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Nephroprotective Effect Of Ethanolic Extract Of Cissus Quadrangularis Linn Fruits In Gentamicin Induced Nephrotoxicity: In Vitro Cell Viability And In Vivo Models

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Abstract

Nephrotoxicity is known to be a major complication during cancer patients. The present study was undertaken to evaluate nephroprotective activity of ethanolic extract of *Cissus quadrangularis linn* (EECQ) fruits in Wistar albino rats and in human embryonic kidney (HEK)-293 cells. Ethanolic extract of *Cissus quadrangularis linn* against Gentamicin induced renal damage was evaluated using in-vitro human embryonic kidney (HEK)-293 cells. Randomly selected animals were divided into five groups of six animals each. The test extracts were administered orally at a dose of 100mg/kg & 200 mg/kg. Gentamicin was administered at a dose of 80 mg/kg i.p. to the rats for 8 days. On eighth day all the animals were sacrificed and blood was collected. Elevation of urea and creatinine level in the serum was taken as the index of nephrotoxicity. Histopathological examinations of kidneys of all the groups were carried out. Co-treatment of HEK-293 cells with Gentamicin and EECQ extract at varying concentrations resulted in significant enhancement of cell growth compared to EECQ treatment indicating the cytoprotective activity of EECQ. The findings also revealed that EECQ possesses nephroprotective activity. The elevations of serum urea and creatinine produced by Gentamicin were considerably reduced and showed histopathological changes in the kidneys to normal. The study concludes that *Cissus quadrangularis Linn* possess promising nephroprotective activity due to its potent chemical constituents.

Keywords: *Cissus quadrangularis linn,* Gentamicin, nephroprotective activity, HEK-293 cells, ANOVA.

Introduction

Recognition of drug-induced nephrotoxicity as a significant contributor to kidney disease including acute kidney injury (AKI) and chronic kidney disease (CKD) has gained increasing momentum in recent times. Nephrotoxicity constitute a whole gamut of disorders reflecting damage to different nephron segments as a consequence of individual drug mechanisms. Consequences of drug toxicity might include both glomerular and tubular injuries leading to acute or chronic functional changes (Awdishu & Mehta, 2017). The frequency of drug induced nephrotoxicity is approximately 14-26% in adult populations as detailed in previous prospective cohort studies (Hoste et al., 2015). Aminoglycosides, a commonly used group of antibiotics top the causality chart in drug induced nephrotoxicity (Swain & Kaplan-Machlis, 1999). Aminoglycosides constitute an important part of our

arsenal against many life threatening infections especially against gram negative bacterial infections (Mingeot-Leclercq & Tulkens, 1999). They have survived against all odds despite the introduction of highly potent, wide spectrum antibiotics because of certain properties such as rapid concentration dependent bactericidal effects, clinical effectiveness, a low rate of true resistance, synergism with other beta lactam antibiotics and low cost of therapy (Begg & Barclay, 1995; Edson & Terrel, 1999). However, nephrotoxicity induced by them continue to be a challenge as it results in kidney damage by a direct dose dependent mechanism (Khoory et al., 1996; Rougier et al., 2004). Gentamicin induced acute renal failure has proved to be an excellent working animal model for exploring the pathogenesis of drug induced acute renal failure and has resulted in an impetus to develop therapeutic approaches to minimize or prevent its harmful effects in humans (Murakami et al., 1999). Renal toxicity caused by gentamicin is an elaborate phenomenon, the key features of which include an increase in plasma creatinine and urea levels with severe proximal renal tubular necrosis, with progressive deterioration and renal failure (Cuzzocrea et al., 2002). Generation of reactive oxygen species (ROS) in the kidney have been implicated as the culprits for nephrotoxicity induced by aminoglycosides (Al-Majed et al., 2002; Reiter et al., 2002). The cellular antioxidant status plays an important role in determining the susceptibility to oxidative damage which might alter in response to oxidative stress(Halliwell & Gutteridge, 2000; Abdel-Raheem et al., 2009). Several studies have claimed antioxidant property of drugs as crucial for their nephroprotective effects in gentamicin induced renal damage (Yaman & Balikci, 2010; Harlalka et al., 2007; Saxena, 2016).

Cissus quadrangularis reaches a height of 1.5 m (4.9 ft) and has quadrangular-sectioned branches with internodes 8–10 EECQ (3–4 in) long and 1.2–1.5 EECQ (0.5–0.6 in) wide. Along each angle is a leathery edge. Toothed trilobe leaves 2–5 EECQ (0.8–2.0 in) wide appear at the nodes. Each has a tendril emerging from the opposite side of the node. Racemes of small white, yellowish, or greenish flowers; globular berries are red when ripe.

Cissus quadrangularis is an evergreen climber growing to 5 m (16 ft) by .5 m (1.6 ft) at a fast rate. It is hardy to zone (UK) 10. Suitable for: light (sandy), medium (loamy) and heavy (clay) soils, prefers well-drained soil and can grow in nutritionally poor soil. Suitable pH: acid, neutral and basic (alkaline) soils and can grow in very acid and very alkaline soils. It cannot grow in the shade. It prefers dry or moist soil and can tolerate drought.

