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Ameliorative effects of hydroalcoholic extract of *Lavandula officinalis* L. on cyclophosphamide-induced nephrotoxicity in mice

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ABSTRACT

Background: Cyclophosphamide (CP) is amongst the most effective alkylating anticancer drugs which regardless of its harmful side effects including nephrotoxicity, hematotoxicity, mutagenicity and also immunotoxicity, commonly raised for the treatment of a number of cancers and autoimmune ailments.

Objectives: This study was undertaken to investigate the ameliorative effect of *Lavandula officinalis* L extract (LOE) in nephrotoxicity induced by CP in mice.

Materials and Methods: In this experimental study, 35 male Swiss albino mice (20–25 g) were randomly divided into 5 groups, each group consist of 7 mice. Mice were pretreated with LOE orally in doses of 100, 200 and 400 mg/kg for 5 consecutive days and CP (200 mg/kg, ip) was administrated on the fifth day 1 hour after the last dose of extract. Then on the sixth day, animals were sacrificed. Blood samples were collected to determine serum creatinine (Cr) and blood urea nitrogen (BUN) levels. Malondialdehyde (MDA), glutathione (GSH) levels and catalase (CAT) and superoxide dismutase (SOD) activity were assayed in kidney tissue. The kidney was maintained in formalin for histological examination.

Results: Results showed a significant increase in the levels of MDA, Cr and BUN, and decrease of GSH, CAT and SOD by CP administration. Pre-treatment with LOE showed reduction in the levels of MDA, Cr and BUN and increase of GSH, CAT and SOD in all doses but the most significant alteration was observed at doses of 200 and 400 mg/kg ($P<0.05$). Additionally the nephroprotective effect of the LOE was established by the histological examination of the kidneys.

Conclusions: Our results indicated that LOE has produced amelioration in biochemical indices and oxidative stress parameters against CP-induced nephrotoxicity.

Original Article

Implication for health policy/practice/research/medical education:
In an experimental study, we found that *Lavandula officinalis* L. extract as an antioxidant agent protects kidney against cyclophosphamide induced nephrotoxicity. We believe that this protective effect is probably mediated by antioxidant and GSH preservation effects of *L. officinalis* L. extract.

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1. Background

Kidney performs a vital function in the excretion of metabolic waste products and in the adjustment of extracellular fluids amount, electrolyte composition, and acid–base balance (1). Toxic effects on the kidney related to medications are both common and expected, and could disrupt any or all of these functions (2). A wide variety of drugs, environmental chemicals, and some heavy metals, constitute serious reasons for acute and chronic renal damage (3). Nephrotoxicity due to cyclophosphamide (CP) has been well documented in both humans and animals (4).

CP leads side effects of the renal tissues because of its toxic metabolites. The two active metabolite of CP is phosphoramide mustard (PAM) and acrolein (ACR). CP antineoplastic effects are related to PAM, although ACR is responsible for its toxic unwanted side effects (5).

The ACR leads the cellular injury after binding with the glutathione (GSH) and also decreased their amount in the cell. ACR damaged the GSH dependent antioxidant system as well as enhanced free radical generation (6). ACR interferes with the tissue antioxidant defense system and resulted in necrosis of tubular epithelial cells. Antioxidant agents detoxify the toxic effect of ACR (7-9).

Therefore, it is important to understand the role played by antioxidants agents during drug-mediated toxicity to determine if they can show protective effect against oxidative stress induced by reactive intermediates (10,11). In recent years there is growing evidence that reactive oxygen species (ROS) contribute to organ injury in many systems (12). All natural antioxidants from vegetables and fruit are documented to provide considerable protection that decreases the process of oxidative damage due to ROS (13). Furthermore, preconditioning with bioactive compounds have been found to protect against damage induced by oxidative stress in kidneys (14-18) and other tissues (19). They mostly act through reducing oxidative stress and elevation of tissue antioxidant capacity.

Lavandula officinalis (Lamiaceae), commonly known in Iran as "Ostokhoddous"(20), is a medicinal plant largely used and included for centuries in the pharmacopoeia of several countries, such as Iran (21). The chemical composition and pharmacological evaluation of *L. officinalis* have been the subject of several studies over the years (22).

