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Nephroprotective activity of *Annona squamosa* leaves against paracetamol-induced nephrotoxicity in rats: in vitro and in vivo experiments

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Abstract

Background: Paracetamol (PCM), being extensively adapted analgesic and anti-inflammatory drug all over the world, beyond therapeutic dosages, the oxidative stress-involved nephrotoxicity has been evidenced. However, herbal plants are the windfall for the humankind providing solution for most of the wellness breakdowns. *Annona squamosa* (AS) is one of such plants with enormous therapeutic and nutraceutical potencies. The main aspiration of the current investigation is to evaluate the nephroprotective ability of ethanolic extract of *Annona squamosa* (EEAS) leaves against paracetamol-induced nephrotoxicity using in vitro human embryonic kidney (HEK)-293 cells and in vivo experiments in Wistar rats through biochemical parameters, oxidative parameters, and histopathological findings.

Results: When HEK-293 cells were incubated with PCM, an increased cell death associated with alterations in the morphology of normal cells was observed. At variable concentrations, HEK-293 cells co-treated with PCM and EEAS extracts gave a significant improvement in cell growth on comparing with PCM treatment showing cytoprotective feature of EEAS with an IC_{50} 28.75 μ g/mL. In vivo nephroprotective property was assessed from the amount of blood urea nitrogen (BUN) along with creatinine and uric acid which were reduced ($P < 0.001$) within serum and compact levels of glutathione, catalase, and superoxide dismutase which were termed as GSH, CAT, and SOD, respectively, were increased ($P < 0.001$) in kidney tissue homogenate in the treated groups than the PCM alone group. Results were additionally supported by histopathological observations.

Conclusion: The results exhibited that EEAS has impending benefits against PCM-induced nephrotoxicity through in vitro and in vivo experiments.

Keywords: Nephrotoxicity, Paracetamol, *Annona squamosa*, HEK-293, Oxidative stress

Background

Acetaminophen, most commonly acknowledged with its generic name, paracetamol (PCM), is the last among the threesome of derivatives of para-aminophenol which was introduced at the end of the nineteenth century [1, 2]. PCM is a potent analgesic as well as an antipyretic drug

with lesser side effects than aspirin [3]. Even today, there is exactly no alternative to this particular drug in terms of treating fever and mild pains in both children and adults. Since most of the pharmaceutical outlets do not require a prescription to trade with consumers, the abuse is very common. PCM is usually formulated with two strengths; regular strength of 325 mg and higher strength of 500 mg along with this higher dose PCM is also available. In large dose consumption, PCM is known to result in acute kidney and liver necrosis in mammalian species [4–6].

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However, beyond therapeutic doses, unsafe behaviors arose, wherein the toxic *N*-acetyl *p*-benzoquinone imine (NAPQI), which is an active intermediate metabolite during oxidation of PCM, depletes glutathione (GSH), leading to an increased amount of NAPQI reactive intermediates resulting in deprivation of tissues [7]. NAPQI is known to form covalent adducts with renal cellular proteins promoting the generation of higher levels of reactive oxygen species (ROS) and weakens the ATP, besides leading to apoptosis, promoting renal necrosis with relentless organ dysfunction [8–10].

Over and above, the kidneys are eminently vulnerable for such side effects since, the immense volume of blood course through and filtered in larger amounts aiding the removal of toxins. Nephrotoxicity drives an imbalance in body fluid along with electrolytes leading to hormonal imbalance as well [11]. Herein, we propose a plant source for the protective effect for PCM-induced nephrotoxicity.

Plant sources/medicinal plants are a boon for the human community. Ancient literature suggests numerous medicinal herbs that could alleviate or ease various ailments associated kidneys. To note, several plants have been experimented for their nephroprotective effect with an aid of drug-induced nephrotoxicity in animal models. Herbal extracts from *Eurycoma longifolia* [12], *Curcuma longa* [13–15], *Pimpinella tirupatiensis* [16], *Allium sativum* [17], *Nigella sativa* [14], and many more have proven their potency towards the abovementioned protective effects. Nonetheless, ethanolic extract of leaves of *Annona squamosa* (custard apple) remained unexplored for the same. *Annona squamosa* is a medium-sized, tropical tree growing up to 25 ft. large. The leaves are slender and oblong while the flowers are greenish yellow. The fruit is green and the pulp is yellowish white color with sweet in taste. Chemically, it contains an alkaloid, annonaine, and other constituents like flavonoids, glycosides, terpenes, and tetrahydro-isoquinoline alkaloids [18]. Literature survey demonstrated several pharmacological effects such as anti-diabetic [19], hepatoprotectivity [20], antibacterial [21], antioxidant [22], anti-ulcer [23], insecticidal [24], anti-inflammatory [25], and analgesic [26] activity of an interested plant.

