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RESEARCH ARTICLE

Experimental Evaluation of Nephroprotective Potential of *Calotropis procera* (Ait) Flowers against Gentamicin-Induced Toxicity in Albino Rabbits

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ABSTRACT

Gentamicin-induced nephrotoxicity is associated with free radicals generation in kidney tissue. Medicinal plants contain bioactive constituents that act as natural antioxidants, which can protect the biological system from oxidative stress. In this study, phytochemical constituents of aqueous extract of *Calotropis procera* flowers and its efficacy on gentamicin-induced nephrotoxicity in rabbit model were evaluated. Renal function markers, total oxidant status and catalase activity were assessed along with hematological and renal histopathological analyses. Phytochemical analysis showed the presence of tannins, flavonoids, saponins, glycosides, phenols, alkaloids, steroids and ascorbic acid. Gentamicin administration led to severe changes in serum biochemical markers along with significant alteration in kidney tissue. Co-administration of plant extract with gentamicin reduced the gentamicin-induced toxic effects by restoring the renal function markers *i.e.* blood urea nitrogen, serum urea, serum creatinine and electrolytes indicating the protective potential of plant extract. Likewise, disturbance in hematological parameters induced by gentamicin was attenuated by the administration of plant extract. In addition, *C. procera* extract increased the antioxidant enzyme catalase and decreased the serum total oxidant status along with protective effects evidenced by histopathological evaluation. Aqueous extract of *C. procera* flowers, when co-administered with gentamicin, exerted protective effect against gentamicin-induced nephrotoxicity in rabbits. This nephroprotective potential of *C. procera* aqueous extract might be due to the presence of polyphenols *i.e.* flavonoid and phenol constituents.

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INTRODUCTION

Several antimicrobial agents result in acute renal injury in 60% of hospital acquired cases with considerable morbidity and mortality (Dashti-Khavidaki *et al.*, 2013). Aminoglycosides are frequently used to treat bacterial infections due to their low cost and less chances of antibiotic resistance. Gentamicin is a commonly used aminoglycoside. Routine therapeutic use of gentamicin (80 mg/kg/day) for more than seven days has been a common cause of nephrotoxicity in approximately 30% of patients (Balakumar *et al*., 2010). Acute renal toxicity is characterized by a sudden decrease in kidney function over a period of hours to days, resulting in accumulation of creatinine, urea, and other waste products. It has been shown that nephrotoxicity caused by gentamicin treatment is associated with increased oxidative stress, which might be the major contributing factor towards renal injury (Kang *et al* 2013).

Plants are major source of antioxidants and are used as therapeutic agent in various ailments. Natural antioxidants present in medicinal and dietary plants might be helpful in preventing damage induced by oxidative stress (Hamid *et al*., 2010; Masood *et al*., 2013). The application of external source of antioxidants can help in management of this oxidative stress. Thus, the search for effective and non-toxic plants with anti-oxidative activity has been intensified (Lobo *et al.*, 2010). Co-administration of various medicinal plants possessing nephroprotective activity along with different nephrotoxic agents may attenuate their toxicity (Mishra *et al.*, 2014, Rodrigues *et al* 2014, Shin *et al* 2014).

Calotropis procera (*C. procera*) is an important medicinal plant, commonly known as Arka or Madaar. It belongs to the Asclepiadaceae family, grows in tropical region and is abundant in Bangladesh, India, Burma and Pakistan. *C. procera* flowers contain bioactive compounds *i.e.* glycosides, flavonoids, tannins and triterpenes that may contribute its antioxidant and free radical scavenging activity. Flowers of *C. procera* have a number of pharmacological activities *i.e.* analgesic, antipyretic, hepatoprotective, anti-inflammatory, anthelmintic and antimicrobial (Ramachandra *et al*., 2007). Renal disorders resulting from oxidative stress might be prevented by using natural antioxidants. In view of the pathogenesis of renal injury and the potent anti-inflammatory, antioxidant and free radical scavenging activity of the *C. procera* flowers, the present study was carried out to evaluate the protective effect of aqueous extract of *C. procera* flowers against gentamicin-induced nephrotoxicity.

