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# The protective effect of flaxseed oil on lead acetate-induced renal toxicity in rats

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ABSTRACT

Lead is a toxic metal inducing many destructive effects leading to a broad range of physiological, biochemical, and neurological dysfunctions in humans. Here, we investigated the effects of flaxseed oil (1000 mg/kg) on the outcome of renal cytotoxicity induced by lead acetate (20 mg/kg) in male rats. Lead induced injury of the renal tissue. This was evidenced (i) as increases in lead concentration in the kidney, (ii) as increases in the histopathological damage of the renal tissue, (iii) as increases in uric acid, urea and creatinine, (iv) as increases in lipid peroxidation, nitric oxide and reactive oxygen species, and (v) as lowered glutathione levels and decreased activities of catalase and superoxide dismutase, glutathione reductase, glutathione-S-transferase, and glutathione peroxidase, respectively. All these lead-induced parameters were significantly altered during flaxseed oil treatment. Therefore, our study suggests the role of flaxseed oil in limiting renal cytotoxicity-induced by lead acetate as a model for lead toxicity.


## 1. Introduction

Lead (Pb), is a common environmental toxin that is capable of causing numerous acute and chronic illnesses. Lead is absorbed via the respiratory and gastrointestinal tracts and, occasionally, through the skin. Lead absorption via the respiratory tract is highly efficient, resulting in an average uptake of 40% of the inhaled lead [1]. After absorption, lead is distributed in the blood, bone, and soft tissues. Approximately 99% of blood lead content is bound to red blood cells; only 1% is present in the plasma and is available for exchange with lead contained in the other tissues [2].

The kidney is the critical organ after long-term occupational or environmental exposure to Pb. Excessive exposure to lead may cause acute or chronic nephrotoxic effects. Two types of nephropathy, acute and chronic nephropathy, have been observed in humans. Acute Pb nephropathy is characterized functionally by a generalized deficit of tubular transport mechanisms and morphologically by the appearance of degenerative changes in the tubular epithelium and the nuclear inclusion bodies containing Pb protein complexes [3,4].

In recent years, the clinical importance of herbal drugs has received considerable attention. As many synthetic antioxidants have been shown to have one or the other side effects [5], there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical-induced tissue injury [6]. Numerous plant products have been shown to have antioxidant activity, and the antioxidant compounds of plant origin have been extensively investigated as scavengers of free radicals and inhibitors of lipid peroxidation [7].

Interest in the use of whole flaxseed (*Linum usitatissimum*) and its derivatives (ground flax, flax oil, defatted flax, flax fibre and lignan extracts) as functional food or nutraceutical ingredients and adjuncts to a healthful diet continues to grow, as a result of the increasing body of evidence over the past 20 years, investigating the protective effects of flaxseed against a variety of chronic diseases and risk factors including breast and colon carcinogenesis [8], atherosclerosis [9], damage to pancreatic islet cells in insulin dependent *Diabetes mellitus* and hyperlipoproteinemias [10]. As a functional food, flax is noted to be an excellent source of insoluble and particularly, soluble dietary fibre due to its polysaccharide gum and mucilage content associated with the seed hull, highly polyunsaturated oil rich in alinolenic acid, and as the richest food source of the plant lignan secoisolariciresinol diglucoside [11].

The objective of the present study was to evaluate the antioxidant efficacy of the flaxseed oil against lead acetate-induced renal cytotoxicity in adult male albino rats.

## 2. Materials and methods

### 2.1. Animals and experimental design

Twenty-four healthy male albino rats approximately 8–9 weeks old ranging in weight from 120 to 150 g, obtained from the animal house of the holding company for biological products and vaccines (VACSERA, Cairo, Egypt) were used in the present study. Animals were kept in wire-bottomed cages in a room under standard con-

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dition of illumination with a 12-h light–dark cycle, 55 + 5% relative humidity and at 25 ± 2 °C room temperature. They were provided with balanced standard pellet (VACSERA) as a diet and sterile water ad libitum. The experiments were approved by the state authorities and followed Egyptian rules on animal protection.