Lavandula officinalis contains over 100 ingredients, among which the major substances are linalool and linalyl acetate, α-pinene, limonene, 1,8-cineole, *cis*-andtrans-ocimene, 3-octanone, camphor, caryophyllene, terpinen-4-ol and flavonoids (23). In pharmacological and biological tests, extracts, fractions and also essential oils of *L. officinialis* are documented to include local anesthetic, antioxidant, antispasmoic and soporific, sedative, antitension, CNS-depressant, antibacterial, anticonvulsive as well as mast cell degranulation inhibitory effects (24, 25). Medicinal properties of *L. officinalis* are due to its valuable compounds and their antioxidant properties. Therefore this plant is used as a remedial source (26). In this study, we have made an attempt to investigate the beneficial effects of *L. officinalis* L extract (LOE) against CP-induced nephrotoxicity.

2. Objectives

In this study, we have made an attempt to investigate the beneficial effects of LOE against CP-induced nephrotoxicity.

3. Materials and Methods

3.1. Chemicals

5, 5- dithiobis (2-nitrobenzoic acid) (DTNB), trichloro acetic acid (TCA), reduced GSH, bovine serum albumin (BSA), thiobarbituric acid (TBA) and Coomassie blue G were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA). All of the chemical substances and reagents utilized were analytical grade. CP was purchased from Roche Chemical Company (Germany).

3.2. Extract preparation

Plant was collected from Shiraz, Iran. Samples of the plant were identified by botanist from the Division of Pharmacognosy, Ahvaz Jundishapur University of Medical Sciences. Material was shade-dried, powdered and also drenched in a 70% aqueous-ethanol solution in a big container for three days with occasional properly shaking. The solvent was filtered through a whatman paper and then was removed under vacuum in a rotary evaporator until dryness (27). The percentage yield was 16% for dried hydroalcoholic extract (w/w).

3.3. Animals

Male Swiss albino mice (6–8 weeks old, 20–25 g) were obtained from animal house of Ahvaz Jundishapur University of Medical Science, Iran. Mice were kept in polypropylene cages and given standard rat chow and drinking water ad libitum. The rats were preserved at a regulated condition of temperature (20±2°C) with a 12-hour light: 12-hour dark cycle. Animal were acclimated to the habitat for at least seven days before inclusion in experiment. The investigation was performed according to the Animal Ethics Committee

Guidelines for the use of experimental animals (IR.AJUMS.REC.1395.628).

3.4. Experimental design

Animals were divided in to 5 groups, each group consist of 7 mice. Group 1 as negative control group, received normal saline for 5 days; group 2 received CP (200 mg/kg, ip) (28) as positive control only on the fifth day; groups 3-5 received LOE orally in doses of 100, 200 and 400 mg/kg respectively, during 5 days and CP (200 mg/kg, ip) on the fifth day 1 hour after the last dose of extract administration.

3.5. Sample collection

On the day sixth, 24 hour after the last administration, animals were anaesthetized with diethyl ether and blood samples were collected from the jugular vein. Serum was separated by centrifugation for 10 minutes at 3000 rpm and stored at -20ºC until analysis. Then animals were sacrificed by decapitation and kidneys were isolated, washed with saline quickly. For histological experiments, the right kidney was fixed in 10% phosphate buffered formalin. For evaluation of tissue markers, left kidney was weighed and then homogenized (1/10 w/v) in ice-cold Tris-HCl buffer (0.1M, pH 7.4). Protein content in homogenates was measured by the method of Bradford (29), utilizing crystalline BSA as standard.

3.6. Serum analysis

Creatinine (Cr) and blood urea nitrogen (BUN) levels were assessed utilizing the RA-XT (automated) biochemical analyzer with specific test kits (Technicon, Bayer S .A. Diagnostic).

3.7. Glutathione levels assay

The amounts of GSH in the tissue homogenate was measured following the method described by Ellman (30) according to the creation of a yellow colored complex with Ellman's Reagent (DTNB). Homogenates were instantly precipitated with 0 .1 mL of 25% TCA and the precipitate was eliminated after centrifugation. Free endogenous-SH was assayed in a 3 mL volume glass by addition of 2 mL of 0.5mM DTNB prepared in 0.2M phosphate buffer (pH = 8) to 0.1 mL of the supernatant and the yellow color created was read at 412 nm utilizing a spectrophotometer(UV-1650 PC, Shimadzu, Japan). GSH content was showed as nmol/mg protein.