Material and Methods

Materials

Gentamicin sulfate injection (Piramal Health Care Ltd) was used to induce renal damage. Dimethyl sulfoxide-bio reagent, DMEM growth medium, Fetal bovine serum (FBS), L-Glutamine, MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], MEM-non-essential amino acid solution, reduced glutathione (GSH), Greiss reagent, were procured from Sigma-Aldrich (St. Louis, MO, USA). Commercial reagent kits for determination of creatinine (CRE), urea (UR), uric acid (UA), total protein (TP), albumin (Alb), calcium (Ca^{2+}), Magnesium (Mg^{2+}), phosphorus (P), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (γGT) activities were purchased from Biolabo S.A. (Paris, France). All other chemicals and reagents used were of the analytical grade.

Experimental animals: Adult Wistar albino rats of either sex, weighing 150-200g, inbred in the institutional animal house were used for the study. Animals were housed in polypropylene cages in a controlled environmental condition (22± 30 C, 55 ± 5% humidity and a 12 h light/ dark cycle). The

animals were fed with standard rodent diet and water ad libitum. They were allowed to acclimatize to these conditions for one week.

Methods

Plant collection and authentication: Cissus Quadrangularis Linn Fruits and Michelia Champaea Leaves were obtained from the local places of Tirupati, AP. Cissus Quadrangularis Linn Fruits was authenticated by Dr. K. Madhava Chetty, M.Sc., M.Ed., M.Phil., Ph.D., PG DPD., Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh.

Extraction by Maceration: Fresh leaves of Michelia Champaea and fruits of Cissus Quadrangularis Linn were washed with water to get rid of contaminants like dirt and other impurities and were shade-dried. These dried leaves and fruits were ground and sieved to get a uniform, coarse powder. Powdered plant material was weighed (1Kg) and is immersed in 95% ethanol and kept for maceration for a period of 7 days with occasional stirring. On the 8^{th} day, the solvent was filtered by pressing with a muslin cloth and was evaporated in a rotary evaporator at 40^{o}C. The resultant extract was put in a desiccator to remove any ethanol left in it. The dried ethanolic extract of Cissus Quadrangularis (EECQ) was packed in an air-tight bottle and put in a dry place for further studies.

Qualitative evaluation of Phytoconstituents: The EECQ were screened for the presence of various phytoconstituents like carbohydrates, flavonoids, polyphenolic compounds, saponins, tannins, triterpenoids, etc.

Evaluation of Nephroprotective activity

In-vitro cell line studies

Cell culture: Variety of supplements such as 10% Fetal Bovine Serum (FBS), 1X Penicillin-Streptomycin solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 1500 mg/L sodium bicarbonate were added to Dulbecco's Modified Eagle's medium (DMEM) growth medium and filtered through 0.2 µm filter using a filtration unit fitted to a vacuum pump. Filtered media was stored at 4 °C. Human embryonic kidney cell line (HEK-293; ATCC®, CRL-1573™) obtained from ATCC used for the study. Cells stored in liquid nitrogen were thawed and revived as per recommended methods. Cells were cultured and expanded in complete DMEM growth medium. Sub-confluent mono-layers of cells were harvested, pelleted and re-suspended in growth medium prior to counting on a haemocytometer by Trypan blue exclusion method.
Effect of EECQ extract in gentamicin-induced toxicity in HEK-293 cells HEK-293 cells were cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum in a CO_2 incubator (5% CO_2 in air) at 37 °C. The cells with 70–80% confluency were trypsinized and sufficient media added to inactivate the trypsin activity. The cells were centrifuged at 1200 rpm for 5 min, supernatant was discarded and resuspended the pellet in media prior to counting on a haemocytometer by Trypan blue exclusion method. The cells were diluted in media to get desired number of cells. For cell growth studies, the final seeding density was kept 10,000 cells/ well in a 96-well flat-bottomed micro-titer plate. Post 24 h of cells seeding, cells were untreated, treated or co-treated with

GENTAMICIN (20 µM) and EECQ extract (5, 10, 25, 50, 100 and 200 µg/ mL) for 24 h. After 24 h of treatment, cell viability assay and cell morphological analysis were performed.

Cell viability test: Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) bioassay, which gives a sensitive measurement of the normal metabolic status of cells. Post treatment at 24 h, MTT solution (5 mg/ml) added to the wells of respective treated cells and incubated for 3 h. The dark-blue formazan formed in the well was dissolved in DMSO and the absorbance was measured at 570 nm using a microtiter plate reader.

Cell morphological evaluation: Morphological changes in HEK-293 cells were examined using compound microscope post-exposure to Gentamicin (20 µM) alone, or combination of Gentamicin (20 µM) and EECQ extract (5–200 µg/ml) for 24 h at 37 °C[20].

