CHAPTER-11-

AMELIORATION OF CISPLATIN-INDUCED NEPHRO-TOXICITY BY *A. CALAMUS* EXTRACT AND ALPHA-ASARONE
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11.4. DISCUSSION
11.1. INTRODUCTION

Cis-Diamminedichloroplatinum (II) (cisplatin, CP) a platinum co-ordinate complex, is a widely used antineoplastic agent for the treatment of metastatic tumors of the testis, metastatic ovarian tumors, lung cancer, advanced bladder cancer, and many other solid tumors (Sweetman, 2002). Although higher doses of cisplatin are more efficacious for the treatment of cancer (Di Re, et al, 1990; Gandara et al, 1991) many reversible and irreversible side effects including nephrotoxicity, neurotoxicity, bone marrow toxicity, gastrointestinal toxicity and ototoxicity often limit its utility and therapeutic profile (Antunes et al., 2000; Lynch et al, 2005). Primary targets of cisplatin in kidney are proximal straight and distal convoluted tubules where it accumulates and promotes cellular damage, by multiple mechanisms including oxidative stress, DNA damage, apoptosis and inflammation (Schaaf et al, 2002; Cummings and Schnellmann, 2002; Xiao et al, 2003). Oxidative stress caused by increased generation of free radicals and caspase-mediated apoptosis play a major role in nephrotoxicity and renal dysfunction that progressively develop in response to CP treatment (Arany and Safirstein, 2003).

CP-induced nephrotoxicity is also closely associated with an increase in lipid peroxidation in the kidney tissues. This antitumoral drug also causes generation of reactive oxygen species (ROS), such as superoxide anion and hydroxyl radical that deplete the GSH levels and inhibit the activity of antioxidant enzymes in renal tissue. This ROS may produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage (Kim et al., 1997; Mora et al., 2003). Glutathione, an endogenous free thiol, has been reported to decrease cisplatin nephrotoxicity (Somani et al., 1995). The depletion of GSH seems to be a prime factor that permits lipid peroxidation (Younes and Siegers, 1981). Administration of superoxide dismutase (SOD) and antioxidants (GSH) ameliorates cisplatin nephrotoxicity in experimental animals (Anderson et al., 1990). Superoxide dismutase plays an important role in the dismutation of superoxide anions by catalyzing their conversion to hydrogen peroxide and singlet oxygen. Glutathione peroxidase activity was decreased in the rat renal mitochondria incubated with cisplatin and has been correlated to disturbances in GSH metabolism (Sugiyama et al., 1989).
Previous studies reported that there are several natural agents that may reduce the cisplatin induced nephrotoxicity through the mechanism of free radical scavenging. Extract of medicinal plant Rubia cordifolia has been reported to ameliorate the nephrotoxicity induced by cisplatin (Joy and Nair, 2009). Quercetin, a common antioxidant bioflavonoid in fruits and vegetables, has a potent cytoprotective effects against cisplatin induced nephrotoxicity in cultured renal proximal tubular epithelial cells. (Kaushal et al., 2001). Sugiyama et al. in 1989 have reported that green tea tannin play a major role as antioxidant that scavenges the reactive radicals generated from cisplatin in experimental animals.

Several medicinal plants can be employed to produce extracts exhibiting biological effects. Previous studies reported that the Acorus calamus extract and one of its major active component α-asarone exhibits good antioxidant and free radical scavenging activity. Since CP-induced nephrotoxicity is also closely associated with generation of reactive oxygen species, it could be beneficial by using these antioxidants as nephroprotecting agents against cisplatin induced nephrotoxicity. Hence the present study aims to find out the nephroprotecting property of A.calamus extract and α-asarone against cisplatin induced renal toxicity in mammalian system.

