Hepatoprotective activity of Chenopodium album Linn: in vitro and in vivo studies

Nilesh Kumar Jain, Abhay Kumar Singhai

Abstract

Objective: The hepatoprotective activity of Chenopodium album Linn leaves against carbon tetrachloride (CCL<sub>4</sub>)-induced hepatotoxicity was investigated.

Methods: Rat hepatocyte monolayer culture and rats were used as in vitro and in vivo hepatoprotective screening models. In the in vitro studies, different extracts and fractions of C.album were screened. Silymarin was taken as reference drug. In vitro antioxidant activity was also assessed. In the in vivo studies, hepatotoxicity was induced in wistar rats by administering a mixture of CCl<sub>4</sub>: olive oil (1:1, 2 ml/kg, s.c.). The extent of hepatotoxicity was assessed by measuring the serum enzyme levels. The antioxidant parameters, malondialdehyde, reduced glutathione, superoxide dismutase and catalase of the liver tissue were also assessed.

Results: In the in vitro studies, ethanol extract of C.album was found to be most active than other screened extracts/fractions. Furthermore, ethanol extract was found to be rich in phenolic and flavonoids and showed significant free radical scavenging activity against diphenylpicrylhydrazyl (DPPH) and superoxide ion radicals. In the in vivo studies, ethanol extract at a dose of 100, 200 and 400 mg/kg b.w. exhibited significant protection against CCL<sub>4</sub>-induced hepatotoxicity as evident by prevention of CCL<sub>4</sub>-induced biochemical changes.

Conclusion: The findings of present study concluded the significant hepatoprotective activity of ethanol extract of C.album leaves against CCL<sub>4</sub>-induced hepatotoxicity and suggest its use as potential therapeutic agent for liver diseases.

Key words:
Antioxidants; Carbon tetrachloride; Chenopodiaceae; Chenopodium album; Hepatoprotective

Introduction

Chenopodium album Linn, commonly known as ‘bathua’, ‘fat-hen’ or ‘lamb’s quarters’ belongs to family Chenopodiaceae. Due to high nutritional value, leaves are consumed as vegetables in many Asian countries. Traditionally, C.album is used as a curative medicine for various diseases including hepatic ailments [1, 2]. Chemically, the presence of phenolics, sterols, vitamins, carotenoids, flavonoids, phytoecdysteroids and minerals has been reported in C.album leaves [3, 4].

Many extracts and compounds from C.album leaves have been demonstrated to possess hypotensive [5], anti-inflammatory [6], antihelminthic [7], and anticancer activities [8]. Recently the hepatoprotective activity of C.album against paracetamol-induced hepatotoxicity has been reported [9]. Our present study was aimed to investigate the protective potential of C.album leaves against carbon tetrachloride (CCL<sub>4</sub>)-induced hepatotoxicity and oxidative stress.

Material and methods

Chemicals and reagents
CCL<sub>4</sub>, ethylene glycol tetraacetic acid (EGTA), William’s E medium, collagenase, 2,2-diphenylpicrylhydrazyl (DPPH), thiobarbituric acid (TBA) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were procured from Sigma Chemical Co. (St.Louis, MO, USA). All other chemicals and estimation kits used were of analytical grade and purchased from commercial sources.

Experimental animals
Wistar albino rats (200-250 g) of either sex were used for the experiments. Animal studies were reviewed and approved by the Institutional Animal Ethics Committee (379/01/ab/CPCSEA). The animals were maintained under standard laboratory conditions of temperature (25 ± 2°C) and humidity (55 ± 5%) with 12 h light- dark cycle.

Plant material
The fresh leaves of C.album were procured from market of Sagar and authenticated in the Botany Department of Pharmaceutical Sciences, Dr. Hari Singh Gour Vishwavidyalaya, Sagar, Madhya Pradesh, India.
department of Dr. Hari Singh Gour Vishwavidyalaya, Sagar, India (Herb. No. Bot/Her/890). The fresh leaves were washed with tap water, shade dried and powdered.

**Extraction and fractionation**

The powdered leaf material (800 g) was successively extracted with petroleum ether (60-80°C) and 95% ethanol using Soxhlet extractor. The marc left after the ethanol extraction was macerated with distilled water for 24 h. The solvents were distilled off under reduced pressure below 45°C to afford petroleum ether extract (CAPEE, yield 9.6% w/w), ethanol extract (CAEE, 12.7% w/w) and aqueous extract (CAAE, 11.3% w/w).

