Role of flaxseed oil and silymarin in amelioration of lead-induced kidney injury

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Aim
This study was undertaken to test the effect of flaxseed oil and silymarin on the oxidative stress induced in the renal tissue of male albino rats on exposure to a sublethal dose of lead acetate.
Methods
Adult male albino rats were exposed to oral doses of lead acetate solution (1/20 LD50) for 6 weeks. Flax seed oil was added to second group and a third group received silymarin in addition.
Results
Lead-induced oxidative stress was indicated by elevated malondialdehyde level in kidney homogenates. This level dropped markedly in kidney tissue of rats receiving flaxseed oil, and better results were observed in the group receiving both flaxseed oil and silymarin. Histopathological examination of hematoxylin and eosin-stained kidney sections showed degenerative changes in the kidney tissue, in addition to disorganization of the collecting duct. Renal function was not significantly affected. Less degenerative changes were noted in the group receiving flaxseed oil, and they were nearly abolished in the group receiving both flaxseed oil and silymarin. The normal kidney histology was almost restored in such group. Endogenous antioxidants like reduced glutathione and glutathione peroxidase enzyme levels were reduced with lead acetate intake and raised significantly ($P<0.05$) with flaxseed oil intake. The addition of silymarin resulted in further increase in glutathione peroxidase level ($P<0.05$).
Conclusion
This study revealed the potential protective role of flaxseed oil as a compound rich in phenolic antioxidant components. Silymarin, a potent antioxidant known for its hepatoprotective effect, has a synergistic effect when added to flaxseed oil, ameliorating lead-induced renal damage.

Keywords:
antioxidant, flaxseed oil, lead toxicity, oxidative stress, renal damage, silymarin

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Introduction

Although lead is one of the most toxic heavy element, it has important physicochemical properties, like softness, malleability, ductility, poor conductibility, and resistance to corrosion. This makes its use difficult to give up, but its continuous use has led to its accumulation in the environment with increasing hazards [1]. Multiple evidence showed that lead induces toxicity in multiple target organs such as hematopoietic system, immune system, kidneys, and nervous system. After its absorption into the blood, 99% of lead is bound to erythrocytes and the remaining 1% is delivered to other tissues [2]. Exposure to even low lead levels can affect an individual's health, as there are no safe levels. Thus, it is necessary to eliminate lead from potential sources of exposure to prevent the effects of lead toxicity [3].

Acute renal damage induced by lead exposure is characterized by an impaired tubular transport and morphologically by the appearance of degenerative changes in the tubular epithelium along with the occurrence of nuclear inclusion bodies containing lead protein complexes. No proteinuria occurs, but there can be abnormal excretion of glucose, phosphates, and amino acids, giving rise to what is known as Fanconi syndrome.

The chronic nephropathy can lead to irreversible functional and morphological damage. Glomerular and tubulointerstitial changes take place, resulting in renal breakdown, hypertension, and hyperuricemia [4].

One of the major mechanisms of lead-induced toxicity is oxidative stress. There exist two different pathways occurring simultaneously for this mechanism: reactive

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oxygen species generation and depletion of the antioxidant reserves. Lead inactivates glutathione by binding to its sulfhydryl groups. Moreover, enzymes like glutathione peroxidase (GPx), glutathione reductase (GSH), and glutathione-S-transferase are inactivated by lead. This further depresses glutathione levels [5].

Silymarin, the active ingredient of milk thistle (*Silybum marianum*), is a polyphenolic compound known to be effective in treating liver and gall bladder diseases. It acts as an antioxidant by reducing free radical-mediated tissue damage. It does so through stabilization of membrane permeability by inhibiting lipid peroxidation and preventing glutathione depletion [6].

Flaxseed plays a great role in disease prevention. It is a functional food rich in dietary fibers, plant proteins, polyunsaturated fatty acids, and lignans which are biologically active components [7,8]. The oil component of flaxseed (FO) contains ~57% α-linolenic acid (ALA). The eicosapentaenoic acid and docosahexaenoic acid formed from ALA metabolism can reduce the risk of chronic diseases, such as atherosclerosis, cardiovascular disease, cancer, and hyperlipidemia [9].

Flaxseed oil and secoisolariciresinol diglucoside, which is the main lignan of flaxseed, improve enzymatic antioxidant defenses and GSH levels [10].

