IN VITRO RADICAL SCAVENGING ASSAY AND IN VIVO NEPHROPROTECTIVE EFFECT OF BOERHAAVIA DIFFUSA L. AGAINST CISPLATIN INDUCED NEPHROTOXICITY IN MALE WISTAR ALBINO RATS

Sreedhevi Sasikumar*, 1Maleeka Begum S F. and 2Durgadevi P
1Dept. of Biotechnology, Sri Krishna Arts and Science College, Coimbatore, Tamil Nadu, India.
2Manian Institute of Science and Technology, Coimbatore, Tamil Nadu, India.
*Corresponding Author: sdhevi@gmail.com

ABSTRACT
Twenty four male Wister albino rats were organized into four groups, with six in each group, in order to identify the nephroprotective effect of Boerhaavia diffusa against cisplatin induced nephrotoxicity. The groups were labeled as the control, induced, low dose and high dose respectively. The experiment was carried out for seven days, where on the first day the induced group, low dose group and high dose group animals were administered with cisplatin (5 mg/kg) to induce nephrotoxicity. For the next five days the ethanolic extract of Boerhaavia diffusa was administered to the low dose and high dose groups at a concentration of 200 mg/kg and 400 mg/kg body weight respectively. On the last day the mice were sacrificed and the blood and kidney were collected to identify if the plant extract possessed the nephroprotective activity. Several parameters were assessed on the kidney and serum and it was identified that the ethanolic extract of Boerhaavia diffusa possessed nephroprotective activity against cisplatin. However the high dose showed better protective activity when compared to the low dosage.

Keywords: nephroprotective activity, Boerhaavia diffusa, cisplatin- induced nephrotoxicity

INTRODUCTION
Cisplatin is one of the most commonly used antineoplastic agents for the treatment of solid tumors. The drug is a co-ordinate metal complex with significant chemotherapeutic activity. The anticancer activity of cisplatin is due to its conversion to di-ucl-acquo complex, which forms an interstrand crosslink with double strand DNA to prevent DNA synthesis [1]. Cisplatin is freely filterable at the glomeruli because of its low molecular weight and unchanged character. In addition cisplatin also gets excreted via tubular secretion. Hence the drug, gains access to renal tubular cell by means of secretion or reabsorption. The intracellular concentration of cisplatin is higher than the extracellular concentration in the kidney [2]. Due to this, Cisplatin can induce severe nephrotoxicity and several other severe side effects such as bone marrow suppression, gastrointestinal toxicity, hepatotoxicity and neuropathy [3,4].

Cisplatin therapy has been demonstrated to induce oxidative stress, mainly involving reactive oxygen species (ROS) in the renal tubular cells. Oxidative stress is caused by various free-oxygen radicals, such as hydrogen peroxide, superoxide anion and hydroxyl radicals [5]. The interaction of ROS with cellular components may lead to damage of DNA, protein and lipids. Cisplatin decreases antioxidants and antioxidant enzymes [6] leading to enhanced generation of reactive oxygen metabolites and lipid peroxidation, thus affecting antioxidant defense system and results in oxidative damage in different tissues and reaction with thiols in protein and glutathione, which could cause cell dysfunction [7].

Boerhavia diffusa is an herbaceous plant of the family Nyctaginaceae. Pharmacological studies have demonstrated that B. diffusa exhibits a wide range of properties. Due to the combination of diuretic, antioxidants and anti-inflammatory activities of B. diffusa, it is regarded as therapeutically highly efficacious for the treatment of inflammatory renal diseases and common clinical problems such as nephrotic syndrome, oedema, and ascites.
From these investigations it is believed that *B. diffusa* improves renal function and may protect renal cell against chemical induced nephrotoxicity \[^8\]. The present study was aimed to investigate the possible nephroprotective effect of *B. diffusa* against cisplatin induced nephrotoxicity in rats.

