

Protective effect of *Kalanchoe pinnata* pers. (*Crassulaceae*) on gentamicin-induced nephrotoxicity in rats

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ABSTRACT

Objective: The present study was undertaken to evaluate the aqueous extract of *K. pinnata* for its protective effects on gentamicin-induced nephrotoxicity in rats.

Materials and Methods: Nephrotoxicity was induced in Wistar rats by intraperitoneal administration of gentamicin 100 mg/kg/day for eight days. Effect of concurrent administration of *K. pinnata* leaf extract at a dose of 125 mg/kg/day given by intraperitoneal route was determined using serum and urinary creatinine and blood urea nitrogen as indicators of kidney damage. The study groups contained six rats in each group. As nephrotoxicity of gentamicin is known to involve induction of oxidative stress, *in vitro* antioxidant activity and free radical-scavenging activity of this extract were evaluated.

Result: It was observed that the aqueous extract of *K. pinnata* leaves significantly protects rat kidneys from gentamicin-induced histopathological changes. Gentamicin-induced glomerular congestion, peritubular and blood vessel congestion, epithelial desquamation, accumulation of inflammatory cells and necrosis of the kidney cells were found to be reduced in the group receiving the leaf extract of *K. pinnata* along with gentamicin. This extract also normalized the gentamicin-induced increases in urine and plasma creatinine, blood urea and blood urea nitrogen levels. *In vitro* studies revealed that the *K. pinnata* leaf extract possesses significant antioxidant as well as oxidative radical scavenging activities.

Conclusion: It is proposed that the nephroprotective effect of the aqueous extract of *K. pinnata* leaves in gentamicin-induced nephrotoxicity may involve its antioxidant and oxidative radical scavenging activities.

KEY WORDS: Antioxidant, *Bryophyllum pinnata*, gentamicin, nephrotoxicity

Extensive use of plants belonging to the *Bryophyllum* species in complementary and alternative therapy has been widely reported. These plants are also used in the treatment of certain diseases like urolithiasis, hypertension and diabetes involving altered kidney function.^[1,2] However, systematic and scientific reports on the investigation of *K. pinnata* for its effects on renal function are scarce. In the present study, an effort has been made to evaluate the effects of the aqueous leaf extract of this plant on gentamicin-induced nephrotoxicity in rats.

Gentamicin-induced nephrotoxicity is a model of acute renal failure caused by oxidative stress generated through the induction of superoxide anions.^[3] Hence, *in vitro* antioxidant activity of this extract has been further investigated.

Materials and Methods

Plant material

Fresh leaves were collected from the herbal garden of our institute and authenticated as *K. pinnata* (Lam.) Pers. (*Crassulaceae*) by the Botanical Survey of India, Pune, India. The

voucher specimen (number - 120445) was submitted for future reference. The aqueous extract of the plant was prepared by using the cold maceration process. The extract was dried in a vacuum evaporator below 40°C and stored in air-tight, amber-colored containers at room temperature.

Animals

Healthy, male albino Wistar rats each weighing 150-200 g were used for this study. The rats were housed in polypropylene cages and maintained under standard conditions (12 h light and dark cycles, at 25±3°C and 35-60% humidity). Standard pelletized feed and tap water were provided *ad libitum*. The study was approved by the Institutional Animal Ethical Committee of R. C. Patel College of Pharmacy, Shirpur, India, registered under CPCSEA, India (Registration No. 651/02/C/CPCSEA).

Dose and route of administration

According to earlier reports, the LD₅₀ value for the aqueous extract of leaves of *K. pinnata* is 560 mg/kg i.p. in rats.^[4] The extract has been found to be ineffective orally in mice and rats as it did not produce any gross observable effect even at doses

as high as 2000 mg/kg p.o. Lack of any observable biological effect when administered by the oral route was attributed to inadequate absorption of the active phytoconstituents of this plant.^[5,6] In a preliminary study conducted in our laboratory, the leaf extract of *K. pinnata* did not exert any observable effects in rats when given by the oral route. However, intraperitoneal administration of the same extract was found to cause diuresis. Based on this observation and earlier reports, the dose of 125 mg/kg given by intraperitoneal route, which caused significant diuresis without any observable toxicity, was used throughout this study.

