

Protective Effect of *Celastrus paniculatus* Seed Extract against Lead Acetate Induced Nephrotoxicity in Wistar Rats

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ABSTRACT

Objectives: The plant *Celastrus paniculatus* is used in the traditional medical practices of India to treat a plethora of diseases. Earlier research on the plant revealed several biological properties and interesting bioactive compounds with significant medicinal uses. **Materials and Methods:** In this study, the ethanolic extract of the seeds of the plant (EECP) has been investigated against lead acetate (LA) induced nephrotoxicity in Wistar rats. Thirty rats were divided into five groups ($n=6$) wherein group 1 that contained normal animals served as control while group 2 received LA (30mg/kg b.w/day, p.o.). Animals in groups 3 – 5 received respectively the standard drug N-acetylcysteine (NAC, 200mg/kg b.w/day, p.o.) and EECP in two doses (400 and 800mg/kg b.w/day, p.o.) together with LA (30mg/kg b.w/day, p.o.) for 28 consecutive days. On day 29, all the animals were sacrificed and the blood and kidney were collected for analysis. **Results and Conclusion:** LA significantly decreased the level of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), reduced glutathione (GR), glutathione S-transferase (GST) and glutathione (GSH) and increased the level of hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot) and lipid peroxidation (LPO) as compared to those in control rats. Administration of EECP especially 800mg/kg b.w. significantly downregulated the serum urea, creatinine and KIM 1 levels and also the level of oxidative stress markers when compared to the LA group. Histological and immunohistochemistry (KIM 1) study showed a significant change in the cytoarchitecture of the renal tissue thereby revealing the pro-active role of *C. paniculatus* seeds in nephrotoxicity.

Key words: *Celastrus paniculatus* seeds, Free radical scavenging activity, KIM 1, Lead Acetate, Nephro-toxicity, Wistar rats.

INTRODUCTION

Celastrus paniculatus Willd. (Family: Celastraceae) known as *Maalakaangani* or *Vaaluhuvai* in Tamil and *Jyotishmati* in Sanskrit is used in Ayurveda and Siddha systems of medicine to treat neurological disorders. Various researches carried out on this plant reported analgesic, antidepressant, anti-inflammatory, antioxidant, neuroprotective and nootropic effects.¹⁻³ As the seeds were reported to possess significant antioxidant property, their protective effect against lead acetate induced

nephrotoxicity has been investigated in this study by estimating biochemical, oxidative stress and antioxidant markers, histological and immunohistochemistry of KIM 1 expression.⁴

The kidney is one of the most highly differentiated organs in the body that modulates a variety of physiologic processes especially the removal of drug metabolites. As per the Global Burden of Disease Study (2010), kidney diseases ranked 18th in the list of

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causes of the total number of deaths worldwide that stood 27th in 1990.⁵ About 10% of the population worldwide is affected by kidney diseases and in several countries, its prevalence is increasing, mostly owing to the aging population and changing lifestyles, parallel to the growing prevalence of obesity.⁶ Oxidative stress in renal tissue occurs due to the production of reactive oxygen species (ROS) by activated inflammatory cells, such as macrophages and eosinophils. The superoxides production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in phagocytes and endothelial cells is also the major cause of oxidative stress.⁷ The presence of naturally-occurring and chemically manufactured toxicants in the environment, individuals are exposed frequently over their lifetime to toxicants that can adversely affect the kidney.⁸ Lead Acetate (LA) has been found to negatively impact antioxidant activity by interfering with the metals that are essential for antioxidant enzyme activities.⁹ Prevention, early identification and treatment of the underlying cause are essential strategies to be followed for patients with kidney diseases.

MATERIALS AND METHODS

Chemicals

All chemicals used in the study were of analytical grade procured from SISCO Research Laboratories Private Limited and D. K. Enterprises, India. The reagents for antioxidants and oxidative markers were procured from Sigma Aldrich, USA.

