Hepatoprotective and Antioxidant Activity of Ethanol Extract of Cynoglossum zeylanicum (Vahl ex Hornem) Thurnb ex Lehm in CCl₄ Treated Rats

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ABSTRACT
This study was designed to evaluate the hepatoprotective and antioxidant effect of ethanol extract of whole plant of Cynoglossum zeylanicum on CCl₄ induced hepatotoxicity in rats. Activities of liver marker enzymes, SGOT, SGPT and ALP, total protein, albumin, globulin, total, conjugated and unconjugated bilirubins at an oral dose of ethanol extract of Cynoglossum zeylanicum(50, 100 and150mg/kg) showed a significant hepatoprotective effect. Regarding antioxidant activity, ethanol extract of Cynoglossum zeylanicum exhibited a significant effect showing increasing levels of SOD, CAT, GPx, GSH and GRD by reducing malondialdehyde (MDA) levels.

Key words: Hepatoprotective activity, Antioxidant, CCl₄, Bilirubin, MDA, GGT.

INTRODUCTION
Liver is a key organ regulating homeostasis within the body by various functions. Liver injury caused by toxic chemicals and certain drugs has been recognized as a toxicological problem. Hepatotoxicity is one of the very common ailment resulting into serious deilities ranging from severe metabolic disorders to even mortality (Patel et al., 2008).

Liver is a dual organ having both secretory and excretory functions. Also liver is the only organ that has the remarkable property of self regeneration, due to hepatocytes acting as unipotential stem cells.

Liver is the target organ and primary site of detoxification and is generally the major site of intense metabolism and hence prone to various disorders (Paliwal et al., 2009).

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Usually the prescribed dosages of drugs are tolerated but overdose is the most common cause of drug induced liver diseases. In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systemic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity.

The attention of pharmacologists throughout the world has been focused on finding out safer and potent hepatoprotective drug. The natural products today symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to humans and environment. So people are returning to the natural product with the hope of safety and security (Rachehh et al., 2011).

However so far there is no systematic study on hepatoprotective activity has been reported in the literature. Hence the present study focuses on evaluating the hepatoprotective activity of whole plant of Cynoglossum zeylanicum.
MATERIALS AND METHODS

Plant material

The whole plant of *Cynoglossum zeylanicum* was collected from Kothagiri, Nilagiri Biosphere Reserve, Western Ghats, Tamil Nadu and identified by the Botanical Survey of India, Coimbatore. A voucher specimen was retained in Ethnopharmacology Unit, Research Department of Botany, V. O. Chidambaram College, Tuticorin for further reference.

Preparation of plant extract for phytochemical Screening and Hepatoprotective Studies

The whole plant was dried under shade and then powdered with a mechanical grinder to obtain a coarse powder, which was then subjected to extraction in a Soxhlet apparatus using ethanol. The extract was subjected to qualitative test for the identification of various phytochemical constituents as per standard procedures (Brinda et al., 1981; Anonymous, 1990; Lala, 1993). The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extract were used for hepatoprotective studies.

Animals

Normal healthy male Wistar albino rats (180-240g) were used for the present investigation. Animals were housed under standard environmental conditions at room temperature (25±2°C) and light and dark (12:12h). Rats were fed with standard pellet diet (Goldmohur brand, MS Hindustan lever Ltd., Mumbai, India) and water ad libitum.

Acute Toxicity Studies

Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method), albino rats (n=6) of either sex selected by random sampling were used for acute toxicity study (OECD, 2002). The animals were kept fasting for overnight and provided only with water, after which the extracts were administered orally at 5mg/kg body weight by gastric intubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 50, 100 and 2000 mg/kg body weight.

Experimental Design

In the investigation, a total of 30 rats (25 CCl₄ hepatic toxicity induced rats and 5 normal rats) were taken and divided into five groups of 5 rats each.

