Ameliorative Effect of Ficus hispida Linn. Leaf Extract on Cyclophosphamide-Induced Oxidative Hepatic Injury in Rats

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Abstract: The current study was designed to scrutinize the putative hepatoprotective potential of the methanolic leaf extract of Ficus hispida Linn. (FH) (400 mg kg⁻¹ body weight) on cyclophosphamide (CP) elicited oxidative injury in rat liver. CP administration (150 mg kg⁻¹ body weight, i.p., twice, in 2 consecutive days) caused liver injury, featuring substantial increase in serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase (GGT) and bilirubin levels. In contrast, treatment with FH significantly precluded all these alterations. CP intoxicated rats depicted a remarkable oxidative stress, as evidenced by a significant elevation in lipid peroxidation (LPO) with a concomitant decrease in the GSH activity. These changes were coupled with a marked decline in the activities of enzymic antioxidants [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR)] in the liver tissue of CP-administered rats. FH treated rats displayed a significant inhibition of lipid peroxidation (LPO) and augmentation of endogenous antioxidants. Taken together, these findings emphasize the hepatoprotective effect of F. hispida leaf extract against CP-induced oxidative liver injury. Hence, F. hispida might serve as a promising medicinal herb in complementary chemotherapeutic modalities.

Keywords: Ficus hispida, cyclophosphamide, oxidative stress, hepatotoxicity, antioxidants

INTRODUCTION

The most imperative obstacle in cancer chemotherapy is the non-specific cytotoxic action on both tumor cells and normal healthy cells (Hui et al., 2006). Cyclophosphamide (CP), an oxazaphosphorine-alkylating agent, is extensively used as an antineoplastic drug in chemotherapeutic regimens of lymphoproliferative disorders, certain solid tumors, and as an immunosuppressant in the treatment of autoimmune diseases such as nephrotic syndrome, systemic lupus erythematosus and rheumatoid arthritis (Morais et al., 1999). In addition, CP is of paramount importance as an immunosuppressive agent in organ and bone marrow transplant regimens (Demirel et al., 1996; Zincke and Woods, 1977). Use of CP as an effective chemotherapeutic agent is often restricted because of its widespread adverse side effects and toxicity (DeSouza et al., 2000). An ample literature implicate that elevated therapeutic dose of cyclophosphamide, could cause liver disorders (Bacon and Rosenberg, 1982; Shulman et al., 1980; Snover et al., 1989).

The prime factor for therapeutic and toxic effects of cyclophosphamide is the requirement of biotransformation by hepatic microsomal cytochrome P450 mixed functional oxidase system.
Lindley et al., 2002; Smith and Kehr, 1991). Metabolic activation through the predominant pathway (4-hydroxylation), yields 4-hydroxycyclophosphamide (HCP) that exists in equilibrium with aldophosphamide, which degrades by β-elimination to form the DNA cross-linking agent, Phosphoramidate Mustard (PM) and an equimolar amount of the toxic byproduct, acrolein (Lindley et al., 2002; Pass et al., 2005). PM brings about interstrand cross-links between opposite DNA strands and hampers the replication and transcription processes that characterise the clinical activity of CP (Dong et al., 1995, Paolo et al., 2004). Hence, the therapeutic effect of cyclophosphamide is attributed to PM, while acrolein is associated with unwanted side effects (Colvin, 1999). Bioconversion of CP to these metabolites leads to the formation of high levels of Reactive Oxygen Species (ROS), which result in decreased antioxidative capacity (Stankiewicz et al., 2002). It is well known that excessive production of ROS could eliminate in oxidative stress (Scherz-Shouval and Elazar, 2007). Mounting evidences suggest that oxidative stress play a predominant aetiological role in cyclophosphamide induced hepatotoxicity (Manda and Bhatia, 2003; Selvakumar et al., 2005; Stankiewicz et al., 2002).

Cytoprotectants like amifostine, mesna and dexrazoxane were used to manage the toxic effects of cancer chemotherapy, but these agents are not approved for wide clinical use due to lack of efficacy, gastrointestinal side effects, hypotension, hypersensitivity reactions, anxiety, urinary retention and myelosuppression (Adamson et al., 1995; Hensley et al., 1999; Reinhold-Keller et al., 1992). Limitations to such conventional treatment have spurred the development of new treatment modalities.