Secoisolariciresinol diglucoside forms ~1% of flaxseed dry weight [11]. Secoisolariciesinol, enterodiol, and enterolactone molecules are its products after its metabolism in the intestine and colon. Both molecules have already shown health beneficial effects [12,13].

Some of the previous studies attribute the beneficial effects of flaxseeds to FO and its high ALA concentration [14,15]; however, other studies show that the antioxidant effects of flaxseed are owing to its content of beneficial lignans, especially secoisolariciresinol diglucoside [12,16].

Materials and methods

Chemicals

Thiobarbutiric acid (TBA) was used for measuring malondialdehyde (MDA levels in tissues)

Ellman reagent: 5,5′-dithiobis, 2-nitrobenzoic acid was used for measuring GSH levels in animal tissues. Both chemicals were purchased from Sigma Chemicals (Aldrich Chemicals, St. Louis Missouri, USA).

GPx assay kit was obtained from Cayman Chemical (Ann Arbor, Michigan, USA).

Silymarin capsules were obtained from SEDICO Pharmaceutical Company (Cairo, Egypt).

Lead acetate was purchased from El-Gomhoreya Company (Cairo, Egypt).

Flaxseed oil was obtained from Emtenan Company (Cairo, Egypt).

Animals

Male adult albino rats weighing 90–120 g were housed for 6 weeks in the animal house of Bilharzia Research Center, Faculty of medicine, Ain Shams University. They were kept in groups of three rats per group in cages under controlled conditions of humidity and temperature. Rats were provided with water and balanced diet.

The experiments were performed according to the guidelines of institutional animal ethical committee.

Experimental groups

(1) Group I: three untreated rats serving as control.
(2) Group II: three rats received oral daily doses of lead acetate solution for 6 weeks (1/20 LD_{50} dissolved in 0.5 ml of normal saline [17]).
(3) Group III: three rats received flaxseed oil (0.5 ml) 1 h before the lead acetate dosage.
(4) Group IV: three rats received flaxseed oil 1 h before receiving the lead acetate. A silymarin dose (0.5 ml of 100 mg/kg/day) was added with the lead dose [18].

The rats were decapitated 24 h after the last dose; blood samples (for measuring blood urea and serum creatinine) were collected and centrifuged at 3000 rpm. Plasma was frozen at −20°C till analysis.

Kits obtained from Biodiagnostic Company (Giza, Egypt), were used for assessing kidney function.

Excised kidney tissues were used for two purposes: some pieces were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin for histopathological studies.

Other pieces were homogenized in PBS, for measuring tissue levels of GSH, MDA, and glutathione peroxidase.

Determination of lipid peroxidation

The level of lipid peroxides was determined in kidney homogenate by measuring MDA levels, in the form of TBA reactive substances [19].

Tissue homogenate samples were heated with TBA reagent for 20 min in a boiling water bath. TBA reagent included 20% TCA, 0.5% TBA, and 2.5 N HCl. After cooling, the solution was centrifuged at 2000 rpm for 10 min, and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm against a blank solution. The MDA equivalents of the sample were calculated using an extinction coefficient of 1.56×10^5 mol/l cm.

Measuring glutathione tissue levels

GSH was determined spectrophotometrically by the method previously described by Ellman [20]. Homogenized tissue samples (0.5 ml) were mixed with 2 ml of 5% TCA.

The mixed liquid was precipitated for 5 min. Supernatant (1 ml) was extracted.

To this, 0.5 ml of Ellman's reagent and 3 ml of PBS (1 mol/l, pH 8.0) were added. The absorbance of 5-thio-2-nitrobenzoic acid, product formed when sulphydryl groups reacted with 5,5′-dithiobis, 2-nitrobenzoic acid, was measured at 412 nm against an appropriate blank without sample. GSH content was obtained by a standard curve.

Assay of glutathione peroxidase

Cayman assay kit (cat. no.703102; Ann Arbor, USA) was used to measure the concentration of GPx in kidney homogenate spectrophotometrically.

Tissue samples are first homogenized in a 1-ml homogenization buffer (50 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA and 1 mmol/l DTT) per 100-mg tissue. Tissue homogenates are centrifuged at 10 000*g* 15 min at 4°C. Supernatant is collected to perform the assay. Samples are incubated with assay buffer, co-substrate mixture, NADPH, and hydrogen peroxide according to manufacturer's protocol. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A_{340} is directly proportional to the GPx activity [21].

