Amelioration of alcohol-induced hepatotoxicity by the administration of ethanolic extract of Sida cordifolia Linn.

S. Rejitha, P. Prathibha and M. Indira*
Department of Biochemistry, University of Kerala, Kariavattom, Thiruvananthapuram 695 581 Kerala, India

(Submitted 1 August 2011 – Final revision received 17 October 2011 – Accepted 17 October 2011 – First published online 31 January 2012)

Abstract
Sida cordifolia Linn. (Malvaceae) is a plant used in folk medicine for the treatment of the inflammation of oral mucosa, asthmatic bronchitis, nasal congestion and rheumatism. We studied the hepatoprotective activity of 50% ethanolic extract of S. cordifolia Linn. against alcohol intoxication. The duration of the experiment was 90 d. The substantially elevated levels of toxicity markers such as alanine aminotransferase, aspartate aminotransferase and \( \gamma \)-glutamyl transferase due to the alcohol treatment were significantly lowered in the extract-treated groups. The activity of antioxidant enzymes and glutathione content, which was lowered due to alcohol toxicity, was increased to a near-normal level in the co-administered group. Lipid peroxidation products, protein carbonyls, total collagen and hydroxyproline, which were increased in the alcohol-treated group, were reduced in the co-administered group. The mRNA levels of cytochrome P450 2E1, NF-\( \kappa \)B, TNF-\( \alpha \) and transforming growth factor-\( \beta_1 \) were found to be increased in the alcohol-treated rats, and their expressions were found to be decreased in the co-administered group. These observations were reinforced by histopathological analysis. Thus, the present study clearly indicates that 50% ethanolic extract of the roots of S. cordifolia Linn. has a potent hepatoprotective action against alcohol-induced toxicity, which was mediated by lowering oxidative stress and by down-regulating the transcription factors.

Key words: Alcohol: Sida cordifolia Linn.: Oxidative stress: Hepatotoxicity

Materials and methods
Preparation of ethanolic extract of Sida cordifolia roots
S. cordifolia roots were collected from Trivandrum, India. The plant was authenticated by Dr Valsaladevi, Curator, Department of Botany, Kerala University. The identified and authenticated specimen was deposited in the herbarium of the Department of Botany, University of Kerala (plant no. KU5787). Fresh plant roots (250 g) were collected, washed...
thoroughly and dried in the shade. The root was then crushed 500 ml of 50% ethanol were added in order to extract both hydrophilic and hydrophobic components of the root. It was refluxed in a water-bath for 1-5 h at 60–65°C. Then, it was concentrated using a rotary flash evaporator. The 250 g of the plant root yielded 4.2 g (1.68%) of extract. This extract was named Sida alcoholic extract (SAE).

Male albino rats (Sprague–Dawley strain), weighing 100–140 g, bred and reared in our animal house, were used for the experiment. Weight-matched animals were selected. A total of twenty-four rats were divided into four groups of six rats each: control (group I); alcohol (4 g/kg body weight, group II); SAE (50 mg extract/100 g body weight per d, group III); alcohol (4 g/kg body weight) + SAE (50 mg extract/100 g body weight per d) (group IV).

The animals were housed in polypropylene cages. The cages were kept in a room that was maintained between 28 and 32°C. The light cycle was 12 h light and 12 h dark. The animals were handled using the laboratory animal welfare guidelines. Rats were fed with rat feed (Ashirvad Private Limited). Food and water were given ad libitum. Alcohol (4 g/kg body weight) and SAE (50 mg extract/100 g body weight per d) were given orally by gastric intubation. The dose was taken from the studies and SAE (50 mg extract/100 g body weight per d) were given.

