Pharmacogn. Mag.
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ORIGINAL ARTICLE

Protective Effect of *Carica papaya* Leaf Extract against Mercuric Chloride-Induced Nephrotoxicity in Wistar Rats

Yuvaraj Maria Francis, J Vijayakumar, Gunapriya Raghunath, S. Vijayalakshmi, Senthilkumar Sivanesan[1], R. Vijayaraghavan[1], Ethirajan Sukumar[1]

Departments of Anatomy and [1]Research and Development, Saveetha Institute of Medical and Technical Sciences (Deemed to be University) Chennai, Tamil Nadu, India

Submitted: 08-Jan-2020 Revised: 26-Feb-2020 Accepted: 17-Mar-2020 Published: 28-Aug-2020

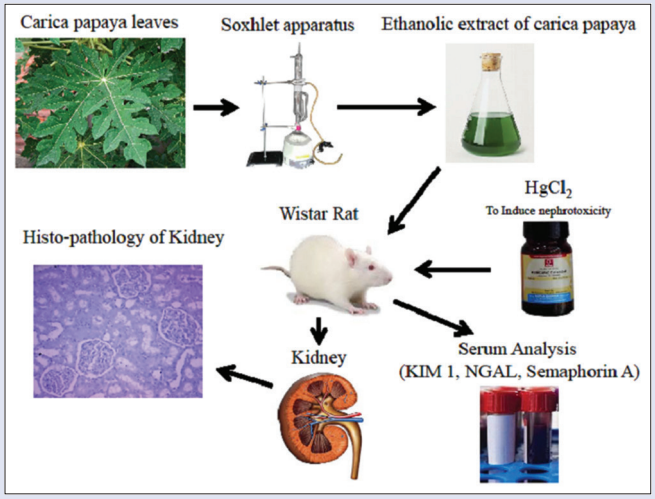
ABSTRACT

Introduction: Chronic kidney disease is the progressive loss of renal function, measured by creatinine, urea and proteinuria levels. Literatures show that renal damages can be ameliorated naturally by medicinal plants, one such plant is *Carica papaya*. Fresh leaves of *C. papaya* were collected, powdered, and extracted with ethanol to form ethanolic extract of *C. papaya* leaves (ECP). **Aim and Objectives:** The aim and objective of this study are to assess the remedial implications of ethanolic ECP leaves on mercuric chloride ($HgCl_2$)-induced nephrotoxicity in female Wistar rats. The objectives of this study were to assess the effectiveness of two different oral doses of ECP using immunohistochemistry, histopathology, and serum biomarkers such as kidney injury molecule-1, neutrophil gelatinase-associated lipocalin, and semaphorin 3A. **Materials and Methods:** The rats were divided into five groups (n = 6): Control (Physiological saline, 2 ml/kg b. w), Negative control ($HgCl_2$, 2.5 mg/kg b. w), Positive control (N-acetyl cysteine [NAC] 180 mg/kg b. w + $HgCl_2$, 2.5 mg/kg b. w), Experimental group (*C. papaya* leaves, 300 and 600 mg/kg b. w + $HgCl_2$, 2.5 mg/kg b. w) for 28 days orally through gavage. Collection of blood and renal tissue was done to determine the serum biomarkers, immunohistochemistry, and histopathology. **Results:** Pretreatment with 300 mg and 600 mg doses of ECP had protective effects slightly lower than NAC and equal to that of NAC on $HgCl_2$-induced nephrotoxicity, respectively. Statistical analysis was performed using the one-way analysis of variance using SPSS version 17.0 with a statistical significance level of $P < 0.001$. Such pretreatment of *C. papaya* leaves, modified the following; (a) levels of serum marker enzymes (b) histopathological changes, and (c) immunohistochemistry expression caused by $HgCl_2$. **Conclusion:** ECP plays a very significant role in the management of nephrotoxicity induced by $HgCl_2$ with equivalent to NAC.

Key words: *Carica papaya*, kidney injury molecule-1, mercuric chloride, nephrotoxicity, neutrophil gelatinase-associated lipocalin and semaphorin 3A

SUMMARY

- Extract of *Carica papaya* (ECP) was selected based on the phytochemical analysis and has free radical scavenging activity
- ECP leaves ameliorate mercuric chloride induced nephrotoxicity in female Wistar rats by reversing oxidative stress. Nephroprotective activity is due to the presence of alkaloids and flavonoids present in *ECP* leaves. Biochemical parameters such as creatinine, urea, serum kidney injury molecule-1 (KIM 1), neutrophil gelatinase-associated lipocalin (NGAL), and semaphroin 3A are analyzed. KIM 1, NGAL, and semaphroin 3A are the most specific biomarkers for renal damage.



