Screening of methanol extract of roots and rhizomes of Smilax zeylanica L for hepatoprotective effect against carbontetrachloride induced hepatic damage

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Abstract
Objective: To evaluate the hepatoprotective potential of methanol extract of roots and rhizomes of Smilax zeylanica L on carbontetrachloride (CCl₄) induced hepatotoxicity in Wistar albino rats.

Methods: Hepatotoxicity was induced in Wistar albino rats of either sex by administration of CCl₄ 0.5 ml/kg p.o. once a day for 7 days. The methanol extract of roots and rhizomes of Smilax zeylanica L were administered p.o. at doses of 200, 400 and 600 mg/kg b.w for 7 days. On the 8th day, biochemical estimations were carried out to measure the serum levels of ALT, AST, ALP, albumin, total proteins and total bilirubin. Histopathological examination of liver sections was also performed to study the extent of damage to hepatic parenchyma. Preliminary phytochemical screening of the methanol extract was carried out to find out the presence of various phytoconstituents. HPTLC fingerprint profiles of the detected phytoconstituents were also obtained.

Results: Administration of CCl₄ produced profound hepatic damage as evidenced by the significant increase in serum levels of ALT, AST, ALP and total bilirubin and decrease in total proteins and albumin. The altered biochemical parameters were brought to near normal levels by the administration of methanol extract of roots and rhizomes of S. zeylanica.

Conclusion: S. zeylanica roots and rhizomes possess significant hepatoprotective properties, which may due to the presence of potential hepatoprotective phytoconstituents present in them.

Key words: Biochemical estimations; Hepatoprotective; HPTLC; Serum enzymes; Smilax zeylanica

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Introduction
The liver is a frequent target of many toxic substances as it is involved in various metabolic functions and detoxification of hazardous substances [1]. CCl₄ is one of the most widely used toxicants for experimental induction of liver toxicity in laboratory animals [2]. CCl₄ produces dose dependent hepatotoxicity by causing lipid peroxidation [3]. It induces lipid peroxidation in experimental animals within minutes of administration and causes severe hepatic injury [4]. Hepatic injury is due to generation of its reactive trichloromethyl radical (•CCl₃) under the action of microsomal cytochrome P450 enzyme (CYP2e11). This highly reactive trichloromethyl radical readily interacts with molecular oxygen to form the trichloromethyl peroxy radical (•CCl₃O₂). The massive generation of free radicals causes degradation of bio membranes which is one of the principle causes of hepatotoxicity [5].

Chopachinee is an important drug in Ayurveda [6]. The accepted botanical source of Chopachinee is Smilax china L, while many other species of Smilax including Smilax zeylanica are used as its substitutes [6, 7]. S. zeylanica (Smilacaceae) is distributed in India, Myanmar and Kampuchea (Cambodia) [6, 8]. S. zeylanica is also a substitute for Indian Sarsaparilla, for which the accepted botanical source is Hemidesmus indicus (L) R.Br., which is known as Sariva in Ayurveda [6]. Root, rhizome and leaf of S. zeylanica are used in epilepsy, fever, venerial and skin diseases, sores, swellings and abscesses [9]. Root is also used for treating rheumatism and pain in the lower extremities [10]. The plant is also used in ritual healing techniques [11] and in bloodless dysentery [12]. S. zeylanica is used in the villages of Bangladesh for the treatment of fever, headache and wounds [13].

Phytoconstituents reported in S. zeylanica are the steroidal saponin glycosides dioscin, diosgenin, smilagenin and sarsapogenin [14]. Antioxidant property of root, rhizome and leaf of S. zeylanica...
has been reported by the same authors [15, 16]. Antiepileptic activity studies have been reported on the roots and rhizomes of *S. zeylanica* [17]. The pharmacognostical characteristics of *S. zeylanica* roots and rhizomes have also been described [18]. Since hepatoprotective properties has been reported on *H. indicus* [19, 20], similar studies were undertaken on this substitute of Indian Sarsaparilla.

**Materials and methods**

**Plant material**

The roots and rhizomes of *S. zeylanica* were collected from the forests of Kanyakumari district, Tamil Nadu during June 2008. The plant material was identified and authenticated by Dr. S.N. Yoganarasimhan, Taxonomist and Research Coordinator at M.S. Ramaiah College of Pharmacy (MSRCP), Bangalore, Karnataka, India. The taxonomic identification was carried out following local floras [21, 22], and the herbarium specimen (No.012) along with crude sample have been deposited at the herbarium and crude drug museum of PG Department of Pharmacognosy, MSRCP.