As mentioned, since ethanolic extracts of leaves of *Annona squamosa* remain unexplored for a protective effect against PCM-induced nephrotoxicity, herein, screening was done with an aid of in vitro analysis using HEK-293 cells and in vivo analysis using Wistar rats. The study highlights the preliminary phytochemical screening of EEAS, the toxicity of EEAS by studying MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, and effect of EEAS on serum parameters such as BUN, creatinine, uric acid, and antioxidant enzymes levels in the kidney tissue along with histopathological studies.

Methods

Chemicals

Paracetamol was obtained from Waksman Selman Pharmaceuticals Pvt. Ltd., Anantapur, India. HEK-293 cells procured from NCCS, Pune. DMEM-high glucose, fetal bovine serum, MTT reagent, biochemical kits for quantification of serum, and antioxidant enzymes were procured from the Himedia Laboratories, Mumbai, India. Dimethyl sulfo-oxide (DMSO) and Silymarin were acquired from Sigma-Aldrich (St. Louis, USA). Ethanol (95%) and other chemicals of analytical grade were utilized in the present study were obtained from standard pharmaceutical companies.

Collection of plant solids

Green leaves of *Annona squamosa* were possessed from fields of Madhavaram, Kadapa Dist, Andhra Pradesh. The identification and authentication of leaves were done by Dr. K. Madhava Chetty, Dept. of Botany, S.V. University, Tirupathi, with a voucher specimen no:0986.

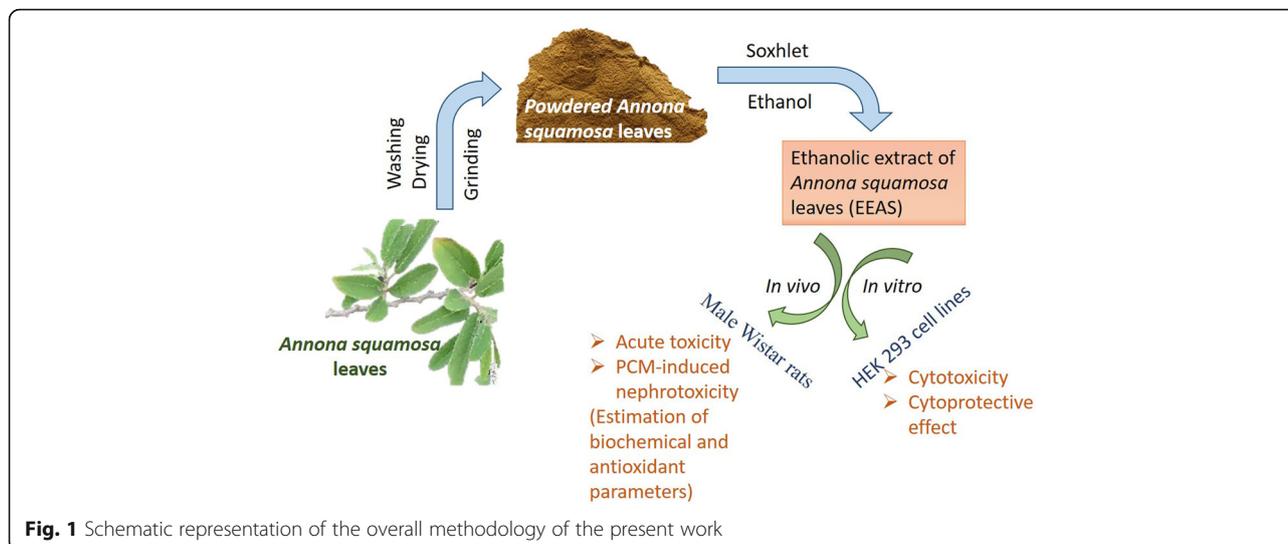
Preparation of ethanolic extract of *Annona squamosa* leaves

The collected fresh plant material was washed thoroughly with tap water to get rid of filth and dried under shade till the leaves became brittle. Once dried, the leaves were crushed coarsely and powdered using a blender. The powder (850 g) was then subjected to defatting using petroleum ether in soxhlet for 18 h followed by drying. The dried powder was again packed within soxhlet for ethanol extraction for 72 h and until a clear solution is obtained in siphon tube and the yield of EEAS was 16.4%. The overall methodology of the work is presented in Fig. 1.

