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

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**ARTICLE HISTORY** (14-579)

Received: November 13, 2014
Revised: February 12, 2015
Accepted: February 21, 2015

**KEYWORDS:**
Antioxidant enzymes
Medicinal plants
Nephrotoxicity
Phytochemistry

**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 which 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, anthelminthic 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 leucocyte 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≤0.05), 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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (CN)</th>
<th>Gentamicin (GM)</th>
<th>Gentamicin + Silymarin (GM+S)</th>
<th>Gentamicin + C. procera 1 (GM+CPA1)</th>
<th>Gentamicin + C. procera 2 (GM+CPA2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC count (×10⁶/µL)</td>
<td>6.8±0.2</td>
<td>4.0±0.2</td>
<td>4.3±0.4</td>
<td>4.4±0.4</td>
<td>4.5±0.4</td>
</tr>
<tr>
<td>TLC count (×10³/µL)</td>
<td>7.4±0.2</td>
<td>9.1±0.2</td>
<td>7.1±0.2</td>
<td>8.2±0.3</td>
<td>6.9±0.2</td>
</tr>
<tr>
<td>HPLC count (×10³/µL)</td>
<td>278.6±12.3</td>
<td>126.0±17.3</td>
<td>279.8±11.3</td>
<td>197.0±13.9</td>
<td>223.4±17.2</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>10.2±0.2</td>
<td>9.2±0.3</td>
<td>9.7±0.2</td>
<td>9.7±0.3</td>
<td>9.9±0.3</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>35.2±0.6</td>
<td>30.7±1.1</td>
<td>33.4±0.5</td>
<td>32.7±1.0</td>
<td>33.5±0.9</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>73.6±0.6</td>
<td>77.6±2.6</td>
<td>73.7±0.9</td>
<td>75.1±1.2</td>
<td>76.2±1.5</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>22.2±0.2</td>
<td>23.1±0.8</td>
<td>21.7±0.2</td>
<td>22.1±0.0</td>
<td>22.2±0.1</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (CN)</th>
<th>Gentamicin (GM)</th>
<th>Gentamicin + Silymarin (GM+S)</th>
<th>Gentamicin + C. procera 1 (GM+CPA1)</th>
<th>Gentamicin + C. procera 2 (GM+CPA2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/dL)</td>
<td>33±1.9</td>
<td>58±3.3</td>
<td>35.9±2.6</td>
<td>41.3±1.1</td>
<td>38.3±2.6</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.7±0.02</td>
<td>0.9±0.05</td>
<td>0.8±0.03</td>
<td>0.8±0.03</td>
<td>0.75±0.02</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>15.6±0.6</td>
<td>16.8±1.3</td>
<td>19.3±0.7</td>
<td>17.9±1.2</td>
<td>12.7±0.3</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>136.8±6.6</td>
<td>131.9±4.5</td>
<td>138.3±0.5</td>
<td>137.8±0.4</td>
<td>136.3±0.6</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>5.5±0.1</td>
<td>4.8±0.1</td>
<td>5.0±1.0</td>
<td>5.3±1.0</td>
<td>5.5±1.0</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>107.0±5.0</td>
<td>104.3±5.0</td>
<td>107.1±5.0</td>
<td>105.5±2.8</td>
<td>106.2±2.8</td>
</tr>
<tr>
<td>TOS (µMol/L)</td>
<td>2.2±0.1</td>
<td>4.1±0.2</td>
<td>3.1±0.0</td>
<td>3.0±0.1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Catalase (KU/L)</td>
<td>39.6±3.9</td>
<td>40.9±4.9</td>
<td>41.2±0.5</td>
<td>41.1±0.5</td>
<td></td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (CN)</th>
<th>Gentamicin (GM)</th>
<th>Gentamicin + Silymarin (GM+S)</th>
<th>Gentamicin + C. procera 1 (GM+CPA1)</th>
<th>Gentamicin + C. procera 2 (GM+CPA2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular congestion</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blood vessel congestion</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Necrosis</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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

inspections of GM group showed severe necrosis in tubular epithelial cells, fragmented glomerules 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
The results of the present study indicated that serum electrolytes decreased absorption of electrolytes (Chen et al., 2009). Gentamicin-induced contraction of glomeruli and the mesangial cells leads to a 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 increases in vasoconstrictor mediators, including increases in angiotensin-II, endothelin-I, thromboxane A2 and ROS, and decreases in vasodilator prostaglandins have 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 calotropigenin, calotropin and calotoxin. Similarly, high molecular weight isolated compounds and the proximity of many aromatic rings of coroglaucegenin, glucofrugoside, calotropside 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 and SJ and JAK analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

**Acknowledgement:** The authors are indebted to Ghulam Muhammad and Muhammad Saqib, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad for facilitating this research. Ahsan Naeem, Institute of Pharmacy, Physiology and Pharmacology, University of Agriculture, Faisalabad helped in critical reading of the manuscript and gave useful suggestions. Muhammad Kashif Saleemi, Department of Pathology, University of Agriculture, Faisalabad helped in histopathological studies.

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