Hepatoprotective and Antioxidant Activity of Root Bark of *Calotropis procera* R.Br (Asclepiadaceae)

1R. Chavda, 1K.R. Vadalia and 2R. Gokani
1Department of Pharmaceutical Chemistry,
Shree H.N. Shukla Institute of Pharmaceutical Education and Research, Rajkot-360001, India
2Department of Pharmacognosy, S.J. Thakkar College of Pharmacy, Rajkot, India

**Abstract:** In the present study, *Calotropis procera* (Asclepiadaceae) was evaluated for its possible hepatoprotective and antioxidant potential. Hepatoprotective activity of the methanol extract (MCP) and phyto-constituents directed three sub fractions hexane (HCP), ethylacetate (ECF) and chloroform (CCF) of the root bark was determined using carbon tetrachloride (CCL₄) induced liver injury in mice. First the MCP extracts and then three sub fractions namely HCP and ECF and CCF from MCP extract evaluated, at an oral dose of 200 mg kg⁻¹. The animals were weighed each and divided in groups of six. Liver damage was achieved by injecting CCL₄ in olive oil (1:1) 0.8 mL kg⁻¹. The treatment groups pretreated with above extracts. Silymarin was used as reference standard drug. At the end of 7 days, blood was collected, liver extracted, weighed, processed for histopathological assessments and for antioxidant activity. The MCP and its sub fractions HCP and ECF exhibited a significant hepatoprotective effect by lowering the elevated serum levels of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), Alkaline phosphatase (ALP) total and direct serum bilirubin, cholesterol and significantly increasing high density lipoprotein (HDL) and moderately increasing total protein and albumin. While, the CCP fraction does not show significant protective effect. These biochemical observations were supplemented by histopathological examination of liver sections. Further, the effects of the active fractions on antioxidant enzymes also have been investigated to elucidate the possible mechanism of its hepatoprotective activity. The fractions exhibited a significant effect by modifying the levels of reduced glutathione, super oxide dimutase, catalyse activity and malondialdehyde equivalent, an index of lipid peroxidation of the liver. These findings suggest the use of this plant for the treatment of liver toxicity in oriental traditional medicine.

**Key words:** *Calotropis procera*, hepatoprotective, Asclepiadaceae, lipid peroxidation, antioxidant, silymarin, hepatotoxicity

**INTRODUCTION**

Liver is a vital organ of the body. It plays a pivotal role in the metabolism, secretion and storage. Any type of injury or impairment of its functions may leads to many type of complication in one’s health. Unfortunately, Hepatic dysfunction due to ingestion or inhalation of hepatotoxins is increasing worldwide. Management of liver disease is still a challenge to the modern medicine (Reddy et al., 1993). Due limited therapeutic options and disappointing therapeutic success of the modern medicine, uses of herbal drugs has increased worldwide (Stickel and Schuppan, 2007). Numerous medicinal plants and their formulations used for liver disorders in ethnomedical practices and in traditional system of medicine in India (Sethumaran et al., 2003). In this modern age it is very important to provide scientific proof to justify the medicinal uses of herbs. Efficacy of the drugs should be tested by standard experimental methods and there should be adequate data from studies to validate the therapeutic potential (Girish et al., 2009). In the present study, in order to search for a new natural remedy for hepatic disorder, the *Calotropis procera* root bark was evaluated for its possible hepatoprotective activity.

The genus *Calotropis* R. Br (Asclepiadaceae) is distributed in tropical and subtropical region of Asia and Africa, while in India it is represented by two species viz., *Calotropis procera* and *Calotropis gigantea*. *C. procera* is large bushy shrub, more common in southwestern and central India and western himalayas (Phonke, 1992). In India the *C. procera* holds pride of place largely because of its other uses and economical values (Ahmed et al., 2005). The plant is also known for its use in folk medicines. Traditionally, the plant has been used as
Antifungal (Larhsini et al., 1997), antipyretic (Al-Yahya et al., 1985) and analgesic activity (Mohsin et al., 1989). Dried leaves used as an expectorant, as antiinflammatory (Kapur and Sarin, 1984), for treatment of paralysis and rheumatic pains (Sebastian and Bhandari, 1984). Dried latex and dried root used as an antidote for snake poisoning. It is also used as an abortifacient (Basu et al., 1992), for treatment of piles (Gupta et al., 1996) and intestinal worms (Singh et al., 1980). The tender leaves of plant are also used to cure migraine (Ahmed et al., 2005). The encapsulated root bark powder is effective in diarrhoea (Ahmed et al., 2005) and asthma (Singh et al., 1980). The previous pharmacological studies include reports of antitricheour (Ahmed et al., 2005), antifungal (Hassan et al., 2006), Antifungal (Ahmed, et al., 2006) activity of C. procera. The flowers of the plant possessed hepatoprotective activity (Setty et al., 2007), antiinflammatory, antipyretic, analgesic, antimicrobial properties and larvicidal activity (Mascolo et al., 1989; Markouk et al., 2000). The latex of the plant was reported to possess analgesic and wound healing activity (Dewan et al., 2000; Rasik et al., 1999), anti-inflammator activity (Arya and Kumar, 2005) and antimicrobial (Sehgal et al., 2005). The roots are reported to have anti-fertility (Kamath and Rana, 2002) and anti-ulcer activities (Basu et al., 1997).