**Preparation of methanol extract of *S. zeylanica* roots and rhizomes**

Coarsely powdered roots and rhizomes (1 kg) were extracted successively with petroleum ether, chloroform and methanol, in a Soxhlet apparatus. The methanol extract (SZRM) was concentrated at reduced pressure to produce a brownish semisolid mass (3.96% w/w). The phytoconstituents present in the methanol extract were identified by qualitative analysis and confirmed by HPTLC fingerprint analysis. The dried extract was suspended in 2% w/v acacia in distilled water and used for the pharmacological studies.

**Chemicals**

Biochemical assay kits used for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) were procured from BioSystems (Barcelona, Spain); kits for albumin, total proteins and total bilirubin were procured from Agappe Diagnostics (Pattimattom, Kerala, India). Silymarin was obtained as gift from Micro Labs (Hosur, Tamil Nadu, India). Solvents used for extraction were obtained from Ranbaxy Laboratories (Gurgaon, Haryana, India).

**Experimental animals**

Albino Wistar rats of either sex (170-200 g) were divided into 6 groups of 6 animals each as follow: *Group I* - normal control, treated with vehicle (2% w/v acacia, 2 ml/kg, p.o.);

*Group II* - positive hepatotoxic control (2% w/v acacia, 2 ml/kg + CCl\(_4\) 0.5 ml/kg);

*Group III* - silymarin (100 mg/kg + CCl\(_4\), p.o.);

*Group IV* - SZRM (200 mg/kg + CCl\(_4\), p.o.);

*Group V* - SZRM (400 mg/kg + CCl\(_4\), p.o.);

*Group VI* - SZRM (600 mg/kg + CCl\(_4\), p.o.).

All treatments were given once daily for 7 days. All groups except the normal control were treated with CCl\(_4\) 0.5 ml/kg (1:1 in liquid paraffin), p.o., once daily for 7 days. On 8\(^{th}\) day, 18 h after the last dose of CCl\(_4\), animals were anaesthetised using anaesthetic ether. Blood was collected from retro-orbital sinus into clean and dry eppendorf tubes and allowed to coagulate. The coagulated blood was then centrifuged at 8000 rpm for 10 min in a micro-centrifuge. The supernatant serum was separated and used for biochemical estimations. Following this the animals were sacrificed by excess of anaesthetic ether and livers isolated. The isolated liver was perfused in ice cold saline, blotted dry and weighed. It was preserved in 10% w/v formalin for the histopathology studies [25].

**HPTLC fingerprint studies**

HPTLC studies were carried out following standard procedures [23]. A Camag HPTLC system equipped with Linomat V applicator, Camag TLC scanner 3 and WinCATS-4 software for interpretation of data were used. Precoated aluminium plates coated with Merck Silica Gel 60 F254 as adsorbent, was used. All the solvents used were of HPLC grade and were obtained from Merck (Darmstadt, Germany).

**Acute toxicity study**

Acute toxicity studies were performed following OECD guidelines [24]. The lethal dose of the methanol extract was found to be more than 2000 mg/kg. The screening doses of 200, 400 and 600 mg/kg were chosen for the subsequent studies.

**CCl\(_4\) induced hepatotoxicity**

Hepatotoxicity was induced by administration of CCl\(_4\) 0.5 ml/kg, p.o., once a day for 7 days. Albino Wistar rats of either sex (170-200 g) were divided into 6 groups of 6 animals each as follow:

*Group I - normal control*, treated with vehicle (2% w/v acacia, 2 ml/kg, p.o.);

*Group II - positive hepatotoxic control* (2% w/v acacia, 2 ml/kg + CCl\(_4\) 0.5 ml/kg);

*Group III - silymarin* (100 mg/kg + CCl\(_4\), p.o.);

*Group IV - SZRM* (200 mg/kg + CCl\(_4\), p.o.);

*Group V - SZRM* (400 mg/kg + CCl\(_4\), p.o.);

*Group VI - SZRM* (600 mg/kg + CCl\(_4\), p.o.).
**Biochemical estimations**

**Serum glutamate pyruvate transaminase – Alanine aminotransferase (SGPT/ALT)**

ALT catalyses the transfer of an amino group from alanine to 2-oxoglutarate, by which pyruvate and glutamate are formed. The catalytic concentration of ALT is determined from the rate of decrease of NADH, measured at 340 nm, by means of the lactate dehydrogenase coupled reaction [26].