Cell viability and in vitro cytotoxicity

Cytotoxicity analysis

Human embryonic kidney 293 cell lines were utilized for the cell cytotoxicity analysis via the standard colorimetric method. The cells cultured in a 96-well plate with a cell density of 20,000 cells/well, in the absence of the test agent was allowed for 24 h. Test agents were added and subjected to incubation for 24 h at 37 °C under CO₂ atmosphere of 5%. The spent media was then taken out and treated with a solution of MTT (0.5 mg/mL of total volume) and again incubated for 3 h. The cell viability was estimated by examining the conversion of MTT into purple formazon crystals by metabolically viable cells. The medium was then removed, and the obtained crystals were quantified for cell viability by dissolving in DMSO (100 μL). ELISA was performed at 570 nm and the reference wavelength was set at 630 nm [27].



Cytoprotective analysis

HEK cell line suspension of 200 μ l cells was taken in a 96-well plate at an appropriate cell density in the absence of a test agent and grew for 24 h. To induce toxicity, a test compound, paracetamol with IC_{25} , IC_{50} , and IC_{75} concentrations were added and subjected to incubation for 24 h under a CO_2 atmosphere of 5% at 37 °C. After incubation, spent media was taken out and MTT was added and incubated for 3 h. With the addition of 100 μ l of solubilization solution, the MTT reagent was removed and allowed to dissolve MTT formazan crystals. With an ELISA reader, cytoprotective activity was analyzed.

Animals and environmental conditions

The animals used for the study were obtained from animal houses, and whole experiments were performed at the NGSM Institute of Pharmaceutical Sciences, Mangaluru, with a written consent. A total of 24 male Wistar rats with a weight of about 200–250 g were considered for the investigation and were quarantined and acclimatized for 14 days. Six rats were housed in every cage with ambient temperature (21 ± 3 °C), controlled humidity ($50 \pm 10\%$), and 12 h of alternative light and dark cycles with an easy access to both food as well as water. The entire experimentations and protocols described in the present study were established by the Institutional Animal Ethical Committee (IAEC) of the NGSM Institute of Pharmaceutical Sciences, Mangaluru (NGSMIPS/IAEC/MARCH-2019/126).

Acute toxicity studies

With an established OECD guideline 425, the acute toxicity of EEAS leaves was tested on various groups each with 10 rats. Every rat received different doses of 50,

100, 200, 400, 800, 1000, and 2000 mg/kg body weight. The number of deaths observed in every group was recorded for 48 h. Up to 2000 mg/kg, there was an absence of mortality and toxicity. Based on these studies, 200 and 400 mg/kg of EEAS were selected for future experimentations.

Experimental design for PCM-induced nephrotoxicity

The study was designed for 14 days, and the procedure was followed as in the previous reference [28]. Male Wistar rats were randomized into four groups of six rats in each group to achieve an average weight between and within the group not exceeding $\pm 20\%$ of the average weights of all rats. The animal grouping was done in the following ways: Group I: (normal control) treated with normal saline p.o.; Group II: (disease control) treated with PCM, intraperitoneally at a dose of 200 mg/kg per day for 14 days; Groups III and IV: received EEAS at a dose of 200 and 400 mg/kg p.o., respectively, along with PCM (200 mg/kg) for 14 days.

Estimation of biochemical and antioxidant parameters

On 15th day, the fasted rats were euthanized with ketamine (100 mg/kg, intramuscularly) and heparinized tubes were used to collect the blood from the retro-orbital plexus. All 24 animals were used for each parameter at the end of the study. Blood plasma and serum were separated by centrifugation. The kidneys were quickly removed, washed with cold water followed by isotonic saline, and then blotted using a filter paper. Tissues were next homogenized using Tris-HCl buffer (0.1 M) of pH 7.4. The quantification of antioxidant enzymes was done using homogenate. Urea, creatinine, and uric acid in serum were determined using an analytical kit (Himedia Laboratories,

Mumbai, India). Oxidative stress was estimated by measuring SOD, GSH, and catalase levels in the kidney tissue.