Earlier chemical examination of this plant has shown the presence of triterpenoids, calotropiseryl acetate and caloprienedenyl; a norditerpenyl ester, calotocrypteryl ester oleane triterpenes like calotropOLEAN ester, procerelian A and B (Ansari and Ali, 2001) and cardiac glycosides calotropigenin, calotropin, uscharin, calotoxin and calactin (Ahmed et al., 2005). The plant also has been investigated for cardenolides (Seiber et al., 1982) and anthocyanins (Ahmed et al., 2005). The root bark also found to possess α-amyrin (Saber et al., 1969), β-amyrin ( Saxena and Saxena, 1979), lupeol, β-sitosterol (Saber et al., 1969) and flavonoids like quercetin-3-rutinoside (Lal et al., 1985). The rich source of phytoconstituents and there are no scientific bases or reports in modern literature regarding usefulness of root bark as hepatoprotective agent prompts us to evaluate root bark of plant for its possible hepatoprotective activity.

In the course of searching for hepatoprotective agents from medicinal plants, the MeOH extract of root bark of C. procera was evaluated against carbon tetrachloride induced hepatic damage. The results instigated us for further pharmacological investigation of phyto-constituents detected fractions from MeOH extract to locate possible active phytoconstituents. The identified active fractions then also studied for ex vivo antioxidant activity to identify the possible mechanism of action.

MATERIALS AND METHODS

Plant material: Fresh, well-developed plants of C. procera were collected from Rajkot, Gujarat, in the month of September 2007. The authenticity of plants was confirmed by a taxonomist of Gujarat Ayurveda University, Jamnagar, Gujarat. Voucher specimen (HNS 11) was deposited in the department of Pharmacognosy, Shri H. N. Shikla Institute of Pharmaceutical Education and Research, Rajkot, Gujarat. Bark of the roots were separated and dried in the sun and reduced to powder (60 #).

Preparation of extract: Dried root bark powder (200 g) was extracted with methanol by soxhlet apparatus for 5 h. The methanolic extract of C. procera (MCP) was tested for qualitative phytoconstituents and indicated the presence of tri-terpenoids and their glycosides, flavonoids, alkaloids and steroids. Hepatoprotective activity of the methanolic extract was studied. Further, phytoconstituents directed fractionation was carried out using concentrated MeOH extract (30 g), suspended in H2O, acidified with 2N H2SO4 and sequentially partitioned with n-hexane and Ethyl acetate. The Acidic layer was basified with dilute ammonium hydroxide (pH 10) and extracted with CHCl3. Preliminary phytochemical testing and thin layer chromatography showed presence of terpenoids and steroids in the hexane fraction (HCP), flavanoids in the ethyl acetate (ECP) and alkaloids in chloroform fraction (CCP). All the three fractions were subjected for detailed hepatoprotective activity and ex vivo antioxidant activity.

Animals: BLAB/c albino mice (22-25 g) of either sex were used. The animals received a standard pellet diet (Lipton, Mumbai), water ad libitum and were maintained under standard temperature and humidity conditions. All the protocols followed for pharmacological assays were duly endorsed by the Institutional Ethical Committee of Smt. R. D. Gardi B. Pharmacy College, Rajkot, Gujarat.