**Serum glutamate oxaloacetate transaminase – Aspartate aminotransferase (SGOT/AST)**

AST catalyses the transfer of an amino group from aspartate to 2-oxoglutarate, forming oxaloacetate and glutamate. The catalyst concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of malate dehydrogenase coupled reaction [26].

**Alkaline phosphatase (ALP)**

ALP catalyses the transfer of phosphate group from 4-nitrophenyl phosphate to 2-amino-2-methyl-1-propanol in an alkaline medium, liberating 4-nitrophenol. The concentration of ALP is determined from the rate of 4-nitrophenol formation by measurement at 405 nm [27].

**Total proteins**

Colorimetric estimation of total proteins was performed based on the principle of the Biuret reaction (copper salt in an alkaline medium). Protein in serum forms a blue colored complex when treated with cupric ions in alkaline solution. The intensity of the blue color formed is proportional to the protein content in the sample. Measurement was done at 546 nm [28].

**Albumin**

Albumin in serum or plasma reacts with the dye bromocresol green and produces a change in color that is proportional to the concentration of albumin. The change in color was measured at 630 nm [29].

**Bilirubin (total)**

The concentrations of direct and indirect bilirubin are approximately equivalent to the conjugated and unconjugated fractions. Both direct and indirect bilirubin couple with diazotized sulphanilic acid forming a colored complex that is measured in the presence of cetrimide at 540 nm [30]. The terms direct and total refer to the reaction characteristics of serum bilirubin in the absence or presence of solubilizing reagents.

**Histopathological studies**

One animal from each group was used for this purpose. The liver specimens were fixed with 10% neutral formalin and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed according to standard procedures [31].

The histological evaluation of the extent of liver injury was carried out microscopically using an image microscope.

**Statistical analysis**

The results were expressed as Mean ± SD and statistically analyzed by One Way Analysis of Variance (ANOVA) followed by Tukey Kramer multiple comparison test.

**Results**

Preliminary phytochemical screening of methanol extract of *S. zeylanica* roots and rhizomes revealed the presence of glycosides, phytosterols, saponins, phenolic compounds and tannins. Earlier studies have reported the presence of diosgenin in the roots and rhizomes of *S. zeylanica* [18]. HPTLC fingerprint profiles of the phytoconstituents were also obtained (Fig.1). The methanol extract of *S. zeylanica* roots and rhizomes did not show any sign or symptoms of toxicity and no mortality was recorded during the study.

![Figure 1. HPTLC fingerprint profiles of (A) β- sitosterol at 425 nm; (B) polyphenols and tannins at 366 nm; and (C) saponins at 254 nm.](image-url)
The effects of methanol extract of *S.zeylanica* (SZRM) were evaluated on CCl₄ induced hepatotoxicity in albino Wistar rats. An increase in liver weight is characteristic of CCl₄ induced hepatotoxicity. The liver weight was calculated and expressed in terms of grams per 100 g body weight. Liver weight increased in the positive control group with daily administration of CCl₄. Treatment with extracts of *S.zeylanica* roots and rhizomes showed significant decrease in liver weight in comparison with the positive control animals. SZRM 600 mg/kg significantly (p < 0.001) prevented an increase in the weight of liver. However the effects of SZRM 200 and 400 mg/kg were not significant (Fig.2).

The serum levels of ALT, AST, ALP, total bilirubin and albumin were estimated and compared with that of the positive control. Levels of ALT, AST, ALP, total bilirubin were significantly increased and that of total proteins and albumin were significantly decreased in the positive control group compared with the normal control animals. Treatment with the extracts produced significant changes in the altered serum parameters. ALT and AST levels decreased significantly (p < 0.001) with all the three doses of SZRM. The results were independent of dose and comparable with that of the standard silymarin. ALP levels also decreased significantly with SZRM 200 (p < 0.05), 400 and 600 mg/kg (p < 0.001).

