Hepatoprotective Effect of Pongamia pinnata Leaves in Ammonium Chloride Induced Hyperammonemiac Rats

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Abstract: Effect of Pongamia pinnata (an indigenous plant used in Ayurvedic Medicine in India) leaf extract (PPEt) on the levels of circulatory ammonia, urea, lipid peroxidation products such as TBARS (thio barbituric acid reactive substances), HP (hydroperoxides) and liver markers such as bilirubin, AST (aspartate transaminase), ALT (alanine transaminase), ALP (alkaline phosphatase), LDH (Lactate dehydrogenase), Gamma glutamyl-S-transferase (GGT) were studied for its hepatoprotective effect during ammonium chloride induced hyperammonemia. Ammonium chloride treated rats showed a significant increase in the levels of circulatory ammonia, urea, bilirubin, AST, ALT, ALP, LDH, GGT, TBARS and HP. These changes were significantly decreased in PPEt and ammonium chloride treated rats. Our in vitro studies have shown that PPEt effectively scavenge reactive oxygen species including superoxide anion, hydroxyl and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Our results indicate that PPEt offers hepatoprotection by influencing the levels of lipid peroxidation products and liver markers in experimental hyperammonemia and this could be due to its ability to detoxify excess ammonia, urea and creatinine and free radical scavenging property (both in vitro and in vivo) by means of reducing lipid peroxidation and the presence of natural antioxidants.

Keywords: Hyperammonemia, Pongamia pinnata, liver markers, DPPH radical

INTRODUCTION

Hyperammonemia is a major contributing factor to neurological abnormalities observed in hepatic encephalopathy and in congenital defects of ammonia detoxication (Rodrigo et al., 2004). Ammonia is a neurotoxin that has been strongly implicated in the pathogenesis of hepatic encephalopathy (Norenberg et al., 2004). Ammonia has also been a major pathogenetic factor associated with inborn errors of urea cycle, Reye’s syndrome, organic acidurias and disorders of fatty acid oxidation (Qureshi and Rama Rao, 1997). It was reported that elevated levels of ammonia causing irritability, somnolence, vomiting, seizures, derangement of cerebral function, coma and death (Murthy et al., 2001; Rodrigo et al., 2004). Ammonia toxicity results in lipid peroxidation and free radical generation, which cause hepatic dysfunction and failure and significantly increase number of brain peripheral benzodiazepine receptors and could increase the affinity of ligands for these receptors that might enhance GABA (gamma amino butyric acid) adrenergic neurotransmission. These changes probably contribute to deterioration of intellectual function, decreased consciousness, coma and death (Kosemko et al., 1997; Lena and Subramanian, 2004).

In recent years of scientific investigations, attention has been drawn to the health promoting activity of plant foods and its active components. Pongamia pinnata (Linn.) Pierre is a medium sized
glabrous tree popularly known as Karanja in Hindi, Indian Beech in English and Pungai in Tamil. Different parts of this plant have been used in Ayurvedic medicine for bronchitis, whooping cough, rheumatic joints and quench thirst in diabetes (Punitha and Manoharan, 2006). Most of the Tamil Nadu physicians of Indian system of traditional medicine Ayurveda and Siddha use *P. pinnata* to treat various kinds of diseases including leucoderma, leprosy, lambsco, muscular, articular rheumatism (Shirwaikar et al., 2003) and diabetes mellitus (Punitha et al., 2006). The leaves are anthelmintic and cure piles, wounds and other inflammations (Punitha et al., 2006). A hot infusion of leaves is used as a medicated bath for relieving rheumatic pains and for cleaning ulcers in gonorrhea and scrofulous enlargement (Srinivasan et al., 2001). Different extracts of roots and seeds of *P. pinnata* have been reported to have anti-inflammatory and antitumor activities (Srinivasan et al., 2001). To our best knowledge no scientific data regarding the hepatoprotective effect during hyperammonemia of *P. pinnata* leaves are available except in the treatise of Ayurvedic medicine. Thus, the present study was undertaken to evaluate the *in vitro* antioxidant properties and the hepatoprotective effect of ethanolic extract of *P. pinnata* leaves in ammonium chloride induced experimental hyperammonemnic rats.

