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RESEARCH ARTICLE

Pharmacological Evaluation of Nephroprotective Activity of Aqueous Flowers Extract of *Calotropis Gigantean Linn* in Gentamicin Induced Nephrotoxicity in Wistar Rats.

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ABSTRACT

Nephrotoxicity is one of the most common kidney problems and occurs when body is exposed to a medicine or chemicals. A number of therapeutic agents can adversely affect the kidney resulting in acute renal failure, chronic interstitial nephritis and nephritic syndrome because increasing number of potent therapeutic drugs like aminoglycoside antibiotics, chemotherapeutic agents and NSAIDS. Nephroprotective agents are the substances which possess protective activity against nephrotoxicity. Medicinal plants have curative properties due to the presence of various complex chemical substances. The present review is about the some of the medicinal plants possessing nephroprotective activity on Gentamicin induced nephrotoxicity. The Nephrotoxic effect of cyclosporine, aminoglycoside antibiotics, cisplatin, amphotericin-B, beta-lactam antibiotics and Indomethacin are reviewed. These drugs were produce produced nephrotoxicity because they are most frequently causes of renal injury in children. In addition, their nephrotoxicity is acquired by altered mechanisms. Several generalizations can be made, however. First, agents which could cause tubular accident tend to be accessory in their baneful effects. This review attempts to portray the discory and development of anesthetic from galenical to genomical, with a focus on the abeyant and role of medicinal plants

Key words: Nephrotoxic, Gentamicin

Introduction

Herbal medicine, also called botanical medicine or phytomedicine, refers to the use of seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. Use of herbs and traditional systems of medicine is becoming more main stream as improvements in analysis and quality control along with advances in clinical research show their value in the treatment and prevention of diseases.

From ancient time, plants have used as the major source of medicine and food for human being, and they have continued to provide mankind with new, novel therapeutic medicine and remedies. Since the last five decades, there has been a remarkably research in the study and use of herbal plants.

This current global interest in the study and use of medicinal plants has led to the characterization and identification of novel lead molecules, and isolation of active chemical compounds of therapeutic importance. The current world scenario of utilization of plant-derived natural remedies hascreated a dire need for accurate and up to date information on the characteristic properties and therapeutic uses,

efficacy, safety and quality of medicinal plant products[1].

Due to toxicity and side effects of allopathic medicines, has led to rapid increase in the number of herbal drug manufacturers for reduced these problems.Herbal drugs/products have reached extensive adequacy as beneficial agents like anti arthritic, sedative, antidepressant, anti anxiety, antispasmodic, analgesic, anti-inflammatory antimicrobial, anti diabetic, anti fertility, anti ageing, etc. Herbal drugs have been recognized for approximately 5000 years. These drugs have survived real world testing and thousands of years of human testing[2].

Lifestyles of populations across the world have changed dramatically in the 20th century. These changes (collectively termed as epidemiological transition) have been brought about by a number of developments in science and technology that now affect every facet of human existence. Most human societies have moved from agrarian diets and active lives to fast foods and sedentary habits.

Nephrotoxicity diseases . Nephrotoxicity is one of the most common kidney problems and occurs when body is exposed to a medicine or chemicals. A number of therapeutic agents can adversely affect the kidney resulting in acute renal failure, chronic interstitial nephritis and nephritic syndrome because increasing number of potent therapeutic drugs like aminoglycoside antibiotics, chemotherapeutic agents and NSAIDS. Nephroprotective agents are the substances which possess protective activity against nephrotoxicity. Medicinal plants have curative properties due to the presence of various complex chemical substances. The present review is about the some of the medicinal plants possessing nephroprotective activity on Gentamicin induced nephrotoxicity[1].

Nephrotoxicity can be authentic as renal disease or dysfunction that arises as a absolute or aberrant aftereffect of exposure to medicines, and environmental or industrial chemicals. Several factors accept been articular which accomplish the kidney accessible to toxic injury due to indigenous medicines. These drugs were produce produced nephrotoxicity because they are most frequently causes of renal injury in children. In addition, their nephrotoxicity is acquired by altered mechanisms. Several generalizations can be made, however.

