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THYMELAEA MICROPHYLLA AMELIORATES OXIDATIVE STRESS AND METALLOTHIONEIN LEVEL INDUCED BY CADMIUM IN RAT KIDNEY

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ABSTRACT

Background and Objective: The present investigation was performed to evaluate the protective effect and the antioxidant power of *Thymelaea microphylla* against cadmium chloride induced oxidative stress and renal toxicity in Wistar rats. **Materials and Methods:** First, antioxidant and radical scavenging effect of *Thymelaea microphylla* was evaluated in vitro with 2,2- diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl (OH) radicals assays. Then, Wistar rats were subjected to oral pretreatment with n-butanolic extract of *Thymelaea microphylla* (BETM) (200 mg/kg) against the renal toxicity induced by oral administration of cadmium chloride $CdCl_2$ (5 mg/kg) for 28 days, vitamin E (250 mg/Kg) was used similarly as standard. Efficiency of *Thymelaea microphylla* against the renal toxicity was evaluated in terms of biochemical estimation of renal function parameters, antioxidant enzymes activities, metallothionein levels and histopathological modifications. *Thymelaea microphylla* antiinflammatory effect was evaluated by carrageenan (1%) induced rat paw edema. **Results:** *Thymelaea microphylla* revealed a high scavenging ability of DPPH and OH radicals. Also, *Thymelaea*

microphylla(200 mg/Kg) pretreatment prevented deteriorative effects caused by cadmium chloride through a protective mechanism that included significant decrease in the levels of Blood Urea Nitrogen, creatinine, gamma-glutamyltranspeptidase (GGT) , potassium, sodium, Myeloperoxidase, Nitric Oxide and Thiobarbituric Acid Reactive Species as well as by restoration of antioxidant enzymes, metallothionein levels and histopathological changes against cadmium chloride administration. Additionally, *Thymelaea microphylla* revealed an important anti-inflammatory effect reducing carrageenan induced paw edema. **Conclusion:** This study proved the antioxidant and protective effect of *Thymelaea microphylla* against cadmium chloride induced oxidative stress and kidney injury.

KEYWORDS: Cadmium chloride, kidney, oxidative stress, *Thymelaea microphylla*, metallothionein.

INTRODUCTION

Cadmium (Cd) is a serious environmental toxicant and pollutant emanating from industrial and agricultural sources,[1] for example, food, water, cigarette smoke and from some industries such as electroplating, plastics, coloring pigments and batteries.[2] Exposure to Cd could induce tubular kidney damage, osteoporosis,[3,4,5] hepatic dysfunction[6,7] and different types of cancer in organs such as the urinary bladder, pancreas, breast, kidney, lung and prostate.[8,9,10,11,12] Cd is mostly retained in the kidney (half-time 10-30 years) and the kidney is an important target organ after chronic exposure to Cd.[13]

After exposure and uptake into the systemic circulation, cadmium is at first bound to albumin in blood plasma followed by its uptake in blood cells.[14] Albumin-bound cadmium in plasma is then transported to the liver, where the complex undergoes degradation with the release of Cd, which in turn induces the synthesis of metallothionein (MT). Cadmium-induced MT (Cd–MT) then binds with it and transports the metal to the kidneys for excretion.[15] Because of their low molecular weight, the Cd- MT complexes are freely filtered at the glomerulus, and are then incorporated into renal proximal tubular cells.[16]

Throughout the last years, many investigations have reported that oxidative stress is an important mechanism of Cd toxicity.[17] It might take part in early proximal tubular kidney damage, which may induce a general transport defect in the proximal tubule.[18] In ordinary conditions, cellular antioxidant defense systems diminish the perturbations brought on by reactive oxygen species (ROS). Moreover, ROS are physiologically advantageous in

controlled amounts: they play an important role in the regulation of many intracellular signaling pathways as activators of signal transduction.[19,20] When its production in increased to an extent that defeats the antioxidant enzymes and molecules, oxidative stress is induced[21] causing damage to DNA, proteins or mitochondria, lipid peroxidation and apoptotic cell death.[20,21,22,23] Cd, which is not a redox active metal, can indirectly be implicated in free radicals generation. It is thought that Cd may play the role of redox active metals such as iron and copper, which participate in free radical generation via Fenton reaction.[24] Cd-induced ROS generation may also be due to depletion in endogenous intracellular radical scavengers such as GSH, to which it binds with high affinity.[25,26] Furthermore an elevation in lipid peroxidation and a diminution in GSH levels were often observed in the liver and the kidney.[27]

Recently, a great deal of interest has focused on the protective effects of naturally occurring antioxidants in biological systems against toxic heavy metals. It has been reported that chelating compounds such as calcium disodium versenate, dimercaprol and mesomercaptosuccinic acid,[28] have been used for cadmium toxicity treatment. Co-administration of antioxidants N-acetylcysteine or vitamin E prevents Cd-induced lipid peroxidation and protects animals from Cd liver and kidney toxicity.[29] Flavonoids contained in fruits, vegetables, nuts, seeds, leaves, flowers and barks of plants[30] were one of the most numerous and widespread group of naturally occurring antioxidants.

The genus *Thymelaea* which belong to thymelaeaceae family includes about 30 species of evergreen shrubs. *T.microphylla* Coss. *Et Dur*. is a medicinal species endemic to Algeria and very common in the arid and desert pastures.[31] The leaves decoction is used in popular medicine to treat abscess, skin diseases, and abdominal pain and for anticancer, anti-inflammatory, and antidiabetic properties.[32,33] Only a few data about the antioxidant activity of the extracts of *T. microphylla*,[33,34,35] as well as on its chemical composition, are available.[36,37] To our knowledge, this study was the first one demonstrating the protective effect of *T. microphylla* on Cd induced nephrotoxicity.

The present investigation was carried out to evaluate the antioxidant and nephroprotective potential of the *n*-butanolic extract of *T. microphylla* (BETM) against cadmium induced oxidative damage in the renal tissue.