Histopathological studies

The kidney tissues of rats from each group were preserved in 10% formalin and processed with paraffin wax. For histopathological examination, very thin sections were considered for staining with hematoxylin followed by eosin for the clear analysis using a light microscope. Two sections from the individual sample were analyzed with the help of standardized protocol to identify chief morphological characteristics associated with PCM-induced nephrotoxicity. With microscope ocular of 22 mm FN, "high power field (40x)" = 0.237 mm² area, hence by investigating at 10 HPF, a total of 2.37 mm²/slide have been studied. The histological changes of glomerular, interstitial, tubular, and endothelial components were studied [29].

Statistical analysis

All the values were expressed as mean \pm SEM ($n = 6$). The obtained data from various parameters was evaluated statistically using one-way analysis of variance (ANOVA) trailed by Tukey-Kramer multiple correlation tests, and the mean values were considered for the respective parameters.

Results

Preliminary screening of phytochemicals

The percent yield of EEAS was 16.40% w/w. The qualitative phytochemical analysis of the extract showed the existence of high amounts of tannins, terpenoids, flavonoids along with phenolic compounds, moderate amounts of carbohydrates, alkaloids, anthraquinones, and lower amounts of steroids.

MTT assay

Cytotoxicity and cytoprotective activity of the test compound for PCM-induced toxicity were estimated by MTT assay with 15 μ M of silymarin (positive control), and the results are exposed in Fig. 2. Figure 2a, b depicts the cytotoxicity for PCM and EEAS, respectively, and Fig. 2c represents the cytoprotective feature of EEAS. Untreated cells were considered as control and STD refers to silymarin. From Fig. 2a, it is obvious that, with an increase in PCM concentration, the cell viability decreases indicating the cytotoxic effect of PCM at higher dosages. However, treatment with varied concentrations of EEAS did not pose a significant effect on the percent cell viability concluding the non-cytotoxic nature of EEAS. So, EEAS was considered as a cytoprotective compound on HEK 293 cells but not PCM alone due to its lower IC₅₀ value. Further, the cytoprotective nature of EEAS against HEK 293 cell lines was evaluated at different dosages (100, 200, and 400 μ g/ml) along with IC₅₀

value of PCM (28.75 μ g/ml). With a treatment of PCM alone, cell viability was significantly reduced ($P < 0.001$). The cytoprotective nature of combinational ones was dose-dependent, for the lower dose, cell viability increased to 17–18 %, for the medium dose, the increase was 6–8%. Further with a higher dosage, the mean cell viability was around 85% endorsing the cytoprotective feature of EEAS. The direct microscopic observations of the untreated group, positive control, negative control, and EEAS+PCM-treated groups are displayed in Fig. 2d, i. The microscopic images are in good agreement with obtained cell viability.

In vivo studies

Effect of EEAS on BUN, creatinine, and uric acid on PCM-induced nephrotoxicity in rats

Quantification of serum biomarkers revealed a sharp rise in BUN, creatinine, and uric acid in the PCM alone-treated groups (Group II) on comparing with the normal group indicating the intraperitoneal administration of 200 mg/kg/day of PCM for 14 days prompted remarkable ($P < 0.01$) increase in all three conventional biomarker levels. Estimated levels of these parameters are tabulated in Table 1. However, in a dose-dependent fashion, the biomarkers level significantly ($P < 0.001$) decreased presenting the potency of EEAS towards nephrotoxicity.

Effect of EEAS on antioxidant enzymes (GSH, SOD, and catalase) on PCM-induced nephrotoxicity in rats

The kidney damage induced by PCM-intoxicated rats and the effect of EEAS on GSH, SOD, and CAT is provided in Table 2. A considerable decline in levels of GSH, SOD, and CAT was observed for the PCM alone-treated group on comparing with the normal group. Contrarily, a significant raise ($P < 0.001$) was noted for antioxidant enzyme activities with an administration of EEAS 200 mg/kg and 400 mg/kg, in the experimental model after inducing nephrotoxicity with PCM, indicating the efficiency of the EEAS with respect to the nephroprotective property.

