Hepatoprotective and antioxidant activities of flowers of *Calotropis procera* (Ait) R. Br. in CCl₄ induced hepatic damage

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Hepatoprotective activity of 70% ethanolic extract of flowers of *C. procera* was studied against CCl₄ induced hepatic injury in albino rats and mice. In addition, antioxidant activity was studied by *in vitro* models. Pre-treatment with 70% ethanolic extract (CPA) reduced the biochemical markers of hepatic injury like serum glutamate pyruvate transaminase, serum glutamate oxaloacetate transaminase, alkaline phosphatase, bilirubin, cholesterol, HDL and tissue glutathione (GSH) levels. Similarly pretreatment with CPA reduced the CCl₄ induced elevation in the pentobarbitone sleeping time. Histopathological observations also revealed that pretreatment with CPA protected the animals from CCl₄ induced liver damage. CPA demonstrated dose dependent reduction in the *in vitro* and *in vivo* lipid peroxidation induced by CCl₄. In addition it showed dose dependant free radical scavenging activity. The results indicate that flowers of *C. procera* possess hepatoprotective property possibly because of its anti-oxidant activity. This property may be attributed to the quercetin related flavonoids present in the flowers of *Calotropis procera*.

**Keywords:** Anti-oxidant, *Calotropis procera*, CCl₄ Hepatoprotective

Liver, the largest organ in the body is being evolved to maintain the body’s internal milieu and also protect itself from the challenges it faces during its functioning. Since it is involved in the biochemical conversions of various endogenous and exogenously administered/ingested substances, there is a possibility of generation of various highly reactive species of free radicals. However, it has an inbuilt system like tissue glutathione (GSH), etc to scavenge them off. Inspite of this the free radicals generated by some hepatotoxins like CCl₄ may overpower the protective mechanism of the liver and cause hepatic damage. Though the modern medicinal system has grown phenomenally, the drug for treating hepatic disease is still a dream. Hence, people are looking at the traditional systems of medicine for remedies to hepatic disorders.

*Calotropis procera* (Ait) R. Br. possesses flavonoids, alkaloids, cardiac glycosides, tannins, sterols and triterpines¹. Flowers of *C. procera* possess anti-inflammatory, antipyretic, analgesic, antimicrobial properties and larvicidal activity²,³. The latex of the plant was reported to possess analgesic and wound healing activity⁴,⁵. The roots are reported to have anti-fertility and anti-ulcer activities⁶,⁷. The flowers are reported to contain flavonoids, quercetin-3-rutinoside, sterols, etc⁸. However, there are no reports regarding the hepatoprotective and anti-oxidant activity of the flowers of this plant. Flavonoids are reported to possess anti-oxidant and hepatoprotective properties⁹. Since, the flowers contain flavonoids, the present study has been undertaken to screen for hepatoprotective activity of the flowers of *Calotropis procera* and to verify the claim using CCl₄ induced hepatic injury model in rats. With a view to establish the mechanism of action, anti-oxidant activity has also been carried out.

**Materials and Methods**

Plant material—The flowers were collected in the month of September from the suburban fields of Harapanahalli. K Prabhu, Department of Pharmacognosy, SCS College of Pharmacy identified the flowers and the herbarium specimen
Preparation of extract—The coarse powder of shade-dried flowers of *C. procera* was extracted with 70% alcohol in a soxhlet extractor for 16 hr. Thus obtained extract was concentrated under reduced pressure in a rotary vacuum evaporator and the yield was 20% (calculated on the basis of volume of solvent added and the weight of the dry powder used).

Animals—Albino rats (125-175 g) and mice (18-25 g) of either sex were obtained from National Institute of Mental Health and Neuro Sciences, Bangalore and were kept in standard plastic animal cages in groups of 6-8 animals, with 12 hr of light and dark cycle in the institutional animal house. The animals were fed with standard rodent diet and provided water *ad libitum*. After one week of acclimatization the animals were used for further experiments. Approval from the institutional animal ethical committee for the usage of animals in the experiments was obtained as per the Indian CPCSEA guidelines.