The ethanol extract (CAEE, 30 g) was suspended in water (300 ml) and fractionated successively with chloroform (4 x 300 ml), ethyl acetate (4 x 300 ml) and n-butanol (4 x 300 ml) to afford chloroform fraction (CACF, 2.5 g), ethyl acetate fraction (CAEF, 4 g) and n-butanol fraction (CABF, 5.13 g), respectively.

**Preliminary phytochemical screening**

Preliminary phytochemical analysis was performed to identify the nature of phytoconstituents in different extracts and fractions [10].

**Determination of total phenolic and flavonoid content**

The total phenolic and flavonoid contents of different plant extracts/fractions were determined [11]. The total phenolic content was expressed as milligrams of gallic acid equivalents/g extract (mg GAE/g of dry mass) and the total flavonoid content was expressed in milligrams of quercetin equivalents/g of extract (mg QE/g of dry mass).

**In vitro antioxidant activity**

**DPPH scavenging assay:** the DPPH scavenging activity was determined according to the method described previously [12]. The absorbance (Abs) was taken at 517 nm. The percent inhibition was calculated according to following formula:

\[
\% \text{ Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100
\]

Ascorbic acid, a known antioxidant was used as positive control.

**Superoxide ion radical scavenging assay:** the superoxide ion radical scavenging activity was determined according to the method of Robak and Gryglewski [13]. The reaction mixture, comprising 3 ml of extract/fraction solution (10-500 µg/ml), 10 µl of phenazine methosulphate (60 µM) and 1 ml of NADH (468 µM) was incubated at 25°C for 5 min and the absorbance was taken at 560 nm.

**In vitro hepatoprotective activity**

Hepatocytes were isolated from rat liver as per the reported method by Jain and Singhai [14]. The isolated hepatocytes were suspended in William's E medium (pH 7.4) and seeded in collagen pre-coated culture plates at a density of 2 to 3 x 10^5 cells/well at 37°C in humidified atmosphere of 5% CO_2 in a CO_2 incubator. After 24 h of culturing, cells were exposed to CCl_4 (2.5 mM) with or without plant extracts/fractions (100 µg/ml) or silymarin (10 µM) and incubated for another 24 h at 37°C in CO_2 incubator. After 24 h incubation, the leakage of alanine transaminase (ALT) [15] and lactate dehydrogenase (LDH) [16] was determined in the culture medium.

**Acute oral toxicity studies**

The acute oral toxicity studies were performed following OECD guidelines [17]. On the basis of these studies, oral doses of 100, 200 and 400 mg/kg b.w. were selected for *in vivo* studies.

**In vivo hepatoprotective activity**

The experiment was conducted according to method described previously [18]. Rats were randomly divided into six groups, each consisting of six rats and treated as follows:

- **Group I (normal control):** distilled water (1 ml/kg, p.o.) daily for 5 days and olive oil (1 ml/kg, s.c.) on days 2 and 3.
- **Group II (CCl_4 control):** distilled water daily for 5 days and CCl_4:olive oil (1:1, 2 ml/kg, s.c.) on days 2 and 3.
- **Group III (positive control):** silymarin (50 mg/kg, p.o.) daily for 5 days and CCl_4:olive oil on days 2 and 3, 30 min after administration of silymarin.
- **Groups IV–VI:** CAEE (100, 200 and 400 mg/kg, p.o., respectively) for 5 days and CCl_4:olive oil on days 2 and 3.

On the 6th day, under ether anesthesia, blood and liver samples were collected and processed for biochemical estimations.

**Biochemical assay**

Activities of aspartate and alanine transaminase (AST and ALT) [15], alkaline phosphatase (ALP) [19] and lactate dehydrogenase (LDH) [20] were determined in serum. Level of lipid peroxidation (LPO) (expressed in terms of malondialdehyde, MDA) [21], glutathione (GSH) [22], superoxide dismutase (SOD) [23] and catalase (CAT) [24] were also determined by the standard methods to assess oxidative stress.

**Statistical analysis**

The results are expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple
comparison test using Graph Pad Prism software; p < 0.05 was considered to be significant.

**Results**

**Preliminary phytochemical screening**

In the preliminary phytochemical analysis, different extracts and fractions of *C.album* showed the presence of various phytoconstituents (Table 1).