**MATERIALS AND METHODS**

**Animals**

Adult male albino Wistar rats, weighing 180-200 g bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, were used. The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, temperature of 22±2°C and humidity of 45-64%. Animals were fed with a standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. All animal experiments were approved by the ethical committee, Annamalai University and were in accordance with the guidelines of the National Institute of Nutrition (NIN), Indian Council of Medical Research (ICMR), Hyderabad, India and this study was conducted September to October 2006.

**Plant Material and Preparation of Extract (PPEt)**

The mature green leaves of *P. pinnata* were collected from Chidambaram, Cuddalore District, Tamil Nadu, India. The plant was identified and authenticated at the Herbarium of Botany Directorate in Annamalai University. A voucher specimen (No. 3670) was deposited in the Botany Department of Annamalai University. The shade-dried and powdered leaves of *P. pinnata* were subjected to extraction with 70% ethanol under reflux for 8 h and concentrated to a semi-solid mass under reduced pressure (Rotavapor apparatus, Buchi Labortechnik AG, Switzerland). The yield was about 24% (w/w) of the starting crude material. In the preliminary phytochemical screening, the ethanolic extract of PPEt gave positive tests for glycosides, sterols, tannins and flavonoids (Trease and Evans, 1959). The residual extract was dissolved in sterile water and used in the investigation. Ammonium chloride was purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used in the study were of analytical grade. Hyperammonemia will be induced in Wistar rats by daily intraperitoneal injections of ammonium chloride at a dose of 100 mg kg⁻¹ body weight for 8 weeks (Essa et al., 2005).

**Experimental Design**

In this experiment, a total of 24 rats were used. The rats were divided into 4 groups of 6 rats each. Group 1 rats were normal untreated. Group 2 were normal rats treated with PPEt orally (300 mg kg⁻¹ body weight) (Srinivasan et al., 2001). Group 3 rats were treated with ammonium chloride intraperitoneally (100 mg kg⁻¹ body weight) (Essa et al., 2005). Group 4 were rats treated with ammonium chloride + PPEt. At the end of 8 weeks, all the rats were killed by decapitation after giving (Pentobarbithone sodium) anesthesia (60 mg kg⁻¹). Blood samples were collected for various biochemical estimations.
Blood ammonia levels were estimated by the method of Wolheim (1984). To 20 µL of the blood, 200 µL of triethanolamine buffered substrate and 150 µL of NADPH/GLDH/buffered substrate were added, mixed well and the absorbance was read at 470 nm. Plasma urea levels were determined by Diacetyl monoxime method (Varley et al., 1998). To 0.1 mL of plasma, 3.3 mL of water was added and mixed. Then 0.3 mL of 10% sodium tungstate and 0.3 mL of 0.67 N sulphuric acid were added, mixed and centrifuged. To 2.0 mL of the supernatant, 2 mL of water, 0.4 mL diacetyl monoxime and 1.6 mL of sulphuric acid-phosphoric acid mixture were added and heated in a boiling water bath for 30 min and cooled and the absorbance was read at 480 nm.

Lipid peroxidation in blood was estimated colorimetrically by TBARS and HP by the method of Niehaus and Samuelson (1968), Jiang et al. (1992), respectively. In brief, 0.1 mL of plasma was treated with 2 mL of (1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. 0.1 mL of plasma was treated with 0.9 mL of Fox reagent (88 mg butylated hydroxytoluene (BHT), 7.6 mg xylene orange and 9.8 mg ammonium ion sulphate were added to 90 mL of methanol and 10 mL 250 mM sulphuric acid) and incubated at 37°C for 30 min. The color developed was read at 500 nm colorimetrically.