Animals were randomly allocated to four groups of six rats each. Group I served as vehicle control and received saline (0.3 ml saline/rat) by oral administration. Group II received intraperitoneal (i.p.) injection of 100 μl of 20 mg/kg lead acetate [12] for 5 days. Group III received 0.3 ml of flaxseed oil by gavage (orally) once daily for 5 days at a dose of 1000 mg/kg [13], and the animals of Group IV received 0.3 ml flaxseed oil by gavage once daily for 5 days at a dose of 1000 mg/kg body weight. An hour after the treatment with flaxseed oil, Group IV was intraperitoneally injected with 100 μl of 20 mg/kg lead acetate for 5 days.

After 24 h of the last i.p. injection of lead acetate, the animals of all groups were cervically dislocated and blood samples were collected. After half an hour the blood samples centrifuged at 500 × *g* for 15 min at 4 °C to separate serum and stored at −70 °C. Part of kidney was weighed and homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50 mM Tris–HCl and 300 mM sucrose, pH 7.4 [14]. The homogenate was centrifuged at 500 × *g* for 10 min at 4 °C. The supernatant (10%) was used for the various biochemical determinations.

### 2.2. Relative kidney weight

At the end of the experimental period, each rat was weighed. The left kidney was then removed and weighed. Finally, the relative kidney weight was calculated by dividing left kidney weight by body weight and then multiplying it by 100.

### 2.3. Lead concentration in renal tissue

Lead concentration was measured in the renal tissue of rats according to Jones and Hopkin [15]. Tissue samples of kidney were dried in oven at 60 °C and combusted at 450 °C for 24 h. Thereafter, the combusted samples were dissolved in hot solution of 1 M $HNO_3$. The samples were transferred into 50 ml volumetric flasks and adjusted with the deionized water to this volume. The appropriately diluted and digested tissue samples were analyzed at 283.3 nm using flame atomic absorption spectrophotometer (PerkinElmer, 3100).

### 2.4. Histology and apoptosis detection by BAX immunochemistry

Small pieces of the kidneys were quickly removed, then fixed in neutral buffered formalin. Following fixation, specimens were dehydrated, embedded in wax, and then sectioned to 5 μm thickness. For histological examinations, sections were stained with haematoxylin and eosin [16].

Immunolocalization technique for Bax was performed on 3–4 μm thickness sections according to Pedrycz and Czerny [17]. For negative controls, the primary antibody was omitted. In brief, mouse anti-Bax (diluted 1:25, Santa Cruz Biotechnology, Santa Cruz, CA, USA), was incubated with sections for 60 min. Primary antibodies were diluted in TBS (Tris buffered saline)/1%BSA (bovine serum albumin). Then a biotinylated secondary antibody directed against mice immunoglobulin (Biotinylated Link Universal – DakoCytomation kit, supplied ready to use) was added for 15 min incubation, followed by horse radish peroxidase conjugated with streptavidin (DakoCytomation kit, supplied ready to use) for15 min incubation. At the sites of immunolocalization of the primary antibodies, a reddish to brown color appeared after adding 3-amino-9-ethylcarbasole (AEC) (DakoCytomationkit, supplied ready to use) for 15 min. The specimens were counterstained with hematoxylinfor1 min and mounted using the Aquatex fluid (Merck KGaA, Germany). Histological damages were scored as follows: 0: absent; + mild; ++; moderate; and +++: severe. In addition, Apoptosis was scored as follows: 0: absent; + mild; ++; moderate; and +++: severe.

### 2.5. Biochemical estimations

#### 2.5.1. Uric acid, urea and creatinine levels

Uric acid, blood urea and creatinine were assayed in serum using kits provided from Biodiagnostic Co. (Giza, Egypt).

#### 2.5.2. Quantification of reactive oxygen species

A modified version of a previously described assay for the intracellular conversion of nitro blue tetrazolium (NBT) to formazan by superoxide anion was used to measure the generation of reactive oxygen species [18] with slight modification. Briefly, 200 μl NBT (1.0 mg/ml) was added to the kidney homogenate of different groups, followed by additional incubation for 1 h at 37 °C. Solutions were then treated with 100 μl KOH (2 M). The absorbance at 570 nm was determined using a spectrophotometric method and expressed as μmol NBT reduced/g tissue.

#### 2.5.3. Determination of nitric oxide and lipid peroxidation

Nitric oxide (NO) and malondialdehyde (MDA) were assayed colorimetrically in renal tissue homogenate according to the method of Berkels et al. [19] and Ohkawa et al. [20], respectively. NO was determined where in an acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide is coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color which can be measured at 540 nm. MDA was determined by using 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% and were then heated in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) formed.