**Total phenolic and flavonoid contents**

Our phytochemical analysis showed the presence of moderate to high concentration of phenolics (64.35 ± 3.05 to 275.93 ± 4.97 mg GAE/g) and flavonoids (77.45 ± 2.49 to 288.85 ± 12.41 mg QE/g) in different extracts and fractions of *C.album*. The highest phenolic and flavonoid contents were found in CAEE (Table 2).

**In vitro antioxidant activity**

In the in vitro evaluation, the CAEE had stronger antioxidant activity compared to other tested extract/fractions. The IC\(_{50}\) values of CAEE against DPPH and superoxide ion radicals were found to be 36.17 and 32.56 µg/ml, respectively. Meanwhile, the ascorbic acid showed potent antioxidant activity with IC\(_{50}\) values of 31.12 and 28.2 µg/ml, respectively (Fig.1).

<table>
<thead>
<tr>
<th>Nature of phytoconstituents</th>
<th>CAEE</th>
<th>CAAE</th>
<th>CACF</th>
<th>CAEAF</th>
<th>CABF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid and triterpenoids</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic and tannins</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Anthraquinone glycoside</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Protein</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 1. Preliminary phytochemical screening of various extracts and fractions of *Chenopodium album* L (CA) leaves

<table>
<thead>
<tr>
<th>S.No</th>
<th>Extracts/fractions</th>
<th>Total phenolic content (mg GAE/g dry extract)</th>
<th>Total flavonoid content (mg QE/g dry extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethanol extract</td>
<td>275.93 ± 4.97</td>
<td>288.85 ± 12.41</td>
</tr>
<tr>
<td>2</td>
<td>Aqueous extract</td>
<td>112.25 ± 8.34</td>
<td>110.61 ± 10.88</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform fraction</td>
<td>96.8 ± 9.19</td>
<td>77.45 ± 2.49</td>
</tr>
<tr>
<td>4</td>
<td>Ethyl acetate fraction</td>
<td>119.92 ± 7.61</td>
<td>142.98 ± 10.43</td>
</tr>
<tr>
<td>5</td>
<td>n-butanol fraction</td>
<td>64.35 ± 3.05</td>
<td>86.18 ± 3.64</td>
</tr>
</tbody>
</table>

Table 2. Total phenolic and flavonoid contents of various extracts and fractions of *Chenopodium album* L leaves

Each value is expressed as mean ± SD (n = 3); GAE, Gallic acid equivalent; QE, Quercetin equivalent

**Figure 1.** Free radical scavenging activity of different extracts and fractions of *Chenopodium album* leaves:
(A) DPPH scavenging assay, (B) superoxide radical scavenging assay. All determinations were performed in triplicate (CAPEE, petroleum ether extract; CAEE, ethanol extract; CAAE, aqueous extract; CACF, chloroform fraction; CAEAF, ethyl acetate fraction; CABF, n-butanol fraction; AA, ascorbic acid).
In vitro hepatoprotective activity

Incubation of hepatocytes with CCl₄ (2.5 mM) resulted in a significant (p < 0.001) elevation of ALT and LDH (3- and 1.7-fold, respectively) in CCl₄ control hepatocytes (Table 3). Treatment with different extracts and fractions of Chenopodium album (100 µg/ml) or silymarin (10 µM) showed a moderate to high hepatoprotective effect as evident by the restoration of ALT and LDH levels. The maximum restoration against enzyme leakage was observed with CAEE (58.7% and 62.3%, respectively for ALT and LDH), while the reference drug silymarin showed protective effect (73.1% and 79.6% restoration, respectively for ALT and LDH). The most active extract, CAEE was selected for in vivo hepatoprotective studies.

Acute toxicity studies

In acute oral toxicity studies, the CAEE did not show any sign and symptoms of toxicity and mortality up to 2000 mg/kg dose, considered relatively safe.

In vivo hepatoprotective activity

The effect of CAEE on serum marker enzymes during CCl₄-induced hepatotoxicity is shown in Table 4. The elevated levels of AST, ALT, ALP and LDH due to CCl₄ intoxication were significantly (p < 0.001) prevented with CAEE treatment when compared with CCl₄ control rats. Maximum activity was found with higher dose. Silymarin also showed significant protective effect against CCl₄-induced alterations.

