Antioxidant and hepatoprotective effect of the ethyl acetate extract of *Enicostemma axillare* (Lam). Raynal against CCl₄-induced liver injury in rats

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*Enicostemma axillare* is used in Indian traditional medicine as a liver tonic. Its ethyl acetate extract has shown potent *in vitro* antioxidant activity and found to contain 7.26% of a bitter secoiridoid glycoside, swertiamarin. Hence, in the present study the ethyl acetate extract was screened for hepatoprotective and antioxidant properties against CCl₄ induced hepatic injury in rats. The hepatoprotection was assessed in terms of reduction in histological damage and changes in serum enzymes and metabolites. The pretreatment with the extract at 100 and 200 mg/kg body weight doses given orally for eight days prior to CCl₄ caused significant restoration of altered biochemical changes due to CCl₄ towards the normal in serum, liver and kidney. The extract treatment at 200 mg/kg body weight was found to be more potent than the standard silymarin at 100 mg/kg body weight in reversing most of the biochemical parameters. Histopathological studies complemented the results of biochemical estimations in providing a proof of hepatoprotective and antioxidant actions of the extract. The study provides a support to the ethnomedical use of *E. axillare* in India.

**Keywords:** Antioxidant, *Enicostemma axillare*, Gentianaceae, Liver protection

*Enicostemma axillare* (Lam.) Raynal (Synonym, *Enicostemma littorale* auct. Non Blume), locally known as Rechki, Chhotacirayata (Hindi) and Nagajivha or Nayi (Sanskrit), family Gentianaceae is a glabrous or procumbent perennial herb found throughout India, commonly in coastal areas. The whole plant is used in medicine as digestive, anti-inflammatory, liver tonic, antimalarial, antipyretic and as a laxative. Its hypoglycemic, antioxidant, hypolipideamic, anti-inflammatory, hepatoprotective and anticancer activities are also reported. In addition to a bitter secoiridoid glycoside, swertiamarin, alkaloids, steroids, saponins, triterpenoids, flavonoids, phenolic acids and xanthones were isolated from *E. axillare*. Swertiamarin is a representative constituent of many crude drugs and formulations, which are marketed in Japan and other countries and these drugs are normally evaluated by their high swertiamarin content. Many plants containing swertiamarin are also known ethnomedically for their potent hepatoprotective properties. Iridoids isolated from many plants are known to possess potent antihepatotoxic potentials. In absence of reliable liver protective drugs in modern medicine, there are a number of medicinal preparations in the Ayurvedic system of Indian medicine recommended for the treatment of liver disorders. Though they are claimed to offer significant relief, their usage is in vogue since centuries. *E. axillare* is one such plant used for the treatment of liver disorders in Indian traditional medicine. Its aqueous and ethanol extracts are reported to possess potent hepatoprotective activity. However, no effort has been made to identify the phytoconstituents responsible for the hepatoprotective activity of the plant. Preliminary studies with four successive (petroleum ether, chloroform, ethyl acetate, methanol) and two crude (methanol and water) extracts for *in vitro* antioxidant properties indicated potent antioxidant activity of the successive ethyl acetate extract of *E. axillare*. The extract was also found to contain 7.26% swertiamarin by HPTLC, which may be the active constituent of the plant. Hence, in the present study hepatoprotective and antioxidant activity of the ethyl acetate extract of *E. axillare* was carried out against CCl₄ induced liver damage in rats.

**Materials and Methods**

**Chemicals**—5,5-dithiobis-2 nitro benzoic acid was obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai. Carbon tetrachloride (CCl₄) and Ecoline kits...
for serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP), total protein, total cholesterol, total bilirubin, triglycerides, creatinine and albumin were obtained from Merck Ltd., Ambemath, India. Standard silymarin was obtained from Ranbaxy (India) Ltd., New Delhi. All the chemicals used were of analytical grade.