DIFFERENT TYPES OF NEPHROTOXICITY

Aminoglycoside nephrotoxicity: Aminoglycosides specially influence the proximal tubular cells. These operators are uninhibitedly separated by the glomeruli and immediately taken up by the proximal tubular epithelial cells, where they are consolidated into lysosomes after first connecting with phospholipids on the brush fringe films. They apply their primary harmful impact inside the tubular cell by adjusting phospholipid digestion system. Consequently, a solitary every day huge measurement is desirable over 3 dosages for each day. One measurement for each day probably causes less amassing in the tubular cells once the immersion point is reached[7-10].

Calcineurin inhibitor nephrotoxicity: Cyclosporine and tacrolimus cause acute kidney injury (AKI) by initiating afferent and efferent arteriolar vasoconstriction. Steady harm can prompt to interstitial fibrosis. Tacrolimus has been appeared to bring about thrombotic microangiopathy therefore of endothelial injury.

Clinical features

- **Pain** is the most common presenting symptom of ureteric calculi and is caused by the stone obstructing the urinary tract. A sudden onset of acute loin pain, often at night when the urine is maximally concentrated. A stone can become impacted along the urinary tract at one of the five anatomical locations. Classical renal colic is characterized by an acute onset of pain in the loin that radiates to the groin and scrotum or labia majora.
- **Infection** may be a causal factor in stone formation or may be secondary to obstruction caused by the calculus. Typically, infection with urease-producing organisms causes alkalinization of the urine, leading to formation of magnesium ammonium phosphate stones which may become large staghorn
- Haematuria**:** blood in the urine, due to minor damage to inside wall of kidney, ureter and/or urethra. Haematuria is present

in 85–90 % of patients with stone and is usually microscopic (although frank blood may be observed). Haematuria may be absent (even on dipstick analysis) in up to 15% of patients[18].

- Pyuria: pus in the urine.
- Dysuria**:** burning on urination when passing stones (rare). More typical of infection.
- Oliguria**:** reduced urinary volume caused by obstruction of the bladder or urethra by stone or extremely rarely, simultaneous obstruction of both ureters by a stone.
- Abdominal distension**.**
- Nausea/vomiting**:** Embryological link with intestine stimulates the vomiting center.
- **Fever and chills.**
- **Hydronephrosis**
- Postrenal azotemia**:** when kidney stone blocks ureter.

Diagnosis of Nephrotoxicity

Table 1: List of analyses carried out as part of a Stone Screen procedure

Sample	Analyses performed
Stone	Quantitative infra-red analysis for mineral constituents
Blood	Urea, creatinine, sodium, potassium, bicarbonate, albumin, calcium, magnesium, phosphate, urate, alkaline phosphatase. PTH and 25(OH)-vitamin D, oxalate (in cases of severe hyperoxaluria)
Spot urine	Creatinine, urea, pH, calcium, phosphate, oxalate, urate, sodium, potassium, magnesium
24-h urines	Volume, pH, creatinine, calcium, magnesium, phosphate, oxalate, citrate, urate, urea, sodium, potassium, protein, qualitative cystine (and quantitative cystine), ornithine, arginine and lysine, if qualitative cystine is positive
Diet diary	Fluids, water, calories, calcium, magnesium, phosphate, oxalate, total protein, animal protein, meat + fish + poultry protein, fruit + vegetable + cereal protein, dairy protein, purine, fat, refined carbohydrates, fibre, sodium and potassium

(Source: Renal stone disease. Medicine 35:8, 418).

A. Imaging (Radiography)

- **A kidney-ureter-bladder (KUB) X-ray**
- **Ultrasound**
- **Intravenous urogram (IVU)**
- **Computed tomography (CT KUB)**

MATERIALS AND METHODS:-

Plant Material

The Aqueous flowers extract of *Calotropis Gigantean Linn* was used by initially making a stock solution of 1mg/ml and thereafter concentrations of 25, 50, 75, 100, 250 and 250 µg/ml were made.

Methods:

In-vitro Antioxidant methods:

DPPH radical scavenging activity
Phosphomolybdenum reduction assay
Nitric Oxide radical-scavenging activity Assay
Reducing power assay

DPPH radical scavenging activity [40]

Materials & Methods

Chemicals:

1, 1- diphenyl-2-picryl hydrazyl (DPPH) AR, Methanol (95%), Butylated hydroxy toluene (B.H.T.).

