Effect of alcoholic extract of roots of *Dichrostachys cinerea* Wight & Arn. against cisplatin-induced nephrotoxicity in rats

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Abstract
The alcoholic extract of roots of *Dichrostachys cinerea* Wight & Arn. (200 and 400 mg/kg, p.o.) was studied for its protective effect against cisplatin-induced renal injury in rats. Cisplatin (6mg/kg, i.p.) significantly elevated serum markers level, increased urinary protein excretion, reduced urine to serum creatinine ratio and creatinine clearance. In curative regimen, the alcoholic extract exhibited dose dependent protection. Animals which received prophylactic treatment also showed partial protection against cisplatin-induced effects. Histopathological studies substantiated the above results. Further, the alcoholic extract showed marked nitric oxide scavenging effect and reducing power suggesting an antioxidant property. A triterpenoid, fatty acid and a steroid were isolated from the n-hexane, ethyl acetate fractions of alcoholic extract. The results suggested that the roots of *D. cinerea* showed protective effect against cisplatin-induced nephrotoxicity which may probably be mediated by its antioxidant property.

Keywords: *Dichrostachys cinerea*, Nephrotoxicity, Cisplatin, Antioxidant

IPC code; Int. cl.8— A61K 36/00, A61P 39/06

Introduction
Cisplatin (Cis-diammine dichloro platinum-II) is a potent anticancer agent with efficacy against a wide variety of tumours. Nephrotoxicity is dose limiting side effect of cisplatin. Although factors responsible for nephrotoxicity are not clearly understood, several mechanisms have been hypothesized for cisplatin-induced renal toxicity i.e., apoptosis, inflammatory mechanism and generation of reactive oxygen species. A number of therapeutic agents are experimentally evaluated against cisplatin-induced nephrotoxicity but none of them exhibited effective protection against cisplatin-induced renal damage. Recent reports revealed that plants such as *Aerva lanata* Juss. ex Schult., *Cassia auriculata* Linn. and *Zingiber officinale* Rosc. exhibited nephroprotective activity against cisplatin-induced nephrotoxicity. Further, various reports also suggested that several plants containing antioxidant principles exhibited nephroprotecting activity against cisplatin-induced renal damage. *Dichrostachys cinerea* Wight & Arn. (Family-Mimosaceae) is one such plant which was reported to contain antioxidant principles such as β-amin, friedelan-3-one, friedelan-3 β-ol, friedelin and α-amin. Roots of the plant are rich in triterpenoids and used to treat rheumatism, urinary calculi and renal troubles by village folk of Rayalaseema (Andhra Pradesh, India). However, there is lack of experimental data to justify its nephroprotective activity. Hence, a systematic pharmacological evaluation against experimentally induced renal damage has been carried out.

Materials and Methods
Plant Material
*D. cinerea* was collected from Tirumala hills and identified by Dr. Madhavachetti, Keeper Herbarium, Department of Botany, Sri Venkateswara University, Tirupati. Voucher specimen was deposited in the herbarium of School of Pharmaceutical Sciences, Tirupati. The roots were chopped and shade dried.

Drugs and chemicals
Cisplatin was obtained as gift sample from Intas Pharmaceuticals, Gujarat, India. Urea, creatinine and serum total proteins were estimated by use of commercial kits (Dr. Reddy’s laboratories). All other chemicals used for study were purchased either from S.D.Fine or Merck (India).

Preparation of plant extract
The roots (2 kg) were powdered in Wiley mill and extracted with rectified spirit (4L×3). The extract was concentrated under reduced pressure (60g).
Phytochemical screening

Preliminary phytochemical screening of alcoholic extract revealed the presence of fixed oils, steroids, flavonoids and phenolic compounds.

The alcoholic extract was successively fractionated with n-hexane and ethyl acetate. The solvents were removed under reduced pressure to get the respective n-hexane (14g) and ethyl acetate (10g, EtoAc) fractions. n-Hexane fraction was subjected to column chromatography using silica gel-G as adsorbent and the column was eluted with n-hexane followed by varying percentages of n-hexane, ethyl acetate. Similar procedure was followed for phytochemical studies on ethyl acetate fraction.

Pharmacological studies

Animals: The study was performed on Wistar strain albino rats of either sex (120 days) weighing 150-200g. They were maintained in standard conditions, diet (Gold Mohur pellets, Bangalore) and water was given ad libitum. The study was conducted after obtaining Institutional ethical committee clearance.