Plant material—The whole plant of E. axillare was collected during full blooming in Sept. 2005 from Guttuvali village, Bidar district, Karnataka and was authenticated by Dr. S. Rajan, Medicinal Plants Survey and Collection Unit, Ootacamund, India, where a voucher specimen (No. 8572) has been deposited for further reference. The whole plant was shade dried, powdered and extracted (250 g) successively and separately with 1.5 L each of petroleum ether (60º-80ºC), chloroform, ethyl acetate and methanol in a Soxhlet extractor for 18-20 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40º-50ºC) in a rotavapor. The ethyl acetate extract was yielded a brown semisolid (7.5 g, 3.0%).

High performance thin layer chromatography—The ethyl acetate extract was subjected to column chromatography (column length: 50 cm, diam: 3 cm) on silica gel (60-120 mesh, 125 g) using the eluents, ethyl acetate, ethyl acetate:acetone and acetone. Fractions (500 ml, 1-4 ethyl acetate; 5-11 ethyl acetate : acetone mixtures; 12-14 acetone) were collected. Fraction 5-11 (4.5 g) on re-chromatography and elution with chloroform:methanol (9:1) gave swertiamarin (0.5 g, 0.20%) as a white powder. The spectral values were identical to those reported13,14.

The methanol solutions (1 mg/ml) of ethyl acetate extract (15 µl) and swertiamarin (10 µl) were applied onto a commercially available precoated TLC plate of silica gel GF254 with a bandwidth of 5 mm using Linomat-IV applicator (CAMAG, Switzerland). The plate was developed in twin-trough chamber using the solvent system, ethyl acetate:butanol (1:1) and scanned using densitometer (Camag, Switzerland) at 254 nm. Based on the peak area of standard swertiamarin, the amount of swertiamarin in the extract was calculated.

Animals—Male Wistar rats (160–200 g) obtained from JSS College of Pharmacy, Ooty, India were maintained at a controlled temperature of 19º-25 ºC with a 12:12 h light/dark cycle and fed a standard diet and water ad libitum. The experiments were conducted according to the Institutional Animal Ethics Committee regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (JSSCP/IAEC/PH. D/PH. CHEM/03/2007-08).

Preparation of suspensions—The ethyl acetate extract of E. axillare and standard silymarin were suspended in distilled water using sodium carboxymethylcellulose (CMC, 0.3%) and administered orally to the animals with the help of an intragastric catheter.

Acute toxicity studies—Acute oral toxicity study in rats was carried out as per the classical method19. Graded doses (200, 500 and 2000 mg/kg body weight) of the ethyl acetate extract were administered orally to various groups of rats containing ten in each group. The animals were observed for mortality, clinical signs and body weight changes daily for a period of 15 days and at the end of the study period, all the animals were subjected to gross necropsy.

Hepatoprotective activity—The rats were divided into following 6 groups of 6 animals each. Group 1 was served as normal and group 3 as CCl4 treated control. Animals in both these groups received sodium CMC (0.3%, 5 ml/kg body weight). Group 2 rats served as extract alone treated and received the ethyl acetate extract at 200 mg/kg body weight. Groups 4 and 5 animals were treated with the extract at the dose levels of 100 and 200 mg/kg body weight. Group 6 was treated with the standard drug silymarin at 100 mg/kg body weight. All these treatments were given orally for 8 days. On the last day of the treatment, all the animals except the normal group 1 and the extract alone treated group 2 were received a single dose of CCl4 in liquid paraffin (1:1), 1 ml/kg body weight i.p after 1 h of the vehicle, extract or standard treatments. On the ninth day, the animals were anesthetized by anesthetic ether and blood was collected from the abdominal artery and kept for 30 min at 4ºC. Serum was separated by centrifugation at 2500 rpm for 15 min at 4ºC and used for the biochemical estimations. Serum marker enzymes such as SGOT, SGPT, SALP, triglycerides, creatinine, total protein, total cholesterol, total bilirubin and albumin were measured in an autoanalyzer using Ecoline kits. Glutathione20, glycogen21, superoxide dismutase22, catalase23 and thiobarbituric acid reactive substances24 were measured spectrophotometrically.