Abbreviations used: CKD: Chronic kidney disease; ECP: Ethanolic Extract of *Carica papaya*; GPX: Glutathione peroxidase; $HgCl_2$: Mercuric chloride; H_2O_2: Hydrogen peroxide; KIM-1: Kidney injury molecule; LPO: Lipid peroxidation; NAC: N-acteyl cysteine; NGAL: Neutrophil gelatinase-associated lipocalin; PBS: Phosphate-buffered saline; RNS: Reactive nitrogen species; ROS: Reactive oxygen species.

Correspondence:

Mr.Yuvaraj Maria Francis,
Department of Anatomy,
Saveetha Institute of Medical and Technical Sciences, Chennai, Tamil Nadu, India.
E-mail: sujinalways@gmail.com
DOI: 10.4103/pm.pm_11_20

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INTRODUCTION

Chronic kidney disease (CKD) is life-threatening and widely prevailing ailment in many developed and developing countries. The morbidity and mortality rates are increasing among people despite the advent of advancements in the field. The overall prevalence and incidence of CKD among Indians is 17.2% that accounts for 48.3 million men and 61.7 million women. It is estimated that >100,000 new cases might enter renal transplant level, every year and expected to reach 138.5 million by the year 2030.[1,2] The primary causative agents for kidney injury are heavy metals, drugs, radiocontrast substances, and micro-organisms. It may also occur

Cite this article as: Francis YM, Vijayakumar J, Raghunath G, Vijayalakshmi S, Sivanesan S, Vijayaraghavan R, *et al*. Protective effect of *Carica papaya* leaf extract against mercuric chloride-induced nephrotoxicity in Wistar rats. Phcog Mag 2020;16:S379-84.

as secondary to non-communicable diseases such as cardiovascular diseases, diabetes mellitus, obesity, and hypertension.[3] Among heavy metals, mercury is the second-most common next to arsenic. The former is found in the environment naturally by erosion of rocks, agriculture, volcanic eruption, and ground water besides commercial products such as thermometers, batteries, paints, fluorescent lamps, and dental preparations.[4] Kidney is the most targeted organ by mercury, which alters its structure and functions. The rate of toxins delivers to the kidney, as a result of high renal blood flow, which approximates 25% of cardiac output. Excessive cellular workload of the kidney in hypoxic environment enhances risk for nephrotoxicity.

Even though many remedial measures were suggested for tackling mercury toxicity, the use of medicinal plants is on the rise due to their accessibility and availability. In addition, they contain a wide variety of secondary metabolites and possess antioxidant property. *Carica papaya* Linn. (Family: *Caricaceae*) is one such plant that is native to tropical countries and has been proved to possess several interesting biological properties.[5] In this work, the ethanolic extract of *C. papaya* leaves (ECP) has been considered to assess its efficacy against mercuric chloride ($HgCl_2$)-induced nephrotoxicity in rat model. The vital parameters of immunohistochemistry, histopathological changes and serum biomarkers such as kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and semaphorin 3A were studied for assessing the protective role of the plant extract against nephrotoxicity. In our earlier report, NGAL has been found to be a reliable biomarker to infer the early onset of nephrotoxic complications in rat.[6]

MATERIALS AND METHODS

Animals

The animals (36 rats) were purchased from Biogen, Bengaluru (Registration number: 2/PO/RcBi/SL/99/CPCS) and the study was carried out in the Centre for Laboratory Animal Research, Saveetha Institute of Medical and Technical Sciences after obtaining proper approval from the Institutional Animal Ethics Committee (SU/CLAR/RD/025/2017). Female rats weighing about 180–210 g were used in the study. They were maintained in clean white polypropylene cages with specified relative humidity (40%–60%) and temperature (25°C ± 2°C) control. The natural 12 h light/dark cycle was maintained and the animals were fed with standard pellet diet and had access to clean drinking water provided *ad libitum*.