#### 2.5.4. Estimation of reduced glutathione and the anti-oxidant enzymes

The renal reduced glutathione (GSH) was determined by the methods of Ellman [21]. The method based on the reduction of Ellman's reagent (5,5′ dithiobis (2-nitrobenzoic acid) "DTNB") with GSH to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm. The level of renal antioxidant as catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) levels were determined by the methods of Aebi [22], Nishikimi et al. [23], Habig et al. [24], Paglia and Valentine [25] and Factor et al. [26], respectively.

### 2.6. Statistical analysis

The obtained data were presented as means ± standard error. One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Duncan's test using a statistical package program (SPSS version 17.0). All $p$ values are two-tailed and $p < 0.05$ was considered as significant for all statistical analysis in this study.

## 3. Results

To estimate renal performance, the relative kidney weight was calculated. Lead acetate (PbAc) induced a significant increase ($p < 0.05$) in relative kidney weight of rats due to a significant decrease in body weight (Table 1). Flaxseed oil could not retain the weight loss of rats after 5 days of treatment (Table 1).

**Table 1**
Effect of flaxseed oil on the body weights, kidney weights and the relative kidneys weights in male rats treated with lead acetate.

| Groups | Body weights (gm) | Kidney weights (gm) | Relative kidney weights (%) |
|---|---|---|---|
| Group I | 100.0 ± 1.70 | 0.35 ± 0.12 | 0.35 ± 0.013 |
| Group II | 92.0 ± 2.21[a] | 0.42 ± 0.18[a] | 0.46 ± 0.027[a] |
| Group III | 109.8 ± 2.91[a] | 0.35 ± 0.15 | 0.32 ± 0.010 |
| Group IV | 94.6 ± 2.02 | 0.41 ± 0.15[a] | 0.43 ± 0.021[a] |

Values are means ± SE ($n = 6$).
[a] Significant change at $p < 0.05$ with respect to Group I.
[b] Significant change at $p < 0.05$ with respect to Group II.

The concentration of Pb in the renal tissue of rats is presented in Fig. 1. The level of Pb was significantly increased in the kidneys of rats received PbAc (11.53 ng/g wet tissue). Flaxseed oil was able to decrease the level of Pb to approximately 50% in the renal tissue of rats administered lead (Fig. 1).

Lead acetate caused a moderate inflammatory response of the kidney as indicated by inflammatory cellular infiltrations, cytoplasmic vacuolation and dilatation of some kidney tubules (Fig. 2). In addition, the kidney tubules apparently contained more apoptotic cells as compared to kidneys of the control rats (Fig. 3). Treatment of rats with flaxseed oil largely prevented the lead-induced histopathological changes in the renal tissue as indicated in Table 2 for the histological score of renal tissue damage.

The BAX antigen was detected in epithelial cells of the kidney tubules (Fig. 3). Staining intensity was more pronounced in renal tissues of rats inoculated with PbAc and the intensity of color was reduced by flaxseed oil treatment.

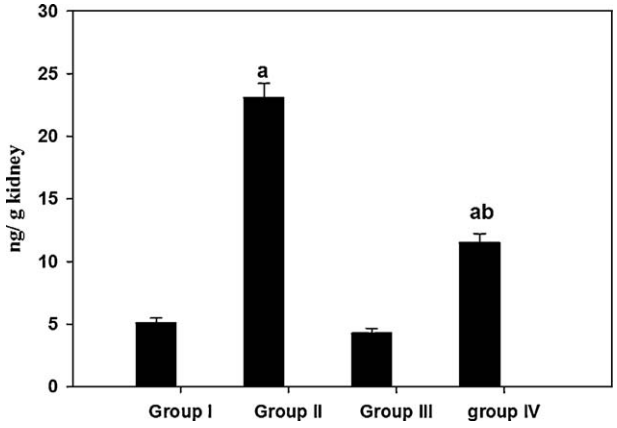


**Fig. 1.** Ameliorative effects of flaxseed oil on lead accumulation in renal tissue of rats treated with lead acetate for 5 days. (a) Significant change at $p < 0.05$ with respect to the Group I as negative control group. (b) Significant change at $p < 0.05$ with respect to the Group II as positive control group.