Procurement and preparation of the plant material

C. paniculatus seeds were procured from M/s. Herbal Care and Cure Centre, Chennai and authenticated by Dr. V. Chelladurai, Pharmacognosist, St. Xavier's College, Tirunelveli. They were shade dried and coarsely powdered using mortar and pestle. About 500g of this was soaked in 1L of 90% ethanol in the aspirator bottle. After 72 h, the solvent was filtered and distilled off. Final traces of the solvent were removed under vacuum to obtain the extract (EECP) for biological studies. Earlier reports revealed that the extract was safe up to a dose of 5000 mg/kg b.w. in rats.¹⁰

Experimental animals

The study was conducted after obtaining approval from the Institutional Animal Ethics Committee (No: SU/CLAR/RD/002/2019) dated 09.08.2019. Thirty female Wistar rats (180 ± 20g) procured from Biogen Animal Facility, Bangalore. During acclimatization and experimental periods, the animals were housed in normal laboratory conditions (25 ± 2°C, relative humidity:

50-70% and 12 h light-dark cycle) and fed with the standard pellet diet and free access to water.

Experimental design

The animals were divided into five groups (*n*=6) as follows and the study was carried out for 28 days.

Group 1: Saline (2 mL/kg b.w/day, p.o.)

Group 2: LA (30mg/kg b.w/day, p.o.)

Group 3: NAC (200 mg/kg b.w/day, p.o.) + LA (30 mg/kg b.w/day, p.o.)

Group 4: EECP (400mg/kg b.w/day, p.o.) + LA (30 mg/kg b.w/day, p.o.)

Group 5: EECP (800 mg/kg b.w/day, p.o.) + LA (30 mg/kg b.w/day, p.o.)

After the study period, on day 29, the animals were anesthetized using 1% isoflurane for blood collection and euthanized to harvest the kidneys which were stored at 80°C for further analysis.

Estimation of serum urea and creatinine

About 2 mL of blood was collected in heparinized Eppendorf tubes from the retro-orbital sinus by a capillary tube and centrifuged to separate serum for biochemical analysis. Renal biomarkers urea and creatinine were determined by standard methods.^{11,12}

Estimation of oxidative stress and antioxidant parameters

The renal tissues were homogenized and analyzed for catalase, glutathione peroxidase, glutathione reductase, glutathione, glutathione S-transferase, superoxide dismutase activities besides estimating hydrogen peroxide, lipid peroxide, hydroxyl radical by established procedures.¹³⁻²¹ Estimation of Serum Kidney Injury Molecule 1 (KIM 1). The presence of KIM-1 in serum were detected using the KIM 1 ELISA kit according to the instructions in the manufacturer's manual. The samples along with coating buffer were mounted to 96-well plates and incubated overnight at 4°C. The remaining protein-binding sites were then blocked by incubating samples for 1.5 h with blocking buffer containing 2% fetal bovine serum at 37°C. Primary mouse anti-rat KIM-1 monoclonal antibodies (dilution, 1:1,000) and goat anti-mouse IgG secondary antibodies (dilution, 1:2,000) were added to bind specifically with the target antigen. Following treatment with 3,3-diaminobenzidine (DAB) solution from the ELISA kit and stop buffer, the plates were read at 490 nm using a microplate reader.

Histopathological Studies

After the experimental period, the animals were euthanized; the kidneys were harvested and fixed in 10%

formalin. The dehydration process was employed by immersing the tissues in a series of ethanolic solutions of increasing concentration to avoid excessive distortion of the tissue. The tissues were embedded in the paraffin embedding medium and the paraffin sections were cut at a thickness of 3-5 μ m with a rotatory microtome. The slides were stained with Hematoxylin and Eosin (H&E) and observed under 100X and 400X magnifications with the compound microscope. The cortex and medullary regions of the kidney were examined for cytoarchitectural changes and photographs were done.

Immunohistochemistry analysis of expression of KIM 1

Immunohistochemistry staining against KIM 1 was performed. After antigen retrieval (100 \times Citrate Buffer) for 20 min in a domestic pressure cooker and blocking non-specific binding sites with protein block, the sections were immunoreacted with primary antibodies against ALA-D (Thermo Fischer Scientific Company) overnight at 4 $^{\circ}$ C, respectively. KIM-1 antibody (R and D Systems, Minneapolis, USA) and AQP-1 rabbit polyclonal antibody (EMD Millipore, Temecula, USA) were applied overnight at 4 $^{\circ}$ C. The hematoxylin stain was used to counterstain the slides. Finally, the sections were incubated with DAB-hydrogen peroxide for 30 min and washed in water, counterstained and viewed under the light microscope. Five 200X microscopic views per slide were selected randomly and photographed using Image J Software.