MATERIALS AND METHODS

Chemicals, plant material and extraction: Gentamicin sulphate (Gentamicin sulphate® injectable solution 40mg/ml) and Silymarin (Silliver® film coated tablet 200 mg) were purchased from Wuhan Grand Pharmaceutical Group Co. Ltd. China and Abbott Laboratories Ltd. Karachi Pakistan, respectively. Flowers of *C. procera* were obtained and authenticated from the Botany Department, University of Agriculture Faisalabad. After washing with distilled water, flowers were dried in shade and finely powdered. One kilogram of *C. procera* flowers was extracted in distilled water for 24 h and concentrated to a thick semi-solid mass using a rotary evaporator. The crude extract of *C. procera* yield was approximately 10% w/w. The extract was water-soluble. It was stored at 4°C till further analysis.

Animals and study design: Thirty-six healthy male albino rabbits were equally divided into six groups. Control group (CN) rabbits received routine diet, Gentamicin group (GM) was daily injected (i.p.) @ 80 mg/kg BW; gentamicin+Silymarin group (GM+S) was administered daily with gentamicin+aqueous solution of silymarin (200mg/kg BW); gentamicin+extract (low dose) group (GM+CPA1) was administered daily with gentamicin + aqueous extract of *C. procera* (150 mg/kg BW); Gentamicin + extract (high dose) group (GM+CPA2) was administered daily with gentamicin + aqueous extract of *C. procera* (300mg/kg BW). *C. procera* extract only group (CPA2) was administered daily with aqueous extract of *C. procera* (300mg/kg BW).

Blood and tissue samples collection: At 15th day, animals were sacrificed. The blood samples were collected for biochemical and hematological studies. Serum was separated after centrifugation at 1507g for 15 minutes. Pieces of kidney were fixed in 10% neutral formalin for histological examination.

Phytochemical analysis and hematological profile: Plant extract was analyzed for the presence of phytochemical constituents *i.e.* saponins, tannins, phenols, glycosides, terpenoids, alkaloids, flavonoids, steroids and ascorbic acid as described previously (Parekh and Chanda, 2007; Khan *et al*., 2011). Hematological analysis was performed by using M-Series Hematology Analyzers manufactured by Clinical Diagnostic Solutions, Inc. USA. Serum samples were analyzed for blood urea nitrogen, serum urea and creatinine by using colorimetric assay kits (Merck Private Ltd, Pakistan). The samples were analyzed for serum Na^+, Cl^- and K^+ levels using Prolyte Electrolyte Analyzer (K102959), Diamond Diagnostics, USA. Serum total oxidant status (TOS) and catalase activity were determined using the previously described methods (Goth, 1991; Erel, 2005).

Statistical analysis: The data were analyzed by one-way analysis of variance and presented as mean±SE. In case of significant differences, the groups were analyzed by Duncan multiple range test. The statistical significance level was accepted at $P<0.05$.

RESULTS

Hematological changes: Gentamicin administration is associated with vasoconstriction and inhibition of erythropoietin production in renal cortex leading to hemodynamic disturbances. In the present study, the administration of gentamicin reduced red blood cell count, hematocrit, platelets count and hemoglobin concentration. While total leukocyte count was increased in gentamicin-treated animals, blood indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) did not vary significantly (Table 1). These hematological parameters were restored when animals were given the plant extract along with gentamicin.