MATERIALS AND METHODS

Chemicals and drugs: Cadmium chloride ($CdCl_2$) was obtained from Sigma , 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylenediamine tetra acetic acid (EDTA), were purchased from Sigma Aldrich ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox), L-ascorbic acid, ferrous chloride ($FeCl_2$), ferric chloride (FeCl3), thiobarbituric acid (TBA), 5,5-dithiobis-2-nitrobenzoic (DTNB), 1-Chloro-2,4-dinitrobenzene (CDNB), trichloroacetic acid (TCA). trihydroxymethylaminomethane (Tris), b-Nicotinamide adenine dinucleotide phosphate (NADPH), deoxyribose , Griess reagent, Carrageenan λ, sodium nitrite (NaNO2), Hexadecyltrimethylammonium bromide (HTAB), Reduced glutathione (GSH) Diclofenac-sodium and all other chemicals were of analytical grade were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Plant material extraction and isolation: *T. microphylla* was collected during the flowering phase in April 2010 in the east of Algeria and was authenticated by Professors Nadra Khalfallah (Constantine 1 university) and Mohamed Kaabache (Setif 1university). A voucher specimen has been deposited at the Herbarium of the VARENBIOMOL research unit, University of Constantine 1.

To prepare the extract, air-dried leaves and flowers (4980 g) of *Thymelaea microphylla* were macerated at room temperature with EtOH–H_2O (70:30, v/v) for 24 h, three times. After filtration, the filtrate was concentrated and dissolved in H_2O (1950 ml) under magnetic agitation. The resulting solution was filtered and successively extracted with $CHCl_3$, EtOAc and n butanol. The organic phases were dried with Na_2SO_4 filtered and concentrated *in vacuo* at room temperature to obtain the following extracts: chloroform (8.20 g), EtOAc (14.94g) and *n*-butanol (56 g). In our investigation we are interested in the *n*-BuOH extract. The *n*-BuOH extract of *T. microphylla* (BETM) was served for the in vitro and *in vivo* experiments.

IN VITRO STUDIES

Antioxidant activities

DPPH$^{\bullet}$ scavenging activity: The effect of BETM on DPPH$^{\bullet}$was assayed using the free radical α, α-diphenyl-b-picrylhydrazyl (DPPH) method with minor modifications.[38] 1 ml of a methanolic solution of DPPH$^{\bullet}$(0.2mM) was added to 1 ml of the different concentrations of BETM- and allowed to react in the dark at room temperature for 30 min.. The absorbance of the mixture was measured at 517 nm. Ascorbic acid, Trolox and ascorbic acid were used as

references. Results were expressed as percentage of inhibition of the DPPH· according to the following equation:

% Inhibition = [(Abs C – Abs S) /Abs C] x100 Where Abs C was the absorbance of the control, and Abs S was the absorbance of the extract or standard.

Hydroxyl radical scavenging assay: Hydroxyl radical scavenging activity of BETM was measured according to a modified method.[39] Stock solutions of EDTA (1mM), $FeCl_3$ (10 M),ascorbic acid (1mM), H_2O_2(10mM) and deoxyribose (10mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml of EDTA, 0.01ml of $FeCl_3$, 0.1mlof H_20_2, 0.36 ml of deoxyribose,1.0 ml of plant extract (50-400µg/ml), 0.33 ml of phosphate buffer (50 mM,pH7.4) and 0.1ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1hr. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA to develop the pink chromogen, measured at 532 nm. The absorbance was noted at 532 nm. The scavenging activity was calculated as follows:

% OH° scavenging capacity = [(Ao− A1)/Ao] × 100, where Ao the absorbance of the control and A1 the absorbance of the fraction or standard (Trolox).

Anti-inflammatory activity (Carrageenan-induced paw edema): The anti-inflammatory activity of BETM on carrageenan-induced paw edema was determined in rats.[40] Rats were divided into four groups of 5 each, Group I served as control (received vehicle, PBS), Group II served as reference (received orally diclofenac sodium, 10 mg/kg),[41] groups III and IV received orally 100 and 200 mg/kg of BETM respectively. Sixty minutes later, paw edema was induced by injecting 0.1 mL of 1% carrageenan into sub plantar tissue of the left hind paw of the rats. The progression of edema (mm, after 1, 2, 3, 4, 5 and 6 hours) was evaluated by measuring the paw edema by using graduated micrometer combined with a constant loaded lever system. The edema was expressed as the increase in ear thickness (mm) due to the inflammatory challenge. The percentage inhibition of the inflammation was calculated from the formula: % inhibition = D_0-D_t/D_0 x 100, where D_0 average inflammation (hind paw edema) of the control group of rats at a given time D_t average inflammation of the drug - treated rats (extract or diclofenac-sodium reference) at the same time.

Animals and experimental design: Adult male Wistar Albinos rats (120-200g) were housed at a temperature of 25°C under a 12 hr. light/dark cycle with free access to water and standard food. The animals were randomly divided into four groups of six rats each as follows:

- Control group:received orally daily 2 mL of distilled water for 28 days.
- $CdCl_2$ treated group: treated daily with an oral dose of $CdCl_2$ (5mg/Kg/day) for 28 days.
- Vit E treated group: received orally and daily Vit E (250mg/Kg/day) and after 90 minutes $CdCl_2$ (5mg/Kg/day) for 28 days.
- BETM treated group: daily co-treated with both a BETM extract (200mg/Kg/day) and $CdCl_2$ (5mg/Kg/day) for 28 days.

In all experiment the $CdCl_2$ co-administration was performed after 90 minutes.

Biochemical Estimation: After the last treatments, rats were fasted overnight. Blood samples were taken by puncture in the recto-orbital venous plexus and the obtained plasma (centrifugation at 4000rpm, 15 minutes) were subjected the renal function markers (creatinine, BUN, GGT, sodium and potassium) using enzymatic methods according to manufacturer instructions.