**Group I:** Rats received normal saline was served as a normal control.

**Group II:** CCl₄ hepatic toxicity induced control: Rats received 2.5ml/kg body weight of CCl₄ for 14 days.

**Group III:** Liver injured rats received ethanol extract of whole plant of *Cynoglossum zeylanicum* at the dose of 50 mg/kg body weight for 14 days.

**Group IV:** Liver injured rats received ethanol extract of whole plant of *Cynoglossum zeylanicum* at the dose of 100mg/kg body weight for 14 days.

**Group V:** Liver injured rats received ethanol extract of whole plant of *Cynoglossum zeylanicum* at the dose of 150mg/kg body weight for 14 days.

**Group VI:** Liver injured rats received standard drug silymarin at the dose of 100mg/kg body weight for 14 days.

Biochemical Analysis

The animals were sacrificed at the end of experimental period of 14 days by decapitation. Blood was collected, sera separated by centrifugation at 3000g for 10 minutes. Serum protein (Lowry, 1951) and serum albumins was determined quantitatively by colorimetric method using bromocresol green. The total protein minus the albumin gives the globulin. Serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) was measured spectrophotometrically by using the method of Reitman and Frankel (1957). Serum alkaline phosphatase (ALP) was measured by the method of King and Armstrong (1934).

Total bilirubin and conjugated bilirubin were determined as described by Balistrei and Shaw (1987). The unconjugated bilirubin concentrations were calculated as the difference between total and conjugated bilirubin concentrations. Gamma-glutamyltransferase (GGT) was estimated by the method of Sazsz (1969). Liver homogenates (10%W/V) were prepared in ice cold 10mM tris buffer (pH7.4). Quantitative estimation of MDA formation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in 10% liver homogenates by the method of Okhawa. Enzymatic antioxidants, superoxide dismutase (SOD) (Mishra and Fridovich, 1972) Catalase (Aebi, 1974; Colowick, 1984) and non enzymatic antioxidant glutathione peroxidase (GPx) (Pagila and Valentine, 1967) glutathione reductase (GRD) (Goldberg and Spooner, 1983) and reduced glutathione (GSH) (Prins and Loos, 1969) were also assayed in liver homogenates.

Statistical Analysis

The data were expressed as the mean ± S.E.M. The difference among the means has been analyzed by one-way ANOVA. p<0.001, p<0.01 and p<0.05 were considered as statistical significance using SPSS Software.

RESULTS

The ethanol extract of whole plant of *Cynoglossum zeylanicum* subjected for phytochemical study showed the presence of alkaloids, coumarins, glycosides, flavonoids, saponins, steroids, phenols, tannins and xanthoproteins. The ethanol extract did not show any sign and symptoms of toxicity and mortality upto 2000 mg/kg dose. The effect of ethanol extract of *Cynoglossum zeylanicum* on body weight of the normal, CCl₄ intoxicated and drug treated rats are shown in Table 1. Table 2 shows the effect of
ethanol extract of *Cynoglossum zeylanicum* on serum total protein, albumin, globulin, A/G ratio, serum transaminases, alkaline phosphatases in CCl4 intoxicated rats.

There was a significant (*p* < 0.01) increase in serum GOT, GPT and ALP levels in CCl4 intoxicated group (Group II) compared to the normal control group (Group I). The total protein and albumin levels were significantly (*p* < 0.01) decreased at the levels of 8.12g/dl and 4.20g/dl respectively in normal group. Ethanol extract of *Cynoglossum zeylanicum* at the dose of 50mg/Kg orally significantly decreased the elevated serum marker enzymes and reversed the altered total protein and albumin to almost normal level.

The effect of ethanol extract of *Cynoglossum zeylanicum* on total, conjugated and unconjugated bilirubin is shown in Table 1. The ethanol extract of *Cynoglossum zeylanicum* at the dose of 50mg/Kg orally significantly decreased the elevated serum marker enzymes and reversed the altered total protein and albumin to almost normal level.