3.8. Malondialdehyde levels assay

The lipid peroxidation was expressed by measuring the amounts of malondialdehyde (MDA) via the TBA color reaction by the modified method of Buege and Aust (31). Briefly, 0.5 mL of kidney homogenate was mixed with 2.5 mL of TCA (10%, w/v), the samples were centrifuged at 1500 g for 10 minutes and 2 mL of each sample supernatant was transferred to a test tube containing 1 mL of TBA solution (0.67%, w/v). The mixture was kept in boiling water for 10 minutes, forming a pink color solution. The mixture was then cooled immediately and the absorbance was measured at 532 nm by spectrophotometer (UV-1650 PC, Shimadzu, Japan). The concentration of MDA was calculated based on the absorbance coefficient of the TBA–MDA complex ($\varepsilon = 1.56\times10^5\ cm^{-1}\ M^{-1}$) and it was expressed as nmol/mg protein.

3.9. Catalase activity assay

Catalase (CAT) activity in the tissue was assayed by following the procedure of Aebi (32). In a cuvette containing 200 µL phosphate buffer and 50 µl of tissue supernatant (obtained after centrifugation of tissue homogenate at12000 g for 20 minutes at 4°C), was added 250 µL of 0.066M H_2O_2 and decrease in OD was measured at 240 nm for 60 seconds. One unit of activity equals to the moles of H_2O_2 degraded (per min), divided by the number of milligrams of protein in the tissue supernatant. The molar extinction coefficient of 43.6 $M^{-1}\ cm^{-1}$ was used to determine CAT activity.

3.10. Superoxide Dismutase activity assay

Tissue supernatant obtained after centrifugation at 12000 g for 20 minutes at 4°C was measured spectrophotometrically by calculating the rate of inhibition of auto-oxidation of hematoxylin for the assay of superoxide dismutase (SOD) according to the method described by Martin (33) and expressed as units/mg protein.

3.11. Histopathological assessments

For the histological examination, the kidneys were fixed in 10% formalin for at least 24 hours. then kidneys tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 µm sections and stained with hematoxylin & eosin dye (H&E stain). These sections were then examined under a photomicroscope for the presence of tubular degeneration, edema, necrosis, mononuclear/ polymorphonuclear cell infiltration, hemorrhage and narrowing of Bowman's capsule space (34).

3.12. Ethical issues

The research followed the tenets of the Declaration of Helsinki. The research was approved by ethical

committee of Jundishapur University of Medical Sciences. Prior to the experiment, the investigation was performed according to the Animal Ethics Committee Guidelines for the use of experimental animals

3.13. Statistical analysis

Results were expressed as mean±SD and all statistical comparisons were made by means of one-way analysis of variance (ANOVA) test followed by Tukey post hoc analysis and *P* value less than 0.05 was considered significant.

4. Results

4.1. Effects of LOE and CP on BUN and Cr levels

Result showed that 24 hours after CP administration mice developed sever nephrotoxicity that reflected by a significant increase ($P<005$) in the levels of BUN and Cr (Figure 1). Pre-treated groups with LOE showed decrease in the level of BUN and Cr in all doses but it was significantly decrease in doses of 200 and 400 mg/kg ($P<005$).

4.2. Effects of LOE and CP on MDA and GSH levels

Figure 2 shows the effect of CP and LOE pretreatment on the level of MDA and GSH in kidney tissue. The results clearly revealed that CP intoxication markedly increase the amount of MDA in rats ($P<0.001$).

As shown in Figure 2, the decrease in MDA level was significantly observed in pretreated rats by LOE (400 mg/kg). GSH level significantly decreased in the kidneys of rats exposed to CP and pretreatment with LOE (200 and 400 mg/kg) significantly inhibited the CP-induced reduction of GSH content.

4.3. Effects of LOE and CP on CAT and SOD activity

As shown in Figure 3, CP significantly decreased SOD and CAT activity in compared to control group ($P<0.001$). Pretreatment with LOE (200 and 400 mg/kg) significantly inhibited the CP-induced reduction of SOD and CAT activity.