After the collection of blood samples, the liver and kidney were excised, rinsed in ice-cold normal saline,
followed by cold 0.15 \( M \) potassium chloride (pH 7.4) and blotted dry. A 10% w/v homogenate was prepared in 0.15 \( M \) potassium chloride buffer with Elvenjan homogenizer fitted with Teflon plunger and centrifuged at 2500 rpm for 15 min at 4°C. The supernatants were used for the estimation of catalase, superoxide dismutase, lipid peroxidation and glutathione in both liver and kidney and glycogen in liver. A portion of the liver and kidney tissues were fixed in 10% formalin, cut into 5 \( \mu \)m thick sections and stained using heamatoxylin-eosin and histopathological observations were made.

**Statistical analysis**—The significance of the in vivo data was analyzed by one-way ANOVA, followed by Tukey-Kramer multiple comparison tests and \( P<0.05 \) was considered as statistically significant.

**Results**

The \( R_f \) value of swertiamarin was found to be 0.45. By HPTLC, the amount of swertiamarin found in the ethyl acetate extract of \textit{E. axillare} was 7.26% (w/w). The HPTLC spectrum of swertiamarin and the ethyl acetate extract are shown in Fig. 1. In the acute toxicity studies, the ethyl acetate extract was found to be nontoxic and no mortality was observed up to 15 days when given a single dose of up to 2000 mg/kg body weight p.o and there were no gross necropsy findings.

In the in vivo studies, the administration of CCl\(_4\) caused a significant increase in the levels of SGOT, SGPT, SALP, triglycerides, total cholesterol, total bilirubin, and thiobarbituric acid reactive substances in serum when compared to normal rats reflecting the liver injury caused by CCl\(_4\) (Table 1 and Fig. 2c). It also caused a significant decrease in the levels of creatinine, total protein, albumin, catalase and superoxide dismutase in serum (Table 1 and Figs 2a and b). The administration of the ethyl acetate extract of \textit{E. axillare} at 100 mg/kg body weight dose to CCl\(_4\) intoxicated rats significantly inhibited the increased levels of SGOT, SGPT, SALP, triglycerides, total cholesterol, total bilirubin and thiobarbituric acid reactive substances towards the normal when compared to CCl\(_4\) treated animals. It also increased significantly in the level of creatinine, total bilirubin and albumin towards the normal. The high dose, 200

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**Table 1**—Effect of the ethyl acetate extract of \textit{E. axillare} on biochemical parameters in CCl\(_4\)-induced toxicity in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SGOT (IU/l)</th>
<th>SGPT (IU/l)</th>
<th>SALP (IU/l)</th>
<th>Triglycerides (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Total bilirubin (mg/dl)</th>
<th>Total protein (g/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Albumin (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>143.66</td>
<td>96.66</td>
<td>179.66</td>
<td>40.83</td>
<td>29.16</td>
<td>0.75</td>
<td>8.33</td>
<td>0.31</td>
<td>3.43</td>
</tr>
<tr>
<td>Extract control (200 mg/kg body weight)</td>
<td>± 6.60</td>
<td>± 1.11</td>
<td>± 9.09</td>
<td>± 1.72</td>
<td>± 1.30</td>
<td>± 0.04</td>
<td>± 0.55</td>
<td>± 0.05</td>
<td>± 0.15</td>
</tr>
<tr>
<td>CCl(_4) control</td>
<td>138.83</td>
<td>140.00</td>
<td>444.16</td>
<td>46.66</td>
<td>54.83</td>
<td>0.75</td>
<td>8.16</td>
<td>0.18</td>
<td>3.13</td>
</tr>
<tr>
<td>Extract (100 mg/kg body weight) + CCl(_4)</td>
<td>± 7.59</td>
<td>± 5.16</td>
<td>± 6.71</td>
<td>± 2.90</td>
<td>± 2.91</td>
<td>± 0.04</td>
<td>± 0.60</td>
<td>± 0.02</td>
<td>± 0.18</td>
</tr>
<tr>
<td>Extract (200 mg/kg body weight) + CCl(_4)</td>
<td>± 8.40</td>
<td>± 2.46</td>
<td>± 10.65</td>
<td>± 1.18</td>
<td>± 1.57</td>
<td>± 0.05</td>
<td>± 0.40</td>
<td>± 0.04</td>
<td>± 0.10</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg body weight) + CCl(_4)</td>
<td>± 3.21</td>
<td>± 2.80</td>
<td>± 9.59</td>
<td>± 2.12</td>
<td>± 1.76</td>
<td>± 0.04</td>
<td>± 0.47</td>
<td>± 0.02</td>
<td>± 0.13</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg body weight) + CCl(_4)</td>
<td>± 4.49</td>
<td>± 4.49</td>
<td>± 3.57</td>
<td>± 3.19</td>
<td>± 1.74</td>
<td>± 0.04</td>
<td>± 0.76</td>
<td>± 0.05</td>
<td>± 0.12</td>
</tr>
</tbody>
</table>

