Role of Cressa cretica on CCl₄-induced liver damage in experimental rats

Dharmendra Singh, PV Arya, Vikram Singh, Dharmendra Arya, Kiran Bhagour and RS Gupta

Abstract
The present study was undertaken to evaluate the role of Cressa cretica aerial part’s extract on tissue-lipid peroxidation, antioxidant status and serum markers in experimental rat liver damage. The liver was damaged in experimental rats with the administration of 1:1 (%v/v) mixture of CCl₄ (carbon tetrachloride) in olive oil at the dose level of 1 ml/kg body weight once a week for 1 week. The methanol extract of Cressa cretica (50mg/kg and 100mg/kg) and standard drug- silymarin (25mg/kg) were given orally to rats from day 1 to 21. The extract of C. cretica produced significant (P<0.001) dose-dependent protective role on the altered hepatic- SOD (superoxide dismutase), CAT (catalase), GSH (glutathione reduced), GPx (glutathione peroxidase), LPO (lipid peroxidation) and cytochrome P-450 enzyme when compared with the toxic controls. Serum was also analyzed for AST, ALT (transaminases), ALP (alkaline phosphatase), γ-GTP (γ-glutamyl transpeptidase), LDH (lactate dehydrogenase), total bilirubin and total protein levels. Cressa cretica extract also play a protective role by decreasing the activity of serum marker enzymes, total bilirubin and by increasing the total protein levels. The results were comparable with the standard drug- silymarin. From the above results, it can be concluded that the observed hepatoprotective role of Cressa cretica extract might be explained by its strong antioxidant capacity and ability of oxidative stress reduction due to the presence of triterpenoid saponins, alkaloids, total phenolics and flavonoids in the extract and/or its isolated purified compounds – n-octacosanol-1, β-sitosterol, 6-hydroxy-3,4-dimethyl coumarin, 6-methoxy-7,8-methylene dioxy coumarin, β-sitosterol-glucoside, quercetin, kaempferol and rutin.

Keywords: Antioxidants, CCl₄, Cressa cretica, liver damage, marker enzymes

1. Introduction
Liver is a vital organ which plays a principal role in the metabolic processes of mammals and has an amazing regenerative capability after its mass loss by the numerous chemicals or infective agents intake within an acceptable range [1]. If intolerable or untreated, the chemicals or infective agents may lead to progressive liver fibrosis and ultimately cirrhosis and then liver failure [1, 2]. Carbon tetrachloride (CCl₄) is widely used as a chemical toxicant for experimental liver damage. The principle cause of CCl₄ is induced hepatic damage through lipid peroxidation, decreased the activities of antioxidant enzymes and generation of free radicals [3]. However, no effective allopathic practice that interrupts the disease progression and complications has yet been found [4]. Although the medicinal plants and their phytoconstituents, belonged to more than 40 families in India have been also explored as liver protective agents. The plant Cressa cretica L. belongs to family Convolvulaceae; commonly known as ‘Rudanti’ or ‘Nadewa’ in Hindi is an erect, small, dwarf shrub, grows in sandy or muddy saline habitats usually in mono specific stands along the landward edge of marshes and distributed throughout the tropical and sub-tropical regions of the world like India, Timor, and Australia (Western Australia, Northern Territory, Southern Australia, Queensland, New South Wales, Victoria) etc. [5, 6]. Traditionally, the plant is used as an anthelmintic, antitubercular, expectorant, stomachic, antilulious, tonic and aphrodisiac purposes, enhance the blood constituents and also beneficial for leprosy, asthma, diabetes, urinary discharges, and constipation [5, 6]. The dry leaves of Cressa cretica crushed with sugar are used as emetic in Sudan and also fruits have been reported to possess antitubercular and antimicrobial activities [5, 6]. Some fractions of Cressa cretica also showed in vitro hepatoprotective [7] and in vitro antioxidant activities [8]. Although, the in vivo hepatoprotective role through antioxidant status of this plant is not experimentally established till today. Therefore, the present study was carried out to determine the hepatoprotective role of Cressa cretica on CCl₄-induced liver damage in experimental rats.
2. Materials and methods
2.1 Plant material
The fresh aerial parts of Cressa cretica L. were collected from the chapparwada dam area, dudu, Jaipur, India, in the month of July to September, 2010. The plant was identified and authenticated by the Department of Botany, University of Rajasthan, Jaipur, India (Herbarium Sheet No. RUBL-17873).