Renal homogenate preparation: After cervical decapitation. The removed kidneys were weighed and washed using chilled saline solution of sodium chloride (NaCl 0.9%). The renal tissue was minced and homogenized (10% w/v) in KCl (1.15%) and centrifuged (1000 rpm, 15min). The resulting supernatant was performed for the lipid peroxidation (MDA estimation. The cytosolic fraction was homogenized with Tris EDTA pH 7.6 (Tris; 50mM + EDTA; 0.1mM). Then, the supernatant obtained by centrifugation at 9600 rpm for 45min was frozen at -80°C for oxidative stress parameters assay.

Renal oxidative stress estimation: The extent of lipid peroxidation i.e. malondialdehyd (MDA) were measured by the method based on the reaction with thiobarbituric acid (TBA).[42] Reduced glutathione (GSH) level was assayed by the method based on the reaction with Ellman's reagent (400mg dithionitrobisbenzoic acid in 100ml of 1% sodium citrate) determine GSH using 5,5-dithiobis(2-nitrobenzoicacid) (DTNB). Absorbance was measured at 412 nm using a spectrophotometer. [43]SOD activity was determined using pyrogallol as a substrate. [44] This method is based on pyrogallol oxidation by the super-oxide anion ($O2^{\cdot-}$) and its dismutation by SOD. Catalase (CAT) activity was measured by measuring hydrogen peroxide decomposition at 240nm. [45] Glutathione S-transferase (GST) activity was determined spectrophotometrically by using CDNB as the substrate.[46]

Metallothionein quantification: MT analysis was performed in renal tissue.[47] It was homogenized on ice in buffer solution (20 mM tris, 1.5 µM phenylmethyl sulfonyl, 10-5mM

ß-mercaptoethanol and 0.5 M sucrose) in 1:3 (w/v) ratio range .The homogenate was centrifuged at 25000 g for 60 min at 4°C. Cold pure ethanol (1.05 mL at -20°C) and 80 µL chloroform were added to 1 mL of supernatant. After centrifugation at 6000 g for 10 min at 4°C, 40 µL of 37% HCl and 3 fold sample volume of ethanol were added. After protein precipitation (storing samples at -20°C), they were centrifuged at 6000 g for 10 min. The pellets were washed in homogenizer buffer containing 87% ethanol and 1% chloroform. The samples were again centrifuged at 6000 g for 10 min at 4°C and pellet dried under a nitrogen gas stream for 10 min. The dry pellets were added to 150 µL NaCl (0.25 M) and 150 µL HCl (1 N) containing 4 mM EDTA. Sodium chloride solution (2 M) containing 43% DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) was mixed with a buffer of Na-phosphate (pH = 8) and added to the samples at the room temperature. At the end, the samples were centrifuged at 3000 g for 5 min. The absorbance of the samples was measured at 412 nm and the concentration of metallothionein was determined using reduced glutathione as the reference standard.

Myeloperoxidase (MPO): MPO activity was measured as it was stated in the method of Bradley et al.[48] The samples were homogenized, frozen and thawed, then centrifuged at 1,500 rpm for10 min at 4°C. MPO activity was measured at 450 nm by adding 100 ml of the supernatant to 1.9 ml of 10 mmol/l phosphate buffers (pH 6.0) and 1ml of 1.5mol/l o-dianisidine hydrochloride containing H_2O_2 (0.0005% w/v).

Determination of NO: Tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production because NO levels estimation in biological specimens is difficult. They were measured by the method based on the Griess reaction.[49] Samples were first deproteinized with Somogyi reagent. Total nitrite (nitrite+ nitrate) was measured after conversion (reduction) of nitrate to nitrite using a spectrophotometer at 545 nm. Serial dilutions of sodium nitrite were used to establish the standard curve.

Histopathological studies: The kidneys obtained from the normal and experimental rats were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5µm thickness were stained with haematoxylin and eosin (H&E) to study the histology of kidney of all experimental rats.

Statistical analysis: For *in vivo* studies(n=7) results are expressed as the mean values ± SD. The *in vitro* experiment were carried in triplicates and results expressed as means ± SD**.** IC_{50}-

value (µg extract/ml) is the effective concentration which proves 50% of activity, was calculated for each assay. The statistical evaluation was carried out by means of analysis of variance (ANOVA). The level of significance between diff erent groups was evaluated by the Student's *t*-test and values were considered to be highly significant at $P < 0.01$ and significant at $P< 0.05$.

RESULTS

DPPH· radical scavenging ability: As shown in (Fig 1), BETM showed a gradual increase in DPPH radical scavenging activity in a dose dependent manner. BETM revealed a high DPPH radical scavenging ability (88.1±0.87- 88.6±0.87 %)at the concentration ranged between 600-800 µg/ml these results were compared to those of ascorbic acid and trolox standards (97.20±0.03%,83.03±0.4%) respectively. According to IC_{50} value, the DPPH radical scavenging ability was in order of ascorbic acid: 6.41 ± 0.61<trolox: 30.08 ±0.83< BETM: 237.22 ± 6.4.

Hydroxyl radical scavenging activity: In the present study BETM exhibited strong scavenging capacity of OH° at 400 µg/mL (74.34±0.51%) as compared to the trolox standard reference which was found to be 83.09±0.73%.The IC_{50} inhibition of hydroxyl radical was found to be 142.01±9.29µg/ml, as compared to the trolox (50.08 ±0.70µg/ml) (Fig 2).

Carrageenan-induced paw edema: For acute inflammation, subplantar injection of λ-carrageenan (1%) induced a progressive swelling of the rat paw (5.068 ± 0.0192mm) and reached a maximum level at the third hour (6.124±0.036mm) , the antiedema effect of BETM was significantly ($P < 0.01$) more pronounced at 200 mg/kg concentration (Fig 3) showed that BETM at 200 mg/kg significantly ($p < 0.01$) reduced the swelling in both the early (3h; 45.5%), and the late (6 h;42.35%)phase of carrageenan-evoked edema, this effect is clearly compared to diclofenac-sodium (10 mg/kg),which were found to be 50.1 % and 43.01% inhibition at the both times respectively.