Acute toxicity and gross behavioural studies: Animals were divided into 6 groups, each group containing 6 animals. The first group was control group and the remaining groups were experimental groups which received different doses (100, 300, 600, 1000 and 3000 mg/kg) of alcoholic extract (suspended in 2% gum acacia) of the roots of D. cinerea to study gross behavioural responses like vocalization on touch, locomotor activity, palpebral reflex, autonomic responses such as tremors, convulsion, salivation, diarrhoea, sleep, coma and observed continuously for 2h and intermittently once every 2h and then monitored for any mortality for the following 14 days.

Renal toxicity: Animals were divided into 7 groups (n=6) and were put on treatment schedule given in Table-1. To induce nephrotoxicity in rats, cisplatin dose selected was 6mg/kg (intraperitoneally, single dose). The alcoholic extract was prepared in 2% gum acacia (40 mg/kg) and the extract was administered orally by gastric intubation.

Assessment of renal function: Blood urea nitrogen (BUN: Diacetyl monoxime method), serum creatinine (SC: Alkaline picrate method) and serum total proteins (S_{TP}: Biuret method) were estimated by using commercial kits. Urine was collected on day 15 (prophylactic regimen), 5 and 16 (curative regimen) for 6h (initiated at 8AM) by keeping the animals in individual metabolic cages and was analyzed for creatinine and protein (Up: sulphosalicylic acid method). Creatinine clearance was calculated by using following formula:

\[
\text{Creatinine clearance} = \frac{\text{Creatinine} \times \text{Urinary volume h}^{-1}}{\text{Serum creatinine}}
\]

Lipid peroxidation in kidney:
Lipid peroxidation was evaluated as malondialdehyde (MDA) production as described by Heath and Backer. The animals were sacrificed by decapitation on day 15 or 16. The kidneys were dissected out, immediately placed in ice cold saline to prevent contamination with blood and then removed and weighed on blotting paper; weighed and homogenized in 1.5% KCl with the help of Teflon homogenizer. To 1ml of homogenate, 2.5 ml of trichloroaceticacid (TCA, 20%) was added and centrifuged at 3500rpm for 10 min. The resulting pellet was dissolved in 2.5 ml of 0.05M H_2SO_4 and then 3ml of thiobarbituric acid was added and incubated at 37°C for 30 minutes. The contents were then extracted into 4ml of
n-butanol and the absorbance was measured spectrophotometrically at 530 nm.

**Histopathological studies**

Two animals from each group were sacrificed on day fifteen or sixteen and kidneys were isolated. The kidney sections were stained with hematoxylin and eosin and observed under light microscope.

**In vitro antioxidant studies**

*Nitric oxide scavenging activity:* Sodium nitroprusside (5mM) was mixed with different concentrations of alcoholic extract of roots (50, 100, 200, 300, 400, 500 µg/ml) and incubated at 25°C for 5 hours. After 5h, Griess reagent (2% o-phosphoric acid, 1g of sulphanilamide, 100 mg of N-naphthylethylenediamine made up to 100ml) was added and absorbance of the chromophore formed was read at 546 nm. Control experiments were also carried out in a similar manner. The experiments were conducted in triplicate. Percentage scavenging effect was calculated21, 22, Fig. 1.

**Reducing power**

The reducing power of alcoholic extract of roots was determined by Oyaizu method23. Different concentrations (10, 50, 100, 200, 300 mg/ml) were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 minutes. TCA (10%) 2.5 ml was added to the mixture which was then centrifuged at 3000 rpm for 10 minutes. The upper layer (2.5 ml) was diluted with distilled water (2.5ml) and 0.5 ml ferric chloride solution (0.1%) was added and absorbance was measured at 700nm. The absorbance of reaction mixture indicates reducing power, Fig. 2.

**Statistical analysis**

The results are expressed as mean ±SEM and the data analysed using one way analysis of variance followed by post hoc Student-Keuls test using SPSS computer software for *in vivo* studies. Student-Keuls test was used for *in vitro* studies. Statistical significance was set at \( P \leq 0.05 \).

**Results**

Phytochemical investigation revealed the presence of n-octacosanol (5% EtOAc in n-hexane eluent of n-hexane fraction), \( \beta \)-sitosterol (10% EtOAc in n-hexane eluent of n-hexane fraction and 5% EtOAc in n-hexane eluent of EtOAc fraction) and \( \beta \)-amyrin acetate (20% EtOAc in n-hexane eluent of EtOAc fraction). These compounds were characterized by comparing the spectral data (IR, \(^1\)H NMR and Mass) with authentic samples.

The alcoholic extract of this plant was found to be safe since no animal died even at the maximum dose of 3000 mg/kg body weight. The extract at 600 mg/kg and above showed mild sedation.