2.2 Extraction, isolation and characterization of compounds
The plant material was shade dried, crushed to a rough powder and treated with petroleum ether for defatting as well as to remove chlorophyll. The powder was packed into a soxhlet apparatus and subjected to hot continuous percolation using methanol as solvent for 48 hours at 58–60 °C. The extract was concentrated under vacuum, dried in a vacuum desiccator, and yielded 6.7% *w*/*w* as a dark greenish-brown solid mass. The solid mass was then powdered, and washed with chloroform to remove the remaining content of chlorophyll as well as fatty portions presented in the extract. Half of the extract was suspended in a proper volume of olive oil to prepare the desired concentration for oral administration to rats.

Rest of the extract (35g) was subjected to traditional column chromatography for fractionation with different solvents. For this purpose, a column (1.4 m × 5 cm) filled with 800g Si-gel (60–120 mesh) was used. The purity of fractions was checked by qualitative thin layer chromatography using different solvent systems. After determining the purity of compounds, it was subjected to detailed spectral analysis (IR, 1H NMR, 13C NMR and MS) to establish the structures of yielded compounds [data not shown]. As a result, the compounds- n-octacosanol-1, β-sitosterol, 6-hydroxy-3,4-dimethyl coumarin, 6-methoxy-7,8-methylene dioxy coumarin, β-sitosterol-glucoside, quercetin, kaempferol and rutin were isolated, purified and characterized [9-14].

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of Compound</th>
<th>Solvent system</th>
<th>Melting point in °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>n-octacosanol-1</td>
<td>Pet ether : Benzene (3:1)</td>
<td>82°</td>
</tr>
<tr>
<td>2.</td>
<td>β-sitosterol</td>
<td>Chloroform : Benzene (1:1)</td>
<td>136°-137°</td>
</tr>
<tr>
<td>3.</td>
<td>6-hydroxy-3,4-dimethyl coumarin</td>
<td>Chloroform : Ethyl acetate (3:1)</td>
<td>219°-221°</td>
</tr>
<tr>
<td>4.</td>
<td>6-methoxy-7,8-methylene dioxy coumarin</td>
<td>Chloroform : Ethyl acetate (1:3)</td>
<td>258°-259°</td>
</tr>
<tr>
<td>5.</td>
<td>β-sitosterol-glucoside</td>
<td>Ethyl acetate</td>
<td>285°-287°</td>
</tr>
<tr>
<td>6.</td>
<td>Quercetin</td>
<td>Acetone : Methanol (1:1)</td>
<td>301°-302°</td>
</tr>
<tr>
<td>7.</td>
<td>Kaempferol</td>
<td>Methanol</td>
<td>274°-276°</td>
</tr>
<tr>
<td>8.</td>
<td>Rutin</td>
<td>Methanol</td>
<td>189°-190°</td>
</tr>
</tbody>
</table>
2.3 Experimental animals
Colony bred healthy, adult male albino rats (wistar strain) (Rattus norvegicus) weighing 150–160g, were used in the present study. The rats were housed in polypropylene cages under controlled conditions of temperature (25±3 °C), humidity (55%-65%) and light (12h light/dark cycle). They were provided with a nutritionally adequate standard laboratory diet (Lipton, India Ltd., Bangalore, India) and tap water ad libitum.

2.4 Ethical aspects
The study was approved by the ethical committee (Protocol No. 1678/G0/a/12/CPCSEA/121) of the University Department of Zoology, Jaipur, India. Indian National Science Academy, New Delhi, (INSA, 2000) guidelines were followed for maintenance and use of the experimental animals.