Statistical analysis

The results are expressed as mean SD for three animals in each group. Differences between groups were assessed by one-way analysis of variance using SPSS, Version 19, Chicago, IL, USA. Intergroup comparisons were done by using the least significant difference test, with the differences considered significant at *P* value less than 0.05.

Results

Malondialdehyde level

The level of MDA in kidney tissue of rats exposed to daily intake of lead acetate solution (group I) was increased significantly by almost four-folds as compared with the control group. This level, however, decreased to 2.2-folds on daily intake of flaxseed oil (group II) and almost back to normal when silymarin was added (group IV). There was a significant difference ($P<0.05$) between groups III and IV (Table 1).

Serum urea and creatinine

Table 2 shows that the kidney function was not affected after daily intake of lead acetate solution (1/20 LD_{50}) for continuous 6 weeks. Flaxseed oil added in group III had no effect on urea or creatinine levels. Moreover, added silymarin dosage in group IV had no effect on neither parameters, as denoted by *P* value more than 0.05.

Glutathione tissue level

GSH level in kidney tissue of lead acetate treated rats (group II) was markedly lowered by 75% compared with the control group. There was a marked improvement in sulfhydryl content in group III,

Table 1 Malondialdehyde levels in kidney tissues of different animal groups

Parameter	Groups			
	Group I (*n*=3)	Group II (lead) (*n*=3)	Group III (lead+FSO) (*n*=3)	Group IV (lead+FSO+silymarin) (*n*=3)
Malondialdehyde (nmol/mg protein)	1.04±0.12	3.89±0.23	2.31±0.46	1.13±0.14
		↑3.7-folds	↑2.2-folds	

Data are expressed as mean±SD of three rats per group. The *P* value between the results of the four groups was calculated as 0.000017. The result is significant at $P<0.05$. Comparing groups II and III, the *P* value is 0.013325. The result is significant at $P<0.05$. Comparing groups II and IV, the *P* value is 0.000114. The result is significant at $P<0.05$. Comparing groups III and IV, the *P* value is 0.025036. The result is significant at $P<0.05$.

Table 2 Serum urea and creatinine levels in different groups

Parameters	Groups			
	Group I (control) (*n*=3)	Group II (lead) (*n*=3)	Group III (lead+FSO) (*n*=3)	Group IV (lead+FSO+silymarin) (*n*=3)
Blood urea (mg/dl)	25.7±3.3	20±1.3	21±2.5	17±3.5
Serum creatinine (mg/dl)	0.53±0.047	0.56±0.047	0.57±0.094	0.5±0.08

Comparing blood urea levels among groups, the *P* value is 0.076757. The result is not significant at $P<0.05$. Comparing serum creatinine among groups, the *P* value is 0.727822. The result is not significant at $P<0.05$.

Table 3 Glutathione reductase levels in kidney tissues of different animal groups

Parameter	Groups			
	Group I (*n*=3)	Group II (lead) (*n*=3)	Group III (lead+FSO) (*n*=3)	Group IV (lead+FSO+silymarin) (*n*=3)
Glutathione reductase (μg/mg protein)	41.3±2.5	10.2±1.3	17.8±4.4	21.5±3.6
		↓75.3%	↓57%	↓48%

Data are expressed as mean±SD of three rats per group. The *P* value between the results of the four groups was calculated as <0.000013. The result is significant at $P<0.05$. Comparing groups II and III, the *P* value is 0.044627. The result is significant at $P<0.05$. Comparing groups II and IV, the *P* value is 0.006915. The result is significant at $P<0.05$. Comparing groups III and IV, the *P* value is 0.327215. The result is not significant at $P<0.05$.

Table 4 Glutathione peroxidase levels in kidney tissues of different animal groups

Parameter	Groups			
	Group I (*n*=3)	Group II (lead) (*n*=3)	Group III (lead+FSO) (*n*=3)	Group IV (lead+FSO+silymarin) (*n*=3)
Glutathione peroxidase (μ/g tissue)	296.6±12.4	97.7±4.02	228.6±14	294.6±5
		↓67%	↓23%	

Data are expressed as mean±SD of three rats per group. The *P* value between the results of the four groups was calculated as <0.00001. The result is significant at $P<0.05$. Comparing groups II and III, the *P* value is 0.0001. The result is significant at $P<0.05$. Comparing groups III and IV, the *P* value is 0.001547. The result is significant at $P<0.05$.

where its level dropped by 57% compared with the control group. With the addition of silymarin in group IV, sulfhydryl content dropped by 48% compared with the control group.

