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Oleuropein protects kidney against oxidative and histopathological damages in subchronic cadmium intoxicated mice

Hedya Jemai[1]*, Anwar Feryéni[2], Asma Mahmoudi[1], Ines Fki[1], Zouhaier Bouallagui[1] & Sami Sayadi[1]

[1]Laboratory of Environmental Bioprocesses, Centre of Biotechnology of Sfax, University of Sfax, PO Box 1177, 3038 Sfax, Tunisia

[2]Laboratory of Animal Ecophysiology. Faculty of Sciences of Sfax. University of Sfax, Tunisia

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Cadmium (Cd) is an environmental contaminant leading to serious health hazard to human and animals, directly or by producing reactive oxygen species. Subsequently, the exploration of antioxidants in Cd toxicity is with great interest. Here, we investigated the protective effects of olive leaves oleuropein in reversing Cd induced oxidative stress in the kidney of mice. For this purpose, laboratory male mice (5 weeks old) were used and divided into three groups: the first group (control), the second received intra-peritoneal injection of Cd as $CdCl_2$, administered in four doses during two weeks (each of 0.5 mg Cd/kg body wt.) amounting to a total dose of 2 mg Cd/kg body wt., i.p.; and the third was simultaneously exposed to the same doses of Cd as the first group and simultaneously received 16 mg/kg oleuropein in four doses by oral gavage. The results showed that exposure to Cd significantly affects the kidney biochemical and antioxidant parameters and its histological organization. Furthermore, oleuropein administration restored significantly all the subchronic Cd intoxication features in the mice kidney.

Keywords: Antioxidants, Cadmium renopathy, Heavy metal toxicity, *Olea europaea*, Olive oil, Oxidative stress

Cadmium (Cd), a ubiquitous toxic element, is a harmful heavy metal to human health. It is known by its bioaccumulation in vital tissue principally, liver and kidney[1]. It is reported to cause oxidative stress in the liver, kidney and testis of rats[2]. It stimulates free radical production, resulting in oxidative damages of lipids, proteins and DNA, and initiating various pathological conditions in humans and animal models[2,3]. The kidneys are major sites of antagonistic interactions between Cd and essential elements and target organ for Cd toxicity. Absorbed cadmium is deposited mostly in the liver where it is bound to and induces the synthesis of metallothionein, and then in the kidneys as metallothionein-cadmium complex[1,4]. It is now confirmed that Cd induces different renal injury, mainly tubular dysfunction, marked reduction of renal energy metabolism, altered essential mineral composition, and many transmembrane transport abnormalities[5]. In the organism, absorbed Cd is bound to the metallothionein and is filtered through the glomerulus then the proximal tubule cells and degraded by the lysosomes, resulting in Cd release. The intracellular release of Cd causes depletion in the levels of reduced glutathione, an alteration in the activities of antioxidant enzymes, as well as a change in the structure of the cellular membrane through a process of lipid peroxidation[1]. Cadmium toxicity is mainly related to its interactions with essential elements or its oxidative stress establishment. It has been reported that treatment with antioxidants, such as polyphenols, during Cd exposure, has protective effects on Cd induced toxicity in various organs and tissues[6]. Recently, Seth *et al*.[7] have demonstrated protective potential of *Aegle marmelos* against Cd induced oxidative stress and neural tissue damage.

Oleuropein, a natural bioactive phenolic compound from olive leaves, is well known for its antioxidant properties[8,9]. Further, it possesses anti-inflammatory and antiapoptotic potential as well[10-12]. In this study, we explored its efficacy in protection against sub-chronic levels of cadmium induced toxicity.

Material and Methods

Oleuropein-rich extract preparation.

Olive (*Olea europaea*) leaves, variety "Chemlali", obtained from the south of Tunisia were dried and

*Correspondence:
Phone: +216 98 660 319
E-mail: hedya.scientist@gmail.com

powdered before extraction. A mixture of methanol and water (200 mL, 4:1 v/v) was added to olive leaves powder (50 g) and the mixture was kept under agitation for 24 h. Subsequently, the solution was filtered using GF/F filter paper. The extract was concentrated by evaporation to dryness at 40°C using a rotary evaporator, and the residue obtained was stored in glass vials, at 4°C in the dark. Four grams (4 g) dry extract were dissolved in 10 mL methanol/H_2O (4:1) and extracted thrice with ethyl acetate (Prolabo, France) (40 mL each time) to prepare the Oleuropein-rich extract. Ethylacetate was then removed under vacuum. Quantitative analysis of Oleuropein in the prepared extract was determined using high performance liquid chromatography (HPLC) with detection at 254 nm (Fig. 1).