11.2. MATERIALS AND METHODS

11.2.1. Preparation of the extract
Aqueous-ethanol extract of Acorus calamus was prepared as described in section 2.2.1.

11.2.2. Animals
Male Swiss albino mice of 6 weeks old weighing 25 ± 2 g were employed for nephrotoxicity studies.

11.2.3. Determination of protection against cisplatin induced nephrotoxicity by Acorus calamus extract and α-asarone

Animals were divided into 4 groups of 5 animals each and treated as follows,

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Untreated control (distilled water)</td>
</tr>
<tr>
<td>II</td>
<td>Cisplatin (12 mg/kg body weight, i.p.) as a single dose</td>
</tr>
</tbody>
</table>
Group III  
*A. calamus* extract (250 mg/kg body weight) + cisplatin

Group IV  
α-asarone (50 mg/kg body weight) + cisplatin

Group I treated with vehicle (distilled water) kept as untreated control. Group II was injected with a single dose of cisplatin (12 mg/kg body weight; i.p, which is equivalent to the dose administered in human situations). The extract was administrated orally to Group III animals, 1 hour before and 24 and 48 hours after cisplatin injection. Similarly Group IV treated with α-asarone (50 mg/kg body weight) 1 hour before and 24 and 48 hours after cisplatin injection. Seventy two hours after cisplatin injection, animals were sacrificed using ether anesthesia, blood were collected directly from the heart, serum separated for urea and creatinine analysis. Kidneys were excised after sacrificing the animals and washed with ice-cold PBS and 10% homogenate was prepared in phosphate buffer (0.05M, pH 7) using a polytron homogenizer at 4°C.

**11.2.4. Assessment of nephrotoxicity**

Serum creatinine was determined by alkaline picric acid method using a diagnostic kit (Agappe Diagnostic Pvt. Ltd; Ernakulam, Kerala, India). Serum urea was determined by diacetylmonoxime (DAM) reagent using Agappe diagnostic kit.

Reduced glutathione (GSH) level was measured colorimetrically using DTNB as the substrate. The concentrations of malondialdehyde (MDA) as indices of lipid peroxidation were assessed according to the method of Buege and Aust (1978). Superoxide dismutase activity was determined by the nitroblue tetrazolium reduction method of Mc Cord and Fridovich (1969). GPx activity was determined by the method of Hafemann et al. (1974), based on the degradation of H₂O₂ in the presence of GSH. Catalase activity was determined from the rate of decomposition of H₂O₂ monitored by decrease of 240 nm following the addition of tissue homogenate (Aebi, 1983). Tissue protein was estimated according to the method of Lowry et al. (1951) using bovine serum albumin as standard.
11.2.5. Histopathological studies
Histopathological examinations of kidney from all the treated groups were evaluated using light microscopy. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin-eosin. The histopathological examinations were carried out at Sudharma Metroplis Pathological Laboratory, Thrissur, Kerala, India.

11.3. RESULTS

11.3.1. Nephroprotection by A. Calamus and Alpha-Asarone

11.3.1.1. Effect on serum creatinine and urea levels
Administration of cisplatin to mice was found to induce a marked renal failure, characterized by a significant increase in serum urea and blood creatinine levels. As shown in Fig 11.1 and Fig 11.2, serum urea and serum creatinine concentrations were significantly increased in the cisplatin (133.29±35.18 and 1.11±0.07) treated group compared to the untreated control group (37.83±3.68 and 0.56±0.03). Treatment with the A. calamus extract showed marked decrease in concentrations of serum urea and creatinine compared to control group (p<0.001). Treatment with 250 mg/kg body weight of the extract restored the urea level to 58.13 ± 9.17 mg/dL and creatinine level to 0.59 ± 0.046 mg/dL in cisplatin administered animals. In α-asarone administered animals, the urea and creatinine concentrations in the serum were restored to 64.35 ± 3.72 mg/dL and 0.65 ± 0.04 mg/dL respectively.

11.3.1.2. Effect on kidney antioxidants and MDA levels
Activities of three major enzymes of the antioxidant defense system namely SOD, catalase and GPx and levels of GSH were significantly decreased while the concentration of malonaldehyde (MDA) was found to be elevated in cisplatin treated group. Administration A. calamus extract and α-asarone were found to significantly elevate the decreased activities of SOD, catalase, and GPx. The activities of renal SOD, CAT, and GPx in the cisplatin alone, cisplatin+ A. calamus extract and cisplatin + α-asarone administered groups were given in table 11.1. Administration of these two also inhibited the cisplatin induced increase in the MDA levels as shown in Fig 11.3. It can be seen from Fig. 11.4 that the decrease in the GSH levels in renal tissues
induced by cisplatin can be prevented by the administration of *A. calamus* extract and α-asarone.