Histopathological studies

Histopathological slides of the normal group, PCM alone group, and two used dosages of PCM and EEAS are presented in Fig. 3. The kidney tissue of normal rats showed no visible signs of degeneration or necrosis and was previously confirmed from biochemical and antioxidant results (Fig. 3a). Extensive kidney tissue degeneration along with tubular necrosis was noted in the PCM alone-treated rats (Fig. 3b). The rats which received 200 mg/kg optimal EEAS showed a tubular pattern through reasonable necrosis and degranulation (Fig. 3c), whereas the rats which received 400 mg/kg EEAS exhibited very

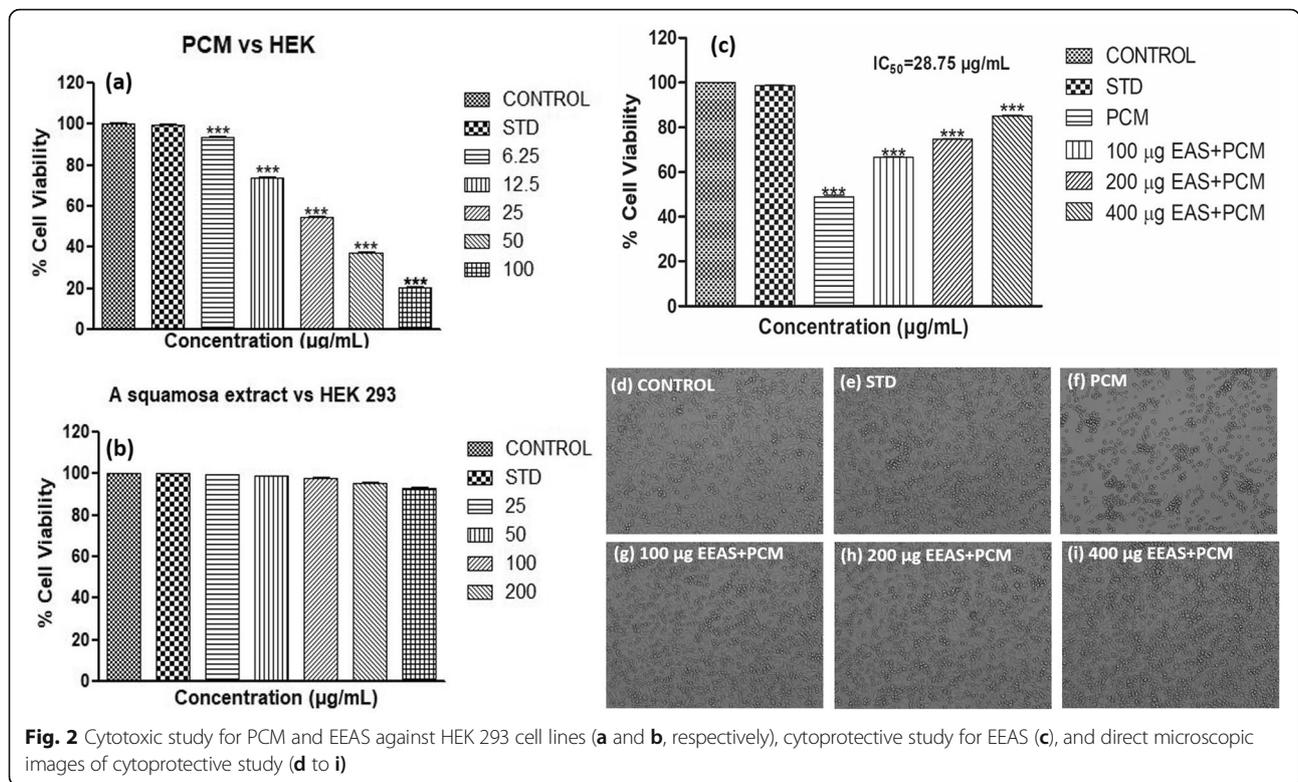


Fig. 2 Cytotoxic study for PCM and EEAS against HEK 293 cell lines (a and b, respectively), cytoprotective study for EEAS (c), and direct microscopic images of cytoprotective study (d to i)

mild swelling, necrosis, and cellular desquamation (Fig. 3d).

Discussion

At therapeutic doses, PCM is well thought-out to be a benign analgesic drug as well as an antipyretic drug. But, overdoses of it cause hepatotoxicity and nephrotoxicity in both humans and experimental animals. In critical overdosage or if daily dosage surpasses for an extended time, the standard conjugative metabolic pathway will be saturated and PCM is metabolized oxidatively by P450 (a diverse oxidase function system) to NAPQI. NAPQI possesses less half-life and is swiftly conjugated with glutathione and also with exhausted contents of cellular GSH. PCM-induced nephrotoxicity may cause tubular injury in the kidneys and is proved by phosphaturia, proteinuria of low molecular weight and can lead to severe renal failure which could be deadly in humans [1].