Plants are arguably poised for a comeback as sources of human health products, mainly due to their enormous propensity to synthesize complex mixtures of structurally diverse compounds, which could provide a safer and more holistic approach to disease treatment and prevention (Raskin et al., 2002). Plant extracts and natural compounds have also shown protective effect on CP-induced toxicity (Haque et al., 2001, 2003; Kumar and Kuttan, 2005; Sharma et al., 2000; Sudharsan et al., 2005).

*Ficus hispida* Linn., is a shrub or moderate sized tree of the Mulberry family (Moraceae), commonly known as Peyatti (Tamil), Bhramhamedi (Telugu), Gobla (Hindi) and Dumoor (Bengali). It is found in damp localities of many parts of India and flowers and fruits practically throughout the year. Almost all parts of *Ficus hispida* are used in Indian folklore medicine for the treatment of various ailments like leucoderma, skin diseases, jaundice and as anti-poisonous. Previous reports reveal that *F. hispida* leaves contain oleancic acid, bergapten, β-amyrin, β-sitosterol (Khan et al., 1991), hispidin (Huong and Trang, 2006) and phentanthroindolizidine alkaloids (Penaza-Sánchez et al., 2002). Interestingly, Mandal et al. (2000) demonstrated the protective effect of *F. hispida* leaf extract on paracetamol-induced hepatotoxicity. In this view, we conjectured that treatment with methanolic extract of *F. hispida* leaves might confer protection against cyclophosphamide-induced oxidative hepatic injury.

**MATERIALS AND METHODS**

**Drugs and Chemicals**

Cyclophosphamide (Lodoxan \( ^{\circledR} \)) was purchased from Dabur Pharma Limited, New Delhi, India. All other chemicals and solvents used were of the highest purity and analytical grade.

**Plant Material**

The leaves of *F. hispida* Linn. (Moraceae) were collected during the month of February 2007 from the herbal garden of Anna Siddha Hospital and Research Centre, Chennai, India. A voucher specimen (PARC/2007/Vel`s/28) was deposited in the Plant Anatomy Research Centre, Pharmacognosy Institute, Chennai, India and was authenticated by Dr. Jyanuman. The leaves were collected and dried under shade and pulverized in a mechanical grinder and stored in a closed container for further use.
Preparation of Extract

The powdered leaves were defatted with petroleum ether (BP 60-80°C) and then extracted with methanol in a Soxhlet extractor. On evaporation of methanol from the methanol extract \textit{in vacuo}, a greenish colored residue was obtained (yield 4.7% (w/w) with respect to the dry starting material) and was stored in a desiccator.

Phytochemical Screening

On preliminary screening, the methanol extract showed positive reaction for triterpenoids (Noller \textit{et al.}, 1942), Shinde test for flavonoids (Markham, 1982), steroids (Liebermann, 1885), tannins, saponins and alkaloids (Kokate, 1988).

Animal Model

The study was conducted on male Wistar rats (140±10 g). Animals were obtained from the Animal House, Vel’s College of Pharmacy, The Tamilnadu Dr. M.G.R. Medical University, Chennai, India. Animals were fed with commercially available standard rat pelleted feed (M/s Pranav Agro Industries Ltd., India) under the trade name Amrut rat/nice feed and water was provided \textit{ad libitum}. The animals were deprived of food for 24 h before experimentation but allowed free access to tap water. The rats were housed under conditions of controlled temperature (25±2°C) and were acclimatized to 12 h light, 12 h dark cycles. Experimental animals were used after obtaining prior permission and handled according to the University and institutional legislation as regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Experimental Protocol

The experimental animals were randomized into four groups of six rats each as follows:

**Group 1**: Control rats received normal saline (1 mL kg\(^{-1}\) body weight), orally for 10 days.

**Group 2**: Rats received CP dissolved in saline, intraperitoneally in a dose of 150 mg kg\(^{-1}\) body weight, twice, in 2 consecutive days (i.e., in the first 2 days of the experimental period).

**Group 3**: Rats received FH extract by oral gavage (400 mg kg\(^{-1}\) body weight for 10 days).

**Group 4**: Rats were administered CP as in Group 2, immediately followed by supplementation with FH extract (400 mg kg\(^{-1}\) body weight) by oral gavage for 10 consecutive days.

After the 10 days experimental period (i.e., on the 11th day), all the animals were anesthetized and decapitated. The liver tissue was immediately excised and rinsed in ice cold physiological saline and then homogenized in 0.01 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the assays. Blood was collected and serum was separated for analysis of biochemical parameters.