**Fig.11.1.** Effect of *A. calamus* extract and α-asarone on serum urea concentrations in mice treated with cisplatin. All Values expressed as Mean ± S.D, (n=5). a-P<0.001 Vs. cisplatin.
**Fig.11.2.** Effect of *A.calamus* extract and α-asarone on serum creatinine concentrations in mice treated with cisplatin. All values expressed as Mean ± S.D, (n=5). a-P<0.001 Vs. cisplatin.

![Graph showing the effect of *A.calamus* extract and α-asarone on serum creatinine concentrations in mice treated with cisplatin.](image)

**Fig.11.3.** Effect of administration of *A.calamus* extract and α-asarone on cisplatin induced lipid peroxidation. All values expressed as Mean ± S.D, (n=5). a-P<0.001 Vs. cisplatin

![Graph showing the effect of *A.calamus* extract and α-asarone on cisplatin induced lipid peroxidation.](image)
Fig.11.4. Effect of administration of *A.calamus* extract and α-asarone on cisplatin induced depletion of renal GSH content. All values expressed as Mean ± S.D, (n=5). α-P<0.001 Vs. cisplatin.
Table 11.1. Effect of p.o. administration of *A. calamus* extract and α-asarone on cisplatin induced decrease in renal antioxidant enzymes. Values are expressed as mean ± SD (n = 5).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GPx  (Unit/mg protein)</th>
<th>SOD  (Unit/mg protein)</th>
<th>CAT  (Unit/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18.84±1.13</td>
<td>13.19±0.4</td>
<td>5.67±0.04</td>
</tr>
<tr>
<td>Cisplatin (12mg/ kg b.w)</td>
<td>10.86±1.60</td>
<td>6.42±2.4</td>
<td>3.06±0.02</td>
</tr>
<tr>
<td><em>A. calamus</em>+Cisplatin (250mg/ kg b.w)</td>
<td>17.32±1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.02±4.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.75±0.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-asarone+Cisplatin (50mg/ kg b.w)</td>
<td>17.44±0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.89±5.27&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.16±0.63&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a-P< 0.001 Vs. cisplatin  
c-P< 0.05 Vs. cisplatin  
d-non significant Vs. cisplatin

11.3.1.4 Histopathological studies

Histopathological investigation showed that, in cisplatin treated mice kidney there is a decreased cellularity of the glomeruli and edema of the lining of epithelial cells in the renal tubules. Moreover, the nuclei of the lining cells show vacullation. The interstitial tissue also showed edema as can be evident from Fig.11.5 (B). The renal tissue architecture of the untreated mice kidney (Fig11.5 (A)) was unaffected with normal glomeruli. The renal tissues of cisplatin treated mice when administered with extract of *A. calamus* or α-asarone after the cisplatin treatment, showed near normal architecture with normal glomerular, renal tubules and interstitial tissue appearance (Fig.11.5 (C) and (D)).
Fig. 11.5. Effect of *Acorus calamus* extract and α-asarone on cisplatin induced renal damage in mice (X 40).

[A] Renal section from untreated control mice shows normal cellular architecture with normal glomureli. [B] Kidney from mice treated with cisplatin (12mg/kg body weight) exhibited decreased cellularity of the glomeruli, edema of the lining of epithelial cells in the renal tubules and nuclear vaculation. [C & D] Kidney treated with *A.calamus* (250 mg/kg) and α-asarone (50 mg/kg) shows near normal architecture with normal glomerular, renal tubules and interstitial tissue appearance.
11.4. DISCUSSION

Cisplatin is one of the most effective cancer therapeutic agents but the major side effect of this chemotherapeutic agent is nephrotoxicity. Boogaard et al. (1991) reported that a minimum dose of cisplatin (5 mg/kg body weight, i.p.) was sufficient to induce nephrotoxicity in rats. A higher dose of cisplatin (10 mg/kg body weight, i.p.) corresponds to the equivalent human dose currently being used in clinical practice. Cisplatin is known to accumulate in mitochondria of renal epithelial cells and induces ROS in renal epithelial cells primarily by decreasing the activity of antioxidant enzymes and by depleting intracellular concentrations of GSH (Sadzuka et al, 1992; Huang et al, 2001; Hanigan and Devarajan, 2003). Cisplatin causes the peroxidation of membrane lipids which may also accounts for its nephrotoxicity (Safirstein et al, 1984).