Statistical analysis

The data obtained from the experiments were analyzed using One-way ANOVA with SIGMA PLOT 13 using SYSTAT software followed by Newman Keul's test for comparison between the groups. The values were expressed as mean \pm SEM and those with $P < 0.001$ were considered statistically significant.

RESULTS

Effects of EECP on serum urea and creatinine

The effect of EECP on serum urea and creatinine are shown in Table 1. The LA treated animals in group 2 showed a noticeable elevation in urea and creatinine when compared to the normal rats in group 1. However, in EECP-treated groups, there was a remarkable decline in their levels as compared to standard-drug treated group 3. The decrease was highly significant in the 800mg treated group.

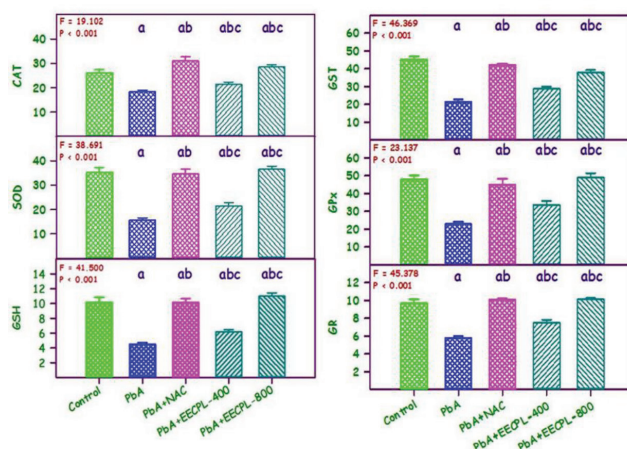


Figure 1: Effect of ethanolic extract of *C. paniculatus* seeds 400 mg/kg (EECP-400) and 800 mg/kg (EECP-800) compared with n-acetylcysteine (NAC 200 mg/kg) in lead acetate toxicity (LA, 30 mg/kg) on antioxidant markers. Data are expressed as mean \pm SE ($n = 6$ each). The 'F' and 'P' values are by one way ANOVA with Student Newman Keul's multiple comparison test. a: Significantly different from the control group, b: Significantly different from LA group, c: Significantly different from NAC group.

Effects of EECP on oxidative stress markers and antioxidant enzymes

The LA-induced nephrotoxic rats showed a significant increase in oxidative stress markers along with decreased levels of antioxidant enzymes in renal tissues as compared to normal animals. The increased levels of oxidative stress markers were found to be reverted to near normal status after the administration of EECP (800mg/kg b.w.). Co-administration of EECP also significantly increased antioxidant enzyme activities in comparison with group 3 rats (Figure 1 and Table 1) (Figure 2 and Table 2). The EECP was found to possess an antioxidant effect in a dose-dependent manner.

Effects of EECP on serum KIM 1

The level of the serum KIM-1 showed in Figure 3. There was a significant up-regulation of KIM-1 in the LA group compared with control animals. Administration of NAC (200 mg/kg b.w.), 400 and 800mg/kg b.w. of EECP were significantly attenuated the up-regulated KIM 1. EECP (800mg/kg b.w.) and NAC offered full protection when compared to control animals, whereas EECP (400mg/kg b.w.) offered partial protection when compared to control animals. It was found to be statistically significant ($P < 0.001$).

Effect of EECP on the histology of kidney

The histology of the renal tissues of control, NAC (200mg/kg b.w.) and EECP (800mg/kg b.w.) showed normal cytoarchitecture which compared with LA

Table 1: Protective effect of *Celastrus paniculatus* seed extract on lead acetate induced changes in antioxidant markers. Value are mean±SEM (n = 6 each).

Group	CAT	GSH	SOD	GST	GPx	GR
Control	26.167±1.22	10.167±0.65	35.333±1.91	45±2.08	47.833±2.22	9.655±0.45
PbA	18.333±0.61	4.5±0.22	15.667±0.88	21.167±1.42	22.833±1.32	5.775±0.17
NAC	31±1.84	10.167±0.47	34.667±1.96	42±0.81	45±3.29	10.075±0.18
EECPL-400	21.333±0.98	6.167±0.30	21.5±1.31	28.833±1.01	33.667±2.04	7.475±0.35
EECPL-800	28.5±0.88	11±0.45	36.5±1.29	37.833±1.537	49±2.30	10.108±0.14