Gentamicin induced nephrotoxicity method

Animal Grouping: The animals were divided into five groups with six animals in each group (n=6).
The treatment is as follows:
Group I-Normal Control: Normal saline (10ml/kg), p.o, OD for 28 days.
Group II-Disease Control: Gentamicin (80mg/kg), i.p, once daily (OD) for 8 days.
Group III-Standard Control: Vitamin E (250mg/kg), p.o, OD for 28 days with intraperitoneal administration of Gentamicin once daily during the last 8 days.
Group IV-Test Control (100mg/kg): EECQ (100mg/kg), p.o, OD for 28 days with intraperitoneal administration of Gentamicin once daily during the last 8 days.
Group V-Test Control (200mg/kg): EECQ (200mg/kg), p.o, OD for 28 days with intraperitoneal administration of Gentamicin OD during the last 8 days (Hamad et al., 2018). 24 hours post final dose administration, body weights were measured and the blood was drawn into Eppendorf tubes via the retro-orbital route. Serum was separated by the centrifugation of blood samples at 3000 rpm for a period of 10mins. Serum samples were prepared and used to evaluate the biochemical parameters like creatinine, urea, uric acid, total protein, Blood Urea Nitrogen (BUN), albumin, and globulin, etc. After collecting blood samples, animals were sacrificed by dislocating the spinal column, dissected, and kidneys were isolated from each animal, where one kidney kept in 10% Formalin for preparing histopathological slides and the other one homogenized using ice-cold KCl to prepare tissue homogenate to evaluate the in vivo antioxidant parameters (Kanna et al., 2015; Singh et al., 2018).

Biochemical evaluation of Serum samples: These are the bio chemical parameters were estimated like serum Creatinine, Uric acid,
Urea, Total protein, Albumin

In vivo Anti-oxidant studies: In In vivo Anti-oxidant studies Lipid peroxidase (LPO) activity, Reduced Glutathione Catalase (CAT) activity was estimated

Histopathological Studies
The isolated kidneys that were preserved in 10% formalin were embedded in paraffin wax and longitudinally sliced by the use of microtome. They were stained using hematoxylin and eosin (H&E) stain and observed under a trinocular microscope.

Statistical analysis: All the results were analyzed by using one-way ANOVA followed by Dunnett's multiple comparison tests.

Results and Discussion

Preliminary Phytochemical Screening: Results of phytochemical screening were elucidated in Table 1**.**

Table 1. Results of Phytochemical screening of EECQ

S. No	Name of the Phytochemical	EECQ
1.	Carbohydrates	+
2.	Amino acids	+
3.	Proteins	+
4.	Alkaloids	+
5.	Cardiac glycosides	+
6.	Triterpenoids	+
7.	Saponins	+
8.	Flavonoids	+
9.	Phenolic compounds	+
10.	Tannins	+
11.	Steroids	-
12.	Gums	-

Where, + means positive and - means negative.

The preliminary phytochemical screening showed the presence of various phytoconstituents like flavonoids, phenolic compounds, triterpenoids, tannins, saponins, amino acids, proteins, and carbohydrates in Ethanolic Extraction of Cissus Quadrangularis (EECQ).

Results of EECQ extract in Gentamicin -induced toxicity in HEK-293 cells

The efficacy of the plant extract (EECQ) was evaluated in Gentamicin induced cytotoxicity in human embryonic kidney (HEK-293) cells. Cell viability was evaluated to assess the cytoprotective effect of EECQ extract in gentamicin treated HEK-293 cells. HEK-293 cells were treated with various concentrations of EECQ extract (5, 10, 25, 50, 100 and 200 µg/ mL) alone or in combination with Gentamicin (20 µM) for 24 h. Treatment with EECQ extract alone did not induce any overt detrimental effect on cell viability. Gentamicin treatment significantly ($P < 0.001$) reduced the cell viability and was associated with morphological changes such as cell shrinkage, rounded cell shape and cytoplasmic vacuolation com-pared to normal control. To measure the effects of EECQ extract on the growth of Gentamicin-treated renal cells, HEK-293 cells were treated with Gentamicin (20 µM) and/or different concentration of EECQ extract. Cell viability was significantly improved when cells were cotreated with EECQ extract and Gentamicin. The cell viability was improved by 7–25% with EECQ treatment. [Figure 1 and 2].