Total proteins increased significantly on treatment with SZRM 600 mg/kg, however there was no significant increase with the lower doses of SZRM. Total bilirubin levels also decreased significantly with SZRM 200 mg/kg (p < 0.01), 400 mg/kg (p < 0.001) and 600 mg/kg (p < 0.001). Serum albumin levels in the positive control group were reduced in comparison with that of normal control. However treatment with the extract increased the serum albumin levels; the effect of SZRM 400 mg/kg was significant (p < 0.001). The results of biochemical estimations are presented in Table 1.

Histopathology studies of liver showed ballooning degeneration, fatty degeneration, congestion, reactive changes like binucleation and loss of normal structure of hepatocytes in CCl₄ control rats, in comparison with the normal control (Fig.3A & B). The extract treated groups showed regeneration of hepatocytes and normalization of fatty changes in hepatocytes. Extracts of *S.zeylanica* root and rhizome showed reduction in fatty degeneration with increase in dose from 200 to 600 mg/kg. Specimens from SZRM 200 mg/kg treated liver showed symptoms of fatty changes, early fibrosis with cirrhotic changes showing nodules of hepatocytes separated by fibrous septae (Fig.3C). The groups treated with the higher doses showed regenerative changes in hepatocytes and decreased fatty degeneration (Fig.3D & E). The silymarin treated group showed regenerative changes and fatty changes were less prominent (Fig.3F). Histopathological grading of the sections were also performed (see Table 2).

![Image](https://via.placeholder.com/150)

**Figure 2.** Liver weight expressed as g/100 g b.w.

### Table 1. Effect of methanol extract of *S.zeylanica* roots and rhizomes on CCl₄ induced hepatotoxicity

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>Positive control</th>
<th>Standard silymarin</th>
<th>SZRM 200 mg/kg</th>
<th>SZRM 400 mg/kg</th>
<th>SZRM 600 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGOT IU/L</td>
<td>140.2±6.7</td>
<td>910.8±110.6</td>
<td>180.2±96.2***</td>
<td>163±21***</td>
<td>153.7±13.4***</td>
<td>147±8.3***</td>
</tr>
<tr>
<td>SGPT IU/L</td>
<td>41±5.7</td>
<td>684±79.8</td>
<td>37.3±6.1***</td>
<td>81.8±38.7***</td>
<td>58.16±17.6****</td>
<td>93.7±69.5***</td>
</tr>
<tr>
<td>ALP IU/L</td>
<td>214.9±76.8</td>
<td>1018.2±116.8</td>
<td>988±9.3</td>
<td>777.3±21.7***</td>
<td>567±84***</td>
<td>502.7±184***</td>
</tr>
<tr>
<td>Total proteins (g/dl)</td>
<td>6.73±0.2</td>
<td>4.85±1.9</td>
<td>6.56±0.5*</td>
<td>5.78±0.4</td>
<td>6.18±0.5</td>
<td>6.28±0.2*</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.43±0.2</td>
<td>1.43±0.1*</td>
<td>0.91±0.2***</td>
<td>1±0.3**</td>
<td>0.75±0.2***</td>
<td>0.67±0.2***</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.82±0.2</td>
<td>2.75±0.2*</td>
<td>3.25±0.3*</td>
<td>3.1±0.2</td>
<td>3.4±0.3***</td>
<td>3.0±0.2</td>
</tr>
</tbody>
</table>

All values expressed as Mean ± SD. Tukey-Kramer Multiple Comparison Test; *p < 0.001 in comparison with the normal control, ***p < 0.001, **p < 0.01 and *p < 0.05 in comparison with the positive control.
Figure 3. Histopathology studies on liver sections; (A) normal control (100x), (B) positive control (100x), (C) SZRM 200 mg/kg (100x), (D) SZRM 400 mg/kg (100x), (E) SZRM 600 mg/kg (400x), and (F) silymarin 100 mg/kg (100x).

Discussion
When cell membrane of hepatocytes is damaged, a variety of enzymes such as ALT, AST and ALP are released into blood from the cytosol [32]. The elevated level of these serum enzymes is an indication of cellular leakage and loss of functional integrity of liver cell membrane [33]. Estimation of these enzymes is a useful quantitative marker for assessing the extent and type of hepatic cell damage [34]. The ability to regenerate is a unique feature of the liver tissue [35]. However, recovery of hepatocytes is inhibited by repeated dosing of CCl4.
[36]. The altered levels of liver transaminases in CCl4-treated rats in the present study corresponded to the extensive liver damage induced by CCl4. The aminotransferases constitute a group of enzymes that catalyse the inter conversion of amino acids and α-ketocids by the transfer of amino groups. These liver specific enzymes are very sensitive and are reliable indices of hepatotoxic as well as hepatoprotective or curative effect of various compounds [37]. Serum levels of both ALT and AST increase following administration of hepatotoxins, affecting the integrity of liver cells [38]. Serum levels of CCl4-treated positive control animals recorded an increase in ALT and AST. The levels of ALT and AST decreased significantly with all the three doses of SZRM; the results were independent of dose and comparable with that of the standard antioxidant silymarin.