Hepatoprotective activity: Animals were divided into four groups each of six animals. Group I and II served as normal and intoxicated control, respectively and received only the vehicle (0.5% Tween-80, 1 ml kg⁻¹ p.o). Group III animals were treated with standard silymarin at an oral dose of 100 mg kg⁻¹ and group IV received the MCP at an
oral dose of 200 mg kg\(^{-1}\). The treatment was continued for 7 days, once daily. On the day of 2nd, 4th and 6th for groups II, III, IV 30 min post-dose of extract administration animals received CCl\(_4\) at the dose of 0.8 mL kg\(^{-1}\) (1:1 of CCl\(_4\) in olive oil) orally. Twenty four hours after CCl\(_4\) administration, blood was obtained from all groups of mice by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and analyzed for various biochemical parameters, Aspartate aminotransferase (SGOT), Alanine aminotransferase (SGPT), Alkaline phosphatase (ALP) and Bilirubin (Total and Direct) using Span diagnostic kits.

**Detail hepatoprotective study of sub fractions from methanolic extract:** Group IV, V and VI received the HCP, ECP and CCP fractions respectively (200 mg kg\(^{-1}\), p.o.) as a fine suspension of 0.5% aqueous Tween-80 and Group VII animals received silymarin (100 mg kg\(^{-1}\), p.o). The treatment was continued for 7 days, once daily. On the day of 2nd, 4th and 6th for groups III-VI received CCl\(_4\) (1:1 of CCl\(_4\) in olive oil) 0.8 mL kg\(^{-1}\) i.p. 30 min after the dose of extracts administration. The animals were sacrificed after 36 h of administration of acute dose of CCl\(_4\). The blood was collected and serum was separated out and used for estimation of aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), alkaline phosphatase (ALP), albumin, bilirubin (Total and direct), total protein (TP), cholesterol and HDL using Span diagnostic kits. The liver was immediately dissected out and the liver-tissue was used for estimation of malondialdehyde equivalent, an index of lipid peroxides (LPO), reduced glutathione (GSH), Superoxide Dismutase (SOD) and Catalase Activity (CAT). A section of liver was processed for histological studies.

**Assessments of oxidative stress**
**Preparation of tissue antioxidant:** The livers were rinsed with ice cold distilled water followed by sucrose solution (0.25 M). And again rinsed with distilled water and immediately stored at -20°C till further biochemical analysis. One gram of liver tissue homogenized in 10 mL of ice cold Tris-hydrochloride buffer. The prepared homogenates were centrifuged and used for the assay of determination lipid peroxidation (LPO) by measuring the release of malondialdehyde (MDA) by the method of Slater and Sawyer (1971) and the estimation of reduced glutathione enzyme (GSH) (Moron et al., 1979).

**Post Mitochondrial Supernant preparation (PMS):** The homogenates were centrifuged at 800 rpm for 5 min at 4°C to separate debris. The supernatant so obtained was centrifuged at 10,500 rpm for 20 min at 4°C to get the post mitochondrial supernatant (PMS) which was used to assay catalase (CAT) (Aebi, 1984) and superoxide dismutase enzyme(SOD) activity (Misra and Fridovich, 1972).

**Histopathological study:** The tissues of liver were fixed in 10% formalin and embedded in paraffin wax. Sections of 4-5 microns thickness were made using rotary microtome and stained with haematoxylin-eosin. Histological observations were made under light microscope (Galigher and Kozloff, 1976; Luna, 1968).

**Statistical analysis:** The results are expressed as Means±SD. The differences between experimental groups were compared by one-way ANOVA (toxic control versus treatment, tukeys method, using Graph pad prism statistical software, version 5.0) and were considered statistically significant at p<0.05.