**MATERIALS AND METHODS**

**Collection and Processing of *B. diffusa***

Fresh leaves of *Boerhaavia diffusa* were collected from areas in and around Coimbatore, Tamil Nadu. The leaves were washed in running tap water to remove adhering dust and wiped to dryness. The leaves were then dried under shade. The shade dried leaves were finely ground using a mechanical blender. The powder obtained was used for ethanol extraction in a soxhlet extractor.

**In vitro Radical Scavenging Assays of Ethanolic Extract of *B. diffusa* Leaves**

The scavenging activity of ethanolic extract of *Boerhaavia diffusa* leaves on hydroxyl radicals and superoxide radicals were measured according to the methods of Klein *et al.* and Beauchamp and Fridovich respectively. The standard used was gallic acid. The % hydroxyl radical scavenging activity was calculated as follows:

\[
\%\text{HRSA} = \left(\frac{\text{control OD-sample OD}}{\text{control OD}}\right) \times 100
\]

The percentage inhibition of superoxide anion generation was calculated as:

\[
\%\text{Inhibition} = \left(\frac{\text{control OD-sample OD}}{\text{control OD}}\right) \times 100
\]

**Free Radical Scavenging Activity on DPPH**

by Dot Blot Assay

Dot Blot assay was performed according to the method of Soler-Rivas *et al.* A Thin Layer Chromatography plate was flooded with DPPH solution. The excess of solution was removed with a tissue paper and dried by blowing cold air. The stained silica layer reveals a purple colour. Various concentrations (10 - 50µg) of *Boerhaavia diffusa* in ethanol was loaded in the order of increasing concentration on the DPPH coated TLC plate. The zone of clearance was measured using a scale.

**Evaluation of Protective Effects of *B. diffusa* against Cisplatin Induced Nephrotoxicity**

**Experimental animals**

Male albino Wistar rats (150-200 g) used in the present study were procured from the small animals breeding station, Mannuthy, Kerala, India. They were housed in polypropylene cages (38 x 23 x 10cm) with not more than six animals per cage and maintained under standard environmental conditions (14h dark /10h light cycles; temp 25±2°C; 35-60% humidity, air ventilation) and were fed with standard pellet diet (M/s. Hindustan Lever Ltd., Mumbai, India) and fresh water *ad libitum*. The animals were acclimatized to the environment for two weeks prior to experiment use. Animals were fasted over night before the experimental schedule, but have free access for water *ad libitum*. The experiment was carried out according to the guidelines prescribed by Animal Welfare Board and with the prior approval of animal ethic committee.

**Experimental design**

The nephroprotectivity of the ethanolic extract of *Boerhaavia diffusa* leaves were evaluated against Cisplatin induced nephrotoxicity in rat model following the method of Naghizadeh *et al.* (2008) \[^9\]. The rats were segregated into 4 groups of six animals each. The experiment was designed as follows:

- **Group I**: Normal control group.
- **Group II**: Received a single dose of cisplatin (5mg/kg) only at the first day of experiment.
- **Group III**: Single dose of cisplatin (5mg/kg) on the first day + received ethanolic extract of
In vitro Radical Scavenging Assay and in vivo Nephroprotective Effect of Boerhaavia diffusa I. against Cisplatin Induced Nephrotoxicity in Male Wistar Albino Rats

Boerhaavia diffusa (200 mg/kg b.w., respectively) for four consecutive days.

Group IV: Single dose of cisplatin (5 mg/kg) on the first day + received ethanolic extract of Boerhaavia diffusa (400 mg/kg b.w., respectively) for four consecutive days.

Cisplatin injections were carried out intraperitoneally. At the seventh day, all animals were euthanised with chloroform; blood samples were taken out by cardiac puncture and the kidneys were immediately removed, washed in saline, blotted between filter paper fold to dryness and weighed. The kidneys were then homogenized in Tris Hcl buffer (pH – 7.4) to give a 10% homogenate and used for measuring malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), vitamin C, glutathione peroxidase (GPx), glutathione S transferase (GST), glutathione reductase (GR) and total reduced glutathione (TRG).

