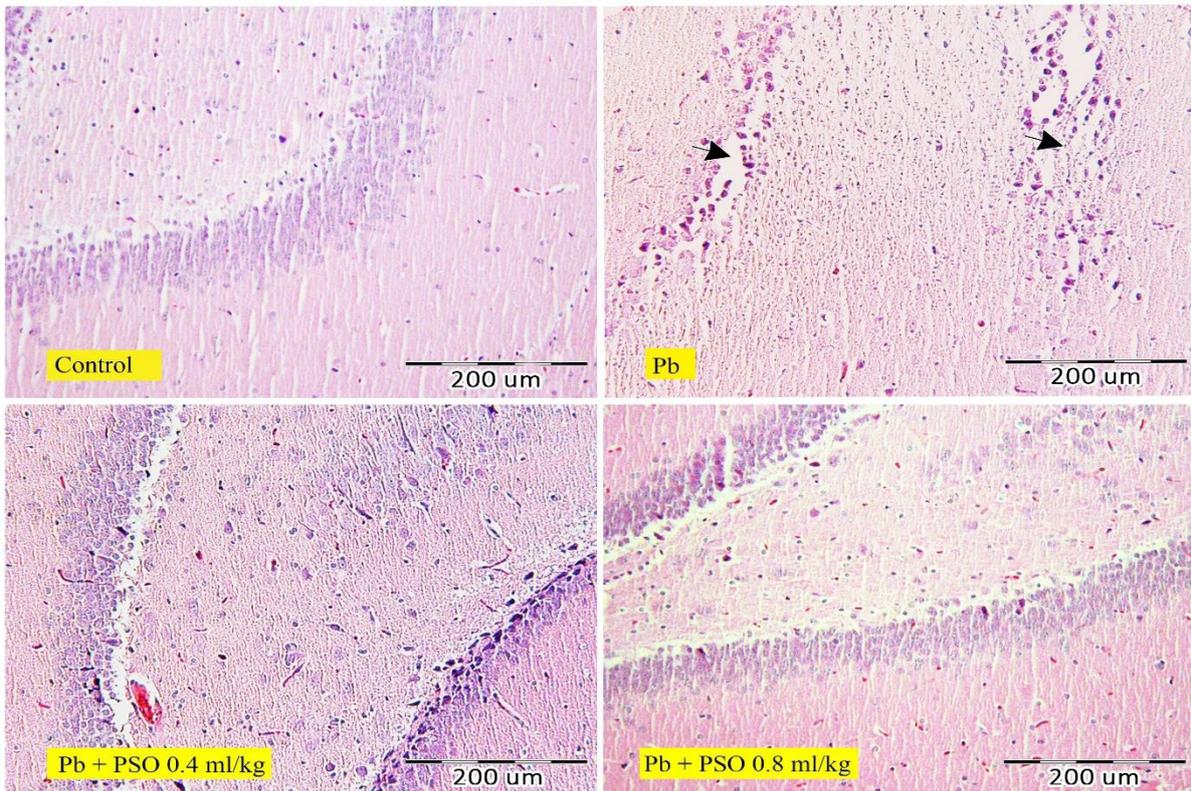
The current study determined that PSO can protect the bone, bone marrow, and hippocampus against the toxicity caused by lead acetate. Our results revealed that 0.8 ml/kg of PSO significantly decreased MDA in bone, bone marrow, and hippocampus. It also could increase thiol in serum and SOD in bone marrow.

Lead acetate has been used as an ingredient in hair dyeing products in the cosmetics industry. Lead is recognized for its detrimental effects on several organs in the human body, such as the brain, bone marrow, liver, and kidneys. Oxidative stress significantly contributes to the harmful impact of lead on organs. This indicates that lead decreases the levels of antioxidant enzymes and increases amount of reactive oxygen species (ROS) (Aydın 2024).

Lead is recognized for causing direct or indirect harm to mitochondria by reducing the endogenous thiol-containing antioxidant glutathione. It exhibits a high affinity for the sulfhydryl groups of numerous enzymatic and non-enzymatic antioxidants, hence reducing their efficacy (Abdel-Moneim et al. 2015). Furthermore, it competes with and replaces certain essential ions, including zinc and copper, that are essential cofactors in the activities of SOD and catalase (Flora, Gupta and Tiwari 2012). This will elevate ROS that induce oxidative stress. The present study observed increases in lipid peroxidation, which are consistent with the lead-induced peroxidation of membrane lipids in the bone marrow (Haleagrahara et al. 2011).

The bone marrow's hematopoietic system which is crucial for blood cell formation, is greatly affected by lead toxicity. Haleagrahara and coworkers investigated the effect of lead exposure (200-600 ppm in drinking water for 21 days) in rats bone marrow (Haleagrahara et al. 2011). A significant increase in lipid peroxidation and a reduction in bone marrow total antioxidants, as well as SOD, glutathione peroxidase, and catalase enzyme levels, were associated with chronic lead exposure (Haleagrahara et al. 2011). The number and differentiation status of bone marrow-derived precursors isolated from rodents were decreased by lead as well (Banijamali, Rabbani-Chadegani and Shahhoseini 2016). Administration of lead nitrate resulted in moderately decreased cellularity in the bone marrow along with moderately increased fat vacuoles in rats (Ehimigbai et al. 2015). Lead toxicity resulted in normocytic normochromic anemia with substantial leukocytosis and lymphocytosis, as reported by Abdelhamid et al. Lead may also cause oxidative damage to the hepatic and testicular tissues, as proven by a great increase in MDA level and a significant decrease in the antioxidant system (Abdelhamid, Mahgoub and Ateya 2020).