Renal function markers: Animals treated with $CdCl_2$ (5mg/Kg) showed a significant ($p<0.05$) increase in creatinine and BUN levels (Table 1) as well as in GGT activity, sodium and potassium levels in comparison with the control group. Animals received the combined treatment of Cd (5mg/Kg) and BETM (200mg/Kg) showed a significant ($p<0.01$) improvement in renal function markers, as evidenced by a significant ($p < 0.01$) reduction in both creatinine (61.91 %) and BUN (70.09 %) levels. There was also a significant ($p < 0.01$)

modulation in GGT activity (74.13 %), sodium (70.85 %) and potassium (79.73 %) levels (Table 2) when compared to the standard reference, VitE-group (83.59 %, 85.65 % and 81.96 %) respectively.

Renal oxidative stress and metallothionein level: The extent of lipid peroxidation (MDA) was significantly ($p<0.05$) elevated in the $CdCl_2$-rats. The co-treatment of BETM (200mg/Kg) with $CdCl_2$ (5mg/kg) significantly ($p<0.05$) reduced (86.07 %) the lipid peroxidation. A significant ($p<0.05$) decrease in the renal antioxidant defense system; SOD, CAT, GST activities, and depletion of GSH level was observed in cadmium-treated rats. Administration of BETM (200mg/kg/day) along with cadmium significantly ($p<0.05$) restored the levels of the renal antioxidant defense system (72.64, 65.95, 59.45, 69.27 %) respectively as compared to the Vit E-group (84.55, 71.049, 72.71, 84.6 %) (Table 3). Metallothionein level, which is also an antioxidant agent, was significantly ($P<0.01$) higher in the cortex renal tissue of $CdCl_2$ -rat (353.1±15 µg/g tissue) than that of the control group (99.35±10.8µg/g tissue.) In BETM --group and Vit E-group the metallothionein level significantly ($P<0.01$) was normalized (79.42 %) in comparison to Vit E (87.76%) (Table3)

Renal MPO and NO levels: MPO activity, which is an indicator of neutrophil infiltration, was significantly ($p<0.05$) higher in the renal tissue of Cd group (87.3±1.98) than that of the control group (28.24±1.028). The NO level of Cd-treated group was increased significantly ($p<0.05$) in renal tissue in comparison with the other groups ($p<0.05$). The combined treatment of $CdCl_2$ (5mg/kg) and BETM (200mg/Kg) significantly ($p<0.05$) restored the MPO and NO levels by (61.91, 70.09 %) respectively as compared to the VitE-group (81.79, 78.70 %) (Table 4).

Histopathology examination: The histopathological analysis of the cortical region of rats kidneys revealed morphological alterations of renal parenchyma. In the Cd -group, Cd caused severe and widespread damage evidenced by tubular lumen dilatation, necrotic tubule cells, degenerative epithelium, congested blood vessels and interstitial damage with variable degrees of tubular necrosis and inflammatory cell infiltration (Fig. 5B1, B2) in comparison with control rats kidneys (Fig 5A). These alterations were also characterized by disrupted renal parenchyma namely severe glomerular degeneration.

In the BETM -treated group, there was a remarkable improvement in the histological features of the kidney and a moderate degree of histopathological alteration (The reduced tubular

damage and interstitial inflammation VitE co -treated group and BETM - pre-treatment group showed slight degeneration at a few places and a moderate degree of histopathological alteration (Fig 5 C, D).

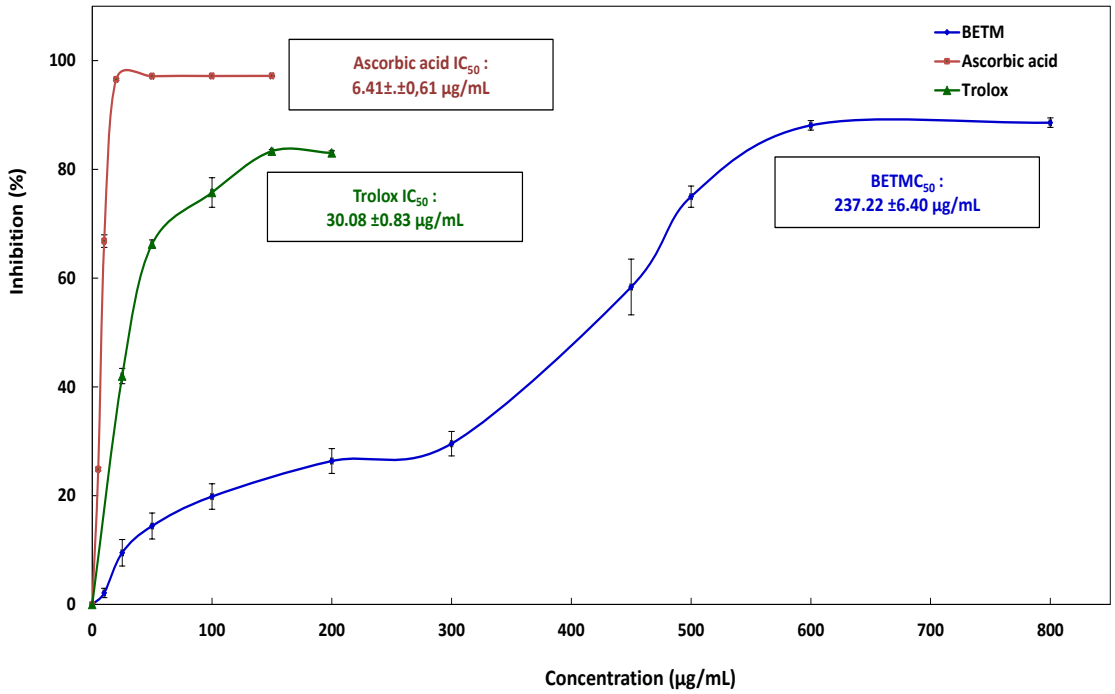


Figure 1: DPPH• scavenging activity of BETM and standards. Values are means ± SD (n=3) P<0.05.