2.5 Toxicological study
The extract of plant material was administered to all the test groups in graded doses ranging up to 2g/kg body weight and the rats were observed for signs of toxicity and mortality for 30 days afterward. The extract was found to be practically non-toxic when given orally to rats and its LD50 value was found to be higher than 2g/kg body weight [data not shown] [15]. The minimum dose levels viz. 50mg and 100mg/kg body weight were used for oral administration to rats during experimentation.

2.6 Chemicals
All chemicals were analytical grade and chemicals required for all biochemical assays were obtained from Sigma Chemicals Co., St. Louis, MO, USA.

2.7 Silymarin
Silymarin was purchased from MP Biomedicals, France and it was dissolved in olive oil for oral administration to rats during experimentation at the dose level-25 mg/kg body weight/day [15].

2.8 Experimental design
After acclimatization of 15 days, the rats were divided into the following groups containing 06 animals in each group:

Group I: Vehicle treated rats were kept on normal diet and served as control for 21 days.

Group II: Rats intoxicated with CCl4 (1 ml/kg body weight/once a week with olive oil, 1:1, intra-peritoneally) for 21 days.

Group III: Rats orally received Cressa cretica extract at 50 mg/kg body weight/day, and ccl4 as Group II for 21 days, simultaneously.

Group IV: Rats orally received Cressa cretica extract at 100 mg/kg body weight/day, and ccl4 as Group II for 21 days, simultaneously.

Group V: Rats orally received silymarin at 25 mg/kg body weight/day, and ccl4 as Group II for 21 days, simultaneously.

2.9 Autopsy schedule
After the last dose-delivery, rats of each group were kept on starvation for 24h and after that anaesthetized under mild ether anesthesia. Blood samples were collected by cardiac puncture of each animal in sterilized vials and allowed to clot at 37 °C. Serum was separated by centrifugation then stored at 4 °C until assayed.

After the collection of blood, The entire liver was perfused immediately with cold physiological saline and thereafter carefully removed, trimmed free of extraneous tissues, weighed on an electrical balance and immediately frozen (at −20°/−70 °C) for biochemical analysis.

2.10 Serum biochemistry
Biochemical assay of marker enzymes, viz. AST (aspartate transaminase), ALT (alanine transaminase), ALP (alkaline phosphatase), γ-GTP (gamma glutamyl transpeptidase), LDH (lactate dehydrogenase), along with total bilirubin and total protein were performed by using colorimetrically kit methods. AST (batch no. 61105), ALT (batch no. 60805) γ-GTP (batch no. 34004) kits were purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India. LDH (lot no. 6854), ALP (lot no. 7093), total bilirubin (lot no. 6801), and total protein (lot no. 6808) kits were purchased from Span Diagnostic Pvt. Ltd., Surat, India.

2.11 Liver biochemistry
The quantitative estimation of SOD (superoxide dismutase) [16], CAT (catalase) [17], GSH (glutathione reduced) [18], GPx (glutathione peroxidase) [19], and LPO (lipid peroxidation) [20] were performed in liver homogenate. Further, a liver microsomal fraction was prepared and the cytochrome P-450 content in this fraction was measured from a reduced carbon monoxide difference spectrum [21, 22], respectively.

2.12 Statistical analysis
The results obtained in the present study were expressed as the mean ± SEM for each parameter and statistically processed by applying student “t” test. p values ≤0.05 were considered as significant.

3. Results
The results of biochemical parameters revealed that the administration of carbon tetrachloride (CCL4) to rats caused significant (P≤0.001) hepatic damage through oxidative stress as evidenced by hepatic antioxidant defense system and serum marker enzymes after 21 consecutive days of experiment (Table I and II).

Table I depicts that the hepatic SOD, CAT, GSH, and GPx contents were elevated significantly (P≤0.001) after the experimental rats treated with Cressa cretica extract at the dose level-100 mg/kg body weight/day (group IV) but exhibited non-significant to significant (P≤0.05 & P≤0.001) elevation in the contents of all above antioxidant parameters at the dose level-50 mg/kg body weight/day (group III) whereas CCL4-intoxicated group II was shown highly significant (P≤0.001) decrease in the all above antioxidant contents as compared to normal controls after 21 successive days of experimentation.