Serum bilirubin (Malloy and Evelyn, 1937) and the activities of serum aspartate transaminase (AST, EC 2.6.1.1), alanine transaminase (ALT, EC 2.6.1.2), alkaline phosphatase (ALP, EC 3.1.3.1) and lactate dehydrogenase (LDH, EC 1.1.1.27) were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Sigma Diagnostics (I) Pvt. Ltd., Baroda, India). Gamma glutamyl transferase (GGT, EC 2.3.2.2) activity was determined by the method of (Rosalki et al., 1970) using γ-glutamyl-p-nitroanilide as substrate. The plant extract was also studied for its in vitro anti radical activity in terms of hydroxyl radical (Halliwell et al., 1987), DPPH radical (Braca et al., 2001) and superoxide anion radical (Nishikimi et al., 1972) scavenging assays. BHT (Butylated hydroxy toluene) was used as a positive control for comparing the effect of PPEt on superoxide anion scavenging activity.

**Statistical Analysis**

Statistical analysis was carried out by analysis of variance (ANOVA) and the groups were compared using Duncan's Multiple Range Test (DMRT).

**RESULTS**

Table 1 shows the levels of circulatory ammonia, urea and bilirubin of control and experimental animals. Circulatory ammonia, urea and bilirubin levels increased significantly and the levels reduced significantly in ammonium chloride and PPEt treated rats. Normal rats treated with PPEt showed no significant differences in levels of ammonia, urea and bilirubin when compared with control rats (Table 1).

The levels of circulatory TBARS, HP, AST, ALT, ALP, LDH and GGT increased significantly in ammonium chloride treated rats (Table 2). Ammonium chloride and PPEt-treated rats showed significantly low levels of circulatory TBARS, HP, AST, ALT, ALP, LDH and GGT when compared

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood ammonia (µmol L⁻¹)</th>
<th>Urea (mg dL⁻¹)</th>
<th>Bilirubin (mg dL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>88.2±6.7a</td>
<td>10.9±0.8b</td>
<td>0.40±0.03b</td>
</tr>
<tr>
<td>Normal+PPEt (300 mg kg⁻¹)</td>
<td>83.9±6.3b</td>
<td>11.5±0.88b</td>
<td>0.42±0.03b</td>
</tr>
<tr>
<td>AC (100 mg kg⁻¹)</td>
<td>331.2±25.22c</td>
<td>21.8±1.66b</td>
<td>0.97±0.07b</td>
</tr>
<tr>
<td>AC+PPEt (300 mg kg⁻¹)</td>
<td>139.7±16.7b</td>
<td>13.08±1.09b</td>
<td>0.53±0.04b</td>
</tr>
</tbody>
</table>

ANOVA followed by Duncan's multiple range test. Values not sharing a common superscript (a, b, c) differ significantly at p<0.05. AC: Ammonium Chloride

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Table 2: Effect of PPEI on changes in the levels of circulatory liver markers and TBARS, HP of normal and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (IU L⁻¹)</th>
<th>ALP (IU L⁻¹)</th>
<th>ALT (IU L⁻¹)</th>
<th>GGT (IU L⁻¹)</th>
<th>LDH (IU L⁻¹)</th>
<th>TBARS (nmol mL⁻¹)</th>
<th>HP (±10⁻⁵ nmol dL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>71.5±0.54</td>
<td>85.5±1.53</td>
<td>51.1±2.31</td>
<td>0.7±0.05</td>
<td>10.7±0.19</td>
<td>1.5±0.09</td>
<td>8.4±0.78</td>
</tr>
<tr>
<td>Normal+PPEI (300 mg kg⁻¹)</td>
<td>70.0±2.33</td>
<td>74.3±0.60</td>
<td>23.0±1.75</td>
<td>0.6±0.05</td>
<td>10.9±0.36</td>
<td>1.5±0.02</td>
<td>8.19±0.54</td>
</tr>
<tr>
<td>AC (100 mg kg⁻¹)</td>
<td>116.3±8.86</td>
<td>138.7±12.92</td>
<td>61.3±4.24</td>
<td>0.9±0.07</td>
<td>156.2±11.9</td>
<td>3.0±0.23</td>
<td>14.4±1.29</td>
</tr>
<tr>
<td>AC+PPEI (300 mg kg⁻¹)</td>
<td>80.9±6.17</td>
<td>81.7±6.72</td>
<td>28.7±2.19</td>
<td>0.8±0.06</td>
<td>123.0±9.37</td>
<td>1.8±0.13</td>
<td>9.70±0.55</td>
</tr>
</tbody>
</table>