Antioxidant activity—Reducing power of 70% ethanolic extract of *C. procera* (CPA) was carried out as per Oyaizu\(^\text{10}\). Different doses (5, 10, 25, 50 and 100 μg) of CPA were prepared and 1 ml of each solution was mixed with phosphate buffer (2.5 ml., 0.2 M, pH 6.6) and potassium ferricyanide \([K_3Fe(CN)_6; 2.5 \text{ ml } 1\%]\). The mixture was incubated at 50°C for 20 min. To this mixture 2.5 ml of 10% trichloroacetic acid was added and then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl\(_3\) (0.5 ml, 0.1%) was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The % increase in the absorbance was calculated.

The measurement of superoxide anion scavenging activity of CPA was carried out using the method of Nishimiki modified by Ilhams et al\(^\text{11}\). Nitroblue tetrazolium (NBT) solution (1 ml 156 μM NBT in 100 mM of phosphate buffer, pH 7.4), nicotinamide adenine dinucleotide (NADH) (1 ml, 468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of CPA (5, 10, 25, 50, 100 μg) in water were mixed. The reaction was initiated by adding 10 μl of phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm. Decrease in absorbance of the reaction mixture indicated increase in superoxide anion scavenging activity. The % decrease in absorbance of the reaction mixture was calculated.

Hydroxyl radical generation by phenyl hydrazine was measured by the 2-deoxyribose degradation assay of Hathwell and John Gutteridge method\(^\text{12}\). Solutions of 1 mM deoxyribose and 0.2 mM phenyl hydrazine hydrochloride were prepared in 50 mM phosphate buffer (pH 7.4). Deoxyribose 0.6 ml (1 mM) and 0.4 ml of CPA (varying doses 5, 10, 25, 50 and 100 μg) or sodium metabisulphate (25 μg, standard) were taken and phosphate buffer was added to make the volume up to 1.6 ml. After 10 min of incubation, 0.4 ml of 0.2 mM phenyl hydrazine hydrochloride was added. Incubation was terminated after 1 and 4 hr. To this mixture 1 ml each of 2.8% TCA and 1% w/v thiobarbituric acid were added and the mixture was heated for 10 min in a boiling water bath. The tubes were cooled and absorbance was taken at 532 nm. Decrease in absorbance indicated the increase in hydroxyl free radical scavenging activity. The % of decrease in absorbance was calculated.

The inhibition of *in vitro* lipid peroxidation was determined by using the method of Fairthurst et al\(^\text{13}\). A solution of 30% rat liver homogenate in ice cold KCl (0.15 M) was prepared by using homogeniser and 0.5 ml of the homogenates were transferred to small conical flasks. To the homogenates 10 μl of CCl\(_4\) was added. To these mixtures 1.5 ml of 0.15M KCl and different doses of CPA (10, 25, 50 and 100 μg) were added. To the control flask only 0.5 ml of vehicle (phosphate buffer) was added. The flasks containing mixtures were incubated at 37°C in constant shaker bath (150 cycles/min) for 45 min. After incubation the reaction was stopped by addition of 4 ml of 10% w/v trichloroacetic acid. The mixtures were centrifuged; 2 ml of thiobarbituric acid (0.68% w/v) was added to 2 ml of supernatant prior to heating in water bath for 15 min. The colour was stabilized with KOH and the absorbance was measured at 543 nm. Decrease in the elevated absorbance due to addition of CCl\(_4\) indicates the reduction in the lipid peroxidation. The % reduction in the absorbance was calculated which is proportional to the reduction in the lipid peroxidation.

To evaluate the hepatoprotective activity of CPA, the method of Suja et al\(^\text{14}\) was adopted. In this experiment 24 hr fasted animals were randomly divided into 5 groups of 6 animals each. Animals of
group I and II received 1 ml of distilled water, whereas animals of group III, IV and V received silymarin (100 mg/kg), CPA (200 mg/kg) and CPA (400 mg/kg) respectively for 5 days orally. Animals of group I received 1 ml/kg of liquid paraffin on 2nd and 3rd day, whereas animals of group II, III, IV and V received 2 ml CCl₄: paraffin (1:1) subcutaneously on 2nd and 3rd day 30 min after treatment with vehicle, silymarin or test extract. Animals were sacrificed under mild ether anesthesia. Blood samples and liver tissue were collected.

