Chapter 7

Ameliorative effect of *Pseudarthria viscida* on toxicity induced by cyclophosphamide
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7.1 Introduction

Cyclophosphamide is a nitrogen mustard related alkylating agent, widely used as a chemotherapeutic agent against different cancers, including multiple myeloma, lymphomas and leukaemia (Mythili, 2007). It alkylates DNA strand and thereby interfere with DNA replication mechanism. It is also used in treating autoimmune diseases (Devine et al., 2012). In liver cyclophosphamide get converted into 4-hydroxy cyclophosphamide by cytochrome P450 enzymes and this newly formed active metabolite acts as the active component for chemotherapy. Cyclophosphamide is also known to have several side effects and even lead to life threatening conditions. It is known to cause a steady increase in the rate of malignancy. Chances for skin cancer, bladder cancer, leukaemia, lymphoma etc. are very high when exposed to cyclophosphamide (Baker et al., 1987). It is also known to cause reduced fertility or infertility. Ovarian follicles are highly sensitive to cyclophosphamide treatment and resultant tissue damages are found to be associated with depletion in GSH level in ovarian follicles (Lopez and Luderer, 2004). Herbal preparations are known to have an ameliorative effect on several chemically induced toxicities. P. viscida has the ability to reduce oxidative stress and hence improve the availability of antioxidant molecules such as GSH, which has a crucial role in cyclophosphamide metabolism. Thus, we investigated the ameliorative activity of P. viscida against cyclophosphamide induced toxicity.

7.2 Materials and Methods

7.2.1 Animals

Male BALB/c mice (25-30) were purchased from the Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were maintained under standardized environmental conditions (22-280C, 60-60% relative humidity, 12hr dark/light cycle) and they were fed with normal mouse chow (Sai Durga Feeds,
Bangalore) and water *ad libitum*. Experiments were conducted according to the rules and regulations put forwarded by Animal Ethics Committee, Government of India and followed the guidelines of Institutional Animal Ethics Committee.

### 7.2.2 Drug preparation

*P. viscida* whole plant extracts were dissolved in desired volume of distilled water before administering to the experimental animals.

### 7.2.3 Experimental design

BALB/c mice were divided into four groups, each group having 10 mice. Group I consisted of normal animals. Group II consists of control animals which were injected intraperitoneally with cyclophosphamide at a dosage of 25 mg/kg body weight for 10 days. Groups III and IV mice were administered orally with *P. viscida* extract at the doses of 100 mg/kg and 250 mg/kg body weight. There was a 10-day pre-treatment with *P. viscida* before starting of cyclophosphamide (25 mg/kg) injection and continued for another 10 days along with administering of cyclophosphamide. The normal and control groups received distilled water. On the 20th day of experiment animals were anesthetised with ether and sacrificed by cervical dislocation.

### 7.2.4 Determination of Total WBC count

Four animals from each group were subjected to tail bleeding. Blood collected on every 3rd day of the experiment was used to conduct WBC counting. Blood was collected in heparinized tubes, and a volume of blood was diluted with Turk’s fluid. Cells were counted using a haemocytometer.

### 7.2.5 Determination of bone marrow cellularity, serum enzymes and endogenous antioxidant levels for cyclophosphamide induced liver, kidney and intestinal toxicity

The blood samples collected by heart puncturing were centrifuged at 2500 rpm for 10 minutes to separate the serum. Serum aspartate transaminase (SGOT) (2.2 Chapter 2), serum glutamate pyruvate transaminase (SGPT), alanine phosphatase (ALP), bilirubin, creatinine and urea levels were assayed using commercial reagent kits. Liver and kidney samples were excised and
rinsed thoroughly in ice-cold saline to remove blood clots and then gently blotted between the folds of a filter paper and weighed in an analytical balance. Homogenate of 25% was prepared in 0.05 M phosphate buffer (pH 7.0) using a polytron homogeniser maintained at 4°C. A part of this homogenate was used for the determination of reduced glutathione, glutathione peroxidase and lipid peroxidation. Rest of the homogenate was centrifuged at 10,000 rpm for 30 min and the supernatant collected was used for the estimation of superoxide dismutase and catalase. A portion of the jejunum part of the small intestine was cut opened. Mucosal lining was scraped with a sterile blade and 25% homogenate was prepared in triss buffer and centrifuged at 10,000 RPM for 30 minutes. The supernatant was taken to analyse GSH level. Femur bone was obtained from the mice (five mice per group) for the determination of bone marrow cellularity. Bone marrow was collected from the femur bones by flushing thoroughly with 1 ml PBS containing 10% goat serum solution using a gauge needle in a 1 ml syringe. The cells collected in sterile tube were diluted before bone marrow cellularity was determined microscopically with a haemocytometer.