ANOVA followed by Dunn's multiple range test, Values not sharing a common superscript (a, b, c) differ significantly at p≤0.05. AC: Ammonium Chloride

Table 3: Hydroxyl, DPPH radical and superoxide anion scavenging activity of PPEI (in vitro) at various concentrations

<table>
<thead>
<tr>
<th>Concentration of PPEI (µg mL⁻¹)</th>
<th>Scavenging of hydroxyl radical (%) by PPEI</th>
<th>Scavenging of superoxide anion (%) by PPEI</th>
<th>Scavenging of superoxide anion (%) by BHT</th>
<th>Scavenging of DPPH radical (%) by PPEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>9.9±0.19</td>
<td>8.6±0.09</td>
<td>21.9±0.10</td>
<td>18.2±0.04</td>
</tr>
<tr>
<td>100</td>
<td>19.0±0.24</td>
<td>18.3±0.14</td>
<td>38.9±0.13</td>
<td>37.4±0.07</td>
</tr>
<tr>
<td>150</td>
<td>40.9±0.26</td>
<td>33.3±0.19</td>
<td>51.3±0.09</td>
<td>59.1±0.06</td>
</tr>
<tr>
<td>200</td>
<td>51.0±0.28</td>
<td>43.1±0.22</td>
<td>62.4±0.18</td>
<td>70.0±0.21</td>
</tr>
<tr>
<td>250</td>
<td>74.3±0.34</td>
<td>77.0±0.12</td>
<td>86.7±0.22</td>
<td>87.6±0.20</td>
</tr>
</tbody>
</table>

The data were means±SD from three repeats from independent experiment for each group.

with the corresponding ammonium chloride group. Normal rats treated with PPEI alone showed no significant differences in levels of TBARS, HP, AST, ALT, ALP, LDH and GGT when compared with control rats (Table 2).

The extract exhibited hydroxyl, DPPH radicals and superoxide anion scavenging at various concentrations. About 74.36, 87.69 and 77.01% of hydroxyl radical, DPPH radical and superoxide anion scavenging activities were observed in the study (Table 3). BHT was used as a positive control for superoxide anion scavenging activity and the effect of PPEI in scavenging was similar to that of BHT.

**DISCUSSION**

Ammonia is removed either in the form of urea in perportal hepatocytes and/or as glutamine in perivenous hepatocytes in liver (Nelson and Cox, 2000). An increased level of circulatory ammonia might indicates a hyperammonemic condition in the rats treated with ammonium chloride (Lena and Subramaniam, 2003, 2004; Essa et al., 2005; Essa and Subramaniam, 2006). Decreased levels of blood ammonia, urea in PPEI and ammonium chloride treated rats show the significant anti-hyperammonemic activity of this plant and it was reported that *P. pinvata* normalized the levels of ammonia, urea and creatinine during hyperammonemic and nephrotoxic conditions (Shirwaikar et al., 2003; Essa and Subramaniam, 2006). Present findings have an in agreement with these reports and the exact mechanism remains to be explored.