HPLC analysis

A reverse phase high performance liquid chromatography method was developed to identify and quantify the major phenolic compounds contained in the olive leaves extracts. Concentrations were calculated based on peak areas compared to those of authentic standards. A Schimadzu HPLC apparatus consisting of a binary pump LC-10ATvp, anSPD-10Avp detector, a CTO-10Asvp column oven and a SIL-10Advp auto sampler was used. The separation was performed on a C18 column (4.6 mm ID × 250 mm, 5 µm particle size; Shim-pack, VP-ODS). The column oven temperature was maintained at 40°C. The flow rate and the injection volume were 0.5 mL/min and 20 µL, respectively. Compounds were eluted using a gradient of phosphoric acid 0.1% in water (A) and 70% acetonitrile in water (B) for a total running time of 50 min where the gradient changed as follows: solvent B started at 10% and increased to 25% in 25 min. During the next 10 min, solvent B raised up to 80%. Then, acetonitrile reached 100% at 37 min and was maintained for 3 min. Finally, the column was reconditioned for 10 min (40-50 min) with a linear gradient of 100-10% B.

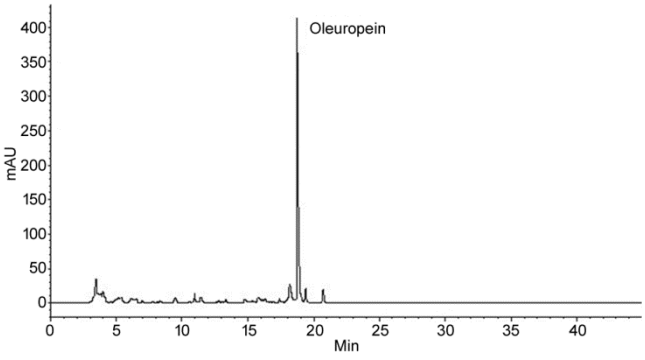

Fig. 1—HPLC chromatogram at 254 nm of olive leaf extracts based on oleuropein.

Experimental protocol

Male laboratory mice of 70±20 g initial body weight were purchased from the Central Pharmacy (SIPHAT, Tunisia). They were housed in stainless steel wire bottom cages at 23±2°C and photoperiod of 12 h day/12 h night. The animals were fed mice pellet diet and water *ad libitum* and they have a period of one week of acclimatization to the laboratory conditions

The experimental design of Cd intoxication was conducted following Jemai *et al.*[13]. The laboratory mice were divided into three groups: Group Cd: received intraperitoneal injection of Cd as $CdCl_2$, administered in five doses (each of 0.4 mg Cd/kg body wt.) on days 5, 10 and 15, giving a total dose of 2 mg Cd/kg body wt., i.p.; the second: Group Cd + OL was simultaneously exposed to the same doses of Cd as the first group and received oral oleuropein solution (16 mg/Kg); and the third group was control: Group C which received 0.5 mL of physiological saline in an identical manner.

Animals were sacrificed by decapitation under anesthesia. Fresh blood was immediately collected into heparinized tubes (BD Vacutainer System) and centrifuged at 2200 ×g for 15 min to separate red blood cells and plasma. The kidneys were removed, weighed, washed with saline and fixed in 10% buffered formalin. Kidney and serum samples were stored at −80°C for later biochemical and histological analysis.

Serum biochemical analysis

Serum samples were collected for creatinine and urea analysis. In these samples, mineral and enzymatic analyses were done according to Roche laboratories protocols using Hitachi 912 analyzer.

Kidney cytosol extraction

Kidneys were utilized for cytosol extraction. Cells fraction was realized after adding 10 mL of KCl (1.15%) to 1.0 g of kidney tissue by using ultra-turrax at 4°C.

Catalase, SOD and GPx activities

Catalase activity was assayed by the method of Claiborne[14]. Briefly, the assay mixture contained

2.4 mL of phosphate buffer (50 mM, pH 7.0), 10 µL of hydrogen peroxide (19 mM) and 50 mL enzyme source. The decrease in absorbance was measured immediately at 240 nm against reagent blank at 10 s intervals for 3 min on a Systronics Spectrophotometer. The activity of enzyme was expressed as µmol of hydrogen peroxide consumed/min/mg protein at 37°C.

Superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich[15]. The reaction mixture contained 50 mM of tissue homogenates in potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 µM nitroblue tetrazolium (NBT). The developed blue colour in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as units per mg of protein.

GSH-Px activity was assayed by subsequent oxidation of NADPH at 240 nm with t-buthyl-hydroperoxide as substrate[16]. GPx units (U/mg protein) were defined as micromoles of NADPH oxidized per second per milligram protein.

Total antioxidant capacity in the kidney

The Trolox equivalent antioxidant capacity (TEAC) assay is measuring the reduction of the ABTS radical cation by antioxidants. ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS stock solution with 140 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For the study, ABTS+ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. One mL diluted ABTS+ solution was added to 50 µL cytosol samples or Trolox standard. The reaction mixture was incubated for 2 min in a glass cuvette at 30°C. The decrease in absorbance was recorded at 734 nm. All measurements were performed in triplicate. The free radical scavenging capacity of the biological samples, calculated as inhibition percentage of ABTS+, was equated against a Trolox standard curve prepared with different concentrations (40-200 mol/L). The results were expressed as mM of Trolox equivalents[10].

Determination of TBARS

As a marker of lipid peroxidation, the TBARS (thiobarbituric acid-reactive substances) concentrations were measured in kidney cytosols using the method of Park *et al.*[17]. For this, 200 µL cytosolic sample was mixed with 600 µL of distilled H_2O and 200 µL of 8.1% (w/v) SDS, vortexed, and incubated for 5 min at 25°C. The reaction mixture was heated at 95°C for 1 h after the addition of 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% (w/v) thiobarbituric acid (TBA). After incubation the reaction was cooled and 1 mL of distilled water and 5 mL of butanol:pyridine (15:1) solution were added. The vortexed mixture was centrifuged at 1935 ×g for 15 min and the resulting colored layer was measured at 532 nm using malondialdehyde (MDA) made by the hydrolysis of 1,1,3,3-tetramethoxypropane as standard.

Histopathological analysis

The washed kidney tissues (fixed in 10% buffered formalin) were dehydrated in the increasing grades of ethanol and finally cleared in toluene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5µm thicknesses and stained with hematoxilin eosine. The sections were analyzed with light microscopy. The sections were analyzed with light microscopy. Cell necrosis in glomerulus and tubules was considered for assessment. Semi-quantitative assessment of kidney injury was performed using scores ranging from 0 to 4 as follows: representing a 25% loss (1+), representing a 50% loss (2+), representing a 75% loss (3+) and representing more than 75% loss (4+)[18,19]. The score were determined in each section selected at random and 20 fields were examined using 100, 400 and 1000X magnification

Statistical analysis

Results were expressed as mean ± standard deviation (mean ± SD). All analyses were carried out with Graph Pad Prism 6 for Windows (Graph Pad Software, San Diego, CA). Significant differences between treatment effects were determined by one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons with statistical significance.

Results

Creatinine and urea evaluation

Blood biochemical parameters reflecting the renal status, showed important changes and perturbation in the Cd intoxicated mice. In fact, Urea and Creatinine levels increased significantly (P <0.001) in comparison with the control group. Oleuropein administration shows significant restoration of the

adverse effects of cadmium on all analyzed parameters (P <0.01) (Fig. 2).

Catalase, Superoxide dismutase and Glutathione peroxidase activities

Cd intoxicated mice showed, in comparison with the controls, a significant decrease in the CAT, SOD and GPx activities in the kidney (P <0.001). The oleuropein administration restored significantly these values. Therefore, no significant differences noticed between control and Oleuropein treated groups in the antioxidant enzymes activities (Fig. 3).

Kidney total antioxidant capacity

The Trolox equivalent antioxidant capacity (TEAC) reflecting the total antioxidant capacity in the kidney, showed a significant decrease in Cd treated mice compared to the control group (P <0.001). The Oleuropein administration restored significantly the kidney total antioxidant capacity (P <0.001) (Fig. 3).

Kidney lipid peroxidation level (TBARS)

TBARS are the lipid peroxidation indicator as result of oxidative stress. The mice Cd intoxication caused a significant increase in TBARS levels in the kidney (P <0.001). On the other hand, oleuropein inhibited lipid peroxidation significantly in the kidney of Cd+OL group mice and as a result, no significant difference was noticed comparing with the control group (Fig. 3).