Nephroprotective activity

Eighteen male Wistar albino rats were assigned to three groups: control group, Gentamicin-treated group and Gentamicin- as well as *K. pinnata*-treated group, each group containing six rats. The gentamicin-treated group received 100 mg/kg/day gentamicin (Hi Media Laboratories, India) by the intraperitoneal (i.p.) route.^[7] The *K. pinnata*-treated group received 100 mg/kg/day gentamicin i.p. and 125 mg/kg/day of the aqueous extract of *K. pinnata* i.p. for eight days. Rats in the control group were given sterile saline solution i.p. for the same number of days. After dosing on the 8th day, individual rats were placed in separate metabolic cages for 24 hours for urine collection to determine urine output and urine creatinine content.^[8] Blood samples were collected via retro-orbital puncture at the end of these 24 hours. The serum was rapidly separated and processed for determination of blood urea nitrogen (BUN) and serum creatinine using commercially available kits of Span Diagnostics Ltd, India.^[4] Changes in body weight were recorded. Three rats per group were sacrificed and both kidneys were isolated from each rat.^[9] The kidneys were weighed and processed for histopathological examination.^[10]

Histopathological examination

The kidneys were sectioned longitudinally in two halves and were kept in 10% neutral formalin solution.^[11] Both kidneys were processed and embedded in paraffin wax and sections were taken using a microtome. The sections were stained with hematoxylin and eosin and were observed under a computerized light microscope (Motic images 2000, version 1.3, China).

In vitro antioxidant activity

For all the *in vitro* antioxidant models mentioned below, ascorbic acid was used as a reference standard. The concentrations of ascorbic acid were 10, 20, 30, 40, 50 µg/ml and that of extract were 50, 100, 150, 200, 250 µg/ml.

DPPH free radical-scavenging activity

To determine the antioxidant activity of the leaf extract, a method based on the reduction of a methanolic solution of the colored free radical 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was used.^[12] The methanolic solution of DPPH (0.1 mM, 1 ml) was incubated with 3 ml of different concentrations of the leaf extract ranging from 50 to 250 µg/ml. Incubation was carried out at room temperature (25°C) for 30 min. For each concentration, the assay was run in triplicate. At the end of the incubation period, the optical density of each sample was determined at 517 nm.^[13] Ascorbic acid (Loba Chemie, India) solution was used as a standard. EC₅₀ values (concentration required to scavenge 50% of the free radicals) for both

Ascorbic acid and the leaf extract were determined. The radical scavenging activity of the tested sample was expressed as an inhibition percentage (IP).

$$\text{DPPH Scavenged (\%)} = (A_{\text{DPPH}} - A_{\text{test}} / A_{\text{DPPH}}) \times 100$$

where A_{DPPH} is the absorbance of the 0.1 mM of DPPH solution and A_{test} is the absorbance in the presence of the extract or Ascorbic acid.

Nitric oxide radical-scavenging activity using the Griess Illosvay reaction^[12,14]

Free radical-scavenging activity was evaluated by studying the inhibition of the generation of Nitric Oxide from Sodium Nitroprusside. An aqueous solution of sodium nitroprusside at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions. The nitrite ions thus produced can be quantified using their reaction with Griess reagent that leads to formation of a chromophore, the concentration of which is proportional to that of the generated nitrite ions. Scavengers of nitric oxide compete with oxygen leading to a reduced production of nitric oxide.

In this assay, 1.0 ml of Sodium nitroprusside (5 mM) in phosphate-buffered saline (PBS) was mixed with 3.0 ml of different concentrations (50-250 µg/ml) of the extract dissolved in the distilled water. The assay mixture was then incubated at 25°C for 150 minutes. These solutions were treated with Griess' reagent and the optical density of the resultant chromophore determined spectrophotometrically at 546 nm and compared with the absorbance of standard solutions of ascorbic acid simultaneously run in identical assay units. The experiment was run in triplicate. As a blank, the assay mixture similarly run in the absence of the extract or ascorbic acid was used.

