Antioxidant and Hepatoprotective Activity of Ethanol Extract of
*Indigofera trita* Linn. on CCl4, Induced Hepatotoxicity in Rats

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**Abstract:** The ethanol extract of *Indigofera trita* (EIT) was studied for its antioxidant and hepatoprotective properties. The ethanol extract exhibited potent *in vitro* antioxidant activity as evidenced by the low I$_{50}$ values in the scavenging of ABTS, DPPH and hydroxyl radical methods. The I$_{50}$ values obtained were 9.50±0.50 and 19.91±1.73 μg mL$^{-1}$, respectively for ABTS and DPPH methods. The I$_{50}$ values obtained for hydroxyl radical scavenging by p-NDA and deoxyribose methods were found to be 104.50±4.50 and 99.60±0.28 μg mL$^{-1}$, respectively. The treatment with the EIT at 200 and 400 mg kg$^{-1}$ body weight showed a significant and dose dependent decrease in the levels of SGOT, SGPT, ALP and TBARS and significant increase in the levels of albumin, total protein, SOD and catalase, when compared to CCl4 treated rats. The treatment with EIT exhibited better results than the standard vitamin-E treatment in some of these parameters. Thus, the EIT showed significant antioxidant and hepatoprotective activity. These results were also confirmed by the histological observation.

**Key words:** *Indigofera trita*, Fabaceae, antioxidant, hepatoprotective, carbon tetrachloride

**INTRODUCTION**

Free radicals play an important role in various pathological conditions such as tissue injury, inflammation process, neurodegenerative diseases, cancer and aging. The compounds that can scavenge free radicals have great potential in ameliorating these disease processes (Coban et al., 2003). Recently natural products and drugs as antioxidant agents have received much attention and herbal medicine has been improved in developing countries as an alternative solution to health problems and costs of pharmaceutical products. Many plant extracts and plant products have been shown to have significant antioxidant activity (Coulidis et al., 2003; Lee et al., 2001). The antioxidant activity of the inhibition of the generation of free radicals is important in providing protection against hepatic damage. A number of plants have been shown to possess hepatoprotective property by improving antioxidant status. Thus, the efficiency of the plant would be preventative and passive for defending against damages.

Plants and plant products have been shown to play an important role in the management of various liver disorders. *Indigofera trita* Linn. (Family: Fabaceae) is an under shrub with wide distribution, mostly found in India, Ceylon, South Africa and North Australia. The plant is known as Kattuvan and Punal Murungai in Tamil. The entire plant is traditionally used for various ailments including liver disorders and tumors (Nadkarni, 1996; Kirtikar and Basu, 1993). It is found to be active
against transplantable tumors. Plants containing flavonoids and phenolics are known to possess strong antioxidant and hepatoprotective properties (Tripathi et al., 1996). Hence, the aim of the present study is to evaluate the antioxidant and hepatoprotective activity of ethanol extract of Indigofera trita (EIT) on carbon tetrachloride induced hepatotoxicity in rats.

MATERIALS AND METHODS

Collection and Extraction

Entire plant of Indigofera trita were collected in and around the foothill of Shevaroys in Salem district, Tamil Nadu, India, in the month of February 2006 and authenticated by Dr. R. Gopalan, Botanical Survey of India, Coimbatore, Tamilnadu, India. The entire plants were shade dried and pulverized. The powder was treated with Petroleum ether for dewaxing and removal of chlorophyll. Later, it was packed (250 g) in a Soxhlet apparatus and subjected to continuous hot percolation for 8 h using 450 mL of ethanol (95% w/v) as solvent. The extract was concentrated under vacuum and dried in a dessicator (yield, 11.25 g, 4.5% w/w).

Animals

Male Wistar albino rats (150-200 g) and Swiss albino mice (20-25 g) were procured from Venkatashwara Enterprises, Bangalore, Karnataka, India and used throughout the study. They were housed in microlon boxes in a controlled environment (temperature 25±2°C and 12 h dark/light cycle) with standard laboratory diet and water ad libitum. The experiments were performed in accordance with the guidelines established by the European community for the care and use of laboratory animals and were approved by Institutional Animal Ethical Committee (IAEC).

Acute Toxicity Studies (LD₅₀)

Acute Oral Toxicity (AOT) of EIT was determined using Swiss albino mice. The animals were fasted for 3 h prior to the experiment and were administered with single dose of extracts dissolved in 5% gum acacia (doses ranges from 500-2000 mg kg⁻¹ at various dose levels) and observed for mortality up to 48 h (short term toxicity). Based on the short-term toxicity, the dose of next animal was determined as per OECD guideline 425. All the animals were also observed for long-term toxicity (14 days). The LD₅₀ of the test extract was calculated using ‘AOT 425’ software provided by Environmental Protection Agency, USA.