Method [30]

The free radical scavenging activity of the extract was assessed on the basis of the radical-scavenging effect of the stable 1,1- diphenyl-2-

picryl hydrazyl (DPPH) free radical . A series of extract concentration in the same extraction solvent was prepared (25, 50, 100, 150, 200 & 250 µg/ml). Then, 3 ml of extract at different concentrations was mixed with 1000 µl of 0.004% DPPH in methanol. The disappearance of DPPH was read spectrophotometrically at 517 nm after 30 min of incubation at room temperature in the dark. A purple to yellow colour change is observed.

Calculation:

Free radical scavenging capacity was expressed as percentage inhibition of DPPH radical and was calculated by the following equation:

I (% inhibition) = 100 × (1-Absorbance of sample/Absorbance of control)

From the obtained values, the IC_{50} (defined as the concentration of extract at which 50% of maximum scavenging activity was recorded) was calculated for each extract.

IN-VIVO PHARMACOLOGICAL INVESTIGATIONS

DIURETIC ACTIVITY [44]

Diuretic activity is the preliminary requirement for the antiurolithiatic activity. Urine volume increases in dieresis. The increased urine volume in this decreases the super saturation of the calcium and oxalate and prevents nucleation, aggregation and crystal growth.

Material & Methods

Chemicals: Urea, NaCl

Animals: Twenty four inbred male Wistar Albino rats (180-200g body weight) were used in this study. Animals were procured from Institutional Animal House of RKDF college of Pharmacy, Bhopal, M.P.. All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27^{0}C and humidity 60-65% with 12:12 light: dark cycles). Food was provided in the form of dry pellets and water *ad libitum*. The animals were acclimatized under laboratory conditions for 7 days before the commencement of the experiment. All experiments involving animals complies with the ethical standards of animal handling and were approved by the Institutional Animal Ethics Committee.

Ethical clearance: Ethical clearance has been obtained from institutional Animal Ethics committee (IACE). Protocol used in this study for use of rats as rats model for nephroprotective research was approved by Institutional Animal Ethical committee (IAEC) of RKDF college of Pharmacy, Bhopal-M.P (Ref. No. RKDFCP/IAEC/2022/53) which having CPCSEA (Committee for the purpose of Control and supervision of Experiments on Animals) registration number-**780/CPCSEA**. The copy of the ethical clearance certificate obtained from institutional Animal Ethical committee (IAEC) is attached.

Acute toxicity studies:

Acute toxicity studies for aqueous flowers extract of *Calotropis Gigantean* was conducted as per OECD guidelines 423 using albino Wistar rats. Each animal was administered the aqueous solution of the extract by oral route. The animals were observed for any changes continuously for the first 2h and up to 24 h for mortality.

Extracts:

Aqueous flowers extract of *Calotropis Gigantean* at doses of X mg/kg b.w. was used to evaluate nephroprotective activity. Stock solution of the extracts was prepared in the range of X mg/ml in water according to the need of study.

Selection of Dose: The dose selected to perform the pharmacological studies were within 1/15th to 1/10th of the LD_{50} Dose.

Preparation of the dose: *Calotropis Gigantean Linn* aqueous extract (250 mg/kg & 500 mg/kg) dissolved in normal saline.

Treatment Protocols:

Group 1: Normal rats received 10 mg/kg normal saline by gastric lavage are served as normal control.

Group 2: Received 250 mg/kg body weight flowers extract of *Calotropis Gigantean* (*FECG*). At the end of experimental period, all the animals were sacrificed under diethyl ether anaesthesia. **Group 3:** Received 500 mg/kg body weight *Calotropis Gigantean* aqueous flowers extract (*FECG*).

Group 4: Received standard urea (960mg/kg in normal saline orally).

The sodium and potassium contents in the urine were determined by using the Crest Biosystem diagnostic kit.

Table: 1 Preparation of sample for Sodium determination

Addition Sequence	S (ml)	T (ml)
Precipitating Reagent (L1)	1.0	1.0
Na^+ / K^+ Standard (S)	0.02	_
Sample	_	0.02

Table: 2 Preparation of Colour development for Sodium determination

Additional Sequence	B (ml)	S (ml)	T(ml)
Acid reagent (L1)	1.0	1.0	1.0
Supernatent from Step1.	_	0.02	0.02
Precipitating Reagent	0.02	_	_
Colour Reagent (L3)	0.1	0.1	0.1

Table: 5 Preparation of Sample for Potassium determination

Additional Sequence	B(ml)	S (ml)	T (ml)
Potassium Reagent	1.0	1.0	1.0
Deionised water	0.02	_	_
Na^+ / K^+ Standard (S)	_	0.02	_
Sample	_	_	0.02

2. They were mix well and incubated at R.T. for 5 min. The absorbance of the Test Sample (Abs.T) and Standard (Abs.S) was measured against Blank, within 15 min.