The isolated RNA was used for RT-PCR to study the expression of cytochrome P450 2E1 (CYP2E1), NF-kB, TNF-α, transforming growth factor β1 (TGF-β1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total tissue RNA (2 μg) was primed with 0.05 μg oligo dT and reverse-transcribed by omniscript RT using a cDNA synthesis kit (Qiagen). PCR was carried out using an Eppendorf thermocycler (model 5332). Primer sequences are given in Table 1. The primer sequences for GAPDH, CYP2E1 and NF-kB were designed using primer 3 software and those for TNF-α and TGF-β1 were taken from a previous study. The PCR mixture contained 10 mM-Tris (pH 8.3), 50 mM-KCl, 1.5 mM-MgCl2, deoxy nucleoside triphosphate (dNTP) (20 mM each), gene-specific primers (0.5 mM each) and Taq polymerase (0.025 units/μl). After an initial denaturation step at 94°C, thirty-five amplification cycles were performed. Each cycle included an initial denaturation step at 94°C for 45s, annealing at 56°C for NF-kB, 55°C for TGF-β1, 61°C for TNF-α, 55°C for CYP2E1 and 62°C for GAPDH. A final extension step of 5 min at 72°C was performed in order to complete the PCR. The amplified product was analysed by electrophoresis on 2% agarose gel containing ethidium bromide. Then, the gels were subjected to densitometric scanning (Bio-Rad Gel Doc) to determine the optical density of each, and then normalised

**Table 1. Primer sequences used for RT-PCR analysis**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Melting temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-TGA CAA CTC CCT CAA GAT TGT CA-3'</td>
<td>63.7</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGC ATG GAC TGT GGT CAT GA-3'</td>
<td>64.7</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Forward: 5'-GCC ACC CTC CTC GTC ATA TC-3'</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCA GAC AAT CAG AAA TGT GG-3'</td>
<td>60.0</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Forward: 5'-CAC CAA AGA ACC ACC TCA GC-3'</td>
<td>58.9</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCA GCG CAT TCA AGT CAT AG-3'</td>
<td>59.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5'-GAA CAA CCC TAG GAG CAC CT-3'</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGG TAG TTT GGC TGG GAT AA-3'</td>
<td>59.4</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Forward: 5'-CCG CAA CAA CCC AAC AAT CTA TG-3'</td>
<td>61.4</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGC CCT GTA TCC CCG TCT CTT-3'</td>
<td>62.4</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYP2E1, cytochrome P4502E1; TGF-β1, transforming growth factor β1.
against an internal control, GAPDH using Quantity One imaging software (BioRad).

Histological analysis

For histopathological studies, liver was fixed in Bouin’s fixative and sections were sliced using a microtome. The sections were stained using haematoxylin and eosin. The pathological changes were examined using a sensitive light microscope.

Statistical analysis

The results were analysed using a statistical program SPSS/PC+, version 11.5 (SPSS, Inc.). A one-way ANOVA was employed for comparison among the six groups. Duncan’s post hoc multiple comparison tests of significant differences among the groups were determined. A *P* value *< 0.05* was considered to be significant.

Results

Body weight

The body weight of the animals (Fig. 1) was recorded on the 1st and 90th day of the experiment. We found that body weight was decreased in the alcohol-treated group when compared with the other groups. No mortality was observed during the entire study.

Biochemical analysis

The activities of γ-glutamyl transferase in the serum and those of alanine aminotransferase and aspartate aminotransferase in the liver and serum (Table 2) increased significantly in the alcohol-treated rats compared with the control group, and their activities were reduced to a near-normal level in the co-administered group. There was no change in the activities of these enzymes in the SAE-alone-treated group.

The concentration of GSH decreased significantly in the alcohol-treated group (Table 2) compared with the control group, and it was increased significantly in the co-administered group compared with the alcohol-treated group.

The activities of catalase, SOD, glutathione peroxidase, glutathione reductase and GSH content (Table 3) were significantly decreased in the liver of the alcohol-treated group compared with the control group. There was also a significant increase in the activities of catalase, SOD, glutathione peroxidase, glutathione reductase and GSH content in the groups administered with SAE and alcohol + SAE compared with the control group.

The level of lipid peroxidation products, malondialdehyde, hydroperoxides and conjugated dienes, in the liver and that of the protein carbonyls in the serum (Table 3) were increased significantly in the alcohol-treated group compared with the control group, and their concentrations were reduced significantly in the co-administered group when compared with the alcohol-treated group.