Blood samples collected from the above mentioned group of animals were used for the estimation of biochemical markers of liver damage like serum glutamate pyruvate transaminase (SGPT)₁⁵, serum glutamate oxaloacetate transaminase (SGOT)₁⁶, alkaline phosphatase (ALP)₁⁷, serum bilirubin₁₈, cholesterol₁⁹ and HDL₂₀.

Livers slices were collected from the above groups of animals were subjected to the determination of tissue glutathione (GSH) level and lipid peroxidation. Tissue GSH levels were estimated by using the method of Ellamn and modified by Aykae et al²¹,²². Similarly inhibition of CCl₄ induced tissue lipid peroxidation was done using the method of Buege and Steven²³.

**Effect on pentobarbitone sleeping time**—This test was performed to find out the influence on the CCl₄ altered functional performance of the liver. Albino mice of either sex were randomly divided into 6 groups of 6 animals each. The animals of groups I were administered pentobarbitone (40 mg/kg ip) and noted the duration of sleep (duration of loss of righting reflex) was determined. The animals of group II, III, IV, V and VI received ip, CCl₄ (CCl₄: paraffin, 1:1, 4 ml/kg), silymarin (100 mg/kg), CPA 100, 200 and 400 mg/kg respectively. Half an hour after the respective treatments, animals of group II, III, IV, V and VI were administered with pentobarbitone (40 mg/kg ip) and sleeping times were determined.

**Statistical analysis**—Results were expressed as mean ± SE. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Student’s ‘t’ test. P value less than 0.05 was considered to be statistically significant when compared to control.

**Histopathological observations**—Liver tissue collected were used for the preparation of histopathological slides by using microtome and were suitably stained and observed under microscope for architectural changes seen during CCl₄ challenge in CPA treated and control groups.

**Results and Discussion**

Antioxidant activity of CPA in four in vitro models was studied. CPA demonstrated dose dependant antioxidant property in all the models. However, CPA showed better reducing power than the standard i.e. sodium metabisulphate (25 μg). In all other models (i.e. superoxide anion scavenging, hydroxyl free radical scavenging and inhibition of in vitro lipid peroxidation) antioxidant activity of even 100 μg of CPA was less than that of standard (sodium metabisulphate, 25 μg). (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reducing power</th>
<th>Superoxide anion scavenging</th>
<th>Hydroxyl radical scavenging</th>
<th>In vitro lipid peroxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1173 ± 0.015</td>
<td>0.6885 ± 0.012</td>
<td>0.3496 ± 0.0211</td>
<td>0.2465 ± 0.015</td>
</tr>
<tr>
<td>CCl₄ 10 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.3515 ± 0.055</td>
</tr>
<tr>
<td>Std. (25 μg)</td>
<td>0.2163 ± 0.013*</td>
<td>0.1757 ± 0.015*</td>
<td>0.1096 ± 0.0192*</td>
<td>0.0570 ± 0.011*</td>
</tr>
<tr>
<td>CPA (5 μg)</td>
<td>0.1824 ± 0.020*</td>
<td>0.4887 ± 0.018*</td>
<td>0.2700 ± 0.0193*</td>
<td>0.2213 ± 0.012*</td>
</tr>
<tr>
<td></td>
<td>(+84.39)</td>
<td>(-74.28)</td>
<td>(-68.64)</td>
<td>(-83.37)</td>
</tr>
<tr>
<td>CPA (10 μg)</td>
<td>0.1644 ± 0.025*</td>
<td>0.4484 ± 0.020*</td>
<td>0.2580 ± 0.0199*</td>
<td>0.2018 ± 0.019*</td>
</tr>
<tr>
<td></td>
<td>(+55.58)</td>
<td>(-29.01)</td>
<td>(-22.76)</td>
<td>(-37.04)</td>
</tr>
<tr>
<td>CPA (25 μg)</td>
<td>0.1903 ± 0.027*</td>
<td>0.3676 ± 0.020*</td>
<td>0.2022 ± 0.0221*</td>
<td>0.1912 ± 0.020*</td>
</tr>
<tr>
<td></td>
<td>(+62.31)</td>
<td>(-46.59)</td>
<td>(-42.16)</td>
<td>(-45.60)</td>
</tr>
<tr>
<td>CPA (50 μg)</td>
<td>0.2294 ± 0.019*</td>
<td>0.3154 ± 0.012*</td>
<td>0.1715 ± 0.0300*</td>
<td>0.1521 ± 0.021*</td>
</tr>
<tr>
<td></td>
<td>(+95.65)</td>
<td>(-54.19)</td>
<td>(-50.94)</td>
<td>(-56.72)</td>
</tr>
<tr>
<td>CPA (100 μg)</td>
<td>0.3105 ± 0.011*</td>
<td>0.2193 ± 0.025*</td>
<td>0.1614 ± 0.0310*</td>
<td>0.1023 ± 0.025*</td>
</tr>
<tr>
<td></td>
<td>(+165.13)</td>
<td>(-68.13)</td>
<td>(-53.30)</td>
<td>(-70.89)</td>
</tr>
</tbody>
</table>