Enzymatic Indices of Cellular Damage

Aspartate transaminase (AST), alanine transaminase (ALT), Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were estimated by the method of King (1965a, b, c). Gamma-glutamyl transpeptidase (GGT) was measured by the method of Szasz (1969). Bilirubin was estimated by the Malloy and Evelyn method (1937). Protein content was estimated by the method of Lowry \textit{et al.} (1951).

Lipid Peroxidation

Tissue lipid peroxide level was determined as MDA (Ohkawa \textit{et al.}, 1979). The absorbance was measured photometrically at 532 nm and the concentrations were expressed as \(\mu\)mol malonaldehyde (MDA) min\(^{-1}\) mg\(^{-1}\) protein.
Assay of Antioxidants

SOD was assayed by the method of Misra and Fridovich (1972). Catalase (CAT) level was estimated by the method described by Sinha (1972). Glutathione peroxidase (GPx) was assayed by the method of Rotruck et al. (1973). Glutathione-S-transferase (GST) was assayed by the method of Habig et al. (1974). Glutathione Reductase (GR) was assayed by the method of Staal et al. (1969). Total reduced glutathione (GSH) was determined by the method of Ellman (1959).

Statistical Analysis

The results were expressed as mean±Standard Deviation (SD) for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS 13.0 software package for Windows. Post hoc testing was performed for inter-group comparisons using the Least Significance Difference (LSD) test. p-values<0.05 have been considered as statistically significant.

RESULTS

In the present study, cyclophosphamide administration induced severe biochemical changes as well as oxidative damage in liver. There was a significant (p<0.05) rise in the levels of diagnostic marker enzymes (AST, ALT, ALP, LDH and GGT) and bilirubin in the serum of Group 2 CP intoxicated rats as compared to that of Group 1 control rats (Table 1). The administration of Ficus hispida leaf extract to Group 4 animals restored these enzyme as well as bilirubin levels to near normalcy (p<0.05) as compared to those Group 2 CP-injected rats. In F. hispida alone administered rats (Group 3) versus controls, no significant changes were observed.

Injection of CP induced a significant (p<0.05) increase in the level of lipid peroxidation (LPO), which was paralleled by significant (p<0.05) reduction in the level of GSH (Table 3) in the liver tissue of Group 2 animals as compared to normal controls. Glutathione plays an important role in the regulation of variety of cell functions and in cell protection from oxidative injury. In this study, the treatment with F. hispida (Group 4) significantly (p<0.05) counteracted the CP-induced lipid peroxidation and restored the hepatic GSH level to near normal level in Group 4 rats as compared to that of Group 2 animals.

Activities of glutathione-dependent antioxidant enzymes (GPx, GST and GR) and anti-peroxidative enzymes (SOD and CAT) were significantly (p<0.05) lower in the liver tissue of Group 2 CP-injected rats as compared to that of Group 1 normal control rats (Table 2). The observed reduction in the activities of GPx, GR and GST in CP-induced liver damage might be due to decreased availability of its substrate, reduced glutathione (GSH). In the present study, the treatment of Group 4 rats with F. hispida, significantly (p<0.05) reversed all these CP-induced alterations in the activities of antioxidant enzymes (SOD, CAT, GPx, GST and GR) to a near normal status. The normal rats receiving F. hispida alone (Group 3) did not show any significant change when compared with control rats, indicating that it does not per se have any adverse effects.

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
<th>LDH (IU/L)</th>
<th>GGT (IU/L)</th>
<th>Bilirubin (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>58.3±1.84</td>
<td>41.2±1.29</td>
<td>82.1±3.31</td>
<td>116.5±5.65</td>
<td>0.62±0.02</td>
<td>0.74±0.05</td>
</tr>
<tr>
<td>Group 2 (CP)</td>
<td>225.1±4.06</td>
<td>97.1±6.74</td>
<td>185.3±12.11</td>
<td>178.8±9.33</td>
<td>1.35±0.07*</td>
<td>4.08±0.31**</td>
</tr>
<tr>
<td>Group 3 (FH)</td>
<td>60.0±1.77**</td>
<td>39.7±1.58*</td>
<td>79.5±3.08**</td>
<td>122.0±5.31**</td>
<td>0.66±0.02**</td>
<td>0.81±0.04**</td>
</tr>
<tr>
<td>Group 4 (FH + CP)</td>
<td>67.2±4.78*</td>
<td>45.7±3.25*</td>
<td>90.3±6.65*</td>
<td>130.6±7.5**</td>
<td>0.68±0.03**</td>
<td>1.42±0.07**</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD for 6 rats. Comparisons are made between Group 1 and Group 2. *Statistically significant (p<0.05); NS: Non-Significant