Values are statistically significant at \( P<0.05 \). \(^{a}\)\( P<0.05 \), \(^{b}\)\( P<0.01 \) and \(^{c}\)\( P<0.001 \) between normal and ethyl acetate extract alone treated and CCl\(_4\) control groups. \(^{d}\)\( P<0.05 \), \(^{e}\)\( P<0.01 \) and \(^{f}\)\( P<0.001 \) between CCl\(_4\) control and treated groups.

Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Serum alkaline phosphatase (SALP).
mg/kg body weight also exhibited a significant reversal of all the changes caused by CCl₄ administration towards the normal (Table 1 and Fig. 2c). The standard silymarin treatment at 100 mg/kg body weight dose also showed the similar results.

The CCl₄ treatment showing decreased levels of catalase, superoxide dismutase and glutathione and increased levels of thiobarbituric acid reactive substances in liver and kidney when compared to normal animals. It also caused a significant decrease in the levels of glycogen in liver (P<0.01, when compared to normal animals). Whereas in the extract treated groups at 100 and 200 mg/kg body weight to CCl₄ intoxicated rats showing decreasing level of catalase, superoxide dismutase and thiobarbituric acid reactive substances in both liver and kidney and glutathione in kidney (Fig. 2d) towards the normal when compared to CCl₄ control. The high dose of extract (200 mg/kg body weight) also produced potential changes in all these biochemical parameters in both the tissues along with a reversal of glycogen in liver (Fig. 2e). The standard silymarin (100 mg/kg body weight) also showed similar results, except the thiobarbituric acid reactive substances in kidney and glycogen in liver. The extract treatment at 200 mg/kg body weight was found to be more potent than the standard silymarin in reversing most of the biochemical parameters towards the normal. The treatment with the ethyl acetate extract alone to normal rats caused an increase in the levels of SGPT, SALP and total cholesterol in serum and a decrease in the levels of creatinine in serum and glycogen and glutathione in liver was also observed. All these values were found to be significant.

Histopathological examination of liver sections of normal animals showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein (Fig. 3A). The liver sections of the rats of CCl₄ control group showed disarrangement of normal hepatic cells with high degree of damage, characterized by the centrilobular necrosis, focal necrosis and bile duct proliferation (Fig. 3C). The
sections of the rats treated with the ethyl acetate extract (100 and 200 mg/kg body weight) and intoxicated with CCl₄ exhibited less centrilobular necrosis and bile duct proliferation compared to the CCl₄ control (Figs. 3D and E). Dose dependent results were observed and the higher dose showed better activity. However, the standard silymarin at 100 mg/kg body weight and CCl₄ treated animals showed...
almost normal architecture of the liver with few centrilobular fatty changes and bile duct proliferation (Fig. 3F). However, the treatment with the ethyl acetate extract alone at 200 mg/kg body weight exhibited slight centrilobular fatty changes, necrosis and bile duct proliferation indicating its mild toxicity (Fig. 3B).