Histological study

Light microscopic observation revealed that the control kidney tissue showed normal histologic organization with developed glomeruli and intact brush border in proximal and distal tubules. We have noticed clearly histopathological damage in the kidneys of cadmium intoxicated mice. Cd affected the glomeruli especially the glomerular capillaries in favour of Bowman's space and engender contraction of proximal tubules. Moreover, Cd produces atrophy of some glomeruli with significant necrosis areas in different histological levels confirmed by calculated scores. Oleuropein largely restored the kidney histological organization. Moreover, in proximal and distal convoluted tubules, Cd clearly affects the brush border and the oleuropein administration clearly reverse these toxic effects. Subsequently, the kidney tissue of mice receiving oleuropein show similar appearance with those of control (Fig. 4).

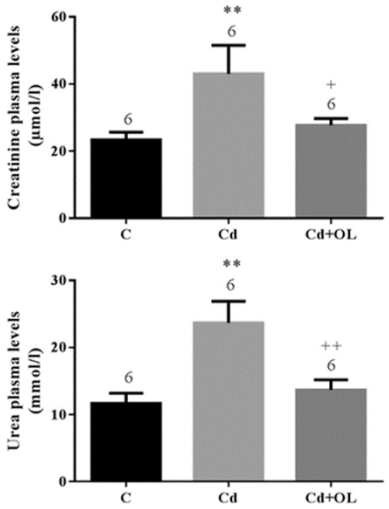

Fig. 2—Urea (mmol/L) and Creatinine (µmol/L) levels in control, Cd intoxicated (Cd) and Cd intoxicated + oleuropein (Cd+OL) groups. [*vs.* control: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$; and *vs.* (Cd): +++ $P \leq 0.001$, ++ $P \leq 0.01$, + $P \leq 0.05$]

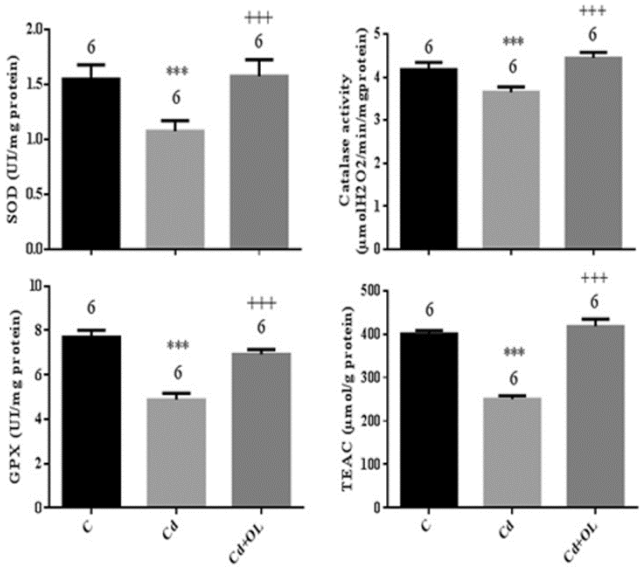

Fig. 3—(A) SOD activity (Unit/mg protein); (B) CAT activity (µmol H_2O_2/min/mg protein); (C) GPx activity (Unit/mg protein); (D) Liver Trolox equivalent antioxidant capacity (nmole/100 g tissue); and (E) Liver TBARS levels (nmole/100 mg protein) in control, Cd intoxicated (Cd), and Cd intoxicated + oleuropein (Cd+OL) groups. [*vs.* control: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$; and *vs.* (Cd): +++ $P \leq 0.001$, ++ $P \leq 0.01$, + $P \leq 0.05$]

Discussion

Cadmium is one of the most dangerous environmental heavy metal. The kidney are nowadays confirmed to be one of the prior target of cadmium intoxication where it causes functional and histological changes[1,5]. The present data determined for the first time the renal protective effects of