*Significant at P<0.001 Compared to control, Std. = Sodium. metabisulphate
In the control group (+ve control) CCl₄ significantly enhanced the biochemical markers like SGPT, SGOT, ALP, cholesterol, and bilirubin and reduced the levels of HDL. Pretreatment with CPA (200 and 400 mg/kg) reduced the elevated levels of all the above mentioned biochemical indicators and enhanced the HDL levels (Table 2). Similarly CCl₄ reduced the liver tissue GSH levels and increased the tissue lipid peroxidation. Pretreatment with CPA enhanced the tissue GSH levels and reduced lipid peroxidation (Table 3).

Histopathological observations reveal that CCl₄ treatment has damaged the liver architecture (i.e. fatty degeneration of hepatic cells as indicated by ballooning of hepatocytes, fatty cyst, infiltration of lymphocytes and proliferation of Kupffer cells. Liver sinusoids were congested) and pretreatment with CPA prevented/reversed the CCl₄ induced liver damage in a dose dependant manner (Fig. 1).

CCl₄ treatment enhanced the pentobarbitone sleeping time and pretreatment with CPA reversed it (Fig. 2).

CCl₄ induced hepatic damage is due to its cytochrome P-450 enzyme system catalyzed hepatic conversion into highly reactive trichloromethyl radical (CCl₃*), which upon reaction with oxygen radical gives trichloromethyl peroxide radical (OOCCl₃*). This radical forms covalent bond with sulfhydryl group of several membrane molecules like glutathione, which is considered as the initial step in the chain of events leading to lipid peroxidation and hepatic tissue destruction²⁴-²⁷. Extent of decrease in tissue GSH and tissue lipid peroxidation is a measure of tissue destruction²⁹. CPA demonstrated potent superoxide and other free radical scavenging property. Therefore, it may be inferred that antioxidant property of the study plant may prevent the formation of trichloromethylperoxide radical. Thereby inhibit the lipid peroxidation and offer hepatoprotection against CCl₄ challenge. Prevention of tissue GSH depletion by CPA treatment also indicates that the natural inbuilt tissue protective mechanism is kept intact and oxidative degeneration of tissue is prevented. However it is necessary to rule out the inhibitory effect on the cytochrome P-450 enzyme system.