Reducing power assay^[15]

As a measure of antioxidant activity, the reducing power of the extract was also determined as follows: 1 ml of different concentrations of the *K. pinnata* extract solutions were added to 2.5 ml of 1% potassium ferricyanide in different test tubes and the resultant mixture incubated at 50°C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid was added to each tube. The tubes were centrifuged for 10 min at 3000 rpm. The supernatant from each tube (2.5 ml) was taken in a separate test tube and 2.5 ml of distilled water and 0.5 ml (0.1%) ferric chloride solution were added to each tube. The absorbance of these assay mixtures was measured at 700 nm. Increase in the absorbance of the reaction mixture was considered to be the reducing power of the extract.

In parallel to this, the reducing power of ascorbic acid was also determined for comparison.

Anti-lipid peroxidation

Decomposition of the lipid membrane of cells leads to the formation of Malondialdehyde (MDA) along with other aldehydes and enols as end-products. Malondialdehyde (MDA) formed during lipid peroxidation then reacts with thiobarbituric acid (TBA) to form a colored complex which can be spectrophotometrically measured at 532 nm.^[16]

Anti-lipid peroxidation in liver homogenate^[17,18]

Preparation of liver homogenate

Rat liver was perfused with ice-cold 0.15 M KCl through the portal vein. The perfused liver was isolated and 10% (w/v)

Table 1**Parameters studied for the nephroprotective activity of the aqueous extract of leaves of *Kalanchoe pinnata***

Parameter	Control (Mean ± SE)	Gentamicin (Mean ± SE)	Gentamicin and <i>K. pinnata</i> (Mean ± SE)	Statistics One-way ANOVA
Body weight (g)	178 ± 2.4	152 ± 1.7*	173 ± 1.8	F = 49.1 P < 0.0001
Weight of kidney (g)	0.52 ± 0.021	0.67 ± 0.04	0.54 ± 0.023	F = 23 P < 0.0001
Urine volume (ml)	2.8 ± 0.17	2.5 ± 0.12	6.2 ± 0.17*	F = 112 P < 0.0001
Urine creatinine (mg/dl)	94 ± 1.6	247 ± 1.5*	94 ± 1.3	F = 3600 P < 0.0001
Serum creatinine (mg/dl)	0.68 ± 0.012	1.0 ± 0.020*	0.63 ± 0.017	F = 145 P < 0.0001
Blood urea (mg/dl)	40 ± 0.67	106 ± 0.51*	43 ± 0.72*	F = 3460 P < 0.0001
Blood urea nitrogen	18 ± 0.32	50 ± 0.25*	20 ± 0.33*	F = 3320 P < 0.0001

n = 6 in each group; df = 15, 2., *P < 0.05 when compared to control

homogenate was prepared in PBS using a tissue homogenizer below 4°C. This homogenate was used to study *in vitro* lipid peroxidation.

The assay mixtures contained 0.5 ml of homogenate, 1 ml of 0.15 M KCl and 0.5 ml of different concentrations of the extract. Lipid peroxidation was initiated by adding 100 µl of 1 mM ferric chloride. The reaction mixture was incubated for 30 minutes at 37°C. After incubation, the reaction was stopped by adding 2 ml of ice-cold 0.25 N HCl containing 15% trichloroacetic acid and 0.38% TBA as well as 0.2 ml of 0.05% butylated hydroxyl toluene. The reaction mixture was heated for 60 min at 80°C, cooled to room temperature and centrifuged at 5000 rpm for 15 minutes. Optical density (O.D.) of the supernatant from each tube was measured at 532 nm against a blank which contained all reagents except liver homogenate and plant extract. Identical experiments were performed to determine the normal (without drug and ferric chloride) and induced (without drug) lipid peroxidation. The percentage of anti-lipid peroxidation effect (% ALP) was calculated by following formula:

$$\% \text{ ALP} = (\text{Ferric chloride O.D.} - \text{Sample O.D.} / \text{Ferric chloride O.D.} - \text{Normal O.D.}) \times 100$$

Statistical analysis

The data obtained was analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. *P* < 0.05 was considered significant.