Among the main approaches used to ameliorate or protect against cisplatin induced nephrotoxicities, the most consistent effects have been observed with the use of antioxidant agents (Mingeot-Leclerq and Tulkens, 1999). Some antioxidant agents that have been used to ameliorate cisplatin induced nephrotoxicity in rats include deferoxamine, methimazole, Vit E, Vit C diethyl dithiocarbomate, L-histidinol, thymoquinone, Naringenin (Mingeot-Leclerq and Tulkens, 1999; Badary et al, 1997a & 1997b; Badary et al,2005). None of these compounds has proved to be clinically efficacious as complete protection in patients.

A recent interesting development is the attempt to use extracts from medicinal plants with antioxidant properties or natural antioxidants to ameliorate cisplatin induced nephrotoxicities. These natural antioxidants may offer comparatively safer alternatives to synthetic antioxidants, which may cause serious or unacceptable adverse side effects.

The present study demonstrates that cisplatin treatment caused significant increase in blood creatinine and serum urea concentrations compared to normal, which clearly indicates the intrinsic acute renal failure. Administration of A.calamus extract (250 mg/kg body weight) and α-asarone (50 mg/kg body weight) significantly prevents the increase of cisplatin induced serum creatinine and urea concentrations, which explains their significant nephroprotective effect. However, treatment with α-asarone was not that much effective when compared to the A.calamus extract.
The present study reported that cisplatin revealed a significant reduction in the renal antioxidant status, such as SOD, CAT, GPx activities, and reduced GSH concentration. But administration of *A. calamus* extracts or α-asarone help to restore the levels of these antioxidant status in a significant manner. These observations support the evidence that the inhibition of enzymes is reversible and part of the mechanism of nephrotoxicity in cisplatin-treated rats is related to depletion of antioxidant system. Thus the inhibition of antioxidant system by cisplatin and the attenuation of this inhibitory effect by the administration of *A. calamus* extract or α-asarone reveal their nephroprotecting property.

GSH, a tripeptide synthesized by glutathione synthase, is thought to be one of the most effective primary antioxidants against oxidizing agents (Shang et al., 2003). Reduced glutathione maintains cellular sulfhydryl groups and other structural proteins in the stable form. The superoxide radical is the most well-known oxygen-derived free radical (Yu, 1994) and can lead to the formation of additional reactive species \( \text{H}_2\text{O}_2 \). Because of its non-ionized state, is able to diffuse through hydrophobic membranes and can form hydroxyl radicals that react with organic lipids to act like highly reactive free radicals. These can cause cellular damage and cell death. Reduced renal GSH can markedly increase the toxicity of cisplatin. The depletion of GSH also seems to be a prime factor that permits lipid peroxidation in the cisplatin treated group. But the administration with the extract or α-asarone in cisplatin treated mice rendered protection due to the increase in GSH concentration and could protect the renal cells from oxidants attack.

Animal bodies possess intracellular defence against both hydrogen peroxides and superoxide anion. The first line of defence against these products is SOD. The function of this enzyme is to convert superoxide radicals into oxygen and hydrogen peroxide (Gaetani et al., 1989). Our results in renal tissues showed that cisplatin induced decline in SOD activities. The decreased SOD activity could cause the initiation and propagation of lipid peroxidation. Moreover reduction in the activity of GPx during cisplatin administration also increases in the levels of peroxides. The decreased activity of CAT and GPx could enhance the lipid peroxidation (Amudha et al., 2006). Thus the levels of MDA, as a result of lipid peroxidation, increased in the cisplatin treated animals. Treatment with *A. calamus* or α-asarone prevented the lipid peroxidation by enhancing the renal CAT, SOD and GPx activities.
In conclusion, the present study showed that administration of either *A. calamus* or α-asarone resulted in amelioration of cisplatin induced nephrotoxicity. The protective effect of the extract or α-asarone is associated with their antioxidant properties, as they showed significant biological activities. Even though the potential usefulness of this herbal extract or phytocutical is evident in cisplatin therapy from the present work, further studies are needed for the therapeutic application.