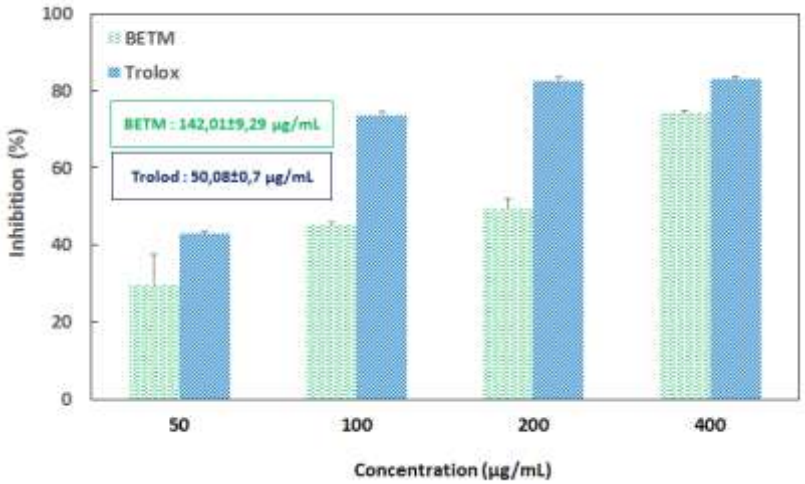


Figure 2: OH• scavenging activities of BETM and standard. Each value represents a mean ± SD (n=3), P<0.05.

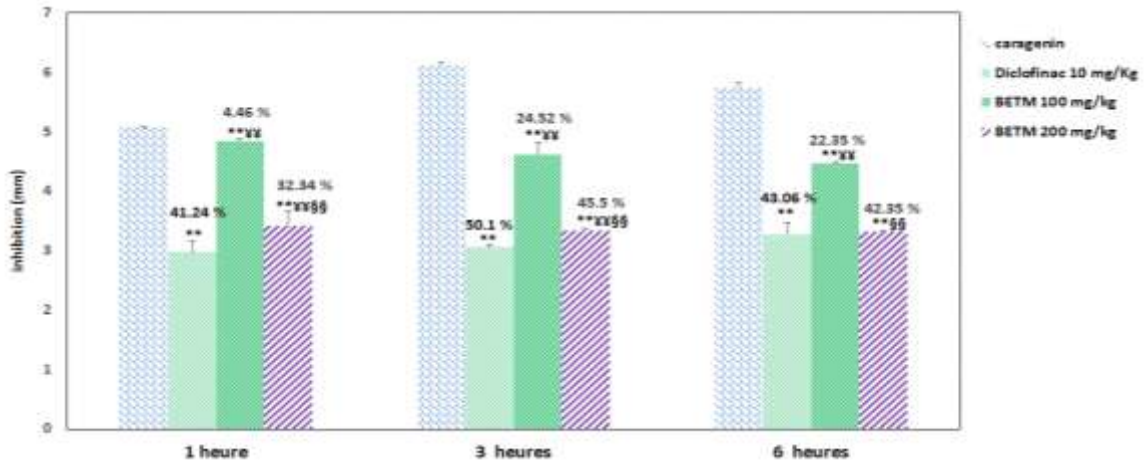


Figure 3: Effect of BETM on carrageenan induced- paw edema in rats. Each value is the mean± SD (n=5); (*: p < 0.05; **: p < 0.01), comparison vs corresponding control; (a: p < 0.05; b: p < 0.01, BETM (100 mg/kg) vs BETM (200 mg/kg); Diclofenac-sodium (10 mg/kg) vs BETM (200 mg/kg. Student's test. Values given at the top of each bar represents percentage of inhibition.

Table 1: The protective effect of BETM on the renal dysfunction induced by cadmium in rat (BUN and creatinine). **Comparison between control and treatments. ŧŧ Comparison between Cadmium and treatments. ¥¥ Comparison between Vit and Extract.

	BUN (mg/dl)	Creatinin (mg/dl)
Control	14,816±0,703	1,198±0,199
Cadmium	87,74±4,040**	3,25±0,27**
Vit E + Cd	34,24±0,97** ŧŧ (**78,70**)	1,75±0,36* ŧŧ (**81,79 %**)
Cd + Extrait	41,18±2,22** ŧŧ ¥¥ (**70,09 %**)	1,32±0,02 ŧŧ ¥¥ (**61,91 %**)

Table 2: Effect of BETM (200mg/Kg) on renal dysfunction induced by $CdCl_2$ (Na^+, K^+ GGT) Values are mean ± SD (n = 7), G2 vs G1; G3, G4 vs G2; **P<0.01; *P<0.05; values in parentheses indicate percent protection. The % of protection is calculated as: 100 x (values of $CdCl_2$)-values of samples/ (values of $CdCl_2$)-values of control, (P<0.001).

Parameters	Control	CdCl2	BETM	Vit E
Na^+ (mEq/L)	152.46±7.96	167.66±1.92**	156.89±3.89§§ (**70.85 %**)	154.64 6.66§§ (**85.65 %**)
K^+ (mEq/L)	6.028±0.33	7.37±0.37**	6.3±0.66§§ (**79.73 %**)	6.27 ±0.7§§ (**81.96 %**)
GGT (UI/L)	2.284±038	4.82±0.37**	2,94±0.67§§ (**74.13 %**)	2.70± 0.32§§ (**83.59 %**)

Table 3: The protective effect of BETM on the oxidative stress induced by cadmium in rat. ** comparison between control and treatments, ŧŧ comparison between Cadmium and treatments, ¥¥ comparison between Vit E and Extract. Values are mean ± SD (n = 7), **P<0.01; *P<0.05; values in parentheses indicate percent protection.