4.4. The light microscopic findings

The histopathological study of kidney in the negative control group showed a normal architecture. In this group, the structure of glomerular was clear, capsular space were small, the structure of epithelial cells in proximal convoluted tubules and distal convoluted tubules was normal, the boundaries of the visceral layer and parietal layer of renal capsule were clear (Figure 4 A). In the CP-intoxicated group, the glomerular were atrophied and disintegrated, capsular spaces became expanded obviously, the visceral layer and parietal layer of renal capsule were damaged and some of them were vanished, the epithelial cells in proximal convoluted tubules and distal convoluted tubule were inflamed several epithelial cells had vacuolization, the structure of epithelial cells was not clear, there have been some cell pieces in the tubules (Figure 4 B).

Pretreatment with the LOE at the doses of 200 and 400 mg/kg showed considerable improvement in

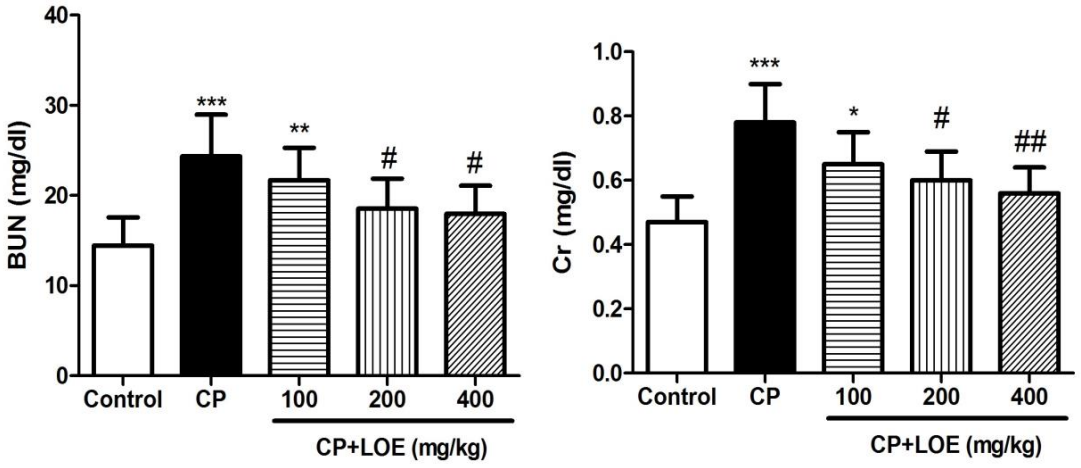

Figure 1. Effect of pretreatment with LOE on serum levels of BUN and Cr in CP-induced nephrotoxicity BUN and Cr levels increased significantly in CP-treated group. Pretreatment with LOE (200 and 400 mg/kg) decreased significantly CP-induced BUN and Cr levels.

Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA test followed by Tukey's post hoc test for multiple comparisons.

Significant difference in comparison with the control group ($P<0.05$, **$P<0.01$, ***$P<0.001$).

#Significant difference in comparison with the CP group (#$P<0.05$, ##P< 0.01).

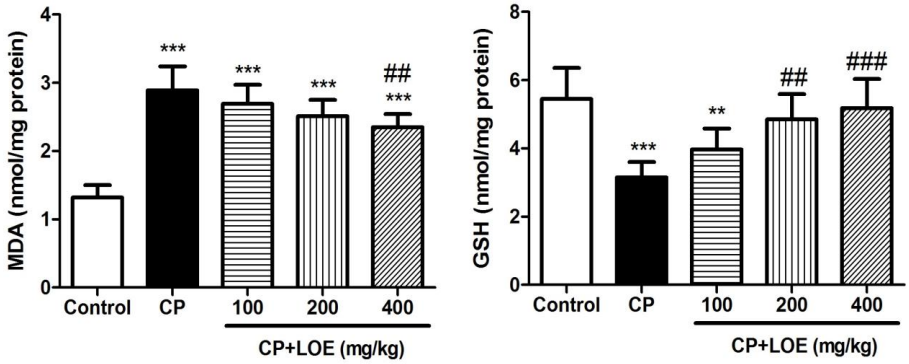

Figure 2. Effect of pretreatment with LOE on MDA and GSH levels in kidney tissues CP-induced nephrotoxicity
MDA level increased and GSH content decreased significantly in CP-treated group. Pretreatment with LOE decreased MDA and increased of GSH levels.
Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA test followed by Tukey's post hoc test for multiple comparisons.
*Significant difference in comparison with the control group (**P< 0.01, ***P< 0.001).
#Significant difference in comparison with the CP group (##P< 0.01, ###P< 0.001).