The effects of ethanol extract of *Cynoglossum zeylanicum* on lipid peroxidation (LPO), Glutathione peroxidase (GPx), glutathione reductase (GRD), superoxide dismutase (SOD) catalase (CAT) and reduced glutathione (GSH) activity was shown in Table 2. Lipid peroxidation level was significantly (*p* <0.01) increased and glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase activity were significantly (*p* < 0.01) decreased in CCl4 intoxicated rats when compared with those of the animals in normal control group. Rats treated with ethanol extract of *Cynoglossum zeylanicum* at the doses of 50 mg/kg significantly decreased the elevated lipid peroxidation levels and restored the altered glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase and reduced glutathione levels towards the normal levels in a dose dependent manner. The results are well comparable with silymarin (standard drug) treated group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Initial Body weight (Gm)</th>
<th>Final Body weight (Gm)</th>
<th>Mean weight Gain (G↑ / loss↓) (Gm)</th>
<th>% of Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0% Saline</td>
<td>23.25±4.46</td>
<td>268.54±8.55</td>
<td>6.29↑</td>
<td>2.65</td>
</tr>
<tr>
<td>Group II</td>
<td>0% Saline</td>
<td>224.50±6.54</td>
<td>194.45±3.54</td>
<td>26.05↑</td>
<td>11.60</td>
</tr>
<tr>
<td>Group III</td>
<td>50(mg/Kg)</td>
<td>231.56±7.64</td>
<td>219.16±4.85</td>
<td>12.40↓</td>
<td>5.35</td>
</tr>
<tr>
<td>Group IV</td>
<td>100(mg/Kg)</td>
<td>229.16±8.17</td>
<td>226.15±4.54</td>
<td>3.01↓</td>
<td>1.30</td>
</tr>
<tr>
<td>Group V</td>
<td>150(mg/Kg)</td>
<td>230.50±6.35</td>
<td>235.11±7.65</td>
<td>6.61↑</td>
<td>2.86</td>
</tr>
<tr>
<td>Group VI</td>
<td>200(mg/Kg)</td>
<td>219.16±4.76</td>
<td>228.55±5.45</td>
<td>9.39↑</td>
<td>4.28</td>
</tr>
</tbody>
</table>

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test. *P* <0.05 as compared with normal control to liver damaged control

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/Kg)</th>
<th>T-Protein (g/dl)</th>
<th>Albumin (g/dl)</th>
<th>Globulin (g/dl)</th>
<th>A/G Ratio</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.9% Saline</td>
<td>8.12±0.26</td>
<td>4.20±0.11</td>
<td>3.92±0.52</td>
<td>1.07:1</td>
<td>21.66±1.56</td>
<td>24.16±1.16</td>
<td>121.3±1.88</td>
</tr>
<tr>
<td>Group II</td>
<td>0.9% Saline</td>
<td>6.88±0.24**</td>
<td>3.65±0.17</td>
<td>2.76±0.17</td>
<td>1.49:1</td>
<td>98.54±3.66**</td>
<td>109.16±3.27**</td>
<td>216.35±4.84**</td>
</tr>
<tr>
<td>Group III</td>
<td>50(mg/Kg)</td>
<td>5.68±0.16**</td>
<td>3.98±0.36</td>
<td>2.60±0.14</td>
<td>1.53:1</td>
<td>24.65±1.84 aa</td>
<td>21.59±2.69 aa</td>
<td>134.33±2.08 aa</td>
</tr>
<tr>
<td>Group IV</td>
<td>100(mg/Kg)</td>
<td>7.12±0.54*</td>
<td>4.16±0.33</td>
<td>2.96±0.21</td>
<td>1.40:1</td>
<td>35.13±3.66*aa</td>
<td>37.92±4.16*aa</td>
<td>178.39±4.67*aa</td>
</tr>
<tr>
<td>Group V</td>
<td>150(mg/Kg)</td>
<td>6.73±0.16ns</td>
<td>4.33±0.16</td>
<td>3.30±0.10</td>
<td>1.31:1</td>
<td>40.56±1.93*aa</td>
<td>47.33±6.54*aa</td>
<td>209.36±5.29*aa</td>
</tr>
<tr>
<td>Group VI</td>
<td>200(mg/Kg)</td>
<td>8.11±0.27a</td>
<td>4.66±0.19</td>
<td>3.45±0.21</td>
<td>1.35:1</td>
<td>16.36±1.39aaa</td>
<td>18.67±2.16aaa</td>
<td>126.59±3.16**aaa</td>
</tr>
</tbody>
</table>