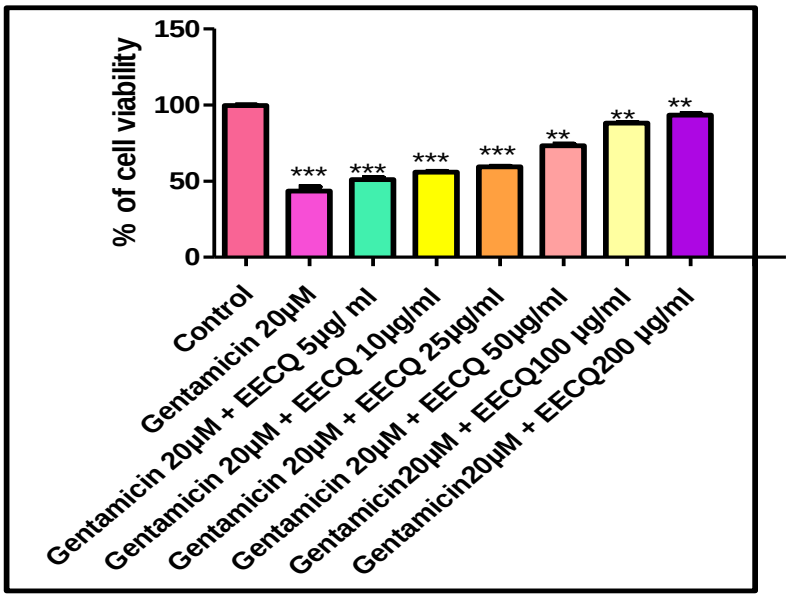
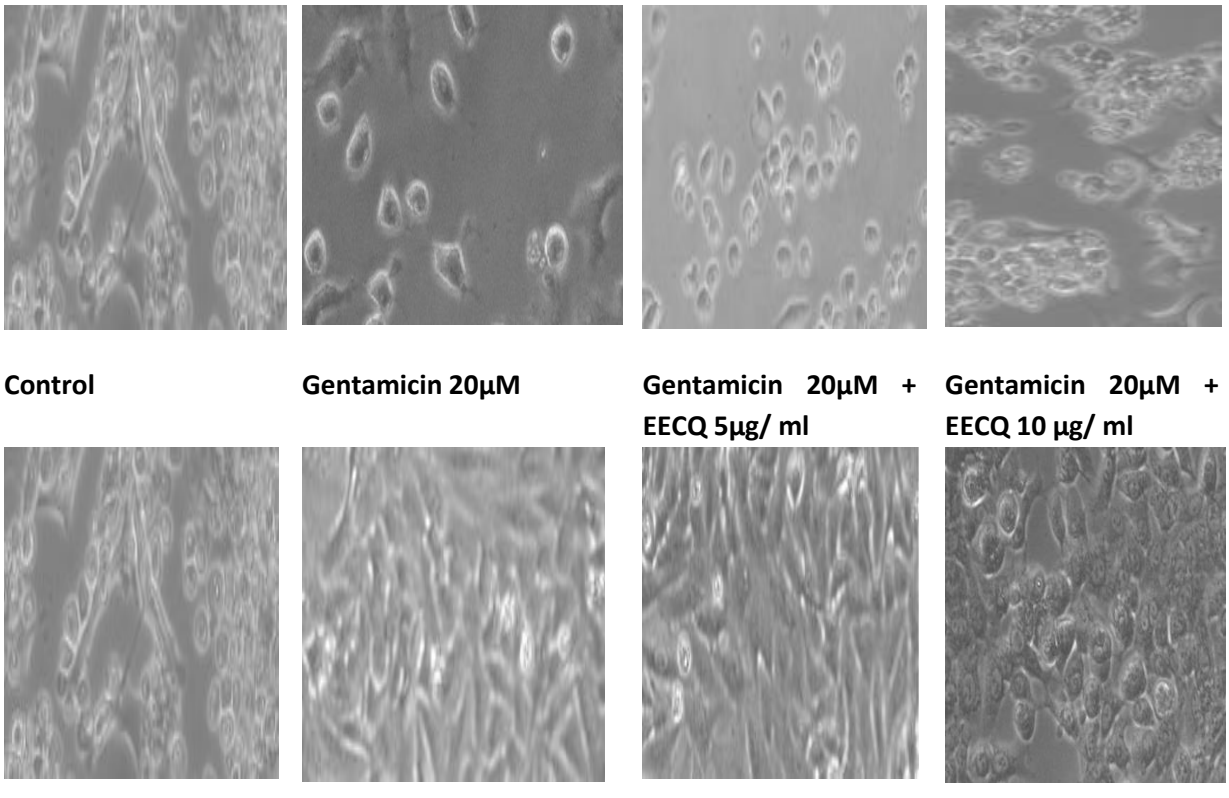


Figure 1. HEK-293 cells were treated with Gentamicin (20 µM) and EECQ extract (5–200 µg/mL) for 24 h and evaluated for cytotoxicity by MTT assay.



Control | **Gentamicin 20µM** | **Gentamicin 20µM + EECQ 5µg/ ml** | **Gentamicin 20µM + EECQ 10 µg/ ml**

Gentamicin 20µM + EECQ 25 µg/ ml | **Gentamicin 20µM + EECQ 50 µg/ ml** | **Gentamicin 20µM + EECQ 100 µg/ ml** | **Gentamicin 20µM + EECQ 200µg/ ml**

Figure 2. Microscopic images of HEK-293 cells morphology after treatment with Gentamicin and EECQ for 24 h.

Results of change in body weights

The significant values of body weights of normal, disease, standard, EECQ 100mg/Kg and EECQ 200mg/Kg were reported. There is no increase in body weights of animals treated with EECC 100mg/Kg and 200mg/Kg when compared to disease and normal control. There is a significant decrease in kidney weights of animals treated with EECQ 100mg/Kg and 200mg/Kg when compared to disease and normal control. [Table 2 and Figure 3].