Chemicals

All the chemicals and reagents used in this study were of analytical grade and purchased from Sigma Chemical Company, St. Louis, MO and U.S.A; Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K. and Sisco Research Laboratories, Mumbai, India.

Preparation of *Carica papaya* extract

Fresh leaves of *C. papaya* were collected from Marthandam, Kanyakumari District of Tamil Nadu State (India) in September on a sunny day. They were thoroughly rinsed with water and shade dried. About 500 g of dried leaves were coarsely powdered and extracted with 950 mL of ethanol by cold percolation method. After 72 h, the contents were filtered using Whatman No. 1 filter paper and distilled over boiling water bath. Traces of solvent were removed *in vacuo* and the final extract (ECP, 32 g) has been utilized for animal studies.

Experimental design

After proper acclimatization, the rats were divided into five groups with six rats each and treated as follows for 28 consecutive days.

- Group I (Control): Saline (2.0 mL/kg b. w, p. o.)
- Group II (Negative control): $HgCl_2$ (2.5 mg/kg b. w, p. o.)
- Group III (Positive control): Std. Drug; N-acetyl cysteine (NAC) (180 mg/kg b. w, p. o.) +
- $HgCl_2$ (2.5 mg/kg b. w, p. o.)
- Group IV (Experimental Group 1): ECP (300 mg/kg bw, p. o.) + $HgCl_2$ (2.5 mg/kg b. w, p. o.)
- Group V (Experimental Group 2): ECP (600 mg/kg b. w, p. o.) + $HgCl_2$ (2.5 mg/kg b, w, p. o.).

Collection of serum and tissues

On day 29, overnight fasted rats were anesthetized using isoflurane and the blood was collected by retro-orbital venous plexus in ethylenediaminetetraacetic acid tubes. A midline incision was made to visualize the kidneys and they were perfused well. The right kidneys were stored in 10% formalin for histological studies and the left kidneys were used to analyze the oxidative stress markers. Serum analyses were performed for various biochemical parameters.

Estimation of renal function parameters

The renal function parameters were assessed by estimating urea and creatinine present in the blood by standard procedures.[7,8] The biomarkers KIM-1, NGAL, and semaphorin 3A levels in serum were measured using ELISA with commercially available kits (CUSOBIO Life Sciences). The instructions provided in the catalogue were used to estimate the above. Readings were taken with micro plate reader at 490 nm.

Estimation of lipid peroxidation and antioxidant parameters

Lipid peroxidation (LPO), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in kidney tissues were determined by the methods mentioned in the literature.[9-11]

Histopathological studies

The stored kidney tissues were fixed in 10% formalin for 48 h and dehydrated gradually with different grades of alcohol, cleared in xylene, and embedded in paraffin wax. Sections of 4-µm thickness were made and stained with eosin and hematoxylin.[12]

Expression of kidney injury molecule-1 proteins by immunohistochemical assay

Immunolocalization of KIM-1 in tissue cells was carried out by `indirect peroxidase' method incorporating the procedure of Hsu and Rain (1981) with modifications of Rowley *et al.*[13]

Statistical analysis

The data obtained were analyzed using one-way analysis of variance with SPSS version 17.0 (IBM Corp, Armonk, NY). followed by the Newman–Keuls test for comparison between the groups. The results were expressed as mean + standard error and the values with $P < 0.001$ were considered statistically significant.

RESULTS

Effects of extract of *Carica papaya* on renal function markers

Administration of $HgCl_2$ to animals increased the renal function parameters urea and creatinine levels in the serum (Group II) while the same have exhibited near normal values with $P < 0.0001$ in the

standard drug (Group III) and ECP-treated rats in Groups IV and V when compared to normal animals in Group I [Figure 1]. Similarly, the renal biomarkers KIM-1, NGAL, and Semaphore 3A have shown upregulation in $HgCl_2$-treated rats (Group II) by 1.2, 1.9, and 2.5-folds, respectively, when compared to control group, whereas pre-treatment with NAC and two doses of ECP revealed downregulation in their expressions [Figure 2]. The latter values were found to be statistically significant ($P \leq 0.001$). The mean values are expressed in Table 1.