Serum biochemistry: Increased levels of urea and creatinine in the serum are the indicators of nephrotoxicity (Afzal *et al*., 2004). As predicted, in the present study, the serum levels of urea, creatinine and blood urea nitrogen (BUN) in gentamicin-treated group were significantly high ($P\leq0.05$), indicating nephrotoxic effects of gentamicin. This increased serum urea, creatinine and BUN levels by gentamicin were antagonized by *C. procera* flowers extract in a dose-dependent manner (Table 2). Gentamicin administration resulted in significant decrease in serum electrolytes *i.e.* sodium, potassium and chloride levels (Table 2). Co-administration of *C. procera* restored the serum electrolytes levels towards normal values. In order to study, whether the protective effect of *C. procera* was due to its antioxidant property, it was found that gentamicin administration significantly increased total oxidant status (TOS) that was counteracted by simultaneous administration of silymarin, as expected. Interestingly, the plant extract along with gentamicin also reduced the oxidative stress. Moreover, a significant decrease in catalase enzyme was observed in gentamicin-treated group that was restored by co-administration of silymarin or plant extract (Table 2).

Renal histopathology: Results of the qualitative analysis of histopathological changes are presented in Table 3. In sharp contrast to the control group, histopathological

Table 1: Hematology parameters of albino rabbits with induced gentamicin toxicity and amelioration with *Calotropis procera* (Ait) flowers

Parameters	Control (CN)	Gentamicin (GM)	Gentamicin + Silymarin (GM+S)	Gentamicin + *C. procera* 1 (GM+CPA1)	Gentamicin + *C. procera* 2 (GM+CPA2)	*C. procera* 2 (CPA2)
RBC count ($\times10^6$/µL)	4.6 ± 0.1^{AB}	4.0 ± 0.2^{C}	4.5 ± 0.1^{B}	4.4 ± 0.1^{B}	4.5 ± 0.1^{AB}	4.8 ± 0.1^{A}
TLC ($\times10^3$/µL)	7.4 ± 0.2^{C}	9.1 ± 0.2^{A}	7.1 ± 0.2^{C}	8.2 ± 0.3^{B}	6.9 ± 0.2^{C}	7.3 ± 0.2^{C}
PLT count ($\times10^3$/µL)	280.6 ± 12.1^{A}	126.0 ± 17.3^{D}	275.8 ± 11.3^{A}	197.0 ± 13.9^{C}	223.4 ± 17.2^{B}	240.5 ± 15.8^{B}
Hemoglobin (g/dL)	10.2 ± 0.2^{AB}	9.2 ± 0.3^{C}	9.7 ± 0.2^{BC}	9.7 ± 0.3^{BC}	9.9 ± 0.3^{B}	10.8 ± 0.2^{A}
HCT (%)	35.2 ± 0.6^{A}	30.7 ± 1.1^{C}	33.4 ± 0.5^{AB}	32.7 ± 1.0^{BC}	33.5 ± 0.9^{AB}	35.5 ± 0.7^{A}
MCV (fL)	76.3±0.9	77.6±2.6	73.7±0.9	75.1±1.2	76.2±1.5	74.6±1.0
MCH (pg)	22.2± 0.2	23.1±0.8	21.7±0.2	22.1±0.0	22.2±0.1	22.7± 0.3
MCHC (g/dL)	29.1±0.2	29.8±0.4	29.5±0.3	29.6±0.5	29.3±0.6	30.4±0.3

Values (mean±SE) with different superscripts in a row differ significantly (P≤0.05). Control (CN): vehicle; Gentamicin (GM): 80mg/kg/day; Gentamicin + silymarin (GM+S): 80mg/kg/day + 200mg/kg/day; Gentamicin + *C. procera* 1 (GM+CPA1): 80mg/kg/day + 150mg/kg/day; Gentamicin + *C. procera* 2 (GM+CPA2): 80mg/kg/day + 300mg/kg/day; *C. procera* (CPA2): 300mg/kg/day.