The levels of uric acid, urea and creatinine were significantly elevated in the blood serum of rats of Group II by approximately 35.7%, 55.6% and 32.0%, respectively (Table 3). On the other hand, rats administered with flaxseed oil only showed a non-significant change in uric acid and creatinine levels, while blood urea was significantly decreased by about 27.0% ($p < 0.05$). Rats administered with PbAc and flaxseed oil have shown a significant decrease in the levels of the parameters mentioned above.

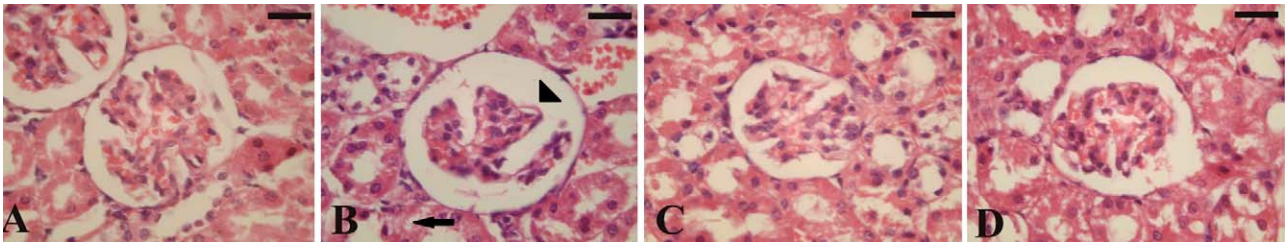


**Fig. 2.** Flaxseed oil improves PbAc renal tissue damage. (A) Control kidney sections appeared with normal architecture. (B) Rats treated with lead acetate. Sections appeared with tubular dilatation, vacuolated tubules (arrow), pockets of hemorrages and shrunken glomeruli (arrow head). (C) Rats treated with flaxseed oil. (D) Rats treated with the lead acetate and flaxseed oil. Sections were stained with hematoxylin and eosin. Bar = 25 μm.

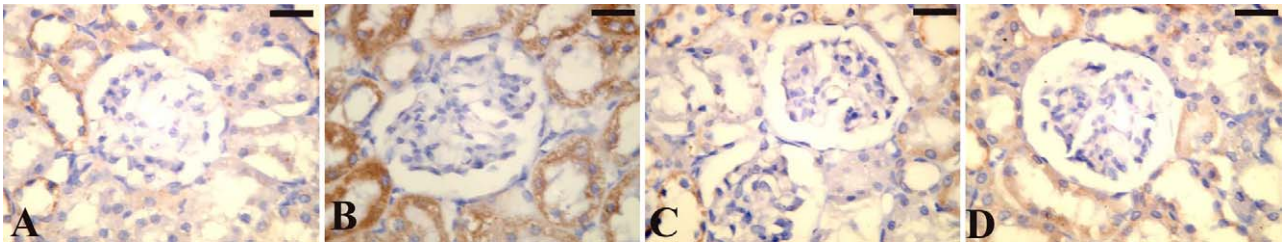


**Fig. 3.** Immunohistochemical localization of BAX antigen in the renal tissue of rats. (A) Control kidney sections. (B) Rats treated with lead acetate. Sections appeared with more staining of BAX. (C) Rats treated with flaxseed oil. (D) Rats treated with the lead acetate and flaxseed oil. Bar = 25 μm.

**Table 2**
Flaxseed oil improves the histopathological kidney damages induced by lead acetate.

| Microscopic observation | | | | | |
|---|---|---|---|---|---|
| Groups | Tubular vacuolization | Hydropic degeneration change | Glomerular damage | Inflammatory cellular infiltration | Apoptosis |
| Group I | + | 0 | 0 | 0 | + |
| Group II | +++ | +++ | +++ | +++ | +++ |
| Group III | + | 0 | 0 | + | + |
| Group IV | ++ | + | + | + | ++ |

0: absent; +: mild; ++: moderate; +++: severe.

**Table 3**
Effect of flaxseed oil on the level of uric acid, blood urea and creatinine in blood serum of albino rats treated with lead acetate.

| Groups | Uric Acid (mg/dl) | Blood urea (mg/dl) | Creatinine (mg/%) |
|---|---|---|---|
| Group I | 4.70 ± 0.05 | 3.92 ± 0.19 | 1.22 ± 0.02 |
| Group II | 6.38 ± 0.14[a] | 6.10 ± 0.40[a] | 1.61 ± 0.06[a] |
| Group III | 4.54 ± 0.09 | 2.86 ± 0.23[a] | 1.13 ± 0.02 |
| Group IV | 5.35 ± 0.29[a,b] | 4.48 ± 0.28[a,b] | 1.19 ± 0.03[b] |

Values are means ± SE ($n = 6$).
[a] Significant change at $p < 0.05$ with respect to Group I.
[b] Significant change at $p < 0.05$ with respect to Group II.