Calculation

Sodium assay: Sodium in mmol/l = Abs. B – Abs. T/ Abs. B – Abs.S x 150

Potassium assay: Potassium in mmol /l = Abs.T/ Abs.S x 5

EXPERIMENTAL PROCEDURE

Induction of Nephrotoxicity by Gentamicin.

Five groups of six rats each were used for the study.

Group I: Animals were orally administered with saline for 23 days.

Group II: animals were treated with only gentamicin (40mg/kg body wt., s.c.) for 13 days, blood was withdrawn on the 14th day.

Group III: Administered with gentamicin for 13 days (40mg/kg body wt., s.c.) and treated with plant extract 250mg/kg per oral dose for 13 days

Group IV: Administered with gentamicin for 13 days (40mg/kg body wt., s.c.) and treated with plant extract 500 mg/kg per oral dose for 13 days

Group V: Administered with Standard drug formulation Neeri for 13 days (40mg/kg body wt., orally.) only

The 3rd and 4th groups are studied for preventive regimen whereas 5th group is studied for standard regimen. Blood was withdrawn from the rat by retro orbital puncture method and animals were sacrificed for isolation of organs on 14th day. Then Serum was separated by centrifuging at 2500 rpm for 15 min and analyzed for various biochemical parameters. At the end of experimental period, all the animals were sacrificed under diethyl ether anaesthesia for further histopathological study.

Biochemical parameters

On respective day of completion of studies, blood was collected from rats by retro orbital puncture method and subjected to Biochemical parameters i.e., Estimation of Blood urea, Creatinine, Uric acid, Total protein were analyzed estimations by using prietest biochemical kits by ROBONIK biochemical analyzer.

HISTOPATHOLOGICAL EXAMINATION [52]

Procedure:

1. ZAfter deep ether anaesthesia, animal was dissected by cutting on the ventral side.
2. Kidney tissue was fixed in neutral buffered formalin (10% formaldehyde in Phosphate buffered saline) over night.
3. After fixation, the tissues placed in 70% isopropyl alcohol for 3 hours and then in

each ascending strength (80%, 90%, 100% isopropyl alcohol) for 2 hours each. The amount of alcohol used should be 15 times of the size of the tissue.

4. Then the tissue was dipped in acetone for a period of 1 – 2 h with periodical shaking.
5. After removing the acetone, xylene was added to check for the appearance of milkyness. If milkyness appears then repeat the dehydration procedure.
6. The dehydrated tissue was impregnated in paraffin wax (m.p. = 56 ^{0} C) for a period of 1h at 58 – 60 ^{0} C.
7. Molten paraffin poured into L-block along with the tissues and allowed it to become hard.
8. The tissue was sectioned into very thin (2–8 or 5 – 10 micrometer) sections using a microtome.
9. The tissue Mounted on the slides with Mayer's albumin solution (a mixture of equal parts of egg white and glycerin, beaten and filtered with the addition of 1% sodium salicylate) and incubated in warm oven for 2 h at 60ºC.
10. Slides containing paraffin sections were placed on a slide holder.
11. Slides were deparaffinized with Xylene for 30 minutes and the excess xylene blotted.
12. The tissue was rehydrate successively with 100%, 90%, 80% isopropyl alcohol for 2 – 3 min. each and put it into water for 3 min.
13. The excess water blotted; the tissue was kept into Haematoxylin stain for 1 – 2 min.
14. Then again kept into tap water for 1 – 2 min.
15. The slides containing tissue sections dipped into 1N HCl followed by Scott's water (Sodium Bicarbonate 3.5 g, Magnesium sulphate 20 g, distilled water 1 litre) for 1 min each.
16. The tissue was immersed in Eosin stain for 30 seconds.

Dehydrate the tissue successively with 80%, 90%, 100% isopropyl alcohol and finally with Xylene for 20 – 30 min.

Place cover slip on the slides using one drop of DPX (Dextrine-polystyrene xylene), taking care to leave no bubbles and dry overnight to make the permanent slide.