Effects of extract of *Carica papaya* on oxidative stress markers and antioxidant enzymes

The effect of ECP on oxidative stress markers and antioxidant enzymes in the renal tissues of $HgCl_2$-treated rats has been studied by estimating LPO and H_2O_2 and also antioxidants enzymes such as GP_X and SOD. There was significant increase in the levels of LPO and H_2O_2 in Group II animals as compared to that of control group. Their elevated levels were substantially brought down in NAC and ECP administered group rats. There was also a concomitant decrease in GPX and SOD negative control group which has been ameliorated in NAC and ECP pretreated groups [Figures 3 and 4].

Histological studies

In the microscopic examination of the kidney tissues of $HgCl_2$-treated group, atrophy of glomerulus and dilatations were seen in Bowman's capsule, whereas the tubules showed dilatation, degeneration, hyaline cast, and coagulative necrosis. In control as well as NAC and ECP (600 mg/kg) pretreated animals, normal cyto-architecture characterized by well-organized glomerulus with intact Bowman's capsule, normal renal tubules, and collecting ducts in the cortex and medulla were observed. In ECP (300 mg/kg) group, shrunken glomerulus, minimal inflammation, and degeneration in tubules and mild coagulation necrosis in renal tissue were seen. The details of histopathological findings are presented in Figure 5.

Effect of extract of *Carica papaya* on kidney injury molecule-1expression in renal tissue

The microscopic examination of kidney tissue with control group shows no immunoreactivity KIM-1 gene. In negative control group

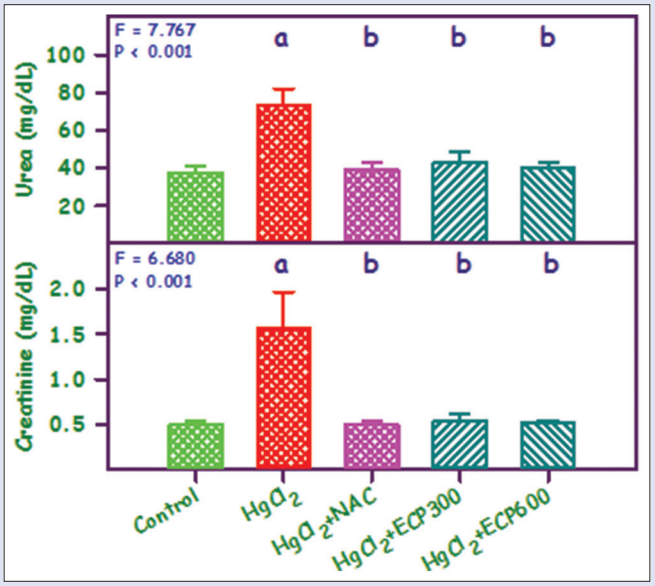

Figure 1: Estimation of urea and creatinine levels in the following groups: Group I (control), Group II ($HgCl_2$), Group III (180 mg/kg NAC+ $HgCl_2$), Group IV (300 mg/kg ECP+ $HgCl_2$) and Group V (600 mg/kg ECP+ $HgCl_2$). Values are mean ± standard error (n = 6 each). The "F" and "P" values are by one-way analysis of variance with student Newman–Keuls multiple comparison test. [a]Significantly different from control group, [b]Significantly different from mercuric chloride group, [c]Significantly different from n-acetyl cysteine group

