ANTI HEPATOTOXICITY STUDIES OF CRUDE EXTRACT OF FERONIA LIMONIA IN CCL₄ INDUCED TOXICITY

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ABSTRACT

The antioxidant activities of Feronia limonia fruit pulp methanolic extract (MEFL) was investigated by and in vivo assay. The effect of the extract in reducing carbon tetrachloride induced (CCL₄) oxidative stress in rats was evaluated. Hepatoprotective activity of MEFL was studied by estimating serum enzyme activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), Serum Lactate Dehydrogenase (LDH), total protein (TP) and total bilirubin (TB). The treatment with MEFL showed a dose-dependent reduction in CCL₄ induced elevated serum levels of enzyme activities with parallel increase in total protein and bilirubin content indicating the extract could preserve the normal functional status of the liver. MEFL at the doses of 200 and 400 mg/kg body weight significantly increased the glutathione (GSH), super oxide dismutase (SOD) and catalase (CAT) activities in a dose dependant manner. Therefore, the results of this study illustrate that the Feronia limonia methanol extract can shield the liver against CCL₄-induced oxidative damage in rats.

Keywords: Feronia limonia, antioxidant activity, phenolic content, CCL₄

INTRODUCTION

Feronia limonia commonly known as wood apple is an important plant in Ayurveda, the traditional system of Indian medicine. This fruit is recommended for the treatment of tumors, asthma, wounds, diarrhea, dysentery, cardiac dysfunction, hepatitis, sore throat and is considered as tonic, astringent (when unripe), antiscorbutic, and alexiformic agent [1,2]. Feronia limonia fruit has also been reported to possess hypoglycemic and hypolipidaemic and wound healing properties[3,4].
Antioxidant activity is a fundamental property important for life. Many of the biological functions, such as antimutagenicity, anticarcinogenicity, and antiaging, among others, originate from this property[5]. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions [6]. It has been suggested that natural antioxidants like L-ascorbic acid are more safe and healthy than synthetic antioxidants such as butylated hydroxytoluene (BHT) [7]. Hence, there is need for safe, cost effective and less toxic antioxidants with high activity from natural sources to replace the synthetic chemicals. The importance of the antioxidant constituents of plant materials in maintaining health and protection from coronary heart disease. Cancer is another deadly disease raising interest among scientists and food manufacturers towards the search of functional food with specific health effects [8]. Potential sources of antioxidant compounds have been searched in several types of plant materials such as fruits, leaves, seeds, barks, roots and crude plant drugs [9]. Crude extracts of plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The antioxidative effect is mainly due to flavonoids and phenolic acids present in the plants. Many of the natural antioxidants, especially phenolics, exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory actions [10].

Liver diseases, especially viral hepatitis occurs principally with an enormous impact on public health. Carbon tetrachloride (CCl₄) is widely used in animal models to induce acute liver injury [11]. It is commonly believed that the toxicity of CCl₄ results from its reductive dehalogenation by the cytochrome P₄₅₀ enzyme system into the highly reactive free radical trichloromethyl radical [12-14]. Antioxidant action has been reported to play a crucial role in the hepatoprotection [15]. In the present study, an attempt has been made to explore the effect of MEFL in CCl₄ induced hepatic damage pertaining to biochemical marker enzymes & histopathology. The result of this In Vivo study will support the plant as a good herbal antioxidant agent.

MATERIALS AND METHODS

Chemicals

CCl₄ were purchased from Sigma-Aldrich (Mumbai). Liv 52 from Himalaya drugs Ltd. All other chemicals and reagents were of analytical grade, and they were used as received.
Plant material and extraction
Fruits of *Feronia limonia* L were collected from local market Bellary, Karnataka, India during the month of March, 2012. It was authenticated by the Department of P.G studies and Research in Botany, Gulbarga University, Gulbarga. Crude fruit pulp extract was prepared by Soxhlet extraction method. About 50gm of powdered plant material was packed in a thimble and extracted successively with 350ml of petroleum ether, chloroform, and methanol. The process of extraction is carried out until the solvent in siphon tube of an extractor become colorless. The extract was taken in a petriplates and kept in hot air oven and heated at 30-40ºC till the solvent got evaporated. Solvent free methanolic extract dissolved in 1% Dimethyl sulphoxide (DMSO) was used for the *In Vivo* studies.