Statistical calculations:

The data expressed are mean ± standard error of mean (SEM) and the median inhibitory concentration (IC_{50}value) with 95% confidence intervals. All statistical comparisons between the groups are made by means of One Way Analysis of Variance with post hoc Dunnett's test or by Student's t-test. The *p* value less than 0.05 is regarded as significant. The concentration-response curves were analyzed by non-linear regression using Graph Pad Instate 3

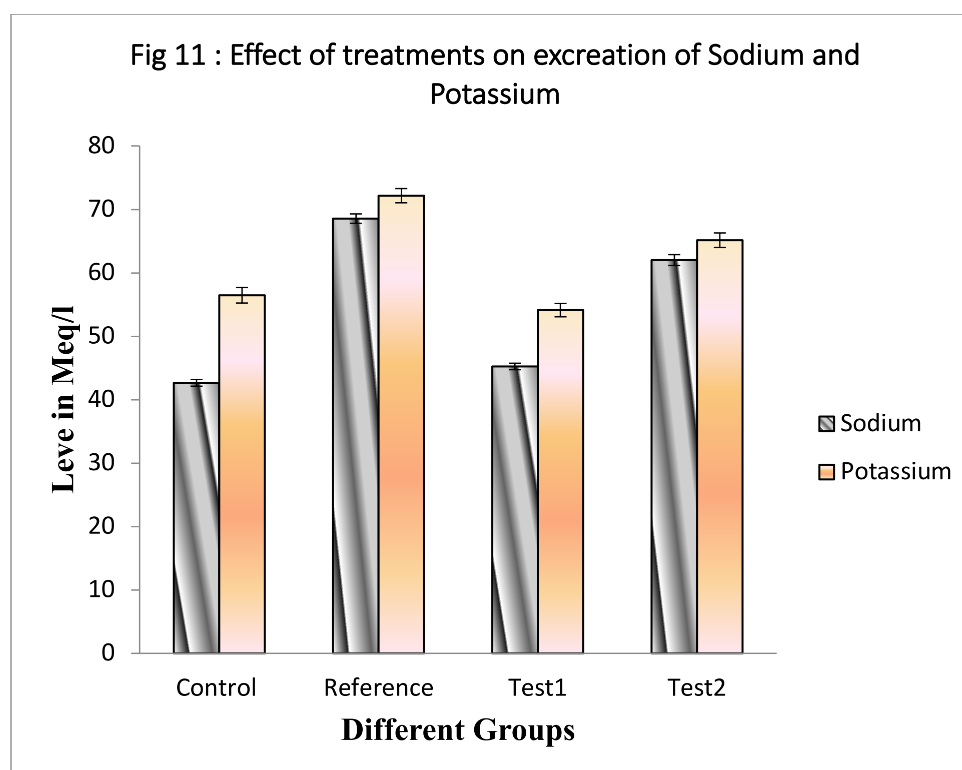
RESULTS:

Sl No.	Groups	Concentration (µg/ml)	Log Conc.	%Scavenging Acitivity (Mean ± S.E.M)	Probits of % Scavenging Acitivity
1.	B.H.T	25	1.39	39.21 ± 0.031	4.71
		50	1.69	62.28 ± 0.015	5.30
		100	2.00	65.76 ± 0.020	5.14
		150	2.17	75.34 ± 0.075	5.66
		200	2.30	78.23 ± 0.042	5.76
		250	2.39	85.47 ± 0.013	6.03
2.	FECG	25	1.39	20.36 ± 0.247	4.16
		50	1.69	35.46 ± 0.526	4.61
		100	2.00	42.38 ± 0.432	4.80
		150	2.17	48.47 ± 0.324	4.95
		200	2.30	65.26 ± 0.447	5.39
		250	2.39	71.31 ± 0.534	5.57

Table: Effect of aqueous flowers extract of *Calotropis Gigentean* on sodium, potassium excretion in urine and mean weight loss.