In several organs, cell damage is followed by release of a number of cytoplasmic enzymes to the blood, a phenomenon that provides the basis for clinical diagnosis (Sundberg et al., 1994). In present study, the increased activities of AST, ALT, ALP and LDH in serum obviously indicate that liver is susceptible to ammonium chloride induced toxicity and also this may be due to the liver damage caused by ammonia-induced free radical generation. Serum GGT has been widely used as an index of liver dysfunction. Recent studies indicating that serum GGT might be useful in studying oxidative stress-related issues. The products of the GGT reaction may themselves lead to increased free radical production (Lee et al., 2004). In group III rats, the significant elevation of serum GGT might be related
to increased oxidative stress developed during experimental hyperammonemia. Another sensitive indicator of liver damage is total bilirubin, which also increased in group III rats and the increase of total bilirubin is correlated with the oxidative damage of other organs resulting from oxidative stress. PPEt may stabilize the hepatic cellular membrane and protect the hepatocytes against toxic effects of ammonium chloride, which may decrease the leakage of the enzymes into blood stream.

Present study shows that PPEt effectively scavenge reactive oxygen species including superoxide anion and hydroxyl and DPPH radicals under in vitro conditions at various concentrations and this effect corroborate with previous findings (Shirwaikar et al., 2003; Prabha et al., 2003; Punitha et al., 2006; Punitha and Manoharan, 2006). Addition of PPEt scavenges hydroxyl radical by competing with deoxyribose for hydroxyl radical and diminishes chromogen formation at a defined time of reaction. This scavenging ability of PPEt might be due to the active hydrogen donating ability, which can neutralize the hydroxyl radical generation.

Evidences point out that the oxidative stress and free radical production could be involved in the mechanism of ammonia toxicity (Kosanko et al., 1997; Lera and Subramanian, 2004). The observed increase in the level of plasma TBARS and HP in hyperammonemic conditions is generally thought to be the consequence of increased production and liberation of tissue lipid peroxides into circulation due to the pathological changes in tissues (Kosanko et al., 1997; Essa and Subramanian, 2006). Reports have shown that excess ammonia induces nitric oxide synthase, which leads to enhanced production of nitric oxide, leading to oxidative stress and liver damage (Kosanko et al., 1997; Schliess et al., 2002) and it was reported that increased lipid peroxide concentration was found in blood during hyperammonemic conditions (Essa and Subramanian, 2006).

PPEt administration significantly decreased the lipid peroxidation, which may be resulting from the scavenging of free radicals generated during hyperammonemia (Essa and Subramanian, 2006). Previous reports have shown that PPEt is an effective free radical scavenger (Prabha et al., 2003; Punitha et al., 2006; Punitha and Manoharan, 2006). It was reported that in vitro studies of the alcoholic extract of P. pinnata flowers revealed marked nitric oxide scavenging activity, suggesting potent antioxidant property (Shirwaikar et al., 2003). This may be due to presence of a number of bioflavonoids and phenolic phytochemicals (such as kaempferol, quercetin, karanjin, kanjone, etc., (Satyavati et al., 1987) in PPEt which offers possible role in offering hepatoprotection and reducing the oxidative stress caused by ammonium chloride by means of reducing the lipid peroxidation products and liver markers. It was reported that the various extracts of P. pinnata tend to reverse the change in lipid peroxidation activity, indicating decreased lipid peroxidation and damage to cells suggesting potent antioxidant property (Prabha et al., 2003; Punitha et al., 2006).

In conclusion, the results suggest that PPEt reverts the hepatotoxicity influenced by ammonium chloride induced hyperammonemia and decreased liver markers, lipid peroxidation by its free radical scavenging (both in vivo and in vitro) and antioxidant activities. This influence of PPEt might be considered as reflective of their protective activity against hyperammonemia. Hence, PPEt might play a beneficial role to reduce the toxic effects developed during ammonium chloride induced hyperammonemia.

REFERENCES