Before proceeding to in vivo nephrotoxicity studies, it is important to know the in vitro cell protective effect of the drug. For which MTT assay was carried out and significant percent cell viability was observed. MTT assay stands for enzymatic conversion of MTT into MTT formazan crystals which could be estimated by colorimetric method. The whole process was supported by succinate dehydrogenase produced from mitochondria of viable cells which depend on the respiration of mitochondria assessing the energy of the cell. In the current study, PCM-induced nephrotoxicity was treated with EEAS and found to be efficient enough to increase the percent cell viability.

With respect to in vivo nephrotoxicity studies, “gold standard” biomarkers were analyzed. The kidneys excrete BUN which is commonly found in liver proteins, diet, or tissue origin obtained from the breakdown of creatine. Creatinine is a derivative of endogenous tissue sources. In kidney ailments, the serum urea gets

Table 1 Effect of EEAS on BUN, creatinine, and uric acid on PCM-induced nephrotoxicity in rats

Group	BUN (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Normal	30.89 ± 0.25	5.12 ± 0.008	1.22 ± 0.06
PCM alone	72.17 ± 0.192*	9.13 ± 0.077*	2.05 ± 0.13*
PCM + EEAS(200 mg/kg)	54.2 ± 0.044@@	3.13 ± 0.112@@	1.70 ± 0.06@@
PCM + EEAS (400 mg/kg)	38.5 ± 0.03@@	4.57 ± 0.82@@	1.23 ± 0.03@@

All values were expressed as mean ± SEM, with six animals per group

*PCM-induced one compared with the normal one (P < 0.01)

@@Two different experimental groups compared with the PCM-induced group (P < 0.001)

Table 2 Effect of EEAS on GSH, SOD, and catalase on PCM-induced nephrotoxicity in rats

Group	GSH (nmol/g protein)	SOD (U/g protein)	Catalase (U/g protein)
Normal	68.16 ± 0.24	51.24 ± 3.11	55.01 ± 0.53
PCM alone	36.80 ± 0.14*	29.17 ± 1.58*	21.21 ± 0.09*
PCM + EEAS (200 mg/kg)	43.36 ± 0.73 ^{@@}	40.33 ± 2.61 ^{@@}	38.58 ± 0.19 ^{@@}
PCM + EEAS (400 mg/kg)	60.95 ± 0.08 ^{@@}	47.79 ± 2.14 ^{@@}	49.54 ± 0.14 ^{@@}

All values were expressed as mean ± SEM with six animals in a group

*PCM-induced group compared with the normal group ($P < 0.01$)

^{@@}Two different experimental groups compared with the PCM-induced group ($P < 0.001$)

deposited as the urea production rate exceeds the clearance rate. Any elevation of urea, creatinine, and also uric acid levels in serum are considered as the indexes of nephrotoxicity. But the concentration of BUN is regarded as a dependable kidney function predictor than creatinine. In the present study, rats having PCM-induced nephrotoxicity displayed a substantial ($P < 0.01$) rise in BUN, creatinine, and uric acid levels on comparing with the normal group. EEAS oral administration (200 and 400 mg/kg) dosage considerably ($P < 0.001$) reduced BUN, creatinine, and uric acid levels in a dose-dependent manner, which showed the potency of regeneration of renal cells. Along with serum biomarkers, administration of PCM nephrotoxic dose (200 mg/kg/day) has distorted kidney tissues oxidative status, causing oxidative stress by production of reactive oxygen and nitrogen species, respectively. These species further lead to necrosis of the renal tissue. Earlier research

documentations exposed that GSH is the chief aqueous-soluble cellular nonenzymatic antioxidant serving as the first line of defense in fighting free radicals. This also plays a key role in detoxification by dropping hydrogen peroxide as well as lipid hydroperoxides directly into H_2O and oxidized glutathione was formed [30]. Rats pre-treated with EEAS (200 and 400 mg/kg) have evidently displayed the nephroprotective effect than critical PCM intoxicated rats. Mammal cells also have endogenous antioxidant enzymes like SOD and CAT which could perform detoxification of free radicals. These enzyme levels are controlled within the cells to make sure of upholding the redox balance of the body [31]. The results displayed in Table 2 have evidently showed a significant rise in the levels of SOD and CAT in EEAS pretreated rats, establishing a nephroprotective effect of considered leaves. The effective refurbishment of the activities of the abovementioned enzymes compared with