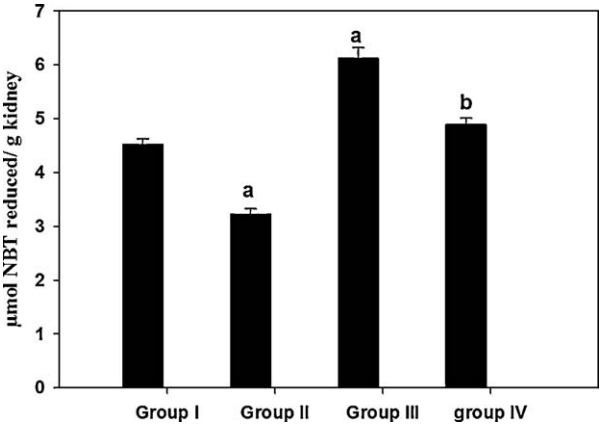


**Fig. 4.** Ameliorative effects of flaxseed oil on reactive oxygen species produced in kidney tissue of albino rats treated with lead acetate for 5 days. (a) Significant change at $p < 0.05$ with respect to the Group I as negative control group. (b) Significant change at $p < 0.05$ with respect to the Group II as positive control group.

Lead acetate significantly induced oxidative stress through the production of oxygen free radicals in the renal tissue of rats (−31.9%; $p < 0.05$) as indicated by NBT reduction assay (Fig. 4). Flaxseed oil reduced ROS significantly (35.4%) when administered in rats. Flaxseed oil treatment after PbAc injection caused a reduction in NBT where the ROS production in Group IV (8.2%) was returned to control value with a significant diminish in ROS production when compared with PbAc group (Group II).

Also, PbAc induced a highly significant increase in renal NO and MDA by approximately 60.7% and 56.0%, respectively (Fig. 5). In blood serum, increases in NO and MDA were less pronounced (Fig. 5). Again, flaxseed oil treatment significantly lowered the PbAc-induced increase in both NO and MDA, respectively.

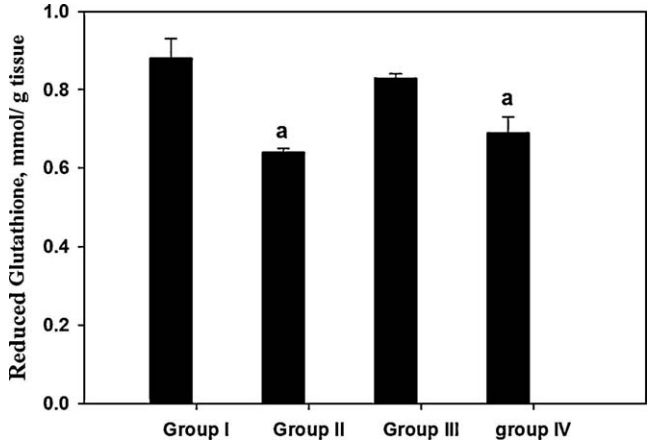


**Fig. 6.** Flaxseed oil alters the reduced glutathione level in renal tissue of rats treated with lead acetate. (a) Significant change at $p < 0.05$ with respect to the Group I as negative control group. (b) Significant change at $p < 0.05$ with respect to the Group II as positive control group.

Fig. 6 showed that flaxseed oil could significantly elevate the GSH in renal tissue of rats inoculated with PbAc for 5 days. In addition, activity of the antioxidant enzymes, CAT, SOD, GST, GPx and GR, measured in the renal homogenate of rats were decreased by approximately −44.4%, −30.4%, −42.9%, −35.7% and −36.4%, respectively on day 5 post-PbAc treatment (Table 4). These previous enzymes were significantly elevated upon treatment with flaxseed oil.