Chemicals

2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2, 2’-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma Aldrich Co., St. Louis, USA. Rutin and p-nitroso dimethyl ariline (p-NDA) were obtained from Acros Organics, New Jersey, USA. Naphthyl Ethylene Diamine Dihydrochloride (NEDD) was obtained from Roch-Light Ltd., Suffolk, UK. All chemicals used were of analytical grade.

Preparation of Extracts and Standard

The extract and standard antioxidants, ascorbic acid, rutin and butylated hydroxyl anisole were dissolved in distilled dimethyl sulfoxide (DMSO) separately and used for the in vitro antioxidant testing using six different methods except the hydrogen peroxide method (where, DMSO interferes with the test). For the hydrogen peroxide method, the extract and standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain the lower dilutions. For in vivo experiments, the suspension of EIT was prepared in 5% gum acacia. Vitamin E standard was dissolved in pure arachis oil and used.
In vitro Antioxidant Activity
Scavenging of ABTS Radical Cation
To 0.2 mL of various concentrations of the extract or standard, added 1.0 mL of distilled DMSO and 0.16 mL of ABTS solution. Absorbance of these solutions was measured spectrophotometrically, after 20 min at 734 nm against the corresponding blank solution. IC_{50} values were calculated. IC_{50} value is the concentration of sample required to scavenge 50% free radical (Re et al., 1999).

DPHP Radical Scavenging Method
DPHP scavenging activity was measured by the spectrophotometric method (Sreejayan and Rao, 1996). Various concentrations of the extract or standard solution (10 μL) were added to DPHP in methanol solution (200 μL) in a 96-well microtiter plate. An equal amount of ethanol was added to the control. After 20 min, the decrease in the absorbance of the test mixture (due to quenching of DPHP free radicle) was read at 517 nm and the percentage inhibition calculated by using the formula

\[
\text{Inhibition} (\%) = \left[ \frac{\text{control} - \text{test}}{\text{control}} \right] \times 100
\]

Nitric Oxide Radical Inhibition Assay
This assay was performed according to the method of Sreejayan and Rao (1997). Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction (Green et al., 1982; Marcocci et al., 1994). Sodium nitroprusside (5 mM in standard phosphate buffer solution was incubated with different concentrations of the extract or standard solutions (1 mL) were incubated at 25°C for 5 h. Control experiments without the extract, but with equivalence amounts of buffer were conducted in an identical manner. After 5 h, 0.5 mL of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride) The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylethylene diamine was read at 546 nm. The experiment was repeated in triplicate.

Scavenging of Hydroxyl Radical by p-NDA Method
To a solution mixture containing ferric chloride (0.1 mM, 0.5 mL), EDTA (0.1 mM, 0.5 mL), ascorbic acid (0.1 mM, 0.5 mL), hydrogen peroxide (2 mM, 0.5 mL) and p-NDA (0.01 mM, 0.5 mL) in phosphate buffer (pH 7.4, 20 mM), were added various concentrations of extract or standard in distilled DMSO (0.5 mL), to give a final volume of 3 mL. Absorbance was measured at 440 nm (Barry et al., 1987).

Scavenging of Hydroxyl Radical by Deoxyribose Method
To the reaction mixture containing deoxyribose (3 mM, 0.2 mL), ferric chloride (0.1 mM, 0.2 mL), EDTA (0.1 mM, 0.2 mL), ascorbic acid (0.1 mM, 0.2 mL) and hydrogen peroxide (2 mM, 0.2 mL) in phosphate buffer (pH. 7.4, 20 mM), were added 0.2 mL of various concentrations of extract or standard in DMSO to give a total volume of 1.2 mL. The solutions were then incubated for 30 min at 37°C. After incubation, ice-cold trichloro acetic acid (0.2 mL, 15% w/v) and thiobarbituric acid (0.2 mL, 1% w/v), in 0.25 N HCl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and absorbance was measured at 532 nm (Barry et al., 1987).

Scavenging of Super Oxide Radical by Alkaline DMSO Method
Super oxide was generated according to the alkaline DMSO method, (Badami et al., 2003). The reduction of NBT by super oxide was determined in the presence and absence of the extract. To the reaction mixture containing 0.1 mL of NBT (0.1 mg) and 0.3 mL of extract or standard in DMSO, added 1 mL of alkaline DMSO (1 mL, 1% water, 5 mM NaOH) to give a final volume of 1.4 mL and the absorbance was measured at 560 nm.
Scavenging of Hydrogen Peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of the extract or standard in methanol (1 mL) were added to 2 mL of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm, after 10 min against a blank solution that contained extract or standard in PBS without hydrogen peroxide (Jayaprakasha et al., 2004).