7.2.6 Statistical analysis
The values were expressed in Mean ± SD, for 10 animals in each group. All groups were analysed for one way anova by Dunnetts test using GraphPad InStat software. The groups with ‘p’ value less than 0.05 were considered statistically significant.

7.3 Result
Level of serum GOT, GPT, ALP, bilirubin, creatinine and urea (Table 7.1) were determined for the 30th day of experiment. Animals treated with cyclophosphamide alone showed a comparatively higher concentration of GOT which is 213.54 ± 18.82. Normal group has a GOT level of 122.2 ± 15.99. P. viscidida treated animals showed significant (p<0.01) reduction in the level of serum GOT for concentrations 100 mg/kg (179.4 ± 4.62) and 250 mg/kg
Serum GPT was also significantly (p<0.01) reduced to 57.33 ± 4.43 (100 mg/kg) 50.37 ± 6.96 (250 mg/kg) compared to 107.16 ± 19.10 of cyclophosphamide alone treated group. GPT level was very high in control group (107.16 ±19.10) in comparison to normal animals (63.97 ± 8.95). Treatment with *P. viscida* 100 mg/kg (57.33 ± 4.43) and 250 mg/kg (50.37 ± 6.96) significantly (p<0.01) reduced GPT level in serum. ALP level was found significantly (p<0.01) reduced in 100 mg/kg (70.23 ± 3.78) and 250 mg/kg (56.72 ± 4.01) of *P. viscida* extract treated animals compared to control group. Bilirubin level was also lowered in *P. viscida* administered groups of 100 mg/kg (0.69 ± 0.05) and 250 mg/kg (0.54 ± 0.5) in comparison to control group (0.82 ± 0.09). Similarly there was reduction in serum creatinine in animals treated with 100 mg/kg (0.625 ± 0.06) and 250 mg/kg (0.58 ± 0.01) of *P. viscida* compared to cyclophosphamide alone treated group (0.632 ± 0.05).

Liver tissue samples from experimental animals were homogenized and evaluated for SOD, GPx, GSH and MDA levels (Table 7.2). SOD activity showed declining trend in control group (1.11± 0.20) in comparison to normal group (2.52 ± 0.14). However, treatment with *P. viscida* 100 mg/kg (1.81 ± 0.21) and 250 mg/kg (2.04 ± 0.12) had recovering effect on SOD level in significant (p<0.01) and dose dependent manner. GPx activity also significantly (p<0.01) improved in 100 mg/kg (21.14 ± 0.62) and 250 mg/kg (23.08 ± 0.33) of *P. viscida* while considering that of control group (17.24 ± 0.42). Presence of GSH was lowered significantly (p<0.01) in control group (14.05 ± 0.62) than normal group (21.12 ± 0.73) and showed significant (p<0.01) improvement when treated with 100 mg/kg (17.43 ± 0.83) and 250 mg/kg (19.74 ± 0.59) of *P. viscida* in dose dependent manner. MDA level was
significantly (p<0.01) higher in control animals (2.02 ± 0.08) than in normal group (0.94 ± 0.06) and showed significant reduction when treated with 100 mg/kg (1.51 ± 0.04) (p<0.05) and 250 mg/kg (1.20 ± 0.06) (p<0.01) of P. viscida. SOD, GPx, GSH and MDA levels in kidney samples of experimental animals were evaluated (Table 7.3). SOD activity was reduced in control group (1.42 ± 0.08) than normal group (1.98 ± 0.03) which was found significantly (p<0.01) elevated when treated with 100 mg/kg (1.68 ± 0.18) and 250 mg/kg (1.93± 0.04) P. viscida. Control group showed (1.21 ± 0.68) reduced activity of GPx and showed recovery of significant level when treated with P. viscida of concentrations 100 mg/kg (3.65 ± 0.12) and 250 mg/kg (5.14 ± 0.28). GSH level was 4.83 ± 0.95 for normal group and was reduced to 1.54 ± 1.36 in control group. GSH showed significant improvement, when administered with 100 mg/kg (3.21 ± 1.21) and 250 mg/kg (4.27 ± 2.41) P. viscida. There was significant (p<0.01) reduction in MDA level when treated with P. viscida of concentrations 100 mg/kg (0.95 ± 0.04) and 250 mg/kg (0.82 ± 0.02) compared to control group (1.86 ± 0.02) which was significantly (p<0.01) higher than that of normal animals (0.69 ± 0.02).