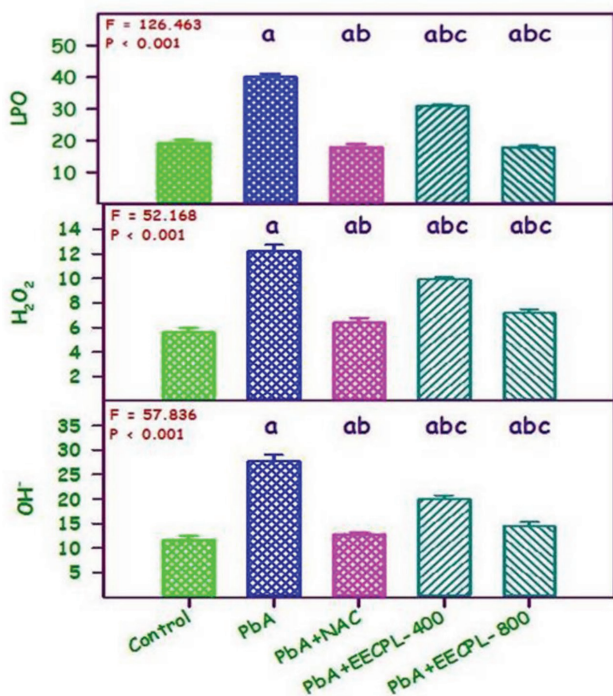


Figure 2: Effect of ethanolic extract of *C. paniculatus* seeds 400 mg/kg (EECPL-400) and 800 mg/kg (EECPL-800) compared with n-acetylcysteine (NAC 200 mg/kg) in lead acetate toxicity (PbA, 30 mg/kg) on oxidative stress markers. Data are expressed as mean + SE (n = 6 each). The 'F' and 'P' values are by one way ANOVA with Student Newman Keul's multiple comparison test. a: Significantly different from the control group, b: Significantly different from PbA group, c: Significantly different from NAC group.

Table 2: Protective effect of *Celastrus paniculatus* seed extract on lead acetate induced changes in oxidative markers. Value are mean±SEM (n = 6 each).

Group	LPO	H ₂ O ₂	-OH
Control	19.167±1.04	5.585±0.53	11.667±0.80
PbA	40±1.06	12.197±0.53	27.667±1.33
NAC	17.833±1.04	6.368±0.43	12.833±0.40
EECPL-400	30.833±0.54	9.91±0.18	20±0.77
EECPL-800	17.833±0.53	7.173±0.30	14.5±0.76

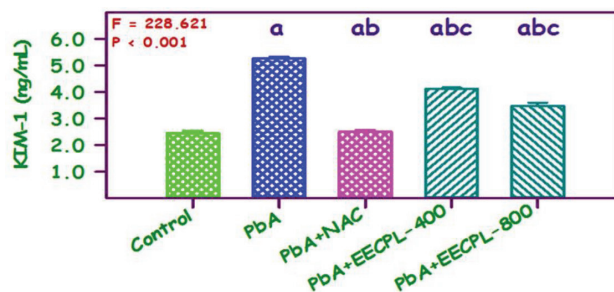


Figure 3: Effect of ethanolic extract of *C. paniculatus* seeds 400 mg/kg (EECPL-400) and 800 mg/kg (EECPL-800) compared with n-acetylcysteine (NAC 200 mg/kg) in lead acetate toxicity (PbA, 30 mg/kg) on serum KIM 1 markers. Data are expressed as mean + SE (n = 6 each). The 'F' and 'P' values are by one way ANOVA with Student Newman Keul's multiple comparison test. a: Significantly different from the control group, b: Significantly different from PbA group, c: Significantly different from NAC group.

(30mg/kg b.w) and EECPL (400mg/kg b.w). The micro-anatomy of the kidney with normal glomeruli having well-structured bowman's capsule, distal and proximal convoluting tubules in the cortex and collecting duct in the medulla in control, NAC (200mg/kg b.w) and EECPL (800mg/kg b.w). In LA (30mg/kg b.w) administered animals showed atrophy of glomeruli and dilatations were seen in Bowman's capsule, the renal tubules are also dilated with coagulative necrosis. Co-administration of LA and EECPL (400mg/kg b.w) showed shrunken glomeruli, inflammation and degenerative changes in renal tubules and mild coagulation necrosis in renal tissue (Figure 4).