Table 2—Effect of 70% ethanolic extract of flowers of CPA on biochemical parameters of CCl₄ induced hepatic injury
[Values are mean ± SE from 6 animals in each treatment]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SGPT (U/l) ± SE</th>
<th>SGOT (U/l) ± SE</th>
<th>ALP (U/l) ± SE</th>
<th>Total bilirubin (mg/dl) ± SE</th>
<th>Direct bilirubin (mg/dl) ± SE</th>
<th>Cholesterol (mg/dl) ± SE</th>
<th>HDL (mg/dl) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline 0.5 ml po x 7 days)</td>
<td>048.55 ± 2.25</td>
<td>095.30 ± 1.59</td>
<td>126.15 ± 1.82</td>
<td>0.99 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>104.76 ± 3.19</td>
<td>47.43 ± 1.47</td>
</tr>
<tr>
<td>CCl₄ (CCl₄ 2 ml/kg sc)</td>
<td>296.46 ± 1.46</td>
<td>481.00 ± 1.80</td>
<td>250.50 ± 1.80</td>
<td>4.23 ± 0.02</td>
<td>1.49 ± 0.03</td>
<td>167.62 ± 4.34</td>
<td>27.60 ± 1.87</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg po, 7 days) + CCl₄</td>
<td>057.77 ± 2.10*</td>
<td>131.75 ± 1.61*</td>
<td>091.79 ± 2.92*</td>
<td>1.24 ± 0.02</td>
<td>0.38 ± 0.02*</td>
<td>118.10 ± 5.04*</td>
<td>44.80 ± 1.54*</td>
</tr>
<tr>
<td>CPA (200 mg/kg po x 7 days) + CCl₄</td>
<td>117.21 ± 2.30*</td>
<td>221.91 ± 1.90*</td>
<td>122.99 ± 2.06*</td>
<td>2.08 ± 0.05*</td>
<td>0.72 ± 0.03*</td>
<td>137.14 ± 4.17*</td>
<td>33.85 ± 1.87</td>
</tr>
<tr>
<td>CPA (400 mg/kg po x 7 days) + CCl₄</td>
<td>89.09 ± 0.87*</td>
<td>152.60 ± 3.16*</td>
<td>81.84 ± 1.90*</td>
<td>1.55 ± 0.01*</td>
<td>0.55 ± 0.01*</td>
<td>120.95 ± 4.08*</td>
<td>40.62 ± 1.80*</td>
</tr>
</tbody>
</table>

*P < 0.01 Vs CCl₄ group, NS = non significant

Table 3—Effect of 70% ethanolic extract of CPA on the liver tissue levels of GSH and lipid peroxidation in the CCl₄ treated rats
[Values are mean ± SE from 6 animals. Figures in parentheses are % increase (+) decrease (-) in absorbance]

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Tissue levels of GSH</th>
<th>Tissue lipid peroxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Saline</td>
<td>0.5 ml</td>
<td>0.9354 ± 0.05</td>
<td>0.2640 ± 0.02</td>
</tr>
<tr>
<td>CCl₄</td>
<td>2 g/kg, po</td>
<td>0.4390 ± 0.04</td>
<td>0.3743 ± 0.06</td>
</tr>
<tr>
<td>Silymarin + CCl₄</td>
<td>100 mg/kg, po</td>
<td>(+91.79)</td>
<td>0.1460 ± 0.02* (−6 0.09)</td>
</tr>
<tr>
<td>CPA + CCl₄</td>
<td>200 mg/kg, po</td>
<td>0.8420 ± 0.05* (−54.37)</td>
<td>0.1833 ± 0.05* (−51.02)</td>
</tr>
<tr>
<td>CPA + CCl₄</td>
<td>400 mg/kg, po</td>
<td>0.6777 ± 0.06* (−55.87)</td>
<td>0.1383 ± 0.04* (−63.05)</td>
</tr>
</tbody>
</table>

*P < 0.01 versus CCl₄ group
Fig. 1—Effect of *C. procera* extract on liver architecture in normal and CCl4 treated rats. [A: liver architecture of negative control (1: central vein, 2: portal tract), B: liver architecture after CCl4 challenge (+ve control) (1: ballooning of hepatocytes, 2: fatty cysts, c: congestion of sinusoids); C: liver architecture after Silymarin (100 mg/kg) + CCl4 challenge (1: lymphocytic infiltration); D: liver architecture after CPA (200 mg/kg)+ CCl4 challenge (1: lymphocytic infiltration, 2: hepatocytes); E: liver architecture after CPA 400 mg/kg + CCl4 Challenge (1: regeneration of hepatocytes, 2: normal hepatic architecture)
are present in CPA and flavonoids are reported to possess antioxidant and hepatoprotective properties. However, further studies are needed for confirmation.

In conclusion, the present study demonstrates that the flowers of Calotropis procera possess hepatoprotective property. In addition, the hepatoprotective property may be attributed to the antioxidant principles of the plant, namely quercetin related flavonoids, tannins and other polyphenolic compounds. Further study is warranted to isolate, characterize and screen the active principles from the flowers of CPA that possess antioxidant and hepatoprotective properties.

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