**RESULTS**

In the present study, it was seen that administration of CCl\(_4\) elevates the levels of serum marker enzymes SGPT, SGOT and ALP (Table 1). It can also be seen from the Table 1 that the animals pretreated with methanolic extract of *C. procera* (200 mg kg\(^{-1}\); p.o.) showed significant (p<0.001) decrease in the serum enzyme values compared to those of toxic control values. Motivated by these results, phytoconstituent directed sub fractionation of the methanolic extract was done to identify the active fractions. Three fractions from methanolic extract, the HCP, ECP and CCP were studied for detailed hepatoprotective activity assay. The results are shown in Table 2. The animals treated with toxic doses of CCl\(_4\) showed markedly elevated values of the serum SGPT, SGOT, ALP, total and direct bilirubin, cholesterol and decreased total protein, albumin and HDL compared to normal mice, indicating acute hepato-cellular damage. Pretreatment with HCP and ECP (200 mg kg\(^{-1}\); p.o.) fraction significantly (p<0.001) decreased the value of SGOT, SGPT, ALP, bilirubin (total and direct) and cholesterol and prevented diminution of HDL value. It suggested clear indication of the improvement of the functional status of the liver cells. Both the fraction showed marginal improvement in the values of total protein and albumin. The CCP did not exhibit significant improvement in serum enzyme values.
Table 1: Effect of MCP on CCl₄-induced hepatotoxicity in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>SGOT</th>
<th>SGPT</th>
<th>ALP</th>
<th>Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
<td>107.2±7.2</td>
<td>65.4±5.0</td>
<td>13.6±3.1</td>
<td>0.3±0.03</td>
</tr>
<tr>
<td>II</td>
<td>CCl₄ treated</td>
<td>399.2±33.1 *</td>
<td>684.2±29.2 *</td>
<td>48.2±7.6 *</td>
<td>1.0±0.2 *</td>
</tr>
<tr>
<td>III</td>
<td>MCP+ CCl₄</td>
<td>224.9±10.8 †</td>
<td>170.8±12.1 †</td>
<td>16.3±1.8 †</td>
<td>0.3±0.06 †</td>
</tr>
<tr>
<td>IV</td>
<td>Silymarin + CCl₄</td>
<td>165.8±14.4†</td>
<td>112.5±11.3†</td>
<td>14.3±2.5†</td>
<td>0.5±0.03†</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SD of six animals in each group. ANOVA Statistical comparisons are as follows: *p<0.001 as compared with group I. †p<0.001 as compared with group II.

Table 2: Effect of various extracts on CCl₄-induced hepatotoxicity in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>SGOT</th>
<th>SGPT</th>
<th>ALP</th>
<th>Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>112.5±7.0</td>
<td>73.7±7.4</td>
<td>12.1±2.3</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>II</td>
<td>CCl₄</td>
<td>203.3±29.7</td>
<td>733.4±61.4</td>
<td>45.0±6.3</td>
<td>0.8±0.08</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>A***</td>
<td>A***</td>
<td>A***</td>
<td>A***</td>
</tr>
<tr>
<td>III</td>
<td>HCP+</td>
<td>156.5±11.0</td>
<td>153.3±20.1</td>
<td>12.2±0.6</td>
<td>0.2±0.014</td>
</tr>
<tr>
<td></td>
<td>CCl₄</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
</tr>
<tr>
<td>IV</td>
<td>ECP +</td>
<td>146.2±13.0</td>
<td>207.2±37.3</td>
<td>15.1±2.9</td>
<td>0.19±0.010</td>
</tr>
<tr>
<td></td>
<td>CCl₄</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
</tr>
<tr>
<td>V</td>
<td>CCP+</td>
<td>174.2±21.1</td>
<td>682.8±32.6</td>
<td>38.2±3.3</td>
<td>0.4±0.126</td>
</tr>
<tr>
<td></td>
<td>CCl₄</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
</tr>
<tr>
<td>VI</td>
<td>Silymarin +</td>
<td>128.2±18.0</td>
<td>78.8±18.7</td>
<td>14.9±2.5</td>
<td>0.21±0.021</td>
</tr>
<tr>
<td></td>
<td>CCl₄</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
<td>B***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SD (n=6); *p<0.05, **p<0.01, ***p<0.001, ANOVA Statistical comparisons are as follows: a vs. Group I; b vs. Group II.

Fig. 1: Microphotograph of normal control mice liver section (×200)

Further, the results also supported by histopathological examination of liver sections of normal control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Fig. 1). Disarrangement of normal hepatic cells with centrilobular necrosis, vacuolization of cytoplasm and fatty degeneration were observed in CCl₄ intoxicated animals (Fig. 2). The liver sections of the mice treated with HCP (Fig. 3), ECP (Fig. 4) and Silymarin (Fig. 5) followed by CCl₄ intoxication showed a sign of protection as it was evident by the absence of necrosis and vacuoles.