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Table 2: Effect of cyclophosphamide and *F. heptica* on the activities of liver enzymatic antioxidants

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GST</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>11.23±0.44</td>
<td>144.33±7.09</td>
<td>116.50±5.96</td>
<td>12.85±0.59</td>
<td>2.42±0.13</td>
</tr>
<tr>
<td>Group 2 (CP)</td>
<td>7.98±0.74**</td>
<td>105.67±8.38**</td>
<td>72.33±6.28**</td>
<td>6.42±0.69**</td>
<td>1.20±0.13**</td>
</tr>
<tr>
<td>Group 3 (FH)</td>
<td>11.65±0.81**</td>
<td>141.88±6.72**</td>
<td>114.17±7.17**</td>
<td>13.18±0.53**</td>
<td>2.56±0.17**</td>
</tr>
<tr>
<td>Group 4 (FH + CP)</td>
<td>10.47±0.61**</td>
<td>136.00±6.08**</td>
<td>109.28±6.64**</td>
<td>11.78±0.59**</td>
<td>2.35±0.19**</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD for six rats. Units: SOD: Units/mg protein, one unit is equal to the amount of enzyme that inhibits auto-oxidation of epinephrine by 50% at 24°C; CAT: μmoles H₂O₂ consumed min⁻¹ mg⁻¹ protein; GPx: μmoles GSH oxidized min⁻¹ mg⁻¹ protein; GST: μmoles CDNB (1-chloro-2,4-dinitrobenzene) conjugated min⁻¹ mg⁻¹ protein; GR: μmoles NADPH oxidized min⁻¹ mg⁻¹ protein. Comparisons are made between: a: Group 1 and Group 2; b: Group 3 and Group 4. *: Statistically significant (p<0.05); NS: Non-Significant.

Table 3: Levels of GSH and LPO in the liver of the experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>4.68±0.26</td>
<td>3.71±0.17</td>
</tr>
<tr>
<td>Group 2 (CP)</td>
<td>2.88±0.21**</td>
<td>6.98±0.67**</td>
</tr>
<tr>
<td>Group 3 (FH)</td>
<td>4.92±0.25**</td>
<td>3.95±0.11**</td>
</tr>
<tr>
<td>Group 4 (FH + CP)</td>
<td>4.10±0.29**</td>
<td>4.61±0.32**</td>
</tr>
</tbody>
</table>

Results are given as mean±SD for six rats. Units: GSH: μg mg⁻¹ protein; LPO: μmoles of MDA formed min⁻¹ mg⁻¹ protein. Comparisons are made between: a: Group 1 and Group 2; b: Group 3 and Group 4. *: Statistically significant (p<0.05); NS: Non-Significant.

**DISCUSSION**

Cyclophosphamide (CP) is a widely prescribed non-cell-cycle-specific antineoplastic drug which is known to cause toxic effects including hepatotoxicity (Dellve, 1996). In the present study, rats intoxicated with CP displayed a substantial increase in the activities of diagnostic marker enzymes (AST, ALT, ALP, LDH and GGT) and bilirubin levels in serum, which obviously reflect a significant damage in the structural integrity of liver. Recent findings suggest that serum GGT might be a useful marker of oxidative stress (Lee et al., 2004). When the liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol leak into blood stream. Their estimation in the serum is a useful quantitative marker for the extent and type of hepatocellular damage (Ansari et al., 1991). Administration of FH attenuated the increased levels of the serum enzymes and bilirubin, produced by CP and caused a subsequent recovery towards normalization, which is fairly in line with the previous study (Mandal et al., 2000). This was an indication of stabilization of plasma membrane which might be attributed to the membrane stabilizing effect of the phytoconstituents like oleic acid and β-sitosterol, present in FH (Tang et al., 2005; Yokota et al., 2006).

Free radicals exert their cytotoxic effect by peroxidation of membrane phospholipids leading to change in permeability and loss of membrane integrity (Meerson et al., 1982). In our present study, intraperitoneal administration of CP resulted in an increase in lipid peroxidation (LPO) [measured by the level of malondialdehyde (MDA)] in liver. FH treated rats showed decreased MDA level, due to significant inhibition of LPO which is in line with earlier studies (Mandal et al., 2000). This might be ascribed to the presence of oleic acid, hspidin and β-sitosterol which have been reported to exhibit anti-lipid peroxidation and/or free radical scavenging properties (Balanehru and Nagarajan, 1991; Park et al., 2004; Yokota et al., 2006).