Treatment	Dose (mg/kg i.p.)	Sodium (Meq./ l)	Potassium (Meq./ l)	Mean weight Loss (gms)
Control (Norma-saline)	10 ml	42.65 ± 0.53	56.45 ± 1.22	2.44 ± 0.26
Reference(Urea)	960 mg	68.54 ± 0.74**	72.15 ± 1.12**	15.66 ± 0.57**
Calotropis Gigantean Extract1	30 mg	45.23 ± 0.50*	54.11 ± 1.05*	8.67 ± 0.58*
Calotropis Gigantean Extract2	50 mg	62 ± 0.86**	65.13 ± 1.15**	12.67 ± 0.86**

Values represent the mean ± S.E.M. of six animals in each group. Significantly different from normal control, *p<0.05, **p<0.01, ***p<0.005



GENTAMICIN INDUCED MODEL

In gentamicin treated group (2nd) of animals the concentration of serum Urea, Creatinine, Uric acid, Total protein and Urine Urea, uric acid, Creatinine were considerably increased than the normal animals (group 1) which indicates severe nephrotoxicity. *FECG* treating groups (group 3 & 4) with aqueous extract of *FECG* showed significant decrease (p<0.001) in concentration of Serum Urea , Creatinine , Uric acid , Total protein and Urine Urea, Uric acid, Creatinine compared to gentamicin treated group(2nd). Considerably decrease in activity of SOD and glutathione peroxidase in gentamicin treated animals (2nd) when compared to normal animals (group 1).

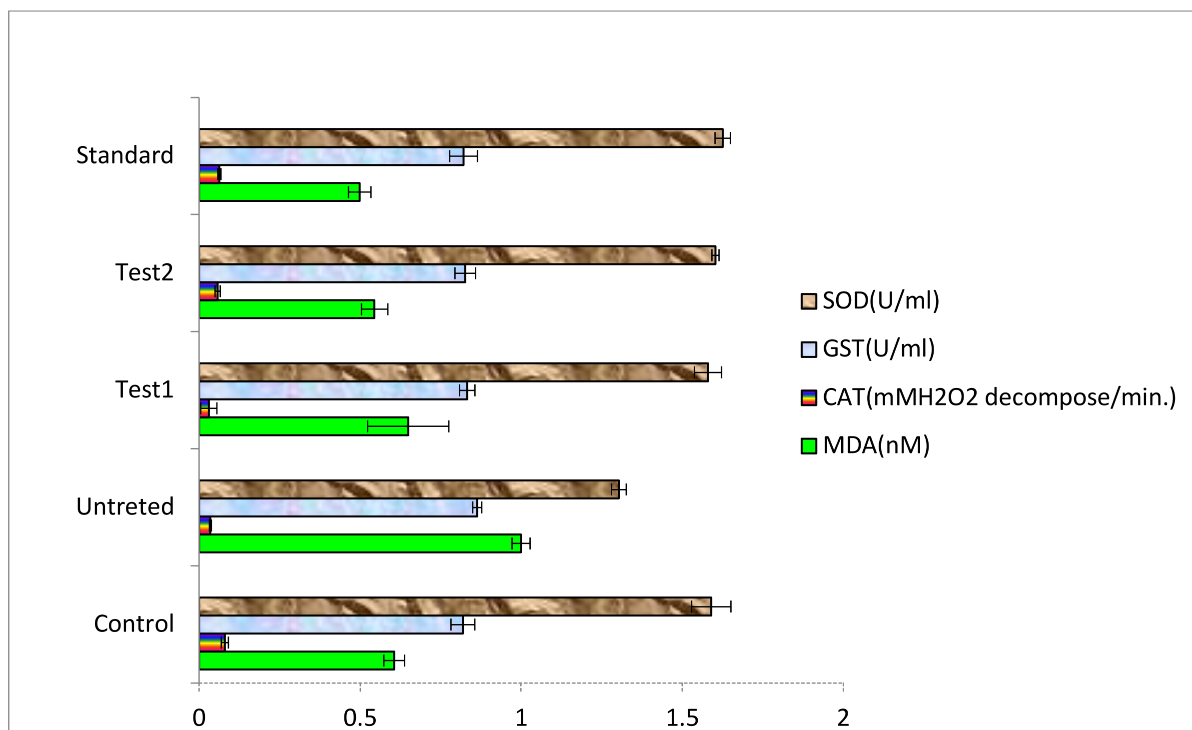


Fig: 25 Effects of Treatments on Antioxidant Marker in Kidney Homogenate Analysis

Histopathological examination of Kidneys

When observed under microscope, many histological preparations were seen in tubules of all regions of kidneys: cortex, medulla and papilla, of all the animals in the nephrotoxic induced group. Control rats showed normal glomerular and tubular histology whereas gentamicin induced group was found to cause glomerular, peritubular and blood vessel congestion and result in the presence of inflammatory cells in kidney sections. Concurrent treatment with the *Calotropis Gigantean* flowers extract was found to reduce such changes in kidneys histology induced by gentamicin.