Effect of EECPL on the expression of KIM 1 using immunohistochemistry

The immunohistochemistry of the expression of KIM-1 in the renal tissues was shown in Figure 5. The renal tissue of the LA (30mg/kg b.w) administered group showed immunoprecipitation of KIM-1 in the glomeruli and renal tubules, more commonly in the proximal tubules. Moreover, in EECPL (400mg/kg b.w) administered animals showed minimal immunoprecipitation when

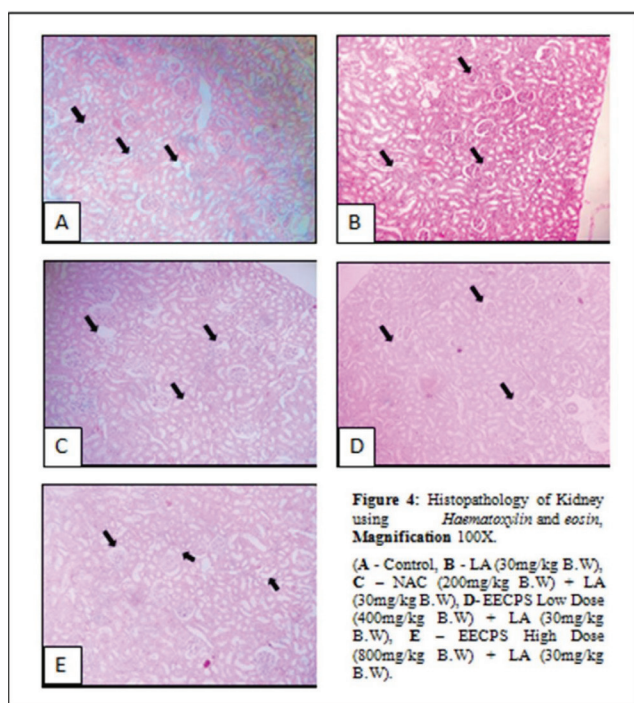


Figure 4: Histopathology of Kidney using Haematoxylin and eosin, Magnification 100X.

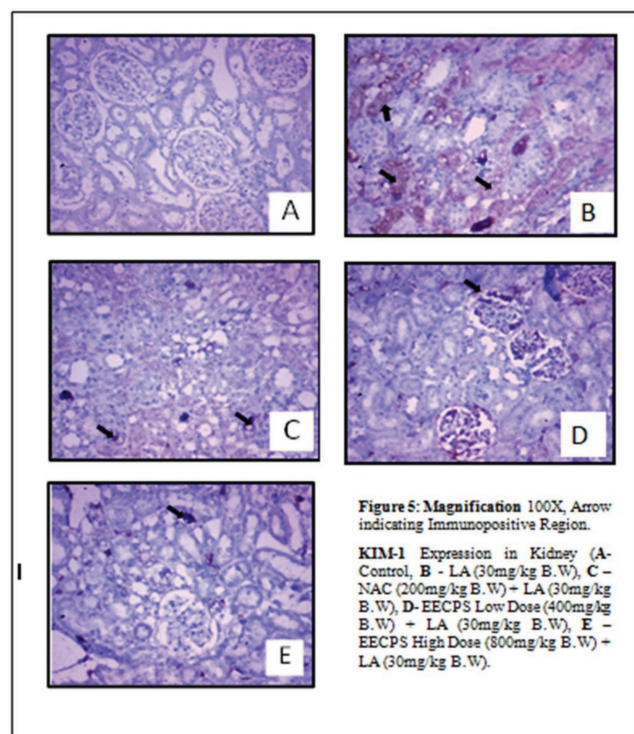


Figure 5: Immunohistochemistry KIM 1 Expression, Magnification 100X, Arrow indicating Immunopositive Region. KIM-1 Expression in Kidney (A- Control, B - LA (30mg/kg B.W), C -NAC (200mg/kg B.W) + LA (30mg/kg B.W), D- EECPS Low Dose (400mg/kg B.W) + LA (30mg/kg B.W), E – EECPS High Dose (800mg/kg B.W) + LA (30mg/kg B.W).

compared to LA administrated group. No immunoreactivity of KIM-1 in the NAC (200mg/kg b.w) and EECPS (800mg/kg b.w) groups which is similar to the control group that also showed no immunoreactivity of KIM 1.