Kidney sections of normal animals showed normal histological appearance (Fig. 4A) and the CCl₄ control group showed high degree of tubulointerstitial
nephritis (Fig. 4C). The sections of the animals belonging to the ethyl acetate extract at both the doses and the standard silymarin treatment groups showed normal histological appearance (Figs. 4D-F). These findings clearly indicate that the liver and kidney tissues, which were damaged by CCl₄ intoxication showed recovery with these ethyl acetate extract and silymarin treatments. However, the ethyl acetate extract at 200 mg/kg body weight alone treated animals showed mild toxicity in kidney (Fig. 4B).

**Discussion**

In order to provide a scientific explanation for the folk use of *E. axillare* as a liver tonic in India, in the present study hepatoprotective and antioxidant effects of its ethyl acetate extract was carried out. Liver injury induced by CCl₄ is characterized system of the xenobiotic induced hepatotoxicity and is a commonly used model for the screening of hepatoprotective activity. When the liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released into the blood stream. Their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage.

The increased levels of SGOT, SGPT and SALP in CCl₄ treated rats in the present study interpreted as a result of the liver cell destruction or changes in the membrane permeability indicating severity of hepatocellular damage induced by CCl₄. The rise in SGOT activity is almost always due to hepatocellular damage and is usually accompanied by the rise in SGPT. An increase in SALP reflects the pathological alterations in biliary flow. Pretreatment with the ethyl acetate extract attenuated the increased activities of these enzymes in serum caused by CCl₄. Recovery towards the normalization suggests that the extract caused parenchymal cell regeneration in liver, thus protecting membrane fragility and thereby decreasing enzyme leakage.

Hepatocellular damage causes a modest hypertriglyceridemia, which is due to the biochemical changes inferring with the transport of triglycerides out of liver. The same is evidenced in the CCl₄ induced rats. A significant increase in cholesterol was observed in CCl₄ induced rats, which may be due to the inability of the diseased liver to remove cholesterol from circulation. The treatment with the ethyl acetate extract at both the doses caused a significant reversal of the altered triglycerides and total cholesterol levels towards the normal.

Determination of serum bilirubin represents an idea for the assessment of hepatic functions and any abnormal increase in the levels of bilirubin in the serum indicate hepatobiliary disease and severe disturbance of hepatocellular function. The extract mediated suppression of the increased bilirubin level caused by CCl₄ suggests the possibility of the extract being able to stabilize biliary dysfunction. The decrease in the level of total protein observed in CCl₄ treated rats may be associated with the decrease in the number of hepatocytes, which in turn may result in decreased hepatic capacity to synthesize protein. The extract at both the doses caused a significant reversal of total protein towards the normal indicating the increased hepatic capacity of the liver.

Hypoalbuminemia is caused by liver diseases and abnormally high levels of creatinine indicate possible malfunction or failure of the kidneys. A significant reversal was observed by the extract treatment towards the normal when compared to CCl₄ treated animals indicating the protection of those organs by the extract. It has been suggested that glycogen serves as an energy buffer capable of providing rapid and short-term energy. The elevation of depressed glycogen stores by the ethyl acetate extract in CCl₄ treated rats may be attributed to either an inhibition of hepatic glucose output improvement in plasma insulin levels or by synthetase responsible for the incorporation of glucose moieties into pre-existing glycogen chains. These results lend credence to the use of *E. axillare* as antihyperglycemic agent.

Superoxide dismutase and catalase form a mutually supportive team of defence against ROS. In CCl₄ induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost and oxidative stress results. Due to this, deregulation of cellular functions takes place leading to hepatic necrosis. The reduced activities of superoxide dismutase and catalase due to the administration of CCl₄ were reversed significantly in serum, liver and kidney of the extract treated animals indicating strong antioxidant activity of the extract.