The elevated MDA and reduced level of enzymatic and non-enzymatic antioxidants (SOD, CAT and GSH, respectively) as observed in CCl₄ control rats (Table 5), were significantly (p < 0.001) prevented in CAEE treated groups, indicating remarkable antioxidant effect.

Discussion

CCl₄, a well-known hepatotoxin, has been widely used to screen new hepatoprotective agents [25, 26]. This xenobiotic is rapidly transformed by cytochrome P450 2E1 to a trichloromethyl radical which is converted into a peroxyl radical in the presence of oxygen. These radicals may interact with cellular macromolecules and initiate the peroxidative degradation of lipid membranes [27]. Enhanced production of reactive oxygen species (ROS) and proinflammatory cytokines by activated Kupffer cells is also involved in liver damage initiated by CCl₄-derived radicals [28].

Table 3. Effect of various extracts and fractions of Chenopodium album (CA) leaves and silymarin (SIL) on ALT and LDH in CCl₄-induced toxicity in rat hepatocyte monolayer culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Restoration</th>
<th>ALT</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CCl₄ control</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SIL (10 µM)</td>
<td>73.1</td>
<td>79.6</td>
<td></td>
</tr>
<tr>
<td>CAEPEE (100 µg/ml)</td>
<td>42.6</td>
<td>48.2</td>
<td></td>
</tr>
<tr>
<td>CAEE (100 µg/ml)</td>
<td>58.7</td>
<td>62.3</td>
<td></td>
</tr>
<tr>
<td>CAEE (100 µg/ml)</td>
<td>33.5</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>CACF (100 µg/ml)</td>
<td>24.4</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>CAEAF (100 µg/ml)</td>
<td>28.9</td>
<td>27.4</td>
<td></td>
</tr>
<tr>
<td>CABF (100 µg/ml)</td>
<td>18.3</td>
<td>37.9</td>
<td></td>
</tr>
</tbody>
</table>

Each value represent the mean ± SEM (n = 4). The % restoration of ALT and LDH were 52.36 ± 2.67 and 46.28 ± 3.7, respectively. Each value represent the mean ± SEM (n = 4). The % restoration was calculated as 100 x (value of CCl₄ control – value of sample) / (value of CCl₄ control – value of normal control). (ALT, alanine transaminase; LDH, lactate dehydrogenase; CAEPEE, petroleum ether extract; CAEE, ethanol extract; CAEE, aqueous extract; CACF, chloroform fraction; CAEAF, ethyl acetate fraction; CABF, butanol fraction)

Table 4. Effect of ethanol extract of Chenopodium album L (CA) leaves (CAEE) and silymarin (SIL) on AST, ALT, ALP and LDH in CCl₄-induced hepatotoxicity in rats

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Group I Normal</th>
<th>Group II CCl₄ olive oil (1:1, 2 ml/kg) + CCl₄</th>
<th>Group III SIL (50 mg/kg) + CCl₄</th>
<th>Group IV CAEE (100 µg/ml) + CCl₄</th>
<th>Group V CAEE (200 mg/kg) + CCl₄</th>
<th>Group VI CAEE (400 mg/kg) + CCl₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>51.17 ± 5.47</td>
<td>185.38 ± 4.23*</td>
<td>89.13 ± 8.11*</td>
<td>122.53 ± 4.83***</td>
<td>103.38 ± 2.27**</td>
<td>94.55 ± 3.16**</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>26.65 ± 2.23</td>
<td>158.03 ± 7.09*</td>
<td>58.3 ± 3.2*</td>
<td>106.63 ± 6.93**</td>
<td>87.83 ± 5.26*</td>
<td>65.18 ± 2.56*</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>34.45 ± 1.89</td>
<td>123.43 ± 2.78*</td>
<td>65.87 ± 1.73*</td>
<td>128.57 ± 11.16**</td>
<td>103.63 ± 3.47**</td>
<td>85.64 ± 5.71*</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>109.99 ± 3.77</td>
<td>243.1 ± 14.72*</td>
<td>154.8 ± 9.05*</td>
<td>211.31 ± 3.94**</td>
<td>167.97 ± 9.92*</td>
<td>174.31 ± 2.89*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM (n = 6); *p < 0.001 as compared with the normal group (vehicle only); **p < 0.01, ***p < 0.01, and ****p < 0.05, respectively as compared with the group treated with CCl₄ alone; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.
In the present study, rats treated with CCl₄ alone, developed a significant hepatic damage and oxidative stress, as evident by the massive elevation in the enzyme activities of AST, ALT, ALP and LDH compared with normal control rats. This is an indicative of cellular leakage and loss of functional integrity of hepatocyte cell membrane [18]. Reduction in the enzyme activities towards the respective normal values by CAEE at different dose levels (100, 200 and 400 mg/kg) is an indication of stabilization of plasma membranes and repair of liver tissue damage.