	CAT (µmol/min/mg protein)	GSH (nmol/g tissue)	GST (nmol/g protein)	SOD (u/mg protein)	MDA (nmol/….)
Control	59,376±2,91	32,24±1,96	5,982±0,611	16,462±0,48	34,52±1,132
Cadmium	20,496±0,88**	12,496±0,63**	2,376±0,44**	7,9±0,65**	79,76±7,79**
Vit E + Cd	48,12±2,07** ŧŧ (**71,049 %**)	29,2±1,25 * ŧŧ (**84,6 %**)	4,998±0,12** ŧŧ (**72,71 %**)	15,14±0,43** ŧŧ (**84,55 %**)	46,54±2,18** ŧŧ (**73,43 %**)
Cd + Extrait	46,14±1,7** ŧŧ (**65,95 %**)	26,17±1,19** ŧŧ ¥¥ (**69,27 %**)	4,52±0,26** ŧŧ ¥¥ (**59,45 %**)	14,12±0,51** ŧŧ ¥¥ (**72,64 %**)	40,82±1,14** ŧŧ ¥¥ (**86,07 %**)

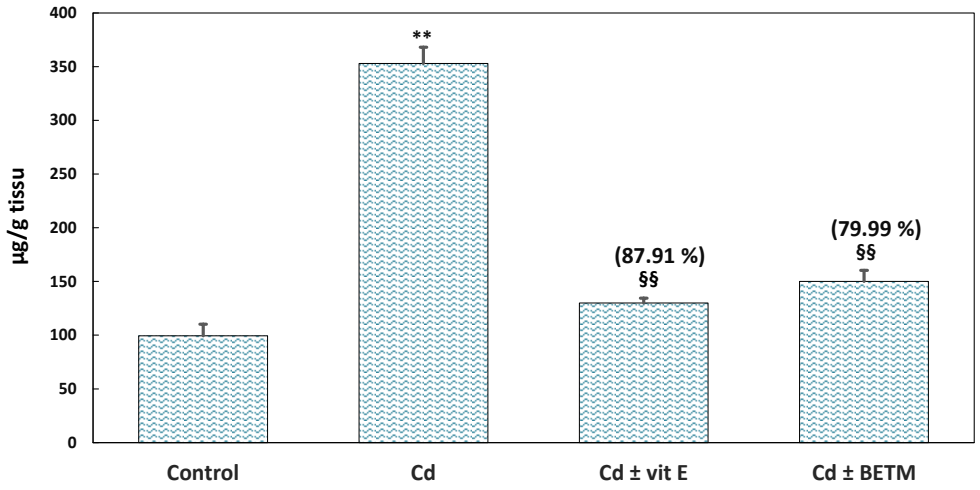
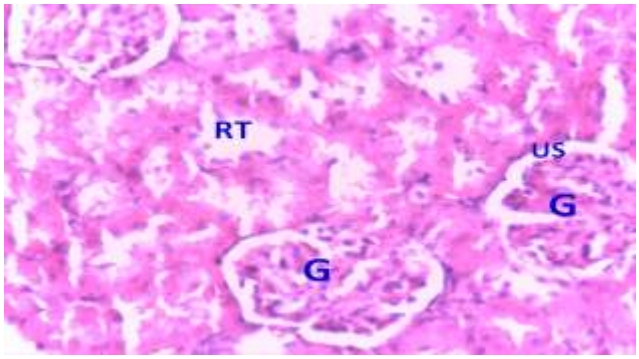


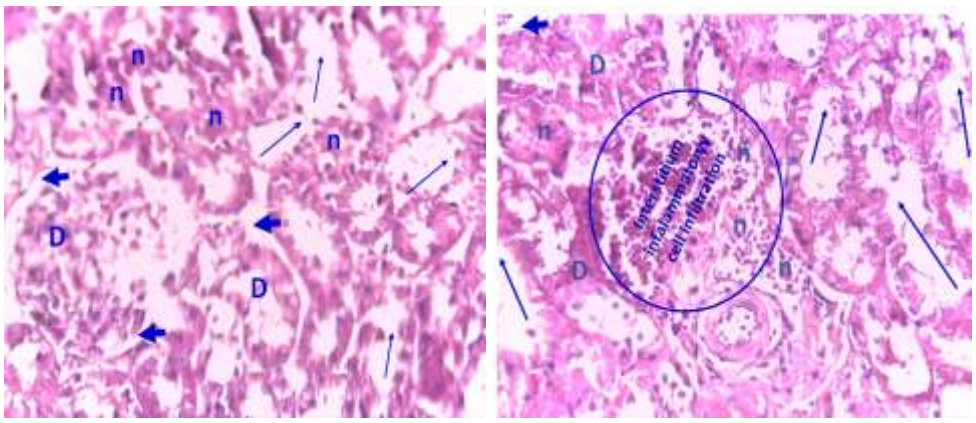
Figure 4. Effect of BETM (200 mg/Kg) on metallothionein level, induced by $CdCl_2$. The % of protection Vit E and BETM on metallothionein induced by $CdCl_2$ values indicates percent protection. The % of protection is calculated as: 100 x (values of $CdCl_2$ – values of samples) / (values of $CdCl_2$-values of control).

Table 4: BETM effect on the oxidative inflammatory markers induced by cadmium in rat. ** Comparison between control and treatments, ŧŧ comparison between Cadmium and treatments, ¥¥ comparison between Vit E and Extract. Values are mean ± SD (n = 7), **P<0.01; *P<0.05; values in parentheses indicate percent protection.

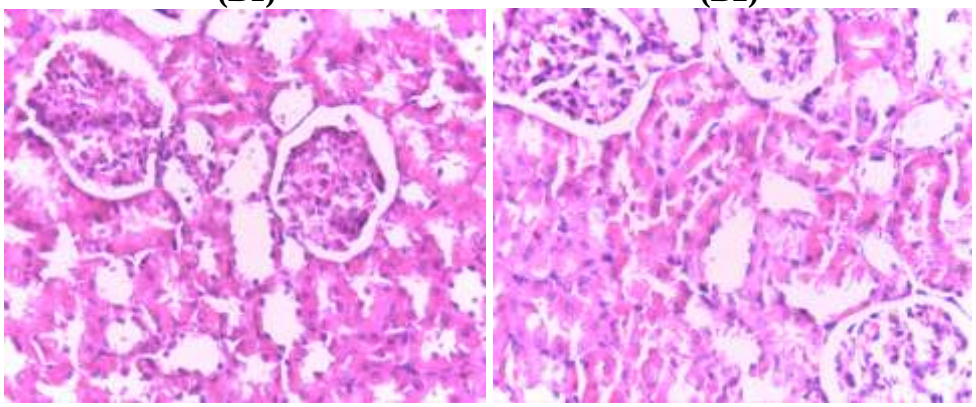
	NO (µg/g tissue)	MPO (nmol/g tissue)
Control	37,98±0,717	28,24±1,028
Cadmium	86,81±2,41**	87,3±1,98**
Vit E + Cd	48,38±1,35** ŧŧ **(78,70 %)**	38,99±1,96** ŧŧ **(81,79 %)**
Cd + Extract	52,59±2,07** ŧŧ ¥¥ **(70,09 %)**	50,74±1,13** ŧŧ ¥¥ **(61,91 %)**