Table 2: Renal function markers of albino rabbits with induced gentamicin toxicity and amelioration with *Calotropis procera* (Ait) flowers

Parameters	Control (CN)	Gentamicin (GM)	Gentamicin + Silymarin (GM +S)	Gentamicin + *C. procera* 1 (GM+CPA1)	Gentamicin + *C. procera* 2 (GM+CPA2)	*C. procera* 2 (CPA2)
Urea (mg/dL)	33.3 ± 1.9^{C}	58.0 ± 3.3^{A}	35.9 ± 2.62^{C}	41.3 ± 1.18^{B}	38.3 ± 2.67^{BC}	27.1 ± 1.24^{D}
Creatinine (mg/dL)	0.72 ± 0.02^{BC}	0.93 ± 0.05^{A}	0.80 ± 0.03^{B}	0.8 ± 0.03^{B}	0.75 ± 0.02^{BC}	0.70 ± 0.02^{C}
BUN (mg/dL)	15.6 ± 0.6^{CD}	27.1 ± 1.5^{A}	16.8 ± 1.2^{C}	19.3 ± 0.7^{B}	17.9 ± 1.2^{BC}	12.7 ± 0.5^{D}
Sodium (mEq/L)	136.8 ± 0.6^{AB}	131.9 ± 0.4^{C}	138.3 ± 0.5^{A}	137.8 ± 0.4^{AB}	136.3 ± 0.4^{B}	136.5 ± 0.9^{B}
Potassium (mEq/L)	5.6 ± 0.1^{A}	4.8 ± 0.1^{D}	5.0 ± 0.1^{C}	5.3 ± 0.1^{B}	5.5 ± 0.1^{AB}	5.5 ± 0.1^{AB}
Chloride (mEq/L)	107.0 ± 0.5^{A}	104.3 ± 0.5^{C}	107.1 ± 0.5^{A}	105.5 ± 0.3^{B}	106.2 ± 0.4^{AB}	106.3 ± 0.5^{AB}
TOS (µMol/L)	2.2 ± 0.1^{C}	4.1 ± 0.2^{A}	1.9 ± 0.1^{D}	3.1 ± 0.1^{B}	2.0 ± 0.1^{CD}	1.8 ± 0.1^{D}
Catalase (KU/L)	39.8 ± 0.3^{B}	35.5 ± 0.9^{C}	42.0 ± 0.8^{A}	39.6 ± 0.6^{B}	40.9 ± 0.4^{AB}	41.1 ± 0.5^{AB}

Values (mean±SE) with different superscripts in a row differ significantly (P≤0.05).

Table 3: Histopathological features of albino rabbits with induced gentamicin toxicity and amelioration with *Calotropis procera* (Ait) flowers

Parameters	Control (CN)	Gentamicin (GM)	Gentamicin + Silymarin (GM + S)	Gentamicin + *C. procera* 1 (GM+CPA1)	Gentamicin + *C. procera* 2 (GM+CPA2)	*C. procera* 2 (CPA2)
Glomerular congestion	-	+++	-	-	-	-
Blood vessel congestion	-	+++	-	+	-	-
Inflammatory cells	-	+++	-	-	-	-
Necrosis	-	++	-	+	-	-

(-) normal; (+) little effect; (++) appreciable effect; (+++) severe effect

inspections of GM group showed severe necrosis in tubular epithelial cells, fragmented glomerulus and sever infiltration at peritubular spaces, indicating renal damage (Fig. 1A and 1B). Rabbits belonging to the group GM+S showed normal epithelial cell lining of the tubules with intact nucleus showing nephroprotective effect of silymarin (Fig. 1C). Histomorphology of kidneys in the GM+CPA1 group displayed mild to moderate epithelial degeneration of renal tubules with minute peritubular infiltration, congestion and mild coagulative necrosis (Fig.1D). The GM+CPA2 treated group showed intact epithelial cells of kidney with regeneration of cells, showing protective and regenerative property of plant extract in gentamicin-induced tissue damage (Fig. 1E). Photomicrographs of kidney tissues of CPA2 group showed intact epithelium with normal cell lining (Fig. 1F). The results were comparable with the control group, indicating that *C. procera* extract alone did not affect the morphology of kidney. No glomerular lesion was observed by light microscopy in controls or animals receiving aqueous extract of *C. procera* flowers alone (Fig. 1). However, in rabbits injected with gentamicin alone showed severe and diffuse tubulopathy, the glomeruli seemed either normal or seemed hypertrophied with a urinary chamber that was apparently too large and that had a collapsed glomerular tuft.