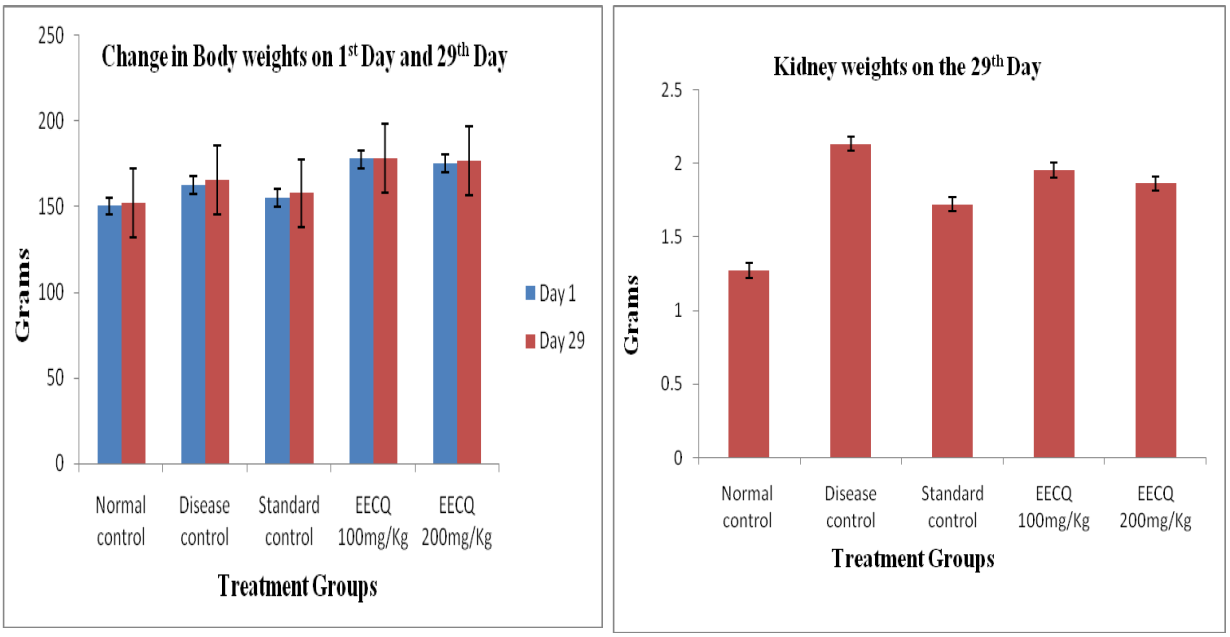


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Figure 3. (A) Change in Body weights on 1st and 29th Day (B) Kidney Weights on 29thday

Table 2. Change in Body weights on 1st Day and 29th Day of EECQ

S.No	Treatment Groups	Body weights on Day 1 (in grams)	Body weights on Day 29 (in grams)	Kidney weights on Day 29 (in grams)
1.	Normal control	150.05±6.050	151.85±6.061	1.273±0.042
2.	Disease control	162.50±8.067	165.20±6.071	2.136±0.060
3.	Standard control	155.02±6.051	157.50±8.098	1.723±0.041***
4.	EECQ 100mg/Kg	177.50±6.065	178.12±8.110	1.954±0.053**
5.	EECQ 200mg/Kg	175.12±6.056	176.51±8.076	1.865±0.043**

Values are represented as Mean ± SD.

Results of EECQ on serum parameters

There is a significant decrease in serum creatinine of animals treated with EECQ 100mg/Kg and 200mg/Kg when compared to disease control. Significant decrease in serum uric acid of animals treated with EECQ 100mg/Kg and 200mg/Kg compared to disease control. There is a significant decrease in urea of animals treated with EECQ 100mg/Kg and 200mg/Kg when compared to disease control. Significant decrease in BUN of animals treated with EECQ 100mg/Kg and 200mg/Kg when

compared to disease control. There is a significant increase in protein levels of animals treated with EECQ 100mg/Kg and 200mg/Kg when compared to disease control. Significant increase in albumin of animals treated with EECQ 100mg/Kg and 200mg/Kg compared to disease control There is a significant increase in globulin in of animals treated with EECQ 100mg/Kg and 200mg/Kg when compared to disease control. [Table 3 and Figure 4].