Results

Nephroprotective Activity: Urine creatinine, serum creatinine, blood urea, blood urea nitrogen and the weights of the kidneys were found to be significantly increased in rats treated with only gentamicin; whereas treatment with the aqueous extract of the leaves of *K. pinnata* was found to protect the rats from such effects of gentamicin. As shown in Table 1, urine volume was found to be significantly increased in the rats treated with *K. pinnata* leaf extract.

The body weights of the rats treated with gentamicin were also found to be significantly reduced as compared to control

group and *K. pinnata*-treated rats.

Histopathological examination

Control rats showed normal glomerular and tubular histology whereas gentamicin was found to cause glomerular, peritubular and blood vessel congestion and result in the presence of inflammatory cells in kidney sections from the gentamicin-treated group. Concurrent treatment with the extract was found to reduce such changes in kidney histology induced by gentamicin [Figure 1 and Table 2].

In vitro antioxidant activity

DPPH method

EC₅₀ value for the Standard Ascorbic Acid Solution was found to be 11.25 µg/ml whereas EC₅₀ for the aqueous extract of leaves of *K. pinnata* was found to be 116.25 µg/ml [Table 3].

Nitric oxide radical-scavenging activity

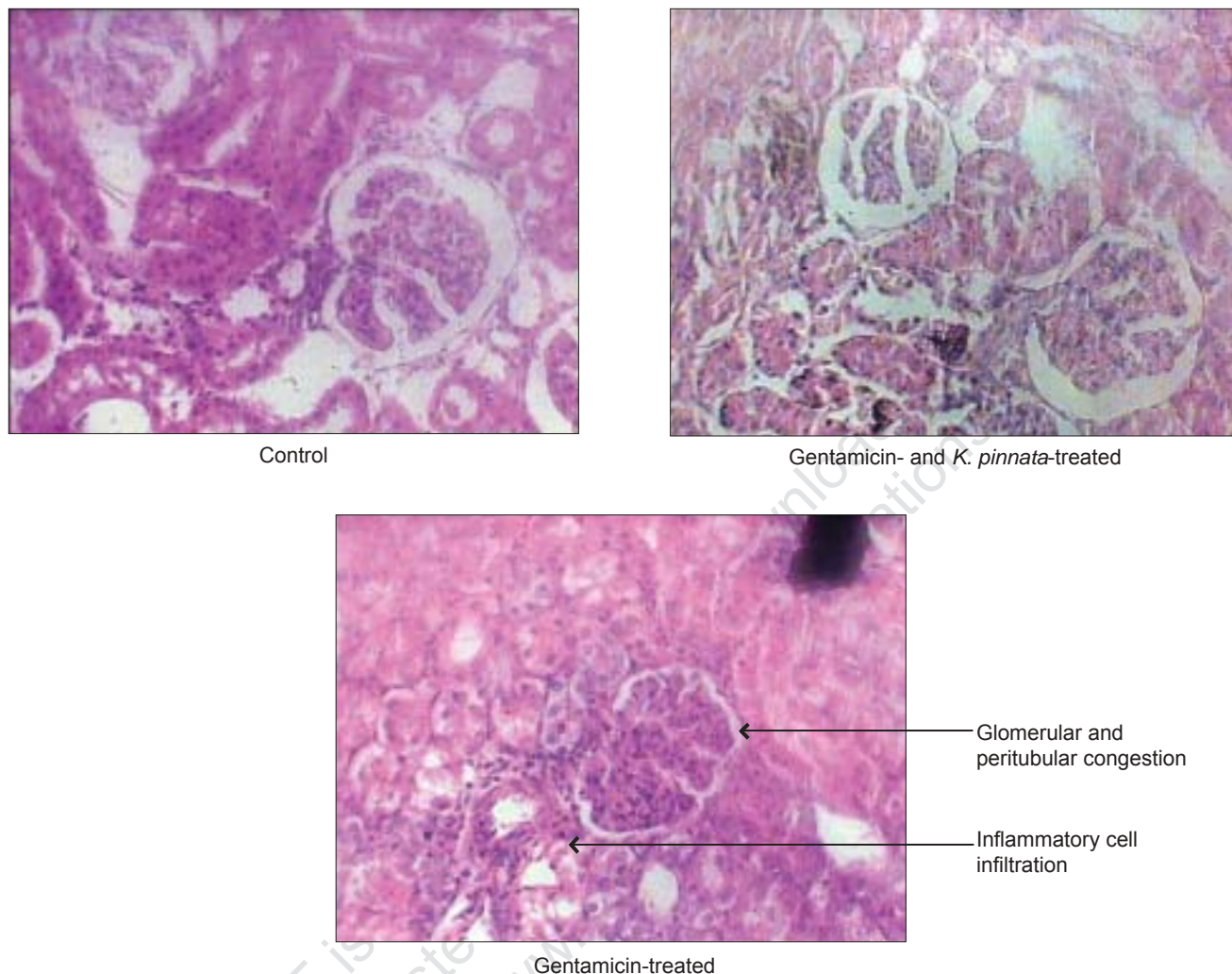
EC₅₀ of the Standard Ascorbic acid solution was found to be 15.5 µg/ml while it was 90 µg/ml for the extract [Table 3].