The dose level-100 mg/kg body weight/day of Cressa cretica extract was showed as most effective dose level but the elimination of hepatic oxidative stress was not as high as in silymarin treated rats of group V (Table I). Table I also showed a significant (P≤0.001) increase in the level of hepatic lipid peroxidation (LPO) in CCL4 intoxicated rats as compared to normal controls. In contrast, treatment with Cressa cretica extract showed a notable (P≤0.05; P≤0.001) inhibition in the rise of LPO content at both the

~ 1370 ~
dose levels (group III & IV). The silymarin treated group V also showed statistically similar decline in the level of hepatic LPO as compared to higher dose level (100mg/kg) of Cressa cretica extract treated group IV after 21 consecutive days of experimentation.

The treatment with CCl₄ declines the hepatic enzyme-cytochrome P-450 level in group II as compared to group I. The significant (P<0.001) protection against CCl₄ induced alterations in this enzyme level was achieved with Cressa cretica extract and silymarin extract (Treatment Table I). The significantly equal protection was showed in Cressa cretica extract and silymarin treated groups against CCl₄ induced hepatic anomalies.

Table II illustrates that the activities of AST, ALT, ALP, γ-GTP, LDH, total bilirubin and total protein levels in serum were significantly (P≤0.001) normalized after the Cressa cretica extract treated experimental rats at the dose level-100 mg/kg body weight/day (group IV) but showed non-significant to significant (P≤0.05; P≤0.01; P≤0.001) protection in the contents of all above serum-enzymes, total bilirubin and total proteins at the dose level-50 mg/kg body weight/day (group III) whereas CCl₄-intoxicated group II was showed highly significant (P≤0.001) alterations in the all above serum markers as compared to normal controls after 21 successive days of experimentation. Therefore, the dose level-100 mg/kg body weight/day of Cressa cretica extract also exhibited again as a most effective dose level but the regularization of all above altered serum parameters was not as high as in silymarin treated rats of group V (Table II).

Table 1: Showing role of Cressa cretica extract and silymarin on CCl₄-induced liver damage in experimental rats through antioxidants defense and enzymatic levels

<table>
<thead>
<tr>
<th>Treatment design</th>
<th>SOD (µ mole/ mg protein)</th>
<th>CAT (µ mole H₂O₂ consumed/min/ mg protein)</th>
<th>GSH (n mole/g tissue)</th>
<th>GPx (n mole NADPH consumed/min/ mg protein)</th>
<th>LPO (n mole MDA/ mg protein)</th>
<th>Cytochrome-P-450 (n mole/ mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle treated) Group I</td>
<td>12.28 ± 0.44</td>
<td>65.32 ± 2.87</td>
<td>5.22 ± 0.27</td>
<td>15.39 ± 0.26</td>
<td>1.92 ± 0.08</td>
<td>5.21 ± 0.18</td>
</tr>
<tr>
<td>CCl₄ (1 ml/kg b wt, ip, once a week with olive oil, 1:1) Group II</td>
<td>5.82 ± 0.16a</td>
<td>36.10 ± 1.98a</td>
<td>2.32 ± 0.13a</td>
<td>8.42 ± 0.19a</td>
<td>5.12 ± 0.18a</td>
<td>2.08 ± 0.14a</td>
</tr>
<tr>
<td>CCl₄ + C. cretica extract (50 mg/kg b wt/day, orally) Group III</td>
<td>7.14 ± 0.13a</td>
<td>44.12 ± 2.12c</td>
<td>2.99 ± 0.33as</td>
<td>10.12 ± 0.29a</td>
<td>4.59 ± 0.14c</td>
<td>3.21 ± 0.15a</td>
</tr>
<tr>
<td>CCl₄ + C. cretica extract (100 mg/kg b wt/day, orally) Group IV</td>
<td>10.58 ± 0.21a</td>
<td>55.15 ± 2.24a</td>
<td>4.98 ± 0.27a</td>
<td>12.75 ± 0.26a</td>
<td>3.10 ± 0.10a</td>
<td>4.29 ± 0.22a</td>
</tr>
<tr>
<td>CCl₄ + Silymarin (25 mg/kg b wt/day, orally) Group V</td>
<td>11.68 ± 0.32a</td>
<td>61.21 ± 2.12a</td>
<td>6.30 ± 0.34a</td>
<td>14.82 ± 0.27a</td>
<td>2.21 ± 0.12a</td>
<td>4.89 ± 0.15a</td>
</tr>
</tbody>
</table>