Although, the comprehensive mechanism for the hepatotoxic activity of CP remains obscure, the causal association of oxidative stress and hepatotoxicity has been supported by the observation that antioxidant therapy ameliorates hepatotoxicity (Manda and Bhatia, 2003; Selvakumar et al., 2005; Stanickiewicz et al., 2002). In the present study, free radical-induced increase in LPO is accompanied by contemporaneous decline in the activities of cellular antioxidants. This may be owing to the inactivation of cellular antioxidants by lipid peroxides and ROS, which are produced due to CP
intoxication. FH administration restored these enzyme levels near to normalcy by bolstering the antioxidant defense system, which might be ascribed to the free radical scavenging/antioxidant properties of the phytochemical constituents present in FH.

GSH plays an important role in the regulation of variety of cell functions and in cell protection from oxidative injury. It is well known that depletion of GSH results in enhanced lipid peroxidation and excessive lipid peroxidation can cause increased GSH consumption, as observed in the present study. The dramatic decline in GSH level caused due to CP exposure may be attributed to the direct conjugation of CP’s metabolites with free or protein bound-SH groups (Yousefipour et al., 2005; Yuan et al., 1991), thereby interfering with the antioxidant functions. Activities of the anti-oxidative enzymes (CAT, SOD and GPx) were significantly reduced in GSH depleted condition due to pronounced oxidative stress and amassing of H₂O₂, making the cells more exposed to oxidative stress (Rajasekaran et al., 2002). Treatment with FH reinstated the GSH level to a near normal status. The presence of triterpenoid constituents in FH may at least in part account for this restorative effect (Liu et al., 1993a, 1995; Oliveira et al., 2005).

CP administration diminished the activities of GSH metabolizing enzymes, GST and GR. The decreased availability of GSH partly might be accountable for the reduced activity of GST and also because of its oxidative modification in its protein structure (Senthilkumar et al., 2006). GR is an important redox enzyme, which plays a major role in regenerating endogenous GSH from GSSG. This enzyme contains one or more sulphhydryl group residues, which are essential for catalytic activity and are vulnerable to free radical mediated inactivation (Gutierrez-Correa and Stoppani, 1997). The selective reaction of acrolein with the active site sulphhydryl eysteine provides an additional evidence for the reduced GR activity (Esterbauer et al., 1991). FH administration augmented these antioxidant levels near to the normal status.

Interestingly, previous reports suggest that oleic acid has protective effect against cyclophosphamide-induced toxicities (Liu et al., 1995). Numerous studies corroborate the hepatoprotective effect of oleic acid on various models (Liu et al., 1993a, b; Kim et al., 2005; Abdel-Zahe et al., 2007). A recent fascinating report by Oliveira et al. (2005) demonstrate the hepatoprotective effect of β-aminin through diminution of oxidative stress. Literature citations show that β-sitosterol possess antioxidant and plausible hepatoprotective properties (Nakamura et al., 1992; Yoshida and Niki, 2003). As cited by Malathi and Gomez (2007) phenanthroindolizidine alkaloids might have a significant role in the hepatoprotective effect. Prodigious amounts of literature data suggest that triterpenoids, flavonoids, tannins possess significant antioxidant/hepatoprotective effects (Augusi et al., 2005; Bai et al., 2007; Buniatian et al., 1998; Daniel et al., 2003). Synergistic action of these aforesaid phytoconstituents present in F. hispida leaf extract might be responsible for the alleviation CP induced hepatic damage.

CONCLUSION

In summary, intoxication of rats with cyclophosphamide impinged oxidative stress and liver damage, which is illuminated by dramatic elevation in the pathological parameters with a substantial drop in the antioxidant parameters. Indeed, the data presented here reveal the hepatoprotective role of F. hispida leaf extract, which is evidenced by the normalization of the pathological and antioxidant parameters. The present study thus pharmacologically validated the folkloric use of F. hispida in the treatment of liver diseases and also highlights the claim that F. hispida may be considered as a potentially useful candidate in the combination chemotherapy with CP to combat oxidative stress mediated liver injury. Further investigation to elucidate the protective role of Ficus hispida in cyclophosphamide-induced toxic manifestations is underway.
REFERENCES