The collected blood samples were allowed to clot, centrifuged to separate serum and this serum was used for analysis of kidney markers like total protein, albumin, urea, creatinine, uric acid, blood urea nitrogen (BUN) and serum electrolytes (Na⁺, K⁺, Cl⁻).

RESULTS AND DISCUSSION

Dot blot assay of B. diffusa

The five different concentrations of ethanolic extract of Boerhaavia diffusa tested were 10-50 µg whereas that of standard gallic acid were 0.4-2.0 µg. The maximum zone of clearance was observed for 40 and 50 µg which was 1.3 cm. However, the standard gallic acid was able to produce maximum zone even at a very less concentration of 2.0 µg (1.1 cm).

Hydroxyl radical and superoxide radical scavenging assay of ethanolic extract of B. diffusa

The hydroxyl radical scavenging and the superoxide radical scavenging activity activity of ethanolic extract of Boerhaavia diffusa and standard gallic acid were carried out. The extract exhibited percentage activity in a concentration dependent manner.

Effect of ethanolic extract of B. diffusa on enzymatic antioxidants of kidney tissue

Cisplatin induction resulted in a significant decrease (p< 0.001) in the activities of enzymatic antioxidants like SOD and CAT. On treatment with ethanolic extract of Boerhaavia diffusa, the SOD and CAT activities were found to significantly increase in a dose dependent manner (Table 1).

Effect of ethanolic extract of B. diffusa on non-enzymatic antioxidants of kidney tissue

The pattern in which the non-enzymatic antioxidants were observed among the groups was in a similar fashion to that of the enzymatic antioxidants. The levels of vitamin C and total reduced glutathione were decreased significantly (p< 0.001) in induced group animals compared to the control group animals. Treatment with ethanolic extract of Boerhaavia diffusa significantly (p< 0.001) elevated back the reduced levels of vitamin C and total reduced glutathione to normal levels (Table 1).

Effect of ethanolic extract of B. diffusa on glutathione related enzymes of kidney tissue

The activities of glutathione related enzymes like GPx, GST and GR were decreased significantly in cisplatin induced groups compared to the control groups. The ethanolic extract of Boerhaavia diffusa significantly (p<0.001 for GPx and GST; p<0.01 for GR) increased the activities of all the three enzymes in a dose dependent manner (Table 2).

Effect of ethanolic extract of B. diffusa on lipid peroxidation of kidney tissue

Lipid peroxide levels were in inverse proportion to that of the antioxidant levels. The induction of cisplatin resulted in a significant increase in the
levels of lipid peroxides from 14.50 ± 1.83 µ moles/mg protein to 23.41 ± 1.55 µ moles/mg protein. On treatment with ethanolic extract of Boerhaavia diffusa, there was a significant decrease (p< 0.001) in the lipid peroxide levels (Table 3).

**Effect of ethanolic extract of Boerhaavia diffusa on protein, albumin of serum**

A significant decrease (p< 0.001) in the levels of protein and albumin were observed in the cisplatin induced group of animals compared to the control group of animals. This reduction was altered to normal values by treatment with ethanolic extract of Boerhaavia diffusa. The increase in total protein and albumin levels in serum were in a dose dependent manner (Table 3).

**Effect of ethanolic extract of Boerhaavia diffusa on serum kidney markers**

Cisplatin induction resulted in a disastrous increase in the levels of urea (135.02 ± 11.87 mg/dl), creatinine (1.34 ± 0.31 mg/dl), uric acid (11.16 ± 0.83 mg/dl) and BUN (51.14 ± 1.36 mg/dl) compared to the control group (urea – 51.11 ± 2.83 mg/dl; creatinine – 0.14 ± 0.04 mg/dl; uric acid – 2.93 ± 0.41 mg/dl; BUN – 30.26 ± 1.66 mg/dl). Ethanolic extract of Boerhaavia diffusa had the ability to bring back the abnormal elevations in these serum markers to normal values (Table 4).