P value was significant (<0.05) when comparing GSH levels among the four groups, whereas there was no significant difference between groups III and IV (Table 3).

Glutathione peroxidase activity

The antioxidant enzyme activity was shown to drop by 67% in lead-treated group (group II) compared with the control group. With the addition of flaxseed oil in group III, the enzyme level dropped by 23% compared with the control group. Further significant rise in enzyme level (almost back to normal) was noted with addition of silymarin in group IV (Table 4).

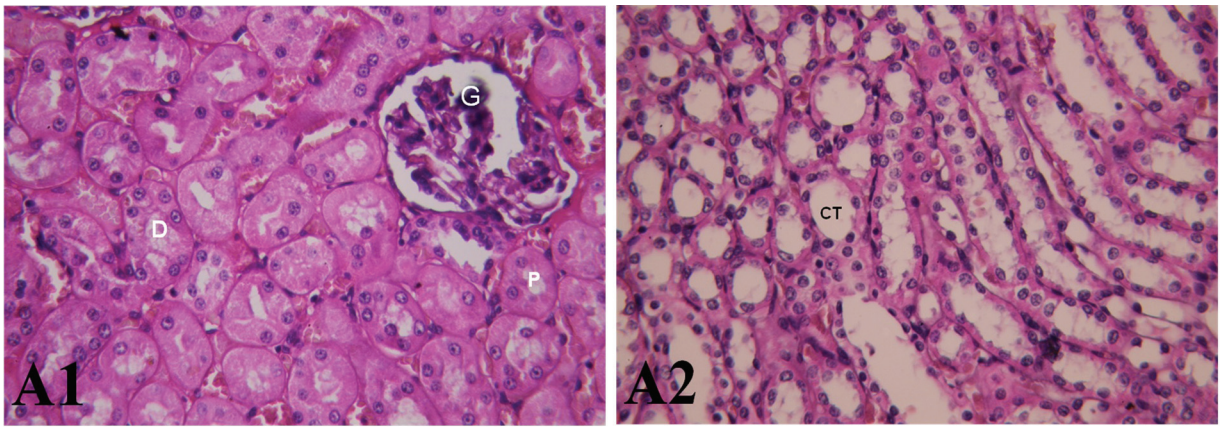
Histopathological studies

(1) Group I: in this study, kidney specimens obtained from the control rats served to study the normal microscopic histological structure of the kidney. Fig. 1 shows the normal histological picture of the glomerulus, the proximal tubule, and the distal tubule. Moreover, the medullary part was examined to show the normal histological picture of the collecting tubules.

(2) Group II: microscopic examination of the kidney specimens of rats of this group in Fig. 2 revealed the toxic effects of lead acetate on renal tissue in which the renal corpuscle shows dilatation of peri-glomerular space, congested capillaries, pyknotic nuclei, and wide spaces; in addition, the proximal tubule shows dilatation, vacuolation, and disorganization. Furthermore, the distal tubule shows degeneration, pyknotic nuclei, and congestion. Moreover, the collecting duct shows dilatation, vacuolation, and disorganization.

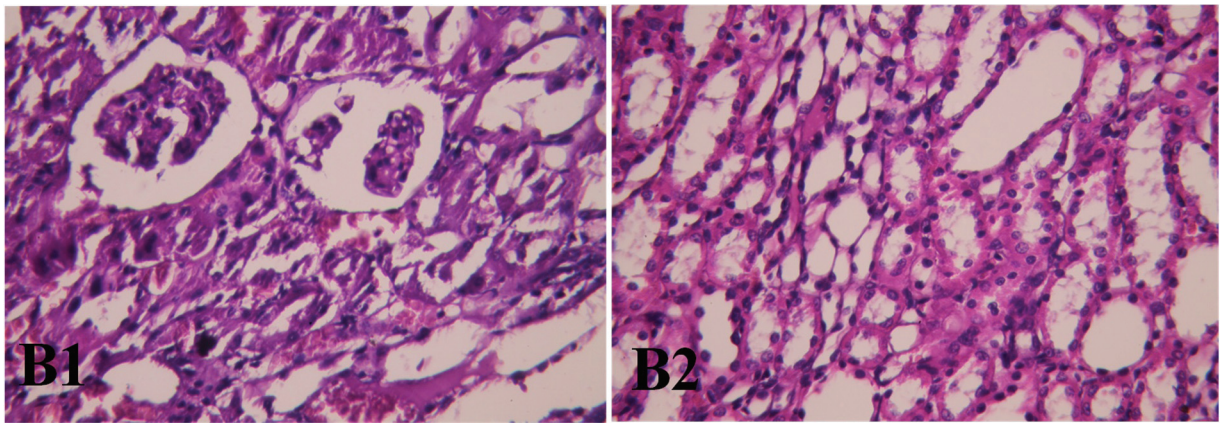
(3) Group III: microscopic examination of the kidney specimens of rats of this group in Fig. 3 shows less lead damaging effects. In that respect, the renal corpuscle shows less dilatation of peri-glomerular space, and some of the proximal tubules show dilatation, vacuolation, and disorganization.