Animals
Wistar strain Albino rats of inbred colony weighing about 150 – 175 g were used. The protocol was approved by the Institute’s Animal Ethical Committee (IAEC Reg No. 34800/ CPCSEA Dated: 19.08.2001). Animals were kept in animal house at an ambient temperature of 25ºC and 45 – 55% relative humidity, with 12 h each of dark and light cycles. They were fed with a balanced diet as described by Central Food and Technological Research Institute (CFTRI, Mysore) and water ad libitum [16].

Acute toxicity experiment
Albino rats were divided into control and test groups (6 animals each). Control group received the vehicle (3% Tween 80) while the test groups got graded doses (100–4000 mg/kg) of MEFL orally and were observed for mortality till 48 h and the LD$_{50}$ was calculated.

Induction of experimental hepatotoxicity
30% CCl$_4$ was prepared in olive oil. Animals of group 2, 3, 4 and 5 were given single dose of CCl$_4$ at 1ml/kg body weight (bw) intraperitoneally (i.p). Methanolic extract of *Feronia limonia* at the dose level of 200 mg/kg bw and 400 mg/kg bw as low dose and high dose were administered to animals of group 4 and 5 orally with the aid of an intragastric catheter for 7 days. Liv 52 (50 mg/kg bw) was used orally as a standard drug to group 3 at a single dose. Rats were divided into five groups as following protocol.

GROUP 1: Normal control (n=6, the animals were given saline at 1ml/kg body weight)
GROUP 2: Hepatotoxic control (n=6, the animals were given CCl$_4$ at 1ml/kg body weight)
GROUP 3: Positive control (n=6, the animals were given CCl₄ + Liv 52 for 7 days)
GROUP 4: Treatment group (n=6, the animals were given CCl₄ + MEFL low dose for 7 days)
GROUP 5: Treatment group (n=6, the animals were given CCl₄ + MEFL high dose for 7 days)

At the end of the experimental period, blood sample from each rat (2 ml) was withdrawn by cardiac puncture and collected in previously labeled centrifuge tubes and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 10,000 rpm for 5 min.

Assessment of hepatotoxicity
Liver functions were evaluated by measuring the serum activity of ALT and AST following the method of [17] while the activities of ALP and LDH were estimated. The serum concentrations of TB estimated according to [20] and TP as described by [21].

Assessment of oxidative stress
Liver tissue was homogenized in 10 volume of 100 mM KH₂PO₄ buffer containing 1 mM EDTA (pH 7.4) and centrifuged at 12,000 rpm for 30 min at 4°C. The activities of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were assayed in the hepatic tissue homogenate of the control and experimental rats according to the methods of [22,23]. GSH tissue content was also measured [24].

Histopathological study
Liver was dissected out and divided into two parts. One part was kept in liquid nitrogen for determination of antioxidant status and the other part was immediately fixed in buffered formalin 10% and was used for histopathological examination using the standard micro technique [25].

Statistical analysis of data
Data were presented as means ± SD of three experiments. Analysis of variance was performed on the data obtained. Significance of differences between means was determined by least significant differences (LSD) at \( P \leq 0.05 \).

RESULTS
Acute toxicity study
The dose selection for MEFL was based on the acute toxicity study. The study did not show any adverse effect of doses up to 4000 mg/kg. Accordingly, experimental oral doses of 200
and 400 mg/kg equal to one-twentieth and one-tenth of the feasible dose of the extract that did not cause mortality in rats were selected.

Assessment of hepatotoxicity

The effects of methanol extract of *Feronia limonia* at dose levels of (200 and 400 mg/kg bw) on serum marker enzymes are shown in Table 2. Hepatic injury induced by CCl₄ has caused significant rise in marker enzymes such as ALT, AST, ALP activities and decrease in serum TP, TB levels (*P* < 0.01). Administration of methanol extract of *Feronia limonia* at two different dose levels attenuated the increased levels of the serum enzymes, produced by CCl₄, and caused a subsequent recovery towards normalization almost like that of Liv 52 treatment.