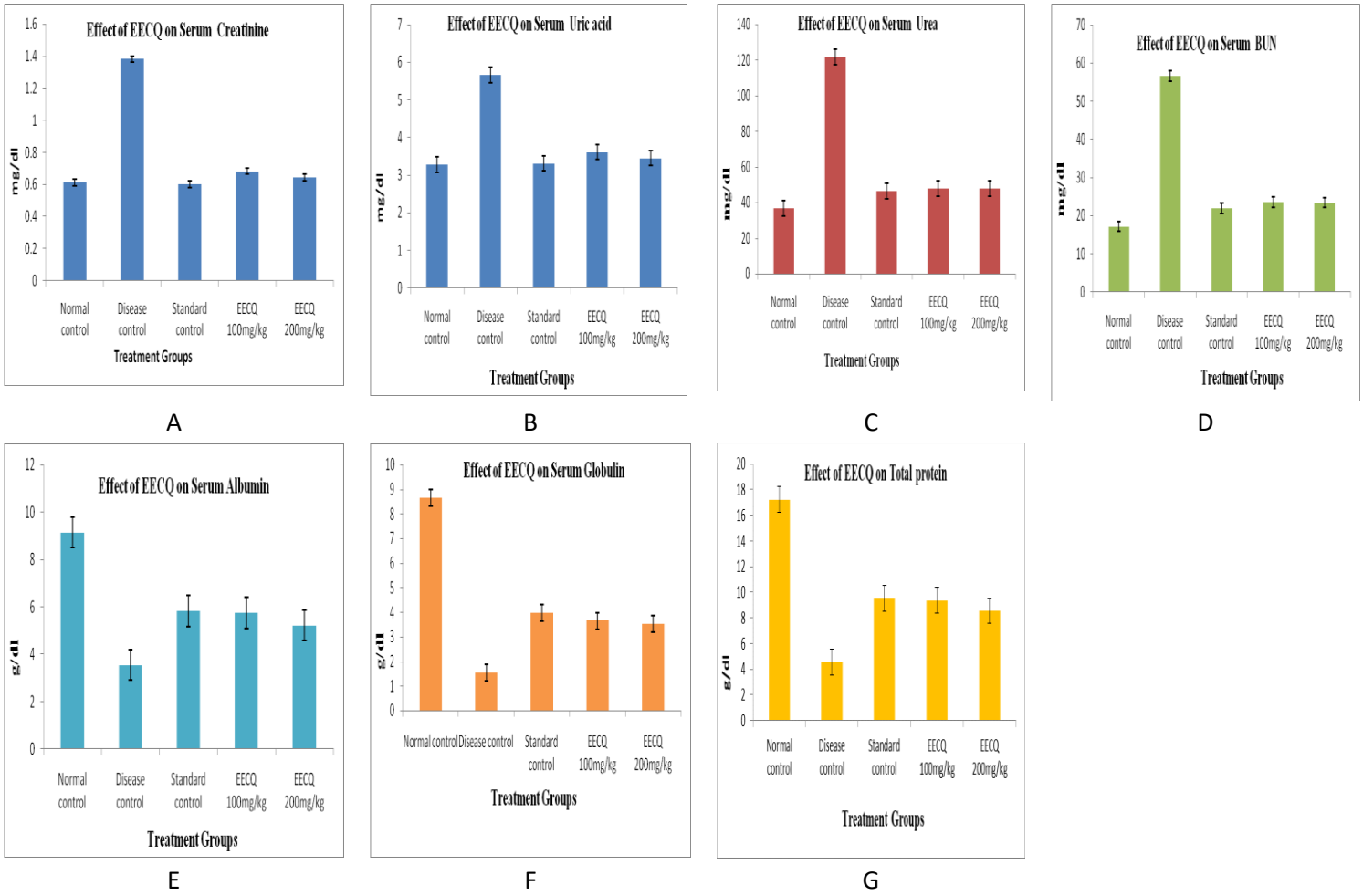

Figure 4. Effect of EECQ on serum Urea levels, BUN levels, Protein levels, Albumin levels and Globulin levels.

Table 3. Effect of EECQ on Serum parameters.

S.No	Treatment Groups	Creatinine (mg/dl)	Uric acid (mg/dl)	Urea (mg/dl)	BUN (mg/dl)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
1.	Normal control	0.613± 0.0213	3.281± 0.289	36.640±4 .321	17.123± 1.453	17.233± 0.545	9.143± 0.432	8.656± 0.321
2.	Disease control	1.382± 0.0324	5.658± 0.163	121.54±4 .396	56.591± 2.133	4.567± 0.653	3.543± 0.332	1.543± 0.432
3.	Standard control	0.602± 0.0354***	3.312± 0.215***	46.460±4 .032***	21.870± 1.376***	9.543± 0.876***	5.832± 0.344***	3.965± 0.443***
4.	EECQ 100mg/kg	0.682± 0.0241***	3.615± 0.268***	48.021±5 .834***	23.534± 2.543***	9.354± 0.896***	5.754± 0.438***	3.654± 0.452***

5.	EECQ 200mg/kg	0.643± 0.0265***	3.452± 0.190***	47.712±3 .321***	23.423± 1.254***	8.543± 0.541***	5.215± 0.264**	3.515± 0.343***

Values are represented as Mean ± SEM. Statistical analysis was done by one way ANOVA followed by post hoc Dunnett's multiple comparison tests. ***p<0.0001, **p<0.001, and *p<0.05 vs Disease control.

In vivo antioxidant studies of EECQ

There is a significant decrease in LPO levels of animals treated with EECQ 100mg/Kg and 200mg/Kg compared to disease control. significant increase in GSH levels of animals treated with EECQ 100mg/Kg and 200mg/Kg compared to disease control. Significant increase in CAT levels of animals treated with EECQ 100mg/Kg and 200mg/Kg compared to disease control. [Table 4 and Figure 5].

Table 4. Effect of EECQ on LPO, GSH, and CAT

S.No	Treatment Groups	Lipid peroxidation (in µM/mg tissue)	Reduced glutathione (in µM of GSH/mg tissue)	Catalase (in units/mg protein)
1.	**Normal control**	2.853±0.087	4.227±0.104	0.720±0.049
2.	**Disease control**	5.233±0.214	2.907±0.136	0.450±0.031
3.	**Standard control**	2.965±0.164***	3.610±0.083***	0.637±0.022**
4.	**EECQ 100mg/Kg**	3.303±0.082***	3.550±0.109***	0.598±0.032*
5.	**EECQ 200mg/Kg**	3.590±0.085***	3.410±0.083**	0.603±0.052*

Values are represented as Mean ± SEM. Statistical analysis was done by one way ANOVA followed by post hoc Dunnett's multiple comparison tests. ***p<0.0001, **p<0.001, and *p<0.05 vs Disease control.