Figure 1



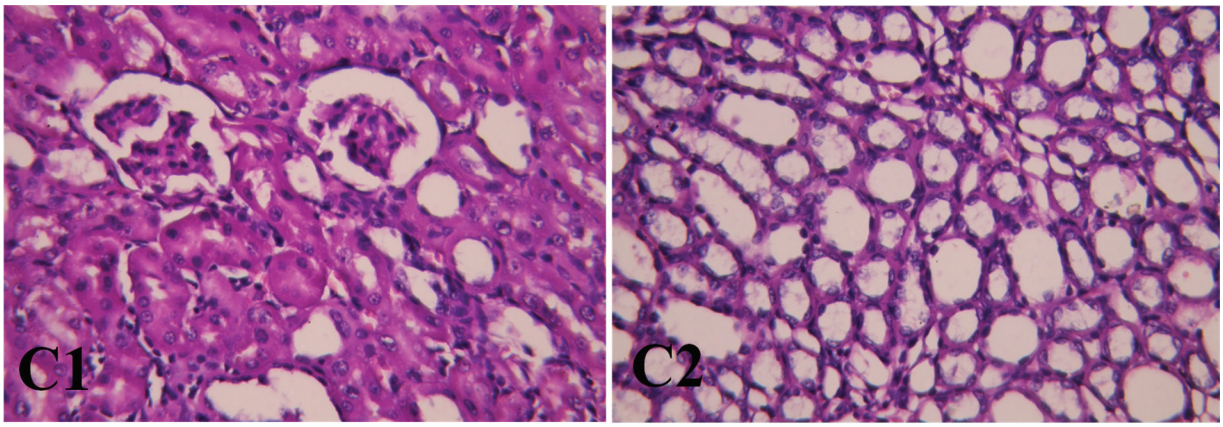
Photomicrographs of sections of the kidney of control rat stained with hematoxylin and eosin. (a1) The normal histological picture of the cortical part contains the glomerulus (G), the proximal tubule (P), and the distal tubule (D). (a2) The medullary part shows the collecting tubules (CT) (×100).

Figure 2



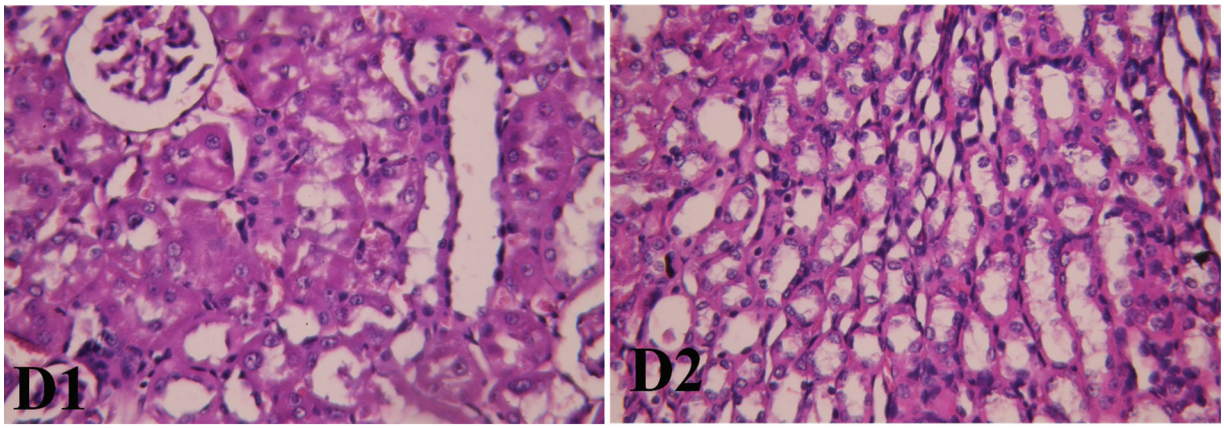
Photomicrographs of sections of the kidney of lead-treated group (group II) stained with hematoxylin and eosin. (b1) Renal corpuscle shows dilatation of peri-glomerular space, congested capillary, pyknotic nuclei, and wide spaces. Proximal tubule shows dilatation, vacuolation, and disorganization. Distal tubule shows degeneration, pyknotic nuclei, and congestion. (b2) Collecting duct shows dilatation, vacuolation, and disorganization (×100).