Table 1 Effect of methanol extract of *Feronia limonia* on serum marker enzymes, total protein and total bilirubin

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>ALP (IU/L)</th>
<th>TP (µmol/L)</th>
<th>TB (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>64.18 ± 0.23</td>
<td>81.56 ± 5.54</td>
<td>107.69 ± 1.33</td>
<td>8.14 ± 0.37</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>HC</td>
<td>170.22 ± 0.14</td>
<td>134.91 ± 7.33</td>
<td>152.56 ± 1.07</td>
<td>4.11 ± 0.3</td>
<td>1.81 ± 0.09</td>
</tr>
<tr>
<td>CCl₄+ Liv 52 (50mg/kg bw)</td>
<td>91.31 ± 0.42</td>
<td>96.07 ± 4.38</td>
<td>120.01 ± 1.25</td>
<td>7.00 ± 0.24</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>CCl₄+ MEFL (200mg/kg bw)</td>
<td>142.10 ± 0.25</td>
<td>126.72 ± 7.16</td>
<td>144.30 ± 1.66</td>
<td>5.01 ± 0.35</td>
<td>1.43 ± 0.06</td>
</tr>
<tr>
<td>CCl₄+ MEFL (400mg/kg bw)</td>
<td>117.35 ± 0.31</td>
<td>115.80 ± 7.43</td>
<td>132.78 ± 2.01</td>
<td>6.25 ± 0.65</td>
<td>1.09 ± 0.05</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M.; ⁵ P < 0.01, compared with NC group; ⁶ P < 0.05, ⁷ P < 0.01, compared with CCl₄-treated group.

Note: NC (normal control), HC (hepatotoxic control).

Assessment of oxidative stress

CCl₄ treatment also resulted in the depletion (*P*<0.01) of the hepatic antioxidant enzymes. The activities of GSH, SOD, CAT were depleted to 1.01 ± 0.12, 17 ± 0.17 and 23.16 ± 1.27 respectively of the hepatotoxic control (Table 3). The decline in the activities were noticeably attenuated (*P*<0.01) by administration of 200 mg/kg bw and 400 mg/kg bw in MEFL treated rats. Treatment with MEFL enhanced liver antioxidant enzymes yet after CCl₄ treatment.
Table 2  Effect of methanol extract of *Feronia limonia* on GSH levels, CAT and SOD activities

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (mg/g prot)</th>
<th>CAT (U/g prot)</th>
<th>SOD (U/g prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.47 ± 0.43</td>
<td>45.02 ± 1.33</td>
<td>26.54 ± 0.33</td>
</tr>
<tr>
<td>HC</td>
<td>1.01 ± 0.12 b</td>
<td>23.16 ± 1.27 b</td>
<td>17.00 ± 0.17 b</td>
</tr>
<tr>
<td>CCl₄ + Liv 52 (50mg/kg bw)</td>
<td>1.32 ± 0.27 d</td>
<td>39.58 ± 1.07 d</td>
<td>22.03 ± 0.25 c</td>
</tr>
<tr>
<td>CCl₄ + MEFL (200mg/kg bw)</td>
<td>1.16 ± 0.22 d</td>
<td>27.20 ± 1.74 c</td>
<td>18.21 ± 0.66 d</td>
</tr>
<tr>
<td>CCl₄ + MEFL (400mg/kg bw)</td>
<td>1.24 ± 0.26 d</td>
<td>34.10 ± 1.45 d</td>
<td>20.95 ± 0.21 d</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M; b P < 0.01, compared with NC group; c P < 0.05, d P < 0.01, compared with CCl₄-treated group.