**Effect of ethanolic extract of Boerhaavia diffusa on serum electrolytes**

Cisplatin induction resulted in a reduction in the serum levels of electrolytes like Na⁺, K⁺ and Cl⁻ ions. The levels of these electrolytes in the control group of animals were found to be 143.00 ± 4.20 mmol/L, 3.31 ± 0.27 mmol/L and 119.67 ± 7.12 mmol/L respectively which were then significantly decreased (p<0.001) to 102.83 ± 5.85 mmol/L, 2.89 ± 0.07 mmol/L and 102.50 ± 5.72 mmol/L after cisplatin induction. The altered electrolyte levels were raised back to normal levels after treatment with ethanolic extract of Boerhaavia diffusa.

---

**Table 1: Effect of ethanolic extract of B. diffusa on enzymatic and non-enzymatic antioxidants of kidney tissue**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vitamin C µg/mg protein</th>
<th>SOD Units/min/mg protein</th>
<th>CAT µ moles of H₂O₂ consumed/min/mg protein</th>
<th>TRG µg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.88 ± 3.35</td>
<td>1.11 ± 0.07</td>
<td>34.11 ± 1.34</td>
<td>42.64 ± 1.78</td>
</tr>
<tr>
<td>Induced</td>
<td>45.07 ± 2.32***</td>
<td>0.48 ± 0.07***</td>
<td>10.37 ± 1.45###</td>
<td>20.20 ± 1.96###</td>
</tr>
<tr>
<td>Low dose (200 mg/kg b.w.)</td>
<td>64.30 ± 3.81***</td>
<td>0.74 ± 0.05***</td>
<td>33.36 ± 2.29***</td>
<td>35.81 ± 2.00***</td>
</tr>
<tr>
<td>High dose (400 mg/kg b.w.)</td>
<td>78.37 ± 4.11***</td>
<td>0.96 ± 0.12***</td>
<td>34.80 ± 1.48***</td>
<td>44.48 ± 2.94***</td>
</tr>
</tbody>
</table>

#Change in activities at P<0.05 when induced compared to control, ## P<0.01, ### P<0.001; *Change in activities at P<0.05 when low dose and high dose compared to induced, ** P<0.01, *** P<0.001; Values are expressed as mean±SD (n=6).
In vitro Radical Scavenging Assay and in vivo Nephroprotective Effect of Boerhaavia diffusa l. against Cisplatin Induced Nephrotoxicity in Male Wistar Albino Rats

Table 2: Effect of ethanolic extract of B. diffusa on glutathione related enzymes of kidney tissue

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPx</th>
<th>GST</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µ moles of GSH oxidized/min/mg protein</td>
<td>µ moles of CDNB conjugation formed/min/mg protein</td>
<td>µ moles of glutathione reduced/min/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>35.62 ± 2.16</td>
<td>240.53 ± 13.26</td>
<td>108.09 ± 5.01</td>
</tr>
<tr>
<td>Induced</td>
<td>17.67 ± 1.54###</td>
<td>163.31 ± 4.48###</td>
<td>63.99 ± 3.11###</td>
</tr>
<tr>
<td>Low dose (200 mg/kg b.w.)</td>
<td>25.08 ± 1.37###</td>
<td>232.68 ± 9.00###</td>
<td>74.79 ± 3.94**</td>
</tr>
<tr>
<td>High dose (400 mg/kg b.w.)</td>
<td>33.78 ± 1.84***</td>
<td>237.11 ± 11.31***</td>
<td>101.85 ± 8.02***</td>
</tr>
</tbody>
</table>

#Change in activities at P<0.05 when induced compared to control, ## P<0.01, ### P<0.001; *Change in activities at P<0.05 when low dose and high dose compared to induced, ** P<0.01, *** P<0.001; Values are expressed as mean±SD (n=6).