Animals which received the alcoholic extract alone (group VI) for ten days exhibited no change in serum markers level and urinary functional parameters. Hence, the alcoholic extract of roots did not show any deteriorative effects on kidney. In curative regimen blood and urine were collected from animals of group II, III and III on day 5 to assess whether cisplatin at the given dose induced nephrotoxicity (Data shown in Tables 2 and 3). To assess curative activity, the data obtained from the treated groups (III, III) was compared with respective curative control group (II). Similarly, prophylactic activity (V) was assessed in comparison with prophylactic control (group IV).

Table 2 lists the effect of alcoholic extract on cisplatin-induced nephrotoxicity. Administration of cisplatin at 6mg/kg i.p. caused significant elevation of BUN, SC and STP in group II and IV animals, when compared to normal control animals (group I). In curative regimen, on administration of alcoholic extract in group III and III animals, a significant dose dependent reduction in the levels of BUN, SC and serum total protein was observed when compared to group II animals. The serum markers levels were reduced significantly on administration of extract in group V animals when compared to respective control group (IV).

The deterioration of renal functions induced by cisplatin and the effect of oral administration of the alcoholic extract are given in Table 3. Administration of cisplatin in group II and IV animals caused significant reduction in the urine to serum creatinine ratio (Ucr / Scr), creatinine clearance and increased excretion of urinary protein.

Treatment with alcoholic extract in group III and III animals significantly increased the urine to serum creatinine ratio, creatinine clearance and reversed the elevated levels of total protein excretion caused by cisplatin when compared to respective control (II). Animals pretreated with alcoholic extract (group V) showed partial but significant protection against cisplatin-induced...
decreased ratio of Ucr/Scr, moderate protection against cisplatin-induced reduction in creatinine clearance and reduced the protein excretion.

Kidneys were isolated from the animals to estimate the amount of MDA which was expressed in nmol/mg protein. Animals which were treated with cisplatin alone (group II and IV) exhibited elevated levels of MDA (group II: 2.92±0.35, group IV:3.96±0.15), when compared with normal control animals (group I: 0.51±0.01), Group IIIa (2.71±0.25) IIIb (0.71±0.04) animals (curative regimen) and group V animals (2.42±0.25) (prophylactic regimen) exhibited decreased levels of MDA, when compared to respective controls. Results are shown in plates 1-6.

Plate-1 represents the section of rat kidney exhibiting normal architecture of kidney. Plate 2 is representing the section of rat kidney showing infiltration of cells, tubular congestion and glomerular atrophy indicating acute renal necrosis on day 5. The sections of the kidney isolated from rats treated with cisplatin showed marked congestion of the glomeruli with glomerular atrophy (curative control, Plate-3) and congestion of inter-tubular...
blood vessels (prophylactic control, Plate-5) indicating marked cisplatin-induced renal injury. In the curative regimen treatment with extract (400mg/kg), glomerular congestion was reduced and degenerated necrotic epithelial cells were not observed indicating that curative regimen showed marked protection against the renal injury caused by cisplatin (Plate-4). Sections of the rat kidney belong to preventive regimen showed partial protection characterized by some regenerative changes in tubular epithelial cells (Plate-6).

Even at lower concentration (50µg/ml), alcoholic extract showed significant nitric oxide scavenging activity (Fig.1) and alcoholic extract also exhibited good reducing power which was concentration dependent (Fig.2).

### Discussion

Cisplatin is a widely used antineoplastic drug that exhibits therapeutic activity against several tumours, such as bladder, testis, ovary, breast, lung and other solid tumours25,26. Cisplatin is potentially more effective if higher doses could be administered safely27. However, high doses of cisplatin are generally contraindicated because of its various side effects, including hematological, renal, neurological and hearing dysfunction28. Of these adverse effects, the major dose limiting toxicity of cisplatin is its dose-related cumulative nephrotoxicity29,30. The precise mechanism of cisplatin-induced nephrotoxicity has not been elucidated, but it has been suggested that oxygen free radicals play an important role31-33. This is further proved by plants containing antioxidant principles and they were reported to possess protection against cisplatin-induced nephrotoxicity11,12.

Cisplatin caused acute renal failure, characterized by elevation of BUN, SC, marked drop in urinary SC ratio, creatinine clearance, which is also evidenced by earlier studies. In curative regimen, the alcoholic extract exhibited marked protection against the renal and functional impairment induced by cisplatin at both the doses tested. However, the protection is more significant.