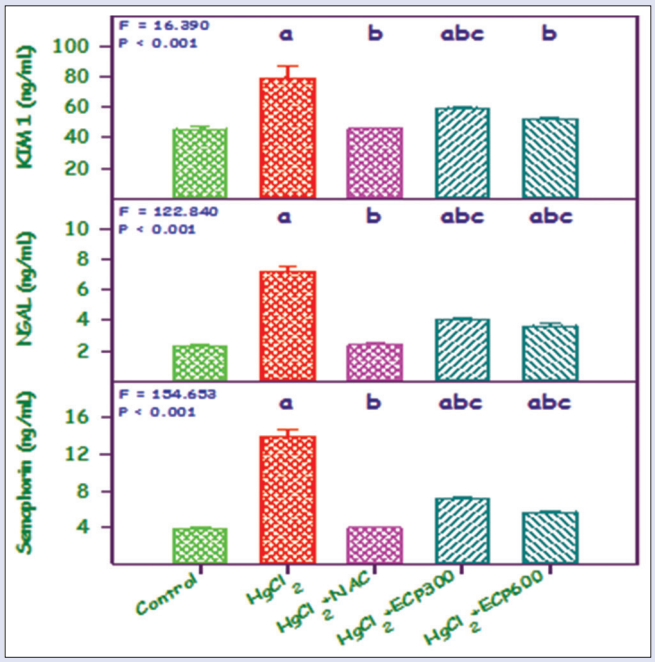

Figure 2: Estimation of KIM-1, NGAL and semaphorin 3A levels in the following groups: Group I (control), Group II ($HgCl_2$), Group III (180 mg/kg NAC+ $HgCl_2$), Group IV (300 mg/kg ECP+ $HgCl_2$) and Group V (600 mg/kg ECP+ $HgCl_2$). Values are mean ± standard error (n = 6 each). The "F" and "P" values are by one-way analysis of variance with student Newman–Keuls multiple comparison test. [a]Significantly different from control group, [b]Significantly different from mercuric chloride group, [c]Significantly different from n-acetyl cysteine group

Table 1: Effect of *Carica papaya* leaf extract on biochemical parameters in $HgCl_2$-induced nephrotoxicity

Treatment	Urea	Creatinine	KIM-1	NGAL	Semaphorin3A
Control (saline 2 mL/kg, p.o.)	37.66±3.49	0.50±0.03	45.70±1.15	2.33±0.11	3.92±0.11
$HgCl_2$ (2.5 mg/kg, p.o.)	73.16±8.69	1.56±0.39	79.33±7.54	7.24±0.26	14.02±0.71
Standard drug (NAC 180 mg/kg, p.o.)	39.33±3.90	0.50±0.03	45.16±0.48	2.39±0.12	3.95±0.06
ECP (300 mg/kg, p.o.)	42.50±5.70	0.53±0.08	59.56±0.37	4.03±0.18	7.18±0.14
ECP (600 mg/kg, p.o.)	40.00±3.00	0.51±0.03	52.31±0.48	3.63±0.17	5.67±0.14

Values are expressed as mean and SE for six animals in each group. The significance at the level of P<0.01. SE: Standard error, KIM-1: Kidney injury molecule-1; NGAL: Neutrophil gelatinase-associated lipocalin; ECP: Ethanolic extract of *Carica papaya* leaves; NAC: N-acetyl cysteine

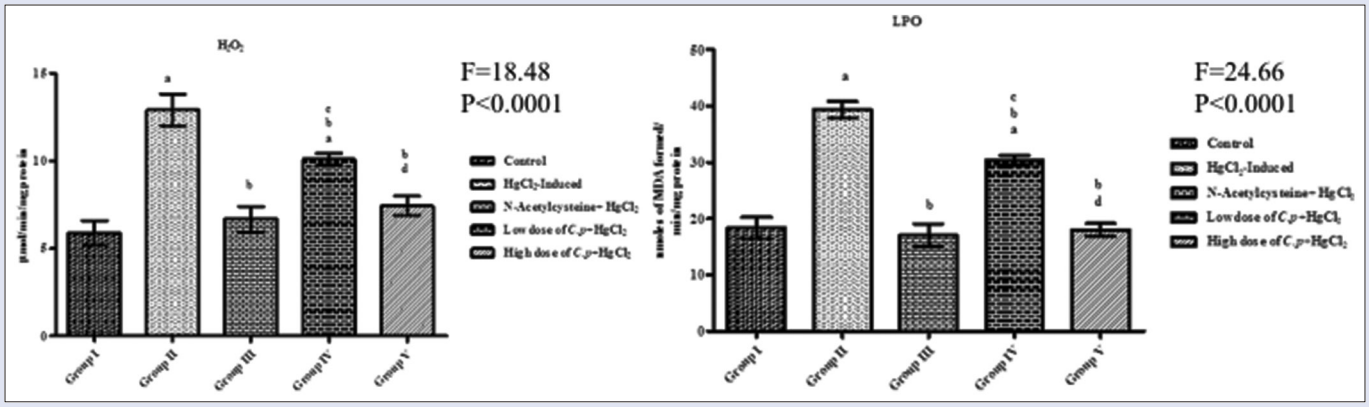

Figure 3: Estimation of H_2O_2 (Bar Graph -Left) and LPO (Bar Graph -Right) levels in the following groups: Group I (control), Group II ($HgCl_2$), Group III (180 mg/kg NAC+ $HgCl_2$), Group IV (300 mg/kg ECP+ $HgCl_2$) and Group V(600 mg/kg ECP+ $HgCl_2$). Values are mean ± standard error (n = 6 each). The "F" and "P" values are by one-way analysis of variance with student Newman–Keuls multiple comparison test. [a]Significantly different from control group, [b]Significantly different from mercuric chloride group, [c]Significantly different from n-acetyl cysteine group