Bone marrow cellularity (Fig 7.1) was significantly reduced in control group (7.64 ± 0.24) on 30th day than normal group (17.68 ± 1.06) and showed significant (p<0.01) recovery in dose dependent manner when treated with 100 mg/kg (13.98 ± 0.35) and 250 mg/kg (14.91 ± 0.242) concentrations of P. viscida. GSH level in intestinal mucosa (Fig 7.2) was lowered in control group (21.03 ± 0.81) in comparison to normal group (35.51 ± 0.92) and showed significant elevation on treatment with 100 mg/kg (27.02 ± 1.04) and 250 mg/kg (30.81 ± 1.11) concentrations of P. viscida. Body weight of animals showed recovery on 30th day when treated with 100 mg/kg (23.21 ± 1.24) and 250 mg/kg (24.68 ± 1.01) concentrations of P. viscida in dose dependent manner when compared to control group (20.14 ± 0.77). WBC count on 30th day for 100 mg/kg (8148 ± 393) and 250 mg/kg (8781 ± 342) P. viscida treated.
groups showed better WBC count than cyclophosphamide alone treated group (6723 ± 408).

7.4. Discussion
Cyclophosphamide destabilize the DNA replication mechanism by alkylating DNA strands and hence affect cell division and cause cytotoxicity and tissue injury which could lead to multiple organ damage (Oboh and Rocha, 2007). The haematopoiesis process of the patients having cyclophosphamide chemotherapy usually suffered. This is akin to our observation from this study that the cyclophosphamide control group had low WBC count. However, there was a significant (p<0.01) increase in the WBC count of animals treated with *P. viscida*. Similarly, there was a recovery in bone marrow cellularity as well in comparison to control group which showed a drastic depletion (Ballas, 1986).

Reduced level of endogenous antioxidants of both liver and kidney tissues in control group indicates the oxidative stress exerted by cyclophosphamide metabolism. However, there was a marked increase in the level of tissue SOD in both liver as well as kidney samples from *P. viscida* treated animals. It is to be noted that tissue GSH levels had found a significant elevation in both organs when administered with *P. viscida* and this could be attributed to the free radical scavenging activity of *P. viscida* (Lopez and Luderer, 2004). Reduced glutathione is one of the major providers of thiol groups in the cellular environment. It has a direct role as a free radical scavenger and also acts as an essential factor for glutathione peroxidase (Rahman and Mac Nee, 2000). GSH not only scavenge reactive oxygen species like superoxides but also help in rejuvenating other antioxidant. As there is increased level of antioxidant protection in such circumstances, the chances for lipid peroxidation becomes less. Thus, there was a reduced level of MDA in *P. viscida* treated animals compared to cyclophosphamide alone treated control
group (Oboh and Rocha, 2007). GPx play a major role in neutralizing hydrogen peroxide and thereby reducing chances for further extension of free radical chain reaction (Brigelius-Flohe and Maiorino, 2013). Increase in GPx level of *P. viscida* treated animals showed reduction in oxidative stress in significant manner as well as normalization of physiological functioning of liver and kidney. Epithelial lining in the intestine is highly susceptible to chemo toxicity induced by cyclophosphamide. However, marked increase in the level of intestinal GSH suggests the protective effect of *P. viscida* treatment. Treatment of cyclophosphamide is marked by a reduction in the body weight (Jang *et al.*, 2013). Nevertheless, animals treated with *P. viscida* showed recovery in their body weight towards normalization in a dose dependent manner.

Increased presence of GOT, GPT, ALP, bilirubin, urea and creatinine in serum samples of the cyclophosphamide control group indicated the extent of tissue damage in kidney and liver tissues and which affect the normal metabolic functioning of the above said organs. However protection against oxidative stress exerted by *P. viscida* might be considered as the reason for reduced levels of tissue damage markers in serum, in extract treated animals (Senthilkumar *et al.*, 2006).

From this study it is evident that *P. viscida* is effective in ameliorating the toxic effect of cyclophosphamide. *P. viscida* helped in rejuvenating innate antioxidants such as reduced glutathione. Moreover, there was a significant reduction in the rate of lipid peroxidation in *P. viscida* treated animals. There was a decrease in the tissue damage level as well when treated with *P. viscida*. As an antioxidant *P. viscida* could reduce the impact of cyclophosphamide induced oxidative stress. Further studies could be recommended for the effective utilization of *P. viscida* as a chemoprotective agent against cyclophosphamide.
Table 7.1  Effect of *P. viscida* extract on serum parameters in serum GOT, GPT, ALP and bilirubin against cyclophosphamide induced toxicity