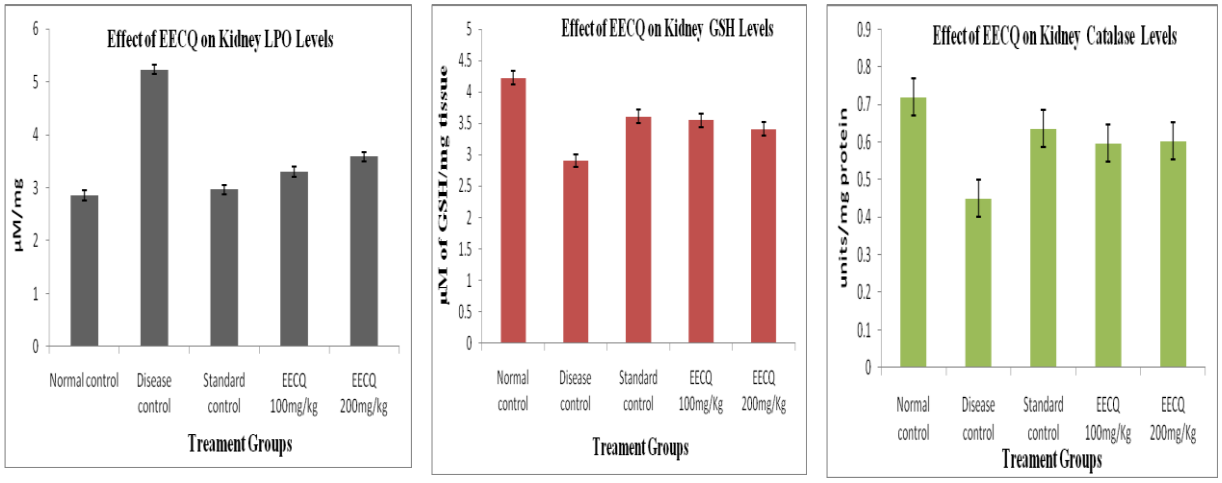

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Figure 5. Effect of EECQ on Kidney LPO, GSH levels, CAT levels.

Discussion

The present study indicates phytochemical and pharmacological evaluation of nephroprotective action of Ethanolic Extraction of Cissus Quadrangularis linn fruits in the doses of 100 and 200 mg/kg

body weight. Preliminary phytochemical screening of EECQ indicated the presence of alkaloids, flavonoids, saponins, carbohydrates, proteins, amino acids, tannins, and phenolic compounds.

Gentamicin (GM) which is a widely used aminoglycoside antibiotic, is recognized for possessing significant nephrotoxic potential in humans and experimental animals. GM-induced nephrotoxicity is characterized by elevated levels of urea, creatinine, uric acid, and BUN in plasma. The nephroprotective study was carried out and serum parameters like creatinine, uric acid, urea, BUN, total protein, albumin, and globulin were measured to assess the nephroprotective activity of EECQ. There are two factors on which serum and urine parameters depend. One is the GFR and the other is the degree of tubular reabsorption. The observed effect may be attributed to a mechanism like increasing the renal blood flow and the attendant increase in GFR.