In vivo Antioxidant Activity

Animals were divided into five groups comprising of six animals in each group. Group I served as normal, Group II served as CCl₄ treated control. Animals of these two groups received 5% gum acacia. Group III and IV received 200 and 400 mg kg⁻¹ body weight of the EIT, respectively. Group V received 50 mg kg⁻¹ body weight of vitamin-E. All these treatments were given orally for 7 days. On the 7th day, after 1 h of sample administration, except for group I, all other group animals received 1.0 mL kg⁻¹ body weight of CCI₄ intraperitoneally. On the 8th day, the animals were sacrificed by decapitation and the blood was collected by heart puncture. It was kept at 37°C in the incubator for 30 min and cold centrifuged at 2000 rpm for 15 min to get a clear supernatant serum, which was used for the biochemical estimations. The liver and kidneys were removed, weighed and homogenized immediately in ice chilled 10% KCl solution (10 mL g⁻¹ of tissue). After centrifugation at 2000 rpm for 10 min, clear supernatant was used for the biochemical estimations. The liver tissue was processed for histopathological studies.

Catalase was estimated by following the breakdown of hydrogen peroxide according to the method of Beers and Sizer (1952), superoxide dismutase (SOD) was assayed according to Misra and Fridovich (1972) based on the inhibition of epinephrine auto-oxidation by the enzyme. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content following the thiobarbituric acid method of Ohkawa et al (1979). The liver enzymes SGOT, SGPT, ALT and total protein and albumin were estimated by standard procedures.

Statistical Analysis

All values were expressed as mean±SEM. Statistical analysis was performed with Student’s t-test. p<0.05 were considered to be statistically significant.

RESULTS

Acute Toxicity Studies (LD₅₀)

The extract treated animals were observed for mortality up to 48 h (short term toxicity) and for long-term toxicity (14 days). Based on the results the extract did not produce any mortality up to 2000 mg kg⁻¹.

In vitro Antioxidant Activity

The EIT exhibited potent antioxidant activity with low IC₅₀ values in the scavenging of ABTS, DPPH and hydroxyl radical methods. The IC₅₀ values obtained were 9.50±0.50 and 19.91±1.73 µg mL⁻¹ respectively for ABTS and DPPH methods (Table 1). The IC₅₀ values obtained for hydroxyl radical scavenging by p-NDA and deoxyribose methods were found to be 10.45±1.50 and 99.00±0.28 µg mL⁻¹, respectively. The extract also showed moderate antioxidant activity in the hydrogen peroxide and nitric oxide radical inhibition methods. However, these IC₅₀ values were found to be higher or comparable with those obtained for the standards used. In the other methods, the extract showed high IC₅₀ values indicating moderate to low activity.
Table 1. In vitro antioxidant activity of EIT using different methods

<table>
<thead>
<tr>
<th>Total material</th>
<th>DPPH</th>
<th>ABTS</th>
<th>1,1-Diphenyl-2-picrylhydrazyl (DPPH)</th>
<th>2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)</th>
<th>Superoxide radical scavenging by aktinide DMPG</th>
<th>Hydroxy radical scavenging by Vit E analogue</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIT</td>
<td>9.54±0.50</td>
<td>15.94±1.73</td>
<td>194.56±4.50</td>
<td>99.0±0.28</td>
<td>348.2±6.12</td>
<td>64.0±0.05</td>
</tr>
<tr>
<td>Acetone</td>
<td>11.2±0.55</td>
<td>17.4±2.27</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.5±0.01</td>
<td>0.6±0.19</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>150±0.5</td>
<td>100±0.1</td>
</tr>
<tr>
<td>Butylated hydroxyanisole (BHA)</td>
<td>-</td>
<td>-</td>
<td>&gt;1000</td>
<td>74.6±0.45</td>
<td>-</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