Figure 3



Photomicrographs of sections of the kidney of group III stained with hematoxylin and eosin stain. (c1) Renal corpuscle shows less dilatation of peri-glomerular space; some proximal tubules show dilatation, vacuolation, and disorganization. Some distal tubules show degeneration, pyknotic nuclei, and congestion. (c2) Few collecting ducts show dilatation, vacuolation, and disorganization (×100).

Figure 4



Photomicrographs of sections of the kidney of group (group IV) stained with hematoxylin and eosin. (d1) A picture similar to that of control group with few proximal tubules showing dilatation, vacuolation, and disorganization. Few distal tubules show dilatation, vacuolation, degeneration, and pyknotic nuclei. (d2) Few collecting ducts show dilatation and vacuolation (×100).

Moreover, some distal tubules show degeneration, pyknotic nuclei, and congestion. In addition, few collecting ducts show dilatation, vacuolation, and disorganization.

(4) Group IV: microscopic examination of the kidney specimens of rats of this group in Fig. 4 shows more or less histological picture similar to that of the control group. However, few of the proximal tubules show dilatation and vacuolation. Moreover, few distal tubules show degeneration and congestion. In addition, few collecting ducts show dilatation, vacuolation, and disorganization.

Discussion

Lead is considered a toxic pollutant to the environment, which is very difficult to be degraded or eliminated from the body [22]. It targets different organs of the body causing alterations, with the kidney regarded as one of the main target organs. The main renal toxic effects of lead reside in the kidney tubules and are manifested by excessive urinary excretion of glucose, amino acids, in addition to intranuclear inclusion bodies [23]. There are no specific findings in chronic lead-induced nephropathy but acute gouty arthritis may be present. With progression of the disease, uremia can take place [24].

Oxidative stress, which results from increased production of reactive oxygen species such as superoxide and hydroxyl radicals and depletion of the cell's major antioxidants, deteriorates biological macromolecules [25]. As a response to a certain level of oxidative stress, autophagy can be activated as a survival strategy. Lead poisoning can activate this pathway [22].

Antioxidants are either natural or synthetic compounds. They can delay some types of cell damage and chelate reactive radical species formed during some metabolic pathways that comprise oxidative reactions. Antioxidants include phenols, polyphenols, carotenoids, anthocyanins, and tocopherols, which are the main group of phytochemicals found in plants [26]. The basic structure of phenol contains a hydroxyl group (–OH) linked to the aromatic ring. The biological activates of phenolic compounds differ depending on the position and number of phenolic groups and their locations [27].

Safer and natural antioxidants are generally required for food, biological, and pharmaceutical systems. Plant constituents with antioxidant activity and free radical scavenging effects are regarded as the safe source for these antioxidants, as it was reported that synthetic antioxidants have shown adverse effects including mutagenic, carcinogenic, and toxic effects [28,29]. Phenolic compounds, which can be obtained from flaxseed (*Linum usitatissimum* L.) oil, are the most widely occurring chemicals, having strong antioxidant properties [30]. Phenolic compounds exhibit their antioxidant activity owing to their redox property. p-hydroxybenzoic acid, ellagic acid, p-coumaric acid, ferulic acid, and ascorbic acid are the main phenolic acids in flaxseeds [31].

Moreover, this plant has been cultivated for fiber as well as a functional food because of its beneficial chemical composition, such as tocopherols oils, fats

enriched in ω3 fatty acids, crude fiber, protein, and antioxidants in its seed. These components are probably also responsible for its antioxidant activity [32].

In this study, we investigated the effect of flaxseed oil and the concomitant addition of silymarin, on lead-induced renal injury in male albino rats.

Measuring tissue level of MDA, a product of lipid peroxidation was used as an indicator for oxidative stress. Measurement of GSH and GPx tissue levels was used as a marker of the antioxidant status of renal tissues.