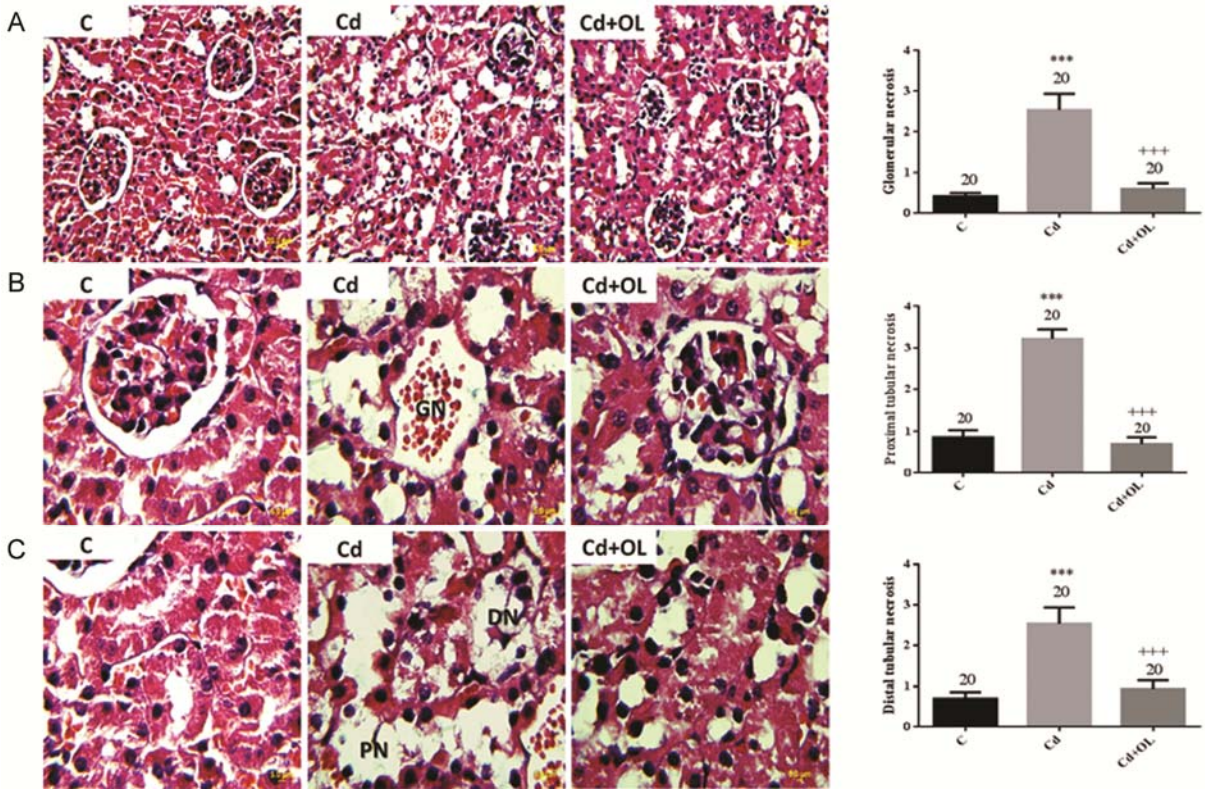

Fig. 4— Histological aspect and semi-quantitative scores of liver injuries of mice in control, Cd intoxicated (Cd) and Cd intoxicated + oleuropein (Cd+ OL) groups. (A) Glomeruli; and (B & C) Proximal and Distal convoluted tubules, respectively. [*vs.* control: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$; and *vs.* (Cd): +++ $P \leq 0.001$, ++ $P \leq 0.01$, + $P \leq 0.05$. Glomerular, GN; Proximal, PN; and Distal Necrocrosis, DN]

Oleuropein in Cd intoxicated mice. In fact, we suggested that the use of a phenolic compound Oleuropein with confirmed antioxidant capacity[12], is recommended as protective molecule in such intoxication.

The biochemical determination of urea and creatinine in our experimentation showed a significant increase in Cd intoxicated mice compared to the control group. In fact urea and creatinine are indicator of the kidney functional and anatomic status. These enhanced values suggesting the severe damage in glomeruli function. These results are largely confirmed as a response to cadmium exposure because it concentrates in the kidney, particularly inducing proteinuria and renal dysfunction[20-22]. In fact, Cd is a well-known nephrotoxic agent. It induces renal tubular dysfunction and decreased glomerular filtration leading to renal failure confirmed biochemically via urea and creatinine values[22]. In the other hand, Oleuropein administration (16 mg/Kg body wt.) during two weeks attenuated cadmium-induced nephrotoxicity. This protective effect of oleuropein may be due to its indirect membrane stabilization properties such all the other polyphenols[23].