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test. *P* <0.05; **P <0.01; ***P <0.001 as compared with Normal Control to liver damaged control. *P* <0.05; *P* <0.01; **P <0.001 as compared with liver damaged control to drug treated animal: ns not significant.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/Kg)</th>
<th>Total Bilirubin (Mg/dl)</th>
<th>Conjugated Bilirubin (Mg/dl)</th>
<th>Unconjugated Bilirubin (Mg/dl)</th>
<th>GGT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.9% Saline</td>
<td>0.71±0.08</td>
<td>0.20±0.02</td>
<td>0.51±0.03</td>
<td>8.12±0.27</td>
</tr>
<tr>
<td>Group II</td>
<td>0.9% Saline</td>
<td>2.94±0.14***</td>
<td>2.12±0.14***</td>
<td>0.82±0.05**</td>
<td>26.84±1.17***</td>
</tr>
<tr>
<td>Group III</td>
<td>50(mg/Kg)</td>
<td>1.14±0.31*</td>
<td>0.69±0.11*a</td>
<td>0.45±0.03nsa</td>
<td>12.71±1.42**</td>
</tr>
<tr>
<td>Group IV</td>
<td>100(mg/Kg)</td>
<td>1.69±0.16**</td>
<td>0.80±0.17**</td>
<td>0.79±0.02*</td>
<td>14.36±1.26*a</td>
</tr>
<tr>
<td>Group V</td>
<td>150(mg/Kg)</td>
<td>1.88±0.03**</td>
<td>1.00±0.02***</td>
<td>0.88±0.01**</td>
<td>16.26±1.37***</td>
</tr>
<tr>
<td>Group VI</td>
<td>200(mg/Kg)</td>
<td>0.93±0.02nsaa</td>
<td>0.24±0.06aaa</td>
<td>0.69±0.03sa</td>
<td>10.27±0.98nsaaa</td>
</tr>
</tbody>
</table>

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test. *P* <0.05; **P <0.01; ***P <0.001 as compared with Normal Control to liver damaged control. *P* <0.05; *P* <0.01; **P <0.001 as compared with liver damaged control to drug treated animal: ns not significant.
DISCUSSION

Liver is largest organ and it is target for toxicity of its role in clearing and metabolizing chemicals through the process called detoxification (Larrey, 2003). Drug induced liver disorders occurred frequently can be life threatening and mimic all forms of liver diseases (Watkins and Seef, 2006). CCl₄ produces an experimental damage that histologically resembles viral hepatitis. Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures (Recknagel, 1983). The toxic metabolite, CCl₃ radical is produced and further reacts with oxygen to give trichloromethyl peroxy radical. Cytochrome P₄₅₀ is the enzyme responsible for this conversion. This radical binds covalently to the macromolecule and causes peroxidative degradation of lipid membrane of the adipose tissue, which leads to leakage of serum marker enzymes. It is possible that hepatocellular damage occurs when the free radicals generation exceeds the cellular radicals scavenging capacity(Jadhav et al., 2010). Assessment of liver toxicology was done by measuring the marker enzymes such as SGOT, SGPT and ALP, which are originally present in high concentration in the cytoplasm. When there is hepatic injury these enzymes leak into blood stream inconformity with extent of hepatotoxicity. Whole plant extract of Cynoglossum zeylanicum at the doses 50mg/kg significantly restored the elevated levels of serum marker enzymes. The normalization of serum markers by Cynoglossum zeylanicum whole plant suggests that they are able to condition the hepatocytes so as to protect the membrane integrity against CCl₄ induced leakages of marker enzymes into the circulation . The above changes can be considered as an expression of the functional improvement of hepatocytes.