Phytochemical evaluation of *C. procera* flowers: These hematological, serological and histopathological changes, which have not previously been described in gentamicin-induced nephrotoxicity, prompted the analysis of aqueous extract of *C. procera* flowers for the presence of different phytochemicals. Phytochemical analysis of aqueous extract of *C. procera* flowers showed the presence of glycosides, terpenoids, phenols, flavonoids, tannins, saponins, alkaloids, steroids and ascorbic acid. Together, these results showed that nephroprotective effects of *C. procera* flowers aqueous extract might be mediated by its phytoconstituents having antioxidant potential.

DISCUSSION

The mechanism by which gentamicin induces nephrotoxicity is unknown; however, gentamicin has been shown, by both *in vitro* and *in vivo* studies, to enhance the generation of reactive oxygen species (ROS). Abnormal production of ROS may result in cellular injury and necrosis through peroxidation of membrane lipids, protein denaturation and DNA damage (Parlakpinar *et al.*, 2005). Oxidative stress by overproduction of reactive oxygen species is one of the main mechanisms involved in acute and chronic renal pathologies (Suganya *et al*., 2011). Natural antioxidants have a variety of biochemical actions *i.e.,* inhibition of reactive oxygen species production, scavenging of free radicals and modification of intracellular redox activity (Abdollahi *et al*., 2005). In this study, gentamicin has significantly increased oxidative stress that was clear from increased total oxidant status in gentamicin-treated group. The administration of silymarin or plant extract along with gentamicin significantly

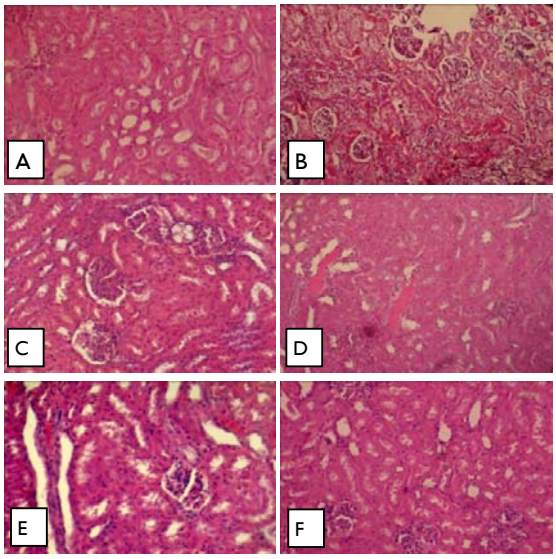

Fig. 1: Photomicrographs of kidney of rabbits after 15 days of treatment. Control group (A), gentamicin treated group (B), gentamicin + silymarin treated group (C), gentamicin + *C. procera* 1 low dose group (D), gentamicin + *C. procera* 2 high dose group (E), *C. procera* 2 alone high dose group (F). H & E; 40x.

reduced total oxidant status. The stimulating factors of gentamicin nephrotoxicity are increased level of hydrogen peroxide and hydroxyl radicals, and decreased amount of intracellular glutathione level (Parlakpinar *et al.,* 2005). The results showed the severe kidney damage by administration of gentamicin, most expected by ROS mediated mechanism as evident by decreased activity of catalase. The observed decrease in the level of catalase may be due to its increased utilization for scavenging gentamicin and/or oxygen derived radicals. The administration of aqueous extract of *C. procera* restored the catalase level to base line, indicating antioxidant activity of the plant extract.

Gentamicin caused a significant increase in creatinine and BUN. This result is in conformity with previous reports (Saleemi *et al*., 2009; Reddy *et al.*, 2012) attributing these changes to nephrotoxicity induced by gentamicin. The mechanism behind elevated serum urea and creatinine might be that the gentamicin increases the entry of Ca^{+2} in the mesangial cells leading to reduced glomerular filtration rate (Stojiljkovic *et al*., 2008). There is an inverse relationship between the quantity of urea absorbed and the rate of tubular urine flow (Kaneko *et al*., 2008). This mechanism may clarify the increase in BUN in the present study. The administration of graded doses of *C. procera* reduced the levels of raised serum urea and creatinine in a dose-dependent manner suggesting that the contents of *C. procera* protected the integrity of kidney tissue.