The concentration of total collagen and hydroxyproline were increased significantly in the alcohol-treated group compared with the control rats (Table 3), and their concentrations

---

**Table 2. Activity of toxicity marker enzymes in the liver and serum of rats**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Alcohol</th>
<th>SAE</th>
<th>Alcohol + SAE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/mg protein)</td>
<td>19.78</td>
<td>1.90</td>
<td>78.52*</td>
<td>7.53</td>
</tr>
<tr>
<td>ALT (U/mg protein)</td>
<td>15.51</td>
<td>1.49</td>
<td>98.81*</td>
<td>7.53</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/mg protein)</td>
<td>185.27</td>
<td>17.80</td>
<td>351.20*</td>
<td>33.69</td>
</tr>
<tr>
<td>ALT (U/mg protein)</td>
<td>51.68</td>
<td>4.96</td>
<td>107.25*</td>
<td>10.29</td>
</tr>
<tr>
<td>GGT (U/mg protein)</td>
<td>17.43</td>
<td>1.59</td>
<td>62.40*</td>
<td>5.99</td>
</tr>
<tr>
<td>GSH (U/mg protein)</td>
<td>518.76</td>
<td>49.87</td>
<td>392.12*</td>
<td>37.61</td>
</tr>
</tbody>
</table>

SAE, Sida alcoholic extract; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT-γ, glutamyl transferase; GSH, reduced glutathione.

* Mean values were significantly different from the control group (*P* < 0.05; one-way ANOVA).
† Mean values were significantly different from the ethanol group (*P* < 0.05; one-way ANOVA).
‡ µmol pyruvate liberated/min.
§ µmol oxaloacetate liberated/min.
|| µmol P-nitroaniline liberated/min.
were reduced significantly in the co-administered group when compared with the alcohol-treated group.

The mRNA expressions of CYP2E1, NF-κB, TNF-α and TGF-β1 were evaluated by RT-PCR. In the alcohol-treated rats, the PCR products had a marked increase compared with the control rats. The treatment with SAE reduced the levels of expressions of CYP2E1 (Fig. 2), NF-κB (Fig. 3), TNF-α (Fig. 4) and TGF-β1 (Fig. 5) genes. There was no significant change in their expression in the SAE-treated rats.

**Histological analysis**

The histological features of the liver in the control (Fig. 6(A)) and SAE-alone-treated groups (Fig. 6(C)) showed a normal liver architecture and cell structure. After ethanol administration, liver sections showed extensive hepatocellular damage, as evidenced by ballooning of hepatocytes, steatosis, vacuolisation and dilation of sinusoids (Fig. 6(B)). These changes were alleviated by treatment with the plant extract (Fig. 6(D)).

**Discussion**

Alcohol appears to increase the metabolic rate significantly, thus causing more energy to be burned rather than stored in the body as fat. The present study has shown that chronic alcohol ingestion results in a decrease in body weight, which is in line with earlier findings(27). The alcohol metabolism via alcohol dehydrogenase and CYP2E1 results in the formation of cytotoxic aldehyde, which in turn is oxidised into acetate by aldehyde oxidase or xanthine oxidase, giving rise to reactive oxygen species, which can damage the biomembrane, resulting in the leakage of liver marker enzymes into the circulation(28). Treatment with 50% ethanolic extract of *S. cordifolia* effectively down-regulated the expression of CYP2E1 and decreased the activities of these enzymes in the liver and serum, indicating hepatoprotective activity of the extract. This is in agreement with Rao & Mishra(29) who showed the hepatoprotective activity of the whole plant of *S. cordifolia* against CCl₄, paracetamol- and rifampicin-induced hepatotoxocities in rats. Fumaric acid isolated from *S. cordifolia* also showed hepatoprotective activity, which was comparable with silymarin(30).

**Table 3. Effect of Sida alcoholic extract (SAE) supplementation on antioxidant status, lipid peroxidation products, protein carbonyls, total collagen and hydroxyproline content in the liver**