In order to elucidate the possible mechanism of the hepatoprotective activity, the effect of HCP and ECP on antioxidant enzymes have also been investigated as the level of these enzymes has been found to be of great importance in the assessment of liver damage. The CCl₄ treated animals had increased tissue lipid peroxide values and decreased SOD, CAT and GSH (Table 3).
Table 3: Effect of extracts on LPO, antioxidant enzymes and GSH in livers of CCl₄-induced hepatotoxic mice \textit{in vivo}

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>LPO⁴</th>
<th>SOD²</th>
<th>CAT¹¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>4.5±0.7</td>
<td>16.3±1.89</td>
<td>2271.9±155.8</td>
</tr>
<tr>
<td>II</td>
<td>CCl₄ treated</td>
<td>33.7±2.6²</td>
<td>3.7±0.87²</td>
<td>536.7±138.9²</td>
</tr>
<tr>
<td>III</td>
<td>HCl+CCl₄</td>
<td>7.0±0.6³</td>
<td>10.4±0.79³</td>
<td>860.3±175.9³</td>
</tr>
<tr>
<td>IV</td>
<td>ECP+CCl₄</td>
<td>11.9±0.9⁴</td>
<td>10.7±0.59⁴</td>
<td>1536.1±178.0⁴</td>
</tr>
<tr>
<td>V</td>
<td>Silymarin+CCl₄</td>
<td>8.4±0.7⁵</td>
<td>12.5±0.55⁵</td>
<td>2294.0±47.6⁵</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SD of six animals in each group. ANOVA statistical comparisons are as follows: *p<0.001 as compared with group I, †p<0.001 as compared with group II (except †† where, p<0.01). K: mol of MDA/mg of protein, L: Units mg⁻¹ of protein, M: µmol of H₂O₂ consumed/min of protein. N: µg mg⁻¹ of protein.

improvement was seen in catalase activity. Hence, protection against liver necrosis could be obtained through antioxidant effect of HCP and ECP.

**DISCUSSION**

Although \textit{Calotropis procera} is reported to possess varied medicinal uses as discussed earlier, there is no previous report about the hepatoprotective activity of the root bark of the plant. The present investigation reports the hepatoprotective activity of the MCP and its sub-fractions HCP and ECP.

In the present study, hepatotoxicity model in mice was successfully produced by administering CCl₄ (1:1 in olive oil, 0.8 mL kg⁻¹) intraperitoneally. It is well established that hepatotoxicity by CCl₄ is due to enzymatic activation to release CCl₄ radical in free state, which in turn disrupts the structure and function of lipid and protein macromolecules in the membrane of the cell organelles (Mujumdar \textit{et al.}, 1998). CCl₄ also plays a significant role in depletion of Intracellular antioxidant reduced glutathione (GSH), increased lipid peroxidation, membrane damage, depression of protein synthesis and loss of enzymes activity (Recknagel \textit{et al.}, 1989). As the damage marker enzymes SGOT and SGPT are cytoplasmic in location (Sallie \textit{et al.}, 1962) they get released in serum (Chenoweth and Hake, 1962). So increase in the level of SGPT, SGOT, ALP, total and direct bilirubin, cholesterol and HDL is conventional indicator of liver injury. CCl₄ challenges significantly decrease the levels of SOD, GSH and catalase in liver. The level of MDA which is produced as a result of lipid peroxidation is significantly increased.

As discussed in results, MCP, HCP and ECP showed significant hepatoprotective activity on CCl₄ induced hepatotoxic animals. In preliminary study, MCP significantly reduced the elevated levels of the different enzyme values. Further, in order to find out active fraction detailed study was done with the treatment of phytoconstituent directed three fractions, of which HCP and ECP showed significant hepatic protective activity. In addition, HCP and ECP also showed appreciable increase...
in the levels of GSH, SOD and catalase whereas decreased the lipid peroxidation. Plant demonstrated superoxide scavenging activity there for it may be inferred that the antioxidant property of the plant may prevent formation of free radical and so inhibit the lipid peroxidation and offers the hepatoprotection against CCl_{4} toxicity. Further, the improvement of GSH level by HCP and ECP treatment also indicate the natural tissue protection mechanism is kept intact and oxidative degeneration of the liver tissues prevented.

The results for antioxidant study suggest that the reason for hepatoprotective effect of the extracts may be that C. procera contains terpenoids (Sunita et al., 2001) and flavonoids (Janbaz et al., 2002) which might have scavenged the free radical offering hepatoprotection.

In conclusion, the present study scientifically demonstrates that the root bark of Calotropis procera possess hepatoprotective property. In addition the hepatoprotective property may be attributed to the antioxidant principles of the plant.

REFERENCES