(A) Control



(B1) **(B2)**



(C) Vit E **(D) BETM**

Figure 5: Photomicrograph of Histopathological analysis Renal Cortex of Mice (H&E X400): A (control): normal glomeruli (G) and renal tubules RT (the proximal tubule and distal tubule), B1 and B2 (Cd-treated rats): disrupted renal parenchyma showing loss of structural arrangement of renal tubules (arrows). Severe degeneration in atrophied glomeruli, dilation of Bowman's space (head arrow), widespread proximal tubular necrosis (n), C (Vit E- rats): Vit E pretreatment preserved glomerular architecture and showed a regular epithelial cell of some tubules, moderate tubular necrosis, D (BETM - rats): showed slight degeneration in proximal tubule with a moderate degree of histopathological alteration with some healthy tubules.

DISCUSSION

Kidney is vulnerable to damage because of larger perfusion and the high concentration of excreted compounds in renal tubular cells.[50] Cadmium induces a broad spectrum of toxicological effects and biochemical dysfunctions to health. Many studies have demonstrated that cadmium-induced nephrotoxicity is associated with the proximal tubular damage which results in proximal tubular epithelial cell necrosis in the kidney of rats.[29,51,52,53,54]

Carrageenan is the phlogistic agent of choice for testing antiinflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects. Moreover, the experimental model exhibits a high degree of reproducibility.

In the carrageenan-induced paw edema, the BETM induced significant reduction of paw edema in rat at the dose of 200 mg/Kg as compared to the control.

In the present study increased serum creatinine and BUN reflect the renal failure. Administration of BETM (200mg/kg/day) significantly restored the kidney function and protects the kidney function from Cd intoxication.

In Cd-exposed rats, sodium and potassium serum levels significantly increased. According to several studies, electrolyte disturbances are due to cadmium toxicity in renal tubules.[55] Cadmium increased the retention of sodium and decreased water excretion. In fact, Cd increases sodium reabsorption.[56,57,58] Cadmium also induces cell membrane depolarization and decreases cell membrane resistance; stimulating potassium channels and increasing extracellular potassium concentration.[59] Another mechanism that increases sodium and potassium level is the decrease in functioning nephrons that triggers multiple adaptive processes in the hyper functioning remaining nephrons including augmented rates of electrolyte reabsorption.[60]

Our study also reveals that GGT, was significantly increased in kidney after 28 days of cadmium consumption. This up-regulation presumably enhances the renal GSH uptake from the blood stream, and this mechanism may contribute to the decrease of the GSH concentration in plasma. The kidney is characterized by the highest GGT activity, nearly 900 times higher than in the liver.[61] Furthermore, the very rapid turnover of cellular GSH has been demonstrated in the kidney and it is considered fundamental for this organ.[62]

In the present study, the increased level of TBARS observed in the renal tissues of rats exposed to Cd (8mg/kg) indicated a high degree of lipid peroxidation and oxidative stress.

It is well known that cadmium may interferes with antioxidant defense mechanisms together with the production of ROS, which may act as a signaling molecule in the induction of cell death.[63] Reactions of these ROS, which consist mainly of O_2° H_2O_2 and OH°,[64] with cellular biomolecules have been shown to lead to lipid peroxidation, membrane protein damage, altered antioxidant system, altered gene expression, DNA damage and apoptosis.[65,66] Although cadmium it indirectly generates various radicals such as superoxide radical, nitrogen species such as peroxynitrite, nitric oxide and hydroxyl radical thus causing damage consistent with oxidative stress.[67,68] Suppression of free radical scavenging function and the enhancement of ROS, contribute to Cd-induced oxidative stress and its associated toxic effects.[69,63] On the other hand, increased ROS levels in renal cells induce the gene expression of the multidrug resistance transporter gene which protects proximal tubule cells against apoptosis.[70] Lipid peroxidation, though indirectly involved in the generation of free radicals, is considered as the primary mechanism for Cd-induced toxicity.[71,72] Cadmium is thought to induce lipid peroxidation, lipid hydroperoxides and protein carbonyl contents and this has often been considered to be the main cause of its deleterious influence on membrane dependent function.[73,74,75] Several experimental studies indicate that Cd exposure rapidly induces membrane damage in kidney cells through the process of lipid peroxidation A study has demonstrated that the highest increase of MDA in rat kidney occurs 24 h after *i.p.* application of Cd (2.5 mg Cd/kg),[76] while a two times higher Cd dose applied via the same route application caused an increase in MDA after 72 h.[77]Experimental studies confirmed increased levels of TBARS/MDA, in the kidney of Cd-treated animals exposed to Cd by different routes and over different periods of time (1-12 weeks).[78,79,80,52,81,82]

The high MDA level mentioned in Cd-group our investigation was associated with a marked depletion of renal GSH by Cd exposure due to the direct binding of Cd to enzyme active sites, if it contains –SH groups.[83] Significant decrease in the levels of these non-enzymatic antioxidants in Cd toxicity could lead to increased susceptibility of the renal tissue to free radical damage .GSH is a non-enzymatic antioxidant which acts as a first line of defense against oxidative stress.[84] The significant reduction in GSH levels that was recorded can be attributed to the increased use of GSH (by the renal tissues) to mop up excessive ROS that

were generated during the process of Cd-induced renal injury.[66] The depletion of GSH also seems to be a prime factor that permits lipid peroxidation in the Cd-treated group.