*(Mean values and standard deviations)*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Alcohol</th>
<th>SAE</th>
<th>Alcohol+SAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (Ut/mg protein)</td>
<td>81±17</td>
<td>29±45*</td>
<td>82±39</td>
<td>76±93</td>
</tr>
<tr>
<td>Catalase (Ut/mg protein)</td>
<td>63±68</td>
<td>45±83*</td>
<td>61±23</td>
<td>60±61</td>
</tr>
<tr>
<td>GPx (U/l/mg protein)</td>
<td>13±37</td>
<td>6±04*</td>
<td>13±70</td>
<td>12±39</td>
</tr>
<tr>
<td>GR (U/l/mg protein)</td>
<td>20±27</td>
<td>5±16*</td>
<td>21±62</td>
<td>18±65†</td>
</tr>
<tr>
<td>MDA (mmol/100 g tissue)</td>
<td>0±50</td>
<td>0±99*</td>
<td>0±48</td>
<td>0±57†</td>
</tr>
<tr>
<td>HP (mmol/100 g tissue)</td>
<td>8±98</td>
<td>18±61*</td>
<td>8±77</td>
<td>9±97†</td>
</tr>
<tr>
<td>CD (mmol/100 g tissue)</td>
<td>60±63</td>
<td>146±47*</td>
<td>53±63</td>
<td>73±79†</td>
</tr>
<tr>
<td>Protein carbonyls (nmol/mg protein)</td>
<td>0±88</td>
<td>3±80*</td>
<td>0±94</td>
<td>1±1†</td>
</tr>
<tr>
<td>Total collagen (mg/100 g protein)</td>
<td>9±66</td>
<td>118±20*</td>
<td>58±72</td>
<td>83±99†</td>
</tr>
<tr>
<td>Hydroxyproline (mg/100 g protein)</td>
<td>10±90</td>
<td>16±74*</td>
<td>9±5</td>
<td>10±98†</td>
</tr>
</tbody>
</table>

SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; MDA, malondialdehyde; HP, hydroperoxides; CD, conjugated dienes.

*Mean values were significantly different from the control group (P<0.05; one-way ANOVA).
†Mean values were significantly different from the ethanol group (P<0.05; one-way ANOVA).
‡Enzyme concentration required to inhibit chromogen produced by 50% in 1 min.
§Velocity constants.
|| 1 µM-NADPH oxidised/min.
Alcohol-induced toxicity is mediated through oxidative stress (31). Oxidative injury induced by alcohol can be monitored by detecting lipid peroxidation products (32). In the present study, we observed increased levels of malondialdehyde, hydroperoxides and conjugated dienes in the liver of the alcohol-administered group. Treatment with SAE resulted in decreased levels of malondialdehyde, hydroperoxides and conjugated dienes in the liver and protein carbonyls in the serum. This is in agreement with reports that the leaves of *S. cordifolia* have significant antioxidant activity during myocardial injury (33).

GSH is a tripeptide antioxidant critical for cellular protection such as detoxification of reactive oxygen species. Depletion of GSH in tissue leads to impairment of the cellular defence against reactive oxygen species and may lead to peroxidative injury. The levels of GSH reduced significantly in the alcohol-treated rats. This is consistent with previous reports (34). Administration of alcohol induces lipid peroxidation and depletes GSH reserves, but there are events that occur after the formation of alcohol metabolites. The reactive oxygen intermediates generated during the metabolism of alcohol lead to GSH oxidation (35), resulting in the depletion of GSH. In the present study, low levels of GSH and decreased activities of glutathione reductase and peroxidase were
observed in the alcohol-treated group. *S. cordifolia* exerts an antioxidant effect by decreasing lipid peroxidation, increasing GSH level and maintaining a normal level of antioxidant enzymes. This may cause accumulation of \(O_2^-, H_2O_2\) or the products of its decomposition. Catalase acts as a preventive antioxidant and plays an important role in the protection against the deleterious effects of lipid peroxidation. Reports have shown that catalase activity has been significantly reduced during deleterious effects of lipid peroxidation. Reports have shown and plays an important role in the protection against the stress leads to the phosphorylation and degradation of inhibitory \(κB\) molecules. Alcoholic-induced oxidative stress leads to the phosphorylation and degradation of inhibitory \(κB\). As there is a decrease in the mRNA expression of NF-κB, there may also be a decrease in the activation of NF-κB. However, this can be confirmed only by further protein translocation studies of NF-κB from the cytosol to the nucleus. The decreased mRNA level of NF-κB in the co-administered group indicates that SAE can down-regulate the expression of NF-κB. Thus, intragastrically administered ethanol induces the endotoxin-mediated activation of NF-κB in Kupffer cells, which accounts for an increased synthesis of the pro-inflammatory cytokine TNF-α, chemokines and TGF-β.