Numerous adverse effects of lead appear to be associated with its capacity to induce cell death and apoptosis in diverse tissues (Sharifi et al. 2011). In accordance with our finding, Sharifi et al. showed that lead acetate could induce apoptosis and DNA fragmentation in mesenchymal stem cells isolated from rat bone marrow (Sharifi et al. 2011). Moreover, administration of lead acetate for 14 days caused significant fibrosis and hypoplasia in rat bone marrows (Tham et al. 2013).

The biochemical results in this work agreed with the study by Yousef et al (Yousef et al. 2019). The researchers examined the neuroprotective effects of coenzyme Q10 against neurotoxicity produced by lead acetate. Lead greatly increased cortical lipid peroxidation while decreasing glutathione content, SOD, and catalase activity. Coenzyme Q10, a benzoquinone compound, decreased the neurotoxicity of lead by anti-apoptotic, antioxidant, and anti-inflammatory mechanisms (Yousef et al. 2019).

In addition to suppressing oxidative stress, natural antioxidants enhance the body's defense mechanism against lead toxicity (Abdrabou et al. 2019; Al-Megrin

et al. 2020). The antioxidation, free radical scavenging, and anti-inflammatory properties of PSO have recently attracted increased interest (Liu et al. 2022).

PSO was found to have a protective effect against lead toxicity in the results of the current study on oxidative biomarkers in serum, bone marrow, and hippocampus of rats. Administration of PSO 0.8 ml/kg concomitant with lead acetate in drinking water, significantly decreased MDA in the bone, bone marrow, and hippocampus. PSO also could increase thiol in serum and SOD in bone marrow. We previously found that PSO could reverse oxidative stress by increasing thiol content and improving SOD activity and decreasing MDA in various animal models of gentamicin (Boroushaki et al. 2014a), cisplatin (Boroushaki et al. 2015), diazinon (Boroushaki et al. 2013) and hexachlorobutadiene (Bouroshaki et al. 2010) -induced nephrotoxicity, tacrolimus-induced cardiotoxicity (Hosseini et al. 2022a) and streptozotocin-induced diabetes (Mollazadeh et al. 2016). Furthermore, PSO attenuated mercuric chloride caused nephrotoxicity (Boroushaki et al. 2014b) and hepatotoxicity (Boroushaki et al. 2016) in rats.

PSO showed powerful antioxidant activity due to the presence of flavonoids. PSO had significant antioxidant activity due to the presence of flavonoids. The metal-chelating ability of flavonoids allows them to bind with metals and create a metal-flavonoid complex. Researchers displayed that lutein and curcumin supplementation significantly reversed lead-caused hepato- and nephrotoxicity in rats (Abdel-Moneim et al. 2015; Al-Megrin et al. 2019). Chrysin is a flavonoid present in passion fruit, honey, and propolis. By reducing the imbalance in redox status, chrysin demonstrated protective potential against the harm resulting by the lead acetate in the testicles of rats (Ileriturk et al. 2021).

PSO is also abundant in unsaturated fatty acids, including punicic acid as the primary component, as well as palmitic acid, stearic acid, oleic acid, and linoleic acid. Lipid concomitants in PSO mainly contained squalene, β-sitosterol, and tocopherols (Aruna, Manohar and Singh 2018; Schubert, Lansky and Neeman 1999).

The main form of vitamin E is α-tocopherol and a component of β-, δ-, and γ-tocopherols. Cell membranes are protected against lipid peroxidation by α-tocopherol, which scavenges superoxide radical anions and lipid peroxyl free radicals (Khodamoradi et al. 2015). Squalene showed protective effects in the liver and kidneys after lead intoxication (Kim 2003; Kim, Lee and Lee 2004). Research on animals indicates that α-tocopherol can alleviate the adverse health impacts of lead exposure by decreasing oxidative damage (Das et al. 2015; Patra, Swarup and Dwivedi 2001). Supplementation with α-tocopherol led to a reduction in levels in lead-exposed workers, but oxidative damage markers were not altered significantly (Kasperczyk et al. 2016; Kasperczyk et al. 2017).

There are some limitations for the current study, which are the ground for future studies. First, using a chelator agent as positive control or combination therapy with a chelating agent and PSO. Another limitation of this study is the estimation of bone marrow cellularity and hematological parameters such as red blood cells (RBCs), white blood cells (WBCs), platelets, hemoglobin (Hb) counts, granulocytes, lymphocytes, and monocytes via an automated hematoanalyzer.

PSO has beneficial effects against the hippocampus and bone marrow toxicity caused by lead acetate. PSO could attenuate oxidative stress by reducing lipid peroxidation and improving thiol content and SOD activity in lead intoxication.

Acknowledgment

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Ethical Considerations

All animal procedures under the guidelines of the National Institutes of Health (NIH) and the National Laws for the use and care of experimental animals were approved by the University Ethics Committee.

Code of Ethics

IR.MUMS.AEC.1402.039.

Authors' Contributions

Conceptualization and Methodology, MTB; Investigation, MSA, AH, FT, MRB; Software and Formal analysis, ZT and FT; Writing original draft, MSA, FT; Writing-review and editing, MTB, MSA. All authors have read and approved the manuscript.

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