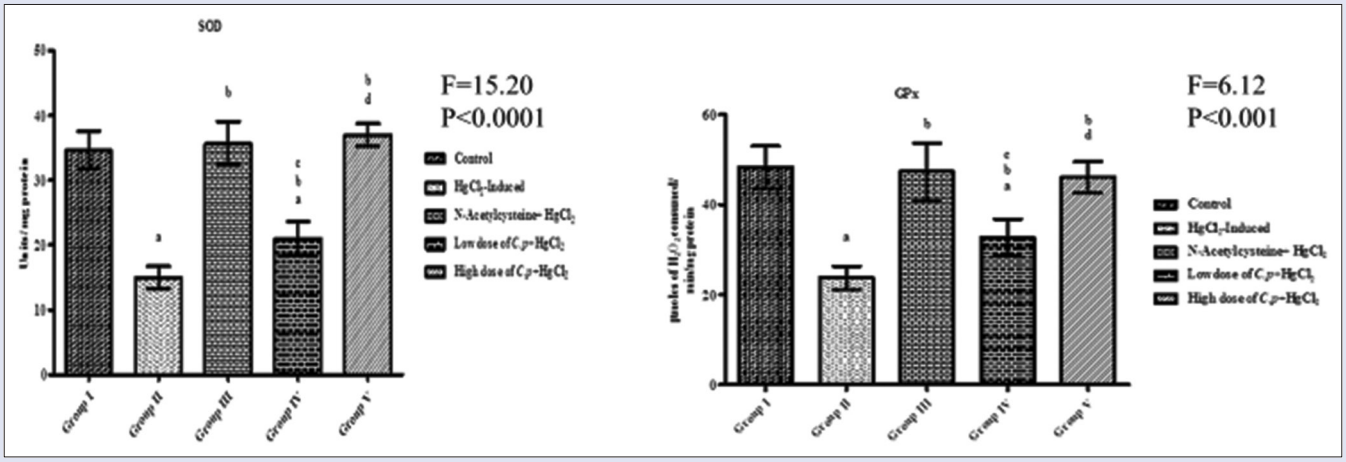

Figure 4: Estimation of SOD (Bar Graph -Left) and GPx (Bar Graph -Right) levels in the following groups: Group I (control), Group II ($HgCl_2$), Group III (180 mg/kg NAC+ $HgCl_2$), Group IV (300 mg/kg ECP+ $HgCl_2$) and Group V (600 mg/kg ECP+ $HgCl_2$). Values are mean ± standard error (n = 6 each). The "F" and "P" values are by one-way analysis of variance with student Newman–Keuls multiple comparison test. [a]Significantly different from control group, [b]Significantly different from mercuric chloride group, [c]Significantly different from n-acetyl cysteine group

showed immunoprecipitation of KIM-1 in the tubules of the kidney. Moreover, in ECP (300 mg/kg) administered rats showed very minimal immunoprecipitation of KIM-1 when compared to negative control group. No expression of KIM-1 gene in the NAC and ECP (600 mg/kg) group and it closely resembled the control group. The expression of KIM-1 in diverse groups is shown in Figure 6.

DISCUSSION

Nephrotoxicity is one of the major complications induced by mercury. It is widely employed in many industries dealing with batteries, electrical equipment's, medicine, and agriculture.[14] Mercury induces toxicity by binding with thiol-containing biomolecules such as proteins, cysteine, and Glutathione (GSH),[15,16] leading to oxidative stress that develops when there is imbalance between reactive oxygen species (ROS) formation and detoxification process. The increase in ROS levels lead to structural and functional changes in the cells. An overall increase in the synthesis of ROS and reactive nitrogen species (RNS) affects renal, central nervous, cardiovascular, and reproductive systems.[17-19]