*average of three determinations

Table 2. In vivo antioxidant activity of EIT in rats

<table>
<thead>
<tr>
<th>SOD</th>
<th>Catalase</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Kidney</td>
<td>Serum</td>
</tr>
<tr>
<td>(Unit/ml)</td>
<td>(Unit/ml)</td>
<td>(Unit/ml)</td>
</tr>
<tr>
<td>Normal</td>
<td>1.0±0.08</td>
<td>1.0±0.06</td>
</tr>
<tr>
<td>CCl4 Control</td>
<td>0.8±0.06</td>
<td>0.7±0.05</td>
</tr>
<tr>
<td>EIT, 200 mg kg⁻¹</td>
<td>0.9±0.07</td>
<td>0.8±0.06</td>
</tr>
<tr>
<td>EIT, 400 mg kg⁻¹</td>
<td>0.9±0.07</td>
<td>0.8±0.06</td>
</tr>
<tr>
<td>Vitamin E, 50 mg kg⁻¹</td>
<td>1.0±0.07</td>
<td>1.0±0.06</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM (n = 6); p<0.05, p<0.01, p<0.001, when compared to normal group, p<0.05, p<0.01, p<0.001, when compared with CCl4 treated control, p<0.05, p<0.01, p<0.001, when compared to standard vitamin E (Student’s t-test)

Table 3. Effect of EIT on CCl4 induced hepatotoxicity in rats (Liver)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
<th>Albumin (g dl⁻¹)</th>
<th>Total protein (g dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>34.2±4.63</td>
<td>63.4±6.93</td>
<td>164.9±7.59</td>
<td>2.9±0.24</td>
<td>6.0±0.40</td>
</tr>
<tr>
<td>CCl4 Control</td>
<td>418.2±22.3</td>
<td>322.8±31.9</td>
<td>250.9±56.25</td>
<td>1.40±0.24</td>
<td>2.40±0.34</td>
</tr>
<tr>
<td>EIT, 200 mg kg⁻¹</td>
<td>352.0±4.34</td>
<td>207.0±13.03</td>
<td>222.5±5.67</td>
<td>2.0±0.24</td>
<td>3.0±0.24</td>
</tr>
<tr>
<td>EIT, 400 mg kg⁻¹</td>
<td>163.0±4.94</td>
<td>114.0±3.55</td>
<td>166.2±0.95</td>
<td>2.0±0.24</td>
<td>3.0±0.24</td>
</tr>
<tr>
<td>Vitamin E, 50 mg kg⁻¹</td>
<td>197.0±4.94</td>
<td>137.4±2.94</td>
<td>152.3±4.94</td>
<td>2.0±0.24</td>
<td>3.0±0.24</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM (n = 6); p<0.05, p<0.01, p<0.001, when compared to normal group, p<0.05, p<0.01, p<0.001, when compared with CCl4 treated control (Student’s t-test)

Table 4. Effect of EIT on CCl4 induced hepatotoxicity in rats (Liver)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
<th>Albumin (g dl⁻¹)</th>
<th>Total protein (g dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>165.0±15.5</td>
<td>71.6±6.93</td>
<td>198.0±6.41</td>
<td>2.0±0.24</td>
<td>6.3±0.55</td>
</tr>
<tr>
<td>CCl4 Control</td>
<td>248.3±23.5</td>
<td>232.2±44.19</td>
<td>423.7±6.97</td>
<td>1.2±0.24</td>
<td>3.2±0.27</td>
</tr>
<tr>
<td>EIT, 200 mg kg⁻¹</td>
<td>218.4±16.2</td>
<td>181.4±10.13</td>
<td>154.0±3.11</td>
<td>2.3±0.24</td>
<td>4.0±0.14</td>
</tr>
<tr>
<td>EIT, 400 mg kg⁻¹</td>
<td>247.3±19.9</td>
<td>134.9±11.2</td>
<td>273.7±17.5</td>
<td>3.0±0.24</td>
<td>4.0±0.24</td>
</tr>
<tr>
<td>Vitamin E, 50 mg kg⁻¹</td>
<td>214.5±6.74</td>
<td>112.5±4.73</td>
<td>214.3±11.3</td>
<td>3.1±0.24</td>
<td>4.1±0.24</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM (n = 6); p<0.05, p<0.01, p<0.001, when compared to normal group, p<0.05, p<0.01, p<0.001, when compared with CCl4 treated control (Student’s t-test)

In vivo Antioxidant Activity

The effects of the ethanolic extract of I. trita on CCl4 intoxicated rats are shown in Table 2-4. Intoxication of rats treated with CCl4 significantly (p<0.001) increased the levels of SGOT, SGPT, ALP and TBARS in serum and liver tissue homogenates (Table 2-4). The treatment also reduced the levels of albumin, total protein, SOD and catalase significantly. The treatment with the EIT at 200 and 400 mg kg⁻¹ body weight reduced the levels of TBARS highly significantly (p<0.001) in liver, kidney and serum. The treatment at 200 mg kg⁻¹ body weight increased the levels of catalase significantly (p<0.05) in liver and kidney and SOD in liver (p<0.05) and serum (p<0.001). A significant increase in the levels of SOD and catalase was also observed at 400 mg kg⁻¹ body weight treatment (p<0.05 to p<0.001) when compared to CCl4 treated control in liver, kidney and serum. The increase in the levels of SOD at both the doses of the extract treatment in serum and TBARS at 200 and 400 mg kg⁻¹ body weight dose in kidney was found to be more than that observed for the standard vitamin E and is significant (p<0.05 to p<0.001).