Histopathological studies

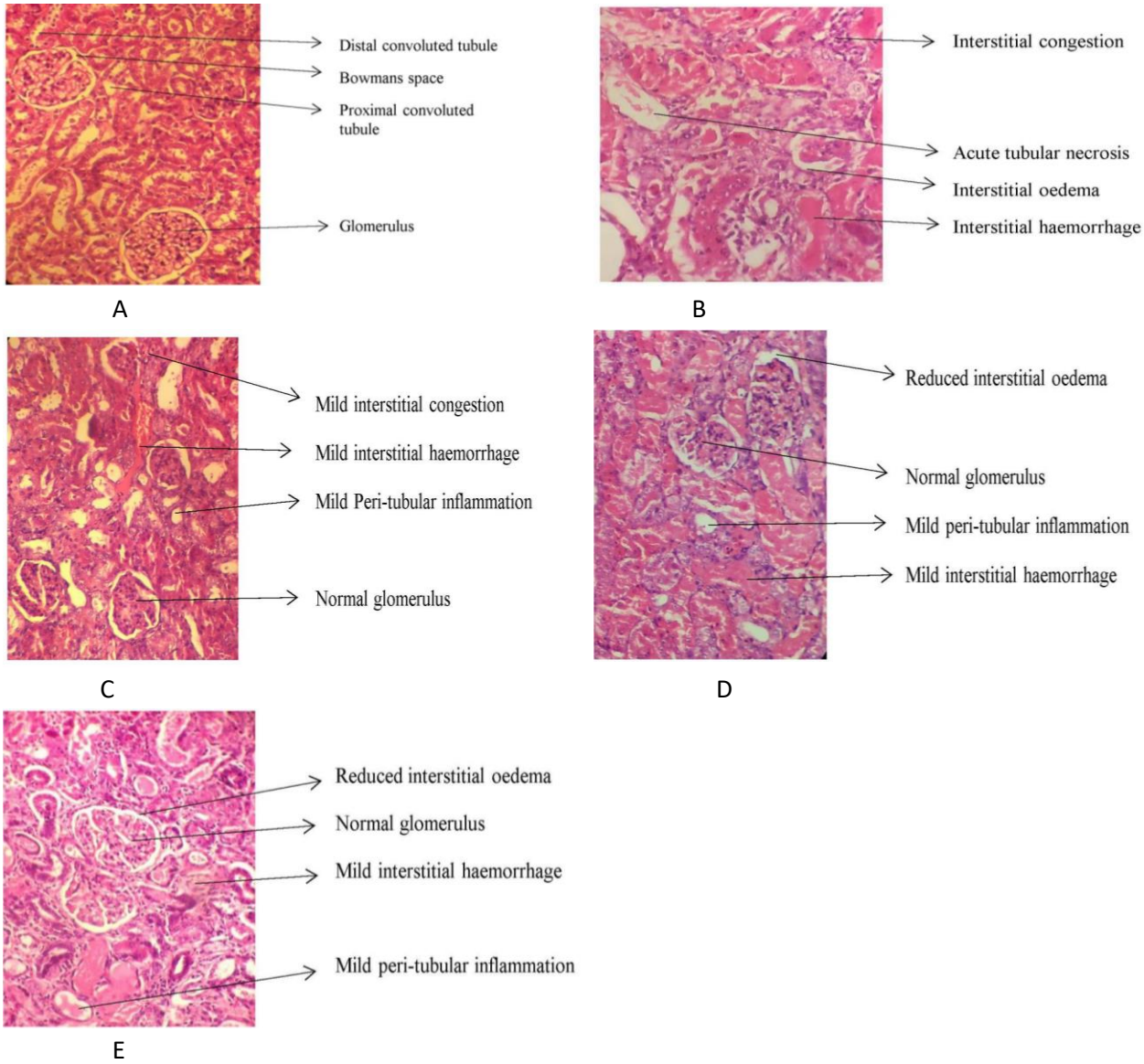

Figure 6. Light microscopic study (H&E) of renal tissue in various experimental groups. (A) Group I-Normal control (Saline 10ml/kg), (B) Group II-Disease control (Gentamicin 80mg/kg),(C) Group III-

Standard control (Vit E 250mg/kg),(D) Group IV-Test control (EECQ 100mg/kg),(E) Group V-Test control (EECQ 200mg/kg).

The present study demonstrates that EECQ significantly increased the GFR compared to disease control animals. EECQ reduced the serum creatinine, uric acid, urea, and BUN and increased the serum protein, albumin, and globulin. These observations indicate an improved renal function by EECQ.

Antioxidant parameters like LPO, GSH, and CAT were also evaluated. Reduced activity of CAT and levels of GSH after treatment with gentamicin suppresses endogenous enzymatic antioxidant machinery. Treatment with EECQ increased the activity of CAT and levels of GSH significantly compared to the disease control animals. It was also observed that an increase in LPO in disease group is due to altered antioxidant machinery and higher susceptibility towards oxidative damage. However, EECQ lowered LPO levels in EECQ treated groups. As per the findings, the secondary metabolites, flavonoids and phenolic compounds are present in the plants which are antioxidant in nature. These may be responsible for kidney protective activity.

The histopathological results obtained correlated well with the biochemical results where standard and EECQ treated groups showed significant improvement when compared to disease control.

Based on traditional medicine uses, chemical compositions and antioxidant EECQ, form the basis for the pre-sent study conducted to examine the protective effects of EECQ against gentamicin-induced toxicity in HEK-293 cells and kidney injury in Rats. Favorably, the study reveals that EECQ extract per se has no cytotoxic effect. However, gentamicin led to a significant increase in cell death with changes in normal cellular morphology in HEK-293 cells. HEK-293 cells treated with gentamicin and EECQ extract resulted in significant enhancement of cell growth compared to gentamicin control indicating the cytoprotective activity of EECQ-extract against gentamicin induced cytotoxicity. In addition, gentamicin-treated rats depicted typical clinical and pathological symptoms such as increased relative kidney weight, altered kidney function parameters such as creatinine, urea, uric acid, total protein, albumin and electrolytes. Further, increased levels of creatinine, urea, and uric acid in animals treated with gentamicin indicated a reduction in glomerular filtration rate. Similarly, the increase in the relative weight of the kidneys is attributed to the retention of urine due to tubular obstruction caused by plasters. Our results are similar to previous findings on gentamicin induced nephrotoxicity. Treatment with EECQ extract resulted in marked amelioration of these altered parameters in HEK-293 cells. Hence, it is proved that EECQ shows nephroprotective activity against Gentamicin-induced acute kidney injury.

Conclusion

Our results demonstrated that EECQ exerts its renoprotective effect against Gentamicin-induced toxicity in both in vivo and in vitro models established in HEK-293 cells. We propose that EECQ can be considered as a safe nephroprotective. EECQ showed significant protective activity against ARF caused by Gentamicin. The renoprotective activity of EECQ may be due to the single or combined effects of flavonoids, terpenoids, saponins, tannins, and phenolic compounds. But there is a high chance that flavonoids or tannins must have shown significant nephroprotective activity because as per literature flavonoids and tannins do possess good action on the urinary system. Thus, we can conclude that Cissus Quadrangularis Linn Fruits possess nephroprotective activity.

Acknowledgement

None

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