The level of lipid peroxides is a measure of membrane damage and alterations in structure and functions of cellular membrane. In the present study increase in the malondialdehyde levels in rats treated with CCl₄ was observed in serum, liver and kidney suggesting enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defence mechanisms to prevent the formation of excessive
free radicals. The extract treatment caused significant reduction in malondialdehyde levels in all the samples tested indicating the reduction in free radical yield and subsequent decrease in harm and damage to the cell membrane and decreasing malondialdehyde production.

Non enzymic antioxidant glutathione is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic glutathione has been shown to be associated with an enhanced toxicity to chemicals including CCl₄. In the present study, a decrease in kidney glutathione level was observed in the CCl₄ treated group. The increase in kidney glutathione level in the rats treated with the extract may be due to de novo glutathione synthesis or its regeneration. Hence, it is possible that the mechanism of hepatoprotection of the extract may be due to its antioxidant action.

Histopathological studies were performed to provide direct evidence of the hepatotoxicity of CCl₄ and of the hepatoprotective effect of the ethyl acetate extract of E. axillare. Marked disruptions of the structure of hepatocytes were noted in liver tissue of rats exposed to CCl₄ only. Minimal disruption of the structure of hepatocytes was noted in liver tissue of rats intoxicated with CCl₄ and treated with the ethyl acetate extract. These results complement the results of the biochemical estimations, where a reversal of effects of CCl₄ towards the normal was observed.

The animals treated with the ethyl acetate extract alone to normal rats caused a significant increase in the levels of SGPT, SALP and total cholesterol in serum and significant decrease in levels of creatinine in serum and glycogen and glutathione in liver. These results indicate slight toxicity of the extract treatment when given alone at 200 mg/kg body weight orally. The histological picture of the liver treated with the extract alone at high dose also exhibited slight centrilobular fatty changes, necrosis and bile duct proliferation supporting the biochemical alterations in serum and liver. However, the extract given along with CCl₄ produced strong hepatoprotective action when given at 100 and 200 mg/kg body weight orally for seven days prior to CCl₄ treatment.

Vasu et al. have shown potent in vivo antioxidant activity of the aqueous extract of E. littorale in hypercholesterolemic rats. The elevated serum cholesterol and triglyceride levels were found to be decreased significantly in the extract treated animals. Restoration of serum cholesterol and triglyceride levels by the aqueous extract of E. littorale in alloxan induced diabetic rats was also observed. The results in the present study are in agreement with these reports.

The preliminary phytochemical analysis of the ethyl acetate extract indicated the presence of secoiridoid glycosides, flavonoids, saponins and phenolics. The HPTLC profile of the extract showed high content 7.26% of swertiamarin in it. Several such constituents are known to exhibit potent hepatoprotective properties. Iridoid glycosides of Scrophularia koelzii are known for their antihepatotoxic properties. They are considered to be the active principles of many other medicinal plants. Hence, swertiamarin may be the active constituent of E. axillare.

In conclusion, the histological and biochemical evidences show that the pretreatment with the ethyl acetate extract of E. axillare effectively protected rats against CCl₄ induced hepatotoxicity. It provides a support for the traditional use of E. axillare in liver disorders in India. The present work and the in vitro antioxidant activity carried out earlier also indicate that the ethyl acetate extract of the plant may be the active extract. Further studies should be conducted to determine the active compounds that are responsible for the hepatoprotective and antioxidant effects and the mechanism of action involved in these.

References
1 Kirtikar K R & Basu B D, Indian medicinal plants. 2nd ed. (Bishen Sing Mahendra Pal Sing, Dehradun, India) 1999, 1655.
11 Mani Senthil Kumar, Raj Kapoor & Kavimani, Protective Effect of Enicostemma littorale in CCl4-induced hepatic damage in rats, Pharm Biol, 43 (2005) 485.
20 Ellman G L, Tissue sulphydryl group, Arch Biochem Biophys, 82 (1959) 70.
24 Ohkawa H, Ohishi N & Yagi K, Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction, Anal Biochem, 95 (1979) 351.