DISCUSSION

Heavy metal toxicity is a serious issue that affects living organisms and causes morbidity and mortality in humans. It is also reported to be of environmental concern in many parts of the world. Lead, a ubiquitous heavy metal and a known toxicant also produces adverse effects on the brain via increased production of reactive oxygen species (ROS) and causes oxidative stress.²² This, in turn, results in oxidative stress at the cellular level causing a reduction in the sarcolemmal calcium pump and $\text{Na}^+ \text{K}^+ \text{ATPase}$ intracellular Ca^{2+} overload. Lead can substitute for calcium ions (Ca^{2+}) and picomolar concentrations of Pb^{2+} can replace micromolar concentrations of Ca^{2+} in a protein kinase C (PKC) enzyme assay, a calcium-dependent process.²³ The literature on lead toxicity also mentions its adverse effect on kidney function due to increased oxidative stress in renal tissues. Various remedies were suggested to tackle this malady in which the use of plants and plant-derived metabolites has been found to play a key role. The utility of plants as a remedial measure is a subtle approach because of their easy availability and accessibility.²⁴ In this investigation, the seed extract of *C. paniculatus* (EECP) has been taken up to find out its free radical scavenging potential in lead acetate induced nephrotoxicity in the rat model. The antioxidant activity of *C. Paniculatus* seeds in rats intoxicated with aluminum was already evaluated and its protective effect on the levels of antioxidants CAT, GSH and SOD were investigated. The results showed that aluminum significantly decreased the above parameters on treatment with *C. paniculatus* seeds at the dose of 200mg/kg b. w. significantly increased.²⁵ The antioxidants present in the plant prevent the ROS-oxidation of vital molecules in the cell and scavenge free radicals thereby reducing the harmful effects.^{26,27}

A similar study on *C. paniculatus* seed extract showed scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.²⁸ The aqueous extract of *C. paniculatus* seeds at the dose of 200 mg/kg b.w for 14 days showed significant improvement in learning and memory in animals. The extract also stimulated a significant decrease in the level of malondialdehyde with a concomitant increase in the levels of GST and CAT. The effects of ethanolic and methanolic extracts

of *C. paniculatus* seed oil against H_2O_2 and glutamate-induced superoxide scavenging were determined using neuronal cell culture. The results showed that *C. paniculatus* seed oil protected cells against H_2O_2 induced oxidative stress by their ability to improve the levels of antioxidant enzymes.²⁹

In an *in-vitro* study, the antioxidant defense of ethanolic extract *C. paniculatus* seeds on murine C_2C_{12} myoblasts cells was experimentally proved. The protective effect of *C. paniculatus* seed extract-treated against t-BHP treatment was confirmed by antioxidant markers of C_2C_{12} muscle cells. The levels of antioxidant markers were reduced in t-BHP treated cells, which were significantly restored in *C. paniculatus* seed extract-treated cells.³⁰

The present study demonstrated that LA ingestion led to a significant increase in H_2O_2 , OH^- and LPO levels in renal tissues. Administration of EECP led to a marked decrease in the elevated levels of these parameters in rat tissues. This is attributed to the antioxidant properties of *C. paniculatus* seeds that protect rat's kidneys from oxidative damage and repair the antioxidant system. The decreased levels of antioxidant enzymes (CAT, GPx, SOD, GR, GST and GSH) was improved near to normal values after EECP administration especially at 800mg/kg b.w. dose when compared to the standard drug-treated animals (Figure 1 and 2).

The Food and Drug Administration and European Medicines Agency recognized KIM-1 to be a sensitive marker for finding renal damage. KIM-1 (25 Kda) is a transmembrane glycoprotein with an immunoglobulin domain and mucin.³¹ Serum KIM-1 is a sensitive marker of kidney damage in the general population, particularly in middle-aged adults. Serum KIM 1 is a potential predictive biomarker when compared with urine KIM1.^{32,33} Hence, in this study, serum KIM-1 was used as the parameter to assess the renal damage.

KIM 1 is a novel marker that is used to analyze the damage in addition to urea and creatinine, which are standard markers. The gentamicin induced nephrotoxicity in rats up-regulated the KIM 1 along with standard renal markers and antioxidant enzymes ameliorated by plant extract.³⁴ A similar result was reported in cadmium-induced nephrotoxicity in male mice.³⁵ In this study, there was significant up-regulation of KIM 1 in LA administrated animals which were attenuated by EECP (800mg/kg b.w) (Figure 3).