A marked decrease in the antioxidant enzymes (SOD, CAT and GST) was also observed in the renal tissues exposed to Cd, which indicates the failure of antioxidant defense system to overcome the influx of ROS on Cd exposure. Cd exposure inactivate a most of the antioxidant enzymes by the direct binding to enzyme active sites,[83] or by the displacement of metal cofactors from active sites.[85] Thus, the inhibition of enzymes involved in free radical removal leads to the accumulation of H_2O_2, which promotes lipid peroxidation and cell death.[68,63]

The present investigation revealed that rat exposure to CdCl2 (5mg/Kg, 28 days) decreased SOD, CAT and GST activities. The same effects on renal CAT activity were observed after prolonged oral exposure to 5 mg/kg of CdCl2 for 4 weeks.[82] Similar effects on blood oxidative status were observed after prolonged exposure to Cd (1 mg/kg; 21 days).[86] As well as oral Cd administration (40 mg/L ;drinking water ; 30 days) revealed significant decreases in the activity of SOD and CAT in serum of rats.[87] Other routes of Cd intoxication induced similar effects: *i.p.* injection of CdCl2 (6.5 mg kg) for 5 days and *s.c.*injection of Cd (1.2 mg Cd/kg) 5 times/week during 9 weeks resulted in significant decreases in renal SOD and CAT activities.[78,52] It was reported that oral exposure to Cd via drinking water (250 mg/L) during 10 and 30 days decreased significantly renal total SOD activity, as well as CAT.[76]

Acute Cd intoxication generally inhibits the activity of antioxidative enzymes SOD and CAT. Many studies demonstrated decreased levels of enzymatic antioxidants in the kidney of animals exposed to prolonged Cd intoxication. However, several studies showed that prolonged Cd exposure decrease the activity of antioxidant enzymes, especially in kidneys, as a result of the effect of Cd on gene expression.[79,88]

Cd-induced nephrotoxicity is thought to be mediated through the Cd–MT complex, which is synthesized in the liver, released into circulation, and taken up by renal proximal tubule cells.[89]In fact, when the synthesis of MT becomes insufficient for binding all Cd ions in the liver, Cd not bound to MT produce hepatocyte injury and a Cd–MT complex is released into the bloodstream. The complex in the plasma is then filtered through the glomeruli in the kidney and taken up by the proximal tubular cells.[90,91,92] Studies conducted on mammals have revealed kidney injury, associated with release of the Metallothionein (Mt)/Cd complex

in peripheral circulation, after liver saturation. On its way through the kidneys, this complex causes injury, mainly in the cortical region, reaching the proximal tubule and causing a gradual loss of the organ's function.[93]

The renal MPO activity elevation may indicate the presence of enhanced polymorph nuclear leukocytes (PNL) recruitment in the inflamed tissue .The neutrophils have a role in oxidant injury via several mechanisms such as the NADPH oxidase or MPO action.[94] Hypochlorous acid is produced largely from stimulated neutrophils via MPO which catalyzes its production. On the other hand, as a secondary damaging agent, hypochlorous acid oxidizes other molecules including proteins, amino acids, carbohydrates, nucleic acids, and lipids.[95] There is increasing evidence that NO, a potentvasodilator, is one of the most important paracrine modulatorsand mediators in the control of renal functions, such asoverall and regional renal blood flow, renal autoregulation, glomerular filtration, renin secretion, and salt excretion.[96,97] NO also plays an important role in the pathogenesis ofseveral renal disease states, such as diabetic nephropathy, inflammatory glomerular disease, acute renal failure, andnephrotoxicity of drugs/metals, conveying both beneficial effectsvia its hemodynamic functions.[96,97] Nitric oxide reacts with superoxide anions and produces peroxynitrite radicals that cause further cell damage and oxidative stress in kidney. Along with ROS induction, experimental studies have confirmed the role of ROS induction, i.e. NO excessive synthesis, in Cd-induced oxidative stress. Intraperitoneal injection of 6.5 mg CdCl2/kg b.w. given to rats for 5 days resulted in elevated levels of NO, which reacts with O_2^-. And produces peroxynitrite radicals that contribute to further oxidative cell damage.[78] The overexpression of NO can be attributed to the up regulation of iNOS in the conditions of the inflammation reaction triggered by Cd.[98]

Our findings showed that biochemical and histopathologic results were in accordance with each other. Histopathological observation in Cd-treated rats showed the tubular necrosis, inflammatory cell infiltration, tubular degeneration, hemorrhage, swelling of tubules and vacuolization. This could be due to the accumulation of free radicals as the consequence of increased lipid peroxidation by free Cd ions in the renal tissues of Cd-treated rats. Our study reported that *Thymelaea microphylla* significantly reduces the histological changes induced by cadmium.

The n- BuoH extract contains 257.4 mg/g of polyphenols which are powerful chainbreaking. These results confirmed the ability of BETM to improve or normalize the renal function

through its organosulfur compounds that could increase the antioxidant effect and decrease lipidperoxide levels by scavenging the free radicals and increasing intracellular concentration of glutathione. This study showed that the BETM is efficient to prevent CdCl2-induced nephrotoxicity in rat; this may be due to the polyphenol content of antioxidant parameters by restoring them to near normal. The antioxidant property and the ability of BETM to inhibit the radical generation could further reduce the oxidative threat caused by cadmium, which could mitigate the consumption of endogenous non-enzymatic antioxidants and increased their levels in the kidney tissue. These findings indicate that different biophenol compounds contained in BETM may have different mechanisms of action against CdCl2 nephrotoxicity.

CONCLUSION

In conclusion, this study demonstrates that *Thymelaea microphylla* has the protective effect against Cd-induced oxidative damage in the kidney of rats. The mechanisms contributing to its effectiveness may be the quenching of free radicals, antioxidant and metal chelating ability of *Thymelaea microphylla*. Recently much attention has been focused on the protective biochemical functions of naturally occurring antioxidants in biological systems against toxic heavy metals.

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