Gentamicin administration is associated with severe necrosis, desquamation in proximal tubules and dysfunction of co-transport systems and channels, leading to decrease absorption of electrolytes (Chen *et al*., 2009). The results of present study indicated that serum electrolytes *i.e.*, sodium, potassium and chloride levels were significantly reduced in gentamicin-treated animals. The decrease in electrolyte level may be due to increased wasting or reduced absorption of electrolytes resulting from kidney damage. The administration of plant extract restored electrolytes levels, indicating nephroprotective activity.

The gentamicin-induced contraction of glomeruli and the mesangial cells leads to reduction in ultrafiltration coefficient and the reduction in glomerular filtration. This would be a logical explanation of the given hemodynamic impairments as a main characteristic of toxic renal failure. Cell necrosis and increase in vasoconstrictor mediators, including increases in angiotensin-II, endothelin-I, thromboxane A_2 and ROS, and decreases in vasodilator prostaglandins have also been proposed to explain gentamicin-induced vascular dysfunction (Kalaidjieva and Iliev, 2000). Hemodynamic disturbances may also be due to the tubular damage by gentamicin. The proximal kidney tubules are involved in erythropoietin production. The tubular damage resulted in decreased erythropoietin production, thus alters the basic hematological parameters (Kalaidjieva and Iliev, 2000). In the present study, the administration of gentamicin reduced hemoglobin, red blood cell count, packed cell volume and platelet count. The gentamicin-treated animals also exhibited higher total leukocyte count and increased erythrocyte sedimentation rate as compared to control animals but there was no significant difference observed in MCV, MCHC and MCH indices among control and experimental groups.

Plants are a rich source of natural antioxidants (Nandave *et al*., 2009). Phytochemical constituents of *C. procera* are responsible for its antioxidant and free radical scavenging activity (Moustafa *et al*., 2010). In the present study, phytochemical investigation of *C. procera* revealed the presence of glycosides, terpenoids, phenols, flavonoids, tannins, saponins, alkaloids, steroids and ascorbic acid. The potential antioxidant properties of this plant might probably due to the active hydrogen donor ability of hydroxylated substitution of calotropogenin, calotropin and calotoxin. Similarly, high molecular weight isolated compounds and the proximity of many aromatic rings of coroglaucegenin, glucofrugoside, calotroposide A and B could be more important for the free radical scavenging activity of this plant extract. These bioactive compounds of *C. procera* might give the possible explanation about this plant to act as reactive oxygen species scavengers.

The present study indicates that administration of gentamicin results in kidney damage, which is counteracted by co-administration of plant extract, showing nephroprotective activity. It has been shown that polyphenolic compounds prevent nephrotoxicity induced by oxidative stress (Wongmekiat *et al*., 2008). The majority of phytochemicals found in *C. procera* are polyphenolic compounds. Based on the above literature, it is proposed that the phytochemical constituents in *C. procera* might be responsible for its antioxidant activity and protective effects in gentamicin-induced nephrotoxicity. However, additional studies are necessary to explore other possible mechanisms.

Conclusion: The antioxidant activity of the aqueous extract of the flowers of *Calotropis procera* through the scavenging of free radicals presented as total oxidant status and catalase activity has been demonstrated. The extract showed a more significant effect on total oxidant

status (TOS). Moreover, the extract has also shown a positive *in vivo* modulatory role of catalase activity. Although, the flowers contained the constituents like alkaloids, cardiac glycosides nevertheless a systematic screening of these plants with special reference to its active phytoconstituents may be of greater benefit to explore the nutraceutical values of this plant.

Author's contribution: SJ and JAK conceived and designed the study. SJ executed the experiments. SJ and JAK analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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