Elimination of free radicals and prevention of LPO is important in the treatment of CCl₄-mediated liver damage [29]. Inactivation, detoxification, removal of ROS and other free radicals depend on enzymatic and non-enzymatic antioxidants. The important enzymatic antioxidants in the tissues are SOD, CAT and glutathione peroxidase (GPx). These antioxidants together with GSH act to prevent the formation of free radicals and thereby prevent oxidative stress [30].

In our experimental conditions, CCl₄ - induced a severe depletion in hepatic GSH, SOD and CAT with respect to normal control group. Moreover this effect was accompanied with a high level of LPO. This would suggest that LPO is an important factor in the pathogenesis of CCl₄-induced liver damage. Rats administered with CAEE showed a significant increase in the levels of GSH, SOD and CAT along with marked reduction in MDA when compared with CCl₄ control rats, indicating remarkable antioxidant effects.

Previous phytochemical investigations on Chenopodium album L (CA) leaves (CAEE) and silymarin (SIL) on hepatic MDA, GSH, SOD, and CAT in CCl₄-induced hepatotoxicity in rats

Table 5. Effect of ethanol extract of Chenopodium album L (CA) leaves (CAEE) and silymarin (SIL) on hepatic MDA, GSH, SOD, and CAT in CCl₄-induced hepatotoxicity in rats

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Group I Normal</th>
<th>Group II CCl₄ olive oil (1:1, 2 ml/kg)</th>
<th>Group III SIL (50 mg/kg) + CCl₄</th>
<th>Group IV CAEE (100 mg/kg) + CCl₄</th>
<th>Group V CAEE (200 mg/kg) + CCl₄</th>
<th>Group VI CAEE (400 mg/kg) + CCl₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nM/mg protein)</td>
<td>0.46 ± 0.04</td>
<td>1.36 ± 0.04*</td>
<td>0.71 ± 0.03*</td>
<td>1.25 ± 0.03***</td>
<td>1.01 ± 0.03**</td>
<td>0.86 ± 0.04*</td>
</tr>
<tr>
<td>GSH (µM/mg protein)</td>
<td>7.8 ± 0.69</td>
<td>3.6 ± 0.38†</td>
<td>6.79 ± 0.32*</td>
<td>5.56 ± 0.24</td>
<td>6.43 ± 0.51**</td>
<td>6.64 ± 0.42**</td>
</tr>
<tr>
<td>SOD (U/mg)</td>
<td>38.9 ± 3.89</td>
<td>20.84 ± 1.23*</td>
<td>33.31 ± 1.6*</td>
<td>25.18 ± 1.79</td>
<td>32.17 ± 1.96*</td>
<td>30.57 ± 1.50**</td>
</tr>
<tr>
<td>CAT (U/mg)</td>
<td>55.35 ± 2.94</td>
<td>29.48 ± 0.65</td>
<td>46.08 ± 2.16*</td>
<td>34.36 ± 1.89</td>
<td>39.95 ± 0.86**</td>
<td>43.66 ± 1.1*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM (n = 6); *p < 0.001 as compared with the normal group (vehicle only). **p < 0.01, ***p < 0.001, and ****p < 0.05, respectively as compared with the group treated with CCl₄ alone (one-way ANOVA followed by Tukey’s multiple comparison test). MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase.

In conclusion, the hepatoprotective activity of Chenopodium album leaves against CCl₄-induced hepatic toxicity was evaluated. Our results demonstrated that ethanol extract (CAEE) possessed significant protection against CCl₄-induced hepatotoxicity, which might be associated with its antioxidant properties through scavenging free radicals to ameliorate oxidative stress and inhibit lipid peroxidation. The phytochemical analysis revealed the high content of phenolics and flavonoids in ethanol extract, which might be responsible for its stronger biological activities.

In vitro studies.

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References