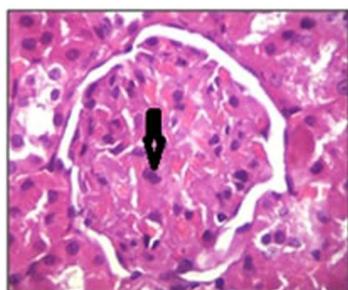


Figure 3a: no visible signs of degeneration or necrosis

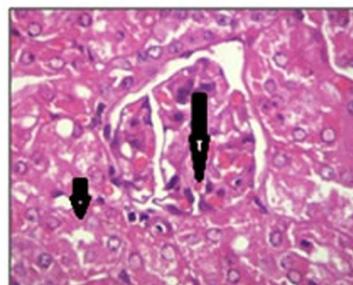


Figure 3b: marked tissue degeneration along with tubular necrosis

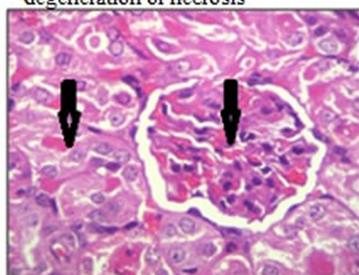


Figure 3c: represents reasonable necrosis and degranulation

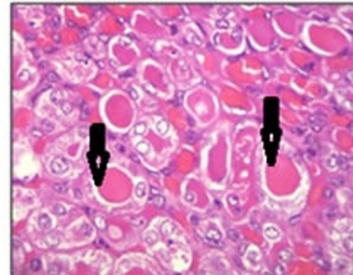


Figure 3d: represents very mild swelling, cellular desquamation

Fig. 3 Histopathological observations of the nephroprotective effect of EEAS against PCM-induced nephrotoxicity in rats where **a** control, **b** PCM alone, **c** PCM + EEAS 200 mg/kg, and **d** PCM + EEAS 400 mg/kg

PCM-intoxicated rats was probably because of the presence of the high levels of flavonoids, tannins, and polyphenols in ethanolic extract of *Annona squamosa* leaves [32]. When compared between a low dose and high dose of extract, EEAS (400 mg/kg) was said to be more effective in restoring serum biochemical parameters similar to normal and also increasing levels of antioxidant enzymes in tissue homogenate.

Similarly, in the present study, the histopathological analysis of PCM intoxicated kidney tissues in rats was acquired by sacrificing animals after 24 h. The analysis displayed acute necrosis and tubular epithelium degeneration. These changes suggested the degeneration of cells along with acute tubular necrosis and were most related to histopathological change. An insignificant swelling or moderate necrosis (Fig. 3c, d) was observed in the pre-treated EEAS (200 and 400 mg/kg) and proved the agreeable renal tissue regeneration and also the results are in well agreement with the control.

Conclusion

The results demonstrated the nephroprotective effect of EEAS against PCM brought toxicity in both in vitro and in vivo models worked upon in HEK-293 cells and rat kidneys, respectively. Hereby, we propose the efficacy of EEAS as a safe nephroprotective agent.

Abbreviations

EEAS: Ethanolic extract of *Annona squamosa*; PCM: Paracetamol; HEK-293: Human embryonic kidney cells; BUN: Blood urea nitrogen; MTT: 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO: Dimethyl sulfoxide; SOD: Superoxide dismutase; CAT: Catalase; GSH: Glutathione

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Authors' contributions

NS and DRP designed all the experiments. NS performed the experiment, analyzed the data, and wrote the manuscript. CSKB provided all the needed information. The authors read and approved the final manuscript.

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Availability of data and materials

All the data and materials are available upon request.

Ethics approval and consent to participate

The animals used for the study were obtained from the animal house, and the whole experiment was performed at the NGSIM Institute of Pharmaceutical Sciences, Mangaluru, with a written consent. Whole experiments and protocols described in the present study were established by the Institutional Animal Ethical Committee (IAEC) of NGSIM Institute of Pharmaceutical Sciences, Mangaluru (NGSMIPS/IAEC/MARCH-2019/126).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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