Levels of significance: Data are mean ± SEM (n = 6)

**Discussion**

This study deals with the liver protective role of Cressa cretica extract against CCl₄ induced hepatic damage in experimental rats. The plant- Cressa cretica, due to the rich source of phytoconstituents like flavonoids, sterols, phenolics and coumarin glycosides, heavy metals, lead, copper, zinc and nickel might be medicinally important and/or nutritionally valuable [6]. It also contains terpenic compounds,
syringaresinol-β-d-deglucoside, triacanontic acid, stigmastanol, ursolic acids, β-amirin, quercetin, n-octacosanol, scopoletin, umbrellerone and edible fixed oil. They are the powerful chain-breaking antioxidants. Therefore, the liver protective activity through antioxidant status observed in this study might be due to a combined effect of some identified and/or unidentified phytoconstituents but according to literature survey, most of the antioxidant activity showed by the presence of phenolic compounds because the phens have strong free radical scavenging ability due to their hydroxyl groups contribution directly to antioxidative action. Therefore, in general, phenolic compounds from plants are known to be good natural antioxidants.

Carbon tetrachloride (CCl₄) does not occur naturally in the environment. It is only a manufactured clear liquid chemical that diffuses very easily in the hydrosphere as well as atmosphere during its production. If carbon tetrachloride consumed directly or indirectly by the animal world, it may cause various health hazards including hepatic oxidative damage through a number of mechanisms. Briefly, CCl₄ can induce liver damage through the formation of reactive free radicals which are catalyzed by P-450 that can bind covalently to cellular macromolecules forming nucleic acid, proteins and lipids or of abstracting a hydrogen atom from an unsaturated lipid, which initiate lipid peroxidation and finally inhibit the protein synthesis resulting inflammation, centriobular steatosis, apoptosis, necrosis and if the damage exceeds the repair capacity of the liver, the liver will progress to fibrosis and cirrhosis. Therefore, the suppression of P-450 can result in a reduction in the level of reactive metabolites, and correspondingly, less tissue injury. The metabolic activation of CCl₄ is believed to be mediated through P 450 2E1.

The inhibitory effect of CCl₄ on cytochrome P-450 level was also compensated by Cressa cretica extract (dose-dependently) and silymarin through maintenance of its standard level. The role of Cressa cretica extract in the defense of CCl₄-mediated loss in cytochrome P-450 content might be deliberated as a sign of improved protein synthesis in the hepatic system.

The lipid peroxidative deprivation of biological membranes is one of the standard causes of liver damage induced by CCl₄. Because the lipid peroxidation is observed as a complex biochemical reaction involving free radicals, oxygen, metal ions and a host of other factors in the biological system of liver cells. Therefore, the measurement of lipid peroxidation in the liver tissue is a convenient method to monitor oxidative cell damage. Inhibition of elevated lipid peroxidation has been observed in Cressa cretica extract (dose-dependently) and silymarin treated groups due to its antioxidant and free radical scavenging activities through reestablishment of biological membranes of liver parenchymal cells.

The antioxidant defense system has been suggested to play a significant role in sustaining the functional physiology of oxygen and hydrogen peroxide and also eradicating peroxides generated from unintended exposure of various toxicants and drugs. Any herbal medicine with antioxidant assets may help in maintaining health when constantly taken as components of dietary food and/or medications. Therefore, the improved level of experimental animal’s antioxidant profile i.e. superoxide dismutase, catalase, glutathione reduced and glutathione peroxidase by Cressa cretica extract (dose-dependently) and silymarin might be endorsed to have biological significance for the removal of reactive free radicals that may distress the regular functioning of liver cells.