Alkaline phosphatase is a membrane bound glycoprotein enzyme present in high concentration in sinusoids and endothelium. It is excreted into the bile and therefore its serum levels are elevated during hepatobiliary diseases [39]. Serum ALP levels are related to the function of hepatic cells [33]. ALP levels in the positive control animals increased significantly compared with the normal control animals. Treatment with the extract decreased the ALP levels significantly in their respective groups.

CCl4 intoxication lowered serum protein levels in the positive control group, compared to the normal control animals. The lowered levels of hepatic proteins in CCl4 intoxicated rats may be attributed to the oxidative damage of some amino acids [40]. The capacity of liver to synthesize proteins especially albumin is adversely affected by hepatotoxins. Methanol extracts of S.zeylanica root and rhizomes elevated the serum protein levels to a significant extent. Studies reveal that in the animals treated with hepatoprotective agents, biosynthesis of proteins in liver continues even with simultaneous CCl4 challenge. The extract enhanced the synthesis of total proteins and albumin which accelerated the regeneration process and afforded protection to liver cells. Therefore, the increased levels of total protein and albumin in the serum of extract treated animals indicate their hepatoprotective activity.

The biotransformation of amino acids in liver may be impaired due to the escape of non-protein and protein nitrogenous substances from injured liver cells as evidenced by the rise in serum levels of ALP, AST and ALT. The protective activity of the extracts may be due to the membrane stabilizing agents present in them, which might have prevented enzyme leakage in hepatic tissues leading to enhanced metabolic transformation of amino acids through synthesis and transformation [41].

CCl4 intoxication also produced a significant elevation in the levels of serum bilirubin. Necrotizing agents like CCl4 produce sufficient injury to hepatic cells causing elevation in serum bilirubin content. Bilirubin levels in serum of treated rats were significantly restored, which may be due to the inhibitory effects of the plant extracts on cytochrome P-450 and/or promotion of its glucuronidation [42].

The hepatoprotective effect was evidenced in the histopathology studies as well. The histopathology studies of liver showed ballooning and fatty degeneration, congestion, reactive changes and loss of normal structure of hepatocytes in CCl4 control rats, in comparison with the normal control. The extract treated groups showed regeneration of hepatocytes and reduction of fatty changes in hepatocytes.

Many of the phytoconstituents present in the plants under this study are reported to possess hepatoprotective potential. Phenolic compounds possess antioxidant [43] and hepatoprotective actions [44, 45]. Hepatoprotective properties of glycosides [46, 47], β-sitosterol [48, 49] and saponins [50, 51] were reported. The presence of the phytoconstituents could have contributed to the hepatoprotective properties of this plant. Further work to isolate the phytoconstituent(s) responsible for the hepatoprotective effect and to elucidate the exact mechanism of action could be undertaken.

### Table 2. Histopathological scores

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Score</th>
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</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0.0</td>
</tr>
<tr>
<td>Positive control</td>
<td>4.0</td>
</tr>
<tr>
<td>Standard (Silymarin)</td>
<td>2.5</td>
</tr>
<tr>
<td>SZRM 200 mg/kg</td>
<td>3.5</td>
</tr>
<tr>
<td>SZRM 400 mg/kg</td>
<td>3.5</td>
</tr>
<tr>
<td>SZRM 600 mg/kg</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Description of histological changes for scoring: 0, normal hepatic architecture and hepatocytes; 1, focal degeneration of hepatocytes; 2, diffuse degeneration of hepatocytes; 3, diffuse degeneration of hepatocytes with periportal fibrosis and inflammatory cells; 4, diffuse degeneration of hepatocytes with periportal fibrosis and inflammatory cells plus fatty vacuoles (vesicular formations).
Acknowledgements

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References

10. Ambasta SP. The Useful Plants of India. NICSAIR, New Delhi, India, p 578, 2006.