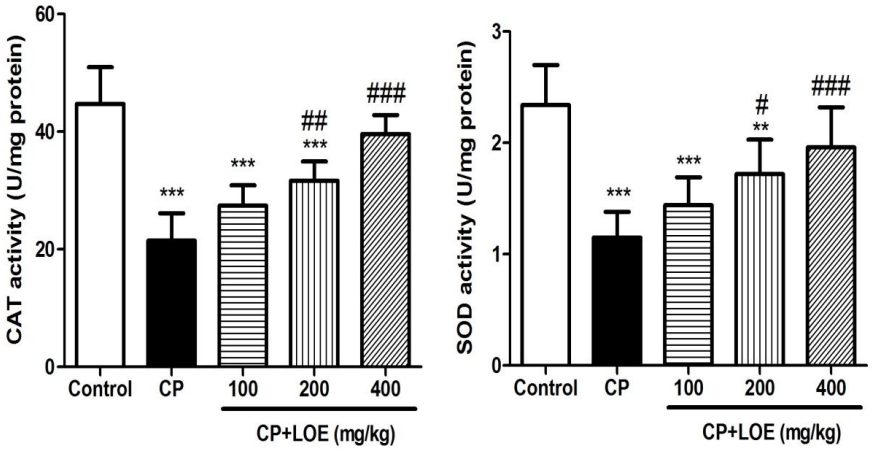

Figure 3. Effect of pretreatment with LOE on CAT and SOD activity in kidney tissues CP- induced nephrotoxicity
CAT and SOD activity decreased significantly in CP -treated group. Pretreatment with LOE (200 and 400 mg/kg) increased significantly CAT and SOD activity.
Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA test followed by Tukey's post hoc test for multiple comparisons.
*Significant difference in comparison with the control group (**P< 0.01, ***P< 0.001).
#Significant difference in comparison with the CP group (#P< 0.05, ##P< 0.01, ###P< 0.001).

proximal and distal convoluted tubules (Figure 4D, E). The atrophy degree of glomeruli was decrease and the capsular spaces were smaller in comparison to the CP treated group (positive control), the boundaries of visceral layer and parietal layer of renal capsule were clear, the epithelial cells in proximal convoluted tubules and distal convoluted tubules were slightly swollen, cell fragments were visible in some tubule.

5. Discussion

CP is a pharmaceutical with an extensive range of medical applications, and it has been proven to be useful in the treatment of cancer (lymphoma, acute and chronic leukemia, and multiple myeloma) as well as non-malignant disorder states such as rheumatoid arthritis (35). It is a well-known bi-function alkylating agent that transfer alkyl residues into a covalent bond with DNA widely used in cancer chemotherapy and expresses its genotoxicity when metabolically activated (36). Normal tissues injury or damage is the major limitation of using CP, which gives rise to numerous side effects, CP treatment also results in the

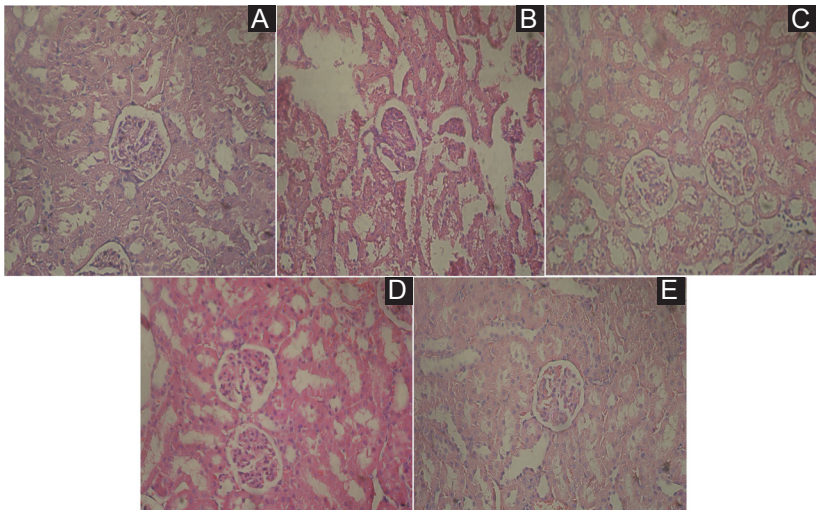

Figure 4. Histopathological observations (kidney sections stained with Hematoxylin & Eosin, magnification × 100) showing effects of LOE on CP-induced nephrotoxicity changes in mouse kidney. (A) Normal, (B) CP treated group, (C), (D) and (E) are CP group pre-treated with 100, 200 and 400 mg/kg of LOE, respectively.