## 4. Discussion

Kidney is a target organ for lead toxicity. The toxic effects of Pb on the kidney appear to be primarily localized in the kidney tubule and are manifested as excessive urinary excretion of amino acids, glucose and phosphate, natriuresis, kaliuresis and intranuclear bodies inclusion [27]. These changes may be related to one or more factors, including increased serum levels of Pb or decreased Pb reabsorption by alteration in tubular transport mechanisms, as well as structural lesions in the nephron [28]. Also, Goyer [29] observed an increase in kidney wet weight in rats given a high dose of lead for protracted periods of time, while O'Flaherty et al. [30] observed increases in both kidney wet weight and dry weight in rats given varying doses of lead. These findings were in agreement with our findings concerning with the increased kidney weight of rats due to lead acetate (PbAc) administration. The current results clearly

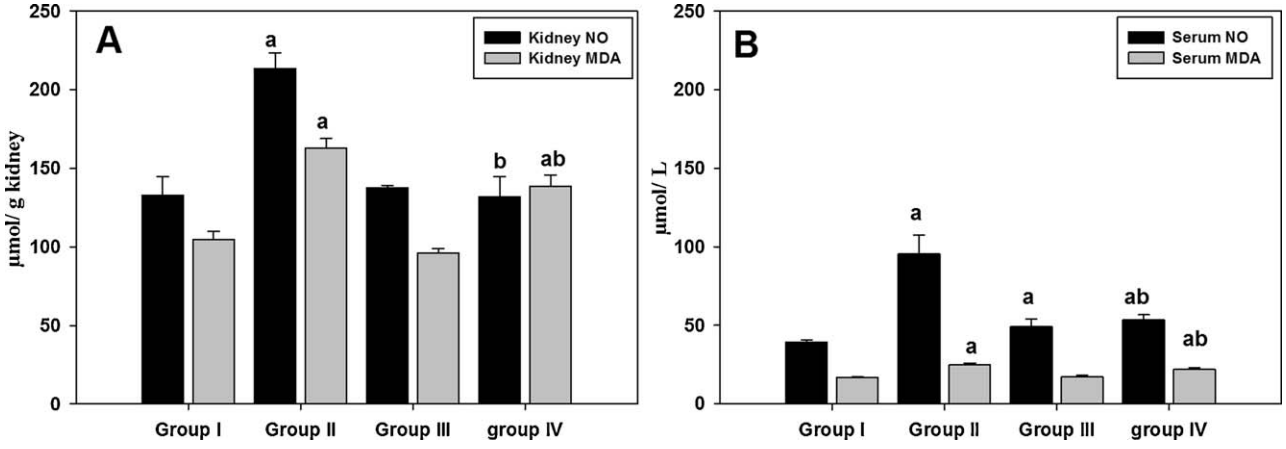


**Fig. 5.** Effects of flaxseed oil on NO and MDA formation in kidney tissue (A) and serum (B) of albino rats treated with lead acetate for 5 days. (a) Significant change at $p < 0.05$ with respect to the Group I as negative control group. (b) Significant change at $p < 0.05$ with respect to the Group II as positive control group.

**Table 4**
Changes in the levels of catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) in kidney of male albino rats treated with lead acetate and lead acetate + flaxseed oil.

| Groups | CAT (U/g) | SOD (U/g) | GST (μmol/h/g) | GPx (U/g) | GR (μmol/g) |
|---|---|---|---|---|---|
| Group I | 1.8 ± 0.1 | 5.6 ± 0.3 | 2.1 ± 0.04 | 1.4 ± 0.04 | 33.9 ± 0.8 |
| Group II | 1.0 ± 0.08[a] | 3.9 ± 0.2[a] | 1.2 ± 0.05[a] | 0.9 ± 0.02[a] | 21.5 ± 1.7[a] |
| Group III | 1.9 ± 0.1 | 5.9 ± 0.3 | 2.2 ± 0.05 | 1.5 ± 0.05 | 38.3 ± 1.3 |
| Group IV | 1.2 ± 0.08[a,b] | 4.21 ± 0.34[a] | 1.3 ± 0.07[a] | 1.0 ± 0.06[a] | 20.8 ± 1.08[a] |

Values are means ± SE ($n = 6$).
[a] Significant change at $p < 0.05$ with respect to Group I.
[b] Significant change at $p < 0.05$ with respect to Group II.

indicated that treatment with flaxseed oil did not induce harmful effects on the animals. Moreover, it succeeded to induce an improvement in body weight [31].