The treatment with the EIT at 200 and 400 mg kg⁻¹ body weight showed significant and dose dependent decrease in the levels of SGOT, SGPT and ALP and increase in the levels of albumin and total protein when compared to CCl4 treated control rats in both liver and serum (p<0.05 to p<0.001). Similar results were observed for the standard vitamin E treatment (p<0.01 to p<0.001).
The histopathological profile of liver of CCl4 treated rats showed many hepatocytes showing fatty changes with increased inflammatory cell infiltrate in the portal areas. Hepatocytes showed early degenerative changes. Liver of rats treated with the EIT at 200 mg kg⁻¹ showed fatty changes of the hepatocytes in few areas along with focal lymphocytic collection and a few degenerated cells. The treatment at 400 mg kg⁻¹ body weight of the extract and vitamin E treatment showed normal structure of the liver.

Similarly, the kidney of CCl4 treated rats showed edema of lining epithelium of renal tubules and lumen of some of the tubules contains eosinophilic material. Interstitial tissue showed scattered lymphocytes and plasma cells. The kidney of rats treated with the ethanol extract at 200 mg kg⁻¹ body weight also showed edema of lining epithelium of the renal tubules along with the collection of lymphocytes and plasma cells in interstitial tissues. The treatment with the EIT at 400 mg kg⁻¹ body weight and vitamin E treatment showed normal renal tubules and minimal edema and focal lymphocytic collection in the interstitial tissue. All these results suggest potent hepatoprotective nature of the ethanol extract of Indigofera trita.

**DISCUSSION**

It is well established that Reactive Oxygen Species (ROS) are implicated in a large number of diseases. Natural antioxidant mechanisms of the body can be insufficient and dietary intake of antioxidant compounds is necessary. There is an inverse relationship between dietary intake of antioxidant rich foods and the incidents of number of human diseases. Therefore, research on determination of natural antioxidant sources is important. In the present study, the EIT exhibited potent antioxidant activity both by in vitro and in vivo methods. Low IC₅₀ values indicating the potent activity were observed in the scavenging of ABTS, DPPH and hydroxyl radical methods. The extract also showed moderate antioxidant activity in the hydrogen peroxide and nitric oxide radical inhibition methods. The difference in the activity in different methods may be due to the different chemical entities of the free radicals and the diverse chemical nature of the extract.

The principle causes of CCl₄ induced hepatic damage is lipid peroxidation and decreased activities of antioxidant enzymes and generation of free radicals. The antioxidant activity is important in providing protection against hepatic damage (Poli, 1993). A number of plants have been shown to possess the hepatoprotective activity by improving antioxidant properties. In the in vivo studies, the EIT exhibited strong antioxidant properties as evidenced by the significant increase in the levels of SOD and catalase and decrease in the level of TBARS, in serum, liver and kidney tissues.

The administration of hepatotoxican CCl₄ increased the serum level of marker enzymes SGOT, SGPT and ALP indicating the induction of hepatotoxicity. The decrease in the level of albumin and total protein observed in CCl₄ treated rats may be associated with the decrease in the number of hepatocytes which in turn may result in the decrease of the hepatic capacity to synthesize protein. The extract mediated the suppression of the increased SGOT, SGPT and ALP activities and the restoration of the total protein and albumin levels suggesting the possibility of the extract to give protection against liver injury. These results suggest that the pretreatment with EIT showed dose dependent protection against the injurious effects of carbon tetrachloride, that may result from the interference with cytochrome P₄₅₀, resulting in the hindrance of the formation of hepatotoxic free radicals (Manjunatha, 2006).

The histopathological study of the liver and kidney of the extract treated rats showed normal structure also confirming the hepatoprotective nature of the extract. Preliminary phytochemical screening shows the presence of phenolic compounds and flavonoids in the plant. A large number of these compounds are known to possess strong antioxidant and hepatoprotective properties (Tripathi et al., 1996). Hence, the observed antioxidant and hepatoprotective activity of the EIT may
be due to the presence of any of these compounds. Further studies to characterize the active principles and elucidate the mechanism of the action of EIT are in progress.

REFERENCES