Besides the biochemical indicators, our results show that Cd intoxication at a dosage of 2 mg Cd/kg BW, i.p for 2 wk, was associated with a significant establishment of oxidative stress in the mice kidney. Cd affects significantly the CAT, SOD, GPx and the TEAC values and engender a significant increase in TBARs. Thus, our findings support and extend previous reports confirming that cadmium intoxication affects the kidney antioxidant defense system and induces lipid peroxidation in experimental animals[13]. These findings are in agreement with previous studies suggesting stress oxidative to be one of cadmium toxicity mechanism[1,5,24]. The significant depletion in the GPx activity in the kidney tissues of Cd-intoxicated mice can be explained by or a direct effect of Cd on the selenium in the GPx structure[25] or by a chemical chelation between Cd and Se at the active site of this antioxidant enzyme[26]. Furthermore, the affectation of CAT and SOD activities can be explained by the cadmium ability to interact with vital elements in organisms[4]. Since CAT contains Fe and SOD contains Zn and Se in their active center, their decreased activities in the kidney of the mice exposed

to Cd might be a result of these element deficiency caused by this metal[27]. Our results showed a significant fall in levels of SOD indicating a potential Cd damage. This results are previously confirmed by Patra et al who demonstrate that the SOD inhibition consists a key factor for inducing oxidative stress in the kidney[28].

As a result of oxidative stress establishment, our study demonstrated that the lipid peroxidation, reflected in TBARS concentration, was significantly elevated in kidney tissues of Cd intoxicated mice compared to control group (P <0.01). This is in a direct relationship with the depletion of the antioxidant enzymes. In fact, these enzymes protect tissues via elimination of reactive oxygen species which could be produced by cadmium via Fenton reaction[29].

Consistent with our findings, several previous studies confirm that cadmium induced significant TBARs levels traducing a high lipid peroxidation[5,13,29].

The administration of olive leaves oleuropein at 16 mg/kg was able to reduce the renal oxidative damage confirmed by biochemical, antioxidant and histological evaluations. Our results indicated that oleuropein administration suppresses oxidative stress in the mice kidney. This fact is proved by the elevation activities of the antioxidant enzymes, CAT, SOD and GPx¸ enhanced total antioxidant capacity (TEAC) as well as depletion of lipid peroxidation products (TBARs). The enhancement of antioxidant statute might be due to the antioxidant power of oleuropein which offers the ability to reduce the accumulation of free radicals generated during cadmium-induced lipid peroxidation[30]. Moreover, this protection can be explained by the fact that oleuropein, like the majority of biophenols could scavenge free radical circulating and increased the expression of antioxidant enzymes at the transcriptional level[31]. Furthermore, previous data indicate that oleuropein has been detected in plasma only in its glycoside form, suggesting that it is absorbed intact from the intestine[32] that is why it presents a high availability in the organism in its active form explaining its high *in vivo* positive impact. Furthermore, oleuropein and its metabolite, hydroxytyrosol, both possess the structural requirement needed for optimum antioxidant and/or scavenging activity[29]. Both its free radical scavenging and metal-chelating activities appear to be responsible for the ability of oleuropein to protect membranes from cadmium initiated lipid oxidation[33].

In the present study, we have explored the histological structure of the kidney of the different experimental groups. In fact, the kidney is well known to be the crucial organ of Cd toxic impact. Furthermore, the segment of the proximal tubule is a major target of Cd deposition, with clinically observable defects in protein and other biomolecules reabsorption resulting from Cd-induced oxidative damage[34,35]. The control mice kidneys show a normal histological organization with normal glomerulus and normal proximal and distal convoluted tubules. Nevertheless, the histological study of the kidneys shows that the Cd intoxication induced severe nephrotoxicity as proved by glomerular and tubular necrosis. This result is with concordance with previous study confirming that Cd has been confirmed as a pro-necrotic metal ion[36,37]. In addition, this is consistent with the earlier works[13,38] in which they showed that cadmium caused kidney damage. These effects could be due to the formation of highly reactive radicals and subsequent lipid peroxidation induced by Cd. The accumulated hydroperoxides can cause cytotoxicity, which is associated with the peroxidation of membrane phospholipids by lipid peroxides. This observation correlates with our biochemical observations, which showed the increased level of lipid peroxidation, creatinine and urea. Treatment with oleuropein reduced the histological alterations induced by Cd. It could be attributed to the antioxidant and chelating properties of oleuropein, which significantly reduced the oxidative stress leading to reduction of histopathological changes.

Conclusion

Our findings substantiate the nutritional potential of olive leaf Oleuropein. We have demonstrated that administration of oleuropein at 16 mg/kg body wt. protects the kidneys from Cd intoxication. Oleuropein reversed the Cd induced renal oxidative stress by improving antioxidant defense system, reducing the levels of lipid peroxides and thereby protecting the kidney tissue integrity.

Conflict of Interest

The authors declare no conflict of interest.

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