Reducing power assay

Proportional increases in reducing power of both Ascorbic

Table 2**Histopathological features of the kidneys of rats of different treatment groups**

Histopathological Feature	Control	Gentamicin-treated	Gentamicin and <i>K. Pinnata</i> -treated
Glomerular congestion	-	+++	+
Peritubular congestion	-	+++	+
Epithelial desquamation	-	+	-
Blood vessel congestion	-	+++	+
Interstitial edema	-	+	-
Inflammatory cells	-	+++	+
Necrosis	-	+	-
Tubular casts	-	+	-

Figure 1: Microscopy of rat kidneys from different treatment groups stained with hematoxylin and eosin magnified using a 45x objective**Table 3**EC₅₀ concentrations of the aqueous extract and ascorbic acid in different antioxidant models

Antioxidant activity	EC ₅₀ of standard ascorbic acid solution	EC ₅₀ of aqueous extract of <i>K. pinnata</i>
DPPH free radical scavenging activity	11.25 µg/ml	116.25 µg/ml
Nitric oxide scavenging activity	15.5 µg/ml	90 µg/ml
Anti-lipid peroxidation activity	14.0 µg/ml	125 µg/ml

acid and the extract of *K. pinnata* were observed with increasing concentrations.

Anti-lipid peroxidation in liver homogenate

EC₅₀ of the standard Ascorbic acid solution was found to be 14.0 µg/ml while EC₅₀ for the aqueous extract of the leaves of *K. pinnata* was found to be 125 µg/ml [Table 3].

Discussion

Our study results show that the aqueous extract of leaves of *Kalanchoe pinnata* possesses potent nephroprotective and *in vitro* antioxidant activity. This plant contains different classes of phytochemicals such as flavonol glycosides like

quercetin-3-L-rhamnosido-L-arabinofuranoside, quercetin-3-diarabinoside and kaempferol-3-glucoside, many alkanes C₂₅-C₃₅ (n-hentriacontane, n-triacontane predominating) and alkanols C₂₆-C₃₄. Pentacyclic triterpenoids like α-amyrin, β-amyrin and sterols like sitosterol have also been isolated from the non-saponifiable fraction. Furthermore, the presence of other phenolic constituents like *p*-coumaric, ferulic, syringic, caffeic and *p*-hydroxybenzoic acids^[19] and organic acids like isocitric^[20] and citric acids has been reported.^[21]

Quercetin and kaempferol are detected in the leaves. An earlier report^[22] suggests that quercetin has a marked protective effect on cadmium-induced nephrotoxicity that results from an

increase in Metallothionein, a small cysteine-rich protein and eNOS (endothelial nitric oxide synthase) expression and the inhibition of COX-2 (cyclooxygenase-2) and iNOS (inducible nitric oxide synthase) expression.

The results of our study suggest that *K. pinnata* contains constituents having nephroprotective and antioxidant activities, which are comparable to that of ascorbic acid. Further investigations using specific fractions of this extract can help to isolate and identify potential nephroprotective and antioxidant constituents.

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