Oral administration of $HgCl_2$ develops oxidative stress by the over production of ROS and RNS that, in turn, alters superoxide anion radicals and peroxide, membranes of lipids, denaturation of proteins.[20,21] Antioxidants generally inhibit the production of ROS and RNS by scavenging free radicals. In recent times, the use of medicinal plants is to treat many ailments. Plant products contain diverse variety of antioxidants and specialized compounds such as alkaloids, phenolic, steroids and the like, which inhibit the production of ROS and RNS and scavenge free radicals.[22,23] Due to this property, phyto products exhibit significant anti-inflammatory, analgesic, anti-hepatotoxic, anti-nephrotoxic, and host of other activities. For example, intake of fenugreek, curcumin, peppermint, parsley, rosemary, garlic, pomegranate, sesame and propolis showed nephroprotective effects against chronic renal injury in humans and mammals.[24] The protective effects against mercury-induced nephrotoxicity have been established by plant products such as *Tribulus terrestris,*[25] *Scopolia tangutica,*[26] flaxseeds,[27] and *Rheum turkestanicum.*[28]

C. papaya is one such potential medicinal plant widely distributed in India and several other countries. Various parts of this plant are used

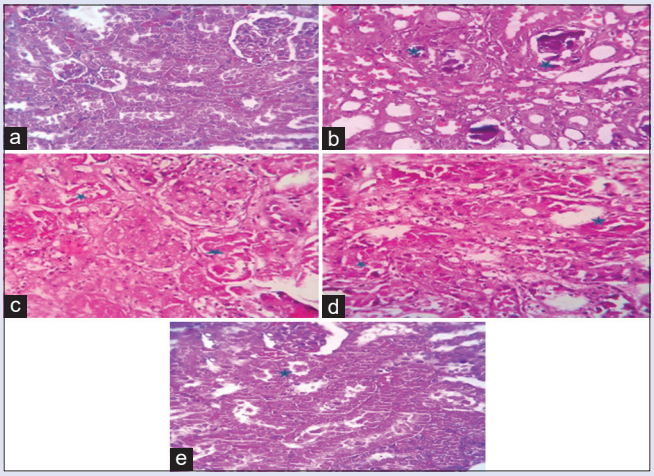

Figure 5: Hematoxylin and Eosin staining of kidney sections of control group I revealed no injury (a). In $HgCl_2$ induced group II, there was loss of brush border, necrosis, and sloughing of cells into the tubular lumen in mercury chloride-treated rats (b). Whereas in group III (180 mg/kg NAC+ $HgCl_2$) administered with standard drug NAC minimal loss of brush border and necrosis (c). Group IV rats (300 mg/kg ECP+ $HgCl_2$) showed well-defined tubular neurosis, loss of cellular integrity (d). Whereas group V rats (600 mg/kg ECP+ $HgCl_2$) showed minimal tubular damage, less prominent cellular derangement

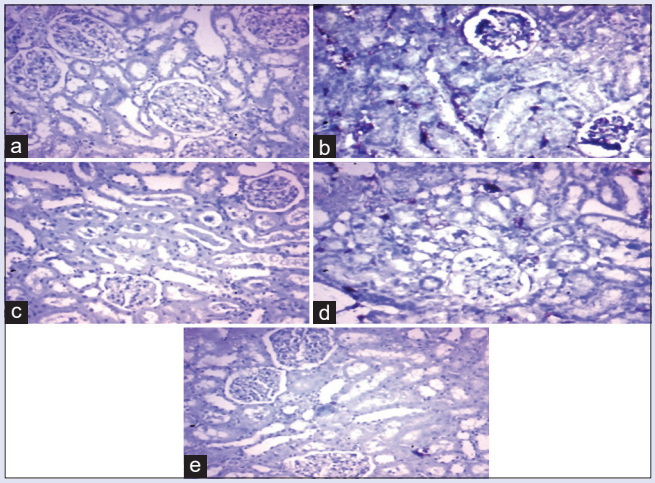

Figure 6: Immunohistochemical staining of kidney sections of control group I revealed no expression of KIM-1(a). In $HgCl_2$ induced group II, damage was observed in tubules of kidney leads to expression KIM-1(b). Whereas in group III (180 mg/kg NAC+ $HgCl_2$) administered with standard drug NAC showed no expression of KIM-1(c). Group IV rats (300 mg/kg ECP+ $HgCl_2$) showed nominal expression of KIM-1 (d). Whereas group V rats (600 mg/kg ECP+ $HgCl_2$) showed no expression of KIM-1 (e)

as food and medicine by the indigenous people. Scientific studies have shown that it possesses a variety of biological properties including significant antioxidant activity.[29] Recent investigations have revealed the protective effect of leaf extracts against streptozotocin and gentamycin-induced nephrotoxicity in rat model.[30,31] In the present study, the leaf extract (ECP) revealed a significant protection against mercury-induced nephrotoxicity as well. This model has been chosen for the study as mercury is a highly persistent and widely prevalent environmental pollutant present in many household products such as batteries, paints, toys, etc. besides being a notorious nephro-toxic agent.