<table>
<thead>
<tr>
<th>Group</th>
<th>SGOT (IU/L)</th>
<th>SGPT (IU/L)</th>
<th>ALP (IU/L)</th>
<th>Bilirubin (mg/dl)</th>
<th>Creatinine (mg/dL)</th>
<th>Urea (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>122.2 ± 15.99</td>
<td>63.97 ± 8.95</td>
<td>48.15 ± 2.03</td>
<td>0.34 ± 0.02</td>
<td>0.58 ± 0.01</td>
<td>35 ± 1.02</td>
</tr>
<tr>
<td>Cyclophosphamide Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. viscida</em> 100 mg/kg</td>
<td>213.54 ± 18.82a</td>
<td>107.16 ±19.10a</td>
<td>82.16 ±2.25a</td>
<td>0.82 ± 0.09a</td>
<td>0.632 ± 0.05b</td>
<td>56.22 ± 4.18a</td>
</tr>
<tr>
<td><em>P. viscida</em> 250 mg/kg</td>
<td>179.4 ± 4.62c</td>
<td>57.33 ± 4.43c</td>
<td>70.23 ± 3.78d</td>
<td>0.69 ± 0.05</td>
<td>0.625 ± 0.06</td>
<td>36.75 ± 3.16c</td>
</tr>
</tbody>
</table>

Values are Mean ± SD; for six animals in each group; (a)p<0.01, (b)p<0.05 as compared to normal. (c)p<0.01, (d)p<0.05 as compared to control.
### Table 7.2 Effect of *P. viscida* extract on SOD, GPx, GSH and MDA levels in liver tissues against cyclophosphamide induced toxicity

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mg protein)</th>
<th>GPx (U/mg protein)</th>
<th>GSH (nmol/mg protein)</th>
<th>MDA (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.52± 0.14</td>
<td>7.07 ± 0.37</td>
<td>21.12 ± 0.73</td>
<td>0.94 ± 0.06</td>
</tr>
<tr>
<td>Control</td>
<td>1.11± 0.20</td>
<td>3.24 ± 0.42</td>
<td>14.05 ± 0.62</td>
<td>2.02 ± 0.08</td>
</tr>
<tr>
<td><em>P. viscida 100mg/kg</em></td>
<td>1.81± 0.21</td>
<td>5.14 ± 0.62</td>
<td>17.43 ± 0.83</td>
<td>1.51 ± 0.04</td>
</tr>
<tr>
<td><em>P. viscida 250mg/kg</em></td>
<td>2.04 ± 0.12</td>
<td>6.08 ± 0.33</td>
<td>19.74 ± 0.59</td>
<td>1.20 ± 0.06</td>
</tr>
</tbody>
</table>

Values are Mean ± SD; for ten animals in each group; (a)p<0.01,(b)p<0.05 as compared to normal. (c)p<0.01,(d)p<0.05 as compared to control.

### Table 7.3 Effect of *P. viscida* extract on SOD, GPx, GSH and MDA levels in kidney tissues against cyclophosphamide induced toxicity

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mg protein)</th>
<th>GPx (U/mg protein)</th>
<th>GSH (nmol/mg protein)</th>
<th>MDA (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.98 ± 0.03</td>
<td>5.63 ± 0.95</td>
<td>4.83 ± 0.95</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>Control</td>
<td>1.42 ± 0.08</td>
<td>1.21 ± 0.68</td>
<td>1.54 ± 1.36</td>
<td>1.86 ± 0.02</td>
</tr>
<tr>
<td><em>P. viscida 100mg/kg</em></td>
<td>1.68 ± 0.18</td>
<td>3.65 ± 0.12</td>
<td>3.21 ± 1.21</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td><em>P. viscida 250mg/kg</em></td>
<td>1.93 ± 0.04</td>
<td>5.14 ± 0.28</td>
<td>4.27 ± 2.41</td>
<td>0.82 ± 0.02</td>
</tr>
</tbody>
</table>

Values are Mean ± SD; for ten animals in each group; (a) p<0.01,(b) p<0.05 as compared to normal. (c) p<0.01,(d) p<0.05 as compared to control.
Fig. 7.1 Effect of *P. viscida* extract on bone marrow cellularity against cyclophosphamamide induced toxicity

Values are Mean ± SD; for ten animals in each group;(a)p<0.01,(b)p<0.05 as compared to normal. (c)p<0.01,(d)p<0.05 as compared to control

Fig. 7.2 Effect of *P. viscida* extract on GSH level in intestinal mucosa against cyclophosphamamide induced toxicity

Values are Mean ± SD; for ten animals in each group;(a)p<0.01,(b)p<0.05 as compared to normal. (c)p<0.01,(d)p<0.05 as compared to control
Fig. 7.3  Effect of *P. viscida* extract on body weight of cyclophosphamide treated mice

Values are Mean ± SD; for ten animals in each group

Fig. 7.4  Effect of *P. viscida* extract on total WBC count in mice against cyclophosphamide induced toxicity

Values are Mean ± SD; for ten animals in each group