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### Table 2: Effect of alcoholic extract of *Dichrostachys cinerea* on cisplatin-induced renal damage

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>BUN (mg/dl)</th>
<th>SC (mg/dl)</th>
<th>S&lt;sub&gt;TP&lt;/sub&gt; (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24.2±1.0</td>
<td>0.6±0.05</td>
<td>6.7±0.6</td>
</tr>
<tr>
<td>II</td>
<td>63.6±3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.2±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>III&lt;sub&gt;a&lt;/sub&gt;</td>
<td>32.6±3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.2±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>III&lt;sub&gt;b&lt;/sub&gt;</td>
<td>25.9±1.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.8±0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.8±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>84.5±10.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.9±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>43.6±4.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.4±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>On day 5</td>
<td>91.0±5.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.9±0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.6±1.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*P<0.05 compared with normal control</sup>

BUN= Blood urea nitrogen (Di acetyl monoxime method); SC = Serum creatinine (Alkaline picrate method); S<sub>TP</sub> = Serum total proteins (Biuret method)

<sup>*P<0.05 compared with curative control</sup>

<sup>*P<0.05 compared with prophylactic control (one-way ANOVA followed by student’s Newman–Keuls post hoc test)</sup>

### Table 3: Effect of the alcoholic extract of roots of *Dichrostachys cinerea* against cisplatin-induced changes in renal functional parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ucr/Scr</th>
<th>Creatinine clearance (ml/h/100g bd.wt)</th>
<th>Urinary protein (mg/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>16.7 ± 0.5</td>
<td>16.0±1.5</td>
<td>7.3±0.8</td>
</tr>
<tr>
<td>II</td>
<td>10.1±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.5±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.8±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>III&lt;sub&gt;a&lt;/sub&gt;</td>
<td>12.0±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.3±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>III&lt;sub&gt;b&lt;/sub&gt;</td>
<td>15.9±1.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.1±1.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.5±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>8.1±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.4±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>14.6±0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.8±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.3±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>On day 5</td>
<td>7.9±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.9±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>Ucr/Scr = Urine to serum creatinine ratio</sup>

<sup>*P<0.05 compared with normal control</sup>

<sup>*P<0.05 compared with curative control</sup>

<sup>*P<0.05 compared with prophylactic control (one-way ANOVA followed by student’s Newman–Keuls post hoc test)</sup>
at the higher dose (400 mg/kg p.o.). This was characterized by changes in serum markers level and urinary functional parameters.

Induction of nephrotoxicity by cisplatin is assumed to be a rapid process involving reaction with proteins in the renal tubules. Because this renal damage occurs in the first hour after administration, it is important that the protective agent be present at sufficient concentrations in renal tissue before the damage occurs. This is the rationale behind the prophylactic treatment of alchoholic extract of selected plant in advance of cisplatin. In prophylactic regimen, pretreatment with the alchoholic extract at 400 mg/kg (p.o.), significantly reversed the effects that are caused by cisplatin.

The reaction of lipid peroxides with TBA has been widely adopted as a sensitive assay method for lipid peroxidation in animal tissues. In the lipid peroxidation studies, animals pretreated with plant extract showed moderate protection against cisplatin-induced elevated levels of MDA. In curative regimen, animals which received 400 mg/kg dose showed good protection on cisplatin-induced elevated levels of MDA. Histopathological studies also substantiated the above results.

Evidence points out that cisplatin induce nephrotoxicity partly via oxidative stress. One of the mechanisms proposed, by which cisplatin induces free radical damage, is by increasing the activity of calcium independent nitric oxide synthase. Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. In vitro studies revealed marked nitric oxide scavenging activity and exhibited good reducing power suggesting a potent antioxidant property. Hence, the possible mechanism by which the alchoholic extract exerts nephroprotection could be attributed to its antioxidant property.

Phytochemical investigation revealed the presence of n-octacosanol, β-sitosterol and β-amyrin acetate. Earlier reports on phytochemical studies revealed the presence of pentacyclic triterpenoids such as β-amyrin, friedelan-3-one, friedelan-3β-ol, friedelin and α-amyrin. Pentacyclic triterpenoids are known to exhibit several biological functions such as analgesic, anti-inflammatory, anti-ulcerogenic, hepatoprotective, anti-hyperglycemic activities. Hence, the presence of such important bioactive constituents might be responsible for marked protection exhibited against cisplatin-induced nephrotoxicity.

**Conclusion**

The alchoholic extract of *D. cinerea* has shown marked nephroprotective activity against cisplatin-induced renal damage. Probable mechanism of nephroprotection may be because of its antioxidant and free radical scavenging activity. Exact mechanism of protection and chemical constituents responsible for the protection need further investigations.

**References**

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