The histopathological finding of this study showed that LA (30 mg/kg b.w) produces significant nephrotoxicity of the kidneys such as shrunken and atrophy of glomeruli, inflammation and degenerative changes in renal tubules and mild to moderate coagulation necrosis

in renal tissue (Figure 4). These findings in LA administrated animals correlate with previous studies.^{36,37}

Apparent renal markers such as KIM-1, NGAL, clusterin, vimentin, uromodulin, nephrin and netrin are investigated using immunohistochemistry. The early detection of renal damage is vital to protect it from progressive and severe complications.³⁸ The animals administrated with LA produced a significant expression of KIM-1 in the renal cortex specifically the apical part of renal tubules showed more expression of KIM-1 indicating damage in the kidney which was similar to the earlier findings.³⁹ Co-administration of LA and standard drug NAC (200 mg/kg b.w) showed no expression of KIM 1, similar to the previous report of cisplatin-induced nephrotoxicity.⁴⁰ EECP (800 mg/kg b.w) showed expression of KIM 1, similar to NAC (200 mg/kg b.w) (Figure 5), Whereas EECP (400 mg/kg b.w) showed minimal expression of KIM-1 in the immunohistochemistry of the renal tissue.

In this study, co-administration of *Celastrus paniculatus* seed (800mg/kg b.w) reverted the serum parameters and microanatomy of renal tissue from LA induced toxicity. The flavonoids in *Celastrus paniculatus* seed modulate the adverse effects of lead acetate and redeem the kidney damage. The results demonstrate that EECP has effectively ceased the LA induced oxidative stress and renal damage by providing free radical scavenging property and by assisting in the synthesis of glutathione. Thus, EECP exhibits nephroprotection by above said two mechanisms which required further studies to explore which might aid in greater characterize the mechanisms.

CONCLUSION

The results obtained in this study suggest that *C. paniculatus* seeds possess significant nephroprotective properties which might be due to secondary metabolites present in them. Identification and isolation of these bioactive compounds will give scope for future research in finding useful drugs to overcome lead toxicity.

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CONFLICT OF INTEREST

The authors don't have any conflicting interests.

ABBREVIATIONS

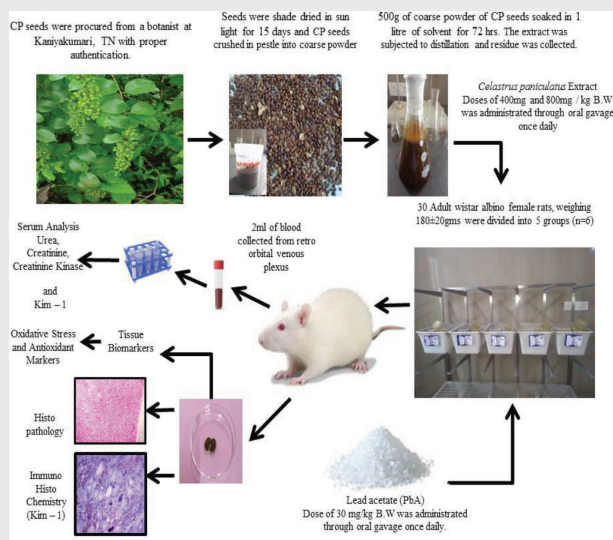
EECP: Ethanolic extract of the *Celastrus paniculatus*; **CP:** *Celastrus paniculatus*; **LA:** Lead acetate; **CAT:** Catalase; **GPx:** Glutathione peroxidase; **SOD:** Superoxide dismutase; **H₂O₂:** Hydrogen peroxide; **GR:** Reduced glutathione; **GST:** Glutathione S-transferase; **GSH:** Glutathione; **OH[·]:** Hydroxyl Radicals; **LPO:** Lipid peroxidation; **KIM 1:** Kidney injury molecule 1; **NGAL:** Neutrophil gelatinase-associated lipocalin; **ROS:** Reactive oxygen species; **NADPH:** Nicotinamide adenine dinucleotide phosphate; **DPPH:** 1,1-diphenyl-2-picrylhydrazyl; **Ca²⁺:** Calcium ion; **Pb²⁺:** Lead ion; **PKC:** Protein kinase C; **ANOVA:** Analysis of variance; **SEM:** Standard error of mean; **mL:** Milli Liter; **°C:** Degree Centigrade; **mg:** Miligram; **kg b.w:** Kilogram body weight.

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PICTORIAL ABSTRACT



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