Protein metabolism is a major project of liver and a healthy functioning liver is required for the synthesis of the serum protein. Hypoproteinemia is a feature of liver damage due to significant fall in protein synthesis. The reduction in the serum albumin and globulin levels in CCl₄ intoxicated group might be due to liver damage. Hepatotoxicity impairs the synthetic function of the liver (David, 1999). Treatment with ethanol extract of Cynoglossum zeylanicum whole plant ameliorated the imbalance.

Serum bilirubin is one of the most sensitive tests employed in the diagnosis of hepatic diseases. Hyperbilirubinemia was observed due to excessive heme destruction and blockage of biliary tract. As a result of blockage of the biliary tract there is a mass inhibition of the conjugation reaction and release of unconjugated bilirubin from damaged and dead hepatocytes (Wolf et al., 1997). Administration of Cynoglossum zeylanicum decreases the level of bilirubin and increased the level of protein suggesting that it offered protection.

γ-glutamyl transferase (GGT) is a microsomal enzyme, which is widely distributed in tissue including liver. The activity of serum γ-glutamyl transferase is generally elevated as a result of liver disease, since γ-glutamyl transferase is a hepatic microsomal enzyme. Serum γ-glutamyl transferase is most useful in the diagnosis of liver diseases. Changes in γ-glutamyl transferase is parallel to those of amino transferases. The acute damage caused by CCl₄ increased the γ-glutamyl transferase level but the same attains the normal after Cynoglossum zeylanicum treatment due to its antioxidant activity.

The body has an effective mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase. When the balance between ROS production and antioxidant defense is lost, oxidative stress results, which through a series of events deregulates the cellular functions leading to various pathological conditions (Castro et al., 1974). Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this type of damage.

Lipid peroxidation (LPO) has been postulated to the destructive process of liver injury due to acetaminophen administration. In the present study the elevations in the levels of end products of lipid peroxidation in the liver of the rat treated with CCl₄ was observed. The increase in melondialdehyde (MDA) levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with Cynoglossum zeylanicum whole plant significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection by ethanol extract of Cynoglossum zeylanicum whole plant due to its antioxidant effects.

Glutathione (GSH), extensively found in cells, protects cells against electrophilic attacks provided by xenobiotics such as free radicals and peroxides GSH deficiency leads to cellular
damage in kidney, muscle, lung, jejunum, colon, liver, lymphocytes and brain (Orhan et al., 2007). The elevation of MDA level, which is one of the end products of lipid peroxidation in the liver tissue, and the reduction in hepatic GSH levels are important indicators in CCl₄ intoxicated rats. In this study, it was ascertained that MAD levels have been suppressed compared to CCl₄ intoxicated group and CCl₄ induced depletion of GSH was prevented.

Superoxide dismutase (SOD), a metallo protein is the most sensitive enzyme index in liver injury and one of the most important enzyme in the enzymatic antioxidant defense system. It scavenges the superoxide anion to from hydrogen peroxide and oxygen, hence diminishing the toxic effect caused by this radical (Cartis et al., 1972). In the present study, it was observed that the ethanol extract of Cynoglossum zeylanicum whole plant significantly increased the SOD activity in CCl₄ intoxicated rats thereby diminished CCl₄ induced oxidative damage.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found to the red cells and in the liver. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals (Chance et al., 1952). Therefore the reduction in the activity of these enzymes may result in the number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. Administration of ethanol extract of Cynoglossum zeylanicum increased the activities of CAT in CCl₄ induced liver damage in rats to prevent the accumulation of excessive free radical and protected the liver from CCl₄ intoxication.

Glutathione peroxide (GPx) is a seleno enzyme, it protect the cells from damage due to free radicals like hydrogen and lipid peroxides (Zaltzber et al., 1999). It catalyzes the reaction of hydroperoxidases with reduced glutathione to form glutathione disulphide and reduction

**REFERENCE**