Table 3: Effect of ethanolic extract of B. diffusa on LPO, protein, albumin of serum

<table>
<thead>
<tr>
<th>Groups</th>
<th>LPO µ moles/mg protein</th>
<th>Total protein mg/dl</th>
<th>Albumin mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.50 ± 1.83</td>
<td>7.51 ± 0.55</td>
<td>3.61 ± 0.70</td>
</tr>
<tr>
<td>Induced</td>
<td>23.41 ± 1.55###</td>
<td>3.39 ± 0.33###</td>
<td>1.27 ± 0.34###</td>
</tr>
<tr>
<td>Low dose (200 mg/kg b.w.)</td>
<td>17.46 ± 2.24***</td>
<td>6.25 ± 0.25***</td>
<td>2.77 ± 0.28***</td>
</tr>
<tr>
<td>High dose (400 mg/kg b.w.)</td>
<td>14.13 ± 1.84***</td>
<td>6.97 ± 0.98***</td>
<td>3.12 ± 0.32***</td>
</tr>
</tbody>
</table>

#Change in activities at P<0.05 when induced compared to control, ## P<0.01, ### P<0.001; *Change in activities at P<0.05 when low dose and high dose compared to induced, ** P<0.01, *** P<0.001; Values are expressed as mean±SD (n=6).

Table 4: Effect of ethanolic extract of B. diffusa on serum kidney markers

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urea mg/dl</th>
<th>Creatinine mg/dl</th>
<th>Uric acid mg/dl</th>
<th>BUN mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.11 ± 2.83</td>
<td>0.14 ± 0.04</td>
<td>2.93 ± 0.41</td>
<td>30.26 ± 1.66</td>
</tr>
<tr>
<td>Induced</td>
<td>135.02 ± 11.87###</td>
<td>1.34 ± 0.31***</td>
<td>11.16 ± 0.83###</td>
<td>51.14 ± 1.36###</td>
</tr>
<tr>
<td>Low dose (200 mg/kg b.w.)</td>
<td>81.84 ± 3.96***</td>
<td>0.72 ± 0.11***</td>
<td>2.98 ± 0.29***</td>
<td>37.75 ± 1.16***</td>
</tr>
<tr>
<td>High dose (400 mg/kg b.w.)</td>
<td>56.62 ± 3.17***</td>
<td>0.25 ± 0.07***</td>
<td>2.59 ± 0.49***</td>
<td>33.77 ± 1.12***</td>
</tr>
</tbody>
</table>

#Change in activities at P<0.05 when induced compared to control, ## P<0.01, ### P<0.001; *Change in activities at P<0.05 when low dose and high dose compared to induced, ** P<0.01, *** P<0.001; Values are expressed as mean±SD (n=6).
It has been shown that administration of cisplatin to rat caused an elevation in urine glucose which correlated with increase in plasma creatinine and urea levels. Also, cisplatin-induced nephrotoxicity was accompanied by an increase in MDA, reduction of total thiol and GSH peroxidase concentrations in kidney tissue. These biochemical parameters were well correlated with the renal histopathological results. It has been suggested that binding of cisplatin to the renal base transport system and the following peroxidation of membrane lipids may account for its nephrotoxicity. There is evidence suggesting that cisplatin exerts its nephrotoxic effects by the generation of free radicals.

The results obtained in the present study were in accordance with the above mentioned reports. A significant (p<) increase in lipid peroxidation, urea, creatinine, uric acid and BUN were observed in cisplatin induced groups (Tables 3 and 4). This increase was altered to normal levels on treatment with ethanolic extract of *Boerhaavia diffusa*. Cisplatin is known to induce ROS primarily by decreasing the activity of antioxidant enzymes and by depleting intra cellular concentrations of GSH and also causes the peroxidation of membrane lipids, GSH reacts with the harmful by products of aerobic life hydrogen peroxide and organic peroxides. In the presence of Cisplatin, GSH forms GSH-platinum complex that is then eliminated from the cell (Florea and Busselberg, 2011). The dose dependent increase in GSH level after treatment with ethanolic extract of *Boerhaavia diffusa* indicated the higher protection of the extract against cisplatin induced toxicity. It is quite possible that the antioxidant constituent of the ethanolic extract of *Boerhaavia diffusa* significantly increased GSH level of the animal provides protection against oxidant attacks created by cisplatin and / or its metabolites. This increase in GSH levels indirectly favors the increase in the activities of enzymes like GPx, GST and GR since GSH forms as the substrate for all these enzymes.