Although, superoxide dismutase and catalase activation is readily suppressed by the reactive oxygen species and/or elevated lipid peroxides induced by CCl₄ hepatotoxicity which results in declined the activities of these antioxidant enzymes. Because, superoxide dismutase is a first step of defense mechanism in the antioxidant system against oxidative stress by catalyzing the dismutation of superoxide radicals (O₂⁻) into molecular oxygen (O₂) and hydrogen peroxide (H₂O₂). Further, H₂O₂ is neutralized by the action of catalase enzyme. Therefore, a noteworthy reduction in the activities of hepatic superoxide dismutase and catalase during CCl₄ hepatotoxicity might be due to the enhanced superoxide radical formation leading to hepatic oxidative damage. Oral treatment with Cressa cretica extract to experimental rats improved the superoxide dismutase and catalase activities by acting as a strong free radical quencher in the hepatic cells. Therefore superoxide dismutase and catalase enzymes are essential to maintain the cellular redox balance of endogenous antioxidant defense system.

Glutathione reduced is a most inter convertible tripeptide, non-enzymatic biological antioxidant and a key factor of the complete antioxidant defense system that protects the membrane protein thiol of hepatic cells from destructive effects of reactive oxygen species. Although, glutathione peroxidase enzyme along with catalase metabolize H₂O₂ to water and other non-toxic ingredients and also consists of glutathione reduced and a range of functionally interrelated enzymes, of which glutathione peroxidase is liable for the renewal of glutathione reduced or from GSSG to GSH, where glutathione peroxidase enzyme work with glutathione reduced in the breakdown of hydrogen peroxide and/or other biological hydroperoxides. Declined Glutathione reduced and glutathione peroxidase enzyme levels during the CCl₄ hepatotoxicity might be due to its extreme exploitation by generated reactive oxygen metabolites in the hepatic cells. However, subsequent dose-dependently retrieval of glutathione reduced and glutathione peroxidase enzyme in the experimental rats treated with Cressa cretica extract has been noticed. It might be due to de-novo GSH synthesis or GSH regeneration (GSSG to GSH) through the Cressa cretica extract’s antioxidant potential by the scavenging of endogenous metabolic peroxides.

According to previous results, our present experimental study showed a momentous increase in the activities of AST, ALT, ALP, γ-GTP and LDH in serum with the exposure of CCl₄ which indicates substantial hepato-parenchymal damage. Oral treatment with Cressa cretica extract attenuated these augmented enzyme profiles created by CCl₄ in serum and a consequent retrieval near to stabilization of these enzymes strongly suggests the possibility of Cressa cretica extract being able to state the hepatic cells so as to cause quicker regeneration of hepato-parenchymal cells and lysosomes, thus defending against lysosomal integrity and cell membrane fragility decreasing the leakage of marker enzymes into the blood circulation. Stabilization near to normal level of serum-total bilirubin and total protein through oral route of Cressa cretica extract is further a strong sign of improvement of functional status of hepato-parenchymal cells.
5. Conclusion
The hepatoprotective role of *Cressa cretica* extract might be explained by its strong antioxidant capacity and ability to oxidative stress reduction due to the presence of alkaloids, triterpenoid saponins, flavonoids and total phenolics in the extract and/or its isolated purified compounds like - n-octacosanol-1, β-sitosterol, 6-hydroxy-3,4-dimethyl coumarin, 6-methoxy-7,8-methylene dioxy coumarin, β-sitosterol-glucoside, quercetin, kaempferol and rutin, by quenching lipid peroxidation and restoration of antioxidant mechanism against oxidative stress due to their hydroxyl group’s contribution directly to antioxidant potential of hepato-parenchymal cells. However, further studies pertaining to possible mechanism of action of isolated pure compounds are required, which are ongoing in our laboratory.

6. Conflicts of Interest: The authors declare no conflict of interest.

7. Acknowledgments: The authors are thankful to the respective authorities for providing the necessary facilities and support.

8. References