Note: NC (normal control), HC (hepatotoxic control)

Histopathology

The histopathological examination displayed significant recovery of hepatocytes in the standard drug and MEFL treated animals, which is again correlated with the biochemical parameters. The results of the liver histopathological studies (Figs 5a-5e) showed hepatocytes swelling and necrosis in CCl₄-treated rats (Fig 5b) in comparison with normal control rats (Fig 5a). Treatment with MEFL exhibited a significant protection against hepatocytes injury and showed complete normalization of the tissues where no fatty accumulation or necrosis was seen (Fig 5d, 5e). The central vein appeared clearly indicating a potent anti-hepatotoxic activity. MEFL was found to exhibit a potent anti-hepatotoxicity compared with standard drug Liv 52 (Fig 5c). The liver section showed the structure of the portal triad and a normal liver parenchyma, and the central vein appeared clearly. There was no lymphocytic infiltration and fatty deposition representing a potent anti-hepatotoxicity for the MEFL under study.
DISCUSSION
Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants due to their scavenging activity are useful for the management
of these diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms [26]. The present study suggests that *Feronia limonia* can be used as a source of antioxidants for pharmacological preparations. Non-phenolic compounds of the plants such as trace elements may decrease the antioxidant activity of the phenolic compounds [27].

Liver injury caused by CCl₄ in rats was first reported [28] and is extensively and effectively used by many investigators. Carbon tetrachloride is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of CCl₃O·, a reactive oxidative free radical, which initiates lipid peroxidation. In the presence of oxidative stress more of lipid peroxidation products are formed due to cell damage. In this study, carbon tetrachloride damage to erythrocytes was confirmed by the increase in SOD, GSH and CAT activities, and decrease in membrane fluidity. SOD is one of the crucial components in the antioxidant defense system for the reduction of reactive oxygen species (ROS) and peroxides produced in the living organism and in detoxification of compounds of exogenous origin, thus playing a primary role in the maintenance of a balanced redox status[29]. The increase of SOD activity suggests that the MEFL has an efficient protective mechanism in response to ROS. Catalase is a very important component of the antioxidant defense system. The increased SOD activity resulted in the accumulation of hydrogen peroxide, which stimulated increases in CAT activity. The MEFL increased the activities of Catalase in CCl₄ induced liver damage rats to prevent the accumulation of excessive free radicals and thus protects the liver from intoxication. GSH is a naturally occurring substance abundant in many living creatures; GSH depletion increases the sensitivity of cells to various aggressions leading to tissue disorder and injury[30]. In the present study we demonstrated the efficiency of the extract by using CCl₄ induced rats and found that exogenous MEFL supplementation elevated GSH levels in rats with CCl₄ treatment and thus might provide a mean of recovering reduced GSH levels to prevent tissue injury. Treatment of experimental animals with the MEFL exhibited an improved free radical scavenging ensuring decrease in activities of enzymes towards normal.

The field of dietary modification and chemoprevention show considerable effective approach against oxidative stress and are the focus of research these days. In the assessment of liver damage by CCl₄, the determination of enzyme levels such as AST, ALP and ALT is largely used. Bilirubin concentration has been used to evaluate chemically induced hepatic injury. The data showed that the control group demonstrated a normal range of AST, ALP, ALT, protein and bilirubin levels, while the CCl₄-treated group showed elevated levels confirming
that CCl₄ caused liver injury, altered membrane integrity and as a result enzymes in hepatocytes leak out [31]. However, after treatment with MEFL, the increase in ALT, AST and ALP were significantly restored. Increase in the level of TP by MEFL indicates hepatoprotective activity, as stimulation of protein synthesis accelerates the regeneration process and production of liver cells. These results indicate that the extract has the ability to protect against CCl₄-induced hepatocyte injury, which is in agreement with the previous study of [32] that reported the protective consequence of polyphenolic compounds against CCl₄-induced liver cirrhosis. Therefore, it is valid to consider that MEFL, possess antioxidant property which is capable of protecting the hepatic tissue from CCl₄-induced injury and inflammatory changes might be due to the presence of phenolics.

CONCLUSION

Our investigations reveals that MEFL hold potent antioxidant activity. The current study demonstrates that MEFL exerts effective protection against acute chemical induced hepatic damage suppressing liver enzyme activities and thus preventing cell membrane damage. Histopathological study revealed morphological evidence and bioassays revealed functional evidence. MEFL can thus be proposed to protect the liver against CCl₄-induced oxidative damage in rats. The in vivo assays indicate that this plant extract is a significant source of natural antioxidant, which might help prevent the progress of oxidative stress. However, the components responsible for the antioxidant activity are currently unclear. Efforts are in progress in our laboratory to isolate and purify the active principle of this medicinal plant.

REFERENCES