Lead may be rapidly absorbed and reached considerable amount in the blood [32]. Han et al. [33] suggested that this element is strongly bound to macromolecules in the intracellular compartment because Pb binding proteins have been isolated from the kidney, liver, blood and brain explained the high concentration of Pb in tissue of kidney in the present study. On the other hand, the efficiency of flaxseed oil to reduced accumulation of PbAc in kidney was perhaps due to the presence of this α-linolenic acid, fibers and lignans. These biologically active compounds might have chelated lead and enhanced its excretion from the body, resulting in reduced Pb accumulation in renal tissue [34].

Histological investigations revealed that PbAc exposure caused progressive glomerular and tubular alterations. These findings are in agreement with results of previous investigations by Lin et al. [35] who recorded alterations in renal histopathology after environmental exposure to Pb. Tubular vacuolization, necrosis and dilation found in the present studies due to PbAc intoxication were reported previously by Karmakar et al. [36]. These tubular alterations caused by PbAc toxicity might be a result of a hydrolic changes in the renal tissue and suggest that PbAc intoxication yields to a partial failure in the ions pump transport of tubules cells which in turn produces tubular swelling and causes necrosis and vacuolization of the tubules. Flaxseed oil could improve to some extent the altered kidney histopathology.

Bax is a pro-apoptotic member of the Bcl-2 family that play key roles in the regulation of intrinsic apoptotic signaling through mitochondrial stress [37]. Our results showed that Bax was highly expressed in the renal tissues of rats inoculated with PbAc (Fig. 3). Oral administration of lead cause significant increase in P53 and BAX expressions in mice model [38]. It was strongly suggest that lead may induce oxidative stress and changes the expression of apoptosis related proteins in rat kidney.

The induced elevation of the uric acid, urea and creatinine due to PbAc administration indicated that the kidney function was affected. Our results proved that, treatment with flaxseed oil significantly improved the kidney function.

Oxidative stress may be a result of excessive reactive oxygen species generation or failure of the cellular antioxidant system. Lead induced an elevation of oxidative stress indicators as NO of the kidney, where it was explained by Gonick et al. [39] that demonstrated that the increased inducible nitric oxide synthase (iNOS) in the kidney cortex of lead-treated rats. Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotics and also it alters physiological and biochemical characteristics of biological systems [40,41]. Valverde et al. [42] found induction of lipid peroxidation and increased reactive oxygen species (ROS) level in tissues of mice after intoxication with PbAc for 1 h. Their findings are in agreement with results obtained by Marchlewicz et al. [43], suggesting that induction of Pb cytotoxicity by indirect mechanisms. Our current investigations showed significantly increased concentration of MDA in serum and kidney homogenate of lead-exposed rats, as well as, ROS generated in kidney that measured be NBT.

Cervello et al. [44] suggested that GST enzyme catalyzes the reaction via the thiol (–SH) group of glutathione, thereby neutralizing and rendering the products more water-soluble. Taking into account mutual relations between GST and GSH in the redox system, the simultaneous decrease in both GST activity and GSH concentration may suggest that the decrease in renal GSH concentration might result, at least partly, from the decrease in GST activity [45]. The decrease in GST activity after the exposure to lead could be caused by lead-induced changes in the enzyme structure as well as by the lack or insufficient amount of GSH, being a substrate for this enzyme [46]. Glutathione reductase, the enzyme responsible for recycling of glutathione from the oxidized form (glutathione disulfide; GSSG) to the reduced form (reduced glutathione; GSH) is also deactivated by lead (Table 2).

Flaxseeds have received increasing attention for their potential role in preventing lipid disorders [31,41]. However, relatively few data are available regarding to the impact of flaxseed oil on blood and kidney. The phenolic lignans and other phytoestrogens have antioxidant activity [45]. The more striking finding in this study is that the presence of flax lignans with PbAc alleviated its harmful effects on the levels of GSH and on the activities of GPx, GR, SOD and GST enzymes. The corrected levels of these parameters were observed likely to near normal values of the control group. Flaxseed is the richest source of lignans, which have also been reported to have antioxidant and hypolipidemic effects [41,45].

In the current study, co-treatment of PbAc and flaxseed oil caused a significant improvement in histopathological picture of the kidney as well as the kidney function and antioxidant status. Therefore, this oil may play a protective role against lead-mediated kidney injury.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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