During renal injury and inflammation, superoxide and peroxide radicals are generated at the site of damage, resulting in the depletion of SOD and CAT activity. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism (Linares *et al.*, 2006). The present study also demonstrated that cisplatin overdose resulted in a decrease in the SOD and CAT activities when compared with normal control rats. Treatment with the extract restored the activity of SOD and CAT significantly (p<). It is also possible for the extract to be mediating its renal antioxidant activities by enhancing the antioxidant defense enzymes mediated through SOD, CAT and replenishing renal glutathione storage. It has already been reported that the aqueous extract of *Boerhaavia diffusa* showed similar effect against acetaminophen induced nephrotoxicity.

The protection offered by *Boerhaavia diffusa* could have been due to the presence of any of the active principles contained in it. Earlier reports suggest the presence of flavonoids, alkaloids, steroids, triterpenoids, lipids, lignans, carbohydrates, proteins and glycoproteins. Flavonoids and other antioxidant constituents of medicinal plants have been reported to inhibit Xenobiotic induced nephrotoxicity in experimental animal models due to their potent antioxidant effects. Any of these or their combination could be responsible for the nephroprotective effect of *B. diffusa*.

CONCLUSION

Nephrotoxicity is one of the most common kidney problems and occurs when body is exposed to a drug or toxin. A number of therapeutic agents can adversely affect the
kidney resulting in acute renal failure, chronic interstitial nephritis and nephritic syndrome because there is an increasing number of potent therapeutic drugs like aminoglycoside antibiotics, NSAID’s and chemotherapeutic agents have been added to the therapeutic arsenal in recent years. Prompt recognition of the disease and cessation of responsible drugs are usually the only necessary therapy. Nephroprotective agents are the substances which possess protective activity against Nephrotoxicity. Antioxidant supplementation strengthens the renal antioxidant system, eliminates oxidation reactions, and prevents cisplatin-induced kidney failure.

The nephroprotective effect of *Boerhaavia diffusa* against Cisplatin induced nephrotoxicity was analysed in Wistar rats by assessing the levels of SOD, CAT, LPO, Vitamin C, TRG, GPx, GR and GST from the kidney of the animals. The serum was taken to assess the levels of urea, uric acid, total protein, albumin, creatinine, blood urea nitrogen and electrolytes. Cisplatin was administered at 5 mg/kg b.w. on the first day in order to induce nephrotoxicity. Treatment with the ethanolic extract of *B. diffusa* was carried out in two dosages (200 and 400 mg/kg b.w.) in group III and IV respectively for the following five days. On the last day the rats were sacrificed by following inhalation euthanasia and the parameters were assessed. The results indicate that the ethanolic extract of *B. diffusa* does contain nephroprotective activity against Cisplatin induced nephrotoxicity. The plant extract had the ability it normalize the elevated levels of urea, creatinine, uric acid, BUN in serum and LPO in kidney. Both the doses of *B. diffusa* boosted up the antioxidant levels which were found decreased in Cisplatin toxicity. However the high dose of ethanolic extract of *B. diffusa* (400 mg/kg b.w.) showed more protective effect when compared to the low dose. This effect of *B. diffusa* could be attributed to its free radical scavenging efficacy which was quite evident from the results obtained for the *in vitro* radical scavenging assays.

Like reduced glutathione, *B. diffusa* could also be co-administered with Cisplatin therapy in Cancer patients since it has got the ability to boost up the glutathione levels and glutathione related enzymes with no adverse effects. But still the mechanism by which *B. diffusa* renders protection against nephrotoxicity has to be studied in detail and also the active principles involved in nephroprotection need to be identified, isolated and its structure has to be elucidated.

**REFERENCES**