Oral administration of $HgCl_2$ brought drastic changes in the animals of Group II by showing increased levels of all the five parameters tested with the serum. In standard drug-treated Group III, however, these values were reverted to near normal while the higher dose of ECP (600 mg/kg) effectively reduced the LPO and H_2O_2 level and increased antioxidant enzymes SOD and GPx in $HgCl_2$-treated animals.

The clinical markers used to evaluate the kidney function are serum urea and creatinine. In kidney injury there is upsurge in the levels of urea and creatinine,[32] which has been observed when $HgCl_2$ was administered to the animals. This increased value of urea and creatinine are due to oxidative-stress induced cellular damage and decreased glomerular filtration rate that indicate the loss of renal function. Pretreatment with the standard drug (NAC) and different doses of ECP significantly restored the altered levels of urea and creatinine that might be due to the repair of renal tissue damage mediated by the antioxidants effects of the above agents.

In addition to the above parameters, of late, more sensitive biomarkers have come to the fore due to developments in the diagnostic science. The Food and Drug Administration and European Medicines Agency recognized KIM-1 to be a highly sensitive biomarker for detecting renal injury. KIM-1 (25 Kda) is a transmembrane glycoprotein with an immunoglobulin domain and mucin. Earlier this biomarker has been studied in urine.[33] As the obtained values were not reliable, recent studies are being carried out in serum. According to Christina-Alexandra *et al*. serum KIM-1 is a potential predictive marker of kidney dysfunction in the general middle-aged population.[34] Hence, in the current study, serum KIM-1 was analyzed which is unexpressed in a healthy kidney. The rats treated with $HgCl_2$ produced upregulation in the expression of KIM-1 due to renal injury and the apical part of renal tubules synthesize more KIM-1 which is in agreement with the earlier findings.[35] Pretreatment with NAC showed no expression while ECP (both 300 and 600 mg/kg doses) downregulated the expression of KIM-1 in serum.

NGAL is a 25 Kda protein, associated with the lipocalin family. This marker was initially identified in activated neutrophils. Multitude of cells such as renal proximal tubular cells, hepatocytes, neurons, cardiomyocytes, and pneumocytes also produce NGAL in response to various injuries.[36] In acute and chronic kidney injury, the expression of NGAL is seen both in serum and urine.[37,38] According to Li *et al.*[39] the serum NGAL is superior to urinary NGAL in the early prediction of renal damage. In this study, the analysis was carried out in serum and the expression of NGAL was predominantly seen in negative control rats (Group II) due to injury to proximal tubules of the kidney. Pretreatment with the standard drug (NAC) showed no expression in NGAL like control group animals whereas ECP treatment down regulated its expression.

Semaphorin-3A is a low-molecular weight protein (about 12 K da) that controls cell migration and neuronal regeneration. It is significantly expressed in kidneys. In normal rat kidneys, Sema3A is expressed in tubules as well as in podocytes. In a transient ischemia or cisplatin induced kidney injury, Sema3A protein expression was up regulated in distal and collecting tubules.[40,41] In the present work, the negative control group animals showed up-regulation as has been observed by earlier workers. Pretreatment with ECP down regulated semaphorin 3A expression in serum, whereas in positive control group, no such expression has been noticed.

CONCLUSION

The present study has proved that dose-dependent nephroprotective activity of the ECP in a rat model of $HgCl_2$-induced nephrotoxicity.

Pretreatment with 600 mg/kg b. w dose ECP provide full protection and 300 mg/kg b. w dose provides partial protection from kidney injury as evidenced by serum renal markers, histopathology, and immunohistochemistry. ECP is a potential nephroprotective agent through its antioxidant and anti-inflammatory property.

Acknowledgements

The author would like to thank the Department of Research and Development, Saveetha Institute of Medical and Technical Sciences, for supporting me to carry out research.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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