Table : Histopathological features of the kidneys of rats of different treatment groups

Histopathological Feature	Group I Normal Control	Group II Gentamicin treated	Group III Gentamicin treated and *FECG* treated (30mg/kg i.p.)	Group IV Gentamicin treated and *FECG* treated (50mg/kg i.p.)	Group IV Treated with (Std)
Glomerular congestion	–	+++	++	+	+
Peritubular congestion	–	+++	++	+	+
Epithelial desquamations	–	+	–	–	–
Blood vessel congestion	–	+++	++	–	–
Interstitial edema	–	+	–	–	–
Inflammatory cells	–	+++	++	–	–
Necrosis	–	+	–	–	–
Tubular casts	–	+	–	–	–

+++ = Highly ++ = moderately, + = mild

DISCUSSIONS

In recent years, there has been a resurgence of interest in studying medicinal plants that are used in the treatment of several human diseases. A wide range of plants are used traditionally for their purported effect on the elimination of urinary problems. So far, no scientific data are available to indicate the beneficial effect of *Calotropis Gigantean* (*FECG*) for patients with kidney problems. Therefore, we used experimentally induced nephrotoxic rats to study the effect of flowers extract of *Calotropis Gigantean* (*FECG*) as an nephroprotective agent for the treatment of kidney problems.The present study showed that the flowers extract of *Calotropis Gigantean* was effective in both male and female animals as nephroprotection at a dose of 500 mg/kg body weight o. p. that produce a significant nephroprotective effect, it did not produce signs of toxicity.

Antioxidants are known to protect the body against free radical mediated toxicities.

Diuretic property is the preliminary requirement of the nephroprotective activity. From literature survey it is evident that diuretic effect of flowers extract of *Calotropis Gigantean* (*FECG*) increased urine output in a dose dependent manner (100- 500 mg/kg body weight, o.p.). Thus diuretic effect of *Calotropis Gigantean* flowers extract is indicated by increases in both water excretion and excretion of sodium and potassium. A previous investigation of the composition of *Calotropis Gigantean* has suggested the presence of flavonoids, saponins, organic acids and steroidal compounds which could be responsible for the plant diuretic effects.

Urine Creatinine, Serum creatinine, blood urea, blood urea nitrogen and the weights of the kidneys were found to be significantly increased ($p<0.01$) in the rats treated with only gentamicin as comparable to normal rats; whereas treatment with the aqueous flowers extract of *Calotropis Gigantean* was found to protect the rats from such effects of gentamicin.

Gentamicin induced nephrotoxicity are often associated with marked elevation in blood urea, serum Creatinine and acute tubular necrosis. So these biochemical parameters have been used to investigate drug induced nephrotoxicity in animals and man in the present study drug induced nephrotoxicity were established by single daily intraperitoneal injection of the gentamicin, for 24 days. In renal diseases, the serum urea accumulates because of the rate of serum urea production exceeds the rate of clearance. Elevation of urea and Creatinine levels in serum was taken as the index of nephrotoxicity. Creatinine derives from endogenous sources by tissue Creatinine breakdown.

The renal tubules were markedly dilated in the entire kidney of all induced rats, and this might have caused by distal obstruction of renal tubular flow by large crystals. The *Calotropis Gigantean* flowers extract treated groups significantly prevented all these effects, thus confirming the nephroprotective effect, as well as, antioxidant potential *in vivo*.

CONCLUSION

The present study demonstrated the dose-dependent nephroprotective activity of the *FECG* in a rat model of Gentamicin-induced nephrotoxicity. Pretreatment with *FECG* dose-dependently prevented kidney injury as evidenced by serum and urine biochemical analysis and kidney histopathology. It protects kidneys from oxidative stress, renal cell injury, diuretic and antioxidant activity. The studies rationalize its medicinal use for urinary diseases. This confirms the utility of the plant in folk medicine against nephrotoxicity. The findings were further substantiated by the histopathological studies which confirmed that *Calotropis Gigantean flowers* aqueous extract protect the renal cells from oxidative stress and renal cell injury. In conclusion, *FECG* is a potential nephroprotective agent against gentamicin-induced nephrotoxicity.

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