Kidney function was not affected after daily intake of lead acetate solution (1/20 LD_{50}) for continuous 6 weeks. Flaxseed oil added in group III had no effect on urea or creatinine levels. Moreover, added silymarin dosage in group IV had no effect on both parameters.

In the lead-exposed group (group II), in the cortex, there was dilatation of peri-glomerular space, congested capillary, pyknotic nuclei, dilatation of proximal tubules, vacuolization, and disorganization. Distal tubules showed degeneration, pyknotic nuclei, and congestion.

The medulla also showed vacuolization and degenerative changes.

These findings are in agreement with another study [33,34], which reported tubular vacuolization, necrosis, and dilatation owing to lead toxicity. In a previous study [35], the kidney tissue of rats exposed to lead acetate showed degeneration of distal and collecting convoluted tubules, vacuolization, shrinkage, and breakage of tissue. Moreover, degeneration of tubular epithelium and swollen nuclei were seen.

When flaxseed oil was added to lead-treated rats (group III), marked improvement of kidney tissue was noticed on histopathological examination. With concomitant addition of silymarin with flaxseed oil to lead-treated rats (group IV), kidney histology appeared to be almost normal.

This result is in agreement with a previous study [36], which reported that flaxseed oil lowered MDA levels about 1.5-folds compared with a group that received high glucose dosage, and increased GSH tissue content about two-folds higher than the glucose only group.

A previous study [33] reported a significant drop in MDA levels of kidney homogenate of rats administered flaxseed oil with lead acetate as compared with lead administered group (elevated by 56%).

Antioxidant enzyme GPx activity decreased by 27% in the lead group, and elevated significantly with addition of flaxseed oil.

GSH is an endogenous antioxidant. It is considered the first line of defense against free radicals [37]. A reduction in GSH levels during lead toxicity has been reported in many studies [38,39]. An increase in blood lead levels is inversely proportional with decline in GSH and GPx concentration as reported in a previous study [40].

GSH levels in kidney homogenates of male albino rats dropped with isolated lead acetate intake (group II) by 75% compared with the control group, rising with addition of flaxseed oil (group III) to show a drop of 57% compared with control group and 48% with addition of silymarin, which significantly improved the tissue sulfhydryl content, hence the antioxidant status.

In a previous study [33], there was a recovery of GSH levels with flaxseed oil administration, after its depletion in kidney homogenate as a result of lead exposure.

In another study [6], hepatic GSH increased significantly when silymarin was added to rats exposed to tamoxifen compared with its depletion (↓52% from control) in tamoxifen-treated rats. The protective action of silymarin is associated with its antioxidant properties, being a scavenger for free radicals, inhibitor of lipid peroxidation, and a plasma membrane stabilizer [41]. It also acts as a preservative of liver GSH content [42].

GPx, selenium-containing metalloenzymes, terminates the chain reaction of lipid peroxidation by removing lipid hydroperoxide from the cell membrane. GPx is most important in removing H_2O_2 because it is in the same sub-cellular compartment (cytosol and mitochondria). It is the only human enzyme requiring the element selenium for its activity, and a selenocysteine residue (-SeH) is present at its active site. Among the antioxidant enzymes, GPx is the most sensitive enzyme [40].

Measurement of GPx enzyme level in kidney homogenate of lead-treated rats (group II) revealed a drop in its concentration by 67% from control value.

Flaxseed oil added in group III significantly decreased the drop in antioxidant enzyme activity (23% lower than control), whereas in the FSO+silymarin-added group (group IV), the enzyme level returned back to normal control value.

In a previously mentioned study [33], there was a recovery of GPx levels with flaxseed oil administration, after its drop by 27% in kidney homogenate as a result of lead exposure.

Decline in GPx level in response to lead concentration is possibly owing to free radical damage by lead and resulting reactive oxygen species. Therefore, prooxidant/antioxidants balance is disrupted [40]. It has been reported that the activity of GPx significantly increased in low blood lead group when compared with control group and decreased when compared with high blood lead level group [43].

Conclusion

In this work, flaxseed oil had an impressive role as an antioxidant, reversing the lead-induced kidney damage. The concomitant addition of silymarin had an exponential protective role, with kidney tissue histology returning back to normal, and level of antioxidants markedly increased, alleviating the oxidative stress.

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Conflicts of interest

There are no conflicts of interest.

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