Among the pro-inflammatory cytokines produced during alcoholic liver injury, TNF-α has been well characterised in animal models and human studies. TNF-α plays a critical role in the initiation and development of alcoholic hepatitis. Kupffer cells are the main source of TNF-α in the liver after alcohol exposure. The present study provides direct evidence that chronic alcohol administration significantly increases intrahepatic mRNA levels of TNF-α and TGF-β1. This may due to the increased activation of NF-κB in the alcohol-treated rat liver. Hepatocytes undergoing oxidative stress due to reactive oxygen species generation and CYP2E1 induction are sensitised to TNF-α-induced apoptosis and necrosis. Furthermore, mediators such as lipopolysaccharide and TGF-β1 activate hepatic stellate cells to proliferate and produce collagen, leading to fibrosis and the progression of liver injury. Co-administration of SAE with alcohol reduced the expression of NF-κB, TNF-α and TGF-β1 mRNA levels, which were elevated by alcohol administration, indicating that SAE can down-regulate the signalling mechanisms in alcohol-induced liver injury.

Hepatic fibrosis is characterised by an abnormal accumulation of extracellular matrix (ECM) proteins, particularly collagen. When hepatic fibrosis occurs, collagen proliferation, mainly collagen types 1 and 3, accounts for 50% of the total protein in fibrotic liver, and collagens are the main components of the ECM. The main collagen-producing cells in the liver are hepatic stellate cells, which proliferate and undergo a process of activation during the development of fibrosis resulting in increased capacity for collagen synthesis. Changes in hydroxyproline content in the liver are considered an index for collagen metabolism and provide valuable information on the biochemical and pathological states of liver fibrosis. The present study demonstrates that administration of SAE prevented the development of hepatic fibrosis in a rat model of alcohol-induced liver fibrosis. The results were confirmed by both liver histology and the quantitative measurement of hepatic hydroxyproline content, a marker of collagen deposition in the liver. From the histological studies of the liver, it was noted that after ethanol administration, liver sections showed extensive hepatocellular damage as, evidenced by ballooning of hepatocytes, steatosis, vacuolisation and dilatation of sinusoids. These changes were ameliorated by treatment with the plant extract, showing that SAE significantly protects the liver cell from damage.

In the present study, we have demonstrated the hepatoprotective actions of SAE. We have not isolated the active principle. However, there are reports that *S. cordifolia* contains many alkaloids, oils, steroids, resin acids, mucin and potassium nitrate. It is also considered to be a potential source of natural antioxidants. Roots of this plant possess diuretic and tonic properties. Cryptolepine is an indoloquinoline alkaloid isolated from *S. cordifolia*. It is known to impart its anti-cancerous effect through arresting the growth of human osteosarcoma cells and activating the p21 promoter through the specific Sp1 site in a p53-independent manner. Tannin or glycoside was not isolated from the plant. The roots of this plant contain alkaloid ephedrine. Recent studies have shown that ephedrine is the major alkaloid present in the aerial parts of the plant. The major flavones isolated from this plant are 5,7-dihydroxy-3-isoprenyl flavone, 5-hydroxy-3-isoprenyl flavone and 5-hydroxy-3-isoprenyl flavone, and even stigmasterol and β-sisterol have been isolated from this plant. The observed beneficial effects of SAE may be due to the concerted actions of alkaloids and flavonoids present in SAE.

In summary, the results show that 50% ethanolic extract of *S. cordifolia* significantly protects the liver cells and reduces the severity of damage caused by alcohol intoxication. The mechanism appears to be by reducing oxidative stress and by down-regulating the expression of transcription factors. However, further detailed studies are required to establish its clinical application.
Acknowledgements

We are thankful to the Council of Scientific and Industrial Research for the financial assistance to carry out the work efficiently. The contributions of each author are as follows: S. R. designed and conducted the experiment, analysed the results and prepared the manuscript; P. P. designed and conducted the experiment and also prepared the manuscript; M. I. was responsible for the very concept and design of the experiment and the interpretation of the data. The authors declare that they have no conflicts of interest.

References