production of ROS, which cause peroxidative damage to kidney and other vital organ (37). A number of studies has been reported that CP or its metabolites leads to acute inflammation of the urinary bladder (cystitis), renal damage and liver damage, thereby limiting the therapeutic use of the drug (38).

These properties motivate us to study protective effects of this extract in nephrotoxicity induced by CP. In similar study Ayhanci et al investigated protective effects of seleno L-methionine (SLM) in nephrotoxicity induced by CP. they reported that intrapritoneal injection of SLM for 6 days restore GSH values close to the control group, furthermore microscopically observations confirm their work (35). In another study Koss and Lavin investigated adverse effects of CP on various organs in the rat. They reported that a single dose of CP (200 mg/kg, intrapritoneal) cause necrosis of tubular epithelium in experimental animals (7).

BUN and Cr are two of the typical evaluation indices for kidney functions and renal structural stability. In our study, enhanced Cr and BUN levels in CP-treated mice demonstrate renal toxicity. This enlargement in Cr and BUN may possibly due to damage generated in kidney tubules which were established by marked changes in kidney tissues in comparison to the control group.

Pretreatment with LOE significantly decreased BUN and Cr in mice treated with CP.

MDA is one of the most commonly markers of lipid peroxidation. Lipid peroxidation is a common procedure of cellular damage in the human body. MDA is a highly reactive three carbon dialdehyde and major oxidative degradation product of membrane unsaturated fatty acid, owning toxic characteristics. Considering that the CP toxicity causes reactive oxygen metabolites in many tissues, mostly kidneys measurement of level of MDA, will be valuable in the diagnosis for toxicity induced by CP.

In the present study, we found that LOE pretreatment significantly decreased MDA formation due to ROS in mice treated with CP. Pretreatment with LOE restored the MDA level, suggesting that LOE might be successful in quenching free radicals, thus inhibiting LPO and protecting against membrane damage from oxidative damage in mice. Our results were parallel to some studies which indicated, the after CP intoxication, significant depletion of the GSH level and also significant increases in MDA, BUN and Cr was evidenced, corroborating the state of oxidative stress (39-42).

The human body is equipped with possesses defense systems against free-radical damage like the non-enzymatic antioxidants such as reduced GSH (41) and endogenous antioxidant enzymes such as GPx, SOD and CAT (43). Hence, generation of high levels of ROS or any disturbance in the oxidant–antioxidant status can result in oxidative damage to macro molecules (DNA, proteins and lipids), tissues or organs (44).

In view of the presented results the activities of antioxidants enzymes; CAT and SOD were significantly ($P < 0.05$) decrease in the kidney tissues of CP-treated mice, in comparison to the control group, which indicated that CP has caused severe oxidative stress. In this study, LOE pretreatment significantly

increased CAT and SOD activity ($P<0.05$). Furthermore the protective effects of LOE was confirmed by histopathological studies of kidney which have shown that considerable improvement in proximal and distal convoluted tubules and atrophy of glomerular in pretreated groups. Our results demonstrated that the protective effects of LOE were dose-dependent, and best results were observed in doses of 200 and 400 mg/kg. We believe it likely that this protective effect is probably mediated by antioxidant and GSH preservation effects of LOE. In conclusion, the results of the present study indicated that hydroalcoholic extract of *L. officinalis* showed protective effects against CP-induced nephrotoxicity in mice.

6. Conclusions

In conclusion, the results of the present study indicated that hydroalcoholic extract of *L. officinalis* showed protective effects against CP-induced nephrotoxicity in mice.

Authors' contribution

AS and MG designed the study and interpretation of the data. MB, SM and GH collected the data, performed experimental analysis and interpretation of the data. ME and MK prepared of the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest

All authors declare no conflict of interest.

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